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Influence of gas management on biochemical conversion of CO₂ 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 CO₂ (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 CO₂ 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 CO₂ 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 CO₂ levels with the aim to improve algal CO₂ fixation and lipid accumulation. Optimal growth of *Nannochloropsis salina* (a marine algae) occurred at 6% CO₂, whilst few cells grew under 20% CO₂. Excess CO₂ resulted in medium acidification, pigment reduction, and growth inhibition. However, the fixation capacity of CO₂ and the production of specific lipids were improved by O₂ 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 CO₂ concentration. Extremely high CO₂ levels (100%) completely inhibited cell growth, but this effect was reversed when air containing atmospheric CO₂ levels was introduced in place of 100% CO₂. 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; CO₂; 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 CO₂ emissions [1]. It is estimated that approximately 33.4 Gt of CO₂ are emitted by fossil fuel power plants each year, accounting for nearly 40% of the total CO₂ emitted into the Earth's atmosphere [2]. Global warming and ocean acidification caused by increasing atmospheric CO₂ 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 CO₂ 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 O₂ 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 CO₂ 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: CO₂, HCO₃⁻, and CO₃²⁻) [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 CO₂ under a majority of experimental conditions [10, 11]. *Thalassiosira pseudonana* prefers CO₂, while *Phaeodactylum tricornutum* prefers HCO₃⁻ [12, 13]. The transfer and uptake of different DIC species in microalgae primarily depends on the microalgae species and the concentration of CO₂. Three major strategies, HCO₃⁻ transportation, conversion of HCO₃⁻ into CO₂, and the direct diffusion of CO₂ 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 CO₂ fixation [15]. In addition, domestication or adaptive laboratory evolution can also be used as strategies to enhance microalgal CO₂ fixation, particularly for the fixation of CO₂ from CO₂-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 CO₂ and/or O₂ supply [16, 17]. O₂ accumulation in closed systems is a serious problem since O₂ can be supersaturated to concentrations as high as 400% in these systems [18]. These supersaturated O₂ concentrations not only inhibit the carboxylase activity of Rubisco, but also strengthen photorespiration. Even in those studies with increased CO₂, the O₂ content in the inlet gas was rarely considered. As such, air is widely used to mix with CO₂ to obtain the desired CO₂ levels [19]. However, the influence of atmospheric O₂ on algal growth is insufficiently characterised and often ignored.

Increasing the CO₂ 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 CO₂ 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% CO₂, 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 CO₂ 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 CO₂ usually exerts a stress on cells [24]. A previous study by the authors showed

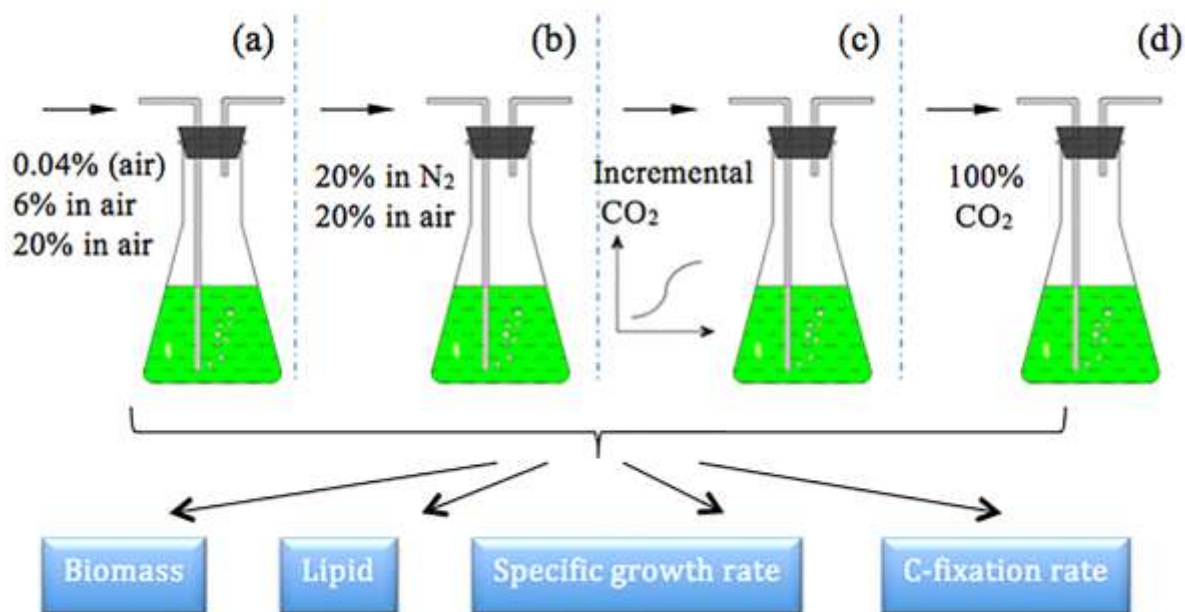
that the growth of *N. salina* could be promoted by increasing the CO₂ concentration to 6% (in air). However, growth was highly inhibited by a CO₂ concentration of over 20% [25].

The present study aimed to improve our understanding of the influence of CO₂ supply at high concentrations and that of O₂ on *N. salina* growth for mitigation of CO₂ emission and for biofuel production,. Despite many reports on microalgal CO₂-acclimation/adaptation mechanisms under low CO₂ conditions, such as CO₂-concentrating mechanisms (CCM) for growth in air, our knowledge on carbon uptake by microalgae under high CO₂ conditions remains limited [26]. A better understanding of microalgal growth under high CO₂ 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 CO₂ fixation and biofuel production by microalgae. The details for each experimental set-up are provided in the following sections.

Gas strategies for CO₂ fixation and biofuel production



Scheme 1. Strategies of gas supply tested in the investigation to study the influence of constant CO₂ in the feed gas (a); O₂ in the feed gas at 20% CO₂ (b); incremental CO₂ in the feed gas (c); and extremely high CO₂ (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 CO₂ 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 $\sim 70 \mu\text{E m}^{-2} \text{s}^{-1}$ by using fluorescent lamps, aerated, and incubated at $24 \pm 2^\circ\text{C}$. The cells were then harvested by centrifugation twice ($3,000 \times g$ for 3 min coupled with $8,500 \times g$ for another 5 min). The resulting supernatant was used for DIC measurement. The cell pellets were frozen at -20°C 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,

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

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,

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

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_{CO_2} , $\text{g L}^{-1} \text{d}^{-1}$) according to the following equation, which was derived from the typical molecular formula of microalgal biomass, $\text{CO}_{0.48}\text{H}_{1.83}\text{N}_{0.11}\text{P}_{0.01}$ [29]

$$\text{CO}_2 \text{ fixation rate } (F_{\text{CO}_2}) = 1.88 \times \text{biomass productivity (BP)} \quad (3)$$

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_{CO_2} , $\text{L L}^{-1} \text{min}^{-1}$) was defined as follows,

$$L_{\text{CO}_2} (\text{L L}^{-1} \text{min}^{-1}) = \text{CO}_2 \text{ concentration } (\%) \times \text{volumetric gas flow rate (vvm)} \quad (4)$$

where L_{CO_2} 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_{CO_2} was used to determine the specific lipid production as follows,

$$S_{lip} \text{ (g min } L^{-1} \text{ d}^{-1}) = \frac{(L_2 - L_1)}{\Delta t \cdot L_{CO_2}} \quad (5)$$

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_{HCO_3}) 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 CO₂, 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,

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

where A is the alkalinity ($\mu\text{mol kg}^{-1}$), $C_{H/OH}$ (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 TCO₂. The data (TCO₂, 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 100°C 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 CO₂ supply in the feed gas, at different concentrations

In order to determine the CO₂ tolerance and fixation capability of *N. salina*, the cultures were aerated in three constant CO₂ concentrations: 0.04% (air), 6%, or 20% (CO₂ in air). For 6% and 20%, pure CO₂ (100%) was mixed with air to obtain the desired CO₂ 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% CO₂, respectively. The corresponding DICs were also monitored.

2.5 Influence of photorespiration

Although the O₂ concentration in the culture can be reduced to avoid oversaturation by sparging a gas, the presence of O₂ in the feeding gas has only been studied scarcely. This may explain why *N. salina* hardly grew under 20% CO₂ in the presence of O₂ (as shown in section 3.1), as reported in our previous investigation [25]. To determine the influence of O₂ on photorespiration and C-fixation, CO₂ was mixed with either air or pure N₂. The flow rates of these gases were controlled using flowmeters to obtain a constant concentration of CO₂ 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 CO₂ levels

A high CO₂ concentration usually results in the overfeeding of algal cells due to the excess CO₂ 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 CO₂ levels may be used to soften the inhibition. To this end, the cultures were sparged with incremental levels of CO₂ over time. Pure nitrogen (100%) was mixed with pure CO₂ (100%) to obtain the desired level of CO₂. The low rate of the mixed gas was fixed at a gas to liquid ratio of 0.5 (vvm), while the CO₂ concentration was adjustable during cultivation. Nitrogen was used to adjust the CO₂ concentration instead of air in order to determine the influence of CO₂ and exclude the influence of oxygen in the air.

2.7 Influence of 100% CO₂

Although an excess supply of CO₂ 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 CO₂ concentration (100%) or a low concentration of 0.04% (air). At a high CO₂ concentration, a low flow rate of the gas was controlled within the range of 0.02–0.04 vvm (L_{CO_2} 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% CO₂ (in the absence of O₂).

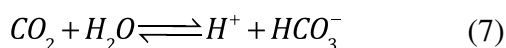
3. Results and Discussion

3.1 Influence of increased CO₂ concentration at a constant gas volumetric flow rate

To determine the capacity of the algal species to sequester CO₂ and produce lipids, the *N. salina* cultures were sparged with a gaseous mixture of air and CO₂ at three constant volumetric proportions: 0.04% (only air), 6%, and 20% CO₂, forming volumetric CO₂ loadings (L_{CO_2}) 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 CO₂ on CO₂ 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 CO₂ 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 CO₂ from 6% to 20% significantly inhibited the algal growth (P<0.05, t-test). The average CO₂ 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% CO₂ 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 CO₂ loading (L_{CO2}) for *Nannochloropsis* sp. was 0.015 L L⁻¹ min⁻¹ (15% CO₂, 0.1 vvm), only half of the 0.03 L L⁻¹ min⁻¹ (6% CO₂, 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 CO₂ pushes the equilibrium towards a decrease in [H⁺], according to the following reaction,



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 CO₂ 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 CO₂ 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 (TCO₂) was in line with the corresponding CO₂ 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 TCO₂ for 0.04%, 6%, and 20% CO₂ were around 0.067 (± 0.007), 0.172 (± 0.017), and 0.390 (± 0.015) g L⁻¹ CO₂ (e), respectively. Given that the fastest CO₂ fixation rate of 0.062 g L⁻¹ d⁻¹ was obtained at 6% CO₂, these TCO₂ 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 CO₂ captured by the medium are not insignificant. It is worth noting that the estimation of the CO₂ 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 CO₂ fixation.

The concentration of carbonate (CO₃²⁻) 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% CO₂. Both CO₂ and bicarbonate (HCO₃⁻) can be utilised through interconversions by carbonic anhydrases [34], where the increase in these two species, as happens at 6% CO₂ sparging, elevates growth. However, the concentrations of these two species were found to be the highest at 20% CO₂, 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 CO₂ concentration may promote the generation of reactive oxygen species, including H₂O₂, 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% CO₂ was around 31.39 (\pm 14.80), 0.60 (\pm 0.15), and 0.018 (\pm 0.017) g min L⁻¹ d⁻¹, respectively. This suggests that the efficiency of CO₂ conversion to lipids declined with the increase in CO₂ loadings. However, a higher lipid content was observed at 6% and 20% CO₂ than at 0.04%, indicating that increasing the CO₂ 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 CO₂ concentration would increase not only the CO₂ fixation but also the efficiency of the conversion and storage of light energy.

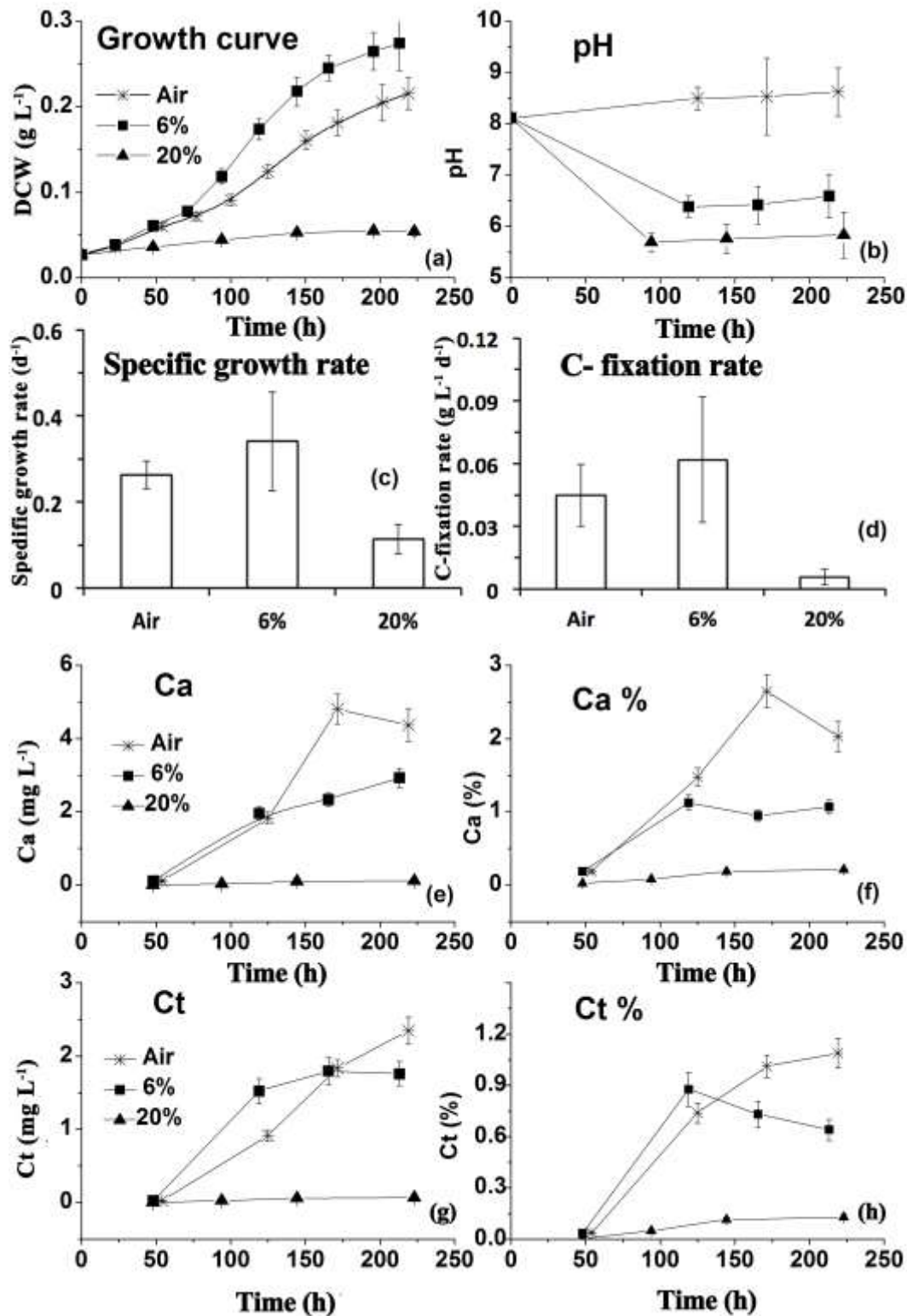


Fig. 1. Effects of different air/CO₂ mixtures (air, 6%, 20% CO₂) at a constant gas flow rate on the growth (a), pH (b), average specific growth rate during active growth (c), maximum CO₂ fixation rate (d) and pigment levels (e-h) in *N.salina* cultivation. Mean of three biological replicates are plotted 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.

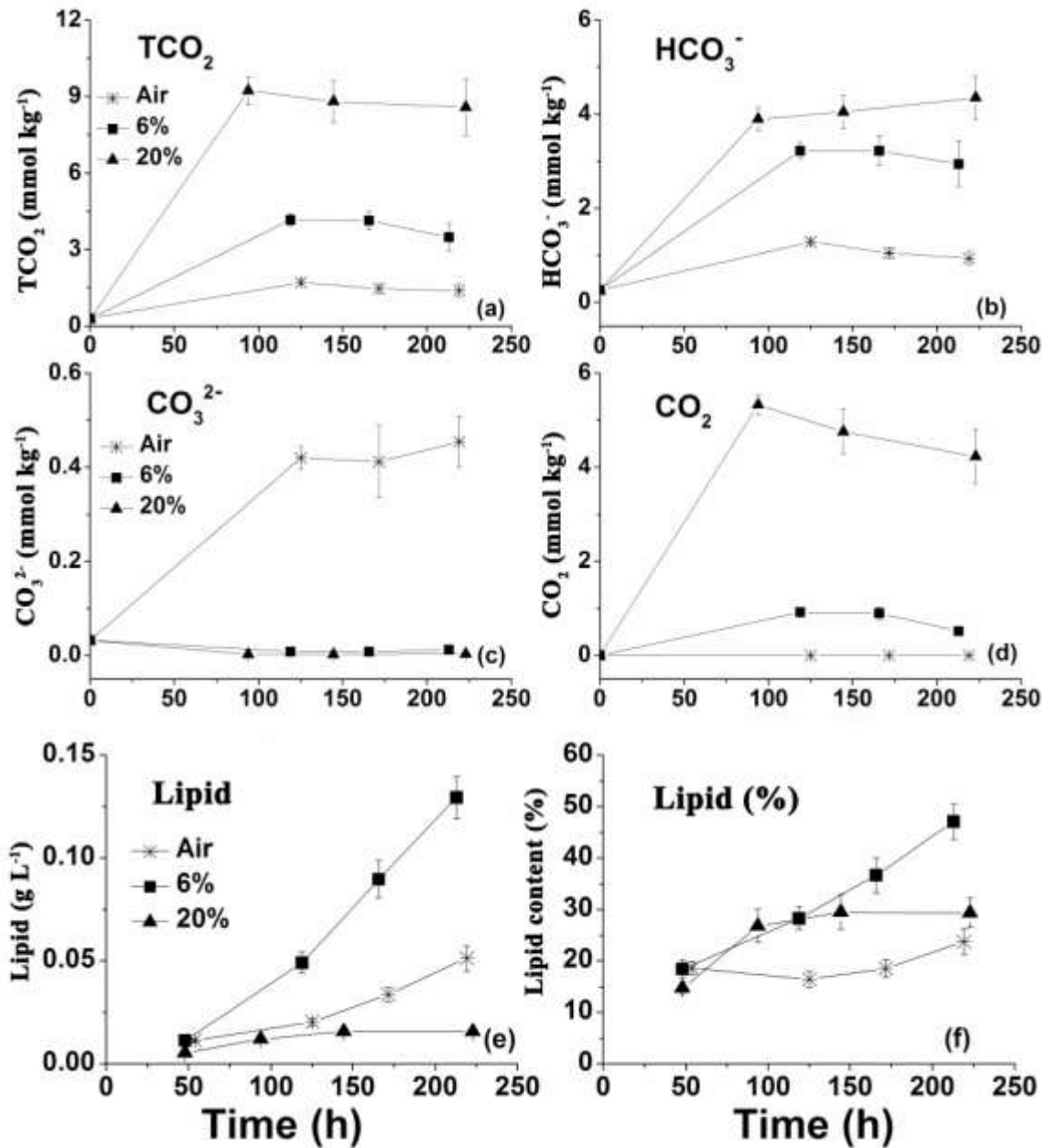


Fig. 2. Effects of different air/CO₂ mixtures (air, 6%, 20% CO₂) at a constant gas flow rate on the medium DIC species and lipids production in *N.salina* cultivations. Mean of three biological replicates are plotted with error bars representing standard error about the mean.

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% CO₂, 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% CO₂ to obtain a CO₂ 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% CO₂ devoid of oxygen (Fig. 3a). However, once the feeding gas was switched to 20% CO₂ in air, the growth rate was inhibited immediately, even becoming negative at times. When the gas was turned back to 20% CO₂ in nitrogen, the growth rate was recovered. The average specific growth rates in the presence of O₂ and N₂ were 0.012 (\pm 0.025) and 0.077 (\pm 0.018) d⁻¹, whilst the average CO₂ fixation rates were 0.005 (\pm 0.009) and 0.029 (\pm 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 CO₂ fixation rate. Therefore, the capacity of CO₂ fixation was enhanced by 4.8-fold after the removal of O₂. The average specific lipid productivities in the presence of O₂ and N₂ was 0.02 (\pm 0.025) and 0.088 (\pm 0.034) g min L⁻¹ d⁻¹, respectively, indicating an increase of 4.4-fold in the CO₂ conversion efficiency to lipids after the removal of O₂. Furthermore, it was observed that the removal of O₂ also improved the lipid percentage by 32.7% (Fig. 3b) compared to 30% (Fig. 2h) of the maximum lipid with 20% CO₂ in air. There appears to be a lag in the response of lipids (% content) in the first few days compared to the biomass.

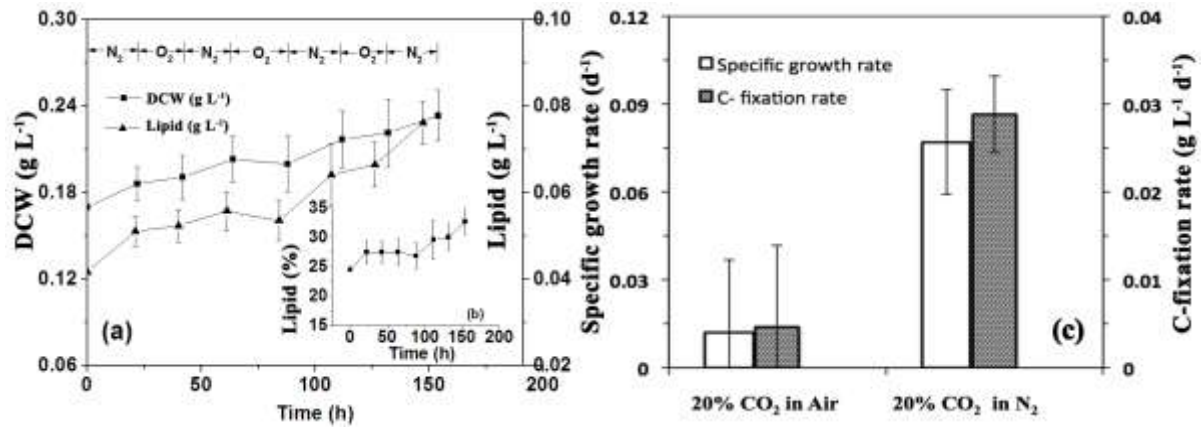


Fig. 3. Influence of oxygen in 20% CO₂ in the presence of oxygen (mixed with air (O₂)), and the absence of oxygen (mixed with nitrogen (N₂)), on the growth/lipid production (a), lipid content (%) (b), average specific growth rate and CO₂ fixation rate (c) of *N. salina*.

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 CO₂-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 CO₂ level as high as 20% and can be expected to sequester CO₂ from exhaust gases. The increased ratio of CO₂/O₂ 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 CO₂ in the feed gas seems to be required to suppress photorespiration, since enhanced photorespiration has been observed only when N₂ was supplied. This has been noted with two marine microalgae, *Tetraselmis gracilis* and *Phaeodactylum tricornutum*, which were aerated with only N₂ and without CO₂, showing a higher photorespiratory flux than that in the cultures aerated with atmospheric air [41]. This is due to the fact that, under N₂ supply without CO₂, O₂ derived from photosynthesis can activate Rubisco oxygenase activity for photorespiration [41]. This is a noteworthy point since, in addition to expelling photosynthetically derived O₂ out of the algal culture, sufficient carbon supplementation is essential. The present study

shows that a high CO₂ concentration (20%) with a medium O₂ concentration (16.76%, i.e. 20.95% × 80%) inhibited the growth of algae. Another scenario is that of a high O₂ concentration with a medium CO₂ concentration. It was found that when the O₂ increased to 84%, an increase of CO₂ 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 O₂ was primarily caused by photorespiration. This is perhaps indicative of the role of pH, when at high CO₂ concentration, such as at 20% CO₂, the pH of the medium drops to 5.5 (Fig. 1). At these pH levels, the absence of dissolved O₂ in the medium (e.g. when 20% CO₂ in nitrogen is supplied) plays a part in facilitating carbon uptake.

3.3 Influence of incremental CO₂ levels

The above experiment shows that the growth of *N. salina* was highly inhibited at 20% CO₂ 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 CO₂ concentration, i.e. a gradual increase in CO₂ 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% CO₂ in nitrogen, 20% CO₂ in nitrogen, and 30% CO₂ in nitrogen, in tandem (Fig. 4). The fastest growth rate was obtained at 6% CO₂. At 20% CO₂, the growth rate remained similar to that at 6%, with only a slight decrease. The growth then plateaued when the CO₂ level was increased to 30%, indicating that growth was limited by the stress induced by a high CO₂ 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% CO₂ were 0.316 (± 0.082), 0.532 (± 0.101), and 0.173 (± 0.070) d⁻¹, respectively. The maximal CO₂ fixation rate was 0.079 (± 0.012) g L⁻¹ d⁻¹ with 6% CO₂. In contrast, the average CO₂ fixation rate with 20% CO₂ in nitrogen was 0.050 (± 0.016) g L⁻¹ d⁻¹, which was

72% higher than that with constant 20% CO₂ in nitrogen (0.029 (± 0.005) g L⁻¹ d⁻¹), and was 733% higher than at constant 20% CO₂ in air (0.006 (± 0.004) g L⁻¹ d⁻¹). The specific lipid productivity at 20% CO₂ 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% CO₂ in nitrogen (0.088 g min L⁻¹ d⁻¹), resulting in an overall increase of 5.5-fold compared to 20% CO₂ in air (0.02 g min L⁻¹ d⁻¹). Both the amount and content of lipids (%) increased during 6% and 20% CO₂ aeration, but decreased when the gas supply was switched to 30% CO₂ in nitrogen. The extremely high concentration of CO₂ had a negative effect on both lipid production and the biomass.

While the effect of CO₂ concentration on microalgal growth has been widely studied, most of these studies have employed continuous or intermittent sparging at fixed CO₂ 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% CO₂ aeration [43]. The maximum rate of CO₂ fixation by *Chlorella vulgaris* P12 reached up to 2.22 g L⁻¹ d⁻¹ with 6.5% CO₂ 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 CO₂, an increase in CO₂ 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 CO₂, especially at the beginning of the cultivation period when the cell numbers are low. Nevertheless, their need for CO₂ 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 CO₂ for *Chlorella vulgaris* UTEX259 was enhanced by a progressive increase in CO₂ concentration. Therefore, in practice, it is recommended that the inlet flow rate of CO₂-enriched gas increase incrementally over time to obtain varying levels of CO₂ at different stages. This allows the cells to gradually acclimate to the high levels of CO₂ and meet their increasing demand for CO₂ caused by an increased cell density.

In fact, phototrophic microalgae have developed special mechanisms to acclimate and adapt to variations in both O₂ and CO₂. Although many reports have been published on CO₂-acclimation/adaptation mechanisms under limited CO₂ conditions, such as CO₂-concentrating mechanisms (CCM), our knowledge of these mechanisms under high CO₂ conditions is limited [26]. Even in reports using high CO₂ 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 CO₂ uptake. The microalgae that show high-CO₂-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 CO₂ 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 CO₂-affinity of Rubisco and are able to maintain pH homeostasis [48]. It is worth pointing out that CO₂ enrichment is not always conducive to increased algal growth, as for those fully low-CO₂-acclimated species, CCM can be induced to enable growth with a near-maximum growth rate under air-CO₂ levels [49]. However, growth can still be considerably enhanced when the cell density is high due to their increased requirements for CO₂ availability [26], indicating that a progressive increase in CO₂ with respect to cultivation time provides a useful strategy for photosynthetic species to avoid carbon deficiency regardless of low- or high-CO₂-acclimated microalgae.

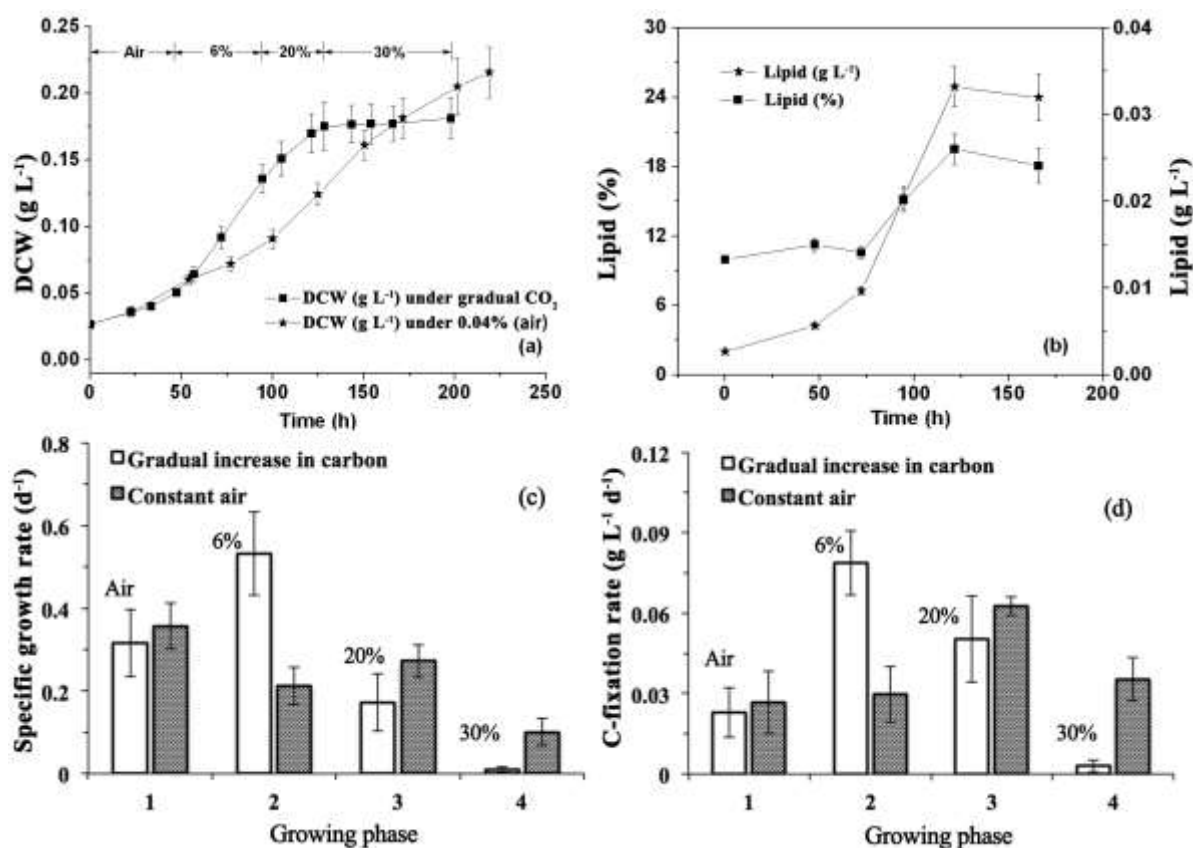


Fig. 4. Effect of gradual increase in CO₂ concentration from air to 30% CO₂ in nitrogen on the growth (a), lipid production/content (%) (b), specific growth rate (c) and CO₂ fixation rate (d) in *N. salina* cultivation. Mean of biological triplicates are plotted with standard error about the mean as error bars.

3.4 Influence of 100% CO₂ feed

The tolerance and fixation capacity of CO₂ can be improved by controlling the gas supply, such as O₂ removal, and appropriate acclimation. However, an extremely high CO₂ concentration can be destructive to the cells. To investigate this, the culture was exposed to 100% CO₂. The resultant growth and pH curves are shown in Fig. 5. Since the CO₂ concentration was too high, the flow rate of CO₂ 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% CO₂ enabling them only a limited acclimatization to the high CO₂ levels. On the second day, the harmful effects of extreme CO₂ levels started to be show as the specific growth rate became negative (-0.183 d^{-1}). A decrease in the flow rate of CO₂ 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.

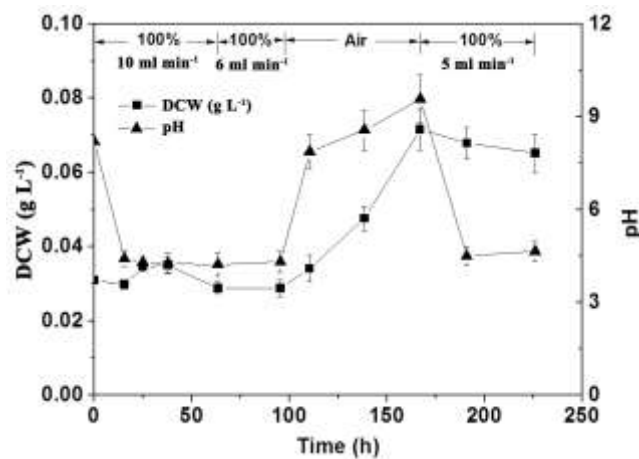


Fig. 5. Effect of 100% CO₂ on the growth and medium pH of *N.salina*.

When the feeding gas was switched to air (0.04% CO₂), a rise in the growth was observed as the stress was removed. This indicates that the inhibitory effect of 100% CO₂ 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% CO₂, 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 CO₂ 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 CO₂ loading, but also by the high percentage of CO₂ (and the resultant change in pH). Significant variations in pH were observed during the alternation of CO₂ and air. When using air (0.04% CO₂), the pH increased from 4.5 to over 9, with an increase in biomass. At 100% CO₂, 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 CO₂ and should have prevented cells from producing pigments for a light harvest.

The extremely high CO₂ 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 CO₂ concentration to relieve stress. The adverse effect of a high CO₂ content on algal growth has been observed in most of the species studied [50, 51]. However, fewer studies have reported algal growth at 100% CO₂. Very few strains are able to survive at such extreme levels of CO₂. *Chlorella* sp. T-1 [52] and *Desmodemus* sp. [53] are two strains that have been shown to grow at 100% CO₂. As reported in the present work, although the growth of *Scenedesmus* was completely inhibited at 100% CO₂, this effect was reversed (i.e. growth resumed) when the concentration of CO₂ 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 CO₂ [48]. Experimenting at 100% CO₂ enables us to develop a better understanding of microalgal CO₂ uptake, as well as assess the suitability of using microalgal cultivations for the sequestering of relatively concentrated sources of pure CO₂, such as from ethanol fermentation.

When the cells were transferred from low- to high-CO₂ conditions, the extracellular CA was found to decrease during acclimation to high-CO₂ 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-CO₂ conditions [56]. This protein acts as a substitute of CA for sensing the CO₂ signal and is the most abundant extracellular

soluble protein (ESP), taking up around 26% of the total ESPs in cells grown under high CO₂ 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 CO₂ conditions [26].

This study investigated different gas management strategies aimed at enhancing CO₂ 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 CO₂ mitigation in future studies. Firstly, the growth of the algae was enhanced by the cheap and abundant CO₂ in the flue gases. Secondly, after combustion, the flue gases only contain residual O₂ (usually between 0.04-6%), which is significantly lower than the O₂ concentration (16.76%) in the gas mixtures that contain 20% CO₂ 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 SO_x and NO_x, 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 CO₂ load) in order to acclimatise the algae and absorb the contents of the flue gas, including CO₂. SO_x and NO_x 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 O₂ input, unless the content of NO is high, which can be oxidised by O₂ to form NO₂⁻. 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 CO₂ regimes, such as flue gases, in order to generate energy concomitantly with a reduction in CO₂ in real life applications. With a constant CO₂ concentration supplied via the inlet gas, the optimal CO₂ concentration for algal growth was generally found to be moderate (6% CO₂ 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 CO₂/O₂ ratio. With gradual increases in the CO₂ concentration in the supply gas, the tolerance of algae to CO₂ was further elevated (up to 20% CO₂ in this study, i.e. 0.1 L L⁻¹ min⁻¹ of L_{CO₂}). The increased CO₂ 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 CO₂ regimes, such as those encountered in flue gases, for the production of biofuels, whilst concomitantly facilitating CO₂ removal. The findings presented here provide a basis for the production of biofuels using other algal species in future studies.

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