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Maximizing biomass productivity and cell density of *Chlorella vulgaris* by using light-emitting diode-based photobioreactor

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ABSTRACT

Green microalgae have recently drawn attention as promising organisms for biofuel production; however, the question is whether they can grow sufficient biomass relative to limiting input factors to be economically feasible. We have explored this question by determining how much biomass the green microalga *Chlorella vulgaris* can produce in photobioreactors based on highly efficient light-emitting diodes (LEDs). First, growth results were improved under the less expensive light of 660 nm LEDs, developing them in the laboratory to meet the performance levels of the traditional but more expensive 680 nm LEDs by adaptive laboratory evolution (ALE). We then optimized several other key parameters, including input superficial gas velocity, CO_2 concentration, light distribution, and growth media in reference to nutrient stoichiometry. Biomass density thereby rose to approximately 20 g dry-cell-weight (gDCW) per liter (L). Since the light supply was recognized as a limiting factor, illumination was augmented by optimization at systematic level, providing for a biomass productivity of up to 2.11 gDCW/L/day, with a light yield of 0.81 gDCW/Einstein. These figures, which represent the best results ever reported, point to new dimensions in the photoautotrophic performance of microalgal cultures.

1. Introduction

The expanding world population is creating a demand for alternative sources to petro-chemically derived products. Photosynthetic microalgae represent such an alternative, as they can utilize either solar energy or artificial light to incorporate CO_2 into biomass compounds. Thus, great interest has developed in biofuel production (Brennan and Owende, 2010; Chisti, 2008; Sheridan, 2009; Vertes et al., 2006; Wijffels and Barbosa, 2010) through microalgae, as well as in their potential for the biofixation of CO_2 and production of commodities (Sydney et al., 2010). Nonetheless, despite the substantial funds and work which have been invested towards developing algal-derived biofuels in recent years (Gardner et al., 2011; Sheridan, 2009; Tang et al., 2011; Vertes et al., 2006), the economic viability of microalgal bioprocesses remains constrained by physical and thermodynamic factors. Processing costs have proved too high and there have been problems with the photosynthetic efficiency (i.e., productivity) of high-density algal cultures. Determining the limits of potential performance is therefore crucial for progress in algal biotechnology.

Photosynthetically grown microalgae are currently used commercially in foods and feeds (Kay, 1991; Lee et al., 2010) as well as in the production of bioactive substances (Lorenz and Cysewski, 2003; Pulz and Gross, 2004). *Chlorella vulgaris* is one of the most widely cultivated microalgae, based on ease and experience of culture and as its chemical composition is favorable for enhancing the nutritional value of conventional foodstuffs (Kay, 1991; Lee et al., 2010). Biomass productivity is a key limit to the economic feasibility of converting CO_2 directly to *C. vulgaris* biomass in practical use. In outdoor systems (Ugwu et al., 2008), for instance in open ponds or enclosed flat-plate photobioreactors (PBRs), vast areas of land are required for large-scale algae cultivation. To discover alternative modes for cultivating algae, various designs and scales of vertical-column PBRs (Park and Lee, 2001; Suh and Lee, 2003; Ugwu et al., 2008) and panel PBRs (Cuaresma et al., 2009) have been tested. While indoor PBRs have been tried by researchers worldwide, their volumetric biomass productivity has remained relatively low, thereby reducing the business promise of a return on production.

This clarifies the need to develop cost-effective PBRs with high algal biomass productivities and low production costs. Light-emitting diode (LED) technology has been used for decades and has the advantages of being highly efficient and reliable, with a long lifetime and low power consumption, when compared to other

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artificial light sources (Yam and Hassan, 2005). The progressive LED technology that is currently emerging has the potential of achieving high conversion efficiency from electricity to light, while providing a narrow spectrum of target wavelengths (Yam and Hassan, 2005). The application of LEDs in PBRs marks a great advance over existing indoor agricultural lighting (Yeh and Chung, 2009). For algal cultures, LEDs allow the grower to control spectral output and adjust the light intensity and frequency in optimizing illumination. Furthermore, LEDs have been established in PBR design since the early 1990s (Lee and Palsson, 1994), though to date they have proven prohibitively expensive. With current and future advances in LED technology, however, LED-based PBRs may soon become practical for producing algal biomass.

We thus set out to examine the optimal relations of biomass productivity and light capture efficiency that can be achieved within the bounds of current LED technology. These performance metrics are essential to assessing the economic feasibility of such a production mode.

2. Materials and methods

2.1. Algal strain and culture conditions

C. vulgaris (UTEX 26, UTEX Culture Collection of Algae) was cultivated using varying CO_2 concentrations in air at $25 \pm 2\,^{\circ}C$. The experiments were mainly conducted in bubble column PBRs that were all equipped with LEDs and had working volumes of 300 ± 5 ml (PBR dimensions: height, ~30 cm; diameter, ~4 cm) and 500 ± 5 ml (PBR dimensions: height, ~60 cm; diameter, ~4 cm). The PBRs utilized in our experiments were the original models bought (Kimble Bomex Glass Co., Ltd., Beijing, China), unless specifically stated otherwise. Adaptive laboratory evolution (ALE) (Palsson, 2011) was conducted by means of a semi-continuous culture system. For ALE, specifically, the cell density in PBRs was kept same at the beginning of each cycle by removal of part of the culture and replacement of fresh medium and the duration of each cycle was three days, unless specifically stated otherwise. The incident average light intensity was 255 $\mu E/m^2/s$ at a 30% duty cycle for ALE, CO_2-dependent growth and optimal growth of *C. vulgaris*. For other growth experiments, different average light intensity conditions were provided.

2.2. Media and reagents

The modified M-8 (Mandalam and Palsson, 1998) and K-8 media were prepared according to the composition described (Table S1). All of the chemicals were obtained from Sigma–Aldrich, and the redesign principal was mainly based on stoichiometric medium formulation. The basic values (Mandalam and Palsson, 1998) for calculating the elemental composition of *Chlorella* (by weight) were as follows: 6.2–7.7% N, 1.0–2.0% P, 0.85–1.62% K, 0.36–0.80% Mg, 0.28–0.39% S, 0.04–0.55% Fe, 0.005–0.08% Ca, 0.002–0.01% Mn, 0.001–0.004% Cu, 0.0006–0.005% Zn. The biomass capacity of M-8 and K-8 media was predicted stoichiometrically (Table S2). The culture pH under K-8 medium was kept stable for short-term cultivation (one day culture or three days culture), at levels between 6.0 and 6.2, mainly by the phosphate buffer system in the medium.

2.3. Artificial light supply and setup

Inexpensive super-red LED arrays (Part number: SSL-LX5093SRC; operating voltage and current are 1.7 V and 20 mA, respectively) with high efficiency and a narrow output spectrum at the peak wavelength of 660 nm (20 nm bandwidth at half peak height) were purchased from LUMEX Inc. (Taiwan, China). The intensity of light supplied to the PBRs was measured using a quantum sensor (SR. NO. Q40526 of QUANTUM, Model LI-1400, LI-COR biosciences, Lincoln, Nebraska, U.S.A.) on the inner surface of each PBR. The experiments were performed with flashing light at different duty cycles but at the same frequency (10 kHz) since it was reported that flashing light enhanced microalgal biomass productivity and photosynthetic efficiency (Park and Lee, 2000). Specifically, each piece of LED panel containing 48 LED was divided into 12 sub-circuits where each sub-circuit consists of 4 LED, a 50 ohm resistor and a BC846AW NPN transistor (Diodes Inc., Plano, Texas, U.S.A.), all connected in series. The current flow through the sub-circuit was controlled by applying a square wave signal to the transistor base terminal. A PIC18f26k20 microcontroller (Microchip Technology Inc., Chandler, Arizona, U.S.A.), was programmed to generate a pulse width modulated signal of the desired frequency and duty cycle. A certain average light intensity could be provided by the specific duty cycle and peak light intensity. For example, a 10% duty cycle with a 10 kHz frequency means that the light was on for only 10% of the duration of one on/off cycle (0.1 ms).

2.4. Biomass determination

Samples were collected every 24 h. Optical density was measured spectrophotometrically at a wavelength of 600 nm. The biomass concentration was determined by correlating the optical density (OD600) with the dry weight. The variances in chlorophyll content have little effect on the correlation coefficient since chlorophylls in *Chlorella* cells have little absorption at 600 nm. More precisely, the relationship between biomass concentration and optical density was as follows:

$$C_b(g/L) = 0.42OD_{600} \quad (R^2 = 0.99) \tag{1}$$

where C_b is the biomass concentration and OD_{600} is measured in the range between 0.1 and 1.0 by diluting cell samples. The samples were then centrifuged at 3000 rpm or 1771 *g*, for 10 min by Eppendorf Centrifuge 5810R (Eppendorf Co., Ltd., Hamburg, Germany), washed with deionized water twice and dried overnight at 80 °C.

2.5. Chlorophyll content measurements

The spectrometer (Biomate 3) used for both basic absorbance measurements and the survey scan was purchased from Thermo Fisher Scientific Inc. (Madison, U.S.A). Chlorophyll was extracted from the samples by using 90% methanol, with which they were diluted 50–100 times, depending on cell concentration. Freshly prepared vials containing the samples were bathed at 50 °C for 50 min and then centrifuged at 12,000 *g* for 5 min. Supernatant absorbance was measured at 650 and 665 nm, respectively. The content of chlorophyll a and b as well as the total chlorophyll content (mg/L) was then calculated using the following equations (Becker, 1994):

$$\text{Chlorophyll } a = 16.5 \times A_{665} - 8.3 \times A_{650} \tag{2}$$

$$\text{Chlorophyll } b = 33.8 \times A_{650} - 12.5 \times A_{665} \tag{3}$$

$$\text{Chlorophyll}(a+b) = 25.5 \times A_{650} + 4.0 \times A_{665} \tag{4}$$

2.6. Calculation of biomass yield on light

The biomass yield in relation to light (quantum/light yield) is defined as the amount of biomass generated per unit of radiance absorbed by the algal culture. The equation for calculating the yield on light (ψ_E) was expressed as follows:

$$\psi_E = \frac{P_b}{F_{vol}} \tag{5}$$

where P_b stands for the volumetric biomass productivity and F_{vol} for the incident photon flux absorbed per unit volume (Molina-Grima et al., 1997). The working volume for a specific bubble column photobioreactor measuring $H = 30$ cm, $D = 4$ cm was 300 ± 5 ml. The incident photon flux absorbed per unit volume (F_{vol}) was calculated by the following equation:

$$F_{vol} = \frac{I_0 \times S_0}{V_0} \quad (6)$$

where I_0 stands for incident light intensity on the culture surface, V_0 for the working volume and S_0 for the surface area of culture (here is 300 cm^2). Eq. (6) was only valid for high density culture in this study. The initial biomass concentration for all experiments conditions was relatively high ($A_{660\,nm} > 1.0$) so that the light scattering and transmission was negligible. It was assumed that the supplied light measured on the inner surface of PBRs was all absorbed by *C. vulgaris* cells during batch culture. Finally, we considered the average volumetric growth rate to be equivalent to the volumetric biomass productivity, P_b.

2.7. Calculation of pneumatic power input per unit volume in photobioreactors (bubble columns)

The equation (Chisti and Moo-Young, 1989) for calculating the specific pneumatic power input in bubble columns was expressed as follows:

$$\frac{P_G}{V_L} = \rho_L g U_G \quad (7)$$

where ρ_L stands for liquid intensity, g for gravitational acceleration, U_G for the superficial gas velocity, P_G for power input due to gas and V_L for liquid volume in the photobioreator.

3. Results

To determine the maximum *C. vulgaris* growth rates that can be achieved with LED-based PBRs, various key variables were examined for their effect on volumetric biomass productivity. These variables included: (i) ALE, (ii) media composition, (iii) the superficial velocity of feed gas and its effect on mixing, (iv) CO_2 concentrations in the feed gas and (v) factors of light delivery. The effect of each variable will now be explained and discussed.

3.1. Adaptive laboratory evolution and media redesign

The red LED (674 nm) achieved the greatest efficiency in regard to light, i.e., it required the least photon flux to stimulate maximum growth in the green microalga *Chlamydomonas reihardtii* (Chang et al., 2011). Previously, optimal growth of *C. vulgaris* had been reported at 680 nm (Lee and Palsson, 1994); however, the most efficient and inexpensive red LEDs emit a shorter wavelength than either of those just mentioned, or 660 nm based on the (Al,Ga) InP system (Krames et al., 2007). ALE has been studied and used to adapt microbial strains to environmental stress (Palsson, 2011). The basic process of ALE was schematically shown (Fig. 1A). We thus began by using ALE to improve *C. vulgaris* growth in LED-based PBRs (Fig. 1B and C) that had a narrow spectrum peaking at the wavelength of 660 nm (see Methods). At this stage in our experiments, *C. vulgaris* was being cultivated in modified M-8 medium at a CO_2 level of 9.5% (v/v) and an input gas flow rate of 45 cm^3/min (corresponding to a superficial velocity of 0.06 cm/s, calculated in the conventional way by dividing the gas flow rate with the cross-sectional area of the PBR; the specific pneumatic power input was 5.9 W/m^3). By applying the ALE process (see Methods and Fig. 1A), the culture's average growth rate and biomass yield were increased gradually over the course of 15 cycles (Fig. 1B) from 0.71 gDCW/L/day to 1.03 gDCW/L/day and from 0.32 gDCW/E to 0.47 gDCW/E (Fig. S1), respectively. Thus, the endpoint growth rate that was finally achieved with the 660 nm LEDs was 1.45 times higher than the initial rate (i.e., performance in the baseline strain), and the cultures had attained a biomass concentration of 5.2 gDCW/L within three days (Fig. S1). At this stage, the nutrients could be limiting factors in this culture since its biomass concentration achieved (5.2 gDCW/L) got close to the predicted biomass capacity of M-8 medium.

Following this initial ALE trial, the M-8 medium was re-designed into a K-8 medium with the predicted capacity of generating 10 gDCW/L of biomass, based on a stoichiometric elemental composition analysis of *C. vulgaris* (see Table S1). In the K-8 medium, ALE was then continued with *C. vulgaris* under 660 nm light and 5.0% (v/v) CO_2 gas introduced at a superficial velocity of 0.18 cm/s (135 cm^3/min), see Fig. 1C. During the next 10–12 cycles of ALE, the average growth rate changed in accordance with the chlorophyll content of the cells (Fig. 1C). Specifically, *C. vulgaris* grew faster during the first 4 cycles and the chlorophyll content increased. During the fifth cycle in the K-8 medium, *C. vulgaris* was cultivated without any $FeSO_4$, in order to test the dependence of chlorophyll on the presence of iron in the medium. After most of the iron was removed, both the cell chlorophyll content and the average growth rate decreased significantly. This shows that the growth rate of *C. vulgaris* depends on iron in ALE, and also shows the necessity of a big amount of iron for synthesizing chlorophyll (Maier and Cattani, 1965; Bibby et al., 2001) in high density culture. When $FeSO_4$ was restored to the medium, the cells' average growth rate increased continuously through ALE, while their chlorophyll content remained relatively stable even after 12 cycles. Given media optimization, ALE thus achieved an overall growth rate of 1.75 gDCW/L/day and 0.79 gDCW/E, which means a 2.5-fold increase in growth rate and biomass yield compared to the starting performance (Fig. S1). The initial and endpoint growth profiles of *C. vulgaris* were also characterized in reference to an entire growth cycle; for comparison, these profiles are presented as curves in Fig. S2.

Through the ALE process, *C. vulgaris* had thus evolved to grow under less expensive and more efficient LEDs, i.e., under 660 nm light instead of 680 nm light. Moreover, the chlorophyll content of adapted *C. vulgaris* displayed the same absorption maximum/peak of 682 nm as the baseline starting strain (the baseline samples were derived from the original *C. vulgaris* that had been cultivated using fluorescent lamps). Also, the chlorophyll extracted in 90% (v/v) methanol from the endpoint and baseline *C. vulgaris* strains had in both cases an absorption maximum/peak of 666 nm, according to a full wavelength scan. On the other hand, the ratio of chlorophyll *a*–*b* did change slightly in the course of ALE (Fig. 1C).

3.2. Varying superficial velocities of the input gas at high CO_2 levels

An average growth rate of 1.75 gDCW/L/day was achieved by an endpoint *C. vulgaris* strain after 114 days of ALE, when it had evolved through a total of 38 cycles (Fig. 1C). Since this growth rate remained the same throughout a further 4 cycles of ALE (Fig. S3), we hypothesized that there might be other constraints on algae performance. We thus studied the effect of varying input gas conditions (Fig. 2). In these experiments, we utilized the *C. vulgaris* strain from the ALE endpoint. The photorespiration was negligible since the provided input CO_2 concentration was at high levels (2.5%, 5.0% and 9.5%). Increasing the levels of CO_2 in a range of 2.5–9.5% (v/v) in the input gas had little impact on the growth rate (Fig. 2). Increasing the superficial gas velocity in a range between 0.06 cm/s and 0.24 cm/s (corresponding to the specific pneumatic power inputs of 5.9 W/m^3 and 23.5 W/m^3, separately) at all these three levels of input CO_2, however, raised growth performances

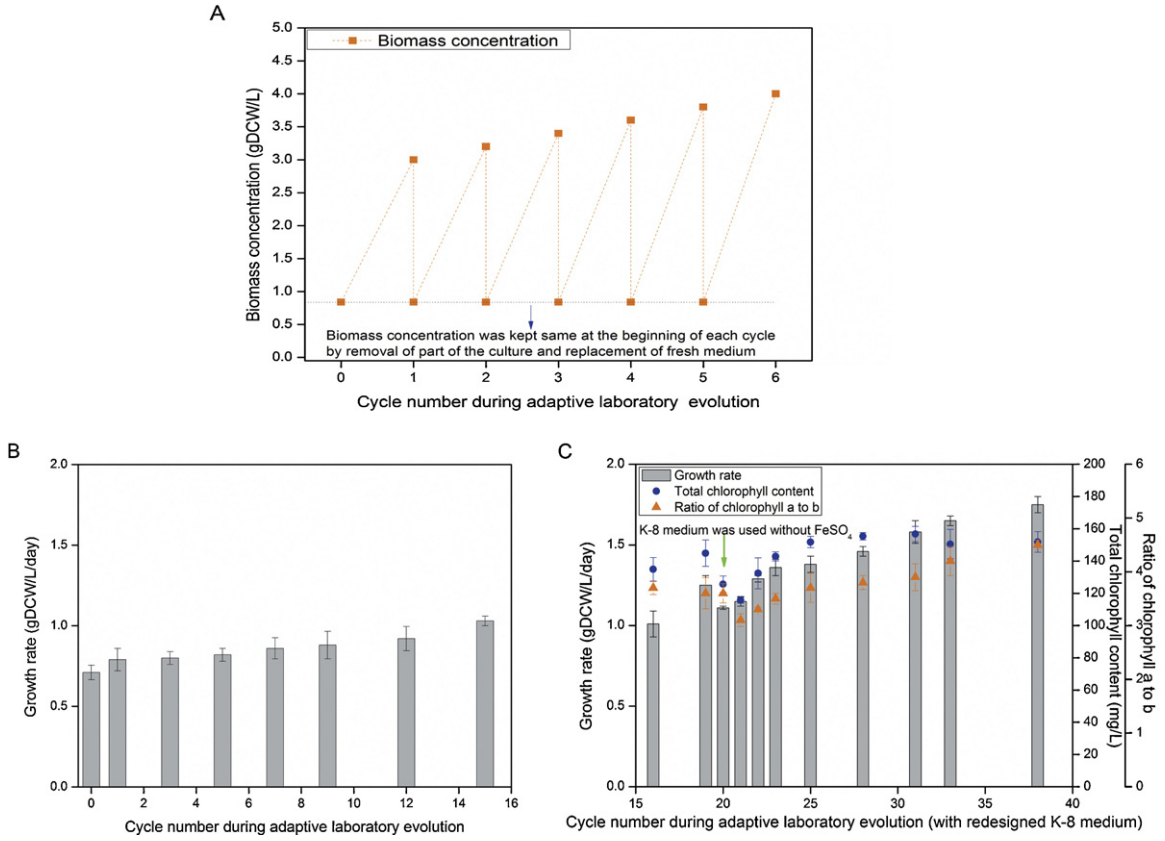

Fig. 1. Adaptive laboratory evolution (ALE) of *C. vulgaris* through 38 cycles (114 days). (A) Schematic process of ALE. (B) Adaptation of *C. vulgaris* to 660 nm light, modifying the M-8 medium by threefold increases in phosphate content. The input gas had a superficial velocity of 0.06 cm/s and a CO_2 level of 9.5% (v/v). Each batch of cells was cultivated from an initial density of around 2.1 gDCW/L for three days. (C) Continued adaptation of *C. vulgaris* in a different modified medium, specifying chlorophyll content. K-8 medium was used with an input gas flow of 0.18 cm/s and a CO_2 level of 5.0% (v/v). Each batch of cells was cultivated from an initial density of around 0.84 gDCW/L for three days. Here, the starting *C. vulgaris* was that which originated from the endpoint of (B). At the twentieth cycle, the culture was refilled with a K-8 medium lacking $FeSO_4$, in order to determine the iron-dependence of *C. vulgaris* growth. While overall adaptation resulted in a 2.5-fold increase in growth, *C. vulgaris* was demonstrated to be iron-dependent. The results presented are values averaged from either two (B) or three (C) independent experiments. Error bars indicate SD.

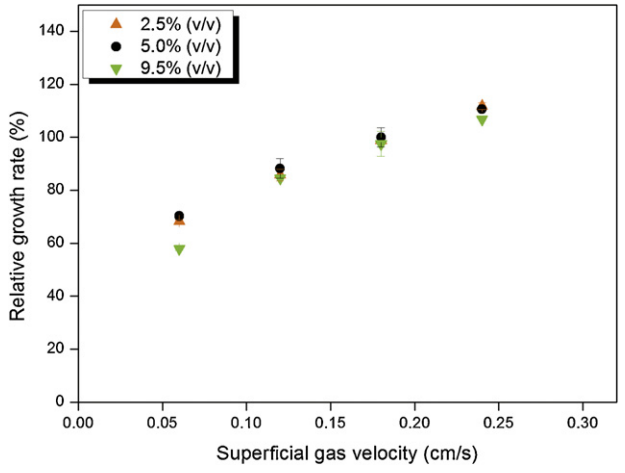

Fig. 2. Effect of varied input gas conditions on growth rates in adapted *C. vulgaris*. The average incident light intensity was 255 $\mu E/m^2/s$, at a 30% duty cycle. The growth rate represents the average volumetric growth rate for 3-day cultures initiated from a biomass concentration of approximately 0.84 gDCW/L. The superficial velocity of the input gas was set at 0.06, 0.12, 0.18, 0.24 and 0.30 cm/s, with varying concentrations of CO_2. Growth rate was normalized to a growth rate of 1.61 gDCW/L/day, determined by growing *C. vulgaris* in air with a 0.18 cm/s superficial velocity, 5.0% (v/v) CO_2 concentration, 255 $\mu E/m^2/s$ average light intensity, and 30% duty cycle. The results are averaged from two independent experiments. Error bars indicate SD.

dramatically (Fig. 2). At 0.24 cm/s and 5.0% (v/v), the growth rate climaxed at 1.94 gDCW/L/day with a corresponding biomass yield of 0.88 gDCW/E. This result prompted us to test an even higher superficial gas velocity, 0.30 cm/s (corresponding to a specific pneumatic power input of 29.4 W/m^3) at 5.0% (v/v), but this did not increase growth rate or biomass yield, with these remaining nearly the same as at a superficial gas velocity of 0.24 cm/s (corresponding to a specific pneumatic power input of 23.5 W/m^3) and 5.0% (v/v) (Fig. S4). Under these conditions, the optimal superficial gas velocity and the specific pneumatic power input had thereby been determined, with performance failing to improve by increasing this velocity (specific pneumatic power input), presumably due to cell fragility (Gudin and Chaumont, 1991) and shear stress (Michels et al., 2010).

The results from increasing the superficial gas velocity suggested that the growth rate of *C. vulgaris* could benefit from the equal and balanced distribution of incident light. A balanced distribution might be achieved through the intensified culture mixing that would occur through an increased gas flow rate, because this would reduce the mixing time (Rubio et al., 2004). The above results also indicated that the growth of *C. vulgaris* was light-limited rather than CO_2-limited at an input CO_2 concentration of 2.5% (v/v). By means of this analysis, the optimal input gas conditions were determined to be a superficial gas velocity of 0.24 cm/s (corresponding to a specific pneumatic power input of 23.5 W/m^3) at a 2.5% (v/v) input CO_2 concentration.

The effect of medium composition was then re-examined at the higher superficial gas velocity in order to determine whether higher cell densities could be achieved. The redesigned K-8 medium (as

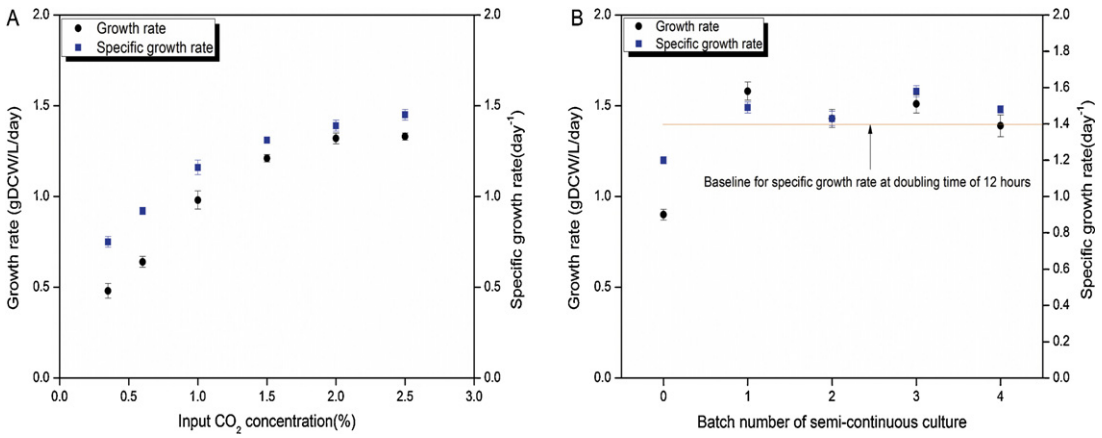

Fig. 3. CO_2-dependent growth and optimal growth of *C. vulgaris*. The input gas superficial velocity was 0.24 cm/s. Initial cell density was around 0.42 gDCW/L, placed in the regime of one day per batch. (A) Effect of CO_2 concentrations on the volumetric growth rate and specific growth rate of batch cultures. One batch of *C. vulgaris* was initially adapted to a specific CO_2 concentration. The effects of each of the CO_2 concentrations in the graph were studied by subjecting a different sample from the initial batch to that particular CO_2 concentration to allow for meaningful comparisons of CO_2 concentration impact. (B) Growth of *C. vulgaris* in semi-continuous culture at an input CO_2 level of 2.5% (v/v). The points above the line indicate instances of optimal growth under this condition. The results presented are values averaged from three independent experiments. Error bars indicate SD.

described above) achieved around 12 gDCW/L of biomass within 9 days of culture (Fig. S5). We further developed the K-8 medium into a K-9 medium by increasing KNO_3 and $MgSO_4$ two- and fivefold, respectively. The new K-9 medium produced even higher biomass concentrations than K-8, and continued its biomass increases to 20 gDCW/L after 15 days of growth, when measurements were hindered by culture sticking (Fig. S5). The increases in biomass concentration were due to the growing time added by the extra nutrients, which permitted growth for 15 days compared to 9 days. These results show that sufficient nutrients in a defined medium can support a high-density biomass concentration, based on a stoichiometric analysis of elemental *C. vulgaris* composition.

3.3. CO_2-dependent growth

The CO_2 delivery rate was further examined to determine its effect on algal growth. The results presented above indicated that a 2.5% (v/v) CO_2 concentration was sufficient for *Chlorella* growth and that increasing the concentration within the range of 2.5–9.5% (v/v) had little effect on growth rates (Fig. 2).

To determine a quantitative relationship between specific growth rate and the input CO_2 level, we needed to create conditions where CO_2 was the sole limiting factor for growth. Therefore, *C. vulgaris* growth was observed at a variety of lower input CO_2 concentrations (Fig. 3A), as applied to a relatively low-density culture (with biomass concentrations of 0.4–2.0 gDCW/L). Under these conditions, which included a K-8 medium and a superficial gas velocity of 0.24 cm/s, the light supply was assumed to be ample, rendering input CO_2 as the single limiting factor for growth. Using equilibrium CO_2 concentrations in the media, we obtained the following Monod growth equation for *C. vulgaris* (see details in Supplementary calculations):

$$\mu = \frac{1.68 S_{CO_2}}{6.56 + S_{CO_2}} \quad (8)$$

where μ is the specific growth rate and S is the equilibrium concentration of CO_2 ($[CO_2]^*$), and where $K_{S_{CO_2}}$ is 6.56 mg/L and μ_{max} is 1.68 day^{-1}, respectively.

Using the above equation, the relevant specific growth rate was calculated to be 1.39 day^{-1} (comparable to the value of μ_{max}). A further significant aspect was that the doubling time of *C. vulgaris*, judging by the batch culture in our study, was around 12 h at exponential phase (Fig. S2), which was in accordance with the doubling times of *C. vulgaris* in previous studies (Griffiths and Harrison, 2009). Based on this analysis, we hypothesized that *C. vulgaris* growth rates could be regarded as optimal whenever they exceeded 1.39 day^{-1}.

Having developed these optimality criteria, we analyzed culture data concerning a semi-continuous culture of *C. vulgaris*. In terms of specific growth rate, we found that a 2.5% (v/v) CO_2 input at a superficial gas velocity of 0.24 cm/s, an 850 $\mu E/m^2/s$ peak light intensity and a 30% duty cycle of flashing light led to optimal *C. vulgaris* growth in semi-continuous culture (Fig. 3B). These values indicate that a 2.5% (v/v) CO_2 input is capable of supporting optimal *C. vulgaris* growth when coupled with an average light intensity of 255 $\mu E/m^2/s$ based on an 850 $\mu E/m^2/s$ peak light intensity and a 30% duty cycle, applied to a semi-continuous regime in relatively low-density culture.

3.4. Light-dependent growth

C. vulgaris growth was also examined in high-density cultures to determine the extent of its dependence on light. Under the conditions of the K-8 medium, a superficial gas velocity of 0.24 cm/s, an input level of 2.5% CO_2 (v/v) and a peak light intensity of 850 $\mu E/m^2/s$, we exposed the cultures to varying duty cycles of flashing light (Fig. 4). In these experiments, the growth rate showed an approximately linear increase, paralleling the increase in average incident light intensity (Fig. 4). These results indicated that light intensity might be a limiting factor in raising high-density cultures and that optimizing the light supply might be an efficient means of achieving high biomass productivity and a profitable biomass yield on light. In the face of these positive indications, however, the biomass yield on input light actually decreased along with increases in the duty cycle, so that the highest biomass yield achieved was 1.32 gDCW/E at a 10% duty cycle, which in contrast produced the lowest growth rate, or 0.97 gDCW/L/day (Fig. 4). This outcome led us to examine further aspects of light intensity by applying different duty cycles with an average light intensity of 170 $\mu E/m^2/s$ (Fig. S6). In these instances, the biomass yield and growth rate turned out to be higher at a low duty cycle (20%) than a higher duty cycle

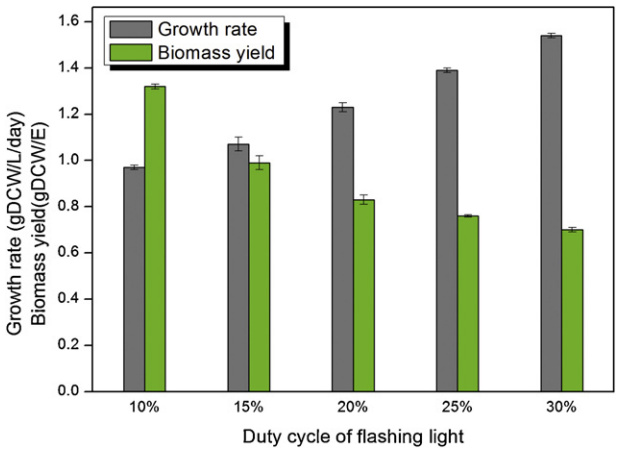

Fig. 4. Effect of varied light intensity on the growth rate of *C. vulgaris* in high-density cultures. The difference in average light intensity was obtained by applying the same peak light intensity (850 μE/m^2/s) at different duty cycles. The superficial velocity of the input gas was 0.24 cm/s, at a 2.5% (v/v) CO_2 level. The growth rate represents an average after 3 days culture, starting from an initial density of 2.52 gDCW/L. The results presented are averaged from three independent experiments. Error bars indicate SD.

(30%) (Fig. S6). These findings show how a lower duty cycle in the light supply can enhance energy conversion efficiency.

3.5. Maximum biomass productivity

Having analyzed the key variables affecting *C. vulgaris* growth, we wanted to analyze further the maximum biomass productivity of the applicable system (i.e., a semi-continuous culture system with LED-based PBRs). To do this, the starting concentration of *C. vulgaris* was investigated as a key variable in the system. Starting concentration enters into the following equation for calculating biomass productivity:

$$P_b = \frac{(C_t - C_0)}{\Delta t} = \frac{C_0[\exp(\overline{\mu}\Delta t) - 1]}{\Delta t} \quad (9)$$

where P_b is the volumetric biomass productivity, C_0 is the biomass concentration at the starting point, C_t is the biomass concentration at time t, Δt is the total duration of the batch culture and $\overline{\mu}$ is the average specific growth rate. This equation shows the relation of P_b to the starting biomass concentration and the average specific growth rate. In an ideal case, $\overline{\mu}$ would achieve the value of μ_{max}, which would be a constant value for *C. vulgaris* (Fig. 3B), and biomass productivity would be proportional to initial cell density. In a contrasting non-ideal case, $\overline{\mu}$ would be a function of incident light and initial cell density, provided that light supply was the sole limiting factor for optimal growth. Further analysis of the data presented in Fig. 5A indicated that the specific growth rate ($\overline{\mu}$) decreased along with increases in the initial biomass concentration (C_0) and that this was probably caused by a shortage of light. The peak growth rate apparently depended on a suitable initial biomass concentration, given light supplied at the same average intensity of 255 μE/m^2/s (Fig. 5A).

Higher light intensity, on the other hand, generated increased growth rates in these experiments. A 20% higher average intensity of incident light (around 300 μE/m^2/s) improved to some extent both the specific and the volumetric growth rate of *C. vulgaris*, using scaled-up PBRs (Fig. 5B). Under optimal conditions, the growth rate and related biomass yield reached 2.11 gDCW/L/day and 0.81 gDCW/E, respectively. These outcomes imply that providing intense illumination without photo-inhibition would be one potential way of achieving high biomass productivity, especially in improved cultures of *C. vulgaris*. Scaling up the approaches in this study remains an engineering challenge related to factors of light delivery and distribution and mass transfer.

4. Discussion

Microalgae are receiving considerable attention as bioprocessing organisms for a variety of applications (Sheridan, 2009; Sydney et al., 2010; Tang et al., 2011; Vertes et al., 2006; Wijffels and Barbosa, 2010). To a great extent, the economic viability of such bioprocesses for biomass and even biofuels production depends on biomass productivity and energy efficiency. This study has provided data on attempts to attain optimal performance in microalgae while using LED-based PBRs. The focus was on volumetric biomass productivity and the biomass yield on light. Firstly, we carried out ALE in order to improve the growth performance of *C. vulgaris* at a more economic wavelength, 660 nm. The results from our first study with microalgae ALE indicate that *C. vulgaris* is capable of adapting to different conditions, as has been observed with other organisms (Applebee et al., 2008; Babu and Aravind, 2006; Ibarra et al., 2002; Teusink et al., 2009), and manages to do so by altering its ratio of chlorophyll a to chlorophyll b. We identified the key bioreactor parameters affecting the performance of *C. vulgaris* at high growth rates and in dense cultures, and we inferred a

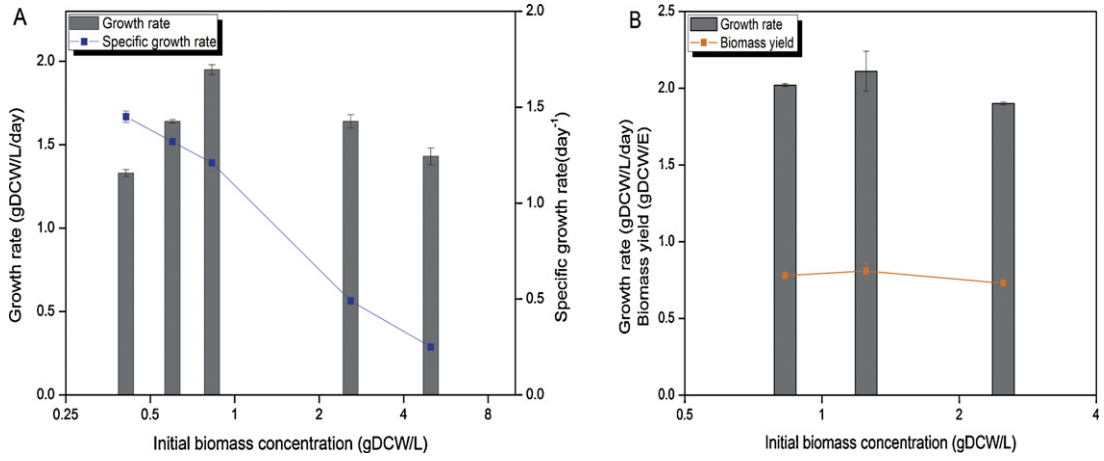

Fig. 5. Effect of different initial biomass concentrations on the growth of *C. vulgaris*. Each batch of cells was cultivated in the regime of one day. (A) The average incident light intensity was 255 μE/m^2/s at a 30% duty cycle, while the input gas had a superficial velocity of 0.24 cm/s and a CO_2 level of 2.5% (v/v). (B) The average incident light intensity was 300 μE/m^2/s at a 25% duty cycle. The superficial velocity of the input gas was 0.40 cm/s at 2.5% (v/v) CO_2. The working volume was 500 ml in scaled-up PBRs (dimensions: height, ~60 cm; diameter, ~4 cm). The results presented are average values from either three (A) or two (B) independent experiments. Error bars indicate SD.

strategy for optimizing biomass productivity in phototrophic cultivation with LED-based PBRs. By designing the media stoichiometrically, we improved biomass productivity, attaining biomass concentrations of up to 20 gDCW/L. The relationship between input CO_2 levels and specific growth rates was also determined, helping us to achieve optimal *C. vulgaris* growth in our system. Finally, solutions were found for optimizing PBR light supplies, thereby achieving a maximum biomass productivity of 2.11 gDCW/L/day, with a high biomass yield on light of 0.81 gDCW/E.

These performance figures provide grounds for discussing economic feasibility. For processes involving LED-based PBRs (Fig. S7), the basic capital expenditure consists roughly of the cost of the LEDs, bubble column tubes, and gas control equipment. While operating costs consist mainly of paying for the electricity consumed in supplying light, there are also minor costs connected to the medium and other materials needed for the growth process. In addition, there are varying costs of labors even if the whole system of production is well-developed and highly automatic and intelligent. Currently, red LEDs are rather expensive, with price quotes ranging from $0.135/LED to $0.27/LED for the LED types used in this research (prices quoted by LUMEX Inc.). Therefore, both capital expenditure and operating costs are dominated by the light supply. While considering possible PBR scale-ups, but above all how light delivery is the key factor limiting growth in a high-density culture, an optimal balance has to be found that weighs the growth rates resulting from a varying distribution and density of LED arrays with the overall objective of keeping the bioprocess cost-effective. Based on our results, light supply is estimated to cost between $20 and $30/kgDCW in 3.6 L PBRs (3.6 L working volume per PBR; see Supplementary Calculations), assuming that the usable LED lifetime will be between 35,000 and 60,000 h (the average lifetime quoted by LUMEX Inc.). Accordingly, our study implies that LED-based PBRs may be economically feasible for the present situation in commercial production, especially if exploited to produce high-end market goods that can significantly exceed the $20–30/kgDCW cost involved in the sold product. Clearly, biofuels have a low-end unit value and cannot be produced economically through the approach under discussion. Emerging LED technology, which is bound to lower the cost of LEDs, lengthen their lifetime and further improve energy efficiency, will probably reduce lighting costs significantly.

Overall, the results of our study show that coupling microalgal biotechnology with LED-based PBR design can make it feasible to fix CO_2 in biomass that is intended for high-quality products. Further improvements in biological performance and micro-electronics could bring significant gains in biomass productivity within a few years, thereby rendering an ever wider range of algae-derived products economical. Rapid progress in the microalgae industry is thus believed to be of significant importance for sustaining global growth through the coming decades.

Authors' contributions

W. F. wrote the manuscript and managed the experiments. O. G. built the PBRs, and calculated the aspects of capex and operating cost. G. H. designed the PBRs. A. M. F. contributed to editing and writing the manuscript. S. B. co-directed the study together with B. O. P., who also wrote the manuscript and conceived the study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2012.07.004.

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