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The Effects of Total Dissolved Carbon Dioxide on the Growth Rate, Biochemical Composition, and Biomass Productivity of Nonaxenic Microalgal Polyculture

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Abstract: The biosequestration of CO_2 using microalgae has emerged as a promising means of recycling CO_2 into biomass via photosynthesis, which could be used to produce biofuels as an attractive approach to CO_2 mitigation. We investigated the CO_2 fixation capability of the native nonaxenic microalgal culture using a 2 L photobioreactor operated in batch mode. The cultivation was carried out at varying concentrations of total dissolved CO_2 (Tco_2) in the bulk media ranging from 200 to 1000 mg $L^{-1,}$ and the temperature and light intensities were kept constant. A maximum CO_2 fixation rate was observed at 400 mg L^{-1} of Tco_2. Characteristic growth parameters such as biomass productivity, specific growth rate, maximum biomass yield, and biochemical parameters such as carbohydrate, protein, and lipids were determined and discussed. We observed that the effect of CO_2 concentration on growth and biochemical composition was quite significant. The maximum biomass productivity was 22.10 ± 0.70 mg L^{-1} day^{-1}, and the rate of CO_2 fixation was 28.85 ± 3.00 mg L^{-1} day^{-1} at 400 mg L^{-1} of Tco_2. The maximum carbohydrate (8.17 ± 0.49% dry cell weight) and protein (30.41 ± 0.65%) contents were observed at 400 mg L^{-1}, whereas the lipid content (56.00 ± 0.82% dry cell weight) was the maximum at 800 mg L^{-1} of Tco_2 in the bulk medium.

Keywords: nonaxenic culture; biomass productivity; polyculture; carbon dioxide; CO_2 biosequestration; microalgae

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1. Introduction

Anthropogenic activities have led to intensive greenhouse emissions, with carbon dioxide (CO_2) being the largest contributor to climate change [1,2]. One of the most crucial current challenges for scientific research is to reduce atmospheric CO_2 levels to pre-industrial levels. Innovative interdisciplinary solutions to rising levels of CO_2 are thus a need of the moment on all fronts. Geological sequestration and microalgal biofixation are two of the most frequently studied ways to capture atmospheric CO_2. Capturing CO_2 in the form of microalgal biomass recycles CO_2 into the biotic carbon pool. Algae convert atmospheric CO_2 into biomass by photoautotrophic mechanism and account for a large proportion (~50%) of the 111–117 billion metric tonnes of carbon per year of the global primary productivity. Therefore, microalgae have the potential to enable the sustainable production of energy, chemicals, and materials [3,4].

Generally, on a lab scale, monocultures of microalgae that are not associated with or contaminated by other species, i.e., axenic microalgal cultures are studied for the production of biofuels and other value-added compounds. Microalgae such as *Botryococcus braunii*,

Chlorella pyrenoidosa, *Dunaliella salina*, *Scenedesmus* sp., and *Chlamydomonas reinhardtii* are some of the organisms which are frequently studied in algal biofuel applications [5–7]. However, the outdoor mass cultivation of microalgae monocultures is often limited by low biomass yields, the susceptibility to crashes caused by contamination with or the natural introduction of invasive species, and low temporal stability to fluctuating conditions.

The challenges faced by large-scale monocultures of microalgae could be overcome by multifunctional polycultures as microalgae naturally function symbiotically with other living organisms present in aquatic ecosystems to utilise dissolved nutrients and organic carbon. In terms of multifunctionality, diverse polycultures also have the potential to better resist damage from grazers and invasions whilst maintaining a stable ecosystem over time [8]. However, the role of biodiversity in improving the multifunctionality of outdoor mass cultivation of algae is poorly understood. For example, Shurin et al. [9] and Stockenreiter et al. [10] have shown that polycultures of algae exhibit a higher lipid concentration and total cell biovolume compared to monocultures. Narwani et al. [8], on the other hand, have shown that diverse cultures do not lead to improved multifunctionality. Apart from being multifunctional in terms of increased culture productivity and stability, polycultures with algal species such as *Ankistrodesmus falcatus*, *Paenibacillus* sp., *Chlorella vulgaris* JSC-7, and *Scenedesmus* sp., could also make the harvesting process more energy efficient by promoting the flocculation of the biomass [11].

Since cultures that are free from pest or predator species rarely develop in a full-scale application, it is crucial to study microalgal polycultures under nonaxenic conditions, i.e., in the presence of other organisms such as bacteria, amoebae, ciliates, and rotifers. Previous studies involving nonaxenic cultures of microalgae have shown that the presence of contaminating microorganism can either be beneficial or detrimental to the overall culture performance. For instance, Watanabe et al. [12] showed that in nonaxenic cultures of *Chlorella vulgaris*, the bacterial contaminants (i.e., in a strict sense, the organisms which make the culture nonaxenic) had both increasing and decreasing effects on the culture lifetime. Such interspecies interactions create complementary environmental niches and contribute to culture stability. Therefore, for our study, we have chosen a microalgal polyculture with microbial composition relevant to outdoor mass cultivation of microalgae [13–15]. As far as we know, studies involving polycultures have not been evaluated for the role of the availability of dissolved CO_2 in the bulk media on the performance of the polyculture in nonaxenic conditions. In this study, we also monitor the change in growth and biochemical composition of algae grown at various concentrations of dissolved CO_2 in the bulk media. Based on the results, we discuss the potential of using mixed microalgal cultures for sustainable CO_2 biosequestration in outdoor mass cultivation.

2. Materials and Methods

2.1. Microalgal Polyculture and Culture Medium

A nonaxenic polyculture of native microalgae reported by Sasongko et al. [13] was collected from the research-based Minamisoma pilot plant, Fukushima, Japan, and was grown at the Algae Biomass and Production facility in Kurihara, Tsukuba, Japan. The species diversity of this nonaxenic microalgal polyculture was previously studied by Demura et al. [16] who reported that it was composed of chlorophytes such as *Ankistrodesmus* sp., *Chlamydomonas* sp., *Coelastrum* sp., *Desmodesmus* sp., *Dictosphaerium* sp., *Eudorina* sp., *Kirchneriella* sp., *Klebsormidium* sp., *Micractinium* sp., *Monoraphidium* sp., *Pediastrum* sp., *Scenedesmus* sp., *Selenastrum* sp., *Staurastrum* sp., *Tetraspora* sp., and *Uronema* sp., Heterokontophytes, Ciliophora, Euglenozoa, Amoebozoa, and Cyanobacteria. The growth media used in this study contained $MgSO_4 \cdot 7H_2O$ 30 mg L^{-1}, $NaNO_3$ 140 mg L^{-1}, $CaCl_2 \cdot 2H_2O$ 10 mg L^{-1}, NH_4NO_3 22 mg L^{-1}, KH_2PO_4 10 mg L^{-1}, K_2HPO_4 5 mg L^{-1}, $CaCO_3$ 10 mg L^{-1}, citric acid 2 mg L^{-1}, Fe-citrate 2 mg L^{-1}, biotin 2 µg L^{-1}, thiamine HCl 10 µg L^{-1}, vitamin B6 1 µg L^{-1}, vitamin B12 1 µg L^{-1}, and Trace element mix A5 with Co (Merck KGaA, Darmstadt, Germany) 5 mL.

2.2. Experimental System

The photobioreactor (PBR) consisted of SIMAX borosilicate glass bottles with diameters of 136 mm and heights of 260 mm (Kavalierglass, Sazava, Czech Republic). The working volume of the PBR was two litres. The PBR was operated in batch mode owing to the simplistic design and flexibility of batch cultivation of microalgae [17]. The media were inoculated with the microalgae mixture collected by centrifugation form a starter culture. The initial dry weight content was set at 200 mg L^{-1}. Pure industrial-grade CO_2 was initially bubbled into the algae media at a flow rate of 0.5 L min^{-1} to reach 200, 400, 600, 800, and 1000 mg L^{-1} of total dissolved CO_2 in the bulk media. The CO_2 flow was cut off when the required concentration of total dissolved CO_2 was reached, pH was adjusted to 8.5 using 5 M of NaOH, and the bottles were then sealed off to reduce CO_2 escaping due to gassing out. The cultures were stirred at 100 rotations per minute by a magnetic stirrer and incubated at 28 ± 2 °C under continuous illumination of fluorescent lamps with a photon flux density of 50 µmol m^{-2} s^{-1}.

2.3. Determination of Total Dissolved Carbon Dioxide

When CO_2 dissolves in the bulk media, it exists as carbonic acid, bicarbonate, and carbonate ions. Thus, total dissolved CO_2, Tco_2 (mg L^{-1}) is the sum of $[H_2CO_3]$, $[HCO_3{}^-]$ and $[CO_3{}^{2-}]$ and is given by the equation:

$$T_{CO_2} = [H_2CO_3] + \left[HCO_3^-\right] + \left[CO_3^{2-}\right] \quad (1)$$

Tco_2 in the bulk media was experimentally determined by lowering the pH of the sampled culture solution to below four by using a citrate buffered ionic strength adjuster where virtually all these ions in the solution become CO_2 gas. A carbon dioxide ion-selective electrode (Handheld carbon dioxide meter CGP-31, TOA DKK, Japan) was then immersed in the sample solution, which enabled CO_2 in the sample solution to diffuse through a gas-permeable membrane until an equilibrium was reached between the partial pressures of CO_2 in the sample solution and the CO_2 in the high concentration sodium carbonate internal filling solution.

2.4. Microscopy and Morphological Analyses

The algae were morphologically observed using an optical microscope equipped with an advanced colour interpolation system (Leica DFC 7000T, Leica Microsystems, Tokyo, Japan). The images were acquired and processed with a LAS X software.

2.5. Measurement of Growth Parameters

Optical density (OD) does not serve as a reasonable estimate of biomass in mixed cultures [18]. Therefore, the biomass concentration was expressed as dry cell weight (DW). 10 mL of culture samples were filtered through dried and pre-weighed GF/C glass microfibre filter papers (Whatman, Chalfont, UK). Excess salts were removed from the filter paper containing biomass by passing 20 mL of 0.5 N NH_4HCO_3 through the filter papers. The filters were dried at 48 °C overnight and weighed. The difference in mass was taken as the dry cell weight (DW).

The biomass specific growth rate, µ (day^{-1}) was calculated using:

$$\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1) \quad (2)$$

where N_1 and N_2 are the biomass at time t_1 and t_2, respectively.

Biomass productivity, P_x was obtained with Equation (3) where X_{max} and X_i are the maximum concentration of microalgae, and initial concentration of microalgae, respectively, and t_f and t_i are the times required to reach X_{max} and X_i, respectively:

$$P_x = (X_{max} - X_i)/(t_f - t_i) \quad (3)$$

The CO_2 fixation rate, R_{CO2} (mg CO_2 L^{-1} day^{-1}) is estimated using the formula as follows:

$$R_{CO_2} = C_{avg} P_x \left(M_{CO_2}/M_C\right) \quad (4)$$

where C_{avg} is the average carbon content of the microalgal cells (% *w*/*w*) measured by an elemental analyser (UNICUBE, Elementrar, Langenselbold, Germany), M_{CO2} and M_C are the molar masses of CO_2 and elemental carbon, respectively.

2.6. Biochemical Characterisation of Microalgal Cells

For the biochemical characterisation of microalgal cells, dry biomass was harvested by centrifugation at 10,000× *g* for 10 min (MX-307, Tomy Digital Biology Co., Ltd., Tokyo, Japan), washed with distilled with H_2O, and freeze-dried (Eyela FDH-2110, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to prevent the degradation of any heat sensitive metabolites and stored at −20 °C till further analysis. The carbohydrate content was estimated and analysed by the Dubois phenol-sulphuric acid method [19]. The total organic carbon and nitrogen were determined by the CHNS elemental analyser (UNICUBE, Elementrar, Langenselbold, Germany) calibrated using sulphanilamide as a reference standard. About 5 mg of lyophilised microalgal biomass were combusted in pre-weighed aluminium capsules with helium as a carrier gas. A total elemental nitrogen to protein conversion ratio of 4.44 was used [20]. The total lipid content was estimated by the Folch chloroform-based lipid extraction method [21].

2.7. Statistical Analysis

All experiments were performed in duplicates, and each measurement was made three times. Hence, all reported values are the mean of six data points. The effect of Tco_2 on biomass growth and biomass composition were statistically evaluated by the analysis of variance (ANOVA) test for equal variances using Minitab 19 (Minitab, LLC, Pennsylvania, PA, USA).

3. Results

3.1. Biomass and Growth Analyses

We studied the effect of various concentrations of Tco_2 in the bulk media on the microalgal polyculture. During the microscopic examination of the polyculture, *Desmodesmus* sp., *Scenedesmus acuminatus*, *Dictyosphaerium* sp., and *Phormidium* sp. were observed as the primary dominant species (Figure 1). Comparing the various growth curves, although a net increase in biomass concentration was observed at all the defined Tco_2, higher levels of Tco_2 were found to be detrimental to the rate of biomass accumulation (Figure 2).

The effect of the different levels of Tco_2 in the bulk media on biomass accumulation was triphasic: an initial period of acclimation and little growth (days 0–3) is followed by a period of rapid biomass accumulation (days 3–7), after which the microalgal population entered a stationary phase from days 7–10. Comparing the specific growth rate at different phases (Figure 3), and the percentage increase in biomass accumulation from the start to the end of each phase (Table 1) elucidates the role of Tco_2 in culture growth. In the first growth phase (1–3 days), the specific growth rate of the culture was highest when the Tco_2 was 600 mg L^{-1} (μ = 0.04 day^{-1}, 15.75% increase in biomass) and was slightly higher than that observed at 400 mg L^{-1} of Tco_2. In the second growth phase (3–7 days), the specific growth rate was highest at 400 mg L^{-1} (74.50% increase in biomass) followed by that at 200 mg L^{-1} of Tco_2 (50.12% increase in biomass). In the third growth phase (7–10 days), while the growth rate vastly slowed down at all concentrations of Tco_2, it was still highest at 400 mg L^{-1} of Tco_2, indicating that CO_2 at this level was the most favourable for growth. At 1000 mg L^{-1} of Tco_2, the highest level tested in our study, the increase in biomass was the least. Thus, the maximum specific growth rate was observed in culture with a Tco_2 of 400 mg L^{-1} at the second phase after an initial acclimation during the first phase.

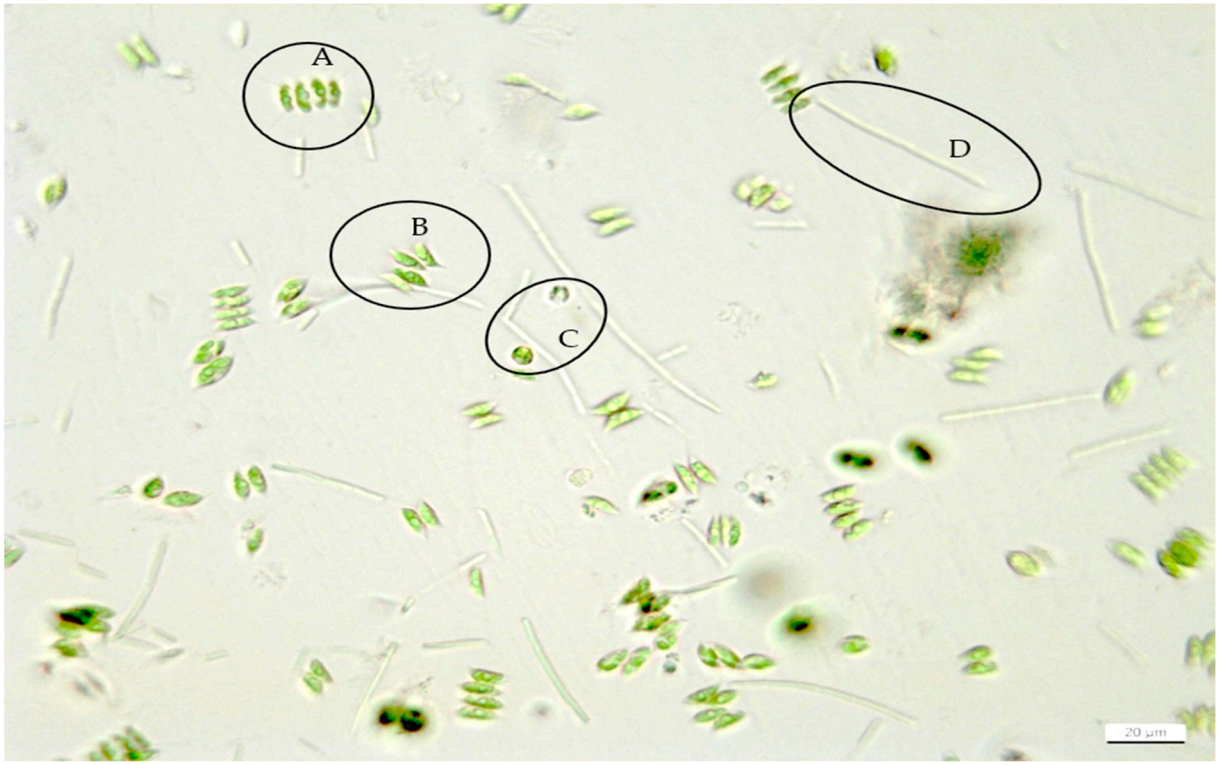

Figure 1. Photomicrograph showing some of the representative species observed in the polyculture. **A**: *Desmodesmus* sp. (4 cells), **B**: *Scenedesmus acuminatus* (4 cells), **C**: *Dictyosphaerium* sp., **D**: *Phormidium* sp.

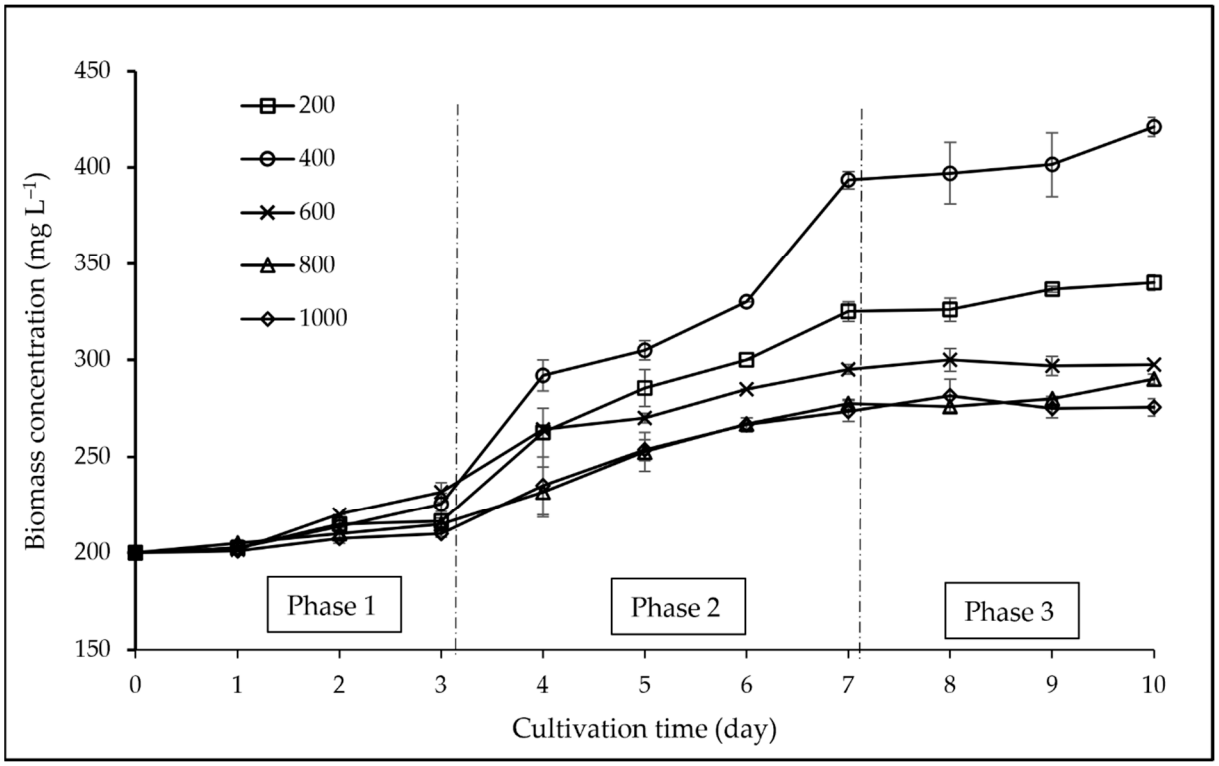

Figure 2. Mean ± standard deviation of the biomass, as measured by the dry cell weight (DW), at different concentrations of total dissolved carbon dioxide (Tco_2) in the bulk media.

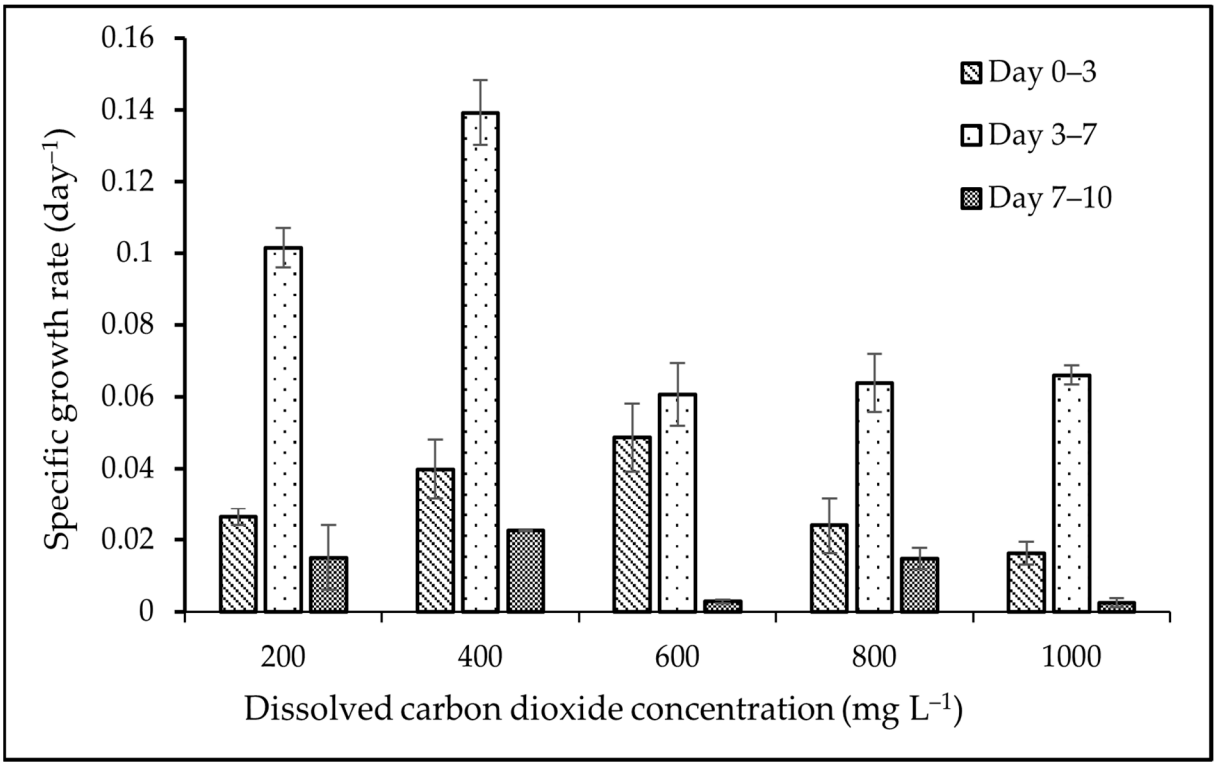

Figure 3. The specific growth rate of the microalgal polyculture within the three phases (0–3 days, 3–7 days, and 7–10 days) of culture under various levels of total dissolved CO_2 (Tco_2).

Table 1. Increase in biomass of the nonaxenic microalgal culture at different concentrations of total dissolved CO_2 at the end of different growth phases.

Tco_2 (mg L^{-1})	Increase in Biomass (%)			
	Phase 1	Phase 2	Phase 3	Overall
200	8.25	50.12	4.62 *	70.00
400	12.75	74.50 *	6.99	110.50 *
600	15.75 *	27.43	0.85	48.75
800	7.50	29.07	4.50	45.00
1000	5.00	30.24	0.73	37.75

* maximum value in each column.

At a Tco_2 level of 200 mg L^{-1}, which showed a 70% overall increase in biomass, the overall productivity and maximum specific growth rate were 14.00 mg L^{-1} day^{-1} and 0.101 day^{-1}, respectively (Figure 3). Whereas at Tco_2 level of 400 mg L^{-1}, which showed a 110.50% overall increase in biomass, the productivity and maximum specific growth rate were 22.10 mg L^{-1} day^{-1} and 0.13 day^{-1}, respectively. The maximum CO_2 fixation rate and biomass productivity were 28.85 ± 3.00 mg L^{-1} day^{-1} and 22.10 ± 0.7 mg L^{-1} day^{-1}, respectively, at 400 mg L^{-1} of Tco_2 whereas 1000 mg L^{-1} of Tco_2 showed the least CO_2 fixation rate of 6.92 ± 1.2 mg L^{-1} day^{-1} and lowest biomass productivity of 7.50 ± 0.4 mg L^{-1} day^{-1} (37.75% overall increase in biomass). The overall increase in biomass at Tco_2 levels of 600 mg L^{-1} and 800 mg L^{-1} was 48.75% and 45%, respectively.

3.2. Effect of CO_2 Concentration on Biochemical Composition

The content of carbohydrates, proteins, and lipids of the microalgae cultivated under different concentrations of Tco_2 was determined and depicted in Figure 4. The carbohydrate content did not change significantly at any of the tested levels of Tco_2.

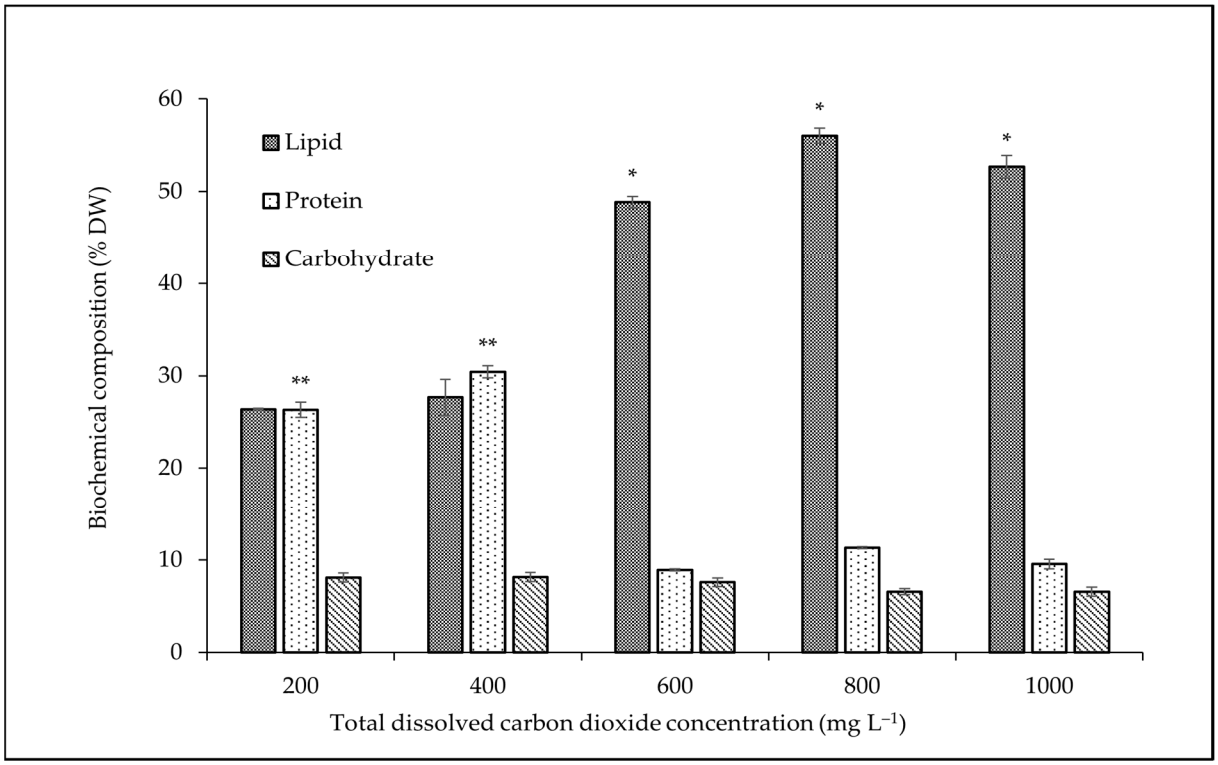

Figure 4. Effect of total dissolved CO_2 (Tco_2) in the bulk media on lipid, protein, and carbohydrate composition. **, and * on top of the columns represent statistical significance at 5%, and 10%, respectively.

Varying Tco_2 concentration significantly affected the protein content of the microalgae. The percentage of accumulated protein at different levels of Tco_2 ranged from 8.92 to 30.41%. The protein content was higher in the groups in which higher growth rates were also observed, i.e., at 200 and 400 mg L^{-1} of Tco_2. The maximum and minimum protein content obtained, 30.41 and 8.92%, were observed at 400 and 600 mg L^{-1} of Tco_2, respectively.

The total lipid content at different levels of Tco_2 ranged from 26.39 (minimum observed at 200 mg L^{-1} of Tco_2) to 56.00% (maximum observed at 800 mg L^{-1} of Tco_2). A gradual increase in lipid content was observed with increasing levels of Tco_2. The lipid content was higher at 600, 800, and 1000 mg L^{-1} of Tco_2 than that observed at 200 and 400 mg L^{-1} of Tco_2. The overall biomass growth was also lower at the levels where lipid content was high. The results indicate that the level of dissolved CO_2 can significantly affect lipid content.

4. Discussion

4.1. Biomass and Growth Analyses

Only a limited number of reported studies were conducted on nonaxenic cultures of microalgal polyculture. As far as we know, most of these studies correlate algal growth parameters to CO_2 concentration in the gaseous phase. CO_2 concentration in the gaseous phase does not reflect the actual level of CO_2 available in the bulk media available for uptake by algal cells, as it is determined by the culture pH and effective mass transfer [22–24]. Anjos et al. [24] also observed this likely lack of dependence of the high biomass accumulation rate on the level of CO_2 in the gaseous phase. Similarly, Yang et al. [25] reported that when operated under continuous mode, *Scenedesmus* sp., and *Desmodesmus* sp., did not show any increase in growth rate beyond 30 and 60 µM of CO_2, respectively.

At higher levels of Tco_2, the microalgae continued to grow but at less than half the specific growth rate at a Tco_2 concentration of 400 mg L^{-1}. Since Tco_2 is the sum of $[H_2CO_3]$, $[HCO_3^-]$ and $[CO_3^{2-}]$, the stimulatory effect of CO_2 is due to the availability of carbon. Thus, for the consortium under study, 400 mg L^{-1} of Tco_2 was ideal for microalgal growth. Below the ideal concentration, algal growth becomes limited, whereas CO_2

abundance influences several key enzymes in carbon metabolism, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and carbonic anhydrase [26,27]. The increased availability of CO_2 increases the carboxylating activity of RuBisCO, leading to increased photosynthesis, but can also adversely affect the activity of extracellular carbonic anhydrase and inhibit cell growth [28–30]. The low values observed for biomass growth parameters of the polyculture indicate that a higher level of Tco_2 is inhibitory. It also points to the presence of CO_2 tolerant species in the culture [31,32]. The high initial cell density and the diversity of cell morphology likely increased the tolerance against high levels of Tco_2 and reduced the long adaptation period [33]. In mixed cultures, a niche partitioning is generally observed in terms of CO_2 assimilation activity and nitrogen-fixing [8,34]. Thus, the growth parameters of the microalgae were significantly influenced by Tco_2, and the microalgae showed a wide range of tolerance to Tco_2. However, further studies are required to identify the optimum combination of traits of algal polyculture which would have the desired effect on biomass production.

4.2. Effect of CO_2 Concentration on Biochemical Composition

The biochemical composition is a crucial factor that determines the applicability of potential algal species for CO_2 biosequestration. The carbohydrate content of the culture did not show any significant change at any of the studied levels of total dissolved CO_2. Our result is contrary to what is generally observed in monocultures [35–37]. In the case of protein content, significant changes were observed with respect to Tco_2. The maximum protein content was at 400 mg L^{-1} of Tco_2. At higher levels of Tco_2, the protein content decreased, likely due to a lack of availability of carbon and nitrogen species. There was a noticeable increase in lipid content at the Tco_2 level of 600 mg L^{-1} and higher. Elevated CO_2 concentration in the bulk media creates a stressful environment for the microalgal cells, leading to the production of lipids [38]. The presence of oleaginous and high CO_2 tolerant *Scenedesmus* sp., and *Desmodesmus* sp., in the polyculture also likely contributed to increased lipid composition [25,26,39]. The biosynthesis of major biochemical constituents such as carbohydrates, proteins, and lipids involves many complex enzymatic reactions such as the light-driven generation of ATPs [40]. Thus, the accurate measurement of metabolic fluxes of CO_2 uptake in photosynthetic microorganisms leading to the formation of carbon backbones is complicated due to the presence of high levels of compartmentalisation [41]. The carbon backbones generated in the first few steps of CO_2 biofixation are utilised for the biosynthesis of carbohydrate during the dark period. The process can be driven towards the synthesis of proteins and lipids by limiting the dark period. Moreover, the enzymes involved in lipid biosynthesis require Mg^{2+} at their active centres. Therefore, we suggest that, by implementing a two-stage cultivation process, comprising a first stage to promote cell growth with nutrient replete condition followed by a nutrient deplete stage whilst supplementing the media with Mg^{2+} ions at pH between 8 and 10 could enhance the accumulation of lipids for the large-scale production of biofuels from microalgae [29,30,42].

5. Conclusions

Our results indicate that (i) the nonaxenic microalgal polyculture was tolerant to all tested total dissolved CO_2 concentrations up to 1000 mg L^{-1}; (ii) even though increased total dissolved CO_2 levels reduced the overall biomass accumulation, it was also found to be the most conducive to lipid accumulation. Our results also show that the growth of polyculture microalgae stabilise over time in nonaxenic conditions even at very high concentrations of total dissolved CO_2 and are suited for large scale biomass cultivation applications in CO_2 biosequestration, and accumulation of carbohydrate, proteins, and lipids. Further research, however, is required to understand the interaction between different species leading to niche partitioning and niche complementarity in nonaxenic microalgal polycultures. In order to develop regimens for the stable and sustainable production of biofuels from microalgae, future research efforts should also focus on identifying the agents responsible for the failure of large outdoor cultivation systems.

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