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Influence of Sulfur and Light Intensity in Nutrient Removal, and Hydrogen and Ethanol Production by Optimized Biomass of *Chlamydomonas Reinhardtii* in Batch Anaerobic Photobioreactors

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Abstract

Hydrogen is a renewable fuel that can be biologically produced by green algae in closed anaerobic photobioreactors with light and organic carbon as energy sources. This research aimed to investigate the influence of sulfur concentration and light intensity on hydrogen and ethanol production, as well as on nutrient removal by *Chlamydomonas reinhardtii* (CC425) in batch cultures. The strain was cultivated in two phases: in the first step, the cultures were maintained under aerobic conditions to obtain biomass; in the second step, the biomass was transferred to closed anaerobic photobioreactors for gas generation under continuous illumination. A factorial design was accomplished to optimize the biomass production in the first step, with light variation, pH, and glucose addition. Afterward, light intensity and sulfur concentration were varied to test hydrogen production in the second step. The best production occurred in photobioreactors without sulfur addition (average increase of 7 times in the production) and under higher light intensity the production was 37% higher than lower light (39.64 ± 2.44 μmol H_2 L^{-1} h^{-1}). There was an effect of sulfur concentration in the ethanol production and under higher light intensity the production was higher (203.20 ± 31.49 mg L^{-1}). In addition, in general, under conditions with the presence of sulfur, there was greater removal of ammoniacal nitrogen (5.3%), phosphate (85.0%), COD (9.1%) and acetic acid (97.2%). This research demonstrates the efficient production of hydrogen and ethanol by *C. reinhardtii* and it shows that the process can be associated with nutrient removal.

1. Introduction

Microalgae are a highly diversified group of photoautotrophic microorganisms, which grow quickly when adapted to the environment and can be cultivated in different conditions from open lagoons and even in closed photobioreactor systems [1]. They have the ability to convert solar energy into chemistry through photosynthesis and accumulate lipids and sugars in their biomass, depending on the species and cultivation conditions, which is a good option for biofuel production, such as biodiesel and methane, from the biodigestion of algal biomass, ethanol and hydrogen [1–4]. The advantage of using hydrogen as an energy source is because it is renewable and clean, generating only water in its combustion, and it is harmless to living beings and the environment [4, 5].

These microorganisms can produce hydrogen through the enzyme hydrogenase by direct or indirect biophotolysis pathways. However, hydrogenase is an oxygen-sensitive enzyme and the presence of oxygen can inhibit hydrogen production [6], therefore alternatives are needed to minimize oxygen evolution and induce the formation of an anaerobic environment for continuous hydrogen production.

Direct biophotolysis occurs from the photolysis of water by photosystem II (PSII) in the chloroplast during photosynthesis, releasing oxygen, electrons and protons which are directed through the plastoquinone to photosystem I (PSI). Indirect biophotolysis occurs from oxidation of organic carbon and directs the flow of electrons to PSI (photofermentation) or to the fermentative pathway in dark, anaerobic conditions (dark fermentation). Thus, the indirect pathway in the PSI occurs regardless of PSII [7–10]. In the presence of oxygen and light energy, the electrons in PSI pass through a transport chain to the final

electron acceptor, ferredoxin, which reduces NADP to NADPH which is then oxidized in the CO_2 fixation reactions in the Calvin cycle [10]. Ferredoxin may also transport electrons to the hydrogenase, where protons are converted to molecular hydrogen, and in the absence of oxygen there is no inhibition of this enzyme [7]. In *C. reinhardtii*, different types of ferredoxins and genes associated with the expression of the hydrogenase structure have been identified [11, 12], and their expression interferes with hydrogen production in different algal strains.

Several researchers have reported that nutritional deprivation, mainly of sulfur, supports the achievement of anaerobiosis [13–15], as this element is a structural part of PSII proteins in the chloroplast and its deprivation causes a decrease in photosynthetic activity [6, 7, 14, 15]. Consequently, there is a decrease in oxygen production during photosynthesis which is then consumed by respiration, a function that is maintained under these conditions. Thus, there is compensation between oxygen consumption and production, and the algal metabolism is responsible for providing an anaerobic environment and consequent hydrogen production [6].

However, for hydrogen production by indirect biophotolysis to occur, it is necessary to grow the microalgae heterotrophically. Some green algae, such as *Chlamydomonas*, have this ability and can grow using acetate as the only carbon source, in addition to having a versatile fermentative metabolism [9, 16] and the potential for production of biofuels under autotrophic, mixotrophic or heterotrophic conditions [7, 17].

Therefore, related to the presence of specific cellular metabolism and the compensatory point between respiration and photosynthesis, there are several factors that can interfere and are important in hydrogen production, such as pH [18], nutritional stress by phosphorus or nitrogen [19], as well as light intensity and the effect of low sulfur concentrations, which are being investigated in order to the improve the process [8, 14, 15, 20]. Furthermore, some researchers reported that greater biomass of microalgae allows higher hydrogen production efficiency [6–8, 21, 22].

As mentioned above, microalgae also have the ability to utilize various organic molecules to generate other products, such as organic acids and ethanol, through dark fermentation pathways [6, 8, 23]. However, since part of the anaerobic metabolism occurs in the chloroplast, the electron flow can be directed to the PSI to reduce hydrogen or to form fermentation by-products, in the process of photofermentation [7, 9]. In addition to being potential hydrogen and by-product producers, green algae can also be used in wastewater treatment, mainly when they are associated with bacteria [24, 25]. In anaerobic digestion, acetic acid bacteria can break down the organic matter into organic acids, such as acetate, by acetogenesis process [26], and microalgae can use this acetate to grow heterotrophically and produce hydrogen by indirect biophotolysis [9, 16] and assist t in removing nutrients.

Therefore, firstly the purpose of this research was to improve the biomass yield of a strain of *C. reinhardtii* (CC425) for the later stage of hydrogen production. Afterward, this research aimed to evaluate the influence of sulfur concentration and light intensity in hydrogen production by this strain of *C. reinhardtii*

in batch anaerobic photobioreactor closed system under continuous illumination, as well as to evaluate the by-product production, such as ethanol and organic acids, and the nutrient removal efficiency by this organism in order to contribute to future research and applications of environmental and economic interest.

2. Material And Methods

2.1. Strain and inoculum maintenance

The strain of *Chlamydomonas reinhardtii* CC425 (cw15 arg2 sr-u-2-60 mt+) was from the National Renewable Energy Laboratory. The strain was maintained in axenic culture Tris-acetate-phosphate, TAP medium [27], with arginine supplementation (100 mg L^{-1}), at 24 ± 0.5 ºC, pH 7.2, 12 h light/dark cycle, and light intensity of 60 µmol m^{-2} s^{-1} obtained with cool white fluorescent tubular lamps in an air-conditioned room. These conditions were used as the control in the biomass optimization experiment for the first experimental step.

2.2. Experimental design and hydrogen production

The experiments were carried out in triplicate simultaneously in an incubator with photoperiod and temperature control (411 FPD Ethiktechnology). The strain was cultivated under two experimental steps in TAP medium [27] with arginine supplementation (100 mg L^{-1}). The first step was utilized to obtain the biomass needed for the second step.

In order to improve the biomass yield in the first step, a factorial design with a central condition (2^4 + 1) was used to generate a matrix with 17 experiments. Experiments were carried out at two levels of temperature (24 and 32°C), pH (6.2 and 8.2), light intensities (200 and 500 µmol m^{-2} s^{-1}), and with or without glucose addition (0 and 1 g L^{-1}), as well as a central condition in the intermediate level of these 4 parameters (28°C, pH 7.2, 350 µmol m^{-2} s^{-1} and 0.5 g L^{-1} of glucose). The experiment was performed with batch cultures in triplicate using glass tubes closed with screw lids (20 mL) the stationary phase was stabilized.

According to the results of the first step for the biomass optimization, the culture was maintained under aerobic growth conditions (continuous illumination of 200 µmol m^{-2} s^{-1} with a cool white lamp; pH 6.2 and 32 ± 0.1 ºC) in 500 mL Erlenmeyer flasks with 300 mL of culture, until the middle of the exponential phase.

The second step was performed to induce hydrogen production through the anaerobic phototrophic process in batch cultures under either sulfur deprivation or sulfur limitation. The biomass of Erlenmeyer flasks with 300 mL of culture from the first step was centrifuged and suspended in TAP medium in 500 mL Duran glass bottles with 300 mL of culture and sealed with a butyl cover (photobioreactor). The temperature and pH from the first step were maintained, and there was a variation of the concentration of the sulfur (0 or 50 µmol L^{-1}) and light intensity (60 or 200 µmol m^{-2} s^{-1}), as shown in Table 1. The

biomass at the end of the first step was centrifuged in 33.3 Hz for 10 min, washed twice with culture medium selected for each condition, and suspended in the respective photobioreactors. The atmospheric conditions of the photobioreactors were changed by adding nitrogen gas for 10 min, previously sterilized using a filter with 0.2 µm of porosity. Experiments were carried out until stabilization of H_2 production.

Aliquots of 500 µL of gas of headspace were collected every 12 h using a syringe and valve (push button valve 22285) to measure the gas composition by gas chromatography (Shimadzu GC-2010) using a thermal conductivity detector (TCD) and argon as a carrier gas. The results obtained are shown in moles of hydrogen and were calculated based on the regression method with calibration curves (r^2 = 0.996). The hydrogen production curves are shown in µmol L^{-1} of hydrogen/mg L^{-1} of chlorophyll *a*.

Table 1
Hydrogen production conditions.

Culture	Temperature	pH	Sulfur	Light	Condition
Medium	**(ºC)**		**(µmol L^{-1})**	**(µmol m^{-2} s^{-1})**	
TAP	32	6.2	0	60	1
organic carbon				200	2
source:			50	60	3
acetic acid				200	4

Furthermore, other parameters were calculated from the data, such as the maximum production of hydrogen per chlorophyll *a* (in µmol L^{-1} of H_2 / chlorophyll *a* in mg L^{-1}, represented by P_{max}) and per mol (in mmol of H_2 / liter of culture, represented by *H*), hydrogen volume (milliliters of H_2 per liter of culture, represented by Vol_{H2}), which were calculated using the ideal gas equation, and hydrogen production rate (μ_{max}) by Gompertz mathematical model [28] (Eq. 1). Equation *H* represents the accumulated production of hydrogen (µmol H_2 L^{-1}), *t* is the hydrogen production time (hours), and *e* is Euler's number (2.71828). The equation results in parameters *A* (hydrogen production potential in µmol L^{-1}), μ_{max} (hydrogen production rate in µmol H_2 L^{-1} h^{-1}) and K (lag phase time). The specific hydrogen production rate, $\mu_{specific}$ (µmol H_2 mg Chl a^{-1} h^{-1}), was calculated from the μ_{max} divided by the initial biomass in chlorophyll *a* (mg L^{-1}) of the photobioreactor.

$$H(t) = A \exp\left\{-\exp\left[\frac{\mu max.e}{A} \cdot (k - t) + 1\right]\right\} \quad (1)$$

2.3. By-product production and nutrient uptake

The ethanol and organic acid concentrations, such as acetic acid, were detected at the end of the second step using gas chromatography in a Shimadzu GC-2010/FID with an HP-INNOWAX capillary column [29]. The ammoniacal nitrogen and phosphate concentrations, as well as chemical oxygen demand (COD) were analyzed from samples taken from the initial photobioreactor and at the end of the hydrogen production process. Nutrient uptake was calculated based on the percentage of the difference between the initial and final concentrations of these compounds. The nutrient analyses were carried out according to APHA [30].

2.4. Biomass and biochemical analysis

The biomass of factorial design in the first step was measured by weighing the suspended solids [30] and the yield was calculated based on the difference between the final and initial biomass values. Biomass of the photobioreactors was estimated at the point when the algae were transferred from the 1st to the 2nd experimental step. The biomass, in terms of cell density was measured by cell counting using a Fuchs Rosenthal chamber in an Olympus BX5 microscope [30], and by the chlorophyll *a* amount, using the extraction method with 80% ethanol [31, 32]. Samples of 3 mL were taken from each photobioreactor for the analyses, and 0.5 mL of the samples were fixed with Lugol's acetic solution and stored in the dark until cell count.

The biochemical characterization of dry biomass was performed with the samples from the initial photobioreactor (sacrifice reactor) and at the end of the hydrogen production phase. The dry biomass was obtained by centrifuging the cultures followed by heat treatment in an oven at 60 ºC for 24 h of the pelletized biomass until a constant weight was achieved. Carbohydrates were determined using the phenol colorimetric method [33] and the total protein was estimated by the total nitrogen analysis TKN [30] with a protein conversion factor of 4.71 [34].

2.5. Analysis of results

The Protimiza Experimental Design software was used to analyze the experimental design of optimization of *C. reinhardtii* biomass in the first step and to find the parameters that have had positive effects on the biomass yield through the Pareto Chart with a significance level of 5%. Furthermore, the Statistica 7.0 software was used for the Analysis of Variance (ANOVA) and Tukey post-hoc tests to differences among the biomass yields. The best biomass yield was selected combining the higher biomass yield averages with Pareto Chart.

The results of the hydrogen production parameters were processed using the Gompertz mathematical model according to Zwietering et al. [28] in the statistical software Origin Pro 8.0. Analysis of Variance (ANOVA) and Tukey post-hoc tests were used to assess the differences among the hydrogen production parameters, such as the hydrogen production rate, maximum production of hydrogen, as well as biomass yields by chlorophyll *a* and cell density. Furthermore, the same statistical comparisons were made of the amount of nutrient uptake (ammoniacal nitrogen, phosphate, COD, and acetic acid) and ethanol produced. The T-Student test was used to compare the initial and final carbohydrate and protein amounts of the biomass in the photobioreactors. We adopted $p < 0.05$ to indicate the significant differences.

3. Results

3.1. Optimization of C. reinhardtii biomass in the first step

The results of experimental design of optimization of *C. reinhardtii* biomass for the first step (Table 2) showed that a higher temperature and more acidic pH improve the biomass yield of *C. reinhardtii*. The ANOVA analysis confirmed that there was a difference in the biomass yield between the conditions tested (17 experiments of design and control) ($F_{(17,46)} = 51.27$, $p < 0.0001$). In general, the best biomass yield conditions, without statistical difference between them, occurred at 32 ºC and pH 6.2, regardless of the light intensity and glucose addition (conditions 10, 11 and 13), except condition 12. The central point (condition 9) also obtained a higher biomass yield and there were no differences among the conditions mentioned above.

The analyze of Pareto Chart (Fig. 1) indicated effect positive of temperature ($t = 11.62$, $p < 0.00001$), effect negative of pH and light intensity ($t = -10.04$ and -3.75, $p \leq 0.0006$), and a positive interaction of variables temperature and pH ($t = 3.28$, $p = 0.0022$). In addition, there was no effect of glucose addition. Although there is no difference in the biomass yield among conditions 9, 10, 11 and 13 (Table 2), the results from the Pareto Chart showed that higher pH values and light intensity can impair the biomass yield. Therefore, condition 10 was chosen as the best one to obtain biomass in the first step (32 ºC, pH 6.2 and 200 µmol m^{-2} s^{-1}) instead of another condition with greater light intensity and glucose addition.

Table 2

Biomass yields (mg L^{-1}) of *C. reinhardtii* from the factorial design and control conditions

Temperature (ºC)	pH	Light (µmol m^{-2} s^{-1})	Glucose addition (g L^{-1})	Condition	Biomass yield (mg L^{-1})
24	7.2	60	0	Control	477.47 ± 23.66
24	6.2	200	0	1	798.77 ± 41.63
			1	2	822.11 ± 50.33
		500	0	3	652.25 ± 40.00
			1	4	785.59 ± 46.199
	8.2	200	0	5	422.21 ± 30.00
			1	6	467.21 ± 34.64
		500	0	7	359.45 ± 44.35
			1	8	364.45 ± 40.00
28	7.2	350	0.5	9	975.79 ± 90.92 #
32	6.2	200	0	10	1060.00 ± 58.88 *
			1	11	1073.33 ± 23.09 *
		500	0	12	892.94 ± 41.63
			1	13	1042.94 ± 80.00 *
	8.2	200	0	14	899.44 ± 60.00
			1	15	776.11 ± 110.15
		500	0	16	749.62 ± 93.81
			1	17	854.62 ± 105.04

Symbols show statistical differences in the best results of biomass yields according to ANOVA

∗ Difference with control, 1, 2,3,4,5,6,7,8,12,15,16 and 17 conditions ($p \leq 0.043$).

Difference with control, 1, 3,4,5,6,7,8,15 and 16 conditions ($p \leq 0.025$).

3.2. Hydrogen and ethanol production

There was no statistical difference in the initial biomass transferred to the photobioreactors between all conditions. The cell density was on average 6.9 ⍰ 10^{-6} ± 0.49 cell mL^{-1} and chlorophyll *a* concentration 13.7 ± 1.09 mg L^{-1}. The experiments were carried out until the H_2 production was stabilized (180 hours).

The best result was obtained from the condition without sulfur and under higher light intensity (condition 2: 285.74 ± 27.01 μmol H_2 mg Chl a^{-1} and 39.64 ± 2.44 μmol H_2 L^{-1} h^{-1}). The second-best result was obtained under the same conditions but under lower light intensity (condition 1: 209.79 ± 2.55 μmol mg Chl a^{-1} and 25.09 ± 0.82 μmol H_2 L^{-1} h^{-1}). The hydrogen production curves (Fig. 2) and the P_{max}, Vol_{H2}, H, and μ_{max} (Table 3) also confirmed these results.

The ANOVA analysis confirmed that there was an effect of light and of sulfur concentration, respectively, in Pmax ($F_{(1,8)}$ = 14.20, p = 0.005; $F_{(1,8)}$ = 720.48, p < 0.0000001), the Vol_{H2} ($F_{(1,8)}$ = 38.84, p = 0.00025; $F_{(1,8)}$ = 2683.91, p < 0.0000001), the μ_{max} ($F_{(1,8)}$ = 163.71, p = 0.000001; $F_{(1,8)}$ = 2707.83, p < 0.0000001), and the $\mu_{specific}$ ($F_{(1,8)}$ = 249.60, p < 0.0000001; $F_{(1,8)}$ = 4939.86, p < 0.0000001). In all these parameters of hydrogen production the second condition was statistically better than all other conditions ($p \leq 0.0008$), and the first condition was better compared to the third and fourth conditions ($p \leq 0.0002$) (Table 3).

The highest ethanol production occurred in the condition with sulfur and light of 200 μmol m^{-2} s^{-1} (203.20 ± 31.49 mg L^{-1}) (Fig. 3), although this number was statistically higher when compared only to conditions 1 (139.30 ± 10.31 mg L^{-1}). According to the ANOVA analysis, only the sulfur concentration affected the ethanol production ($F_{(1,8)}$ = 8.08, p = 0.02). Under all conditions, there was simultaneous production of organic acids, such as isobutyric, butyric and isovaleric acids, but these were below the quantification limit of the method.

Table 3
P_{max} (μmol H_2 mg Chl a^{-1}), H (mmol H_2 L^{-1}), Vol_{H2} (mL H_2 L^{-1}), μ_{max} (μmol H_2 L^{-1} h^{-1}) and $\mu_{specific}$ (μmol H_2 mg Chl a^{-1} h^{-1}), according to Gompertz model.

Cond.	P_{max}	H	Vol_{H2}	μ_{max}	$\mu_{specific}$
1	209.79 ± 2.55 #	2.62 ± 0.002 #	52.46 ± 1.12 #	25.09 ± 0.82 #	2.01 ± 0.10 #
2	285.74 ± 27.014 *	3.57 ± 0.315 *	68.75 ± 2.92 *	39.64 ± 2.44 *	3.16 ± 0.05 *
3	41.09 ± 5.24	0.56 ± 0.027	12.31 ± 1.26	4.31 ± 0.36	0.32 ± 0.06
4	25.38 ± 1.68	0.38 ± 0.017	8.14 ± 0.13	3.72 ± 0.35	0.25 ± 0.01
Conditions 1 to 4 are represented by cond. Symbols indicate statistical differences according to ANOVA.					
* Difference with all conditions ($p \leq 0.0008$).					
# Difference with conditions 3 and 4 ($p \leq 0.0002$).					

3.3. Biochemical composition of biomass

The initial, accumulated in the first step, and final carbohydrate and protein biomass composition in the second step were different under most conditions (Fig. 4). In general, there was a consumption of

carbohydrate in cultures, however only there were statistical differences in conditions under higher light intensity, regardless of the presence of sulfur (average consumption of 38%). The ANOVA analysis confirmed that there was an effect of light in consumption of carbohydrate ($F_{(1,8)}$ = 30.20, p = 0.0006), while in protein consumption this effect occurred only of sulfur concentration ($F_{(1,8)}$ = 133.13, p = 0.000003). However, in all conditions, there was significant protein consumption during the hydrogen production, and this consumption was on average 27% higher in conditions with higher hydrogen production (1 and 2 conditions).

3.4. Nutrient removal

The percentage of ammoniacal nitrogen uptake in the photobioreactors (Fig. 5), occurred only under conditions 3 and 4 (6.11% and 4.57%, respectively), and was affected by the sulfur concentration in the photobioreactor ($F_{(1,8)}$ = 73.45, p = 0.00003), although the decrease in protein levels in biomass may have obscured the results of ammoniacal nitrogen consumption. Phosphate uptake (Fig. 5), in general, was higher under conditions that yielded lower hydrogen production, and it was affected by the sulfur concentration ($F_{(1,8)}$ = 113.42, p = 0.000005), and light intensity ($F_{(1,8)}$ = 69.83, p = 0.000032). These parameters also affected organic carbon uptake (Fig. 5), as measured by COD, according to ANOVA ($F_{(1,8)}$ = 155.55 and 33.71, respectively, $p \leq 0.0004$), and higher organic carbon uptake also occurred under lower hydrogen production conditions. Acetic acid, with an average initial concentration of 910 mg L^{-1}, was consumed in all conditions (Fig. 5), although sulfur conditions had statistically the highest consumption (average consumption of 97.2%) compared to conditions without sulfur (mean consumption of 57.2%), the effect of which was confirmed by ANOVA analysis ($F_{(1,8)}$ = 186.55, p = 0.000001) and there was no light effect.

4. Discussion

The biomass optimization in the first step showed that mainly the pH and the light intensity interfered in the biomass yield, corroborating with previous research that claims that physical and chemical factors affect the growth of microalgae [35–38]. Thus, a higher amount of biomass was obtained in the first step at a lower pH (6.2) and light intensity of 200 µmol m^{-2} s^{-1}. It is believed that this biomass optimization provided the inoculation of a greater amount of biomass in the photobioreactors and favored the hydrogen production variable test as studies have reported that an increased amount of biomass can improve the hydrogen production yield [6–8, 21].

The results described above demonstrate a significant influence of sulfur concentration and light intensity in hydrogen production by *C. reinhardtii* (CC425). The best results were obtained with sulfur-deprived, and higher light intensity supported better hydrogen production, 3.57 mmol L^{-1}, and 2.62 mmol L^{-1} at the lowest light intensity (conditions 2 and 1, respectively) for 180 hours, agree with previous literature that used similar methodology, and were even higher compared to some of the reported results [39, 40].

In order to improve photobiological hydrogen production, there must be a balance between respiration and photosynthesis, and this can be achieved by varying the concentration of nutrients in the culture medium, such as that of sulfur, nitrogen and phosphorus [19]. Sulfur deprivation decreases photosynthetic activity and consequently decreases oxygen production, which is consumed by respiration. There is a compensatory point between photosynthesis and respiration that results in an anaerobic environment [6, 8, 10, 41]. Reports show that low sulfur concentrations (around 12.5 to 100 μmol L^{-1}) can improve the compensatory point between photosynthesis and respiration and obtain better results in hydrogen production [13, 14].

However, other researchers obtained negative results when low sulfur concentration was added compared to complete sulfur deprivation [42] and addition above 50 μmol L^{-1} can delay the onset of hydrogen production and decrease the final yield of hydrogen produced [13]. The same occurred in this research, and when comparing the conditions just by observing the variation in the sulfur concentration (comparison of conditions 1 with 3 and 2 with 4) it was noticed that the hydrogen productivity was on average 6 times higher when there was total sulfur deprivation.

These results could be due to increased photosynthetic rate upon sulfur addition, and consequently an increase in oxygen production, thus resulting in the inhibition of hydrogen production. Another factor associated with sulfur concentration is light intensity, which must be varied in parallel with the sulfur concentration, so that the best compensatory point between photosynthesis and respiration to make the anaerobic environment can be achieved [7, 8, 41]. Wild and mutant strains may also respond differently to sulfur concentrations and light intensities, and variations in this compensatory point may also occur. It has been stated that approximately 27 μmol m^{-2} s^{-1} is the best light intensity to achieve this compensation point with the CC425 strain [39], which is lower than the intensities used in this study and which possibly prevented us from obtaining positive results upon sulfur addition.

In this research, it was evident that the light intensity was another factor that interfered in hydrogen production, mainly in sulfur-deprived conditions in which the production was, respectively, 73.4% higher under 200 μmol m^{-2} s^{-1} compared to 60 μmol m^{-2} s^{-1}. Although higher light intensities can inhibit the hydrogenase due to higher photosynthetic rate [43, 44], in sulfur-deprived cultures there is low evolution of oxygen through photosynthesis, then this inhibition by light is not expected to occur [20]. Several researchers analyzed the influence of light intensity in hydrogen production in sulfur deprived cultures, with different strains of *Chlamydomonas*, and some obtained better results at lower intensities (around 12 to 40 μmol m^{-2} s^{-1}) [20, 45] and others at higher intensities (around 100 to 300 μmol m^{-2} s^{-1}) [6, 46, 47], although researchers affirm that the optimal light intensity is 50 to 200 μmol m^{-2} s^{-1} for hydrogen production [35].

Different light intensities, light:dark cycles, light absence, and sulfur deprivation are also factors that aid in the investigation of metabolic pathways involved in hydrogen and other fermentation by-products. In this study, acetic acid was used as a source of organic carbon and it was consumed in all conditions, although the consumption was higher in the photobioreactors containing sulfur (average consumption of

57.2% under sulfur-free conditions and 97.2% with sulfur addition). As discussed above, under these conditions hydrogen production was lower, probably due to the increase in the photosynthetic rate, as previously discussed. This consumption is one of the factors responsible for maintaining the anaerobic environment and providing reductant for hydrogen production via indirect photoproduction (photofermentation) [10]. Furthermore, acetic acid can also be used by microalgae as a substrate for respiration and starch accumulation, and after 24 hours of anoxia, acetate production by the fermentative acidogenic pathways is observed, and there is a superposition between its consumption and production [8]. Therefore, the results suggest that in the presence of additional sulfur, the higher acetic acid consumption rate is probably due to its lower production by the fermentation pathway, justifying and corroborating the lower hydrogen production that occurs under these conditions.

As the system becomes anaerobic, the ability of microalgae to modify their metabolism to anaerobic fermentation can lead to the generation of organic acids, ethanol, carbon dioxide and hydrogen through multiple fermentation pathways through anaerobic decomposition of pyruvate [6, 8, 23, 48]. However, considering that an important part of the pyruvate anaerobic metabolism occurs in the chloroplast, where the hydrogenase is located, there is competition for a reductant between the hydrogen production and other fermentation pathways [7, 9].

The production of ethanol measured under all experimental conditions in this work confirms the occurrence of the alternative fermentative metabolic pathways. Ethanol production was higher under higher light intensity and with sulfur (average of 203.2 mg L^{-1}). The results of this research were better, compared to those of other researchers that utilized similar methodology and the same strain, who reported 37 mg L^{-1} [8] and 57 mg L^{-1} of ethanol [49], but under lower light intensity (100 µmol m^{-2} s^{-1}) and a more basic pH (around 7.2 to 7.7). It is known that low pH favor ethanol production, which explains the better ethanol production results in this study. However, it has also been shown that high ethanol production decreases hydrogen photoproduction due to the competition for the reductant that occurs between these two pathways [18, 50].

Besides hydrogen and ethanol production by the fermentative pathways, the more acidic pH and nutritional sulfur limitation modify the protein metabolic pathway causing an increase in the degradation of proteins and carbohydrates [18, 51]. It has been reported that the decrease in carbohydrates and protein content occurs concomitantly with hydrogen photoproduction in sulfur-deprived cultures [17, 18, 52], although the carbohydrate accumulation can occur during the hydrogen production due to the presence of acetic acid and mixotrophic metabolism of *Chlamydomonas* [48, 53, 54]. This information corroborates the results obtained in this research, in which there was a decrease in protein concentrations in all photobioreactors, especially under deprived sulfur, which show higher hydrogen production (consumption of protein 111 times higher in deprived sulfur conditions than conditions with sulfur). Carbohydrate consumption, also observed in all conditions, probably occurred for ATP generation needed to conduct metabolic processes that require energy during anaerobiosis [9, 17, 52].

In addition to the efficient hydrogen and ethanol production by the CC425 strain in this research, the results also demonstrated that it is possible to associate the cultivation of microalgae, such as *C. reinhardtii*, with wastewater treatment, as has been recently studied by some researchers, mainly using Chlorophyceae [24, 25, 55]. However, there is a lack of studies on this species in the literature.

The results demonstrated mainly the removal of acetic acid, as previously discussed, and the occurrence of phosphate uptake, with an average removal of 72.8%, which may have occurred by its adsorption to the cell surface and assimilation into biomass [56, 57]. Phosphate enters the cell by active transport through the cell membrane and can be used in the formation of ATP molecules [25]. Nitrogen uptake was not observed at significant levels, possibly due to the increased accumulation of nitrogen compounds in the liquid medium caused by the decrease in biomass protein under all tested conditions. Furthermore, it was observed that the nitrogen removal occurred under the conditions, in which there was less decrease in biomass protein, corroborating the results. Indeed, organic matter uptake was greater under conditions of lower hydrogen production. Under higher hydrogen production conditions, an increase in acetic acid production, as previously discussed, may have occurred due to the acidogenic pathway and may have influenced this result, as previously discussed.

In general, the greatest removal of nutrients occurred under conditions with sulfur presence, in addition to less hydrogen production. These data can be an indication that under these conditions there was a higher photosynthetic rate, and consequently it did not provide a totally anaerobic environment [7, 13]. Thus, it may have contributed to the greater removal of nutrients, since photosynthetic microorganisms can be efficient for this purpose in aerobic conditions, although a high concentration of dissolved oxygen may limit the removal [25, 55, 56]. Therefore, a microaerobic environment may have reduced the hydrogen production, due to the inhibition of hydrogenase, consequently causing metabolic deviations to fermentative pathways and higher production of ethanol in these conditions, in addition to having provided greater nutrient removal.

It has been shown that microalgae biomass bioflocculation, or immobilized-cells cultivation systems, can improve the nutrient removal efficiency [24, 58, 59], and our assays were performed with suspended-cell cultivation systems, which may have decreased the nutrient uptake efficiency. Therefore, research with CC425 with flocculated biomass is the aim of future research in the wastewater treatment to improve the nutrient removal efficiency by this strain, in addition to using these microalgae in association with other microorganisms, which has given positive results for the wastewater treatment [25, 60]. Algal-bacterial consortia can be combined with biohydrogen generation and wastewater treatment and aerobic bacteria can contribute to this process by consuming the oxygen produced by the algae in photosynthesis, providing an anaerobic environment [60]. Furthermore, if there is an anaerobic digestion pathway, fermentative bacteria break down organic matter in wastewater into soluble acids, alcohols, hydrogen gases and carbon dioxide. Acetate can be generated from these products by acidogenic bacteria through the process of acetogenesis during the dark fermentation step [26, 61]. In the second step, microalgae having mixotrophic or heterotrophic metabolism, such as *Chlamydomonas*, are able to grow in closed

systems and use this acetate produced as an organic carbon source to produce hydrogen by photoheterotrophic pathway (indirect biophotolysis) [9, 16, 61, 62].

Therefore, the results of this research confirm that hydrogen production occurs through direct water biophotolysis and from carbohydrates breakdown, from the starch reserve or from acetic acid. The occurrence of each pathway may vary according to the different conditions studied. The relative contribution of each of the electron sources may depend on factors such as the strain, extent of damage of PSII, culture conditions and metabolic restrictions [63], such as light intensity age and sulfur concentration, all of which affected our results. In addition, the results of this research demonstrate applicability of *C. reinhardtii* cultivation (CC425) in anaerobic photobioreactors and provide an important discussion of factors that influence hydrogen production, as well as the contribution of using this strain for other purposes of environmental and economic interest.

5. Conclusion

The biomass optimization in the first step may be an important measure to obtain better hydrogen production yield due to the influence of physical chemical factors in the growth of microalgae. Factors such as sulfur concentration and light intensity influenced the production of hydrogen by *C. reinhardtii* (CC425), and the best results occurred in the absence of sulfur and under higher intensity light, as demonstrated in the previous literature. However, our results also demonstrate that it is possible to produce ethanol and remove nutrients with *C. reinhardtii* in closed anaerobic systems. The most efficient ethanol production occurred in photobioreactors under higher intensity light and in the presence of sulfur, and this result suggests that the higher production may be related to the more acidic pH and fermentation pathway due to sulfur and acetic acid presence. In general, we observed mainly acid acetic and phosphate uptake efficiency, regardless of the condition tested, and more research must be done on the wastewater treatment to confirm these preliminary results. Therefore, this research demonstrates the efficient hydrogen production by strain *C. reinhardtii* (CC425) in closed anaerobic photobioreactors and shows that hydrogen production by this microorganism can be associated with ethanol production and wastewater treatment.

Declarations

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Availability of data and materials

The datasets generated during and/or analysed during the current study are available in the University of São Paulo repository. Persistent web link to datasets: https://teses.usp.br/teses/disponiveis/18/18138/tde-21032017-100636/pt-br.php

Author contributions

All authors contributed to the study conception and design. Conceptualization (Sarah Regina Vargas, Marcelo Zaiat, Maria do Carmo Calijuri); Methodology (Sarah Regina Vargas), Investigation (Sarah Regina Vargas), Formal analysis (Sarah Regina Vargas), Writing- Original draft preparation (Sarah Regina Vargas); Resources (Marcelo Zaiat, Maria do Carmo Calijuri), Supervision (Marcelo Zaiat, Maria do Carmo Calijuri), Project administration (Maria do Carmo Calijuri); Writing- Reviewing and Editing (Marcelo Zaiat, Maria do Carmo Calijuri); Funding acquisition (Maria do Carmo Calijuri). All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors mutually consent for publication this manuscript and state that has not been previously published in another journal.

Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Figures

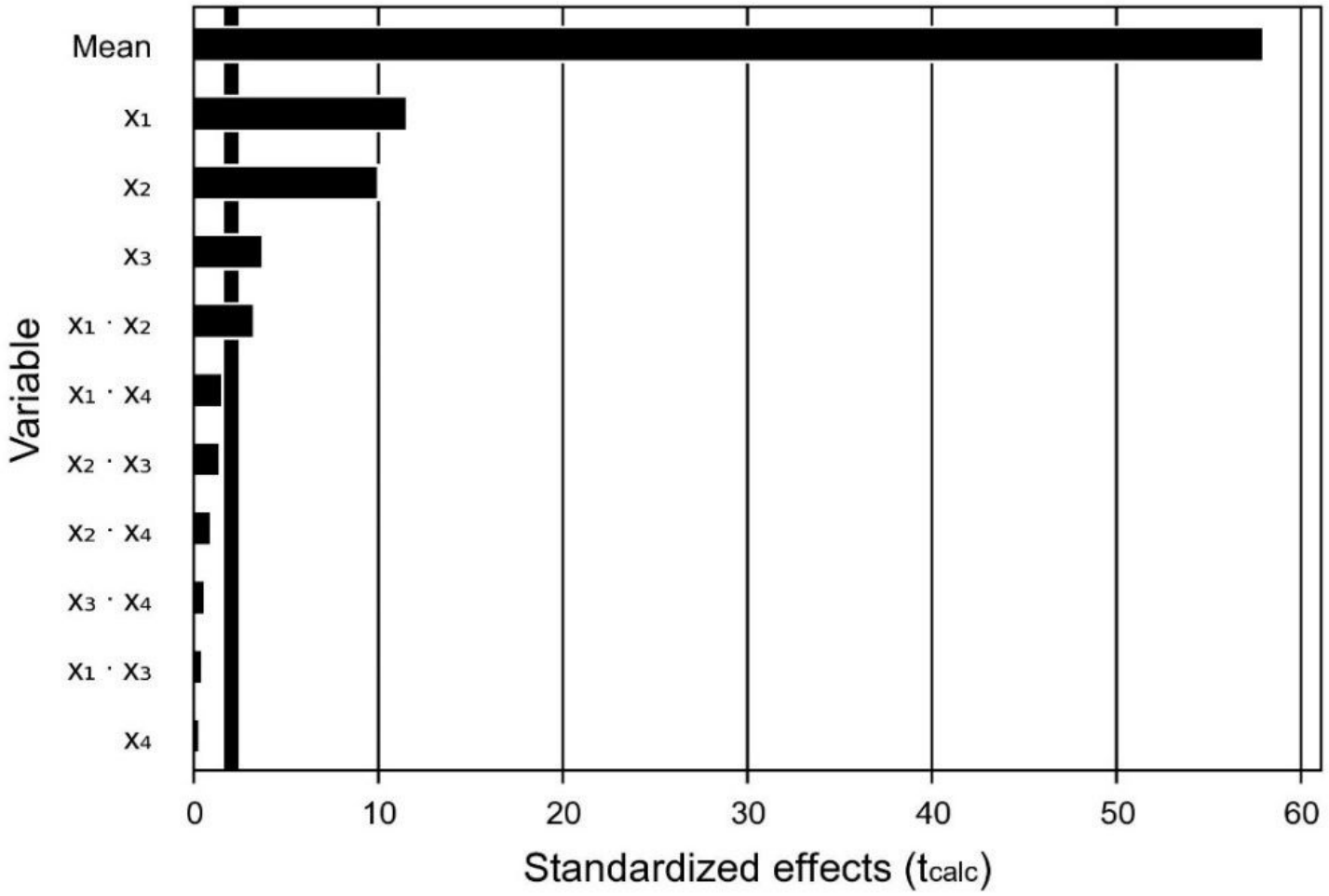

Figure 1

Pareto Chart showing the effect of each factor and their interactions in the optimization of biomass yield of C. reinhardtii. The variables are indicated for X1 (temperature), X2 (pH), X3 (light intensity) and X4 (glucose addition). The vertical bar indicates the significance level of 5%.

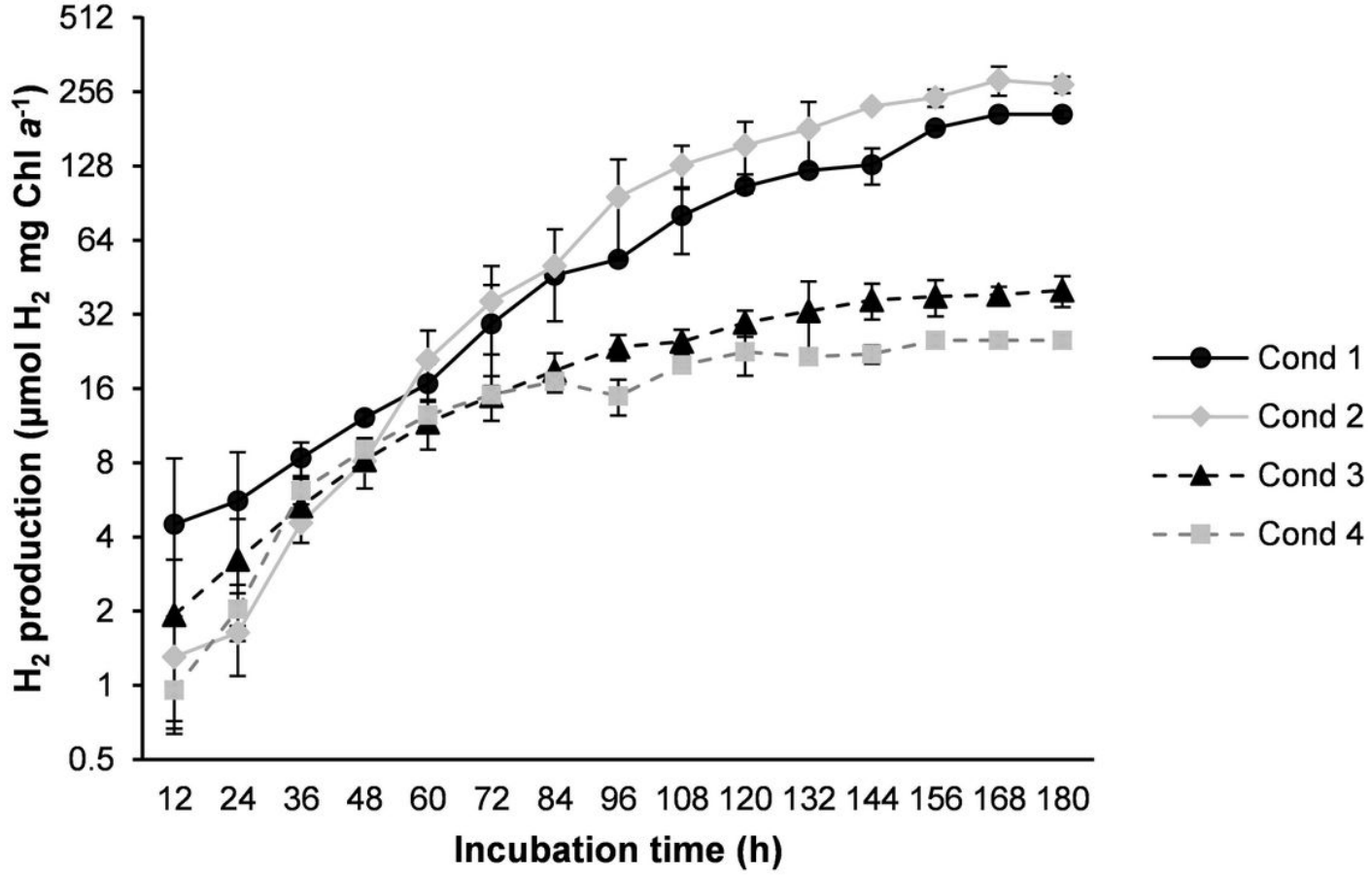

Figure 2

Pmax (µmol H2 mg Chl a-1). Conditions 1 to 4 are represented by cond. and were described in Table 1. The bars indicate the standard deviation (n = 3).

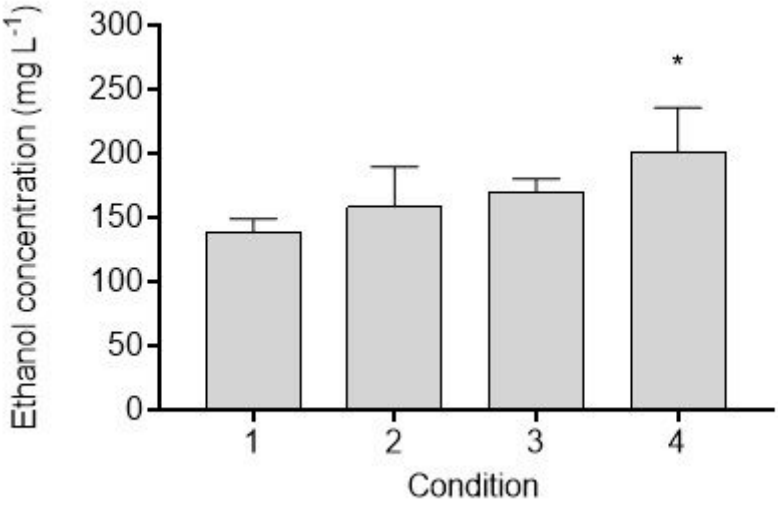

Figure 3

Concentration (mg L-1) of ethanol under conditions 1 to 4. The bars indicate the standard deviation (n = 3). Symbols show statistical differences, according to ANOVA. * Difference with condition 1 (p = 0.034).

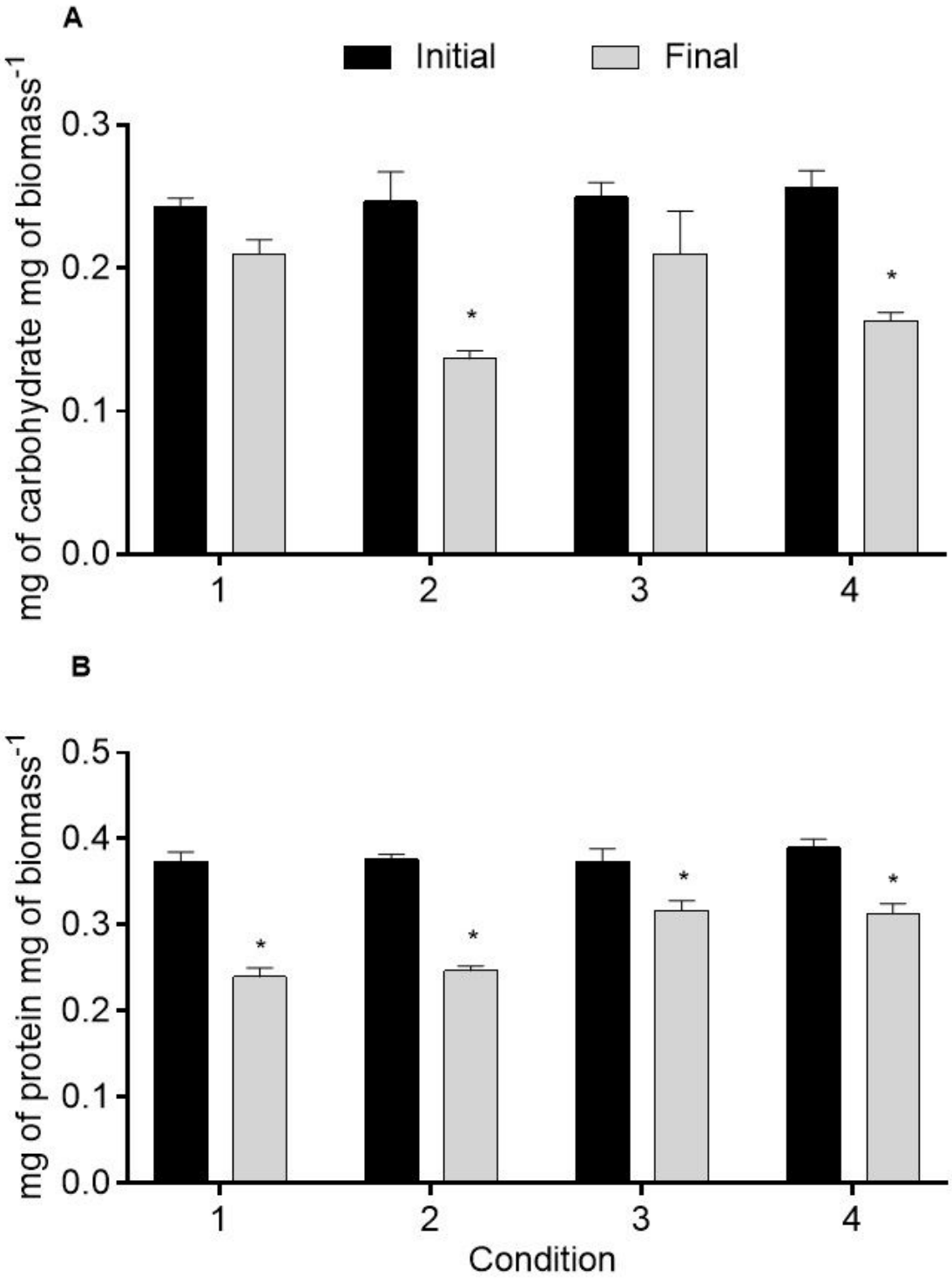

Figure 4

Carbohydrate (A) and protein (B) (mg) per mg of algal biomass under conditions 1 to 4. The biomass compositions accumulated in the first step is represented by initial bars (black), and biomass compositions at the end of the second step (gray bars). The bars indicate the standard deviation (n = 3).

Symbols show statistical differences, according to ANOVA. * Difference between the initial and the final biomass of the bioreactors in each condition ($p \leq 0.013$).

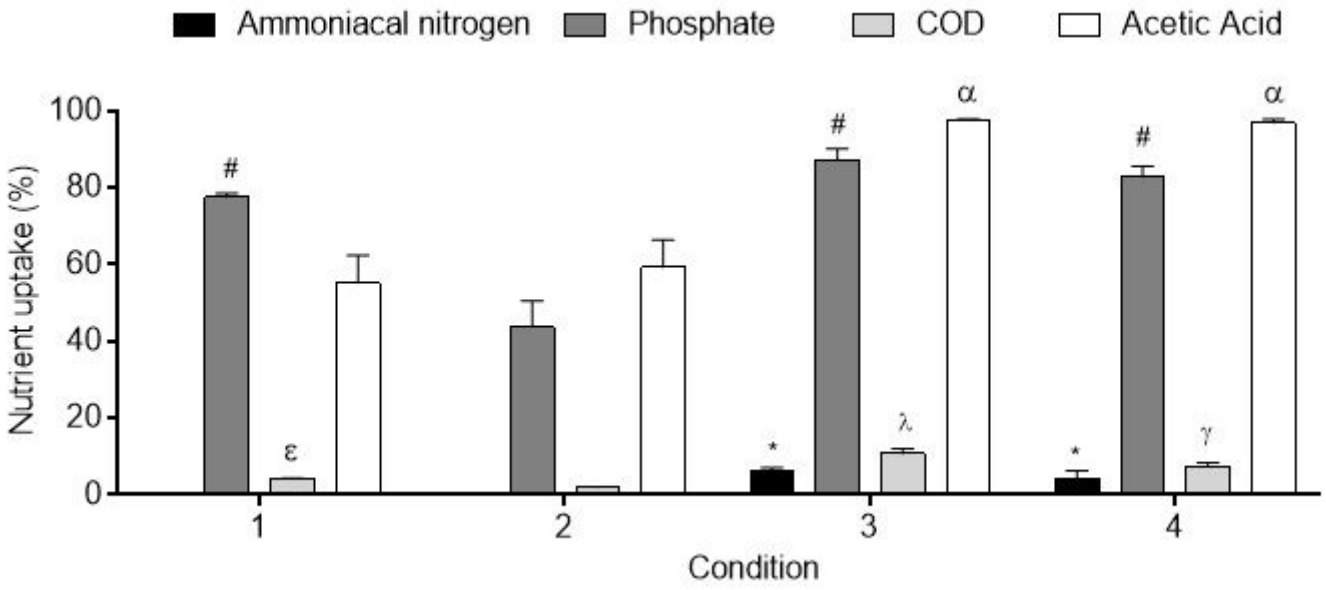

Figure 5

Percentage of nutrient uptake, ammoniacal nitrogen, phosphate, chemical oxygen demand (COD), and acetic acid under conditions 1 to 4. The bars indicate the standard deviation (n = 3). Symbols show statistical differences, according to ANOVA. * Difference with conditions 1 and 2 in the ammoniacal nitrogen uptake ($p \leq 0.004$). # Difference with condition 2 in the orthophosphate uptake ($p \leq 0.0002$). λ Difference with conditions 1, 2 and 4 in the COD uptake ($p \leq 0.006$). γ Difference with conditions 1and 2 in the COD uptake ($p \leq 0.007$). ε Difference with condition 2 in the COD uptake ($p = 0.038$). α Difference with conditions 1 and 2 in the acetic acid uptake ($p \leq 0.0003$).