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Effective CO_2 capture by the fed-batch culture of *Chlorella vulgaris*

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Abstract

Microalgae are promising candidates for CO_2 capture and concomitant biomass production. *Chlorella vulgaris* was grown in the batch and fed-batch cultures to develop a culture strategy targeting maximum CO_2 capture and conversion to biomass. Growth at five different dissolved CO_2 (dCO_2) concentrations in the feeding media (atmospheric, 1.55, 1.62, 1.75 and 1.88 g L^{-1}) was tested to assess the effect of dCO_2 concentration on the growth, biomass productivity, and nutrients removal efficiency of microalgae. Results suggest that fed-batch culture outperformed "standard" batch cultivation with a higher algal growth rate (2.3 times). As expected, the algal growth was limited at low (atmospheric) dCO_2 concentrations and inhibited at high levels (1.75 and 1.88 g L^{-1}). *C. vulgaris* grown with medium containing 1.62 g L^{-1} dCO_2 showed the highest growth rate (0.094 h^{-1}) with the shortest doubling time (7.4 h), maximum biomass productivity, nitrogen and phosphorus uptake rates (222, 7.5 and 1.6 mg L^{-1} d^{-1}, respectively). By pre-dissolving CO_2 into the feeding media and using the fed-batch culture mode, a high CO_2 removal efficiency could be achieved.

***Keywords**:* Batch cultivation, Biomass productivity, *Chlorella vulgaris*, CO_2 capture efficiency, Fed-batch cultivation, Nutrients removal

1. Introduction

CO_2 emission due to fossil fuel combustion nowadays is one of the greatest environmental concerns. To mitigate the global greenhouse process, CO_2 capture has become an urgent issue. Photosynthetic organisms, such as plants and algae, are candidates for sequestering CO_2 via photosynthesis with the added possibility of economically converting CO_2 into high-value biomolecules: polysaccharides, proteins, lipids, pigments, etc. [1]. Compared to higher plants, microalgae-based CO_2 capture appears to have greater prospects because of the higher photosynthetic and CO_2-uptake efficiencies (10 – 50 times) [2–4] due to the rapid growth cycle

of these microorganisms. Microalgae possess the remarkable potential to sequester and convert 513 tons of CO_2 into 280 tons of dry biomass per hectare per year [5], surpassing the peak fixation rate of maize, which stands at 26.5 tons CO_2 ha^{-1} $year^{-1}$ [6].

In nature, the inorganic carbon (C) source for planktonic photosynthesis comes from the air which contains only 0.04% CO_2 [7]. The low dissolved CO_2 (dCO_2) concentration in water at equilibrium (the diffusion coefficient is 10^4 times smaller than in air) generally cannot meet the optimal growth rate and high biomass yield [8–11]. To avoid C limitation, microalgae cultures can be injected with CO_2-enriched air/flue gases [12,13]. The CO_2-enriched gas injected into the photobioreactors promotes algal growth and biomass production, which is largely due to the enhanced photosynthetic performance with an increase of C availability [14,15]. The growth rate and CO_2-fixation efficiency of *Chlorella vulgaris* increased by 120–150% or 6–12 times, respectively, at 10% CO_2 (v/v) [16] although CO_2 becomes toxic to this organism above the concentration of 20% (v/v) in the gas phase [17]. The C availability to cells is dependent on the feeding modes [18]. As the most affordable C source, CO_2 is usually suppled in the gas phase but the insufficient CO_2 mass transfer rate in water may cause a considerable proportion of the gas-phase CO_2 to escape from the culture medium, reducing its utilization efficiency [11,19]. Therefore, the improvement of CO_2 fixation efficiency has become a bottleneck in microalgae-based CO_2 capture technology.

In batch cultures, all the nutrients are present at the beginning of cultures and are used during growth while gaseous nutrients, such as CO_2 or O_2, and light (in the case of photosynthetic cultures) are supplied continuously during the culture period. The growth rate is, therefore, rapidly controlled by the transfer rate of the required gases or the rate of light arrival in the ever-densifying photosynthetic cultures. These phenomena explain the carbon-limitation and/or light limitation of microalgae cultures, and the resulting linear growth phases and low final densities [20,21].

Environmental conditions in the photobioreactor like pH, carbonate chemistry, light and nutrients availabilities to cells alter with the increase in biomass density [22]. These may trigger changes in algal metabolism and physiology, which in turn affect biomass productivity [23,24]. The increasing pH caused by the consumption of dCO_2 and bicarbonate (HCO_3^-) through photosynthesis has been thoroughly explained by Binaghi et al. [25] and Gao [26]. Researchers also found that using NO_3^- as a nitrogen (N) source may cause OH^- release, rising pH [27,28]. Notably, if pH exceeds the optimal threshold for algal development, the intercellular acid-base homeostasis may be disrupted, preventing high biomass densities and productivities. This has been reported for many microalgae such as *C. vulgaris* [29], *Chlorella sorokiniana* [30] and *Chlamydomonas reinhardtii* [31]. To maximize biomass productivity, pH modulation by the injection of acids, buffers and CO_2 may make sense, but these approaches lack economic feasibility due to cost and low CO_2 consumption efficiency [19]. Additionally, using acids and buffers may introduce new ions affecting the salinity and the microalgae growth in some cases.

According to Brune and Novak [32], the dissociated proton concentrated due to CO_2 diffusion mitigates pH increase. Therefore, when nitrate (NO_3^-) is used as a sole N source, there may be a chance to cost-effectively control pH by adding dCO_2-enriched media (eCM), in other words, where CO_2 is pre-dissolved into the growth medium. This process may enhance the CO_2 utilization efficiency through reducing the escaping fraction, and simultaneously mitigate the pH increases caused by photosynthesis and nitrate consumption, consequently stimulating the growth. To the best of the authors' knowledge, this approach has received little consideration. Therefore, to assess the feasibility of this technique, 3N-Bristol medium (in which NO_3^- is the only N source) pre-mixed with CO_2 was intermittently pumped into *C. vulgaris* cultures (fed-batch) to maintain pH, and the growth rate, biomass productivity and nutrients removal capacity of *C. vulgaris* were investigated to compare with those obtained in batch culture. Furthermore, the optimal CO_2 concentration in the feed medium was determined. This study may provide an economic CO_2-feeding strategy to optimize microalgae production systems.

2. Materials and Methods

2.1. Microalgae species and culture maintenance

Chlorella vulgaris CCAP211/e 11B (CCAP, Göttingen) was maintained in Erlenmeyer flasks (250 mL, containing 100 mL 3N-Bristol medium) with a weekly sub-culturing frequency. The medium contains (per litter): 748 mg $NaNO_3$, 28 mg $CaCl_2 \cdot 2H_2O$, 75 mg $MgSO_4 \cdot 7H_2O$, 21 mg FeEDTA, 75 mg K_2HPO_4, 174 mg KH_2PO_4, 20 mg NaCl, 3 mg H_3BO_3, 6 mg $MnCl_2 \cdot 4H_2O$, 303 µg $ZnSO_4 \cdot 7H_2O$, 121 µg $CuSO4 \cdot 7H_2O$, 22 µg MoO_3 (85%, w/w), and 137 µg $CoSO_4 \cdot 7H_2O$ [33]. The flasks were illuminated continuously under a light intensity of 20 µmol photons m^{-2} s^{-1} at the surface of the cultures with a mixing (150 rpm) at 25°C in an orbital shaker incubator (Multitron, Infors HT, Switzerland).

2.2. Experimental procedure

A 6.8 L baffled photobioreactor (GPC bio, France) equipped with 4 pitch-blade impellers (500 rpm) was used to grow *C. vulgaris* in batch (B) or fed-batch (FB) cultures (Fig. 1). The pH probe (EasyFerm Bio Arc 325, Hamilton, Swiss) was calibrated before sterilization (121°C, 20 min) using the standard pH buffers at 7.00 and 10.01 (Thermo Fisher Scientific Inc.); and the dissolved oxygen probe (VisiFerm DO Arc 325, Hamilton, Swiss) was calibrated after sterilization at 0% and 100% air-saturation of sterilized growth medium. Three LED lamps (Ledare 130, 78 lumen, 2700 Kelvin, 27° dispersion angle, IKEA, France) were used to illuminate the cultures from three directions (120° angles to each other) and the total internal light intensity was 185 µmol photons m^{-2} s^{-1} (QSL-2100, Biospherical Instruments, San Diego, CA, USA) at the

internal surface of the glass culture vessel. Temperature-control was by means of a double envelope through which water at 25 °C circulated.

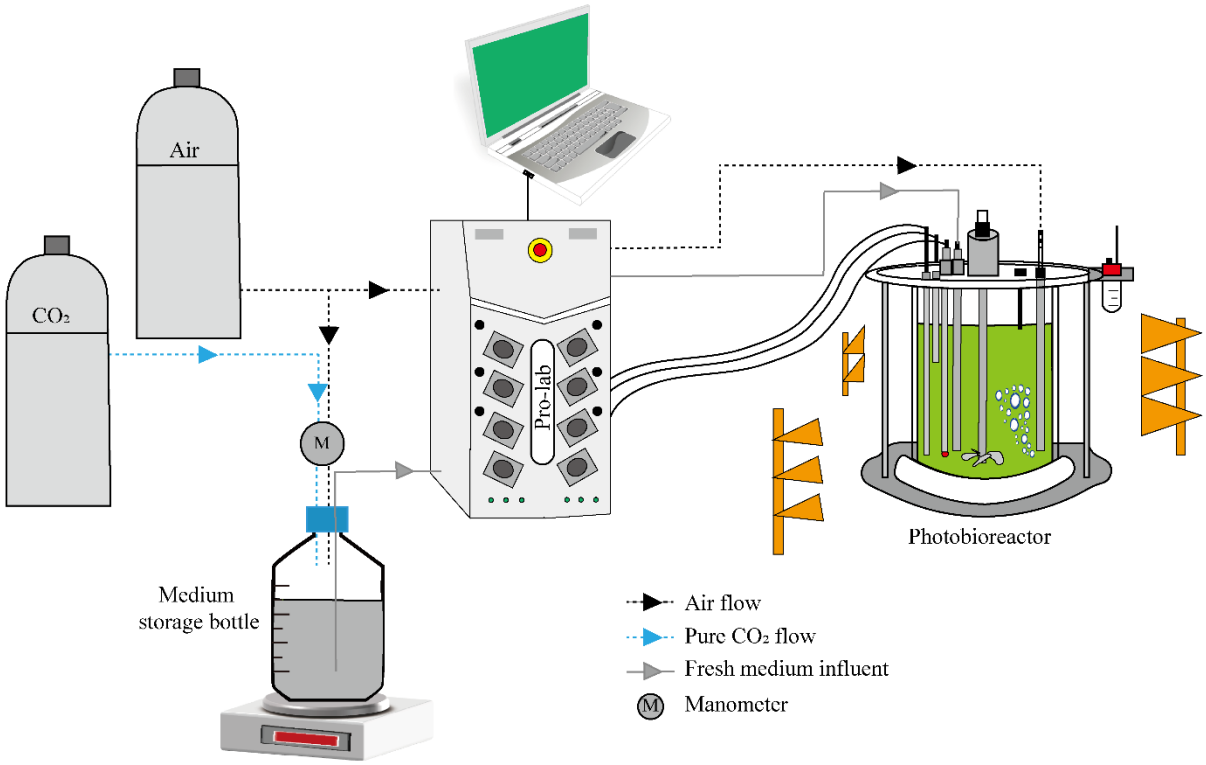

Fig. 1. Experimental set-up for *C. vulgaris* batch and fed-batch cultures.

2.2.1. Batch (B) culture

The "standard" batch culture (5 L, Table 1) was inoculated with 2.1×10^{10} cells (4.2×10^{6} cells mL^{-1}). The algal suspension from section 2.1. was centrifuged at 5000 rpm for 5 min and then re-suspended with sterilized 3N-Bristol medium. The filtrated air (0.2 µm, Sartorius, Germany) was bubbled into the photobioreactor at 500 mL min^{-1} as measured by a mass flow controller (Bronkhorst). During the experiment (8 days), no extra medium was added to the culture.

Table 1. Detailed CO_2/medium feeding regimes in different cultures.

Culture mode	Culture label	pCO_2 in supplied medium (bar)	Culture aeration (mL min^{-1})	*Calculated dCO_2 in growth medium (g L^{-1})	pH control	Initial volume (L)	Final volume (L)
Batch	Batch	Air-equilibrated	500	NA	–	5	5
Fed-batch	FB_blank	Air-equilibrated	0	0.0006	+	5	5
	FB_control	Air-equilibrated	500	NA	+	0.5	5.5
	FB_1.55	1.15	0	1.55	+	0.5	5.5
	FB_1.62	1.20	0	1.62	+	0.5	5.5
	FB_1.75	1.30	0	1.75	+	0.5	5.5
	FB_1.88	1.40	0	1.88	+	0.5	5.5

* represents the calculated dCO_2 using Henry's law, where the constant for CO_2 in water at 25°C is 3.1×10^{-2} M atm^{-1}; "+" represents the culture was under pH-controlled, and "–" indicates the culture had no pH-control; "FB" stands for the fed-batch culture, FB_control and FB_blank respectively represent the fed-batch cultures injected with and without air in the photobioreactor, whereas FB_1.55, FB_1.62, FB_1.75 and FB_1.88 are the fed-batch cultures fed with the media enriched with 1.55, 1.62, 1.75 and 1.88 g L^{-1} dCO_2, respectively; NA, Not applicable.

2.2.2. Fed-batch (FB) culture

For the FB cultures, the bioreactor was initially inoculated with a population of $4.3 \pm 2.8 \times 10^9$ cells (approximately 5 times less than that of the batch culture) in 0.5 L medium, the starting volume of the FB cultures. A 10 L bottle containing 5 L 3N-Bristol medium was connected to the bioreactor through the acid-feeding pump (Fig. 1). The medium in the bottle was previously adjusted to five different absolute pCO2 levels (Table 1): atmospheric (blank and control), and pCO_2-enriched media (eCM) at 1.15, 1.20, 1.30 and 1.40 bar which respectively corresponds to the dCO_2 concentrations of 1.55, 1.62, 1.75 and 1.88 g L^{-1}

(Henry's law). For each target dCO_2-enriched concentration, the growth medium was allowed to reach equilibrium with a different pressure of pure CO_2 gas that was bubbled into the medium. The pressure was maintained at the desired level for the duration of the experiment by means of a permanent connection to a CO_2 gas cylinder. In each experiment, after inoculation, the medium was automatically fed into the bioreactor according to the command of the controller to maintain the culture-pH at 6.5 until the maximal volume (5.5 L) was attained. There was no aeration of the culture for neither the FB 'blank" experiment nor for any of the FB dCO_2-enriched cultures.

2.3. Analysis

2.3.1. Total population

The cell number was determined by particle counting and the associated characterization system (Beckman Coulter Multisizer 4e counter, Beckman, US). Sample preparation involved taking 100 µL of sample with the appropriate dilution (in a total volume of 10 mL). Measurements were performed in triplicates from each sample. Total population (P, cells) in the photobioreactor was calculated by the equation:

$$P = C \times V \quad (1)$$

Where V is the volume (L) of algal culture in the photobioreactor at the time of sampling and C is the cell concentration (cells L^{-1}) of the culture at the same time.

2.3.2. Growth rate and biomass productivity

The specific growth rate (μ, h^{-1}) of cultures was estimated using the equation [34]:

$$\mu = [\ln(P_t) - \ln(P_{t0})] / (t - t_0) \quad (2)$$

Where P_{t0} and P_t are the total populations at the times t and t_0 (h), respectively. The maximal specific growth rate (μ_{max}, h^{-1}) was calculated during the exponential phase and the doubling time (T_d, h) was calculated from the maximal specific growth rate by the equation [35]:

$$T_d = \ln(2) / \mu_{max} \quad (3)$$

The biomass concentration (X, mg L^{-1}) was quantified by dry weight (DW) measurements, which was carried out by a centrifugation of approximately 50 mL sample, followed by a washing protocol using deionised H_2O and transfer to a pre-weighed ceramic crucible. The ceramic crucible was then placed at 105°C for 5 days and re-weighed as soon as possible after cooling. The biomass productivity (P_x, mg L^{-1} d^{-1}) was calculated by the equation:

$$P_x = (X \cdot V - X_0 \cdot V_0) / t \quad (4)$$

Where X_0 and V_0 refer to the biomass concentration and culture volume in the bioreactor at the beginning of the experiments, respectively; and t is the cultivation period (days). Also, the maximum biomass productivity (P_{max}) of cultures under different conditions was calculated depending on the biomass changes during the exponential phase.

2.3.3. Nitrogen/Phosphate removal capacity

In order to assess the nutrients (mainly in terms of nitrogen, N, and phosphorus, P) removal capacity of *C. vulgaris* under different dCO_2 levels, the concentrations of $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ in algal solution were determined using an Ion Chromatography (ICS-5000, ThermoFisher Scientific Inc, USA) at the beginning and end of the experiments. All the samples were filtrated with 0.2 µm filters and stored at – 20 ℃ before analysis. The total nutrients in feed-batch cultures involved two parts: $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ in the bioreactor and those supplied from the medium storage bottle. Therefore, the nutrients uptake rate (N_{uptake}, mg d^{-1} L^{-1}) was calculated by the equation:

$$N_{uptake} = [C_0 (V_0 + V_{add}) - C_{end} V_{end})] / t \quad (5)$$

Where C_0 and C_{end} respectively represent the $N\text{-}NO_3^-$ or $P\text{-}PO_4^{3-}$ concentration (mg L^{-1}) in the bioreactor at the beginning and end of cultivation; V_0 and V_{end} respectively represent the culture volume in the bioreactor at the beginning and end of cultivation, and V_{add} is the volume of medium that was pumped into the bioreactor (where V_{add} is 0 for the batch culture).

2.3.4. pH control

The pH of *C. vulgaris* cultures was measured continuously. For the batch culture, the pH was allowed to change freely. The growth medium-feed for the FB cultures, on the other hand, was linked to the variations in pH. Due to the volume limitation of photobioreactor, the FB growth could be divided into phases: a fed-batch phase (FB-phase), during which the growth medium was added to keep pH constant (6.5 ~ 6.8) [36]; and a batch phase (B-phase), after the addition of all the growth medium (5 L), during which these cultures entered the batch culture mode and the pH was allowed to fluctuate freely.

3. Results

The experiments were designed to compare CO_2 utilization efficiency when CO_2 was pre-dissolved in the growth medium with when it was supplied continuously in gaseous form (aeration in the culture vessel). A batch experiment was performed under the "standard" condition. This is comparable to the FB_control experiment where the medium was fed into an air-aerated bioreactor. In the FB_blank experiment, the growth medium was in equilibrium with the air and fed into the culture using pH control. No aeration was applied in the culture vessel under this experimental setting. The FB dCO_2-enriched experiments used media in equilibrium with pure CO_2 gas at different absolute pressures, achieving a series of CO_2 concentrations

dissolved into the growth media (Table 1). The feeding regime for all FB cultures was based on the addition of growth media through the acid pump to keep the culture pH stable.

3.1 pH changes during growth

In the "standard" batch culture, the pH progressively increased in the first 48h, and then fluctuated between 10.0 and 11.2. In the FB_blank culture, the pH was controlled and remained constant at approximately 6.8. For the other FB cultures, their pH values remained constant at 6.5 (controlling point) while they were being fed with eCMs (at FB-phase). Once the growth media were all added into the culture vessel, the cultures passed into batch mode (at B-phase) and the pH increased sharply and finally plateaued around 9.8.

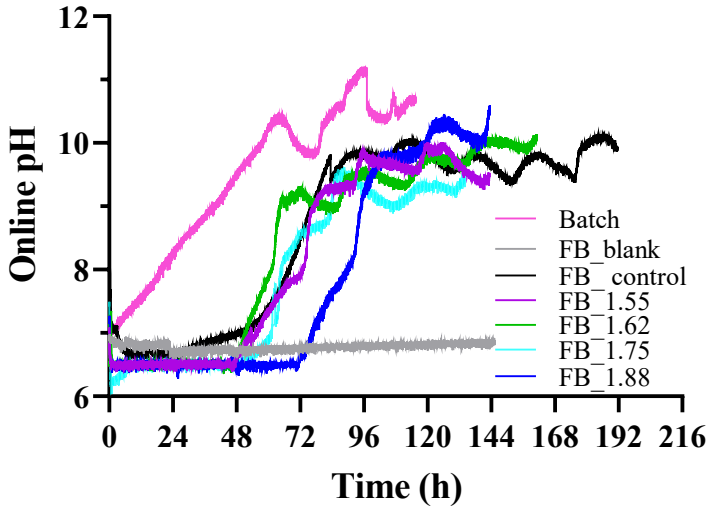

Fig. 2. pH variations of *C. vulgaris* cultures under different feeding regimes ("FB" stands for the fed-batch culture, FB_control and FB_blank respectively represent the fed-batch cultures injected with and without air in the photobioreactor, whereas FB_1.55, FB_1.62, FB_1.75 and FB_1.88 are the fed-batch cultures fed

with the media enriched with 1.55, 1.62, 1.75 and 1.88 g L^{-1} dCO_2, respectively. Same legends also apply to the following figures and tables).

3.2. Growth and productivity of C. vulgaris under different feeding regimes

In batch culture, the growth was linear from the beginning until 48h, yielding a final population of 8.06 × 10^{10} cells and 1140 mg DW at the end (Fig. 3a, Table 2). Considering that the growth stopped at 48h, a maximum biomass productivity (P_{max}) of 79.9 mg DW L^{-1} d^{-1} was obtained (Table 2).

All FB cultures presented a classical growth pattern consisting of the lag, exponential and stationary phase (Fig. 3a). The FB_control culture achieved a final population of 7.67×10^{10} cells (or 1441 mg DW, Table 2), which is comparable to the final biomass of batch culture under the "standard" condition. The final *C. vulgaris* biomass produced in FB cultures was related to the dCO_2 concentrations in the feeding media. The culture fed with growth medium with a dCO_2 concentration at 1.62 g L^{-1} exhibited the highest final population (1.3 ×10^{11} cells, Fig. 3a), 1.6 times higher than that of the batch and FB_control cultures. The FB cultures with lower or higher dCO_2 concentrations had lower final populations.

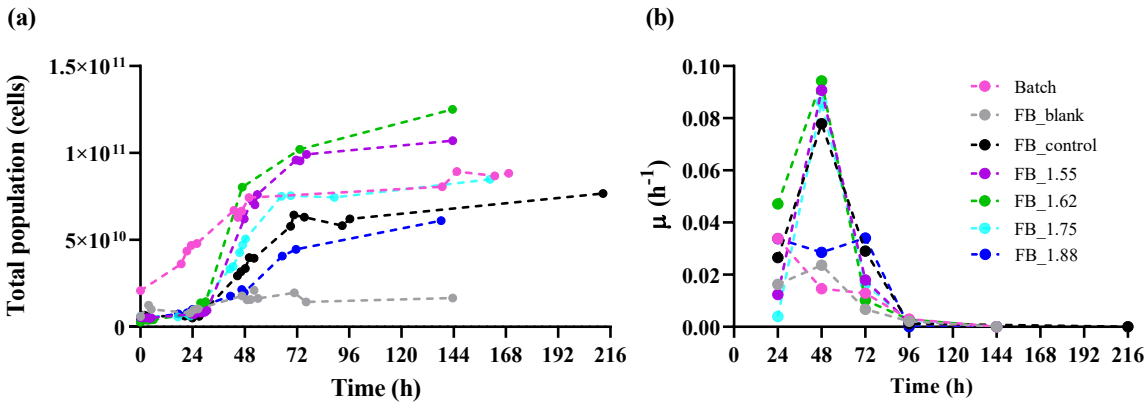

Fig. 3. Total population (a) and specific growth rate (b) of *C. vulgaris* in different cultures (same legends in b also apply to a).

In batch culture, the growth rate (μ) decreased during the culture period with a maximum value of 0.034 h^{-1} determined during the first 24h (Fig. 3b, Table 2). This was 2.3 times lower than the μ_{max} (0.078 h^{-1}) estimated in the FB_control culture. The growth rates of all FB cultures greatly increased on the second day of culture and then decreased progressively, except for the FB culture at 1.88 g L^{-1} dCO_2, with a constant μ at around 0.032 h^{-1} during the first 72 hours. The culture grown at 1.62 g L^{-1} dCO_2 exhibited the highest growth rate (μ_{max} = 0.094 h^{-1}), closely followed by the μ_{max} for the cultures at 1.55 and 1.75 g L^{-1}. After 72h, near-zero growth rates for all cultures were observed (Fig. 3 b).

Table 2. Growth parameters (maximum specific growth rate, doubling time, maximum biomass productivity and final biomass) of *C. vulgaris* cultures under different feeding regimes.

Treatment	Duration of experiments (h)	Maximum specific growth rate (μ_{max}, h^{-1})	R^2 for μ_{max}	Doubling time (T_d, h)	Maximum productivity (mg L^{-1} d^{-1})	Final biomass production (mg)
Batch	169	0.034	0.90	20.4	79.9	1140
FB_blank	144	0.024	0.95	28.9	17.2	264
FB_control	212	0.078	0.97	8.9	86.5	1441
FB_1.55	144	0.091	0.98	7.6	168.2	1744
FB_1.62	139	0.094	1.00	7.4	222.3	2040
FB_1.75	161	0.086	0.99	8.1	123.0	1381
FB_1.88	138	0.034	0.98	20.4	40.8	996

Without continuous air-injection, the FB_blank culture showed the lowest final biomass productivity of 4.5 mg L^{-1} d^{-1} (Fig. 4) while the batch and FB_control cultures at atmospheric dCO_2 concentrations achieved a higher productivity around 25.0 mg L^{-1} d^{-1}. Also, the final biomass productivity in the FB cultures peaked (59.5 mg L^{-1} d^{-1}) at a dCO_2 concentration of 1.62 g L^{-1}. The dCO_2 dosage-dependent effect on the P_{max} of *C. valgaris* cultures was consistent with that on their final biomass productivities (Fig. 4, Table 2).

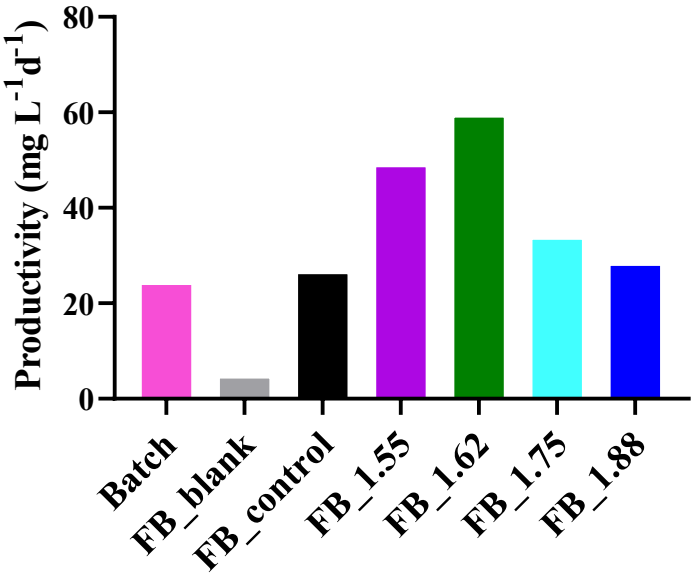

Fig. 4. Biomass productivity of *C. vulgaris* cultures grown under different feeding regimes.

3.4. Effect of dCO_2 on C. vulgaris nutrients uptake

In the FB cultures, the residual N-NO_3^- and P-PO_4^{3-} concentrations at the end of the culture period decreased with the increasing medium dCO_2 up to the value of 1.62 g L^{-1} and then increased with the higher dCO_2 concentrations (Fig. 5). Conversely, the nutrients uptake rates were stimulated by the increasing pCO_2 and

then lowered. The maximum uptake rates of $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ (7.5 and 1.6 mg L^{-1} d^{-1}, respectively) were both observed in the FB_1.62 g L^{-1} dCO_2 culture. On the other hand, the FB_blank culture displayed the lowest nutrients uptake rates. The batch culture showed higher $N\text{-}NO_3^-$ (2.2 times) and $P\text{-}PO_4^{3-}$ (1.1 times) uptake rates than those for the FB_control culture, but significantly lower (0.25 and 0.69 times, respectively) than those for FB_1.62 g L^{-1} dCO_2 culture.

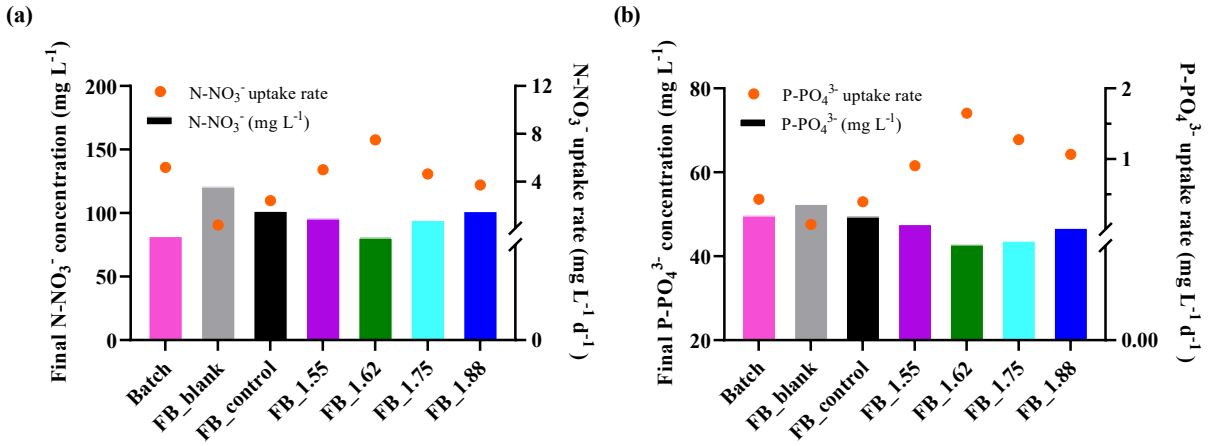

Fig. 5. Final nutrients ($N\text{-}NO_3^-$, a; and $P\text{-}PO_4^{3-}$, b) concentrations and uptake rates of *C. vulgaris* under different feeding regimes.

4. Discussion

4.1. Effect of feeding modes on the growth of C. vulgaris

The pH-controlled FB operational mode proved a successful method for controlling and piloting cultures of *C. vulgaris*. Under atmospheric dCO_2 conditions, FB culture grew faster than B culture, although the final biomass concentration was not any higher (Fig. 2, Table 2). This suggests that the FB feeding mode may

outperform B culture for microalgae production. Similar findings have been reported in green algae *Scenedesmus sp.* and cyanobacteria such as *Microcystis ichthyoblabe* and *Spirulina platensis* [25,37,38]. Wang et al. [13] also reported that under high illumination, the FB culture of *Nannochloropsis oculata* boosted the algal growth as this feeding mode improved algal tolerance to adverse conditions. In our study, the fed-batch culture mode stimulated the algal growth probably by alleviating the negative effects of undesired parameters, such as a greater CO_2 limitation and light attenuation, which may occur in the batch culture.

In order to avoid a growth inhibition by the development of unfavourable factors (e.g. high pH, nutrients starvation and auto-shading effects) at the beginning of the experiments, the FB cultures were inoculated with a fifth of the population than the batch culture (with a small volume of medium, 0.5 L). Indeed, the growth conditions/environmental parameters are highly dependent on the biomass density [26]. The linear growth in the batch culture within the first 48h (Fig. 2) strongly suggests that the algal growth was factor(s)-limited [21]. During this period, the rise of pH in the batch culture (Fig. 2) may suggest declining dCO_2 concentration in the presence of an increasing population as a limiting factor. This might be because the photosynthetic CO_2 removal rate exceeded that of the CO_2 transfer rate from air to liquid [12,26]. The pH of the FB_control culture remained constant for longer than in the batch culture. This could be due to the intermittent addition of growth medium at a lower pH into the culture brought about by the FB mode. Additionally, although the bioprocess of nitrogen assimilation releases OH^-, which normally increases pH, no nutrients limitation for growth was observed in both cultures (Fig. 5).

During the FB-phase, the volume of FB cultures was lower than that of batch culture. One can imagine that the light availability for the FB cultures was greater due to a lower population and volume of liquid [39]. Thus, the photons (energy) accessible to each cell in the bioreactor were likely to be lower in the B culture. This could be another reason for the lower biomass productivity when using the batch culture mode. In order to better understand the influence of feeding mode on the light profiles and the subsequent effect on biomass

accumulation, the availability of light to individual cells in both cultivation modes should be estimated in future studies.

4.2. Effect of dCO_2 concentration on the growth and biomass productivity of C. vulgaris

When CO_2 is used as the sole inorganic carbon source for autophototrophic cultures, two factors influencing algal growth should be considered: 1) carbon availability to cells, and 2) culture pH [40,41]. Before reaching the target volume, all the FB cultures remained at the optimal pH at 6.5 though there was a slight increase in the pH of the FB_control culture (Fig. 2). This was probably because the feed medium in this culture was not acidified enough.

The differences in growth performances of FB cultures during the FB-phase could be attributed to the different carbon availabilities in the growth media. The overall growth profile of *C. vulgaris* under various conditions suggests that algal growth was C-limited at low dCO_2 concentrations and inhibited at high dCO_2 concentrations. This finding is in line with previous studies investigating the impact of CO_2 concentrations on the development of microalgae [10,13,42]. In this study, one may classify the *C. vulgaris* cultures into four groups according to their growth profiles with respect to the CO_2 supplement regimes: strongly C-limited group (FB_blank culture), slightly C-limited group (batch and FB_control cultures), C-saturated group (FB_1.55 and FB_1.62 g L^{-1} cultures) and C-toxic group (FB_1.75 and 1.88 g L^{-1} cultures).

In the FB_blank culture, the rapid consumption of CO_2 by the algae probably resulted in a C-deficient environment that suppressed algal growth. The CO_2 captured through photosynthesis makes up the C-skeleton of macromolecules, such as enzymes, nucleic acids and polysaccharides, that are requisites for algal metabolisms, growth and reproduction [43]. Therefore, the lowest growth and population increase of *C. vulgaris* in this CO_2 feeding regime was likely hampered by a constraint in photosynthetic substrate (CO_2) availability rather than an altered pH. Compared to the FB_blank culture, the growth rate and biomass

productivity for the batch and FB_control cultures were higher, which could be related to higher CO_2 availability in these cultures due to continuous air injection. Despite the greater availability of CO_2 in these two cultures, C limitation was still observed, as indicated by the increasing specific growth rate when *C. vulgaris* was grown in 1.55 and 1.62 g L^{-1} of dCO_2 (μ_{max} of 0.091 and 0.094 h^{-1}, respectively). Limited growth under atmospheric conditions has also been reported in the other *Chlorella* strains [17,44,45], and other microalgae such as *N. oculata* [13] and *Scenedesmus obliqus* [10].

A moderate increase in the availability of CO_2 promotes algal growth [10,17]. In this study, the better media for *C. vulgaris* growth contained 1.55 and 1.62 g L^{-1} dCO_2 where the cultures achieved the highest growth rates, final biomass productions and productivities, as well as the shortest doubling time (Table 2). Increasing dCO_2 further (i.e., 1.75 and 1.88 g L^{-1}) reduced the growth rate, indicating that excess CO_2 supplements may be inhibitory to *C. vulgaris*. Whitney et al. [46] has illustrated that high CO_2 concentration decreased the transmission of photoelectron signals, affecting algal photosynthetic activity and thereby lowering the biomass productivity. High CO_2 concentration has also been found to induce reactive oxygen species and oxidative stress, inhibiting the growth of *Arthrospira platensis* [47] and *S. obliqus* [10]. Despite the CO_2 inhibition at higher levels, the FB_1.75 g L^{-1} culture still had superior growth parameters than those for the FB_control culture. This could be due to that the CO_2 toxicity was offset by the increased carbon availability.

4.3. Effect of pCO2 level on the nutrients uptake capability of C. vulgaris

In the FB cultures, the nutrients (N and P) uptake trends followed those of growth rate and biomass productivity, and peaked at a dCO_2 concentration of 1.62 g L^{-1}. This indicates that a moderately CO_2-enriched medium could improve the nutrients removal capacity of microalgae. The data is in agreement with Almomani et al. [8] who have discovered that with the increase of CO_2 concentration, the N and P

removal efficiencies of *S. platensis* and multispecies-microalgae cultures increased at first (< 10% CO_2, v/v) and then decreased with higher CO_2 concentrations (> 15%). Likewise, Hu et al. [44] also found that *Chlorella sp.* showed low N removal rates (87-89%) under atmospheric and 10% CO_2 conditions, with the greatest rate (96%) at 5% CO_2. CO_2 concentration may modulate the efficiency of photosynthesis, which fuels cellular metabolisms including nutrients absorption and assimilation [48]. It appears that the optimal CO_2 concentration could accelerate photosynthetic activity and then, indirectly enhance the nutrients uptake traits. Notably, *C. vulgaris* grown in batch culture presented higher N/P uptake rates than those for the FB_control culture, which could be related to a higher nutrients consumption due to the larger population at the beginning.

4.4. Feeding CO_2-enriched growth media may enhance the greenhouse gas capture by C. vulgaris

The pH in the FB cultures was maintained at 6.5 during the FB-phase. Under these conditions, the predominant inorganic carbon sources are free CO_2 (aq) and HCO_3^- in equilibrium [32], both of which are the species readily used by photosynthesis. So maintaining this pH (at 25°C) means that over 99% of the carbon species is available to the microalgae [32,49]. The main advantage of pre-dissolving the CO_2 into the feed medium over running a batch culture which has pH control with CO_2 injection is that in the former case, there are no CO_2 losses from the bioreactor. One may therefore suspect a high CO_2 utilization efficiency by pre-dissolving CO_2 into the growth medium, compared to the bubbling systems where a large fraction of CO_2 escapes in gas form [19,50]. A high CO_2 removal rate of up to 96% in *C. vulgaris* culture (in batch) within 1h was determined after CO_2 was pre-dissolved in the growth medium [51].

The CO_2 capture efficiency of *C. vulgaris* was estimated in gas-injecting bioreactors up to 80% [24] (Table 3). To enhance CO_2 bioremediation and mitigate the greenhouse effects, optimization procedures such as improving photobioreactors, gas flow rate, and algal acclimatization to high CO_2 concentrations or species

screening are required (Table 3) [24,50]. This study may provide a new approach for effectively capturing CO_2 through feeding CO_2-enriched media (even with high dCO_2). An additional benefit would be in the startup phase of larger configurations where normally a large volume of starter culture is required. Dissolving the CO_2 in the feed medium also simplifies the optimization of bioprocesses.

Table 3. CO_2 utilization efficiency of *Chlorella* species in gas-injecting photobioreactors.

Species	Bioreactor	Enriched CO_2 concentration	Aeration rate	CO_2 removal efficiency	Reference
Chlorella vulgaris	Bubble column photobioreactors	4*, 12%	1*, 2, 5×10^{-3} m s^{-1}	3.8-14.6%	[52]
Chlorella sp. L166	Erlenmeyer flask	5*, 10%	0.05, 0.10*, 0.15 vvm	< 27%	[44]
Chlorella sp. P12	Open thin-layer photobioreactor	Flue gas (6-8%)*, Pure CO_2	5*-35 m^3 h^{-1}	10-50%	[53]
Chlorella sp.	Semicontinuous photobioreactor	2*, 5, 10, 15%	0.25 vvm	16-58%	[54]
Chlorella sp. NCTU-2	Air-lift photobioreactors	10%	0.125*, 0.25, 0.5 vvm	< 63%	[55]
Chlorella vulgaris	Air-lift photobioreactor	2%	7.5*, 13.3×10^{-3} m s^{-1}	57-80%	[24]

* represents the optimized conditions under which microalgae achieved the highest CO_2 removal efficiency.

Flue gases, containing 3 – 30% v/v CO_2, present a viable and cost-effective alternative carbon source for microalgae cultivation [56,57]. The flue-gases injected cultures for microalgae, such as *Chlorella* [58], *Chlamydomonas* [59] and *Haematococcus* [60], have been enormously documented in previous studies. Compared to those cultures, microalgae cultures feeding with media that are pre-dissolved with the waste gases might potentially achieve superior rates of CO_2 bio-sequestration, according to the explanation above. Nevertheless, a significant challenge lies in the implementation of this strategy for flue gases. The coexistence of inhibitory compounds, such as SOx and NOx, coupled with high temperatures, may create unfavorable conditions that impede microalgae productivity [61,62]. As a consequence of targeting enriched-dCO_2 concentrations by increasing pressure, the concomitant rise of toxic pollutants in the growth medium would limit biomass productivity and subsequent CO_2 fixation efficiency. Hence, in further studies contemplating the adoption of flue gases using the culture technique established in this work, the necessity for screening SOx/NOx/thermal-tolerant species becomes evident.

Overall, the results demonstrated the superiority of the pH-controlled FB culture mode over batch cultivation in biomass production and CO_2 biofixation. This was achieved by dissolving CO_2 into the feeding medium, effectively mitigating CO_2 limitation arising from inadequate mass transfer. Simultaneously, starting with a lower volume of the culture may help alleviate potential light limitation. In this study, an optimal concentration of dCO_2 was also identified for *C. vulgaris*. Maintaining cultures at optimal pH with the addition of dCO_2-enriched media rather than directly injecting gaseous CO_2 may be an effective approach for maximizing CO_2 utilization by the microalgae. This operational mode could be most applicable to closed photobioreactors, particularly in pilot- or large-scale cultivation scenarios.

5. Conclusions

FB cultures with dCO_2-enriched media and feeding based on maintaining a constant culture pH was a promising culture strategy for simultaneously enhancing the biomass productivity and CO_2 removal capacity

of *C. vulgaris*. By dissolving CO_2 into the growth medium, the losses to the atmosphere were greatly reduced and faster growth could be obtained by keeping the culture pH constant. Using this novel technique, the optimal dCO_2 in the medium for *C. vulgaris* growth was determined to be 1.62 g L^{-1}. At this concentration, the μ_{max}, P_{max}, maximum N and P uptake rates determined for *C. vulgaris* were 0.094 h^{-1}, 222 mg L^{-1} d^{-1}, 7.5 and 1.6 mg L^{-1} d^{-1}, respectively. Furthermore, lower growth rate was observed under conditions of low CO_2-availability and high CO_2-toxic. Therefore, the pH-controlled FB culture of microalgae, based on dissolution of CO_2 in the feed medium, provides a promising technology for effective CO_2 capture.

Author Contributions

Conceptualization, B. Taidi and C. C. Wang; Formal analysis, B. Taidi, S. F. Li and C. C. Wang; Funding acquisition, B. Taidi; Methodology, B. Taidi and C. C. Wang; Supervision, B. Taidi; Visualization, B. Taidi and C. C. Wang; Writing-original draft, review and editing, B. Taidi and S. F. Li. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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Conflicts of Interest

The authors declare no conflict of interest.

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