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Research Letter

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Anaerobic phototrophic processes of hydrogen production by different strains of microalgae *Chlamydomonas* sp

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One sentence summary: *Chlamydomonas reinhardtii* had five times higher hydrogen production compared to *Chlamydomonas moewusii*, with cultivation in two different phases and sulfur deprivation; however, it depends on the species and growth conditions.

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ABSTRACT

Hydrogen is an abundant element and a non-polluting fuel that can be biologically produced by microalgae. The aim of this research was to investigate biological hydrogen production by *Chlamydomonas reinhardtii* (CC425) and *Chlamydomonas moewusii* (SAG 24.91) by direct biophotolysis in batch cultures. Strains were cultivated in TAP growth medium (pH 7.2) in two phases: in the first stage, cultures were maintained in an aerobic condition until the middle of the exponential phase; in the second stage, the biomass was transferred to closed anaerobic photobioreactors under sulfur deprived. Gas chromatography and Gompertz model were used to measure the hydrogen production and hydrogen production rate, respectively. We noticed that maximum hydrogen production by biomass of *C. reinhardtii* was 5.95 ± 0.88 μmol mg^{-1} and the productivity was 17.02 ± 3.83 μmol L^{-1} h^{-1}, with hydrogen production five times higher than *C. moewusii*, approximately, though, *C. moewusii* obtained a higher ethanol yield compared to *C. reinhardtii*. The hydrogen production method, with the cultivation of strains in two different phases and sulfur deprivation, was effective for obtaining of biohydrogen for *Chlamydomonas*; however, it depends on the species, strain and growth conditions.

Keywords: green algae; renewable energy; biohydrogen; hydrogenase; anaerobic photobioreactor; ethanol

INTRODUCTION

Energy production from hydrogen is advantageous due to the fact that it is an abundant element in the universe, it is a renewable and inexhaustible source of energy and it is considered clean as it releases only water in its combustion. Therefore, energy production from hydrogen is harmless to humans and the environment (Das and Veziroglu 2008; Dubini and Ghirardi 2015).

Microalgae, cyanobacteria and bacteria can biologically produce hydrogen gas using only light energy, temperature and pressure (Block and Melody 1992; Das and Veziroglu 2008). According to Benemann, the advantages of using microorganisms

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for hydrogen production under laboratory conditions are the ability to convert light energy, the fact that it is easy to cultivate them and the possibility of capturing gas in closed places (Benemann 1997). However, one of the challenges is the low conversion of light energy into hydrogen due to the sensitivity of hydrogenase enzymes to oxygen, which is released during photosynthesis (Benemann 1997; Melis and Happe 2001; Tamburic *et al.* 2011).

Biological production can occur via direct biophotolyses, also called phototrophic process, and can be aerobic or anaerobic; or by indirect biophotolyses through cyanobacteria nitrogen fixation; or via fermentation. The *Chlamydomonas* genus is able to grow heterotrophically using acetate as a carbon source and has versatile fermentative metabolism (Catalanotti *et al.* 2013; Yang *et al.* 2013), thus this genus has the potential to produce biofuel under autotrophic or heterotrophic conditions (Ballester, Jurado-Oller and Fernandez 2015). Rosenbaum and Schröder (2010) found the best results using microalgae in anaerobic phototrophic processes due to the fact that enzyme hydrogenase is sensitive and can be inhibited in the presence of oxygen. An alternative to make the environment anaerobic is to remove sulfur from the culture medium (Benemann 1997; Melis and Happe 2001; Tamburic *et al.* 2011).

The limitation sulfur in a culture medium causes a decrease in photosystem II activity in the photosynthesis process. Sulfur is essential in the constitution of two amino acids: cysteine and methionine, which are part of the protein structure from the center of photosystem II reactions, which is constantly repaired, under normal conditions. Therefore, the water photolysis is inhibited that leads to a decrease in oxygen concentrations. This produces hydrogen by hydrogenase enzymes using light as an energy source (Melis *et al.* 2000; Zhang and Melis 2002; Antal, Krendeleva and Tyystjärvi 2015).

According to the method that was followed, microalgae cultivation was carried out in two steps in order to: (i) produce an efficient concentration of hydrogen; (ii) achieve adequate cell density; (iii) ensure that the biomass loss and consumption of carbohydrates and protein do not affect the hydrogen production (Tamburic *et al.* 2011). For this purpose, in the first step, the cells were cultured in an aerobic condition until the middle of the exponential phase, without the deprivation of sulfur in order to produce energy reserves and increase the biomass. Afterwards, in the second step, the cells were transferred to reactors with culture medium under sulfur deprivation, in whereby the algae use light energy to break down water and carbon molecules and, consequently, release hydrogen through the hydrogenase, without the inhibition of oxygen, as reported in research by Melis and Happe (2001), Zhang and Melis (2002), Tamburic *et al.* (2011) and Saleem *et al.* (2012).

Therefore, there is equilibrium between the production and consumption of oxygen due to the fact that, while the concentration of oxygen decreases gradually, respiration remains the same; therefore, the algae metabolism is responsible for providing an anaerobic environment for hydrogen production, avoiding the hydrogenase inhibition by oxygen (Tamburic *et al.* 2011; Xu *et al.* 2014).

Nevertheless, the two-step method has some problems that we should take into account in order to have better results, such as: the photosynthesis has to be reduced in order to produce low oxygen concentration; this oxygen needs to be consumed by respiration and the light needs to provide sufficient energy for hydrogen synthesis by hydrogenase. Thereby, a higher respiratory rate leads to higher and faster hydrogen production due to an increase in oxygen consumption (Xu *et al.* 2014; Antal, Krendeleva and Tyystjärvi 2015). Besides, the consumption of acetate is important data, due to the fact that mixotrophic conditions lead to higher hydrogen production than autotrophic conditions because of the sensitivity of hydrogenase to oxygen (Tsygankov *et al.* 2006; Kosourov *et al.* 2007; Meuser *et al.* 2009; Ballester, Jurado-Oller and Fernandez 2015).

In this study, we investigated the hydrogen production of two *Chlamydomonas* strains, *Chlamydomonas reinhardtii* and *Chlamydomonas moewusii* by direct biophotolysis using the microalgae cultivation in two different stages: aerobic and anaerobic with sulfur deprivation. *Chlamydomonas* genus has the potential to produce hydrogen; however, it depends on the species, strain and growth conditions. According to this, the aim of research was to compare the potential of this genus and the efficiency of this methodology for these two strains and subproducts production.

MATERIAL AND METHODS

Strains and inoculum maintenance

The strain, *Chlamydomonas reinhardtii* (CC425), was from the National Renewable Energy Laboratory (NREL) and *Chlamydomonas moewusii* (SAG 24.91) was from Sammlung von Algen Kulturen at the University of Goettingen (SAG). Both were maintained in axenic culture medium TAP (Gorman and Levine 1965), pH 7.2, 24°C, 12 h of photoperiod and 60 μmol photons m^{-2} s^{-1} of irradiation.

Experimental design and hydrogen production

The experiments were carried out in triplicate using Duran glass bottles as photobioreactors (500 mL) with 300 mL of culture. The strains were cultivated in a TAP growth medium (pH 7.2 and 24°C) in two steps. In the first step, cultures were maintained in aerobic conditions until the middle of the exponential phase in 200 μmol photons m^{-2} s^{-1}, under continuous illumination in 500 mL Erlenmeyers flasks, with 300 mL of culture. In the second step (hydrogen production phase), the biomass was centrifuged in 33.3 Hz for 10 min, washed 2 times and suspended in the TAP medium with sulfur deprivation in 500 mL Duran glass bottles with 300 mL of culture and sealed with a butyl cover. The atmospheric conditions of the bottles were changed by adding nitrogen for 10 min, previously sterilized using a filter with 0.2 μm of porosity. Cultures were maintained for 204 h in 200 μmol photons m^{-2} s^{-1}, under continuous illumination.

The gas production was measured by gas chromatography (Shimadzu GC-2010) using a thermal conductivity detector and argon as carrier gas. 0.5 mL of headspace gas was collected every 12 h with a syringe and valve (push button valve 22285). The results obtained were shown in hydrogen moles based on regression method with calibration curves ($r^2 = 0.996$). The hydrogen volume (Milliliters of H_2 per liter of culture) was calculated by ideal gas equation.

The biomass was measured by suspended solids and the cell density was performed by cell counting using a Fucsh Rosenthal chamber in an Olympus BX5 microscope, both according to APHA (2012). In the first experimental phase, the inoculum biomass of strain *C. reinhardtii* was 83.3 mg L^{-1} and strain *C. moewusii* was 43.3 mg L^{-1}.

Biochemical analysis

The biochemical characterization of dry biomass was performed in the exponential phase of the first stage, and at the end of

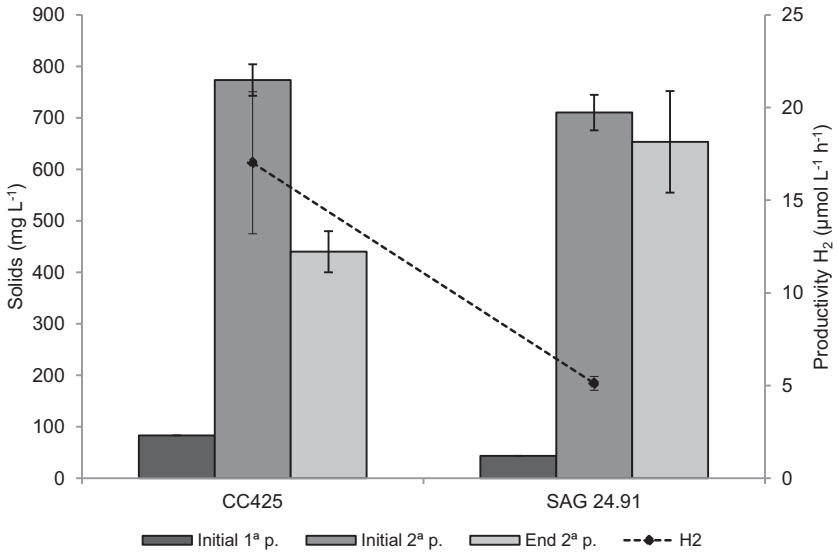

Figure 1. Biomass (total suspended solids; mg L^{-1}) shown in the columns at the beginning of the first experimental phase (Initial 1ª p.), at the beginning of the second experimental phase (Initial 2ª p.) and at the end of the second experimental phase (End 2ª p.). Hydrogen productivity (μmol L^{-1} h^{-1}) of the strains CC425 (*C. reinhardtii*) and SAG 24.91 (*C. moewusii*) shown in the line. Bars indicate standard deviation (n = 3).

the hydrogen production experiments of the second stage. Cultures were centrifuged and the pelletized biomass was dried until a constant weight was achieved in an oven at 60°C for 24 h. Carbohydrates were determined using the phenol colorimetric method, described by Dubois *et al.* (1956), and the total protein was estimated by the total nitrogen analysis TKN (APHA, 2012) with a protein conversion factor of 4.71, suggested for microalgae (Lourenço *et al.* 2004).

Organic acids and ethanol produced by strains *Chlamydomonas* were measured using gas chromatography in a Shimadzu GC-2010/FID with an HP-INNOWAX capillary column, following the procedures described by Adorno, Hirawasa and Varesche (2014).

Analysis of results

The results were processed using the statistical software Origin Pro 8.0. A comparison between the strains was made using the parametric Student-t test. The hydrogen production parameters were obtained using the Gompertz mathematical model according to Zwietering *et al.* (1990). We adopted $P < 0.05$ to indicate significant differences.

RESULTS AND DISCUSSION

At the S-deprivation period, the final *Chlamydomonas reinhardtii* biomass reached 440 ± 40 mg L^{-1}, while that of *Chlamydomonas moewusii* was higher, at 653 ± 98 mg L^{-1}, thus the final biomass yield of *C. moewusii* was higher (Fig. 1, $P < 0.05$), as well as the initial cell densities of *C. moewusii* also showed higher. However, *C. reinhardtii* showed higher hydrogen production by biomass compared to *C. moewusii* (Fig. 2), as well as about 3-fold higher values for maximum H_2 production (mmol L^{-1}), maximum H2 volume collected (mL L^{-1} of culture), hydrogen productivity (umol L^{-1} h^{-1}) and hydrogen production rate, μmax (h^{-1}) (Table 1).

The best *C. reinhardtii* hydrogen production results reported in the literature based on a two-step cultivation method, under normal conditions of temperature and pressure, are: 0.23 mmol L^{-1} in 90 h (Tamburic *et al.* 2011); 4.67 mmol L^{-1} in 80 h (Melis *et al.* 2000); 5.36 mmol L^{-1} in 120 h (Zhang and Melis 2002); 1.48 mmol L^{-1} in 144 h (Xu *et al.* 2014); 9.82 mmol L^{-1} in 140 h (Kim *et al.* 2006); 12.32 mmol L^{-1} in 96 h (Kosourov, Seibert and Ghirardi 2003) and 0.4 mmol L^{-1} in 144 h (Tsygankov *et al.* 2006). The results of maximum hydrogen production and productivity found in the present study are consistent with the referenced results, and this demonstrates that the strain and the methodology adopted in this study are appropriate for obtaining this biogas.

In contrast to our results, previous work comparing the hydrogen production between *C. reinhardtii* (CC124) and *C. moewusii* (SAG 24.91) strains, researchers demonstrated that *C. reinhardtii*, under a sulfur deprivation medium (TAP), achieved the anaerobic condition after 30 h and the hydrogen photoproduction started after 26 h, reaching 120 mL L^{-1} of hydrogen. *Chlamydomonas moewusii*, under the same conditions, showed a slight decrease in oxygen after 80 h of sulfur deprivation and did not have a significant hydrogen production (Meuser *et al.* 2009). Clearly, their S-deprivation period was too short to allow hydrogen production by *C. moewusii* to be detected, as we report here.

Although *C. moewusii* has significant *in vitro* hydrogenase activity, Meuser *et al.* (2009) found that this strain had difficulty in absorbing acetate, therefore this characteristic may have caused an insufficient anaerobic environment or perhaps the oxygen consumption was slower, compared to *C. reinhardtii* (Melis *et al.* 2000). These findings may be the reason for lower hydrogen production by *C. moewusii* in our research. The possible explanation is that *C. moewusii* has lower acetate assimilation and consequently leads to low respiratory activity and thus higher oxygen levels that inhibit the hydrogenase.

The biomass composition showed differences between *C. reinhardtii* and *C. moewusii* in S-deprivation. As shown in Fig. 3, the initial and final protein profile concentration was similar for the two strains, and the protein concentration decreased during the hydrogen production phase in both strains ($P < 0.05$).

This reduction of protein can be a response to the sulfur starvation during the hydrogen production phase. This nutritional limitation can change the protein biosynthesis pathways toward a formation of other molecules, such as carbohydrates and lipids

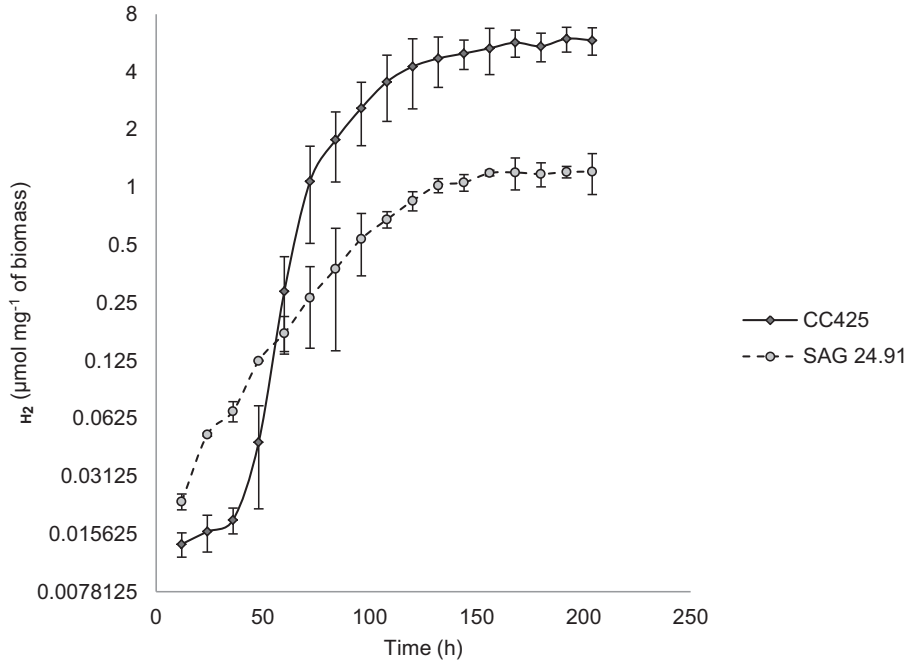

Figure 2. Hydrogen production (μmol mg^{-1} of biomass) of strains CC425 and SAG 24.91 in 204 h. The bars indicate the standard deviation (n = 3).

Table 1. Hydrogen and biomass production parameters of *C. reinhardtii* (CC425) and *C. moewusii* (SAG 24.91) at the beginning and at the end of the experiments.

Parameters		CC425	SAG 24.91
Biomass (mg L^{-1})	Initial	83.33 ± 0.00	43.33 ± 0.00
	End	440.00 ± 40.00	653.33 ± 98.66[a]
Maximum H_2 production (mmol L^{-1})		2.69 ± 0.42[b]	0.78 ± 0.12
Maximum H_2 production by biomass (μmol mg^{-1})		5.95 ± 0.88[c]	1.21 ± 0.29
Maximum H_2 volume (mL L^{-1} of culture)		61.10 ± 7.16[d]	20.67 ± 3.10
H_2 Productivity (μmol L^{-1} h^{-1})		17.02 ± 3.83[e]	5.12 ± 0.37
H_2 production rate—μ_{max} (h^{-1})		0.126 ± 0.015[f]	0.043 ± 0.008

Letters refer to the statistical difference ($P < 0.05$).
[a]Difference in the biomass yield of *C. reinhardtii* and *C. moewusii*.
[b]Difference in the maximum production of hydrogen between *C. reinhardtii* and *C. moewusii*.
[c]Difference in the maximum production of hydrogen by biomass between *C. reinhardtii* and *C. moewusii*.
[d]Difference in the maximum volume of hydrogen between *C. reinhardtii* and *C. moewusii*.
[e]Difference in the hydrogen productivity between *C. reinhardtii* and *C. moewusii*.
[f]Difference in the μ_{max} between *C. reinhardtii* and *C. moewusii*.

due to the fact that sulfur is an element in some amino acids (Rigano *et al.* 1998; Melis *et al.* 2000).

Regarding the carbohydrate, *C. reinhardtii* consumed it during the experiment, whereas *C. moewusii* cultivation increased the carbohydrate concentration significantly (Fig. 3). In the case of *C. reinhardtii*, the carbohydrate consume occurred due to the use of carbon sources for energetic process. The synthesis of carbohydrates indicates that sulfur deprivation was not enough to inhibit photosynthesis to a point of compensation between oxygen production and consumption in *C. moewusii*. Therefore, high energy expenditure for sugar production and storage and an excess of oxygen in the medium might have led to a hydrogenase enzyme inhibition (Rosenbaum and Schröder 2010). Furthermore, *C. moewusii* had the highest dry biomass yield at the end of the hydrogen production phase (Fig. 1).

During the S-deprivation process, apart from hydrogen, the main products generated from the breakdown of pyruvate during the anaerobic phase are ethanol, acetate, formic acid, glycerol, lactate, succinate and carbon dioxide. The ratios of these products differ according to the environmental conditions (Antal, Krendeleva and Tyystjärvi 2015; Yang *et al.* 2015) and to the particular algal species being examined.

In our hands, neither species produced acetic acid at pH 7.7, in agreement with Kosourov, Seibert and Ghirardi (2003), but instead consumed it. Other organic acids were produced during the hydrogen production stage, but in small amounts for both strains, such as propionic acid, isobutyric, butyric, valeric and isovaleric (data not shown).

Diverging results between the two strains were obtained in relation to ethanol production, metabolite generated from the fermentation process of hydrogen production (Fig. 3B). The average yield of *C. reinhardtii* was 67.7 ± 11.7 mg L^{-1}, and *C. moewusii* was 187.3 ± 83.3 mg L^{-1}. *Chlamydomonas moewusii* obtained a higher ethanol yield compared to *C. reinhardtii* ($P < 0.05$). According to a study by Yang *et al.* (2013), which compared the genetic sequencing of *C. reinhardtii* (strain CC124) and *C. moewusii* (several strains, including SAG 24.91), the difference between the two strains, regarding ethanol production and other fermentation products, could be due to the difference in the amount and halotypes of enzymes, and their different expressions.

Indeed, another factor that explains the lower hydrogen production in *C. moewusii* is the competition and co-regulation between the hydrogen reduction and production of fermenta-

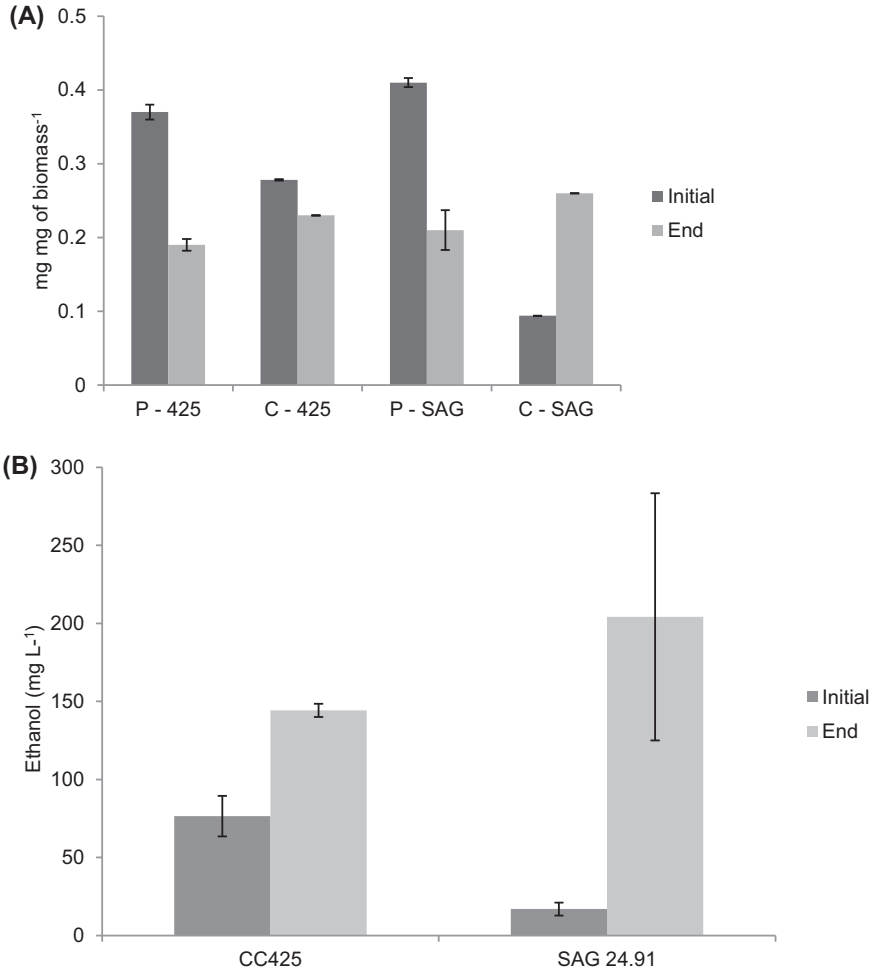

Figure 3. (**A**)Carbohydrate concentration (C) and protein concentration (P; mg mg of biomass^{-1}) of strains CC 425 (425) and SAG 24.91 (SAG). (**B**) Ethanol concentration (mg L^{-1}) of strains CC 425 SAG 24.91 at the beginning of the hydrogen production phase and at the end of the experiment. The bars indicate standard deviation (n = 3).

tive metabolites, which occurs in the chloroplast during the anaerobic metabolism of pyruvate (Catalanotti *et al.* 2013; Antal, Krendeleva and Tyystjärvi 2015) that varies in different algal species.

Both *C. reinhardtii* and *C. moewusii* have metabolically flexible central metabolism that are involved in the production of hydrogen and fermentative products by photosynthesis and anaerobic fermentation under S-deprivation. The difference between these species regarding hydrogenase expression and the anaerobic fermentation pathways involved in balancing electrons from the redox reactions may contribute to different hydrogenase activity and production of secondary metabolites from fermentation (Yang *et al.* 2013).

Meuser *et al.* (2009) also observed that *C. moewusii* had a different profile of secondary metabolite production during fermentation, such as increased ethanol. In addition, they noticed that this species has a fermentative H_2-production activity faster than *C. reinhardtii*, over time in dark conditions. In our experiments, we also found a higher ethanol production and lower hydrogen production in *C. moewusii*.

CONCLUSIONS

This research showed that *Chlamydomonas* genus has the potential to produce hydrogen; however, it depends on the species and growth conditions, which can limit the production of hydrogen due to the sensitivity of the hydrogenase enzyme. The hydrogen production method, with the cultivation of strains in two different phases and sulfur deprivation, was effective, and *C. reinhardtii* had five times higher hydrogen production by biomass compared to *C. moewusii*, approximately, and three times higher productivity. However, in relation to ethanol production, *C. moewusii* obtained a higher yield compared to *C. reinhardtii*.

In summary, the lower production of hydrogen by *C. moewusii* compared to *C. reinhardtii* in this study may be related to the following: (i) *C. moewusii* assimilates lower acetate concentration; (ii) *C. moewusii* culture has a slower decline of oxygen; (iii) there may be a difference in gene expression, in hydrogenase maturation and types of ferredoxin; (iv) *C. moewusii* may have a faster fermentative metabolism, which can compete with hydrogen production. These characteristics highlight the different strategies and metabolic flexibilities, between *C. reinhardtii* and *C. moewusii* under the same condition growth, to maintain the balance of oxide reduction reactions during anoxia.

These results suggest that although both species have the potential for hydrogen photoproduction under sulfur deprivation, more research is needed and genetic engineering techniques according to the specific metabolism of each species, how to improve the compensation point between

photosynthesis and respiration with the sulfur concentration in culture medium, for greater efficiency in converting solar energy into hydrogen.

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Conflict of interest. None declared.

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