frontiers in Plant Science

ORIGINAL RESEARCH
published: 31 October 2018
doi: 10.3389/fpls.2018.01532

A Stepwise $NaHSO_3$ Addition Mode Greatly Improves H_2 Photoproduction in *Chlamydomonas reinhardtii*

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$NaHSO_3$ addition greatly increases the yield of H_2 photoproduction in a unicellular green alga *Chlamydomonas reinhardtii* through removing O_2 and activating hydrogenase but significantly impairs the activity of PSII, an electron source for H_2 photoproduction. Here, a stepwise addition mode of total 13 mM $NaHSO_3$, an optimal concentration for H_2 photoproduction of *C. reinhardtii* identified in a previous one step addition method, significantly improved H_2 photoproduction. Such improvement was believed to be the result of increased residual PSII activity in an anaerobic background, but was at least independent of two alternative electron sinks for H_2 photoproduction, cyclic electron transport around PSI and CO_2 assimilation. Based on the above results, we propose that increased residual PSII activity in an anaerobic environment is an efficient strategy to enhance H_2 photoproduction in *C. reinhardtii*, and the stepwise $NaHSO_3$ addition mode is a case study in the strategy.

Keywords: $NaHSO_3$, stepwise mode, anaerobic environment, PSII activity, H_2 photoproduction, *Chlamydomonas reinhardtii*

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Specialty section:
This article was submitted to Plant Biotechnology, a section of the journal Frontiers in Plant Science

Received: *27 February 2018*
Accepted: *28 September 2018*
Published: *31 October 2018*

Citation:
Wei L, Li X, Fan B, Ran Z and Ma W (2018) A Stepwise $NaHSO_3$ Addition Mode Greatly Improves H_2 Photoproduction in Chlamydomonas reinhardtii. Front. Plant Sci. 9:1532. doi: 10.3389/fpls.2018.01532

INTRODUCTION

With the increasing awareness of fossil fuel depletion and global warming, efforts have been undertaken to develop clean and sustainable energy sources (McKendry, 2002). Molecular hydrogen (H_2) is one of the potential future energy sources (Hansel and Lindblad, 1998; Momirlan and Veziroglu, 2002). *C. reinhardtii*, a unicellular green alga, has been recognized as an ideal system for sustainable H_2 photoproduction under anaerobic conditions; however, this alga cannot efficiently and continuously produce H_2 in an aerobic environment because its H_2ase is extremely sensitive to O_2 (Ghirardi et al., 1997). To activate H_2ase for sustainable and efficient H_2 photoproduction in *C. reinhardtii*, therefore, numerous strategies have been extensively developed mainly through engineering O_2 tolerance in H_2ase (Flynn et al., 2002; Liebgott et al., 2011; Wu et al., 2011) or decreasing O_2 content around H_2ase (Melis et al., 2000; Kruse et al., 2005; Surzycki et al., 2007; Wu et al., 2010; Xu et al., 2011; Jurado-Oller et al., 2015; Xiong et al., 2015; Shu et al., 2018).

Abbreviations: AA, antimycin A; CET, cyclic electron transport around PSI; Chl, chlorophyll; *C. reinhardtii*, *Chlamydomonas reinhardtii*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DO, dissolved oxygen; F_v/F_m, maximal quantum yield of PSII; GA, glycolaldehyde; H_2ase, hydrogenase; Lin, lincomycin; MV, methyl viologen; one step method, a one step addition method of $NaHSO_3$; stepwise mode, a stepwise addition mode of $NaHSO_3$; TAP, Tris-acetate-phosphate.

Meanwhile, our studies demonstrate that $NaHSO_3$ addition strategy is capable of decreased the O_2 content around H_2ase, thereby activating the enzyme activity and promoting H_2 photoproduction (Wang et al., 2010; Ma et al., 2011; Wei et al., 2017). This strategy can result in an approximately 10-fold or 200-fold increase in H_2 photoproduction in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 (Wang et al., 2010) or the unicellular green alga *C. reinhardtii* (Ma et al., 2011; Wei et al., 2017), respectively. Despite these increases, this yield is still not sufficient to meet the requirements of industrial applications. Thus, extensive optimization of this $NaHSO_3$ addition strategy is necessary to increase H_2 photoproduction in *C. reinhardtii* further.

The yield of H_2 photoproduction caused by sulfur deprivation is also not sufficient to meet the requirements of industrial applications. Under sulfur deprivation conditions, therefore, many strategies have been developed to improve the yield of H_2 photoproduction in *C. reinhardtii* via metabolic and genetic engineering (for review, see Esquível et al., 2011; Dubini and Ghirardi, 2015). Among them, increased residual photosystem II (PSII) activity was found to play a vital role in efficient H_2 photoproduction (Zhang et al., 2002; Kosourov et al., 2005; Kim et al., 2010; Volgusheva et al., 2013; Grewe et al., 2014; Steinbeck et al., 2015; Chen et al., 2016), since the PSII activity is significantly impaired by sulfur deprivation (Melis et al., 2000). Similarly, in the $NaHSO_3$ addition background, the PSII activity is also significantly impaired (Wang et al., 2010). To test whether increased residual PSII activity in the $NaHSO_3$ addition background can also enhance H_2 photoproduction, we monitored the accumulated H_2 level and residual PSII activity in the stepwise mode of total 13 mM $NaHSO_3$, an optimal concentration for H_2 production of *C. reinhardtii* identified in a previous one step addition method (Ma et al., 2011). We also measured the content of dissolved O_2 and activities of two alternative electron sinks for H_2 photoproduction, CET and CO_2 assimilation, in the stepwise $NaHSO_3$ addition mode. Our results demonstrate that the stepwise $NaHSO_3$ addition mode evidently enhances the yield of H_2 photoproduction in *C. reinhardtii*; such enhancement is mostly the result of increased residual PSII activity in an anaerobic environment, but is at least independent of two alternative electron sinks for H_2 photoproduction.

MATERIALS AND METHODS

Culture Conditions

Cells of *C. reinhardtii* (CC-503 strain) were cultured at 25°C in TAP medium (Harris, 1989). The medium was buffered with Tris–HCl (20 mM; pH 7.3), bubbled with air under continuous illumination with cool-white fluorescent lamps (40 μmol photons $m^{-2}s^{-1}$), and inoculated with approximately 8.1×10^4 cells mL^{-1} of *C. reinhardtii* (inoculum size, 1%).

Sample Preparation and $NaHSO_3$ Addition

Cells of *C. reinhardtii* were continuously illuminated by growth light of 40 μmol photons $m^{-2}s^{-1}$ and were cultured in 0.5 L of TAP medium for 2 days with bubble aeration (A_{750} = 0.8–1.0), after which a fixed volume of cells containing 300 μg of Chl was transferred to 60 mL serum bottles (30 mL head space and 30 mL cells) with rubber seals. After cells were statically pre-cultured under continuous illumination of 200 μmol photons $m^{-2}s^{-1}$ for 36 h, total 13 mM of $NaHSO_3$ was directly or step by step added to the serum bottles, as indicated in **Figures 1**, **2**, **Supplementary Figure S1**, and described in **Table 1**, with Lin of 5 mM (final concentration) or AA of 10 μM (final concentration) or GA of 2 mM (final concentration) or DCMU of 20 μM (final concentration) or not. Subsequently, the

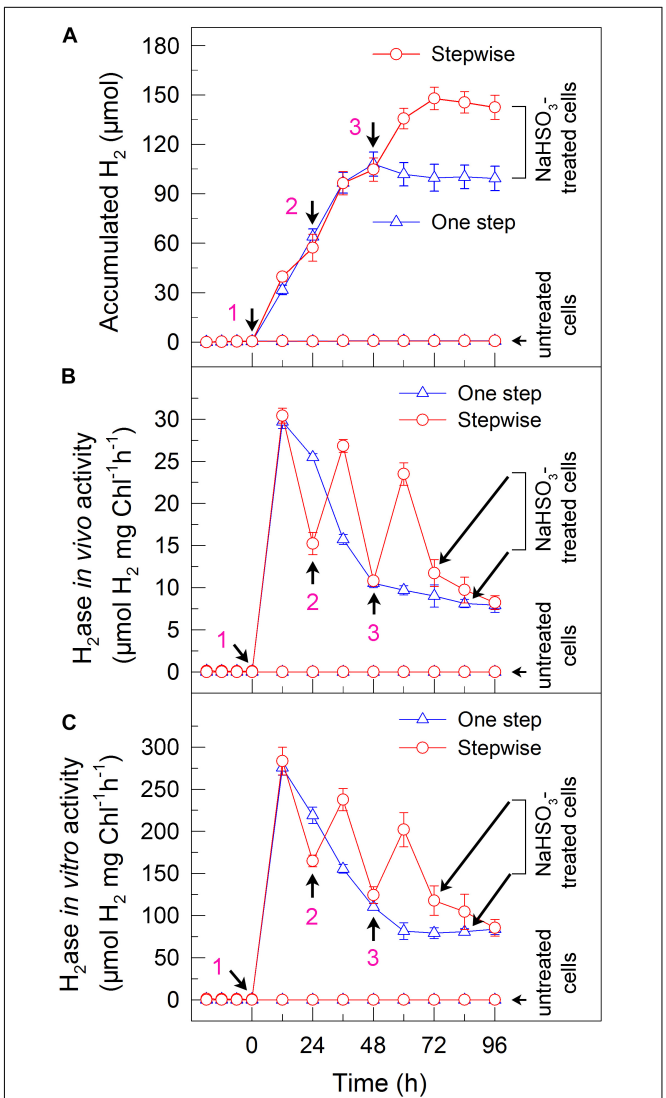

FIGURE 1 | A stepwise $NaHSO_3$ addition mode significantly increases **(A)** H_2 photoproduction, and **(B)** *in vivo* and **(C)** *in vitro* H_2ase activity in *C. reinhardtii*. After cells were statically pre-cultured under continuous illumination of 200 μmol photons $m^{-2}s^{-1}$ for 36 h, total 13 mM of $NaHSO_3$ was directly or step by step added to the serum bottles, as indicated by arrows 1–3 and described in **Table 1**. Values are means ± SD (n = 5).

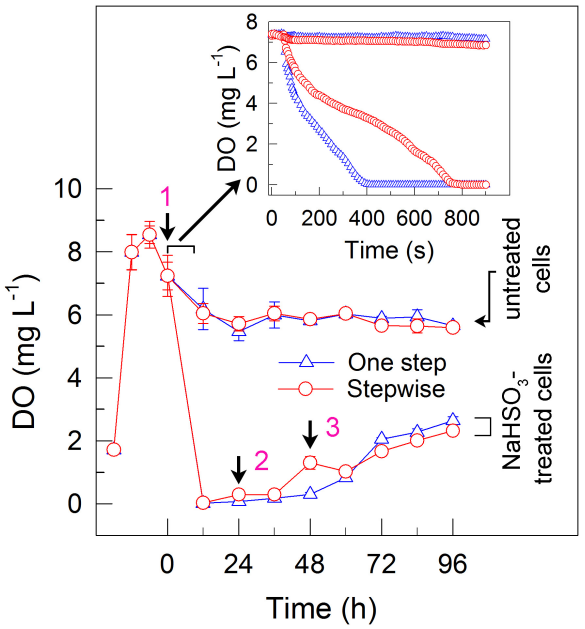

FIGURE 2 | A stepwise $NaHSO_3$ addition mode influences the content of dissolved oxygen (DO) in *C. reinhardtii*. Pre-culture conditions of cells and addition modes of $NaHSO_3$ are shown in the legend of **Figure 1**. Values are means ± SD (n = 5).

TABLE 1 | A table schematically represents one step method and stepwise mode of total 13 mM $NaHSO_3$.

$NaHSO_3$ addition	$NaHSO_3$ (mM)			
	Arrow 1	Arrow 2	Arrow 3	Total
One step method	13	0	0	13
Stepwise mode	9	2	2	13

*Arrows 1–3 shown in **Figures 1–5** and both arrows have a 24 h interval.*

cells were still illuminated at 200 μmol photons $m^{-2}s^{-1}$ or were incubated in the dark to induce the production of H_2.

Monitoring H_2 Photoproduction

At predetermined time intervals, 200 μL of gas samples were withdrawn from the bottles using a gas-tight syringe and injected into a gas chromatograph (Agilent 7890A; Agilent Technologies Inc., United States) with a thermal conductivity detector for determining the concentrations of H_2, O_2, and N_2 simultaneously. The column of the gas chromatograph was a molecular sieve column (type 5Å; 2 m × 1/8 mm), and argon was used as the carrier gas.

H_2ase Activity Assay

In vivo and *in vitro* H_2ase activity was monitored as described earlier (Ma et al., 2011; Wei et al., 2013, 2017) with some modifications. In brief, 1 mL cell suspension samples upon exposure to 200 μmol photons $m^{-2}s^{-1}$ were anaerobically withdrawn from the 60 mL serum bottles at designated time points (see **Figures 1B,C**) and then injected into 10 mL glass vials. To measure *in vivo* H_2ase activity, the cell suspension samples were immediately purged with argon gas for 1 min to eliminate the inhibitory effect of O_2 on the H_2ase activity. The cell suspension samples were then placed in a 25°C water bath for 1 h and shaken continuously (150 rpm) whilst exposed to a constant light of 200 μmol photons $m^{-2}s^{-1}$. To measure *in vitro* H_2ase activity, we used vials containing 1 mL of 10 mM oxidized MV prepared in O_2-free 50 mM Tris buffer (for pH 7.1–9.0) and 0.2% (*w*/*v*) Triton X-100. The reaction was started when MV was reduced by the addition of 100 μL of 100 mM anaerobic sodium dithionite in 0.03 N NaOH. This assay was performed at 37°C in the dark for 20 min. We determined the amount of H_2 produced in the headspace of the glass vial by gas chromatography, and the rate of H_2 production was calculated on the basis of the total Chl content in the glass vial, unless otherwise indicated.

Dissolved Oxygen Measurement

Dissolved oxygen was monitored as described earlier (Wei et al., 2017). In brief, a DO meter (Orion Star A213, Thermo Scientific, Untied States) was used to monitor the DO attenuation process after the addition of $NaHSO_3$ to the cell suspension cultures of *C. reinhardtii*. The DO meter was corrected before each measurement. The DO meter probe was placed in the middle of the cell suspension cultures and the data were recorded at several designated time points.

Chl Fluorescence and P700 Analysis

The yields of Chl fluorescence at a steady-state of electron transport were measured at room temperature using a Dual-PAM-100 monitoring system (Walz, Effeltrich, Germany) equipped with an ED-101US/MD unit (Schreiber et al., 1986; Ma et al., 2008; Wei et al., 2013, 2017). Minimal fluorescence at open PSII centers in the dark-adapted state (F_o) was excited by a weak measuring light (650 nm) at a photon flux density of 0.05–0.15 μmol photons $m^{-2}s^{-1}$. A saturating pulse of red light (600 ms, 10,000 μmol photons $m^{-2}s^{-1}$) was applied to determine the maximal fluorescence at closed PSII centers in the dark-adapted state (F_m). F_v/F_m was evaluated as $(F_m-F_o)/F_m$ (Kitajima and Butler, 1975; Wei et al., 2013, 2017).

The reduction of $P700^+$ in darkness was measured with the aforementioned Dual-PAM-100 fluorometer by monitoring absorbance changes at 830 nm and using 875 nm as a reference. Cells were kept in the dark for 2 min, and 10 μM of DCMU was added to the cell suspension cultures prior to the measurement. The P700 was oxidized by far-red light with a maximum at 720 nm from a light-emitting diode lamp for 30 s, and the subsequent re-reduction of $P700^+$ in the dark was monitored and its half-time was calculated.

Oxygen Evolution Activity

Oxygen production in intact *C. reinhardtii* cells by photosynthesis was determined at 25°C by monitoring the changes in O_2 levels with a Clark-type oxygen electrode (Hansatech Instruments, King's Lynn, United Kingdom). Prior to the measurements, 10 mM of $NaHCO_3$ was added to the

cell suspension cultures. The intensity of light used for the measurements was 1,000 μmol photons $m^{-2}s^{-1}$.

RESULTS

A Stepwise $NaHSO_3$ Addition Mode Significantly Increases the Yield of H_2 Photoproduction in *C. reinhardtii*

To test whether a stepwise $NaHSO_3$ addition mode can enhance the yield of H_2 photoproduction, we monitored accumulated H_2 amounts in the one step addition method and stepwise mode of total 13 mM $NaHSO_3$ (hereafter one step method and stepwise mode, respectively; see **Table 1**), an optimal concentration for H_2 photoproduction of *C. reinhardtii* identified in a previous one step method (Ma et al., 2011). Compared to the one step method, stepwise mode evidently enhanced the yield of H_2 photoproduction (**Figure 1A** and **Table 2**). The H_2 level in stepwise mode was approximately 1.5 times greater than that in one step method and, approximately 350 times greater than that in untreated cells (**Figure 1A** and **Table 2**). This was confirmed by the results of *in vivo* (**Figure 1B**) and *in vitro* (**Figure 1C**) H_2ase activity. We therefore conclude that the stepwise mode considerably improves H_2 photoproduction in the green alga *C. reinhardtii*.

The Stepwise Mode Can Also Establish an Anaerobic Environment

To elucidate the mechanism underlying the increase in the H_2 yield under stepwise mode, we monitored the dissolved O_2 (DO) content in the cell suspension cultures of *C. reinhardtii*. The results indicated that addition of total 13 mM $NaHSO_3$ to the cell suspension cultures in both the one step method and stepwise mode can similarly create an anaerobic environment (**Figure 2**), although the stepwise mode was slightly slow to generate an anaerobic environment when compared to the one step method (insert in **Figure 2**). It is worthy of note that, when an initial concentration of $NaHSO_3$ in stepwise mode was less than or equal to 7 mM, the cell suspension cultures did not enter or maintain an anaerobic environment, which evidently suppressed the increase of H_2 photoproduction in the stepwise mode (data not shown). Based on the above results, we propose that the stepwise mode is necessary to operate in an anaerobic environment as an efficient strategy for H_2 photoproduction in *C. reinhardtii*.

TABLE 2 | Comparison of H_2 photoproduction characteristics in *C. reinhardtii* between one step method and stepwise mode.

$NaHSO_3$ addition	Vmax (μmol H_2 mg $Chl^{-1}h^{-1}$)[1]	Amax (relative; %)[2]	Time (day)[3]
One step method	11.8 ± 0.7	100 ± 6.9	50.2 ± 3.2
Stepwise mode	14.7 ± 1.2	145.6 ± 5.1	76.1 ± 2.8

*[1]Vmax indicates the maximum velocity of H_2 photoproduction. [2]Amax indicates the maximum accumulated H_2 (calculated from **Figure 1A**) and its value in one step method was taken as 100%. [3]Time represents the time of continuous H_2 production, which was calculated from **Figure 1A**.*

The Stepwise Mode Maintains a Relatively High Residual Activity of Electron Source for H_2 Photoproduction

To understand why the stepwise mode can increase the yield of H_2 photoproduction, we measured the activity of PSII, an electron source for H_2 photoproduction. After the addition of total 13 mM $NaHSO_3$ to the cell suspension cultures, the residual activity of PSII was much higher in stepwise mode than that in one step method, as evaluated by the F_v/F_m values (**Figure 3**). This was supported by the results that the stepwise mode maintained a slightly high DO content at an efficient H_2 production stage when compared to the one step method (**Figure 2**). This implies that the relatively high PSII activity under anaerobic conditions is an important reason for improved H_2 photoproduction in the stepwise mode.

The Stepwise Mode Slightly Enhances the Activities of Two Alternative Electron Sinks for H_2 Photoproduction

We also measured the activities of CET and CO_2 assimilation, two alternative electron sinks for H_2 photoproduction. The rates of CET and CO_2 assimilation were slightly faster in the stepwise mode than those in the one step method, as estimated by the rate of re-reduction of $P700^+$ (**Figure 4**), and photosynthetic production of O_2 with $NaHCO_3$ as an artificial electron acceptor (**Figure 5**), respectively. It appears plausible that at least the two alternative electron sinks for H_2 photoproduction do not contribute to enhance the photoproduction of H_2 in the stepwise mode. If this possibility is true, an increase in H_2 photoproduction caused by impaired the activity of either CET or CO_2 assimilation will be higher in the stepwise mode than that in the one step method. The results shown in **Figure 6** support our hypothesis that the increase in H_2 photoproduction

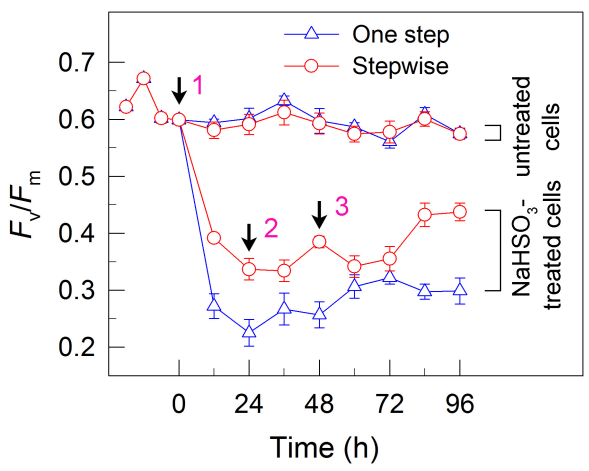

FIGURE 3 | A stepwise addition mode alleviates the inhibitory effects of $NaHSO_3$ on PSII activity in *C. reinhardtii*. The Chl concentration was adjusted to 10 μg mL^{-1} before measurement. PSII activity was evaluated by the F_v/F_m values. Values are means ± SD (n = 5).

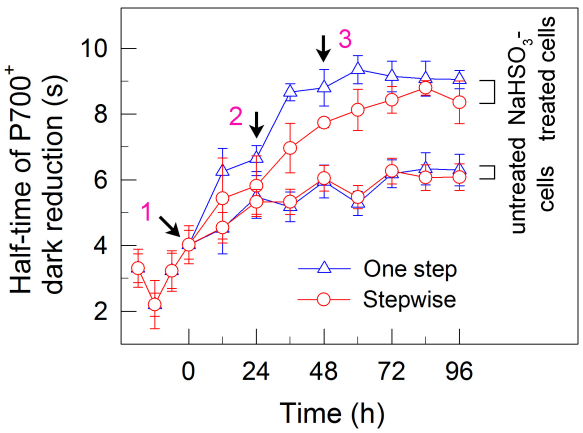

FIGURE 4 | A stepwise addition mode slightly alleviates the inhibitory effects of $NaHSO_3$ on cyclic electron transport around PSI in *C. reinhardtii*. The rate of cyclic electron transport around PSI was judged by half-time of $P700^+$ dark reduction. Values are means ± SD (n = 5).

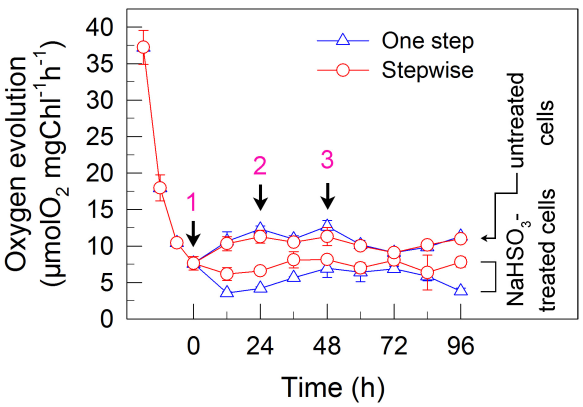

FIGURE 5 | A stepwise addition mode slightly alleviates the inhibitory effects of $NaHSO_3$ on CO_2 assimilation in *C. reinhardtii*. Activity of CO_2 assimilation was assessed by photosynthetic production of O_2 with $NaHCO_3$ as an artificial electron acceptor. Values are means ± SD (n = 5).

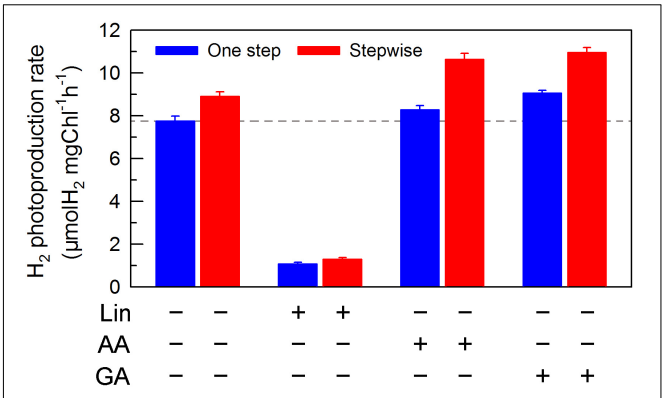

FIGURE 6 | Comparison of the H_2 photoproduction in *C. reinhardtii* between one step method and stepwise mode with multiple inhibitors. After cells were statically pre-cultured under continuous illumination of 200 μmol photons $m^{-2}s^{-1}$ for 36 h, $NaHSO_3$ was added as described in the legend for **Figure 1**, as well as several inhibitors, lincomycin (Lin; 5 mM), antimycin A (AA; 10 μM), and glycolaldehyde (GA; 2 mM) were also added to the serum bottles, respectively. Values are means ± SD (n = 5).

was slightly higher in the stepwise mode than that in the one step method in the presence of either AA that specifically inhibits the CET activity (Tagawa et al., 1963) or GA that disrupts the Calvin–Benson cycle activity via inhibiting the phosphoribulokinase (Rühle et al., 2008). Taking all these results together, we may conclude that in the anaerobic background, increased residual PSII activity can significantly enhance the yield of H_2 photoproduction in *C. reinhardtii*.

If this conclusion is true, impaired PSII activity in an anaerobic environment created by $NaHSO_3$ addition will inevitably decrease the yield of H_2 photoproduction in *C. reinhardtii* at a significant level. As expected, the H_2 photoproduction rate was significantly decreased in the presence of Lin, which impairs the PSII activity via inhibiting the D1 protein synthesis (Vavilin et al., 1995), regardless of either one step method or stepwise mode (**Figure 6**). This consolidates our conclusion that increased residual PSII activity in an anaerobic environment is an efficient strategy to improve H_2 photoproduction in *C. reinhardtii* and the stepwise $NaHSO_3$ addition mode is a case study in this strategy.

DISCUSSION

Whether $NaHSO_3$ addition promotes photosynthesis or H_2 photoproduction depends on its concentrations: $NaHSO_3$ in a low amount improves photosynthesis (Wang et al., 2003) but in a moderate amount can enhance H_2 photoproduction (Wang et al., 2010; Ma et al., 2011). Wang et al. (2003) demonstrate that a low amount (100 μM) of $NaHSO_3$ increases cyclic photophosphorylation and consequently improves photosynthesis via optimizing the ATP/NADPH ratio. By contrast, Wei et al. (2017) demonstrate that a moderate amount (13 mM) of $NaHSO_3$ can remove O_2 efficiently through the reaction of bisulfite with superoxide anion produced at the acceptor side of PSI, especially under sufficient light conditions, consequently activates H_2ase and promotes H_2 photoproduction. The results of this study indicate that a moderate amount of $NaHSO_3$ under a stepwise addition mode can quickly establish an anaerobic environment (**Figure 2**) and significantly improves H_2 photoproduction in a unicellular green alga *C. reinhardtii* (**Table 1** and **Figure 1**). Such improvement is at least independent of two alternative electron sinks for H_2 photoproduction, CET (**Figures 4**, **6**), and CO_2 assimilation (**Figures 5**, **6**) and, most likely the result of maintained a relatively high electron source for H_2 photoproduction, PSII activity (**Figures 3**, **6**).

Under a photon flux density of 200 μmol photons $m^{-2}s^{-1}$, the Mehler reaction is usually considered to also be an important alternative electron sink for H_2 photoproduction. However, we found that the Mehler reaction is almost absent in $NaHSO_3$ addition strategy, regardless of either one step method or stepwise mode (data not shown), possibly because addition of $NaHSO_3$ to

the cell suspension cultures quickly results in entering of cells to an anaerobic environment (less than 800 s) (**Figure 2**). Therefore, the improvement of H_2 photoproduction in *C. reinhardtii* by a moderate amount of $NaHSO_3$ under a stepwise addition mode is also independent of a third alternative electron sink for H_2 photoproduction, Mehler reaction, and consolidating the above mentioned possibility that such improvement is the result of increased residual PSII activity, an electron source for H_2 photoproduction.

Based on different sources of electrons to H_2ase, three pathways for H_2 production have been identified in *C. reinhardtii*. Their sources of electrons to H_2ase come from water photolysis via PSII (Melis et al., 2000; Kosourov et al., 2003), NADPH through type II NAD(P)H dehydrogenase (Baltz et al., 2014) and the fermentative degradation of endogenous compounds (Gfeller and Gibbs, 1985), respectively. We observed that production of H_2 under photon flux densities of 200 μmol photons $m^{-2}s^{-1}$ by $NaHSO_3$ addition was almost completely suppressed in cells incubated in the dark (**Supplementary Figures S1A,B**) or treated with DCMU (**Supplementary Figures S1A,C**). A quick establishment of anaerobic environment by $NaHSO_3$ addition (**Figure 2**) suppresses the acetate uptake (Jurado-Oller et al., 2015) and impairs the mitochondrial respiratory electron transport chain function. Taking all these results together, we propose that in the $NaHSO_3$ addition strategy, the source of electrons for H_2 production predominantly, if not totally, comes from water photolysis via PSII, regardless of either the one step method or the stepwise mode.

The results of this study further indicate that the stepwise mode increased the maximum accumulated H_2 levels, produced a higher maximum velocity of H_2 photoproduction, and prolonged the time length of H_2 photoproduction when compared to the one step method (**Table 2**). We thus propose that the stepwise mode developed in this study is an efficient and sustained strategy for improving H_2 photoproduction in the green alga *C. reinhardtii*.

Although a moderate amount of $NaHSO_3$ can remove efficiently O_2, the activity of PSII, an electron source for H_2 photoproduction, is also significantly impaired (Wang et al., 2010; Ma et al., 2011; Wei et al., 2017). The results of this study observe that in the anaerobic background, a stepwise mode maintains a relatively high PSII activity (**Figure 3**) and consequently promotes H_2 photoproduction (**Figure 1**). The cause and effect of PSII activity and H_2 photoproduction is also present in the sulfur-deprived strategy (Zhang et al., 2002; Kosourov et al., 2005; Kim et al., 2010; Volgusheva et al., 2013; Grewe et al., 2014; Steinbeck et al., 2015; Chen et al., 2016) but the reasons why H_2 photoproduction is terminated in sulfur deprivation and $NaHSO_3$ addition strategies are distinctly different. It is known that H_2 photoproduction is terminated in the sulfur deprivation strategy because of cell death (Nguyen et al., 2008) and in the $NaHSO_3$ addition strategy because of conversion of too much bisulfite to sulfate (Wei et al., 2017). It is worthy of note that the relationship between H_2 production and biomass accumulation in sulfur deprivation and $NaHSO_3$ addition strategies is also distinctly different. Regardless of either one step method or stepwise mode, the simultaneous production of H_2 and biomass is present in $NaHSO_3$ addition strategy, as observed in mixotrophic nutrient-replete cultures under low light conditions (Jurado-Oller et al., 2015), but is absent in sulfur deprivation strategy. Therefore, it appears reasonable that improved PSII activity in the $NaHSO_3$ background is considered to be a better strategy to meet future application requirements in comparison with that in the sulfur-deprived background.

AUTHOR CONTRIBUTIONS

WM designed and supervised the experiments. XL, BF, and ZR performed the experiments and analyzed the data. LW and WM analyzed and interpreted the data and wrote the article.

FUNDING

This work was supported by the Shanghai Science and Technology Committee (Grant No. 17070502900) and National Natural Science Foundation of China (Grant Nos. 31570235 and 31770259).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01532/full#supplementary-material

REFERENCES

Baltz, A., Dang, K. V., Beyly, A., Auroy, P., Richaud, P., Cournac, L., et al. (2014). Plastidial expression of type II NAD(P)H dehydrogenase increases the reducing state of plastoquinones and hydrogen photoproduction rate by the indirect pathway in *Chlamydomonas reinhardtii*. *Plant Physiol.* 165, 1344–1352. doi: 10.1104/pp.114.240432

Chen, M., Zhang, J., Zhao, L., Xing, J., Peng, L., Kuang, T., et al. (2016). Loss of algal proton gradient regulation 5 increases reactive oxygen species scavenging and H_2 evolution. *J. Integr. Plant Biol.* 58, 943–946. doi: 10.1111/jipb.12502

Dubini, A., and Ghirardi, M. L. (2015). Engineering photosynthetic organisms for the production of biohydrogen. *Photosynth. Res.* 123, 241–253. doi: 10.1007/s11120-014-9991-x

Esquível, M. G., Amaro, H. M., Pinto, T. S., Fevereiro, P. S., and Malcata, F. X. (2011). Efficient H_2 production via *Chlamydomonas reinhardtii*. *Trends Biotechnol.* 29, 595–600. doi: 10.1016/j.tibtech.2011.06.008

Flynn, T., Ghirardi, M. L., and Seibert, M. (2002). Accumulation of O_2-tolerant phenotypes in H_2-producing strains of *Chlamydomonas reinhardtii* by sequential applications of chemical mutagenesis and selection. *Int. J. Hydrogen Energy* 27, 1421–1430. doi: 10.1016/S0360-3199(02)00117-9

Gfeller, R. P., and Gibbs, M. (1985). Fermentative metabolism of *Chlamydomonas reinhardtii*: II. Role of plastoquinone. *Plant Physiol.* 77, 509–511. doi: 10.1104/pp.77.2.509

Ghirardi, M. L., Togasaki, R. K., and Seibert, M. (1997). Oxygen sensitivity of algal H_2 production. *Appl. Microbiol. Biotechnol.* 63, 141–151. doi: 10.1007/BF02920420

Grewe, S., Ballottari, M., Alcocer, M., D'Andrea, C., Blifernez-Klassen, O., Hankamer, B., et al. (2014). Light-harvesting complex protein LHCBM9 is critical for photosystem II activity and hydrogen production in *Chlamydomonas reinhardtii*. *Plant Cell* 26, 1598–1611. doi: 10.1105/tpc.114.124198

Hansel, A., and Lindblad, P. (1998). Towards optimization of cyanobacteria as biotechnologically relevant producers of molecular hydrogen, a clean and renewable energy source. *Appl. Microbiol. Biotechnol.* 50, 153–160. doi: 10.1007/s002530051270

Harris, E. H. (1989). *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use*. San Diego, CA: Academic Press.

Jurado-Oller, J. L., Dubini, A., Galván, A., Fernández, E., and González-Ballester, D. (2015). Low oxygen levels contribute to improve photohydrogen production in mixotrophic non-stressed *Chlamydomonas* cultures. *Biotechnol. Biofuels* 8:149. doi: 10.1186/s13068-015-0341-9

Kim, J. P., Kim, K. R., Choi, S. P., Han, S. J., Kim, M. S., and Sim, S. J. (2010). Repeated production of hydrogen by sulfate re-addition in sulfur deprived culture of *Chlamydomonas reinhardtii*. *Int. J. Hydrogen Energy* 35, 13387–13391. doi: 10.1016/j.ijhydene.2009.11.113

Kitajima, M., and Butler, W. (1975). Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. *Biochim. Biophys. Acta* 376, 105–115. doi: 10.1016/0005-2728(75)90209-1

Kosourov, S., Makarova, V., Fedorov, A. S., Tsygankov, A., Seibert, M., and Ghirardi, M. L. (2005). The effect of sulfur re-addition on H_2 photoproduction by sulfur-deprived green algae. *Photosynth. Res.* 85, 295–305. doi: 10.1007/s11120-005-5105-0

Kosourov, S., Seibert, M., and Ghirardi, M. L. (2003). Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H_2-producing *Chlamydomonas reinhardtii* cultures. *Plant Cell Physiol.* 44, 146–155. doi: 10.1093/pcp/pcg020

Kruse, O., Rupprecht, J., Bader, K. P., Thomas-Hall, S., Schenk, P. M., Finazzi, G., et al. (2005). Improved photobiological H_2 production in engineered green algal cells. *J. Biol. Chem.* 280, 34170–34177. doi: 10.1074/jbc.M503840200

Liebgott, P. P., Dementin, S., Léger, C., and Rousset, M. (2011). Towards engineering O_2-tolerance in [Ni–Fe] hydrogenases. *Energy Environ. Sci.* 4, 33–41. doi: 10.1039/C0EE00093K

Ma, W., Chen, M., Wang, L., Wei, L., and Wang, Q. (2011). Treatment with $NaHSO_3$ greatly enhances photobiological H_2 production in the green alga *Chlamydomonas reinhardtii*. *Bioresour. Technol.* 102, 8635–8638. doi: 10.1016/j.biortech.2011.03.052

Ma, W., Wei, L., and Wang, Q. (2008). The response of electron transport mediated by active NADPH dehydrogenase complexes to heat stress in the cyanobacterium *Synechocystis* 6803. *Sci. China C Life Sci.* 51, 1082–1087. doi: 10.1007/s11427-008-0139-0

McKendry, P. (2002). Energy production from biomass (Part 1): overview of biomass. *Bioresour. Technol.* 83, 37–46. doi: 10.1016/S0960-8524(01)00118-3

Melis, A., Zhang, L., Forestier, M., Ghirardi, M. L., and Seibert, M. (2000). Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol.* 122, 127–136. doi: 10.1104/pp.122.1.127

Momirlan, M., and Veziroglu, T. N. (2002). Current status of hydrogen energy. *Renew. Sustain. Energ. Rev.* 6, 141–179. doi: 10.1016/S1364-0321(02)00004-7

Nguyen, A. V., Thomas-Hall, S. R., Malnoë, A., Timmins, M., Mussgnug, J. H., Rupprecht, J., et al. (2008). Transcriptome for photobiological hydrogen production induced by sulfur deprivation in the green alga *Chlamydomonas reinhardtii*. *Eukaryot. Cell* 7, 1965–1979. doi: 10.1128/EC.00418-07

Rühle, T., Hemschemeier, A., Melis, A., and Happe, T. (2008). A novel screening protocol for the isolation of hydrogen producing *Chlamydomonas reinhardtii* strains. *BMC Plant Biol.* 8:107. doi: 10.1186/1471-2229-8-107

Schreiber, U., Schliwa, U., and Bilger, W. (1986). Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynth. Res.* 10, 51–62. doi: 10.1007/BF00024185

Shu, L., Xiong, W., Shao, C., Huang, T., Duan, P., Liu, K., et al. (2018). Improvement in the photobiological hydrogen production of aggregated *Chlorella* by dimethyl sulfoxide. *Chembiochem* 19, 669–673. doi: 10.1002/cbic.201700637

Steinbeck, J., Nikolova, D., Weingarten, R., Johnson, X., Richaud, P., Peltier, G., et al. (2015). Deletion of Proton Gradient Regulation 5 (PGR5) and PGR5-Like 1 (PGRL1) proteins promote sustainable light-driven hydrogen production in *Chlamydomonas reinhardtii* due to increased PSII activity under sulfur deprivation. *Front. Plant Sci.* 6:892. doi: 10.3389/fpls.2015.00892

Surzycki, R., Cournac, L., Peltier, G., and Rochaix, J. D. (2007). Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17548–17553. doi: 10.1073/pnas.0704205104

Tagawa, K., Tsujimoto, H. Y., and Arnon, D. I. (1963). Role of chloroplast ferredoxin in the energy conversion process of photosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 49, 567–572. doi: 10.1073/pnas.49.4.567

Vavilin, D. V., Tyystjärvi, E., and Aro, E. M. (1995). In search of a reversible stage of photoinhibition in a higher plant: no changes in the amount of functional photosystem II accompany relaxation of variable fluorescence after exposure of lincomycin-treated *Cucurbita pepo* leaves to high light. *Photosynth. Res.* 45, 239–247. doi: 10.1007/BF00015564

Volgusheva, A., Styring, S., and Mamedov, F. (2013). Increased photosystem II stability promotes H_2 production in sulfur-deprived *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 7223–7228. doi: 10.1073/pnas.1220645110

Wang, H., Mi, H., Ye, J., Deng, Y., and Shen, Y. (2003). Low concentrations of $NaHSO_3$ increase cyclic photophosphorylation and photosynthesis in cyanobacterium *Synechocystis* PCC6803. *Photosynth. Res.* 75, 151–159. doi: 10.1023/A:1022813402265

Wang, L., Chen, M., Wei, L., Gao, F., Lv, Z., Wang, Q., et al. (2010). Treatment with moderate concentrations of $NaHSO_3$ enhances photobiological H_2 production in the cyanobacterium *Anabaena* sp. strain PCC 7120. *Int. J. Hydrogen Energy* 35, 12777–12783. doi: 10.1016/j.ijhydene.2010.08.115

Wei, L., Li, X., Yi, J., Yang, Z., Wang, Q., and Ma, W. (2013). A simple approach for the efficient production of hydrogen from Taihu Lake *Microcystis* spp. blooms. *Bioresour. Technol.* 139, 136–140. doi: 10.1016/j.biortech.2013.04.026

Wei, L., Yi, J., Wang, L., Huang, T., Gao, F., Wang, Q., et al. (2017). Light intensity is important for hydrogen production in $NaHSO_3$-treated *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* 58, 451–457. doi: 10.1093/pcp/pcw216

Wu, S., Huang, R., Xu, L., Yan, G., and Wang, Q. (2010). Improved hydrogen production with expression of *hemH* and *lba* genes in chloroplast of *Chlamydomonas reinhardtii*. *J. Biotechnol.* 146, 120–125. doi: 10.1016/j.jbiotec.2010.01.023

Wu, X., Liang, Y., Li, Q., Zhou, J., and Long, M. (2011). Characterization and cloning of oxygen-tolerant hydrogenase from *Klebsiella oxytoca* HP1. *Res. Microbiol.* 162, 330–336. doi: 10.1016/j.resmic.2010.12.003

Xiong, W., Zhao, X., Zhu, G., Shao, C., Li, Y., Ma, W., et al. (2015). Silicification-induced cell aggregation for the sustainable production of H_2 under aerobic conditions. *Angew. Chem. Int. Ed. Engl.* 54, 11961–11965. doi: 10.1002/anie.201504634

Xu, F. Q., Ma, W. M., and Zhu, X. G. (2011). Introducing pyruvate oxidase into the chloroplast of *Chlamydomonas reinhardtii* increases oxygen consumption and promotes hydrogen production. *Int. J. Hydrogen Energy* 36, 10648–10654. doi: 10.1016/j.ijhydene.2011.05.130

Zhang, L., Happe, T., and Melis, A. (2002). Biochemical and morphological characterization of sulfur-deprived and H_2-producing *Chlamydomonas reinhardtii* (green alga). *Planta* 214, 552–561. doi: 10.1007/s004250100660

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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