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Chen, Y., Xu, C. and Vaidyanathan, S. orcid.org/0000-0003-4137-1230 (2020) Influence of gas management on biochemical conversion of CO2 by microalgae for biofuel production. Applied Energy, 261. ISSN 0306-2619

https://doi.org/10.1016/j.apenergy.2019.114420

Influence of gas management on biochemical conversion of CO_2 by microalgae for biofuel production

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ABSTRACT

The photosynthetic capacity of algae as a primary producer in nature and the relative ease of its cultivation on a large scale make it attractive to explore opportunities and develop algal technology for simultaneous sequestration of industrial and atmospheric CO_2 (to mitigate climate change), whilst developing sustainable processes for manufacturing renewable fuels alongside biochemicals of value. The development of strategies that maximise algal product yield while optimising the CO_2 gas supply is needed for the appropriate scale-up of algal technology. One of the main targets of this technology is the potential exploitation of flue gases, an inexpensive and carbon-rich source. So far, the growth of microalgae has predominantly been investigated using relatively low CO_2 concentrations that are far from the levels offered by flue gas (6-25%), which are more useful for energy generation with concomitant development of carbon neutral processes. Here, we tested a series of gas supply

strategies to investigate microalgal growth at high CO_2 levels with the aim to improve algal CO_2 fixation and lipid accumulation. Optimal growth of *Nannochloropsis salina* (a marine algae) occurred at 6% CO_2, whilst few cells grew under 20% CO_2. Excess CO_2 resulted in medium acidification, pigment reduction, and growth inhibition. However, the fixation capacity of CO_2 and the production of specific lipids were improved by O_2 removal from the inlet gas by up to 4.8-fold and 4.4-fold, respectively. These parameters were further improved by 72% and 25%, respectively, via a gradual increase in CO_2 concentration. Extremely high CO_2 levels (100%) completely inhibited cell growth, but this effect was reversed when air containing atmospheric CO_2 levels was introduced in place of 100% CO_2. These findings will allow for the future development of more effective strategies using algal biotechnology for producing biofuel while mitigating carbon emissions.

Keywords: Gas management; energy; microalgae; CO_2; lipid; *Nannochloropsis salina*.

1. Introduction

Anthropogenic activities, including the burning of fossil fuels, have greatly contributed to global warming and climate change due to the resulting CO_2 emissions [1]. It is estimated that approximately 33.4 Gt of CO_2 are emitted by fossil fuel power plants each year, accounting for nearly 40% of the total CO_2 emitted into the Earth's atmosphere [2]. Global warming and ocean acidification caused by increasing atmospheric CO_2 levels have already resulted in a series of changes to marine ecosystems, such as mass coral-bleaching episodes in many of the world's reefs [3]. Carbon capture and storage (CCS) technologies and carbon neutral bioenergy have been proposed as counter measures to climate change [4, 5]. Biofuels, such as biodiesel and bioethanol, produced from agricultural crops using existing technologies, cannot sustainably replace fossil-based fuels. However, microalgae have great potential as an alternative feedstock for biofuel production [6]. Microalgae offer a means of

fixing CO_2 and generating biofuels, such as biodiesel, with the scope for developing sustainable processes for biofuel production with minimal recourse to resources. Therefore, microalgae are currently regarded as a promising source of third generation biofuels [7].

Algal photosynthesis is responsible for a large proportion (around 50%) of global carbon fixation and O_2 generation, despite accounting for no more than 1% of photosynthetic biomass. Microalgae can theoretically capture up to 9% of the incoming solar energy via photosynthesis to produce 280 tons of dry biomass ha^{-1} $year^{-1}$, whilst consuming around 513 tons of CO_2 ha^{-1} $year^{-1}$ [8]. The efficiency of the conversion of light energy partially depends on the species characteristics. Many algae use the C3 pathway for the acquisition of dissolved inorganic carbon (DIC: CO_2, HCO_3^-, and CO_3^{2-}) [9]. However, different species of microalgae appear to have different preferences for the carbon species they take up, showing differences in the types and abundance of DIC transporters and carbonic anhydrases (CAs). For instance, *Chlamydomonas reinhardtii* prefers to take up CO_2 under a majority of experimental conditions [10, 11]. *Thalassiosira pseudonana* prefers CO_2, while *Phaeodactylum tricornutum* prefers HCO_3^- [12, 13]. The transfer and uptake of different DIC species in microalgae primarily depends on the microalgae species and the concentration of CO_2. Three major strategies, HCO_3^- transportation, conversion of HCO_3^- into CO_2, and the direct diffusion of CO_2 are known to be employed [14]. Different methods, such as genetic engineering and random mutagenesis, have been developed to modify the genes and the associated enzymes in order to improve of the rates of growth and CO_2 fixation [15]. In addition, domestication or adaptive laboratory evolution can also be used as strategies to enhance microalgal CO_2 fixation, particularly for the fixation of CO_2 from CO_2-rich flue gases [15].

Apart from the species-related factors, there are many cultivation-related factors that can influence microalgae carbon fixation and product yield. Most studies have focused on the nutrients in the medium or other cultivation conditions, such as light, while paying little attention to CO_2 and/or O_2 supply [16, 17]. O_2 accumulation in closed systems is a serious problem since O_2 can be supersaturated to concentrations as high as 400% in these systems [18]. These supersaturated O_2 concentrations not only inhibit the carboxylase activity of Rubisco, but also strengthen photorespiration. Even in those studies with increased CO_2, the O_2 content in the inlet gas was rarely considered. As such, air is widely used to mix with CO_2 to obtain the desired CO_2 levels [19]. However, the influence of atmospheric O_2 on algal growth is insufficiently characterised and often ignored.

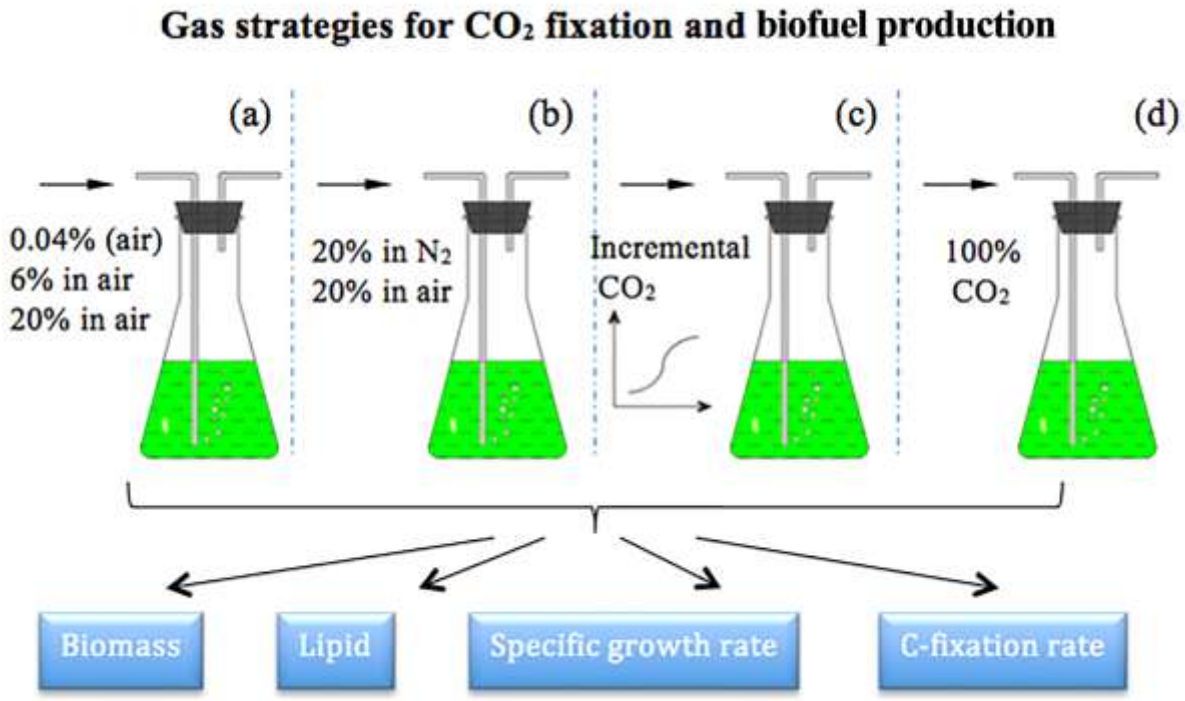
Increasing the CO_2 concentration can affect both lipid productivity and biodiesel quality. Many microalgae, such as *Chamydomonas* sp. [20] and *Nannochloropsis* sp. [21], have been investigated for their ability to capture CO_2 and biofuel simultaneously. *Nannochloropsis* sp. is a yellow green microalgae found in marine habitats that has been found to grow fast and accumulate lipids to high levels [19]. Remarkably, in air containing 1% CO_2, the maximum biomass productivity of *Nannochloropsis* sp. achieved was 0.9 g L^{-1} d^{-1} under high light intensity and complete medium, whilst lipid productivity was able to reach up to 0.297 g L^{-1} d^{-1} under high light intensity and low nitrogen levels [19]. It was found that the CO_2 levels from 2.5 to 10.0% could improve the quality of algal biodiesel to meet the fuel-quality standards [22]. This is due to the fact that chain size and saturation were re-balanced towards the enhancement of biodiesel ignition and cold-flow properties [22]. Moreover, due to their high lipid content, both whole alga and lipid-extracted residues of *Nannochloropsis salina* have been tested in an attempt to produce methane via anaerobic digestion [23]. However, an excess of CO_2 usually exerts a stress on cells [24]. A previous study by the authors showed

that the growth of *N. salina* could be promoted by increasing the CO_2 concentration to 6% (in air). However, growth was highly inhibited by a CO_2 concentration of over 20% [25].

The present study aimed to improve our understanding of the influence of CO_2 supply at high concentrations and that of O_2 on *N. salina* growth for mitigation of CO_2 emission and for biofuel production,. Despite many reports on microalgal CO_2-acclimation/adaptation mechanisms under low CO_2 conditions, such as CO_2-concentrating mechanisms (CCM) for growth in air, our knowledge on carbon uptake by microalgae under high CO_2 conditions remains limited [26]. A better understanding of microalgal growth under high CO_2 conditions will enable the development of the appropriate strategies for applications in energy generation and carbon mitigation.

2. Materials and Methods

Scheme 1 shows the experimental designs of different gas strategies for CO_2 fixation and biofuel production by microalgae. The details for each experimental set-up are provided in the following sections.



Scheme 1. Strategies of gas supply tested in the investigation to study the influence of constant CO_2 in the feed gas (a); O_2 in the feed gas at 20% CO_2 (b); incremental CO_2 in the feed gas (c); and extremely high CO_2 (d).

2.1 Microalgal cultivation and growth monitoring

The phototrophic species of *Nannochloropsis salina* has been found to grow fast while accumulating lipids, making it an ideal candidate for CO_2 sequestration, as well as a source for alternative biofuel production [27, 28]. This species was cultivated in f/2 medium in Erlenmeyer flasks. Three biological replicates were included in each experiment. The cultures were exposed to a light intensity of ~70 µE m^{-2} s^{-1} by using fluorescent lamps, aerated, and incubated at 24 ± 2ºC. The cells were then harvested by centrifugation twice (3,000 ×g for 3 min coupled with 8,500 ×g for another 5 min). The resulting supernatant was used for DIC measurement. The cell pellets were frozen at –20ºC until further analysis. The optical density of cultures at 680 nm (OD_{680}) was measured to monitor the growth curve using a UV/Visible spectrophotometer (Ultrospec 2100 Pro, GE Healthcare). The OD_{680}

obtained was used to deduce the dry cell weight (DCW) on the basis of the following pre-calibrated equation,

$$y = 0.193x + 0.0078 \qquad R^2=0.9914 \qquad (1)$$

where y and x are biomass concentration (g L^{-1}) and OD_{680}, respectively. The specific growth rate (μ, d^{-1}) was measured as follows,

$$\mu = \frac{\ln(W_1/W_0)}{\Delta t} \qquad (2)$$

where W_0 and W_1 are the initial and end cell density, respectively, and Δt is the cultivation time. The resulting DCW was then used for the determination of the CO_2 fixation rate (F_{CO2}, g L^{-1} d^{-1}) according to the following equation, which was derived from the typical molecular formula of microalgal biomass, $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ [29]

$$CO_2 \text{ fixation rate } (Fco_2) = 1.88 \times \text{biomass productivity (BP)} \qquad (3)$$

Since the final CO_2 feeding depends on both the CO_2 concentration (%) and the volumetric gas flow rate (vvm), the real CO_2 loading of the culture (L_{CO2}, L L^{-1} min^{-1}) was defined as follows,

$$L_{CO2} \text{ (L L}^{-1} \text{ min}^{-1}) = CO_2 \text{ concentration (\%)} \times \text{volumetric gas flow rate (vvm)} \qquad (4)$$

where L_{CO2} indicates the real volume of pure CO_2 loaded per volume culture per minute. To determine the effect of CO_2 loading on lipid production, L_{CO2} was used to determine the specific lipid production as follows,

$$S_{lip}\ (\text{g min } L^{-1}\ d^{-1}) = \frac{(L_2 - L_1)}{\Delta t \cdot L_{CO2}} \qquad (5)$$

where S_{lip} is the specific lipid production on the basis of CO_2 loading, L_1 and L_2 are the initial and end lipid content (g L^{-1}), respectively, and Δt is the cultivation time (d). In addition, dissolved inorganic carbon (DIC) was measured (as detailed below) to obtain an indication of the inorganic carbon available to the organism.

The f/2 medium used in the cultivations consisted of (per litre) 33.6 g artificial seawater salts (Ultra Marine Synthetica Sea Salt, Waterlife), 75 mg $NaNO_3$, 5.65 mg $NaH_2PO_4 \cdot 2H_2O$, 1 ml trace elements stock, and 1 ml vitamin mix stock. The trace elemental solution (per litre) included 4.16 g Na_2EDTA, 3.15 g $FeCl_3 \cdot 6H_2O$, 0.18 g $MnCl_2 \cdot 4H_2O$, 10 mg $CoCl_2 \cdot 6H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$, 22 mg $ZnSO_4 \cdot 7H_2O$, and 6 mg $Na_2MoO_4 \cdot 2H_2O$. The vitamin mix solution (per litre) included 100 mg vitamin B1, 0.5 mg vitamin B12, and 0.5 mg biotin.

2.2 Analysis of DIC species

The speciation of the dissolved inorganic carbon and the determination of its abundance were used to estimate the total available inorganic carbon in the culture medium at a given time point. To this end, a simplified "back-titration" technique was employed [25]. This method was based on the principle that the total carbonates (TCO_2) can be derived from carbonic alkalinity (CA). Briefly, the sample pH was adjusted to the bicarbonate equilibrium point (pH_{HCO3}) and subsequently subjected to two titrations, in tandem. The pH was first titrated to

around 3.5 by adding 0.1 M (or 0.5 M) HCl, the amount of which was recorded for calculating the total alkalinity (TA). This is followed by bubbling the sample with nitrogen to remove CO_2, and a second titration to the pH_{HCO3} with the addition of 0.1 M NaOH (or 0.5 M). The acid or base equivalent consumed was used to determine the non-carbonate alkalinity (NA). The alkalinities of TA and NA were calculated using the following equation,

$$A = 10^6 \times C_{H/OH} \times V_{H/OH} / m_o \qquad (6)$$

where A is the alkalinity (μmol kg^{-1}), $C_{H/OH}$ (mol L^{-1}), and $V_{H/OH}$ (L) are the concentration and volume of acid or base, and m_o (kg) is the mass of samples. The difference between TA and NA is CA (i.e. TA–NA), which was used to calculate TCO_2. The data (TCO_2, pH, temperature, and salinity) was fed into the CO2SYS program [30] to calculate the concentration of individual DIC species.

2.3 Analysis of cellular bio-components

The biochemical composition of cells, including pigments, carbohydrates, proteins, and lipids, was measured using a simultaneous assay [31]. Briefly, the harvested cell pellets were ground by glass bead-beating in an alkaline solution using a cell disruptor (DISRUPTOR GENIE®, USA). An aliquot of the sample was used for the carbohydrate assay; meanwhile, the remaining sample was heated at 100ºC for 30 min. This was followed by cooling the mixture to room temperature, after which an aliquot of the saponified sample was taken for the protein assay. Another aliquot of the sample was mixed and vortexed with an organic solvent (chloroform: methanol, 2:1). After centrifuging this mixture, the lower organic phase was used for the total carotenoids and lipid assay, while the supernatant aqueous phase was

used for the chlorophyll assay. Alternatively, a single assay of lipids in other cases was conducted by using a simplified version of the above method [32].

2.4 Influence of a constant CO_2 supply in the feed gas, at different concentrations

In order to determine the CO_2 tolerance and fixation capability of *N. salina*, the cultures were aerated in three constant CO_2 concentrations: 0.04% (air), 6%, or 20% (CO_2 in air). For 6% and 20%, pure CO_2 (100%) was mixed with air to obtain the desired CO_2 concentration by controlling the flow rates using flowmeters. The flow rate of the mixed gas was fixed at a volume ratio of 0.5 (vvm) between the gas (L min^{-1}) and the culture (L). The corresponding L_{CO2} was 2×10^{-4}, 0.03, and 0.1 L L^{-1} min^{-1} for 0.04% (air), 6%, and 20% CO_2, respectively. The corresponding DICs were also monitored.

2.5 Influence of photorespiration

Although the O_2 concentration in the culture can be reduced to avoid oversaturation by sparging a gas, the presence of O_2 in the feeding gas has only been studied scarcely. This may explain why *N. salina* hardly grew under 20% CO_2 in the presence of O_2 (as shown in section 3.1), as reported in our previous investigation [25]. To determine the influence of O_2 on photorespiration and C-fixation, CO_2 was mixed with either air or pure N_2. The flow rates of these gases were controlled using flowmeters to obtain a constant concentration of CO_2 at 20% in the supplied gas. The flow rate of the mixed gas was fixed at a volume ratio of 0.5 (vvm) between the gas (L min^{-1}) and the culture (L), obtaining a constant L_{CO2} of 0.1 L L^{-1} min^{-1}.

2.6 Influence of incremental CO_2 levels

A high CO_2 concentration usually results in the overfeeding of algal cells due to the excess CO_2 supplied, particularly at the beginning of the cultivation period with dilute biomass, leading to growth inhibition [33]. A process of acclimation from a low to a high CO_2 levels may be used to soften the inhibition. To this end, the cultures were sparged with incremental levels of CO_2 over time. Pure nitrogen (100%) was mixed with pure CO_2 (100%) to obtain the desired level of CO_2. The low rate of the mixed gas was fixed at a gas to liquid ratio of 0.5 (vvm), while the CO_2 concentration was adjustable during cultivation. Nitrogen was used to adjust the CO_2 concentration instead of air in order to determine the influence of CO_2 and exclude the influence of oxygen in the air.

2.7 Influence of 100% CO_2

Although an excess supply of CO_2 inhibited the growth of algae, it remains unclear whether this inhibition was fatal or temporary. To examine this, the culture was supplied with either an extremely high CO_2 concentration (100%) or a low concentration of 0.04% (air). At a high CO_2 concentration, a low flow rate of the gas was controlled within the range of 0.02–0.04 vvm (L_{CO2} ranging from 0.02–0.04 L L^{-1} min^{-1}). The inoculum for this experiment was obtained from a culture where the alga was pre-cultured and grew well in 20% CO_2 (in the absence of O_2).

3. Results and Discussion

3.1 Influence of increased CO_2 concentration at a constant gas volumetric flow rate

To determine the capacity of the algal species to sequester CO_2 and produce lipids, the *N. salina* cultures were sparged with a gaseous mixture of air and CO_2 at three constant volumetric proportions: 0.04% (only air), 6%, and 20% CO_2, forming volumetric CO_2 loadings (L_{CO2}) of 2×10^{-4}, 0.03, and 0.1 L L^{-1} min^{-1}, respectively. These experimental

conditions have been described in a previous investigation [25], however, the results were re-interpreted by updating the data with an emphasis on the influence of an increased concentration of CO_2 on CO_2 fixation, pigments, and lipid production. The growth curves and variations in pH, pigments, DIC species, and lipids are presented in Fig. 1 and Fig. 2.

The results indicate that the fastest growth was obtained at a CO_2 concentration of 6% (Fig 1a). The maximum specific growth rates for 0.04% (air), 6%, and 20% were 0.26 (± 0.03), 0.34 (± 0.12), and 0.11 (± 0.03) d^{-1}, respectively (Fig 1c). A further increase in CO_2 from 6% to 20% significantly inhibited the algal growth ($P<0.05$, t-test). The average CO_2 fixation rates during the active growth phase (after the fourth day) for 0.04% (air), 6%, and 20% were 0.045 (± 0.015), 0.062 (± 0.030), and 0.006 (± 0.004) g L^{-1} d^{-1}, respectively (Fig 1d). The growth rate of *N. salina* under air conditions was similar to that reported for *Nannochloropsis* sp. [21], however, the optimal growth of the latter was found to be at 15% CO_2 with a higher growth rate than that of the former at 6%. The initial cell concentration reached 0.17 g L^{-1} for *Nannochloropsis* sp., whereas it only reached 0.05 g L^{-1} for *N. salina* in the present study. This may have been the cause for the differences observed between the two species. In addition, the volumetric CO_2 loading (L_{CO2}) for *Nannochloropsis* sp. was 0.015 L L^{-1} min^{-1} (15% CO_2, 0.1 vvm), only half of the 0.03 L L^{-1} min^{-1} (6% CO_2, 0.5 vvm) for *N. salina* in this study. With 0.04% (air), an increase in pH was observed (Fig. 1b) due to chemical and biochemical reasons. Chemically, the photosynthetic consumption of CO_2 pushes the equilibrium towards a decrease in $[H^+]$, according to the following reaction,

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \qquad (7)$$

The biochemical reason is the light-dependent alkalisation of the medium caused by the utilisation of HCO_3^-, which may occur either by direct uptake or by the conversion of HCO_3^- to CO_2 and OH^- (via the catalysis of carbonic anhydrase) external to the plasmalemma [34]. On the contrary, the pH in the medium dropped quickly to around 6.5 and 5.6 at 6% and 20% CO_2, respectively, due to the increasing build-up of CO_2 in the medium that resulted from a higher supply of CO_2, as compared to its uptake by algae, driving the equilibrium reaction (6) forward.

Although pigments are responsible for light harvesting, the pigment curves were not completely in accordance with the growth curves. Interestingly, cells growing at 0.04% (air) accumulated higher pigments but a lower biomass than at 6% CO_2. This is primarily due to the carbon deficiency at 0.04% CO_2. The pigment production and content (%) at 6% CO_2 was close to that at 0.04% on the first five days, and then decreased (Fig 1e–h). The decrease in the pigment production with the increase in CO_2 concentration is likely associated with a decrease in the pH. Similar results have been reported, showing that the production of chlorophyll *a* and carotenoids in *Dunaliella bardawil* and *Chlorella ellipsoidea* decreased with pH reduction in the range of 4–7.5 [35]. This suggests that the pigments at 6% CO_2 had a higher conversion efficiency of light energy by transferring the excitation energy to fix the increasing availability of CO_2. At higher CO_2 levels (6%), when the dissolved carbon dioxide was relatively sufficient, or even in surplus than required for growth of the algae, feedback inhibition minimised the build-up in intracellular pigment levels. Therefore, the increased growth caused by the increase in CO_2 concentration (e.g. from 0.04% to 6%) appears to result from the enhancement of the energy conversion efficiency by the pigments. A further increase in CO_2 concentration, such as up to 20%, would acidify the medium and inhibit the pigment production, leading to a lower growth rate.

In most studies, DIC speciation and the abundance of carbon species are not reported, and only the overall CO_2 levels in the supplied gas are given [36]. Monitoring the abundance of dissolved inorganic carbon species facilitates our understanding of the effects of different gas compositions on algal CO_2 fixation and the intracellular carbon flux. The profiles of the DIC species and the lipid accumulation are shown in Fig. 2. The total inorganic carbon (TCO_2) was in line with the corresponding CO_2 inlet concentration, which was nearly constant due to continuous aeration. This is due to the fact that a DIC equilibrium was reached between the gas and the medium. The concentrations of TCO_2 for 0.04%, 6%, and 20% CO_2 were around 0.067 (± 0.007), 0.172 (± 0.017), and 0.390 (± 0.015) g L^{-1} CO_2 (e), respectively. Given that the fastest CO_2 fixation rate of 0.062 g L^{-1} d^{-1} was obtained at 6% CO_2, these TCO_2 would require around 1 day, 2.8 days, and 6.3 days to be fixed by *N. salina*, not accounting for gas escape. As such, for large-scale algal farming, the amounts of CO_2 captured by the medium are not insignificant. It is worth noting that the estimation of the CO_2 fixation rate in the present study was based on the dry biomass, which depends on the element composition of cells and varies with species and growth conditions. Moreover, many algae have been found to produce extracellular substances [37], which are not included in the biomass measurement, leading to an underestimation of the CO_2 fixation.

The concentration of carbonate (CO_3^{2-}) is expected to have a lower influence on the accumulation of biomass and lipids since it is not a significant component at 6% and 20% CO_2. Both CO_2 and bicarbonate (HCO_3^-) can be utilised through interconversions by carbonic anhydrases [34], where the increase in these two species, as happens at 6% CO_2 sparging, elevates growth. However, the concentrations of these two species were found to be the highest at 20% CO_2, despite the fact that growth was found to be inhibited at this

concentration due to excess carbon and a decreased pH. Apart from the low pH and low pigment content, oxidative stress could be another reason for the inhibition of growth [38]. It was found that the acidification of the medium and a higher CO_2 concentration may promote the generation of reactive oxygen species, including H_2O_2, phenolic compounds, and lipoperoxides [38]. However, *N. salina* is able to induce antioxidant enzymatic activities, including that of catalase, ascorbate peroxidase, and peroxiredoxine, to mitigate this oxidative stress [38].

The average specific lipid productivities at 0.04%, 6%, and 20% CO_2 was around 31.39 (± 14.80), 0.60 (± 0.15), and 0.018 (± 0.017) g min L^{-1} d^{-1}, respectively. This suggests that the efficiency of CO_2 conversion to lipids declined with the increase in CO_2 loadings. However, a higher lipid content was observed at 6% and 20% CO_2 than at 0.04%, indicating that increasing the CO_2 input can induce lipid accumulation. Lipids and carbohydrates are both energy and carbon reserves, however, the former has a higher energy density than the latter (38 kJ g^{-1} for lipids compared to 17 kJ g^{-1} for carbohydrates) [36]. From this perspective, an increase in CO_2 concentration would increase not only the CO_2 fixation but also the efficiency of the conversion and storage of light energy.

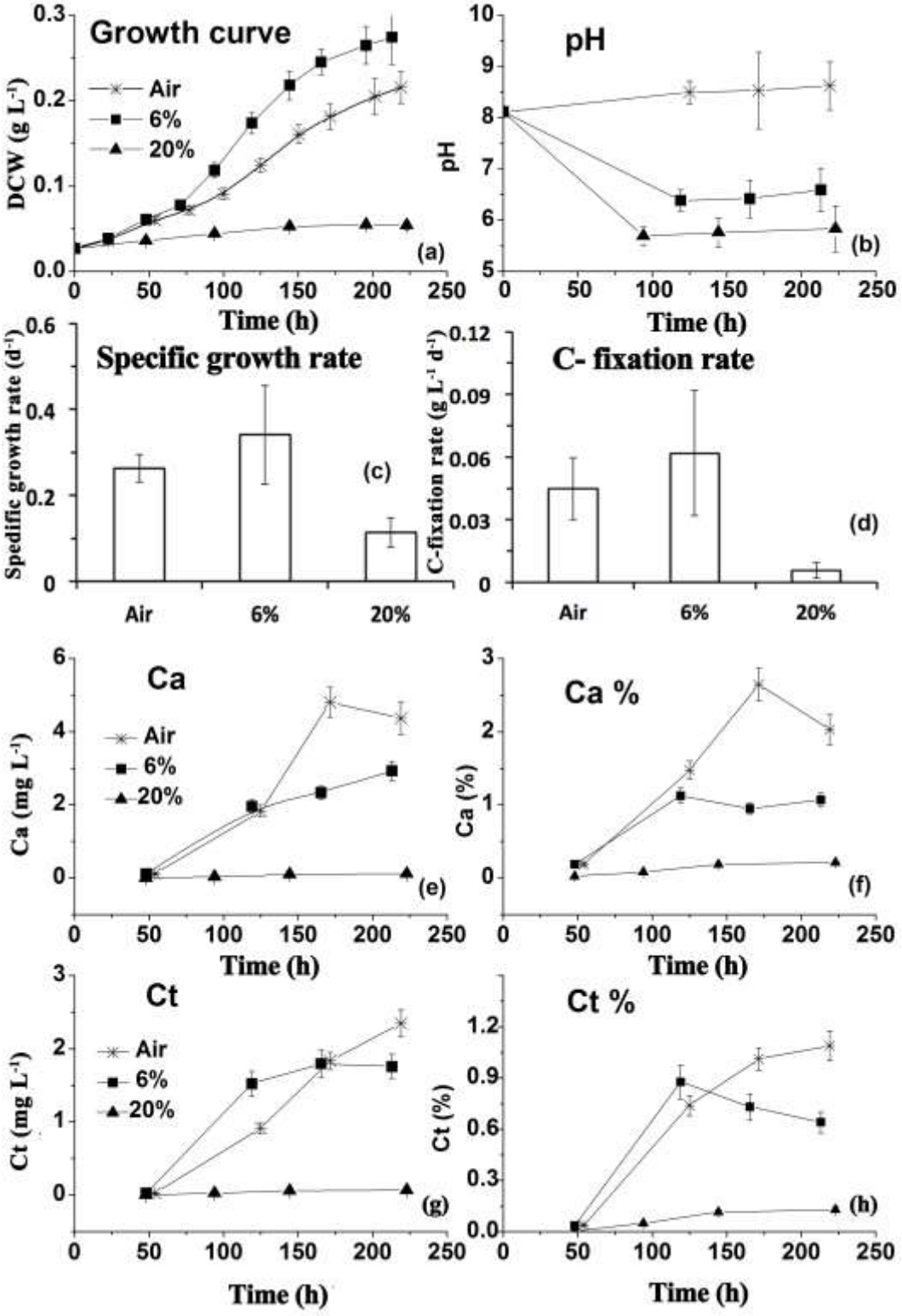


Fig. 1. Effects of different air/CO_2 mixtures (air, 6%, 20% CO_2) at a constant gas flow rate on the growth (a), pH (b), average specific growth rate during active growth (c), maximum CO_2 fixation rate (d) and pigment levels (e-h) in *N.salina* cultivation. Mean of three biological replicates are plotted with error bars representing standard error about the mean. Ca:

Chlorophyll *a*; Ct: total carotenoids; Ca%, Ct% are the corresponding mass content on the basis of DCW.

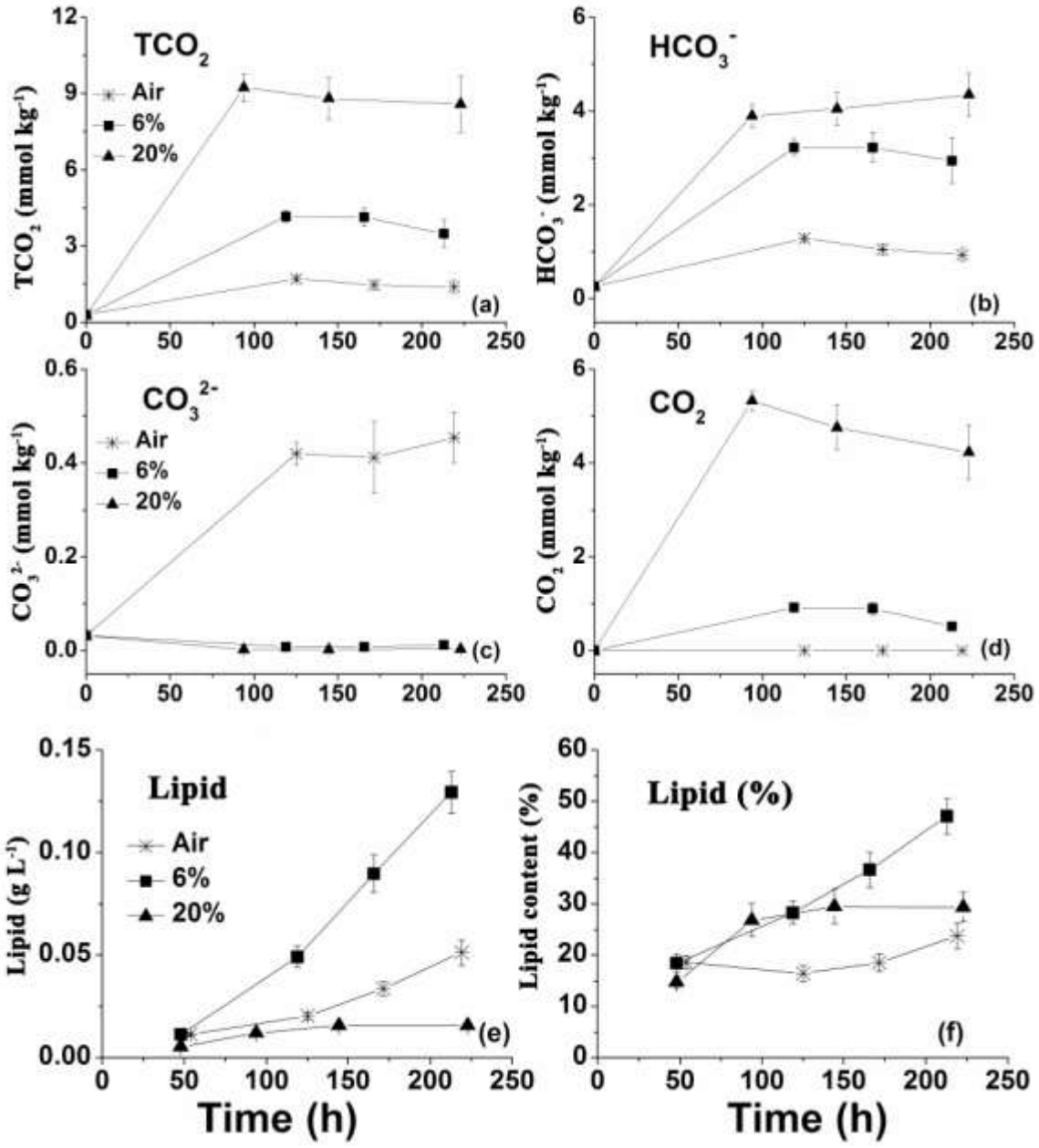


Fig. 2. Effects of different air/CO_2 mixtures (air, 6%, 20% CO_2) at a constant gas flow rate on the medium DIC species and lipids production in *N.salina* cultivations. Mean of three biological replicates are plotted with error bars representing standard error about the mean.

3.2 Influence of photorespiration

Oxygen present in the inlet gas can be a potential factor limiting cell growth [39]. This is related to the process of photorespiration, which involves the consumption of fixed carbon and has been regarded as a waste route during photosynthesis. To determine the influence of oxygen in the feeding gas at 20% CO_2, the *N. salina* culture was supplied alternately with or without oxygen. For the oxygen free condition, nitrogen was used in place of air in the mixture with 100% CO_2 to obtain a CO_2 concentration of 20%.

The growth curves and lipid production are displayed in Fig. 3. It can be clearly seen that the cells were able to grow at 20% CO_2 devoid of oxygen (Fig. 3a). However, once the feeding gas was switched to 20% CO_2 in air, the growth rate was inhibited immediately, even becoming negative at times. When the gas was turned back to 20% CO_2 in nitrogen, the growth rate was recovered. The average specific growth rates in the presence of O_2 and N_2 were 0.012 (± 0.025) and 0.077 (± 0.018) d^{-1}, whilst the average CO_2 fixation rates were 0.005 (± 0.009) and 0.029 (± 0.004) g L^{-1} d^{-1}, respectively. The two-tail t-test showed significant differences between these two scenarios for both specific growth rates and the CO_2 fixation rate. Therefore, the capacity of CO_2 fixation was enhanced by 4.8-fold after the removal of O_2. The average specific lipid productivities in the presence of O_2 and N_2 was 0.02 (± 0.025) and 0.088 (± 0.034) g min L^{-1} d^{-1}, respectively, indicating an increase of 4.4-fold in the CO_2 conversion efficiency to lipids after the removal of O_2. Furthermore, it was observed that the removal of O_2 also improved the lipid percentage by 32.7% (Fig. 3b) compared to 30% (Fig. 2h) of the maximum lipid with 20% CO_2 in air. There appears to be a lag in the response of lipids (% content) in the first few days compared to the biomass.

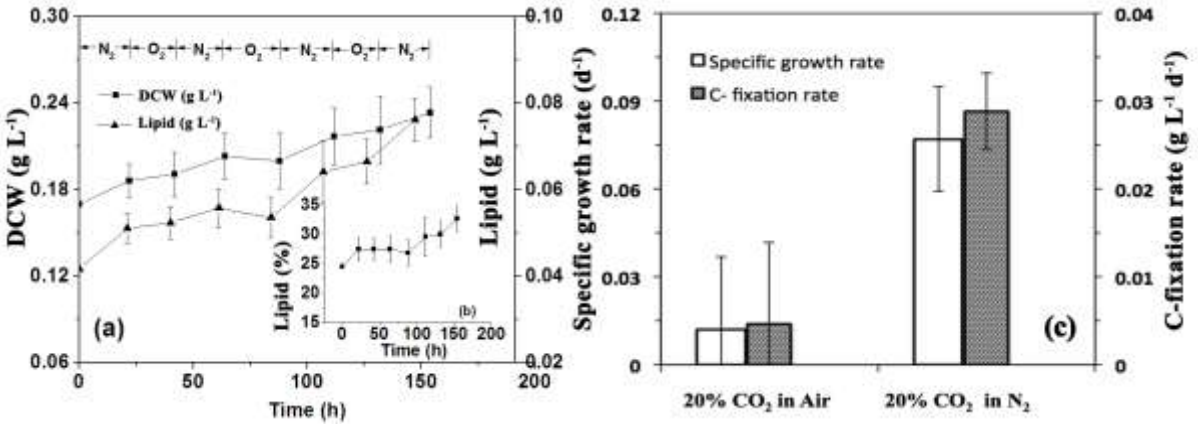


Fig. 3. Influence of oxygen in 20% CO_2 in the presence of oxygen (mixed with air (O_2)), and the absence of oxygen (mixed with nitrogen (N_2)), on the growth/lipid production (a), lipid content (%) (b), average specific growth rate and CO_2 fixation rate (c) of *N. salina*.

This experiment shows that oxygen is indeed a limiting factor for the growth of algae. As such, the flue gases can be expected to be an ideal CO_2-enriched source since most of the oxygen has been consumed for combustion. Without oxygen (or its presence in only trace amounts), *N. salina* is able to grow at a CO_2 level as high as 20% and can be expected to sequester CO_2 from exhaust gases. The increased ratio of CO_2/O_2 should help promote the carboxylase activity of Rubisco and inhibit its oxygenase activity, leading to suppressed photorespiration and enhanced net photosynthesis [40]. Moreover, the presence of CO_2 in the feed gas seems to be required to suppress photorespiration, since enhanced photorespiration has been observed only when N_2 was supplied. This has been noted with two marine microalgae, *Tetraselmis gracilis* and *Phaeodactylum tricornutum*, which were aerated with only N_2 and without CO_2, showing a higher photorespiratory flux than that in the cultures aerated with atmospheric air [41]. This is due to the fact that, under N_2 supply without CO_2, O_2 derived from photosynthesis can activate Rubisco oxygenase activity for photorespiration [41]. This is a noteworthy point since, in addition to expelling photosynthetically derived O_2 out of the algal culture, sufficient carbon supplementation is essential. The present study

shows that a high CO_2 concentration (20%) with a medium O_2 concentration (16.76%, i.e. 20.95% × 80%) inhibited the growth of algae. Another scenario is that of a high O_2 concentration with a medium CO_2 concentration. It was found that when the O_2 increased to 84%, an increase of CO_2 from 0.7% to 2% did not exhibit any positive effects on the growth of the algae [42]. The inhibitory effect resulting from an increased O_2 was primarily caused by photorespiration. This is perhaps indicative of the role of pH, when at high CO_2 concentration, such as at 20% CO_2, the pH of the medium drops to 5.5 (Fig. 1). At these pH levels, the absence of dissolved O_2 in the medium (e.g. when 20% CO_2 in nitrogen is supplied) plays a part in facilitating carbon uptake.

3.3 Influence of incremental CO_2 levels

The above experiment shows that the growth of *N. salina* was highly inhibited at 20% CO_2 when oxygen is present, the removal of which could enhance growth as well as lipid accumulation. Another strategy exists that may be able to further improve the growth of *N. salina* under a high CO_2 concentration, i.e. a gradual increase in CO_2 levels. To this end, the cells of *N. salina* were grown initially at 0.04% (air) during lag phase, followed by the supply of 6% CO_2 in nitrogen, 20% CO_2 in nitrogen, and 30% CO_2 in nitrogen, in tandem (Fig. 4). The fastest growth rate was obtained at 6% CO_2. At 20% CO_2, the growth rate remained similar to that at 6%, with only a slight decrease. The growth then plateaued when the CO_2 level was increased to 30%, indicating that growth was limited by the stress induced by a high CO_2 concentration. Equilibrium appeared to have been reached at the level between the oxidative stress and the antioxidant protection. The specific growth rates at 0.04% (air), 6%, and 20% CO_2 were 0.316 (± 0.082), 0.532 (± 0.101), and 0.173 (± 0.070) d^{-1}, respectively. The maximal CO_2 fixation rate was 0.079 (± 0.012) g L^{-1} d^{-1} with 6% CO_2. In contrast, the average CO_2 fixation rate with 20% CO_2 in nitrogen was 0.050 (± 0.016) g L^{-1} d^{-1}, which was

72% higher than that with constant 20% CO_2 in nitrogen (0.029 (± 0.005) g L^{-1} d^{-1}), and was 733% higher than at constant 20% CO_2 in air (0.006 (± 0.004) g L^{-1} d^{-1}). The specific lipid productivity at 20% CO_2 by this incremental method was found to be around 0.11 g min L^{-1} d^{-1}, which was 125% higher than that at constant 20% CO_2 in nitrogen (0.088 g min L^{-1} d^{-1}), resulting in an overall increase of 5.5-fold compared to 20% CO_2 in air (0.02 g min L^{-1} d^{-1}). Both the amount and content of lipids (%) increased during 6% and 20% CO_2 aeration, but decreased when the gas supply was switched to 30% CO_2 in nitrogen. The extremely high concentration of CO_2 had a negative effect on both lipid production and the biomass.

While the effect of CO_2 concentration on microalgal growth has been widely studied, most of these studies have employed continuous or intermittent sparging at fixed CO_2 levels in the cultivations. For instance, *N. oculata* NCTU-3 was found to produce the maximal biomass and lipid productivity in a semi-continuous system with 2% CO_2 aeration [43]. The maximum rate of CO_2 fixation by *Chlorella vulgaris* P12 reached up to 2.22 g L^{-1} d^{-1} with 6.5% CO_2 at a flow rate of 0.5 vvm (i.e. 0.033 L L^{-1} min^{-1} of L_{CO2}) after seven days of cultivation at 30°C [44]. On the basis of the optimum CO_2, an increase in CO_2 levels causes stress to the cells and limits their growth rate. This is due to the fact that the algal cells do not require much CO_2, especially at the beginning of the cultivation period when the cell numbers are low. Nevertheless, their need for CO_2 increases over time as biomass accumulates. The present study is in agreement with a report [45] that indicated that the tolerance and fixation capacity of CO_2 for *Chlorella vulgaris* UTEX259 was enhanced by a progressive increase in CO_2 concentration. Therefore, in practice, it is recommended that the inlet flow rate of CO_2-enriched gas increase incrementally over time to obtain varying levels of CO_2 at different stages. This allows the cells to gradually acclimate to the high levels of CO_2 and meet their increasing demand for CO_2 caused by an increased cell density.

In fact, phototrophic microalgae have developed special mechanisms to acclimate and adapt to variations in both O_2 and CO_2. Although many reports have been published on CO_2-acclimation/adaptation mechanisms under limited CO_2 conditions, such as CO_2-concentrating mechanisms (CCM), our knowledge of these mechanisms under high CO_2 conditions is limited [26]. Even in reports using high CO_2 conditions, the focus has been mainly on lipid biosynthesis for biofuel production, as well as on how to obtain an optimal productivity [46], rather than on the underlying mechanisms of the algal species' behaviour or on CO_2 uptake. The microalgae that show high-CO_2-acclimation/adaptation, such as *Synura petersenii, Synura uvella*, and *Tessellaria volvocina*, are usually also acclimated to low pH environments where they are isolated and where only CO_2 is predominant as a substrate for photosynthesis [47]. These species appear to lack CCM, as no external carbonic anhydrase on the cell surface is detected and no bicarbonate uptake ability is known. However, they have the high CO_2-affinity of Rubisco and are able to maintain pH homeostasis [48]. It is worth pointing out that CO_2 enrichment is not always conducive to increased algal growth, as for those fully low-CO_2-acclimated species, CCM can be induced to enable growth with a near-maximum growth rate under air-CO_2 levels [49]. However, growth can still be considerably enhanced when the cell density is high due to their increased requirements for CO_2 availability [26], indicating that a progressive increase in CO_2 with respect to cultivation time provides a useful strategy for photosynthetic species to avoid carbon deficiency regardless of low- or high-CO_2-acclimated microalgae.

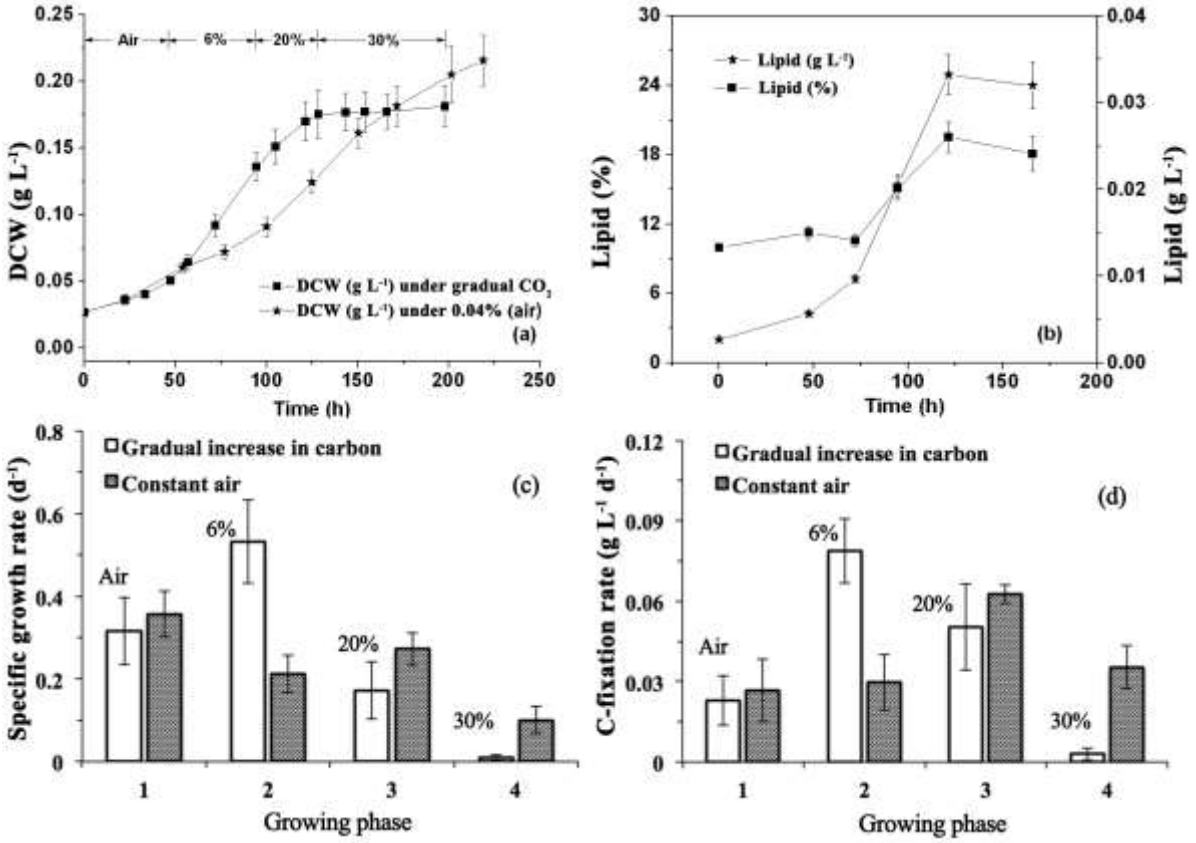


Fig. 4. Effect of gradual increase in CO_2 concentration from air to 30% CO_2 in nitrogen on the growth (a), lipid production/content (%) (b), specific growth rate (c) and CO_2 fixation rate (d) in *N.salina* cultivation. Mean of biological triplicates are plotted with standard error about the mean as error bars.

3.4 Influence of 100% CO_2 feed

The tolerance and fixation capacity of CO_2 can be improved by controlling the gas supply, such as O_2 removal, and appropriate acclimation. However, an extremely high CO_2 concentration can be destructive to the cells. To investigate this, the culture was exposed to 100% CO_2. The resultant growth and pH curves are shown in Fig. 5. Since the CO_2 concentration was too high, the flow rate of CO_2 was controlled at a low level to reach a low volumetric ratio of gas over liquid. As can be seen from the results, the cells only grew on the first day at 10 mL min^{-1} of gas flow rate (i.e. 0.04 L L^{-1} min^{-1} of L_{CO2}), perhaps due to the

pre-cultivation at 20% CO_2 enabling them only a limited acclimatization to the high CO_2 levels. On the second day, the harmful effects of extreme CO_2 levels started to be show as the specific growth rate became negative (–0.183 d^{-1}). A decrease in the flow rate of CO_2 to 6 mL min^{-1} (i.e. 0.024 L L^{-1} min^{-1} of L_{CO2}) slowed the decrease in growth, indicating that the stress was relieved slightly.

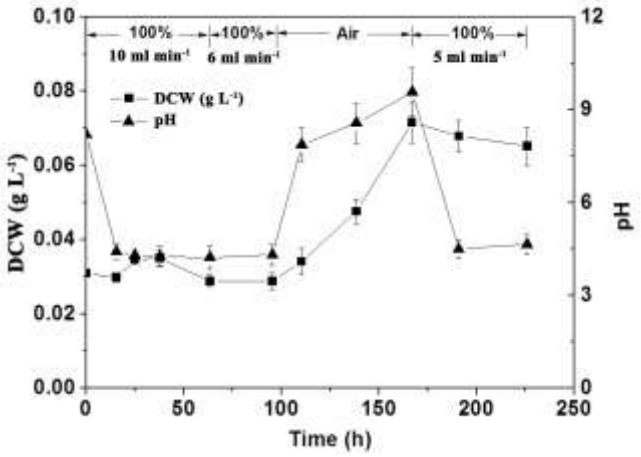


Fig. 5. Effect of 100% CO_2 on the growth and medium pH of *N.salina*.

When the feeding gas was switched to air (0.04% CO_2), a rise in the growth was observed as the stress was removed. This indicates that the inhibitory effect of 100% CO_2 was not fatal to all the cells during the first four days, although a few cells may have died. An aeration period of around three days was followed by a switch to 100% CO_2, which significantly inhibited the growth of the cells again, although the gas flow rate was reduced to 5 mL min^{-1} (i.e. 0.02 L L^{-1} min^{-1} of L_{CO2}), even lower than the optimal CO_2 concentration of 6%, as shown in section 3.1 (0.03 L L^{-1} min^{-1} of L_{CO2}). This indicates that the inhibition of growth is caused not only by the high CO_2 loading, but also by the high percentage of CO_2 (and the resultant change in pH). Significant variations in pH were observed during the alternation of CO_2 and air. When using air (0.04% CO_2), the pH increased from 4.5 to over 9, with an increase in biomass. At 100% CO_2, the pH dropped down to as low as 3.5. This low pH was in favour of

the dissolved inorganic carbon existing in the primary form of aqueous CO_2 and should have prevented cells from producing pigments for a light harvest.

The extremely high CO_2 concentration of 100% inhibited the growth of cells, resulting in a decreased biomass despite a low volumetric ratio. However, this did not kill all the cells, and the growth of cells was recovered by decreasing the CO_2 concentration to relieve stress. The adverse effect of a high CO_2 content on algal growth has been observed in most of the species studied [50, 51]. However, fewer studies have reported algal growth at 100% CO_2. Very few strains are able to survive at such extreme levels of CO_2. *Chlorella* sp. T-l [52] and *Desmodemus* sp. [53] are two strains that have been shown to grow at 100% CO_2. As reported in the present work, although the growth of *Scenedesmus* was completely inhibited at 100% CO_2, this effect was reversed (i.e. growth resumed) when the concentration of CO_2 was returned to 20% [54]. Apart from a low pH, a low pigment content, and oxidative stress, it was found that intracellular acidification caused by intracellular CA is another major reason for the inhibition of photosynthetic carbon fixation when the algae was exposed to an excess concentration of CO_2 [48]. Experimenting at 100% CO_2 enables us to develop a better understanding of microalgal CO_2 uptake, as well as assess the suitability of using microalgal cultivations for the sequestering of relatively concentrated sources of pure CO_2, such as from ethanol fermentation.

When the cells were transferred from low- to high-CO_2 conditions, the extracellular CA was found to decrease during acclimation to high-CO_2 conditions [55]. The loss of CA and active DIC transport systems are strategies employed by algae to avoid the secondary inhibitory effects caused by excess DIC accumulation. However, extracellular 43 kDa protein/Fe-assimilation 1 (H43/FEA1) can be induced under high-CO_2 conditions [56]. This protein acts as a substitute of CA for sensing the CO_2 signal and is the most abundant extracellular

soluble protein (ESP), taking up around 26% of the total ESPs in cells grown under high CO_2 conditions [57]. Moreover, the protein can also be expressed in response to other stressful conditions, such as iron-deficiency and Cd-stress conditions, caused by fast growth under high CO_2 conditions [26].

This study investigated different gas management strategies aimed at enhancing CO_2 fixation and lipid production by algae. Our findings provide a basis for the usage of flue gases as a rich carbon source for biofuel production with concomitant CO_2 mitigation in future studies. Firstly, the growth of the algae was enhanced by the cheap and abundant CO_2 in the flue gases. Secondly, after combustion, the flue gases only contain residual O_2 (usually between 0.04-6%), which is significantly lower than the O_2 concentration (16.76%) in the gas mixtures that contain 20% CO_2 and 80% air (section 3.1). As such, photorespiration was inhibited to an extent when using flue gases. Thirdly, the biggest problem was the content of other gases in the flue gas, including SOx and NOx, which may be toxic to algae. However, this toxicity was primarily attributed to the change in the pH and was alleviated by moderating the pH without a need to pre-treat the algae for these contaminants [58, 59]. Moreover, the algae can be pre-cultured in the flue gases (for example, by gradually the increasing CO_2 load) in order to acclimatise the algae and absorb the contents of the flue gas, including CO_2. SOx and NOx may in fact act as sources of sulphur (S) and nitrogen (N), the latter being an essential nutrient, whose availability in the flue gases will allow for resource recovery options. Fourthly, a mixture of the flue gas with air or another gas resource can reduce the overall toxicity, however, this should be used with caution in order to limit the intensity of the O_2 input, unless the content of NO is high, which can be oxidised by O_2 to form NO_2^-. This is in turn assimilated by the algae, resulting in the detoxification of NO and the promotion of growth [60]. Finally, although the heavy metals may also be present in the flue gas due to the evaporation of the fossil fuels (e.g. coal) after combustion at high

temperatures, the microalgal biomass exhibits high metal-binding capacities for the removal of heavy metals, which would not be a major issue in the biomass if the microalgal biomass is to be used for the generation of biofuels [61].

4. Conclusion

This study offers strategies to cultivate algae for the production of biofuels under high CO_2 regimes, such as flue gases, in order to generate energy concomitantly with a reduction in CO_2 in real life applications. With a constant CO_2 concentration supplied via the inlet gas, the optimal CO_2 concentration for algal growth was generally found to be moderate (6% CO_2 for *N. salina*, i.e. 0.03 L L^{-1} min^{-1} of L_{CO2}). However, the growth and production of lipids was found to be enhanced by the removal of oxygen from the inlet gas, limiting photorespiration, which augurs favourably for the use of flue gases from combustion sources with low oxygen levels and a high CO_2/O_2 ratio. With gradual increases in the CO_2 concentration in the supply gas, the tolerance of algae to CO_2 was further elevated (up to 20% CO_2 in this study, i.e. 0.1 L L^{-1} min^{-1} of L_{CO2}). The increased CO_2 concentration did not induce pigment production, although it caused lipid accumulation. The present work is the first to identify gas supply strategies for a marine species of algae to facilitate the application of high CO_2 regimes, such as those encountered in flue gases, for the production of biofuels, whilst concomitantly facilitating CO_2 removal. The findings presented here provide a basis for the production of biofuels using other algal species in future studies.

Acknowledgment

We gratefully acknowledge EPSRC (UK) (EP/E036252/1), China APEC Foundation (HV01-190101(1)), General Financial Grant from the China Postdoctoral Science Foundation (2016M602055), and Xiamen Marine and Fishery Development Special Foundation (19CZP011HJ08) for the funding support that made this work possible.

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