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Enhancement of *Chlorella vulgaris* cell density: Shake flask and bench-top photobioreactor studies to identify and control limiting factors

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Abstract−Low cell density is a major bottleneck in any microalgal bioprocess that prevents the large scale exploitation of this potential bioresource from commercialization of commodities like biofuels. Control of factors limiting growth is the key to enhancing cell density. Factors limiting photoautotrophic growth of *C. vulgaris* were identified and controlled to a possible extent. Limiting CO_2-transfer rate, light attenuation, scarcity of nutrients, and high pH compounded to retard growth gradually in the basal medium. Analysis of the maximum feasible CO_2 mass-transfer rate and CO_2 fixation rates enabled the assessment of CO_2-limited growth without on-line estimation of dissolved CO_2. Growth (1.4×10^8 cells mL^{-1}, 12.6 g dry wt L^{-1}) was extensively enhanced when limiting factors were staved in a customized 250 mL stirred-tank photobioreactor. Scaling the culture 8 times with constant k_La (volumetric mass-transfer coefficient) and Re_i (impeller Reynolds number) resulted in reduction of biomass titer by 80% because of light attenuation.

Keywords: Microalgae, *Chlorella vulgaris*, Limiting Factors, Photoautotrophic Growth, Photobioreactor

INTRODUCTION

Microalgae have long been an inexpensive source of valuable metabolites [1]. Moreover they have emerged as a potential resource for sustainable production of biofuels capable of meeting the global demand of liquid fuels [2,3]. However, microalgal cultures are prone to several limitations, resulting in low-cell density and biomass productivity. Consequently, the major challenge in any microalgal bioprocess is to produce high-cell density for economic viability of the process-especially for low value products. Also, when product synthesis is not coupled with growth, increasing cell density before subjecting cells to specific stimulus can substantially improve product yield.

Microalgae are photosynthetic organisms; as such they are slow growing and growth is further slowed due to the multitude of changes brought in the culture environment. With the increase in cell density, light available for photosynthesis inside the culture vessel is attenuated due to self-shading, scattering and, absorption by the cells [4]. This problem becomes even more severe as the culture is scaled-up. Moreover, exhaustion of nutrients, changes in pH, oxygen build-up and low availability of carbon dioxide constrain photoautotrophic growth of several important microalgae [5,6]. Ironically, a systematic and complete investigation of these limiting factors all together is never undertaken for any microalga. Since these changes occur simultaneously and are connected, it is hard to study the influence of each separately on growth. This makes the identification of limiting factors more difficult. As an illustration, in an actively growing microalgal culture, the demand of CO_2 is ever increasing and the growth is accompanied by pH changes which shifts the dissolved CO_2 equilibrium, making it difficult to ascertain as to which of these changes would limit the growth and in what capacity. In addition, other factors can further complicate the problem. A clear understanding of this dynamics is necessary to propose suitable control strategies.

Consequences of nutrient deficiencies have been well documented. In general, starvation of macronutrients (nitrogen, phosphorus, and sulphur) blocks biosynthesis of protein and accumulates carbohydrate and neutral lipid [7]. Iron is a key micronutrient for growth of microalgae. Liu et al. reported a significant increase in *C. vulgaris* cell density and lipid content on increasing the iron content of the growth medium [8]. Similar effects of iron have been demonstrated by Concas et al. on experimental validation of their mathematical model describing the effect of iron concentration on growth and lipid accumulation in *C. vulgaris* [9]. Mandalam and Palsson reported a marked increase in cell concentration when the medium components were balanced stoichiometrically based on the elemental composition of *C. vulgaris* [10]. This technique of designing high capacity growth medium for microalgae has been since commonly used. A major challenge in algal cultures is the penetration of light. The rate of photosynthesis and hence specific growth rate of the light-limited cells in dense microalgal cultures decreases with the increase in cell density. However, in response, the cells raise their pigment content in an effort to keep the rate of photosynthesis unchanged [11]. This phenomenon, known as photoacclimatization, is common in photosynthetic organisms [12,13].

Varying pH is another limiting factor in microalgal cultures. It is

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generally believed that as the carbon uptake rate increases, decrease in pCO_2 of the medium increases the pH [14]. Also, nitrogen assimilation changes the pH of medium depending on the nitrogen source and this is evident from the stoichiometry of algal growth [15]. Changes in pH affect metabolism, solubility of nutrients, and concentration of dissolved CO_2. Optimum pH for growth is species dependent. For instance, *C. vulgaris* grows well within the pH range 6.5-7.5 [14], whereas optimum pH for *C. ellipsoidea* is 10 [16]. Sparging CO_2 enriched air in culture buffers pH and ensures a constant availability of this frequently limiting carbon source. Like pH, CO_2 tolerance is also species dependent. Concas et al. reported that *C. vulgaris* can be grown in pure (100% v/v) CO_2 [17], but 5-10% CO_2 is generally used to sustain high cell density in phototrophic cultivation [10]. Chinnasamy et al. observed the maximum growth of *C. vulgaris* at 6% CO_2 [18]. Oxygen, being the by-product of photosynthesis, accumulates quickly in microalgal cultures to inhibitory levels and is a potential threat to growth in the later stage [19]. Inhibition of photosynthesis by high oxygen concentration was first reported by Warburg in *Chlorella* and thereafter found in many other photosynthetic organisms [20,21]. Low pCO_2/pO_2 ratio slows the rate of carbon fixation and promotes photorespiration in addition to the development of oxygen radicals in high light intensities, which can have deleterious effects [5,22].

We conducted experiments on *Chlorella vulgaris*, which is commonly used as a nutritional supplement and has been extensively studied for its suitability for biodiesel production [23,24]. Biomass titer as low as 1 g L^{-1} is generally obtained in batch photoautotrophic cultures of *C. vulgaris*. Cell density or biomass titer of the microalga has been enhanced in many reports, mainly by medium redesigning [10], fed-batch culture [25] and, ultrafiltration [26,27]. However, a systematic investigation and control of growth-limiting factors is never undertaken. Such study will not only enhance cell density and our understanding of the culture dynamics, but will also serve as a framework to identify growth-limiting factors for further improvement of cell density in any established cultivation medium or technique. In the present work, factors impeding photoautotrophic growth of *C. vulgaris* were determined for each experiment from observations and reasoning. These limiting factors were then controlled in the following experiments. The process was repeated until it became impossible to further circumvent the growth-limiting factor.

MATERIALS AND METHODS

1. Microorganism and Growth Medium

Chlorella vulgaris (CCAP 211/11B) used in this research was obtained from Culture Collection of Algae and Protozoa (CCAP), Scottish Marine Institute, United Kingdom. Bold's Basal Medium (BBM), an inexpensive freshwater synthetic medium, without any carbon source and vitamins, was used for maintenance and experimental studies on the strain. Composition of BBM per liter is as follows: $NaNO_3$, 250 mg; $CaCl_2{\cdot}2H_2O$, 25 mg; $MgSO_4{\cdot}7H_2O$, 75 mg; K_2HPO_4, 75 mg; KH_2PO_4, 175 mg; NaCl, 25 mg; $ZnSO_4{\cdot}7H_2O$, 8.82 mg; $MnCl_2{\cdot}4H_2O$, 1.44 mg; MoO_3, 0.71 mg; $CuSO_4{\cdot}5H_2O$, 1.57 mg; $Co(NO_3)_2{\cdot}6H_2O$, 0.49 mg; H_3BO_3, 11.42 mg; EDTA, 50 mg was dissolved in KOH (31 mg L^{-1}); $FeSO_4{\cdot}7H_2O$, 4.98 mg was acidified with 1 mL concentrated sulfuric acid. The pH was adjusted to 6.6 before autoclaving.

2. Light Source

Customized white LED light source of 200W with PWM (Pulse width modulation) based intensity control unit was used to provide the required photosynthetic photon flux density (PPFD, μmol m^{-2} s^{-1}) to microalgal cultures. Quantum sensor (LI-COR LI-190SB, USA) was used to measure the incident PPFD. Maximum PPFD attained from the fabricated light source was 600 μmol m^{-2} s^{-1}.

3. Shake Flask Culture

To photo-acclimatize and protect cells from entering any limiting conditions of light and nutrient, inoculum was developed in the same light intensity at which the shake flask or photobioreactor experiment was performed and the biomass was harvested for inoculation at an absorbance (*Abs*) between 0.3 and 0.4 at 750 nm. Culture flasks were incubated on an orbital shaker set at 150 rpm and the temperature was maintained at 25±1 °C. Constant light was supplied from the bottom of the flask using the LED light source. Under sterile conditions, 15 mL of culture was centrifuged and the biomass recovered was added to the medium to give an Abs_{750} of ca 0.05 (~3×10^5 cells mL^{-1}).

In all shake flask studies, 500 mL baffled conical flasks (Schott Duran, Germany) were used containing 100 mL media inoculated with biomass (as discussed above), incubated at 25±1 °C, 150 rpm in an orbital shaker, and light was supplied from bottom of the flasks. All experiments were performed in triplicate.

4. Photobioreactors Design, Construction and Scale-up

Two glass stirred tank reactors (250 mL and 2 L) were designed and fabricated. Standard stirred tank reactor design with paddle impeller was used (Fig. 1). Reactor dimensions were calculated based on the working volume of 70% and standard geometric proportions from Rushton et al. [28]: $D_T/D_i=3$, $H_L/D_i=3$, $H_i/D_i=1$, $W_b/D_T=0.1$, $W_i/D_i=0.25$, where H_L is the height of liquid, D_T the diameter of tank, D_i the impeller diameter, W_i the width of impeller, H_i the height of impeller from the bottom of tank and W_b the width of baffles.

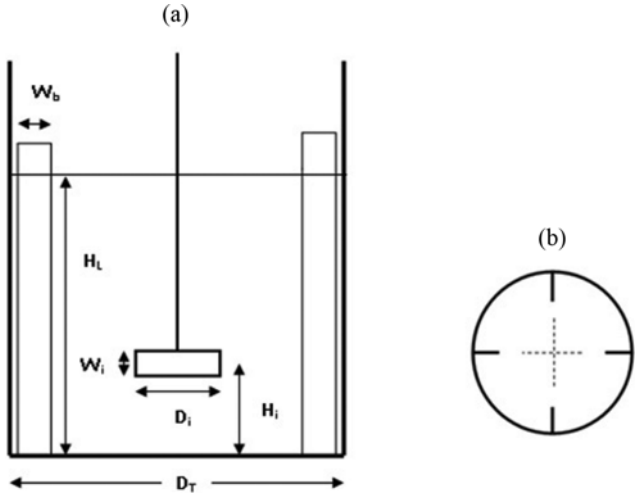

Fig. 1. Schematic diagram of the reactor design. (a) Tank configuration and measurements; (b) top view of tank showing four equally spaced baffles.

Geometric similarity, constant impeller Reynolds number (Re_i) and volumetric oxygen transfer coefficient $(k_La)_{O2}$ were the three criteria used for scale-up. Since both the reactors were designed with the same geometric proportions, they are geometrically similar. Calculated Re_i of 250 mL photobioreactor was 31×10^3 at an operating stirrer speed of 500 rpm. To maintain the same Re_i in the scaled reactor, the stirrer speed of 2 L reactor was set at 250 rpm. The dynamic gassing-out method was used for the measurement of $(k_La)_{O2}$. At 1 vvm air aeration and stirrer speed of 500 rpm, $(k_La)_{O2}$ of 250 mL photobioreactor was 25.5 h^{-1}. To maintain the same $(k_La)_{O2}$ in 2 L photobioreactor, different air flow rates were tested for the required $(k_La)_{O2}$ at 250 rpm in 2 L photobioreactor. Aeration rate of 1.5 vvm was selected for 2 L photobioreactor since $(k_La)_{O2}$ was 28.8 h^{-1} at this air flow rate and 250 rpm stirrer speed.

Light was supplied from bottom of the reactor. Air and CO_2 flow rates were measured and controlled with separate rotameters. Gas composition was determined with exhaust gas analyzer (EGAS-L, Sartorious BBI Systems GmbH) when CO_2 was mixed with air. In experiments where the concentrations of the medium nutrients were required to be maintained during cultivation, sterile medium was circulated through a visking tube suspended inside the photobioreactor. The visking tube (Dialysis membrane-60, HIMEDIA) was boiled and rinsed in distilled water before it was suspended inside the photobioreactor. Complete setup is represented diagrammatically in Fig. 2. All photobioreactor experiments were performed in duplicate.

5. Analytical Methods

5-1. Elemental Analysis of Dry Biomass

Carbon, hydrogen, nitrogen, and sulfur content of dry biomass were determined by element analyzer Vario EL III (Elemantar Analysensysteme, Hanau, Germany). Whereas, potassium, magnesium, calcium, iron, zinc, copper, and manganese content were obtained by inductively coupled plasma optical emission spectroscopy (Optical 2100DV, Perkin Elmer, USA). For phosphorus content, sample was first digested with sulfuric acid followed by persulfate digestion to convert polyphosphates and organic phosphorus compounds to orthophosphate [29]. Orthophosphate estimation was based on the method described by Martland and Robinson [30].

5-2. Growth Estimation

Cell density (cells mL^{-1}) was measured using a hematocytometer (HBG, Germany). Specific growth rate (μ) during a 24 h interval was calculated according to Eq. (1):

$$\mu(day^{-1})=\ln X_{t+24}-\ln X_t \quad (1)$$

where X_t and X_{t+24} are the cell densities at the time t and after 24 h, respectively. Biomass concentration (g L^{-1}) was measured by centrifuging (10,000 rpm for 10 min) a measured volume of culture in a pre-weighed tube and washing the cell pallet with distilled water followed by drying at 80 °C for 24 h.

5-3. Estimation of Chlorophyll Content of Cells and Nitrate in the Broth

Chlorophyll a and b concentrations were determined according to the method of Wellburn [31]. Residual nitrate in the spent broth was estimated by spectrophotometric method described by Cataldo et al. [32]. Supernatant obtained by centrifuging culture was used for the analysis of residual nitrate.

5-4. Protein, Lipid, and Carbohydrate Content of Algal Cells

For protein estimation, cells were lysed in alkali followed by measurement of protein content by the method of Lowry et al. [33]. BSA (50-500 mg L^{-1}) was used as a standard and the absorbance was measured at 750 nm. Lipids were extracted by the method of Folch et al. [34]. For carbohydrate content, biomass was acid hydrolyzed and neutralized followed by the estimation of total carbohydrate by phenol-sulfuric acid method of Dubois et al. [35]. Glucose (20-100 mg L^{-1}) was used as a standard and the absorbance was measured at 490 nm.

5-5. Theoretical Estimation of the Limiting Nutrients

Based on the elemental composition of the strain grown under

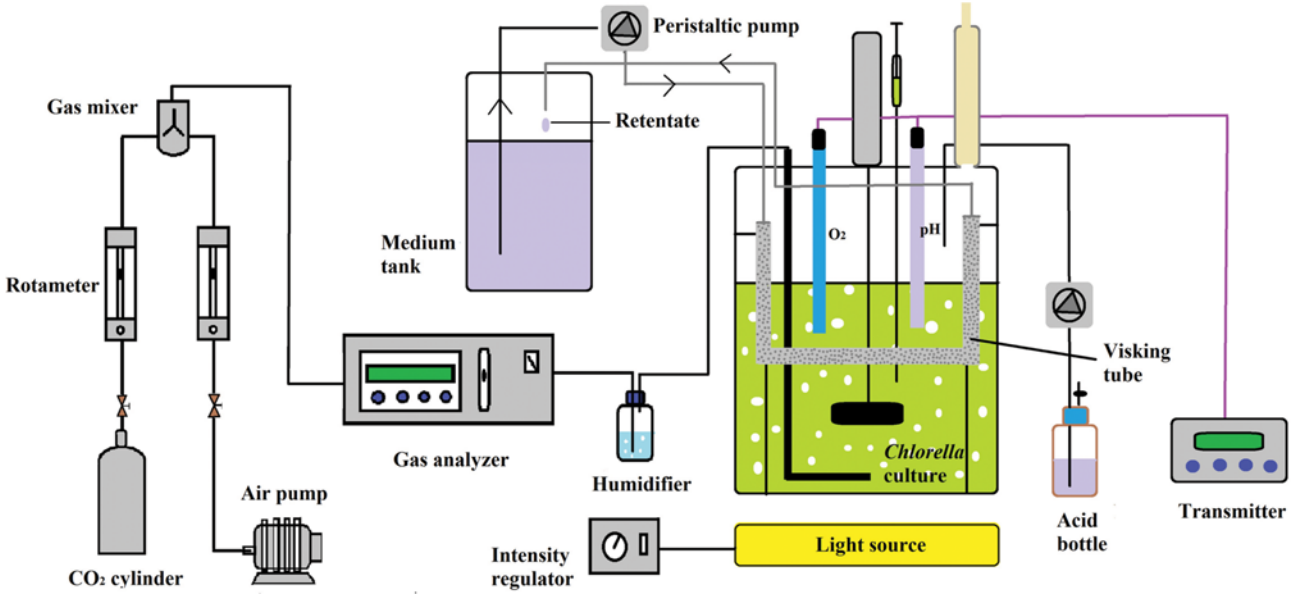

Fig. 2. Schematic layout of the photobioreactor setup.

nutrient and light replete conditions, biomass production capacity of the basal medium was evaluated similar to the analysis performed by Mandalam and Palsson [10]. Biomass, which can be produced from an element, is determined from Eq. (2):

$$\text{Biomass (g L}^{-1}) = \frac{c}{w} \times 10^{-3} \quad (2)$$

where c is the concentration (mg L^{-1}) of the element in the medium and w is the weight fraction of the element in the dry biomass.

5-6. Assessment of CO_2 Limitations

Carbon dioxide is more soluble in water than oxygen but at atmospheric CO_2 concentrations (0.04%), CO_2 equilibrium concentration in water is about 0.6 mg L^{-1} at 1 atm and 25 °C. Carbon content of *C. vulgaris* used in this research is 54% of the dry weight and accordingly 1.98 g CO_2 is required per gram of dry biomass. From these data it can be calculated that just 0.3 mg L^{-1} biomass can be produced on complete assimilation of the dissolved CO_2, assuming nutrient sufficiency and CO_2 consumed is never replaced. It is likely that under inadequate mass transfer conditions, CO_2 can limit growth before any other factor becomes limiting. Assessing CO_2 limitation is therefore important and has been theoretically worked out using the concept of maximum CO_2 transfer rate (CTR_{max}) from sparged gas to broth under the operational conditions and CO_2 fixation rate (CFR) of the algal cells. Mass-transfer of CO_2 is growth limiting when CFR=CTR_{max}. Eq. (3) written for CO_2 transfer rate (CTR) can be used to determine CTR_{max} by substituting dissolved CO_2 concentration (C) equal to zero (Eq. (4)) and replacing volumetric CO_2 transfer coefficient $(k_La)_{CO2}$ with easily measurable $(k_La)_{O2}$ using the relationship proposed by Fair [36] (Eq. (5)):

$$\text{CTR } (g_{CO_2} L^{-1} h^{-1}) = (k_L a)_{CO_2} (C^* - C) \quad (3)$$

$$CTR_{max} \ (g_{CO_2} L^{-1} h^{-1}) = (k_L a)_{CO_2} (C^*) \quad (4)$$

$$(k_L a)_{CO_2} = (k_L a)_{O_2} \left[\frac{D^{CO_2}}{D^{O_2}}\right]^{0.5} = (k_L a)_{O_2} \left[\frac{2.0\times10^{-9} m^2 s^{-1}}{2.4\times10^{-9} m^2 s^{-1}}\right]^{0.5}_{25\,^\circ C} = 0.9(k_L a)_{O_2} \quad (5)$$

where C* is the equilibrium concentration of CO_2 (g L^{-1}) in the broth at the operating gas CO_2 volume fraction, D^{CO2} and D^{O2} are the molecular diffusivities of CO_2 and O_2 in water, respectively. Diffusivities are temperature dependent and their values at 25 °C are substituted in Eq (5).

C* was calculated using Henry's law (Eq. (6)) since the growth medium is alike water:

$$C^* (g\ L^{-1}) = \frac{x_{CO_2, Gas} \cdot P_T \cdot 44}{H_{CO_2}} \quad (6)$$

where $x_{CO_2, Gas}$ is the mole fraction of CO_2 in the gas phase, P_T is total gas pressure (atm) and H_{CO_2} is Henry's constant for CO_2 which is a function of temperature (H_{CO_2} in pure water at 25 °C is 29.41 L atm mol^{-1}).

CFR during a time interval can be estimated from the carbon content of cells using Eq. (7):

$$\text{CFR } (g_{CO_2} L^{-1} h^{-1}) = \frac{\Delta X \cdot w_C}{\Delta t} \times \frac{44}{12} \quad (7)$$

where ΔX is the change in biomass titer (g L^{-1}) during the time interval Δt (h), w_C is the carbon weight fraction of dry algal biomass and the factor (44/12) is multiplied to convert carbon fixation rate to CO_2 fixation rate.

RESULTS AND DISCUSSION

1. Shake Flask Experiments

Microalgal growth is generally limited by insufficient light available for photosynthesis. To avoid light limitation in our experiments, saturating level of PPFD was determined first by growing low cell density cultures (described in section 3 of Materials and Methods) for 24 h at intensities ranging from 100 to 600 μmol m^{-2} s^{-1}. Specific growth rate (μ) calculated from Eq. (1) increased with the incident PPFD and plateaus at 300 μmol m^{-2} s^{-1} (Fig. 3). Since

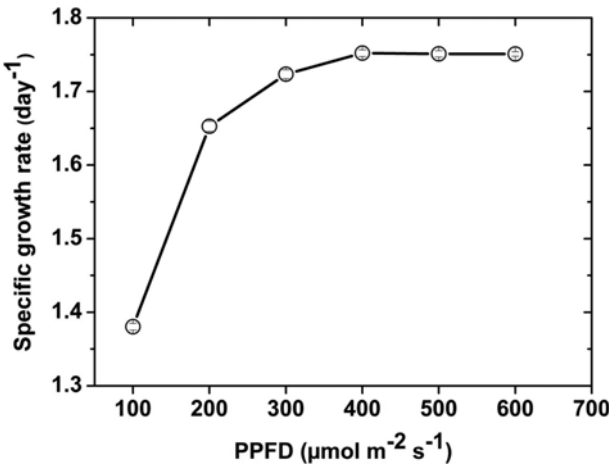

Fig. 3. Effect of light intensity on the specific growth rate of *C. vulgaris* (CCAP 211/11B) incubated for 24 h at low cell density (~3×10^5 cells mL^{-1}) in shake flask.

Table 1. Elemental composition of *C. vulgaris* (CCAP 211/11B) and the calculated biomass production capacity of the basal medium

Element	% In dry biomass (w/w)	Concentration in BBM (mg L^{-1})	BBM capacity to form biomass (g L^{-1})
Carbon (C)	54.00%	-	-
Nitrogen (N)	8.00%	40	0.50
Phosphorus (P)	1.53%	50	3.27
Potassium (K)	1.18%	80	6.78
Sulfur (S)	0.84%	11.51	1.37
Magnesium (Mg)	0.52%	7.39	1.42
Iron (Fe)	0.23%	1.00	0.43
Calcium (Ca)	0.19%	6.97	3.67
Zinc (Zn)	0.02%	2.01	10.05
Copper (Cu)	0.01%	0.40	4.00
Manganese (Mn)	0.01%	0.40	4.00

light is attenuated with the growth, intensities greater than the saturating intensity (supersaturating intensities) are generally preferred to compensate losses with time. In view of this, unless otherwise specified, we used 400 μmol m^{-2} s^{-1} in the following experiments. In addition, using Eq. (2) biomass formation capacity of each element in the basal medium was calculated to estimate limiting nutrient(s) stoichiometrically (Table 1). Low cell density cultures at 400 μmol m^{-2} s^{-1} were used for the elemental analysis of the biomass. The analysis shows that iron and nitrogen (as nitrate) are in limiting amounts in the basal medium.

We grew cells in the basal medium up to stationary phase and changes in the cell density, exogenous nitrate level, chlorophyll content of the cells and pH of the culture were determined every 24 h of growth at 400 μmol m^{-2} s^{-1} (Fig. 4(a)). Continuous decrease in the specific growth rate was observed. There was no change in the cell density after complete exhaustion of nitrate from the broth.

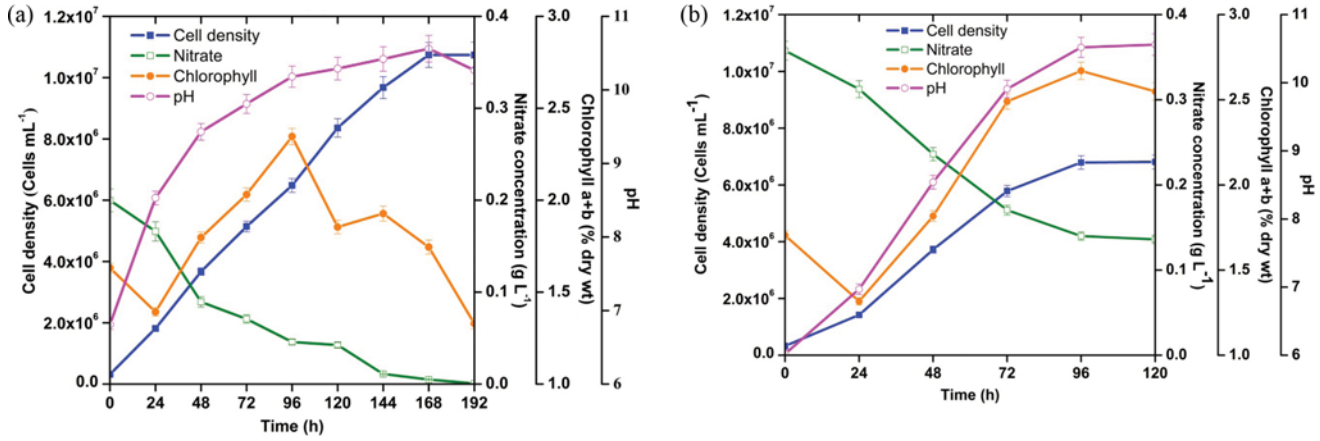

Fig. 4. Time courses of *C. vulgaris* (CCAP 211/11B) shake flask cultures at 400 μmol m^{-2} s^{-1} PPFD. (a) Growth in the basal medium; (b) growth in the nitrate and iron supplemented basal medium. Data are average of three replicates, error bar represents ±SD.

Table 2. Summary of cultivation conditions, limiting factors controlled, growth indices (cell density, biomass titer and productivity), factors that limited growth and, biochemical composition of cells grown up to stationary phase in shake flask and photobioreactor experiments

Culture vessel*	Cultivation conditions**	Factor controlled***	Cell density (cells mL^{-1})	Biomass titer (g L^{-1})	Biomass productivity (g L^{-1} day^{-1})	Growth-limiting factor (s)	Biochemical composition****		
							Carbohydrate (g L^{-1})	Protein (g L^{-1})	Lipid (g L^{-1})
Shake flask	Basal medium 400 μmol m^{-2} s^{-1}	-	1.07×10^7	1.0	0.14	pH NO_3^-, Fe	46.81	24.43	17.40
Shake flask	2 NO_3^-, 5 Fe	NO_3^-, Fe	6.8×10^6	0.6	0.15	pH	22.83	45.05	20.22
250 mL PBR	2 NO_3^-, 5 Fe Air sparging	pH	1.08×10^7	1.0	0.17	CO_2 mass-transfer rate	35.34	34.10	18.32
250 mL PBR+SM	Air sparging	Nutrient levels	9.57×10^6	0.9	0.15	CO_2 mass-transfer rate	21.50	49.08	20.35
250 mL PBR+SM	2% CO_2 enriched air	CO_2 mass-transfer rate	4.72×10^7	4.37	0.73	pCO_2/pO_2 ratio	18.61	50.39	21.21
250 mL PBR+SM	5% CO_2 enriched air	pCO_2/pO_2 ratio	6.29×10^7	5.81	1.16	Light	17.62	51.70	21.05
250 mL PBR+SM	5% CO_2 600 μmol m^{-2} s^{-1}	Light	1.36×10^8	12.62	2.10	Light	17.03	52.91	20.80
2 L PBR+SM	5% CO_2 600 μmol m^{-2} s^{-1}	-	2.91×10^7	2.69	0.54	Light	17.53	51.82	21.05

*SM represents Semi-permeable membrane used in the photobioreactor for maintaining the nutrient levels during growth. PBR: Photobioreactor

**Experiments were conducted in the Basal medium at 400 μmol m^{-2} s^{-1} PPFD. Only modifications in any of these two conditions are mentioned in the second column of the table

***In each experiment, all the factors which were controlled in the preceding experiment were also controlled. However, they were not mentioned in third column to avoid repetition

****Biochemical composition of cells grown in nutrient and light replete conditions is as follows: 21.80% Carbohydrate, 48.48% Protein, 20.51% Lipid. Carbohydrate, protein and lipid titers are the average values

Intracellular nitrogen content was reduced to 4% from 8%, explaining how the biomass obtained was more than double of the theoretical biomass production capacity of the limiting nutrient (Table 1). Carbohydrate content of the cells increased by twofold, whereas protein and lipid content was reduced by 50% and 15%, respectively (Table 2). Pruvost et al. reported similar changes in the biochemical composition of autotrophically grown *C. vulgaris* as a result of nitrogen starvation [37].

Medium became highly alkaline with growth. Consumption of nitrate (NO_3^-) results in the uptake of H^+ ions from the medium, and consequently the medium pH increases. Besides being metabolically detrimental, high pH can affect the medium chemistry by affecting the dissociation of dissolved CO_2 and solubility of nutrient salts. These changes can limit the growth severely at normal cell densities. To investigate the effect of pH on the specific growth rate, cells were grown at different pH values ranging from 5 to 11. A short incubation period of 12 h prevented any significant deviation from the initial pH values in all cases. It is evident from the results (Fig. 5(a)) that the growth is unaffected by the pH in the range 6-8, whereas highly alkaline pH retarded the growth significantly. Since the experiments were conducted at very low cell densities to avert any changes in light and nutrient levels during growth which can perplex the analysis of results, we believe that the effect of alkaline pH at high densities would be more than that observed. Therefore, the initial pH of medium was maintained at 6 in all the following experiments.

After 24 h of growth, chlorophyll content of the cells at first increased to maximum and thereafter it decreased. Increased chlorophyll indicates that the light is attenuated inside the culture, and in response the cells have raised their pigment content. This phenomenon of photo-acclimatization, which is common in microalgae [12], was also experimentally verified by the measurements of chlorophyll content of cells grown for 24 h at different PPFD (Fig. 5(b)). There was no residual iron in the filtered spent broth harvested in the end. Iron and nitrate deficiency seems to have reduced the chlorophyll content in the later phase. Reports on the breakdown of photosynthetic apparatus and pigments with the depletion of nutrients such as nitrogen and iron are available [38-40]. Moreover, precipitation of essential elements for chlorophyll synthesis such as magnesium at highly alkaline pH can also affect the synthesis of chlorophyll in the later stages of growth.

Theoretically, 1 g L^{-1} biomass with normal biochemical composition should be produced if the concentration of $NaNO_3$ is doubled in the basal medium to avert nitrogen limitation. Furthermore, if the shift in biomass composition is considered under nitrogen limitation, as observed earlier, then 2 g L^{-1} biomass should be produced. In addition, iron concentration has to be increased by fivefold to support 2 g L^{-1} biomass. Since high concentrations of micronutrients generally inhibit growth, the effect of high iron concentrations on the growth was investigated separately. The results show that the growth is not affected at iron concentrations up to sevenfold (Fig. 5(c)). EDTA in the basal medium must have chelated the supplemented iron, thereby making it ineffective at high concentrations. After the conviction of iron toxicity at its high

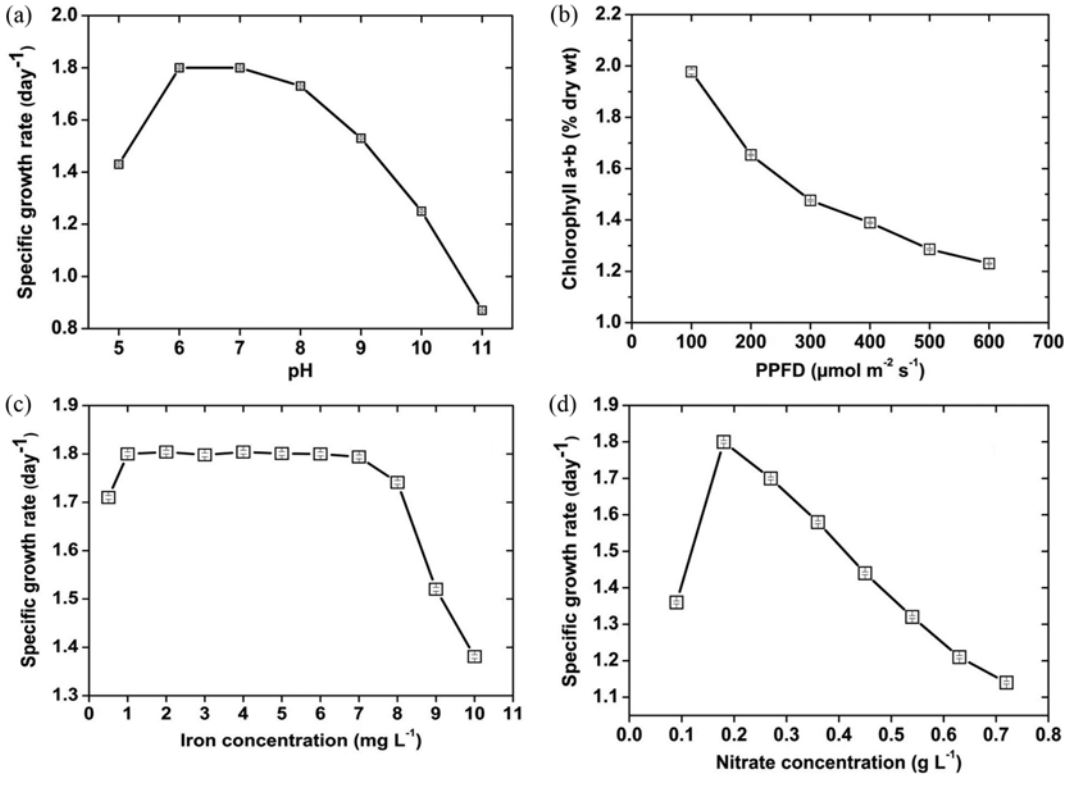

Fig. 5. Effect of pH (a), iron (c) and, nitrate (d) concentration on the specific growth rate of *C. vulgaris* (CCAP 211/11B) incubated for 12 h at 400 μmol m^{-2} s^{-1} PPFD and low cell density (~3×10^5 cells mL^{-1}) in shake flasks. Chlorophyll contents of 24 h grown *C. vulgaris* cells at different light intensities in the basal medium are shown in (b). Data are average of three replicates, error bar represents ±SD.

concentration has been ruled out, cells were grown in the basal medium with nitrate and iron concentrations two and fivefold, respectively. Despite nitrate sufficiency, the growth ceased due to highly alkaline pH (10.5) when only 0.6 g L^{-1} biomass was produced (Fig. 4(b)). Since the growth was not nitrate limited, intracellular nitrogen content (7.84%) was roughly maintained. Increased assimilation of nitrate per unit biomass, evident from the endogenous nitrogen and protein content of the biomass (Table 2), increased the pH to detrimental levels within a short span of time. Also, in the first 24 h of growth, the specific growth rate was lower than that obtained in the original basal medium. Since the increased concentration of iron in the basal medium has been shown to be ineffective on the growth rate (Fig. 5(c)), the twofold increase in nitrate could be inhibiting. To elucidate the effect of nitrate concentration on growth, cells were grown in basal media with nitrate concentrations ranging from 0.5 to 4 times the basal medium concentration (90-720 mg L^{-1} NO_3^-). Nitrate concentrations higher and lower than basal medium concentration (180 mg L^{-1} NO_3^-) suppressed growth (Fig. 5(d)). Low nitrate concentration limited growth, whereas higher concentrations are clearly growth-inhibiting. Growth inhibition at high nitrate concentrations was also reported for green microalga *N. Oleoabundans* [41].

2. Photobioreactor Experiments

Shake flask experiments could not throw any light on the CO_2 availability and O_2 concentration in the broth which can potentially affect growth. Also, uninvited changes in pH and nutrient levels during growth cannot be controlled in a shake flask. Consequently, the culture was scaled to 250 mL photobioreactor to determine and control potential growth-limiting factors. In basal medium with twofold nitrate and fivefold iron, 1 g L^{-1} biomass was produced when the pH was maintained within the optimum range manually (Fig. 6(a)). Growth did not retain even on the addition of depleted salts and increasing the PPFD. Moreover, oxygen was not accumulated to inhibit the growth. From the measurements of the carbon content of biomass sampled periodically, CFR was calculated for each interval of 24 h using Eq. (7). Calculated CFR's for each time interval and CTR_{max} (0.014 g L^{-1} h^{-1}) were of the same order of magnitude. This indicates that the rate of CO_2-transfer is limiting growth. Decrease in the chlorophyll content of cells observed in the later phase of growth could be a manifestation of carbon-limitation. In another experiment, semi-permeable membrane (visking tube) was used to continuously replenish the medium with exhausted nutrient salts (described in section 4 of Materials and Mehods). The original basal medium was used without any supple-

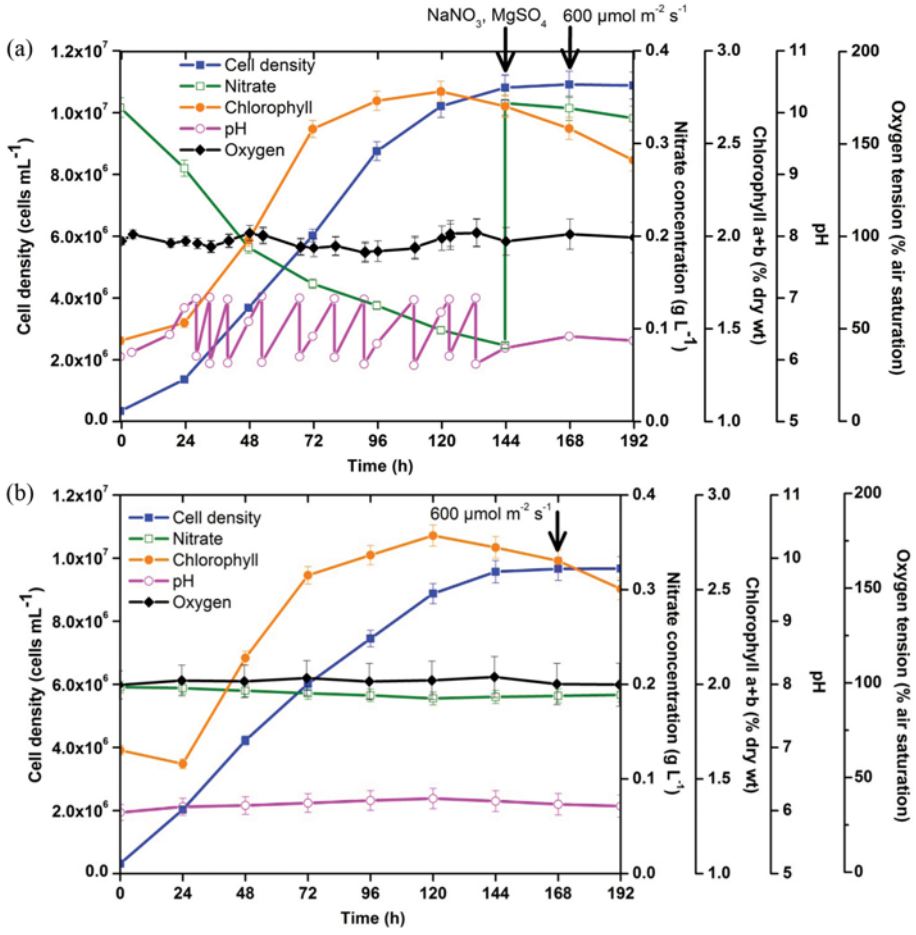

Fig. 6. Time courses of *C. vulgaris* (CCAP 211/11B) air sparged cultures in 250 mL photobioreactor at 400 µmol m^{-2} s^{-1} PPFD. (a) Growth in the nitrate and iron supplemented basal medium; (b) growth in the original basal medium. Semi-permeable membrane for maintaining the level of depleted nutrients was used only in (b). Data are average of two replicates, error bar represents ±SD.

mentation of nitrate or iron. Nitrate level was maintained throughout the course of cultivation, which shows that the diffusion of nutrients through the membrane was fast enough to compensate their exhaustion (Fig. 6(b)). Moreover, pH was also maintained as any increase in the concentration of OH^- in the culture must have been compensated by the diffusion of H^+ and OH^- ions through the membrane like nutrient salts. There was no oxygen accumulation and the growth did not retain on increasing PPFD. CFR calculations show that the growth is limited by CO_2 mass-transfer rate like before.

Improving CTR without changing stirrer speed and aeration rate is only feasible if C* is increased by increasing the mole fraction of CO_2 in the gas phase. From Eqs. (4) and (6), it can be calculated that with just 2% and 5% (v/v) CO_2 in air, CTR_{max} increases by 50 and 125 times, respectively. Also, CO_2-enriched air buffers pH and is generally used as a technique to control pH in algal cultures. Cultures grown in 2% and 5% CO_2 enriched air showed a considerable increase in the specific growth rate compared to air sparged cultures. The same specific growth rate in the first 24 h of growth at both the levels of enrichment suggested that the cells were CO_2 saturated in both cases. Biomass production was enhanced by CO_2 enrichment (Fig. 7(a)). Although CFR's were much below CTR_{max} at both the levels of enrichment, 5% CO_2 produced 1.5 g L^{-1} more biomass than 2% CO_2 (Table 2). High oxygen accumulation was observed in both cases (Fig. 7(a)). Relatively low pCO_2/pO_2 ratio at 2% CO_2 compared to 5% and, significant light attenuation evident from the chlorophyll content of cells due to high biomass titer at 5% CO_2 must have ceased the growth in these experiments. Since it is hypothesized that light limited the growth at 5% CO_2, an experiment was carried out at 600 μmol m^{-2} s^{-1} with 5% CO_2. At 72 h, strength of the growth medium circulating inside the visking tube was increased to 1.5 times to meet the increasing nutrient requirements of the cells evident from the rate of nitrate depletion (Fig. 7(b)). High biomass titer (12.6 g L^{-1}) in a relatively short span of time, despite of the fact that oxygen accumulated to detrimental levels, confirms that the growth was limited by light when 5% CO_2 enriched culture was irradiated with PPFD of 400 μmol m^{-2} s^{-1}. Pure nitrogen was sparged for few minutes to remove the accumulated oxygen when the growth ceased (Fig. 7(b)). No change in cell density was observed in the following 24 h of cultivation. This shows that the growth is primarily limited by light. Very high chlorophyll content of the cells is a clear manifestation of light-limited growth. However, in addition to light attenuation, concurrent accumulation of oxygen must have also contributed in the retardation of growth.

Culture was scaled to geometrically similar 2 L photobioreactor

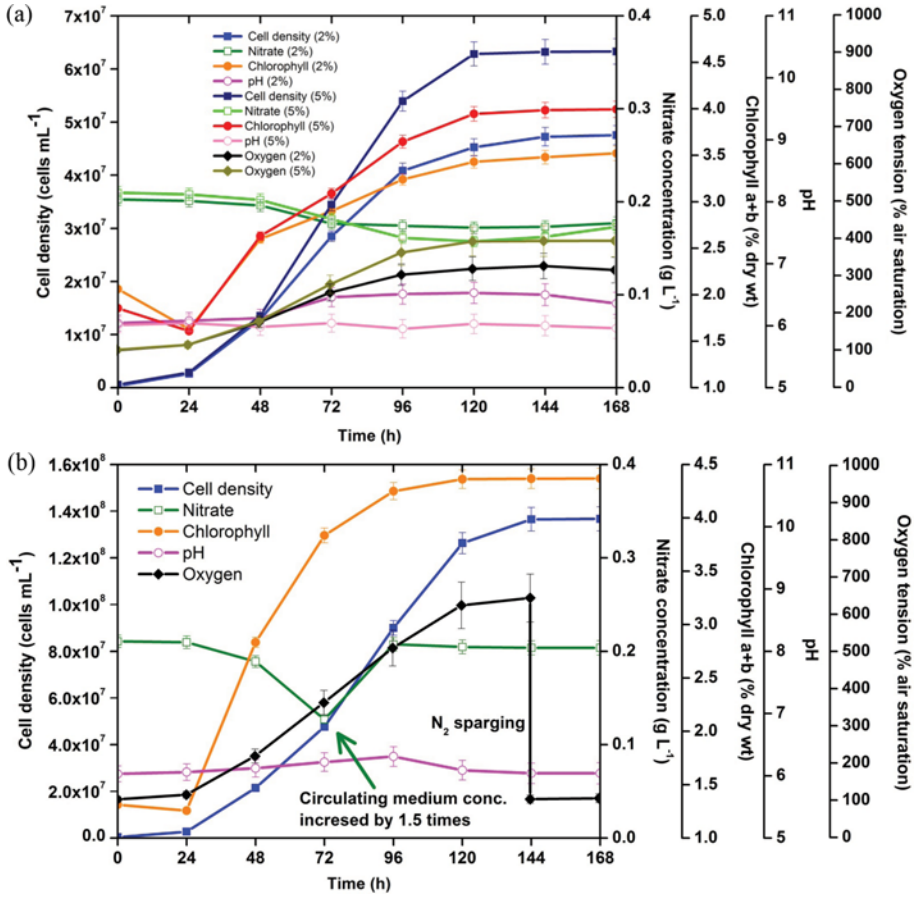

Fig. 7. Time courses of *C. vulgaris* (CCAP 211/11B) CO_2 enriched cultures in 250 mL photobioreactor with semi-permeable membrane. (a) 2% and 5% (v/v) CO_2 enriched air sparged cultures at 400 μmol m^{-2} s^{-1} PPFD; (b) 5% (v/v) CO_2 enriched air sparged culture at 600 μmol m^{-2} s^{-1} PPFD. Data are average of two replicates, error bar represents ±SD.

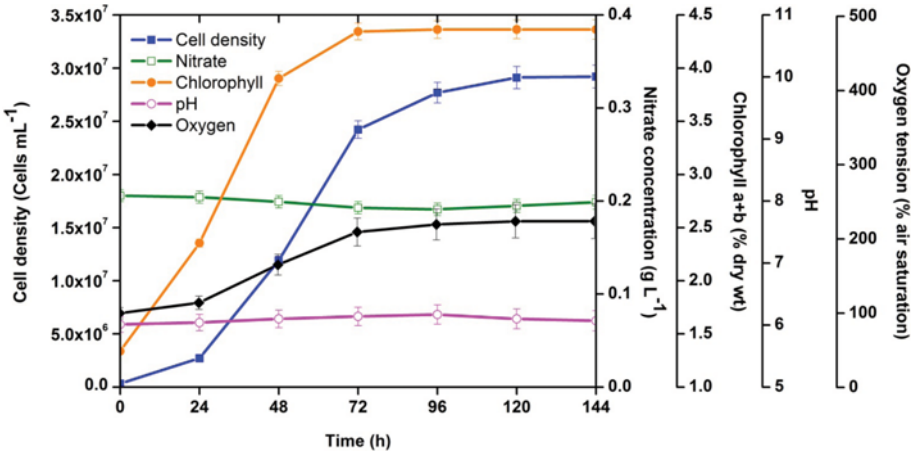

Fig. 8. Time course of *C. vulgaris* (CCAP 211/11B) CO_2 (5% v/v) enriched culture at 600 µmol m^{-2} s^{-1} PPFD in 2 L photobioreactor with semi-permeable membrane. Data are average of two replicates, error bar represents ±SD.

with the same Re_i and $(k_La)_{O2}$ as in 250 mL photobioreactor experiments (scale-up described in section 4 of Materials and Methods). The culture was illuminated with 600 µmol m^{-2} s^{-1} PPFD and sparged with 5% CO_2 en- riched air. The final biomass titer was only 20% of that obtained in 250 mL photobioreactor under same operational conditions (Table 2, Fig. 8). The maximum CFR was just 5% of the CTR_{max}. Relatively low oxygen accumulation was observed as a result of low cell density and increased aeration rate. In such non-limiting environment, light rarefaction should have ceased the growth since the culture depth was doubled on scaling. High chlorophyll content of the cells at low cell density indicates that the light was severely attenuated (Fig. 8). It is therefore reasonable to infer that the scale of the culture has attenuated light sufficiently and is the sole reason for the loss in biomass productivity on scale-up.

CONCLUSIONS

Photoautotrophic growth of microalgae is always constrained by limiting factors. The resulting low cell density forbids large scale production of algal biomass for bioenergy products which are required to be produced inexpensively. Consequently, the major challenge is to enhance cell density and biomass productivity of microalgal cultures. We performed a systematic analysis of factors limiting the growth of a commercially important green microalga *Chlorella vulgaris* in laboratory scale photobioreactors. In a series of strategically designed experiments, factors limiting growth were elucidated using common measurements of specific growth rate, chlorophyll content, biochemical and elemental composition of cells, concentration of residual nitrogen source, dissolved oxygen and pH of the culture. CO_2 mass-transfer analysis together with the calculation of carbon fixation rates enabled the identification of CO_2-limited growth without on-line measurement of dissolved CO_2 with costly CO_2 electrodes. Factor(s) found to be limiting growth were subsequently controlled. A new practice of using a visking tube inside the reactor for maintaining the levels of depleting nutrients has been demonstrated. Cell density was remarkably enhanced in an experiment where all the growth limiting factors were controlled, accept attenuated light. Out of all factors that limited growth in different experiments, light attenuation has been found to be most significant and difficult to circumvent, especially on scaling the culture.

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