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Short communication

Examining the impact of carbon dioxide levels and modulation of resulting hydrogen peroxide in *Chlorella vulgaris*

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ABSTRACT

The eukaryotic green alga *Chlorella vulgaris* UTEX 395 was cultured under carbon dioxide (CO_2) concentrations ranging from 0.04% to 15% in order to examine the effect of CO_2 on algal growth, biomass composition and reactive oxygen species (ROS) accumulation in the culture medium. Supplying 5% CO_2 yielded the highest biomass growth rate ($\mu = 0.35\ day^{-1}$) compared with 0.04% ($\mu = 0.15\ day^{-1}$) and 15% ($\mu = 0.19\ day^{-1}$) CO_2 conditions. Experimental evidence showed that increasing CO_2 levels from 0.04% to 2% and above did not alter overall protein content significantly but did enhance C16:1 and C18:1 monounsaturated fatty acid (MUFA) composition by 3.5 and 2 fold, respectively, reducing C18:3 polyunsaturated fatty acid (PUFA) levels. Interestingly, bubbling 5% and 15% CO_2 increased one type of ROS, H_2O_2 levels, in sterile medium by 1.8 to 2 μM while growing *C. vulgaris* substantially lowered these H_2O_2 levels. The ability to lower H_2O_2 levels, which was reduced for non-viable algal cells, was also observed with *C. protothecoides* UTEX 29 and *C. sorokiniana* UTEX 1230. In order to understand the impact of H_2O_2 directly, 10 μM and 25 μM H_2O_2 were added daily to 0.04% CO_2-bubbled *C. vulgaris* cultures. Periodic H_2O_2 addition did not affect the growth of *C. vulgaris* or change its biomass composition. These findings demonstrate *C. vulgaris* can thrive at elevated concentrations of CO_2 and also showed the capacity of microalgae to reduce the ROS level, specifically H_2O_2, present in a CO_2 bubbling environment.

1. Introduction

Microalgae have emerged as potential candidates to capture carbon dioxide (CO_2) efficiently and to produce lipids that can be used as biofuels or other high value products, such as carotenoids. Members of the *Chlorella* genus are one of the prime candidates for these applications due to their high biomass production rate and lipid content [1]. Indeed, *C. vulgaris*, a widely utilized algal species, was observed to maintain a higher growth rate under photoautotrophic conditions than heterotrophic conditions [2]. Various algal species including *Chlorella*, *Scenedesmus*, *Nannochloropsis* have been studied under different CO_2 levels and demonstrated different tolerance to CO_2 concentrations [3]. In addition, eight different *Chlorella* species have been screened under ambient air and 3% CO_2 conditions. *C. vulgaris* UTEX 395 exhibited the highest biomass productivity and lipid yield when cultured under 3% CO_2 condition [4]. A number of recent studies also investigated the growth of *Chlorella* under different CO_2 conditions [5–8]. One study found the highest growth for *C. vulgaris* was obtained under 5% CO_2 and light intensity of 60 μmol/m^2/s [5]. Two other studies found the highest growth rate under 8% CO_2 conditions [6,8]. A study also reported higher growth rate under 1.75% CO_2 than 9.45% CO_2 conditions [7]. Even though there was high variability in different studies, the optimal CO_2 levels were usually between 2% and 16% for *C. vulgaris*.

The goal of this study was to investigate the growth behavior and biomass composition of *C. vulgaris* under different CO_2 conditions. Meanwhile, this study also wanted to explore the impact of CO_2 on ROS production and determine whether it can also affect *C. vulgaris*.

Under photoautotrophic conditions, chlorophyll absorbs energy from the light and eventually transfers the energy to Calvin cycle to fix CO_2. However, if the energy is not efficiently used, chlorophylls will be

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excited to triplet state (^{3}Chl*) and produce singlet oxygen ($^1O_2$) or other reactive oxygen species (ROS) including superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}$OH), and hydrogen peroxide (H_2O_2) inside the cell [9,10]. These ROS are considered as sources of stress for the metabolism and they can oxidize biomolecules, such as lipids, proteins, and DNA that alters their structure and function thus potentially damage the cells. Lipids, such as polyunsaturated fatty acids, are prone to attack because the electron of ROS can initiate a peroxidation reaction with the double bond in polyunsaturated fatty acids [11]. Higher light intensity increases ROS levels in algae, which can inhibit algal growth. It has been demonstrated that this inhibition can be resolved by using high light intensity-resistant strains [12].

To mitigate the oxidative stress from ROS species, such as H_2O_2, algae have developed different antioxidant defenses, including enzymatic mechanisms involving glutathione S-transferase (GST), glutathione peroxidase (GPX) and catalase (CAT), and nonenzymatic mechanisms related to the presence of carotenoids and glutathione [13]. In *C. reinhardtii*, transcriptomics, metabolomics and proteomic analysis have highlighted the role of higher CO_2 level in algal metabolism [14–16]. Proteomic analysis revealed higher expression of redox related protein in plants including GST and GPX in response to elevated CO_2 conditions, illustrating the antioxidant response to decrease the oxidative stress damage to the cell when CO_2 level increased [16]. Glutamic acid, which is involved in GST and GPX metabolism as well, also increased during growth under high CO_2 conditions [15].

In this study, we explored the impact of different CO_2 concentrations on algal growth and biomass composition. This work also investigated whether CO_2 bubbling would generate an oxidative stress environment and how *Chlorella* strains respond to those changes. Interestingly, we also observed increases in the extracellular H_2O_2 levels in the medium while increasing CO_2 levels even in the absence of algae. Furthermore, this study evaluated the capability of three different *Chlorella* species, *C. vulgaris* UTEX 395, *C. protothecoides* UTEX 29, and *C. sorokiniana* UTEX 1230, to adapt to and to alleviate this oxidative stress and determined this capability likely relied, at least in part, on enzymatic mechanisms in live algae. In addition, we showed that *C. vulgaris* responded to high CO_2 content by altering its fatty acid content. Finally, when an elevated H_2O_2 environment was recreated by direct addition of the chemical to the culture medium, the algae were once again able to reduce ROS concentrations, demonstrating the general capacity of algae to respond to this stress environment. Overall, this study provided insights on algal responses to not only different CO_2 levels but also an oxidative stress environment.

2. Material and methods

2.1. Algal strain and cultivation conditions

Green microalgae *Chlorella vulgaris* UTEX 395, *Chlorella sorokiniana* UTEX 1230, and *Chlorella protothecoides* UTEX 29 were obtained from the Culture Collection of Algae at the University of Texas at Austin and maintained on sterile agar plates (1.5% w:v) containing Bold's Basal Medium (BBM). The BBM recipe is as follows: $NaNO_3$, 250 mg/L; $CaCl_2{\cdot}2H_2O$, 25 mg/ L; $MgSO_4{\cdot}7H_2O$, 75 mg/L; K_2HPO_4, 75 mg/L; KH_2PO_4, 175 mg/L; NaCl, 25 mg/L;; KOH, 31 mg/L; $EDTANa_2$, 50 mg/L; $FeSO_4{\cdot}7H_2O$, 4.98 mg/L; H_3BO_3, 11.42 mg/L; $ZnSO_4{\cdot}7H_2O$, 8.82 mg/L; $MnCl_2{\cdot}4H_2O$, 1.44 mg/L; MoO_3, 0.71 mg/L; $CuSO_4{\cdot}5H_2O$, 1.57 mg/L; $Co(NO_3)_2{\cdot}6H_2O$, 0.49 mg/L. Liquid cultures were inoculated with a single colony in 12.5 mL of sterile BBM. Cells were transferred to 100-mL glass Fernbach flasks or 3 L glass Bellco bioreactors (New Jersey, USA) at 25 °C using BBM with 20 mM Tris buffer. In Fig. 1, the experiment was run in 3 L bioreactors (2 L cultures). In Fig. 2 to Fig. 4, the experiment was run in 250 mL flasks (100 mL cultures). Gas was fed as different CO_2 concentrations from 0.04% (ambient air) to 15% at a flow rate of 13 mL/min for 100 mL cultures and 400 mL/min for 3 L cultures. Cultures were grown under fluorescent illumination (100 μmol photon flux m^{-2} s^{-1}) with 12/12 h light/dark cycle. The growth of the cultures was monitored by measuring optical density (OD) at 750 nm. Each batch culture was inoculated at OD_{750} between 0.05 and 0.06.

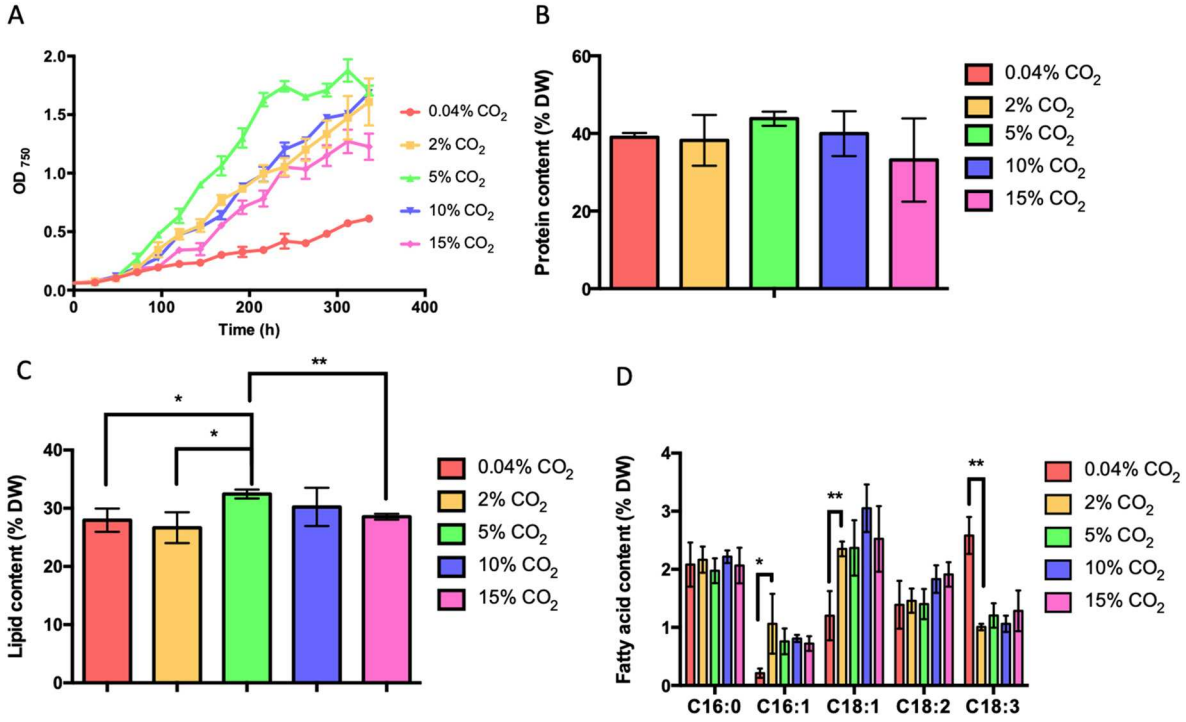

Fig. 1. *C. vulgaris* cultures under 0.04, 2, 5, 10 and 15% CO_2 conditions. (A) OD_{750}; (B) Protein content (% DW); (C) Lipid content (% DW); (D) Fatty acid content (% DW). Error bars: standard deviation of three biological replicates. * $p \leq 0.05$ ** $p \leq 0.01$.

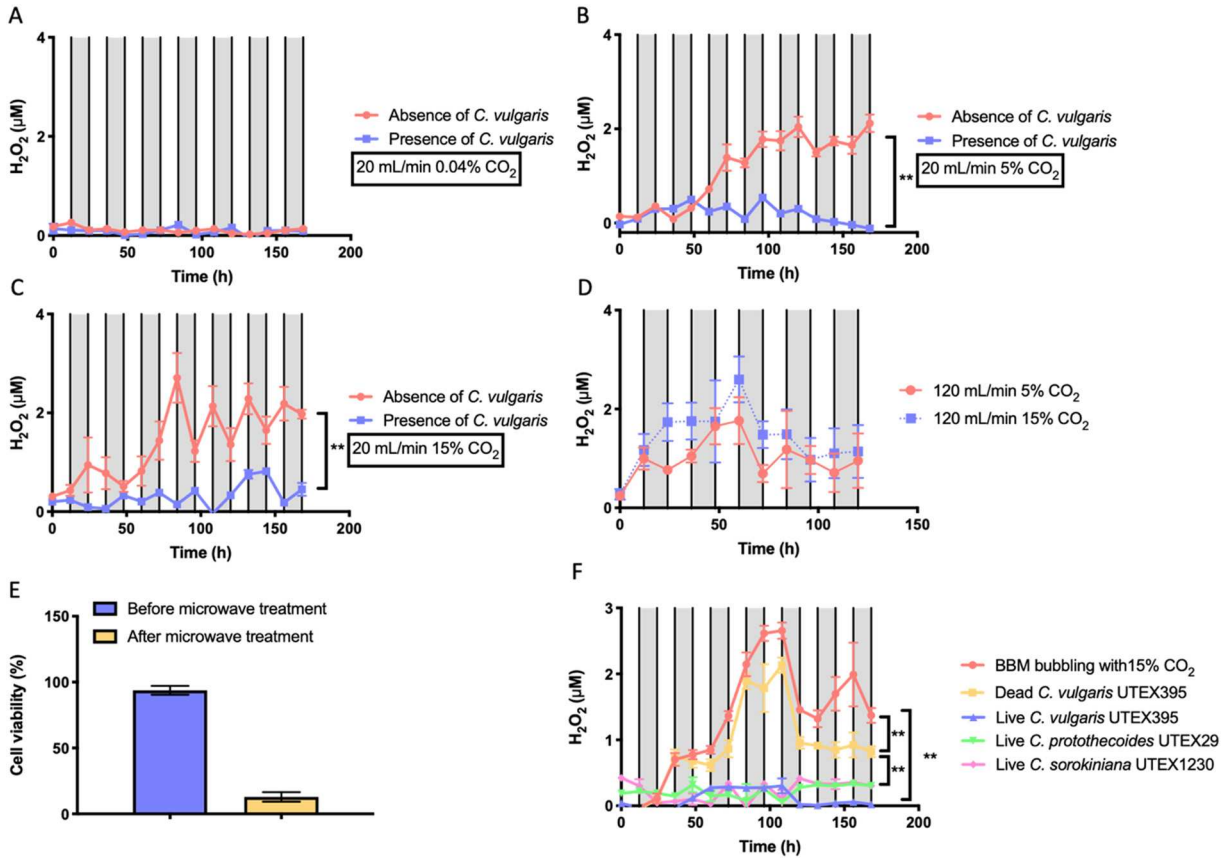

Fig. 2. H_2O_2 levels in the presence and absence of *C. vulgaris* under (A) 0.04%; (B) 5%; (C) 15% CO_2 conditions. (D) H_2O_2 levels in the absence of *C. vulgaris* under 120 mL/min 5% and 15% CO_2 conditions. (E) Cell viability of live *C. vulgaris* UTEX 395 before and after microwave treatments. (F) H_2O_2 levels in 15% CO_2 sparging conditions cultured with live and dead *C. vulgaris* UTEX 395 as well as *C. protothecoides* UTEX 29 and *C. sorokiniana* UTEX 1230 strains. Grey bar represented dark phases and white bar represented light phases. Error bars: standard deviation of three biological replicates. $^{**}p \leq 0.01$.

2.2. Measurement of algal biomass dry weight, total lipid content and protein content

Liquid cultures were harvested at the end of the experiment using a high-speed centrifuge (Beckman J2-21, Baltimore, USA) at 4000 ×*g* for 10 min. The pellets were stored at −80 °C and lyophilized for 24 h at −40 °C. The lyophilized algal dry biomass was weighted gravimetrically using an analytical balance and stored in 4 °C fridge for biomass analysis. After that, the biomass composition was further analyzed by different assays.

Lipid extraction was carried out using the accelerated solvent extraction (ASE) instrument (Dionex ASE 150) and lipids were dried under a stream of nitrogen provided by Tang et al. [17]. The dry biomass samples (150 mg) were extracted with methanol and chloroform (2: 1 v/v). The extraction temperature was 100 °C, with a static time of 5 min, and four static cycles. The extracts were collected in 60 mL tubes and dried. Three biological replicates of total lipid content in the tubes were determined gravimetrically.

Protein extraction followed the sonication procedure provided by Meijer et al. [18]. Biomass samples were first sonicated for 3 cycles of 1 min, which followed the protocol in this reference. In order to completely extract protein from the samples, we added one additional step after sonication. Samples were treated with 4% SDS and heated at 95 °C for 15 min. The samples were centrifuged at 18300 ×g for 5 min and the supernatants were collected. Protein concentration was measured by bicinchoninic acid (BCA) method and the protocol was provided by the vendor (Thermo Scientific 23225-1L).

2.3. Fatty acid methyl ester (FAME) analysis

FAME production followed the procedure provided by Dong et al. [19]. The dry biomass samples (6–7 mg) were mixed with 0.1 mL heptadecanoic (C17:0) fatty acid (5 mg/mL in methanol) as an internal standard, 1 mL of chloroform/ methanol (2:1 v/v) and 1.5 mL of HCl/MeOH (5% v/v). The mixture was heated at 85 °C for 1 h. Then, the mixture was mixed with 1 mL hexane and 5 mL saturated NaCl. The solution was centrifuged at 8450 ×*g* for 10 min in the collect hexane phase. Then, FAME were analyzed using a Shimadzu 2010 Series gas-chromatography (GC) system with discharge ionization detection equipped with a capillary column (Stabilwax-DA, 30 m 0.25 mm ID, film thickness 0.25 mm). GC inlet was set at 250 °C with 1 μL injections. The temperature program started at 50 °C and then increased to 170 °C at a rate of 20 °C min^{-1}, with a plateau for 1 min. After this plateau, the temperature increased from 170 to 220 °C at a rate of 4 °C min^{-1} and then kept constant for 14 min. The total analysis time was 35 min. Helium was used as carrier gas and maintained at 1 mL/min.

2.4. Amino acid content analysis

Amino acid content followed the procedure by Long et al. with modifications [20]. Norvaline was used as an internal standard in this run instead of ^{13}C labeled biomass. Dried algae pellets were hydrolyzed with 500 μL of 6 N HCl at 110 °C for 24 h and then dried under air at 65 °C. The pellets were then added 10 μL 17 mM norvaline (Internal standard), 35 μL pyridine and 50 μL N-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) and 1% *tert*-Butyldimethylchlorosilane (TBDMCS) (Sigma 375,934) and incubated for 30 min at 60 °C. The

mixture was then analyzed by gas chromatography-mass spectrometry (GC/MS). The assay was performed on a Shimadzu GCMS-QP2010 with a Rtx-5MS (30 m × 0.25um × 0.25 mm i.d.) capillary column. The injection temperature was 200 °C, and pressure was held at 65.2 kPa. Helium was used as carrier gas and maintained at 1 mL/min and the ion source temperature was 200 °C.

2.5. Measurement of hydrogen peroxide in the medium

Hydrogen peroxide content in the supernatant was analyzed using an assay kit (Cat no. MAK165-1KT, Sigma). 1 mL samples were taken every light and dark cycle and centrifuged at 10000 ×*g* for 3 min to obtain the supernatant. Prior to the assay kit analysis, the supernatant was kept in the dark at −80 °C before the assay. The assay was conducted within 24 h with duplicate samples.

2.6. Cell viability analysis

Cell viability was analyzed with ethidium homodimer-1 (EthD-1) staining (Cat no. 40014, Biotium). It was performed by incubating cells with 2 μmol/L EthD-1 for 5 min at 37 °C. EthD-1 will enter dead cells with damaged membranes and emit red fluorescence (excitation/emission maxima ~528 nm /617 nm). Images were taken under the fluorescent microscope and analyzed with Fiji software.

2.7. Statistical analysis

All the data are reported as mean ± standard deviation (SD) values. The number of repetitions for each experiment is provided in the figure legend. Software Prism 6 was used to analyze the data and calculate the *p*-value. Statistical significance of the results is indicated by p-value between different groups (* $p \leq 0.05$ and ** $p \leq 0.01$).

3. Results and discussion

3.1. Influence of CO_2 supplementation on cell growth and biomass compositions of C. vulgaris UTEX 395

C. vulgaris UTEX 395 has been widely investigated as a potential biofuel production host. *C. vulgaris* UTEX 265 is able to achieve higher biomass productivities than *C. sorokiniana* UTEX 1230 and *C. protothecoides* UTEX 411 under autotrophic growth with 3% CO_2. In addition, based on the ITS sequences, *C. vulgaris* UTEX 395 is closely related to *C. vulgaris* UTEX 265 [2]. To characterize whether *C. vulgaris* UTEX 395 biomass production depends on CO_2 concentrations in the gas feed under autotrophic conditions, *C. vulgaris* was cultured in 3 L bioreactors at an aeration rate of 400 mL/min with CO_2 concentrations ranging from 0.04% (ambient air) to 15%. The cultures exhibited a lag phase under all CO_2 levels up to 72 h, followed by exponential growth. Under 5% CO_2, *C. vulgaris* grew with the fastest growth rate ($\mu = 0.35 \pm 0.01$ day^{-1}) albeit of shorter duration in which culture density reached stationary phase by 240 h (Table 1 and Fig. 1.A). Above and below 5% CO_2, growth rates declined by 30% (μ = 0.22–0.23 day^{-1}). Similar growth patterns were observed for 2 and 10% CO_2 (Table 1). It took over 300 h to reach an OD_{750} value of 1.6 in 2% and 10% CO_2 and the OD_{750} never exceeded 1.3 in 15% CO_2. The lowest growth rate was observed in 0.04% CO_2, 0.15 ± 0.01 day^{-1}, 2.3 times lower than the maximum growth rate occurring under 5% CO_2 conditions (Table 1). Other studies also reported similar optimal CO_2 levels for different *Chlorella* strains [3]. For example, optimal CO_2 levels for *C. vulgaris* ARC 1, *Chlorella sp* KR-1 and *C. vulgaris* are 6% [21], 10% [22] and 4% [23], respectively. Recent studies also found 8% CO_2 as an optimal condition for *C. vulgaris* [6,8]. Those results are similar to our findings that 5% was the optimal CO_2 level for *C. vulgaris* UTEX 395.

A previous study found that *C. vulgaris* exhibited the highest CO_2_fixation rate of 34% under 0.04% CO_2 condition based on the increase of mass fraction of carbon in the biomass [24]. However, since CO_2 was one of the major carbon sources in our experiment, 0.04% CO_2 (ambient air) may not provide sufficient CO_2 resulting in the lowest growth rate even with a high CO_2_fixation rate. In our experiment, the carbon dioxide pressure should be correlated to the carbon dioxide levels. Based on Henry's law, the dissolved carbon dioxide will increase proportionally to the carbon dioxide pressure in the bioreactor. Therefore, increasing CO_2 levels from 0.04% to 2% should increase the dissolved carbon dioxide. This may help to explain why increasing CO_2 levels from 0.04% to 5% can increase growth rate.

While added Tris buffer helped to maintain the pH between 6.8 and 6.9 for 5% CO_2, the pH eventually dropped to 6.6 after supplying 10% CO_2 (Table 1). The final pH was even lower at 6.4 in 15% CO_2. The pH drop may help to explain the lower growth under 10% and 15% CO_2 conditions. Previous studies also reported the optimal pH value at 7 and 7.5 for *C. vulgaris* [25,26].

C. vulgaris often contains a high protein content [>40% dry biomass (DW)] compared to other unicellular algae, giving it an advantage for large scale production of proteins [27]. When the protein content in *C. vulgaris* was analyzed, it was found to be stable between between 33% and 44% for the five CO_2 concentrations tested (Fig. 1.B). In addition, the lipid content was also found to be stable between 26 and 30% under all the conditions but slightly higher in 5% CO_2 samples, comprising 32% DW ($p \leq 0.05$) (Fig. 1.C). A previous transcriptomics study in *Chlorella sorokiniana* indicated that elevating CO_2 could improve lipid synthesis by enhancing biosynthesis of acetyl-CoA [28]. However, our results indicated that elevating CO_2 availability did not significantly alter the synthesis patterns for protein content in *C. vulgaris* (Fig. 1.B).

Fatty acid content was further analyzed and quantified from the lipid samples extracted from the *C. vulgaris* cultures. The main fatty acids in *C. vulgaris* were distributed as C16:0, C18:1, C18:2, and C18:3 (number of carbon atoms:number of double bonds) shown in Fig. 1.D. Interestingly, unlike the overall protein and lipid content, a major shift was detected in the fatty acid profile going from 0.04% to 2% CO_2. The C16:1 and C18:1 fatty acids increased 3.5 fold ($p \leq 0.05$) and 2 fold ($p \leq 0.01$) respectively. In contrast, there was a 2.5 fold ($p \leq 0.01$) decrease in C18:3 fatty acids. The reciprocal relationship between the increase in C18:1 and decrease in C18:3 content was also found in *C. reinhardtii* going from 0.04% to 1% CO_2 [29] and in *C. vulgaris* from 0.04% to 2% CO_2 [30]. CO_2 also influenced the fatty acid composition in different types of lipids [30]. However, previous studies did not investigate whether this change was sustained under higher CO_2 levels (>2%). From our results, the fatty acid profile was similar after further increases in the CO_2 concentration from 2% to 15%. In this way, we found the major change in fatty acid composition for *C. vulgaris* occurred when the culture condition was altered from 0.04% to 2%. Several studies proposed increasing CO_2 levels or decreasing O_2 levels may regulate desaturase enzymes catalytic activity that resulted in different fatty acid profiles [31,32]. In this way, the proposed regulatory effect may occur when the CO_2 level was altered from 0.04% to 2% for these culture conditions. The dramatic change in the CO_2 level going from ambient air concentrations of CO_2 (0.04%) to 2%, which is 50 fold, may thus affect fatty acid synthesis and fatty acid desaturation in *C. vulgaris*.

Table 1
Growth rate and pH value of *C. vulgaris* cultures under 0, 2, 5, 10 and 15% CO_2 concentrations after 336 h culture. Standard deviation was calculated based on three biological replicates.

CO_2 levels (%)	0.04	2	5	10	15
Growth rate (day^{-1})	0.15 ± 0.01[a]	0.23 ± 0.01[a]	0.35 ± 0.01[b]	0.22 ± 0.01[a]	0.19 ± 0.01[a]
pH	6.93 ± 0.17	6.97 ± 0.06	6.95 ± 0.07	6.59 ± 0.07	6.36 ± 0.06

[a] Growth rate calculated from 48 to 336 h.
[b] Growth rate calculated from 48 to 240 h.

Alternatively, one previous study has linked the change in altered fatty acid compositions at elevated CO_2 levels to the thylakoid membranes as these changes included higher levels of galactolipids, which are major lipids of thylakoid membranes [30]. Increasing CO_2 levels also lowered quantum requirement for photosynthetic oxygen revolution in *Chlorella*, which also suggested some changes in thylakoid membranes [33]. One recent study from cyanobacteria suggested this lipid remodeling may be helpful for the functioning of photosystem II (PSII) under low CO_2 conditions [34].

Oxidation stability is one of the criteria or biodiesel production and the oxidation rate is highly dependent on the number of double bonds in fatty acids. C18:3 is more vulnerable to oxidation than C18:1 because it has more double bonds in its structure. Increasing the ratio between monounsaturated fatty acids and polyunsaturated fatty acids could help to enhance the stability of biodiesel produced from these microalgal lipids [35].

3.2. CO_2-induced H_2O_2 production and modulation by different Chlorella strains

Since the ratio between C18:3 fatty acid and C18:1 fatty acid decreased under high CO_2 levels and C18:3 is more vulnerable to oxidation than C18:1, we evaluated the oxidant levels in the form of H_2O_2 levels under different CO_2 conditions. Bubbling 20 mL/min of 0.04% CO_2 into sterile BBM media, in the absence of *C. vulgaris*, did not result in significant increases in the H_2O_2 levels, which remained very stable between 0.02 and 0.26 μM over 168 h (Fig. 2.A). In contrast, when the CO_2 concentration increased from 0.04% to 5%, the H_2O_2 concentration gradually increased 18 fold from 0.1 μM to 1.8 μM over 96 h (Fig. 2.B). After this time, the H_2O_2 level remained stable until 168 h. However, when *C. vulgaris* was inoculated into the same culture medium, the H_2O_2 level was observed to decrease gradually below 0.2 μM ($p \leq 0.01$) (blue points in Fig. 2.B). Increasing the CO_2 concentration to 15% resulted in H_2O_2 levels around 2 μM (Fig. 2.C). Nonetheless, *C. vulgaris* was still able to reduce the elevated H_2O_2 level significantly between 0 and 0.85 μM ($P \leq 0.01$).

We also explored the effect of changes in the gas flow rates from 20 to 120 mL/min of 5% and 15% CO_2 in order to investigate the relationship between flow rate and H_2O_2 levels (Fig. 2.D). Increasing 5% and 15% CO_2 gas flow rates to 120 mL/min enhanced the rate of accumulation of H_2O_2 up to 1–2 μM within the first 24 h in sterile medium. However, the change in gas flow rate did not substantially alter the maximum concentration of H_2O_2 present in the inoculated medium (Fig. 2.D). Some studies found elevating CO_2 exacerbated ROS formation and stimulated antioxidant responses inside the cell, which confirmed our findings that CO_2 could trigger ROS production [36,37]. Meanwhile, other studies have revealed abiotic generation of H_2O_2 in HEPES buffer in the presence of CO_2 [38]. This H_2O_2 formation was mediated by peroxynitrite and also the presence of CO_2. However, peroxynitrite is not typically present in normal cell culture medium. Therefore, there is likely another component in the medium or environment driving the generation of H_2O_2 and ROS following CO_2 bubbling in this system. Furthermore, we confirmed this oxidative stress production was dictated by the CO_2 levels and not significantly dependent on gas flow rates.

In order to investigate if the H_2O_2 decline in the presence of *C. vulgaris* was due to metabolic activity, half of the algal culture volume (OD_{750} between 0.04 and 0.06) was microwaved to generate heat that inactivated metabolism and enzymatic function and killed the majority of the live cells [39]. Indeed, a 4 min microwave treatment decreased cell viability from 94% to 13% (Fig. 2.E). Microwaved and untreated algae were then placed into the BBM medium which was bubbled with 15% CO_2. Bubbling 15% CO_2 once again produced a high level of H_2O_2 in the medium in the absence of *C. vulgaris* (red dots in Fig. 2.F). Adding dead algae to the medium provided only a minor impact on the H_2O_2 levels, which decreased slightly below those observed without any cells (yellow dots in Fig. 2.F). In contrast, the H_2O_2 level decreased significantly and stabilized at 0.3 μM in the presence of live *C. vulgaris* (blue dots in Fig. 2.F). In order to investigate if other *Chlorella* species can alter the H_2O_2 concentrations in culture, *C. sorokiniana* UTEX 1230 (pink dots in Fig. 2.F) and *C. protothecoides* UTEX 29 (green dots in Fig. 2.F) were also subjected to 15% CO_2 sparging conditions. These organisms exhibited a similar capacity to lower the H_2O_2 levels to approximately 0.3 μM (Fig. 2.F). Collectively, the results indicate that different *Chlorella* species can modulate H_2O_2 that is generated from CO_2 bubbling into the medium. While we cannot exclude the possibility some of the H_2O_2 may be resolved by cell absorption of CO_2, the amount of bubbling CO_2 that was accumulated in the biomass was only 8% for 0.04% CO_2 conditions and less than 1% for all other conditions (Table. 2). This was calculated based on the bioreactor data from Fig. 1 and assumed 48% of final biomass was made of carbon [40].

Furthermore, this effect is dependent on active cellular functions, such as enzymatic activities and metabolism. Several enzymatic and non-enzymatic antioxidative defense systems have been discovered in algae [41]. Catalase, superoxide dismutase and glutathione reductase are three major enzymatic systems. Ascorbate, glutathione and carotenoids are non-enzymatic compounds that can also defend against oxidative stress. Previously, cyanobacteria have been observed to scavenge H_2O_2 in monoculture and co-cultures through the catalase enzyme [42]. In order to protect the cells from oxidative damage during the accumulation of atmospheric oxygen, these species acquired ascorbate peroxidase during evolution [43]. *C. vulgaris* also contains various antioxidant systems, including catalase and superoxide dismutase, to deal with oxidative stress from the environment [13]. Our results suggested that those protective enzymatic mechanisms can be deactivated following microwave treatment. In this way, *Chlorella* loses its capacity to reduce H_2O_2 levels significantly.

3.3. Creating an enhanced H_2O_2 environment at lower CO_2 levels to examine the H_2O_2 effect on C. vulgaris growth and biomass compositions

In order to create an environment with H_2O_2 concentrations similar to those found in 5% and 15% CO_2 conditions (~2 μM), 1 mM of H_2O_2 was added every 24 h along with the 0.04% CO_2 sparging condition in BBM medium. The concentration of H_2O_2 decreased to lower levels by 24 h due to the natural decomposition of H_2O_2 (Fig. 3.A). Pulsing the H_2O_2 resulted in a significant uptick of the H_2O_2 levels, which declined again by the next 24 h time point. However, the overall result was a steady upward trend in H_2O_2 levels, reaching nearly 4 mM H_2O_2 by approximately 100 h. Therefore, the same 24 h pulsing of H_2O_2 was performed by adding 100 μM daily instead, which did not generate the progressively upward trend. Instead, the H_2O_2 concentration oscillated between a maximum of 500 μM at 24 h and minimal levels by 100 h (Fig. 3.A and 3.B). That the level was higher than the added H_2O_2 level is likely due to a chain reaction leading to elevated H_2O_2 levels in the media [44]. Alternatively, adding 10 μM H_2O_2 daily resulted in much lower H_2O_2 levels oscillating between 20 μM and negligible levels (Fig. 3.C). Since this H_2O_2 level of daily addition most closely resembled the levels observed with 5 and 15% CO_2 sparging (approximately 2 μM H_2O_2), experiments were performed in 0.04% CO_2 with 0, 10, and 25 μM

Table 2
Carbon capture efficiency under 0, 2, 5, 10 and 15% CO_2 concentrations after 336 h culture. Standard deviation was calculated based on three biological replicates.

CO_2 levels (%)	0.04[a]	2	5	10	15
Total carbon in the biomass (mg)	125.4 ± 1.5[b]	286.0 ± 8.0	428.4 ± 34.7	277.8 ± 14.8	211.8 ± 16.0
Capture efficiency (%)	7.94 ± 0.09	0.36 ± 0.01	0.22 ± 0.02	0.07 ± 0.01	0.04 ± 0.01

[a] Assume the molar volume of gas was equal to 22.4 L.
[b] Assume 48% of biomass was carbon.

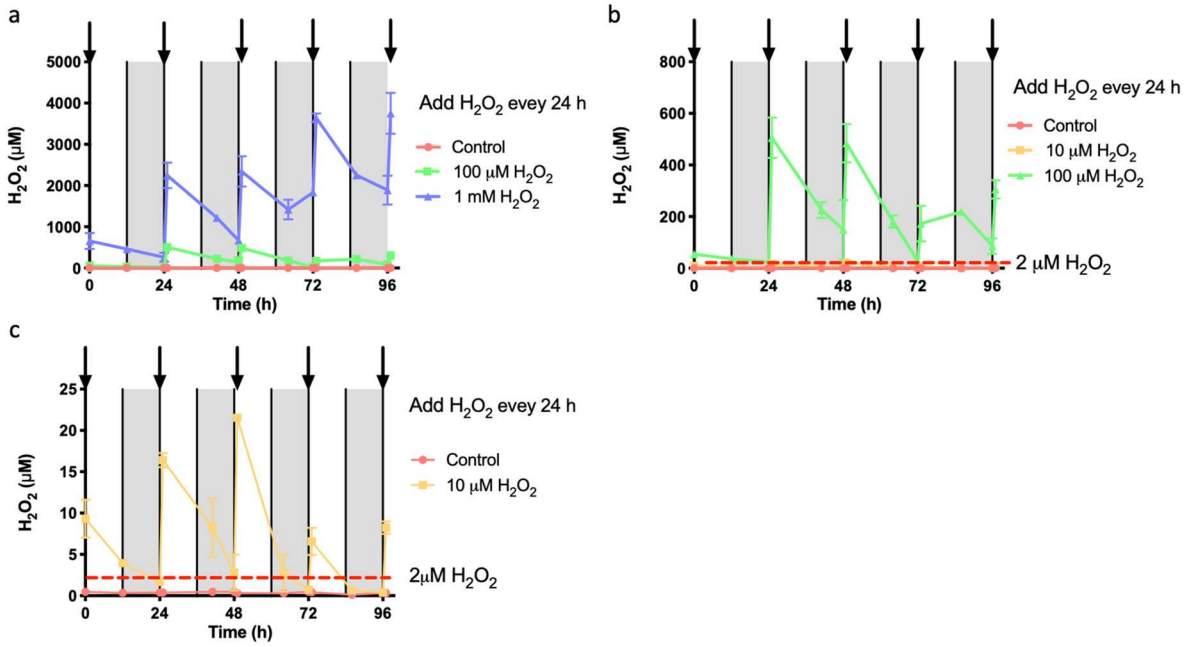

Fig. 3. H_2O_2 levels in the medium by adding different amount of H_2O_2 every 24 h. (A) Control, 100 μM and 1 mM every 24 h; (B) Control, 10 μM and 100 μM every 24 h; (C) Control and 10 μM every 24 h. Grey bar represented dark phases and white bar represented light phases. Error bars: standard deviation of two biological replicates.

daily additions of H_2O_2 to the *C. vulgaris* cultures in order to investigate the effect of oxidative stress we saw in high CO_2 conditions on algal growth (Fig. 4).

C. vulgaris grew from OD_{750} at 0.1 to 0.36 in 192 h under 0.04% CO_2 without any H_2O_2 supplementation. The cultures exhibited similar growth curves, with an OD_{750} between 0.33 and 0.36 by 192 h, when

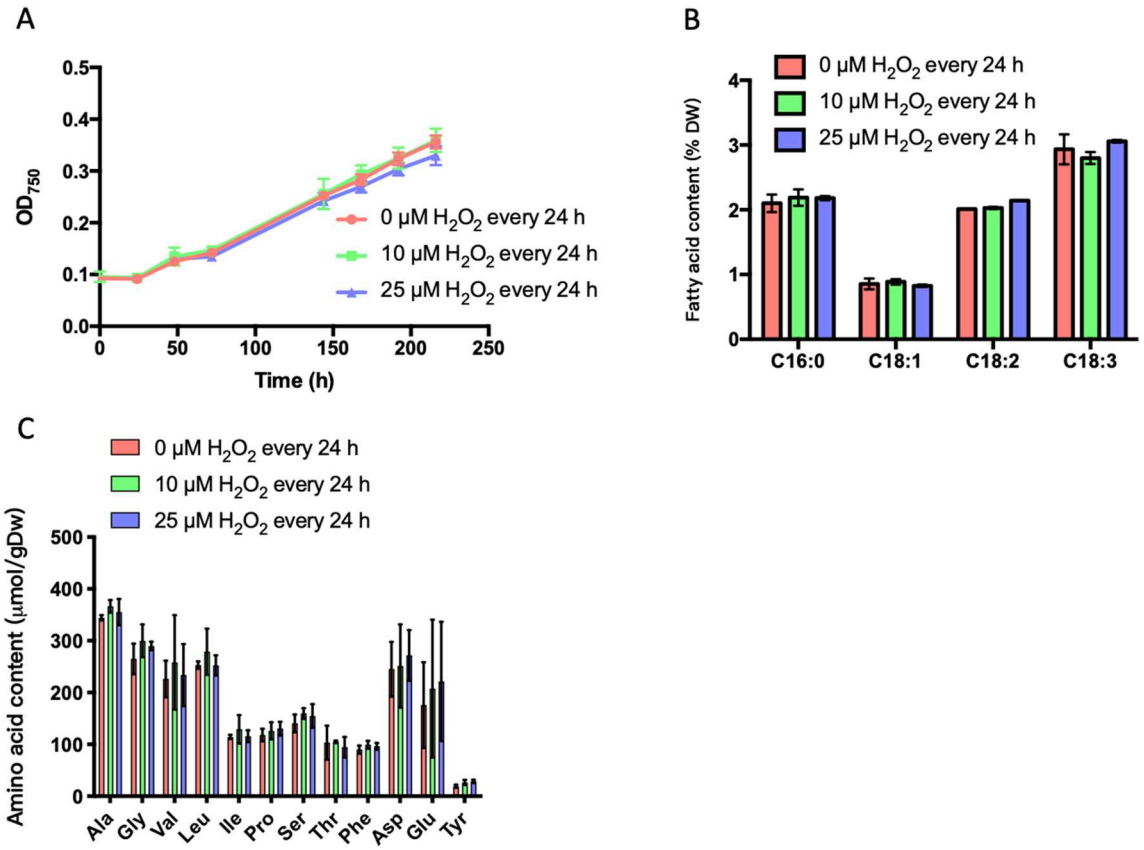

Fig. 4. Growth of *C. vulgaris* by adding 0 μM, 10 μM and 25 μM H_2O_2 every 24 h. (A) OD_{750}; (B) Fatty acid content (% DW); (C) Amino acid content (μmol/gDw). Error bars: standard deviation of four biological replicates for Fig. 4.A and three biological replicates for Fig. 4.B and Fig. 4.C.

supplying 10 μM or 25 μM H_2O_2 to the cultures daily in addition to bubbling of 0.04% CO_2 (Fig. 4.A). Interestingly, previous studies found that biomass concentrations of *C. protothecoides* decreased gradually with increasing H_2O_2 concentrations from 0 to 10 μM [45] while our results indicated that *C. vulgaris* is more robust and resistant to H_2O_2 concentrations similar to those found in CO_2 bubbling conditions.

Next the fatty acid content was examined at 192 h. Surprisingly, unlike the enhanced fatty acid content in C16:1 and C18:1 observed for CO_2 bubbling at 2 to 15%, fatty acid content did not change by adding H_2O_2 daily to algal cultures with the C18:3 fatty acid the most abundant with around 3% DW in all three cultures (Fig. 4.B). Previously, we found an increase in C18:1 and a decrease in C18:3 content when the cultures conditions changed from 0.04% to 5% CO_2 conditions (Fig. 1.D). High CO_2 leads to a reduction in unsaturation of C18 and C16 fatty acids as indicated for *C. vulgaris* in Fig. 1. In this study, we have shown that high CO_2 also causes an increase in H_2O_2 levels. While lipid oxidation can be initiated by free radicals, which were associated with H_2O_2 [46], our study shows that this change is not responsible for the decrease in unsaturated fatty acids seen at elevated CO_2 levels. Thus, H_2O_2 does not appear to be the direct cause of the decline in the desaturation levels observed in this study. Therefore, either the CO_2 levels themselves or other uncharacterized metabolic factor must have caused the shift in saturation content observed when CO_2 levels were enhanced from 0.04 to 5% and above. A previous study showed lipid synthesis was highly correlated to intracellular hydroxyl radical ($^{\cdot}OH$) instead of hydrogen peroxide (H_2O_2) under different stress conditions [47]. Future studies will be required to delineate which metabolic factor or ROS species is the principal cause of the shift in desaturation observed for *C. vulgaris* and other algal hosts.

The amino acid content was also analyzed to investigate whether oxidative stress would affect the resulting amino acid profile. The amino acid content did not change after adding H_2O_2 (Fig. 4.C). Alanine, glycine, valine, leucine and aspartic acid are abundant in *C. vulgaris* biomass under all three conditions. Previous studies observed that bicarbonate can perturb the cellular redox mechanism and induce glutathione peroxidase (GPX) and glutathione S-transferase (GST) expression in plant cells, indicating the connection between CO_2 and oxidative stress [16]. Furthermore, the GPX and GST synthesis pathways are associated with cysteine, glutamate and glycine levels. However, as with the fatty acid profiles, our findings did not exhibit a connection between H_2O_2 levels and a change in the amino acid profile. It is possible that other reactive oxygen species (ROS), instead of H_2O_2, may play an important role in the cellular redox mechanism. Singlet oxygen ($^1O_2$), an excited state of O_2, and hydroxyl radical ($^{\cdot}OH$) represent other ROS species that can play an important role in initiating signaling pathways in algae [13,48]. Indeed, singlet oxygen can damage lipids and proteins, leading to changes in gene expression inside the cells [49]. Therefore, investigating the activation of other ROS species under different CO_2 levels may provide additional insights to understand better the possible effects of CO_2 on ROS and algal growth and metabolism.

4. Conclusion

The goal of this study was to investigate how different CO_2 concentrations can affect cell growth and biomass composition in *C. vulgaris* UTEX 395. Altering the CO_2 levels resulted in changes in the growth rate and lipid composition with an optimum growth rate around 0.35 day^{-1} achieved at 5%. In particular, the C18:3 levels decreased while C18:1 levels increased for CO_2 concentrations of 2% and higher. In addition, we detected elevated levels of H_2O_2 at elevated CO_2 levels, indicative of the production of an oxidative stress environment. This oxidative stress was neutralized by growing different *Chlorella* species while the neutralization capability was lost when using dead algae. Finally, by adding 10 μM and 25 μM H_2O_2 every 24 h for 0.04% CO_2 bubbling, an artificial oxidative stress atmosphere was created, which did not alter either cell growth, fatty acid composition and amino acid composition, indicating H_2O_2, one type of oxidative stress, was not the major driver for the change in fatty acid content observed when increasing CO_2 bubbling conditions from 0.04% to 5% CO_2.

CRediT authorship contribution statement

Chien-Ting Li: Conceptualization, Data curation, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Kevin Trigani:** Data curation, Methodology, Investigation. **Cristal Zuñiga:** Methodology, Writing – review & editing. **Richard Eng:** Writing – review & editing. **Elizabeth Chen:** Data curation, Methodology. **Karsten Zengler:** Writing – review & editing, Supervision. **Michael J. Betenbaugh:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that there are not any potential financial or other interests that could be perceived to influence the outcomes of the research.

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References

[1] C. Zuñiga, C.T. Li, T. Huelsman, J. Levering, D.C. Zielinski, B.O. McConnell, C. P. Long, E.P. Knoshaug, M.T. Guarnieri, M.R. Antoniewicz, M.J. Betenbaugh, K. Zengler, Genome-scale metabolic model for the green alga *Chlorella vulgaris* UTEX 395 accurately predicts hhenotypes under autotrophic, heterotrophic, and mixotrophic growth conditions, Plant Physiol. 172 (2016) 589–602.

[2] J.N. Rosenberg, N. Kobayashi, A. Barnes, E.A. Noel, M.J. Betenbaugh, G.A. Oyler, Comparative analyses of three *Chlorella* species in response to light and sugar reveal distinctive lipid accumulation patterns in the microalga *C. sorokiniana*, PLoS One 9 (2014), e92460.

[3] S. Singh, P. Singh, Effect of CO_2 concentration on algal growth: a review, Renew. Sust. Energ. Rev. 38 (2014) 172–179.

[4] N. Kobayashi, A. Barnes, T. Jensen, E. Noel, G. Andlay, J.N. Rosenberg, M. J. Betenbaugh, M.T. Guarnieri, G.A. Oyler, Comparison of biomass and lipid production under ambient carbon dioxide vigorous aeration and 3% carbon dioxide condition among the lead candidate *Chlorella* strains screened by various photobioreactor scales, Bioresour. Technol. 198 (2015) 246–255.

[5] H. Chang, Y. Zou, R. Hu, N. Zhong, S. Zhao, Y. Zheng, Y. Qin, C. Feng, Kinetics of landfill leachate remediation and microalgae metabolism as well as energy potential evaluation, J. Clean. Prod. 269 (2020), 122413.

[6] M. Molazadeh, S. Danesh, H. Ahmadzadeh, H.R. Pourianfar, Influence of CO2 concentration and N: P ratio on Chlorella vulgaris-assisted nutrient bioremediation, CO2 biofixation and biomass production in a lagoon treatment plant, J. Taiwan Inst. Chem. Eng. 96 (2019) 114–120.

[7] R. Pourjamshidian, H. Abolghasemi, M. Esmaili, H.D. Amrei, M. Parsa, S. Rezaei, Carbon dioxide biofixation by Chlorella sp. in a bubble column reactor at different flow rates and CO2 concentrations, Braz. J. Chem. Eng. 36 (2019) 639–645.

[8] M. Adamczyk, J. Lasek, A. Skawińska, CO 2 biofixation and growth kinetics of Chlorella vulgaris and Nannochloropsis gaditana, Appl. Biochem. Biotechnol. 179 (2016) 1248–1261.

[9] F. Ramel, A.S. Mialoundama, M. Havaux, Nonenzymic carotenoid oxidation and photooxidative stress signalling in plants, J. Exp. Bot. 64 (2013) 799–805.

[10] A. Krieger-Liszkay, Singlet oxygen production in photosynthesis, J. Exp. Bot. 56 (2005) 337–346.

[11] K. Das, A. Roychoudhury, Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants, Front. Environ. Sci. 2 (2014) 53.

[12] B. Förster, C.B. Osmond, B.J. Pogson, Improved survival of very high light and oxidative stress is conferred by spontaneous gain-of-function mutations in *Chlamydomonas*, Biochim. Biophys. Acta 1709 (2005) 45–57.

[13] N. Mallick, F.H. Mohn, Reactive oxygen species: response of algal cells, J. Plant Physiol. 157 (2000) 183–193.

[14] W. Fang, Y. Si, S. Douglass, D. Casero, S.S. Merchant, M. Pellegrini, I. Ladunga, P. Liu, M.H. Spalding, Transcriptome-wide changes in *Chlamydomonas reinhardtii* gene expression regulated by carbon dioxide and the CO_2-concentrating mechanism regulator CIA5/CCM1, Plant Cell 24 (2012) (tpc. 112.097949).

[15] L. Renberg, A.I. Johansson, T. Shutova, H. Stenlund, A. Aksmann, J.A. Raven, P. Gardeström, T. Moritz, G. Samuelsson, A metabolomic approach to study major metabolite changes during acclimation to limiting CO_2 in *Chlamydomonas reinhardtii*, Plant Physiol. 154 (2010) 187–196.

[16] Z. Yin, K. Balmant, S. Geng, N. Zhu, T. Zhang, C. Dufresne, S. Dai, S. Chen, Bicarbonate induced redox proteome changes in *Arabidopsis* suspension cells, Front. Plant Sci. 8 (2017) 58.

[17] Y. Tang, Y. Zhang, J.N. Rosenberg, N. Sharif, M.J. Betenbaugh, F. Wang, Efficient lipid extraction and quantification of fatty acids from algal biomass using accelerated solvent extraction (ASE), RSC Adv. 6 (2016) 29127–29134.

[18] E.A. Meijer, R.H. Wijffels, Development of a fast, reproducible and effective method for the extraction and quantification of proteins of micro-algae, Biotechnol. Tech. 12 (1998) 353–358.

[19] T. Dong, L. Yu, D. Gao, X. Yu, C. Miao, Y. Zheng, J. Lian, T. Li, S. Chen, Direct quantification of fatty acids in wet microalgal and yeast biomass via a rapid in situ fatty acid methyl ester derivatization approach, Appl. Microbiol. Biotechnol. 99 (2015) 10237–10247.

[20] C.P. Long, M.R. Antoniewicz, Quantifying biomass composition by gas chromatography/mass spectrometry, Anal. Chem. 86 (2014) 9423–9427.

[21] S. Chinnasamy, B. Ramakrishnan, A. Bhatnagar, K.C. Das, Biomass production potential of a wastewater alga *Chlorella vulgaris* ARC 1 under elevated levels of CO_2 and temperature, Int. J. Mol. Sci. 10 (2009) 518–532.

[22] K.D. Sung, J. Lee, C. Shin, S. Park, Isolation of a new highly CO_2 tolerant fresh water microalga *Chlorella* sp. KR-1, Renew. Energy 16 (1999) 1019–1022.

[23] V. Bhola, R. Desikan, S.K. Santosh, K. Subburamu, E. Sanniyasi, F. Bux, Effects of parameters affecting biomass yield and thermal behaviour of *Chlorella vulgaris*, J. Biosci. Bioeng. 111 (2011) 377–382.

[24] M.G. de Morais, J.A. Costa, Carbon dioxide fixation by Chlorella kessleri, *C. vulgaris*, *Scenedesmus obliquus* and *Spirulina* sp. cultivated in flasks and vertical tubular photobioreactors, Biotechnol. Lett. 29 (2007) 1349–1352.

[25] J.W. Rachlin, A. Grosso, The effects of pH on the growth of Chlorella vulgaris and its interactions with cadmium toxicity, Arch. Environ. Contam. Toxicol. 20 (1991) 505–508.

[26] C. Wang, H. Li, Q. Wang, P. Wei, Effect of pH on growth and lipid content of Chlorella vulgaris cultured in biogas slurry, Sheng wu gong cheng xue bao=, Chin. J. Biotechnol. 26 (2010) 1074–1079.

[27] E.W. Becker, Micro-algae as a source of protein, Biotechnol. Adv. 25 (2007) 207–210.

[28] Z. Sun, Y.F. Chen, J. Du, Elevated CO_2 improves lipid accumulation by increasing carbon metabolism in *Chlorella sorokiniana*, Plant Biotechnol. J. 14 (2016) 557–566.

[29] N. Sato, Modulation of lipid and fatty acid content by carbon dioxide in *Chlamydomonas reinhardtii*, Plant Sci. 61 (1989) 17–21.

[30] M. Tsuzuki, E. Ohnuma, N. Sato, T. Takaku, A. Kawaguchi, Effects of CO_2 concentration during growth on fatty acid composition in microalgae, Plant Physiol. 93 (1990) 851–856.

[31] E.Y. Artamonova, T. Vasskog, H.C. Eilertsen, Lipid content and fatty acid composition of *Porosira glacialis* and *Attheya longicornis* in response to carbon dioxide (CO_2) aeration, PLoS One 12 (2017), e0177703.

[32] S.K. Butti, S. Venkata Mohan, Photosynthetic and lipogenic response under elevated CO_2 and H_2 conditions—high carbon uptake and fatty acids unsaturation, Front. Energy Res. 6 (2018) 27.

[33] J. Burger, S. Miyachi, P. Galland, H. Senger, Quantum requirements of photosynthetic oxygen evolution and 77 K fluorescence emission spectra in unicellular green algae grown under low-and high-CO2-conditions, Botanica Acta 101 (1988) 229–232.

[34] H. Jimbo, T. Izuhara, T. Hirashima, K. Endo, Y. Nakamura, H. Wada, Membrane lipid remodeling is required for photosystem II function under low CO2, Plant J. 105 (2021) 245–253.

[35] Z. Yaakob, B.N. Narayanan, S. Padikkaparambil, A review on the oxidation stability of biodiesel, Renew. Sust. Energ. Rev. 35 (2014) 136–153.

[36] K. Yangüez, C. Lovazzano, L. Contreras-Porcia, N. Ehrenfeld, Response to Oxidative Stress Induced by High Light and Carbon Dioxide (CO_2) in the Biodiesel Producer Model *Nannochloropsis salina* (*Ochrophyta, Eustigmatales*), 2015.

[37] B. Ezraty, M. Chabalier, A. Ducret, E. Maisonneuve, S. Dukan, CO_2 exacerbates oxygen toxicity, EMBO Rep. 12 (2011) 321–326.

[38] M. Kirsch, E.E. Lomonosova, H.G. Korth, R. Sustmann, H. de Groot, Hydrogen peroxide formation by reaction of peroxynitrite with HEPES and related tertiary amines. Implications for a general mechanism, J. Biol. Chem. 273 (1998) 12716–12724.

[39] S. Horikoshi, K. Nakamura, M. Kawaguchi, J. Kondo, N. Serpone, Effect of microwave radiation on the activity of catalase. Decomposition of hydrogen peroxide under microwave and conventional heating, RSC Adv. 6 (2016) 48237–48244.

[40] C.M. Beal, I. Archibald, M.E. Huntley, C.H. Greene, Z.I. Johnson, Integrating algae with bioenergy carbon capture and storage (ABECCS) increases sustainability, Earth's Future 6 (2018) 524–542.

[41] M. Rezayian, V. Niknam, H. Ebrahimzadeh, Oxidative damage and antioxidative system in algae, Toxicol. Rep. 6 (2019) 1309–1313.

[42] T. Li, C.T. Li, K. Butler, S.G. Hays, M.T. Guarnieri, G.A. Oyler, M.J. Betenbaugh, Mimicking lichens: incorporation of yeast strains together with sucrose-secreting cyanobacteria improves survival, growth, ROS removal, and lipid production in a stable mutualistic co-culture production platform, Biotechnol. Biofuels 10 (2017) 55.

[43] C. Miyake, F. Michihata, K. Asada, Scavenging of hydrogen peroxide in prokaryotic and eukaryotic algae: acquisition of ascorbate peroxidase during the evolution of cyanobacteria, Plant Cell Physiol. 32 (1991) 33–43.

[44] J.F. Perez-Benito, Iron (III)− hydrogen peroxide reaction: kinetic evidence of a hydroxyl-mediated chain mechanism, J. Phys. Chem. A 108 (2004) 4853–4858.

[45] D. Wei, F. Chen, G. Chen, X. Zhang, L. Liu, H. Zhang, Enhanced production of lutein in heterotrophic *Chlorella protothecoides* by oxidative stress, Sci China C Life Sci 51 (2008) 1088–1093.

[46] F. Shahidi, Y. Zhong, Lipid oxidation and improving the oxidative stability, Chem. Soc. Rev. 39 (2010) 4067–4079.

[47] L. Zhang, C. Liao, Y. Yang, Y.-Z. Wang, K. Ding, D. Huo, C. Hou, Response of lipid biosynthesis in *Chlorella pyrenoidosa* to intracellular reactive oxygen species level under stress conditions, Bioresour. Technol. 287 (2019), 121414.

[48] P.M. Mullineaux, M. Exposito-Rodriguez, P.P. Laissue, N. Smirnoff, ROS-dependent signalling pathways in plants and algae exposed to high light: comparisons with other eukaryotes, Free Radic. Biol. Med. 122 (2018) 52–64.

[49] H.K. Ledford, K.K. Niyogi, Singlet oxygen and photo-oxidative stress management in plants and algae, Plant, Cell Environ. 28 (2005) 1037–1045.