Article

Optimizing the growth of *Haematococcus pluvialis* based on a novel microbubble-driven photobioreactor

**Highlights**

- The CO₂ mass transfer demand for optimal *H. pluvialis* growth is identified
- The impact of key parameters on mass transfer are discussed
- Reducing bubble size has pronounced effect on CO₂ mass transfer
- Microbubble photobioreactor demonstrates a high biomass of *H. pluvialis*
Optimizing the growth of *Haematococcus pluvialis* based on a novel microbubble-driven photobioreactor

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**SUMMARY**

*Haematococcus pluvialis*, the richest bioresource for natural astaxanthin, encounters a challenge of achieving high growth rate when it comes to mass biomass production. Based on the substrate consumption model and Redfield ratio, rapid algae growth benefits from a proper carbon supply. However, the conventional cultivation schemes with limited carbon dioxide (CO2) supply and inefficient carbon mass transfer could have constrained the carbon capture and growing ability of *H. pluvialis*. We hypothesize that optimal *H. pluvialis* growth improvement may be achieved by efficient CO2 supply. Here, in this study, we first identified the carbon consumption of *H. pluvialis* during exponential growth. Then, a novel microbubble-driven photobioreactor (MDPBR) was designed to satisfy the carbon demand. The novel microbubble photobioreactor improves the CO2 supply by reducing bubble size, significantly elevating the CO2 mass transfer. With only 0.05 L min⁻¹ of gas flow rate, higher cell growth rate (0.49 d⁻¹) has been achieved in MDPBR.

**INTRODUCTION**

Macroalgae *Haematococcus pluvialis* (*H. pluvialis*) is considered to be the richest bioresource of astaxanthin (Ambati et al., 2014; Kaewpintong et al., 2007). Astaxanthin (C₉₀H₁₂₀O₄, 3,3'-dihydroxy-β,β'-carotene-4,4'-dione) gains its name as a potent antioxidant, widely applied for anti-inflammation, anti-aging, immune system enhancement, cancer prevention, etc. (Fakhri et al., 2020; Kim et al., 2019; Medhi and Kalita, 2021). Involving astaxanthin in diet can significantly reduce MCP-1, TNF-α, IL-6, and ROS in diabetic rats (Chan et al., 2012). Recently, algal-derived astaxanthin has been considered as a potential supplementary medicine for clinical trial against COVID-19 for its anti-oxidative benefit (Talukdar et al., 2020). Consequently, there is a rising interest in developing the bioprocess for *H. pluvialis* cultivation.

During the cultivation process, *H. pluvialis* has two distinctive morphologies–green vegetative cell and red rest cell (Xi et al., 2016). Thus, research communities and industries have mainly resorted to two-stage cultural mode, to obtain high biomass and astaxanthin yield (Osian et al., 2021). During the first stage, cells continuously divide and proliferate. Therefore, full nutrient medium and moderate light intensity, temperature and pH are required (Affalo et al., 2007; Panis and Carreon, 2016). The second stage refers to a red, non-motile resting stage, when cell division stops and astaxanthin content increases. The harvest of *H. pluvialis* vegetative cells was exposed to stress conditions like high irradiance and acetate addition to accumulate astaxanthin (Sarada et al., 2002).

Achieving a high vegetative cell density in a short period of time before being subjected to the stress conditions is a time-saving and cost-effective way to attain high astaxanthin production (Khoo et al., 2019; Nagappan et al., 2019). Although progress has been made in vegetative *H. pluvialis* cultivation, rapidly achieving high biomass during massive cultivation in vegetative stage remains a challenge and most of the photobioreactor (PBR) cultures have been facing the obstacle of low yield/cost ratio (Colusse et al., 2021; Le-Feuvre et al., 2020).

As *H. pluvialis* is a light sensitive species (Kaewpintong et al., 2007), the key solution to this difficulty could lie in efficient nutrient supply. Base on Monod equation (Kampen et al., 2014):

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https://doi.org/10.1016/j.isci.2021.103461

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\[
\mu = \mu_{\text{max}} \left( \frac{C}{C + K_C} \right)
\]

Equation 1

where \( \mu \) is specific growth rate, \( \text{d}^{-1} \) defined as \( (1/X)(dx/dt) \), \( \mu_{\text{max}} \) is maximum value of \( \mu \), \( \text{d}^{-1} \), \( K_C \) is saturation constant, \( g \text{ L}^{-1} \) at \( \mu_{\text{max}}/2 \), and \( C \) is substrate concentration, \( g \text{ L}^{-1} \), where sufficient substrate supply is closely related to algal specific growth rate (\( \mu \)). The chemical formations of algae partly determine the nutrient demand (Hillebrand and Sommer, 1999). Previous studies have focused more on nitrogen (N) and phosphor (P) requirements in the cultivation of H. pluvialis (Nahidian et al., 2018; Zhao et al., 2020). Tocquin et al. declared that a lower N/P ratio (below 1) favors vegetative growth of H. pluvialis (Tocquin et al., 2012), whereas Ding et al. suggested that H. pluvialis had relatively low P uptake efficiency (about 99.5 \( \pm \) 10.1 mmol mg\(^{-1}\text{Chl a}) in both long-term and short-term P uptake experiments (Ding et al., 2019). The inconsistent results indicated that N, P or N/P ratio supplement in medium may not be the sole key parameter of H. pluvialis mass production. Meanwhile, the research on carbon supply and consumption of H. pluvialis are relatively underexplored (Judd et al., 2017).

Generally, it is believed that the ideal chemical elements present in average phytoplankton biomass demonstrate a Redfield ratio (C: N: P = 106: 16: 1) (Redfield et al., 1963). The idealized chemical reaction of phytoplankton can be illustrated as followed equation (Perdue, 2009; Redfield et al., 1963):

\[
106 \text{CO}_2 + 16 \text{HNO}_2 + \text{H}_3\text{PO}_4 + 78 \text{H}_2\text{O} \rightarrow (\text{C}_{106}\text{H}_{175}\text{O}_{42}\text{N}_{19}\text{P}) + 150 \text{O}_2
\]

Equation 2

which indicates the significance of carbon supply on the growth of phytoplankton. For H. pluvialis, the element stoichiometry was reported as 45.6% C, 8.2% H, 6% N, and 0.58% (Garcı´ a-Malea et al., 2006; Razon, 2011), revealing sufficient carbon supply is pivotal for algae biomass enhancement.

Efforts have been made for carbon supply in culture. Carbon supply can be performed under three modes: heterotrophic, mixotrophic, and autotrophic. Heterotrophic cultivation results in low cell concentration, indicating the essentiality of light for high vegetative cell content (Kang et al., 2005). In that case, mixotrophic seems to be a promising method. However, addition of organic carbon source like sodium acetate introduces risk from contamination from other organisms, which could lead to slow growth and even the death of H. pluvialis (Pang and Chen, 2017). In addition, autotrophic pre-culture before organic carbon sources addition allows a better performance for cell growth and astaxanthin accumulation (Pan-utai et al., 2017; Wang et al., 2021). One reason could be that high cell density exhausts the nutrients in the medium which in turn minimizes bacteria contamination (Wen et al., 2020). Thus, autotrophic culture to reach a relatively high biomass is essential for both further mixotrophic culture or astaxanthin induction.

Inorganic carbon supply is crucial for photoautotrophic culture. Carbon dioxide (CO\(_2\)) and bicarbonate (HCO\(_3\)) is considered as the ideal carbon supply. To meet the carbon demand, it requires a constant addition of carbon in batch or fed-batch manner. Nevertheless, continuous addition of bicarbonate can affect the pH and ion level of the medium, causing stress for cell growth (Agranat, 2007). In contrast, the CO\(_2\) supply with a buffer system can avoid the issue and the bubble device is more adjustable and convenient compared with manual addition.

For microalgae mass cultivation, two common systems are open system and close system. Open systems such as open ponds merit flexibility and cost-effective design but suffer CO\(_2\) loss and cell sedimentation (Jerney and Spilling, 2020). Given those issues, PBR, defined as a closed system, was designed. PBRs mainly include stirred tank PBRs, tubular PBRs and bubble column PBRs (Gupta et al., 2015). The stirred tank PBRs require mechanical agitation which tends to generate excess heat and cell damage (Kadic and Heindel, 2014). In tubular PBRs, though they have larger gas-receiving surfaces, the main drawback of them is their poor axial mass transfer—there are significant gas gradients along the tubes (Burgess et al., 2011). H. pluvialis that cultivated in 4 types of tubular PBRs merely had the specific growth rate range from 0.07 to 0.12 d\(^{-1}\) (Lee et al., 2015). Bubble column PBRs are an alternative for the former bioreactors for its low capital cost, high surface area to volume ratio, and satisfactory mass transfer (Gunjal and Ranade, 2016). The maximum biomass productivity of Spirulina (1.03 g L\(^{-1}\text{day}^{-1}\)) was achieved in the bubble column PBR which is significantly higher than race pond (Singh et al., 2016). Nevertheless, sufficient carbon supply remains a challenge for the traditional bubble column PBRs which commonly generate bubble sizes of 3–6 mm (Kazakis et al., 2008). The optimal CO\(_2\) uptake by Dunaliella salina photosynthesis could reach up to 0.3 \( \times \) 10\(^{-4}\) mol L\(^{-1}\text{min}^{-1}\) (Zimmerman et al., 2011), whereas the CO\(_2\) mass transfer rate of conventional
technologies was in a range of $0.4 \times 10^{-6}$ - $0.7 \times 10^{-5}$ mol L$^{-1}$ min$^{-1}$, far away from meeting the CO$_2$ demand for Dunaliella salina culture (Ying et al., 2013a). Thus, we hypothesize that optimal H. pluvialis growth improvement can be achieved by increasing CO$_2$ mass transfer for efficient CO$_2$ supply.

To tackle the mass transfer issue, microbubble technology was developed which has been widely applied in wastewater treatment industries (Rehman et al., 2015; Yao et al., 2016). This technology refers to reducing bubble size of gas injection system in order to achieve both higher mass transfer rate and capture efficiency (Ying et al., 2013a). With microbubble device, threefold improvement of mass transfer rate was achieved, the sufficient mixing and gas flow benefits wastewater treatment process (Rehman et al., 2015). The CO$_2$ mass transfer rate is mainly determined by the volumetric mass transfer coefficient ($K_{La}$) and $K_a$ manifests cubic increase by reducing the bubble size (Erickson, 1990). Studies have shown that the CO$_2$ mass transfer can be significantly elevated by using microbubble technology (Ying et al., 2013b). Cultured in microbubble induced airlift loop bioreactor, the dry biomass of Dunaliella salina was increased from 0.0067 g/L to 0.24 g/L (about 35 folds) after 17 days’ culture (Zimmerman et al., 2011). Based on the feature of high mass transfer, it is possible to improve the H. pluvialis growth by replacing the conventional CO$_2$ feeding technologies (with bubble size around 3–6 mm) with microbubbles.

In this study, we addressed three major issues: (1) How much is the carbon consumption of H. pluvialis during the exponential growth period? (2) How can the bioreactor meet the carbon demand via optimizing the design parameter? (3) How can the theoretical derivations be applied to the microalgae mass production? Based on the results, we designed a novel microbubble-driven photobioreactor (MDPBR) for H. pluvialis rapid cultivation. This technology improves the mass transfer in photobioreactor which is crucial for sufficient carbon supply and can be further applied to microalgae mass production.

**RESULTS AND DISCUSSION**

**H. pluvialis growth and carbon consumption**

*Effect of carbon concentration on carbon consumption and growth of H. pluvialis*

We analyzed how the carbon consumption, biomass, and specific growth rate of H. pluvialis vary under different carbon concentrations. The data in Figure 1 shows that higher carbon concentration (8 mM) contributions to higher carbon uptake and biomass increase. The total carbon consumption achieved 0.2, 0.4, and 0.6 mM within 1.5 days with the initial carbon addition at 2, 4, 8 mM, respectively, indicating a daily consumption rate of 0.13, 0.26, and 0.40 mM d$^{-1}$ (Figure 1A). Meanwhile, the control groups (containing medium of different carbon concentration without algae) were proved that experimental operation had no significant effect on the carbon concentration decrease, indicating that the content changes were caused by algal consumption (Figure 1C). The contribution of carbon consumption for unit biomass increase was about $7 \times 10^{-3}$ mol g$^{-1}$ (the slope of each linear regression), which was nearly identical in each group (Figure 1D). In other words, the yield coefficient (142.9 gCell molC$^{-1}$) remains unaffected with elevating concentration of carbon. It is highly supported by the theoretical assumption that the activities of the enzymes for nutrient assimilation should be constant under a fixed environment including temperature, pH, salinity, etc., (Najafpour, 2015). Hence, the growth of H. pluvialis is adjustable subduing in different carbon concentration conditions.

Higher carbon concentration in the culture was found having a positive effect on the H. pluvialis biomass. The H. pluvialis culture with 2 mM of carbon addition showed the lowest biomass productivity, with the dry weight ascending by 0.033 g L$^{-1}$; however, in 4 mM and 8 mM group, the biomass increased by 0.059 and 0.064 g L$^{-1}$, respectively (Figure 1A). Likewise, the highest specific growth rate was observed in the 8 mM carbon addition group, which is around 78% and 15% higher than the 2 mM and 4 mM group in the first 12 h (Figure 1B). Therefore, the 8 mM carbon addition group outperformed other groups regarding biomass and specific growth rate.

The question worth thinking about is whether it will be beneficial with continuous addition of carbon concentration. Firstly, the response to carbon concentration varies slightly in algal species. Increasing the CO$_2$ concentration from the atmospheric concentration to 2% resulted in a significant improvement in biomass productivity for the cultivation of Halochlorella rubescens (Schultze et al., 2015). Likewise, after comparing the growth of Chlorella sp. with different CO$_2$ concentrations ranging from 0.5% to 5.0%, researchers found that the highest cell density was achieved with 5% CO$_2$ injection (Ryu et al., 2009). However, the results were found opposite in Dunaliella salina. The photosynthetic rate decreased slightly by increasing the CO$_2$ concentration from 2 mM to 8 mM (Ying et al., 2014). Elevating the CO$_2$ concentration to 20 mM resulted in the
cell death. Generally, the discrepancy in those results could mainly be attributed to the various levels of tolerance on carbon concentration for various algal species.

Secondly, higher CO₂ concentration could lead to a lower intracellular pH level, which may inhibit the activities of photosynthetic enzymes (Ying et al., 2014). In our case, the pH for each culture was in the range of 7.0–8.0 during the entire experimental period, which was suitable for H. pluvialis growth (Choi et al., 2017; Wan et al., 2014). Given the points above, the elevation of carbon concentration should be performed within a proper level.

According to our result, the possible upper limit of carbon concentration in this experimental condition is close to 8 mM. On the one hand, attenuation of the improvement on productivity was observed when rising the carbon concentration from 4 mM to 8 mM. The biomass rises by 78% comparing the 4 mM group with the 2 mM group, but only an 8% increase was shown between 4 mM group and 8 mM group (Figure 1A). Similarly, the descending trend was found in specific growth rates. The value dropped by 0.11, 0.16, and 0.22 d⁻¹ of 2 mM, 4 mM, and 8 mM groups, respectively, after cultivation for 36 h. Even though the 8 mM group possesses the greatest specific growth rate, it experiences the greatest decline. Based on the Monod model (Equation 1), the instantaneous specific growth rate is positively correlated to the instantaneous concentration of the limiting substrate (carbon concentration in this case). The culture with higher concentration within the tolerant range of carbon supplements leads to a greater μ. Hence, further increase of carbon concentration above the threshold value of 8 mM could result in growth inhibition. Taken together, ascending carbon concentration to a proper level contributes to carbon consumption enhancement and furthermore boosts productivity.

**Growth and carbon consumption kinetics**

To provide qualified carbon concentration for H. pluvialis, we dug deeper into the relationship between growth rate and carbon consumption. The overall specific growth rate for each culture was calculated,
whereas the corresponding carbon concentration was estimated as an average value for 1.5 days. The reciprocal of overall specific growth rates (1/μ) versus the reciprocal of carbon concentrations (1/C_0) were plotted (data not shown), where the slope and intercept of the linear regression represented the K_c/μ_max and 1/μ_max, respectively, according to the linearized form of Equation 1. The saturation constant on carbon (K_c) and the maximum specific growth rate (μ_max) were therefore found to be 2.0 mM and 0.68 d⁻¹, separately. Based on Equation 1, to attain the maximum specific growth rate, the instantaneous carbon concentration in the medium should be maintained at 0.4 M, approximately 200 times higher than K_c (Erickson, 1990). Nonetheless, carbon is commonly provided via CO₂ bubbling for microalgae culture, with the equilibrium carbon concentration of 0.04 M achievable under 100% CO₂ dosing. Therefore, theoretical μ_max is hardly achieved in real practice.

The first order kinetic model describing the H. pluvialis growth and carbon consumption, including equations and values of the coefficients, was summarized in Table 1. Assuming a) nitrogen and phosphorus are replete and the light limitation does not occur, b) the daily carbon uptake by H. pluvialis growth can be traded off by sufficient carbon supplement achieved through 1% CO₂ dosing, and c) the instantaneous concentration of total carbon in the medium is maintained at around 5 mM for the whole culture period, therefore H. pluvialis would grow at a constant specific growth rate of 0.49 d⁻¹ (about 70% of maximum potential). By using this model, the instantaneous concentration of carbon and biomass for the above experiments were simulated. The computed results were thereafter compared to the experimental results, an identical match was found between them (Figure 2), indicating a strong validity of this model.

The simulation of H. pluvialis culture with conventional photobioreactor (with bubble size around 3 mm) was shown in Figure 2. The growth at the first day was estimated according to the equations listed in Table 1. The growth between day 2 and day 7 for the conventional PBR culture was calculated as Equation 3, where W_{t1} and W_{t2} mean the dry weight of biomass at time t_1 and t_2, respectively. ΔC_{1→2} represents the overall carbon consumption rate between t_1 and t_2, which is limited to the mass transfer rate of conventional bubbles.

\[
W_{t2} = W_{t1} + \frac{\Delta C_{1→2}(t_2 - t_1)}{Y_{w/c}} \quad \text{Equation 3}
\]

In most of photobioreactor cultures, CO₂ was dosed into the medium via conventional bubbles (approximately 3 mm in diameter) at a volumetric flow rate of nearly 5% V/V. CO₂ with 1%–5% mixture gas dosing along with the addition of certain concentration of carbonate in the medium, providing approximately 5–12 mM of equilibrium carbon concentration, could be employed to attain nearly 70–85% of the maximum growth potential and meanwhile to maintain the pH at a suitable range (7.0–7.5) for H. pluvialis growth (Ying et al., 2014). For the case of 5 L culture applying 1% CO₂ conventional dosing, the CO₂ mass transfer coefficient was estimated to be 5.6 × 10⁻⁵ min⁻¹ in a preliminary experiment (data not shown), which could provide a maximal mass transfer rate of roughly 3.2 × 10⁻⁵ mol L⁻¹ day⁻¹ (Equation S8). This mass transfer

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### Table 1. List of equations describing the H. pluvialis growth and carbon consumption

| Equations | Representatives |
|-----------|----------------|
| \[
\frac{dW}{dt} = \mu \cdot W = W_0 \cdot e^{\mu t}
\] | Biomass growth, g L⁻¹ |
| \[
\frac{dC}{dt} = \mu \cdot \frac{W}{Y_{w/c}} \cdot e^{\mu t} \Rightarrow \Delta C = \frac{W_0}{Y_{w/c}} (e^{\mu t} - 1)
\] | Carbon consumption, mol L⁻¹ OR g L⁻¹ |
| \[
\mu = \mu_{max} \left( \frac{C}{C + K_c} \right)
\] | Specific growth rate under carbon limitation, d⁻¹ |
| \[
C = C_0 - \Delta C = C_0 - \frac{W_0}{Y_{w/c}} (e^{\mu t} - 1)
\] | Instantaneous concentration of carbon, mol L⁻¹ OR g L⁻¹ |

| Key parameters | Values |
|---------------|--------|
| μ_max | 0.68 d⁻¹ |
| K_c | 0.002 mol L⁻¹ OR 0.024 g L⁻¹ |
| Y_{w/c} | 142.9 g mol⁻¹ OR 11.9 g g⁻¹ |
The rate could only maintain *H. pluvialis* optimal growth for about 1 day. Thereafter, the *H. pluvialis* would grow linearly rather than exponentially (Figure 2). The ideal growth scenario of *H. pluvialis* (at vegetative stage) along with its corresponding daily carbon consumption was then simulated based on this model, illustrated in Figure 2. Under the ideal conditions the *H. pluvialis* is expected to grow exponentially from initial 0.01 g L\(^{-1}\)\(\cdot\)C\(_0\)\(^1\) to 0.30 g L\(^{-1}\)\(\cdot\)C\(_0\)\(^1\) within 7 days, with the corresponding daily carbon consumption rising from about 4.4 \(\times\) 10\(^{-3}\) mol L\(^{-1}\) d\(^{-1}\) to 1.3 \(\times\) 10\(^{-3}\) mol L\(^{-1}\) d\(^{-1}\). Therefore, to maintain the optimal growth for 7 days, the CO\(_2\) mass transfer rate of 1.3 \(\times\) 10\(^{-3}\) mol L\(^{-1}\) d\(^{-1}\) is required according to a conservative consideration. The third part of this section will discuss a lab-scale experiment which has been done to validate the inference.

**Mass transfer of microbubbles**

The MDPBR designed based on chemical engineering

The CO\(_2\) mass transfer efficiency is the main challenge to tackle. Since the CO\(_2\) equilibrium concentration in the liquid depends on the partial pressure of CO\(_2\) in the mixture gas (i.e. CO\(_2\) volume percentage) according to Henry’s law (Equation 58), the enhancement of K\(_{L\alpha}\) is crucial when the CO\(_2\) volume percentage is fixed. The impact of K\(_{L\alpha}\) is analytically related to the mean bubble size (d\(_B\)) and the gas holdup (\(\varepsilon\)) (Erickson, 1990). To further clarify the gas holdup, it is defined as the volume fraction of gas in the gas-liquid dispersion (Najafpour, 2015), which is a function of bubble rising velocity (V\(_B\)), ratios of height to diameter (H/D), and liquid volume (V\(_L\)). According to Stokes’ law, the mean value of the bubble rising velocity can be simplified as a function of liquid-gas density (\(\rho_L-\rho_G\)), viscosity (\(\mu\)), bubble size (d\(_B\)) and gas flow rate (Q). After simplifications, our model was shown in Figure 4A, indicating that K\(_{L\alpha}\) is correlated to Q, H/D, V\(_L\), and d\(_B\) (Equation 4).

\[
K_{L\alpha} = \frac{432\mu K_L}{II(\rho_L-\rho_G)\rho_L^{1/3}} \cdot \frac{Q}{V_L^{1/3} d_B^3} = \alpha Q(H/D)^{3/2} V_L^{1/3} d_B^3
\]

Equation 4

According to Equation 4, K\(_{L\alpha}\) is supposed to be in direct proportion to the item Q(H/D)^{3/2}/(V\(_L\)^{1/3}d\(_B\)). Among those key parameters, bubble size has the most remarkable influence on mass transfer coefficient, followed by the gas flow rate. By individually increasing the Q and H/D by 10 times, the K\(_{L\alpha}\) could be improved by about 10 times and 10^{2/3} times, respectively, whereas the K\(_{L\alpha}\) could be 1000 times greater by reducing the bubble size by only 10 times.

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**Figure 2. Simulated *H. pluvialis* growth at optimal specific growth rate and the daily carbon consumption**

The dotted line stands for the simulation of the *H. pluvialis* growth in a conventional PBR culture, whereas the solid line with circles is the prediction of *H. pluvialis* growth under the circumstance that daily carbon supply meets or exceeds the consumption demand (Right Y axis). The bar demonstrates the daily carbon consumption (Left Y axis).
The experimental results also support the correlation described in our model. Impacts of microbubble size, flow rate, H/D ratio, and liquid volume on CO₂ mass transfer were shown in Figure 3. Regarding bubble size (dₐ), the KLa in different groups ranges from 0.0035 to 0.02 min⁻¹, 0.0045 to 0.0375 min⁻¹, and 0.007 to 0.0854 min⁻¹ of 554 μm, 464 μm, or 333 μm bubble size respectively. In other words, when the bubble size was reduced by 40%, the KLa increased nearly 4 times. When it comes to flow rate (Q), increasing flow rate also leads to a higher gas hold-up (Ying et al., 2013a). In Figure 3A, by increasing flow rate from 0.15 to 0.5 L min⁻¹, the KLa almost tripled, rising from 0.007–0.03 min⁻¹ to 0.02–0.09 min⁻¹ with the H/D ratio of 5:1. In addition, increasing the H/D ratio can also enhance the KLa, as a longer bubble residence time in the liquid (Erickson, 1990). Nevertheless, the adjustment of H/D does not have the distinctive effect compared to the above parameters. For groups with microbubbles size of 333 μm at 0.5 L min⁻¹ flowrate, a 28.5% increase of KLa was observed after raising the H/D ratio 1.6 times.

On the other hand, the KLa values were reduced nearly by half when the liquid volume was doubled to 10 L (Figure 3B). Therefore, for scale-up cultures, flow rate, and H/D, the bubble size should be adjusted correspondingly to maintain the similar KLa desired for lab scale studies. Taken together, with the same liquid volume, KLa can be improved by either reducing the bubble sizes or increasing the flowrate and H/D ratio.

To further test the accuracy of the model, the theoretical data was compared with the practical data. The diagram of KLa versus the item Q(H/D)²/₃/(VL²/₃dB³) was plotted to find out the proportionality constant (α) in Equation 4 (Figure 4A). The linear regression with R² = 0.89 was obtained, indicating a strong proportional function between KLa and the item Q(H/D)²/₃/(VL²/₃dB³) (Figure 4B). The value of the proportionality constant α was determined to be 6 × 10⁶. As shown in Figure 4C, the deviations of the major computational values are less than 30% in comparison with their corresponding practical values. To be concise, the deviations of half percentage of the data estimated are less than 20%. The main causes of deviation to the real values can be the occurrence of bubble coalescence, bubble entrainment, and internal turbulence, etc., which were not taken in consideration because of the simplifications.

Overall, our model (Equation 4) provided an archetype on the mathematical relationship between KLa and its relevant parameters. Based on the classic theory (such as two-film theory and stocks law) and the proportionality constant obtained from the practical trials, the model is reliable to predict KLa in the cultivation system. Hence, the mass transfer model built in this study could be used as guidance for the engineering design in the scale-up process.

Optimization of mass transfer parameters for H. pluvialis culture

To design the microbubble driven bioreactor, the KLa values were firstly estimated on three mean bubble sizes (350, 450, and 550 μm), four flow rates (0.05, 0.1, 0.15, and 0.3 L min⁻¹), and three H/D ratios (1, 3, 5) by using Equation 4. As discussed in previous section, for a 5 L lab-scale culture, CO₂ mass transfer rate of 1.3 × 10⁻³ mol L⁻¹ d⁻¹ is required to maintain H. pluvialis optimal growth for 7 days, and to achieve a relatively high biomass density (around 0.3 g L⁻¹ or 3 × 10⁵ cells mL⁻¹). The estimated KLa ranged from...
0.61 × 10^{-3} \text{ min}^{-1} to 0.042 \text{ min}^{-1}, capable of achieving 0.35 × 10^{-3} – 2.4 × 10^{-2} \text{ mol L}^{-1} \text{ d}^{-1} of mass transfer rate, under continuous 1% CO2 dosing. We selected five sets of combinations (Table 2) which were close to the minimum carbon supply requirement. (The mass transfer rate of a 5 L-conventional bubble column PBR (Set. 6) with bubble size around 3 mm was also computed.) As mass production, energy consumption was considered. Higher flow rate means with the same amount of time and liquid volume, more gas was injected into the system which will create extra cost (Gil et al., 2010). Meanwhile, larger bubble sizes require lower injection pressure and less energy input (Han et al., 2002). Given energy cost and lower flow rate, larger bubble size (within the microbubble range) are favorable. Set. 5 requires both the smallest flow rate (0.05 L min\(^{-1}\)) and injection pressure, which well balances the mass transfer requirement with energy cost, and therefore was selected as the optimal combination of mass transfer parameters for 5 L-\textit{H. pluvialis} culture.

| No. of set | \(V_L\) | H/D | \(d_B\), \(\mu m\) | \(Q\), L min\(^{-1}\) | \(K_{La}\), min\(^{-1}\) \(\times 10^{-3}\) | Daily mass transfer rate, mol L\(^{-1}\) day\(^{-1}\) \(\times 10^{-3}\) |
|-----------|--------|-----|-----------------|----------------|----------------|------------------|
| 1         | 5      | 1   | 350             | 0.05           | 2.4            | 1.4              |
| 2         | 5      | 1   | 450             | 0.1            | 2.3            | 1.3              |
| 3         | 5      | 3   | 450             | 0.05           | 2.3            | 1.4              |
| 4         | 5      | 3   | 550             | 0.1            | 2.6            | 1.5              |
| 5*        | 5      | 5   | 550             | 0.05           | 1.8            | 1.1              |
| 6         | 5      | 5   | 3000            | 0.25           | 0.056          | 0.032            |

Set.1-5 are the simulations for MDPBR, while Set.6 is the optimistic estimation for conventional PBR. The set being star marked was considered as optimal when balancing the mass transfer with energy cost.

![Figure 4. The mathematical relationship between \(K_{La}\) and its relevant parameters](image)

(A) Schematic diagram demonstrating the relationship between mass transfer coefficient and its relevant parameters. (B) plot of \(K_{La}\) versus the item \(Q(H/D)^{2/3}/(V_L^{2/3}d_B^{3})\). The \(K_{La}\) values were obtained from the experiments, while the values of the item \(Q(H/D)^{2/3}/(V_L^{2/3}d_B^{3})\) were calculated based on the practical values. (C) the comparison between practical and theoretical values of \(K_{La}\). The theoretical values of \(K_{La}\) were calculated using Equation 4.

\[
K_{La} = \frac{432 \mu u}{n \rho u - \rho u} \frac{Q}{\alpha} \left( \frac{d_B}{V_L} \right)^{3/2} \frac{V_L^{2/3}d_B^{3}}{V_L^{2/3}d_B^{3}}
\]
**H. pluvialis** growth in an MDPBR

In our study, we demonstrated an optimized vision of conventional bubble column PBR. With airlift design (Figure S4) used to create proper circulation, a microbubble technique was harnessed to improve carbon supply at a relatively low energy cost.

We designed a 5 L MDPBR which was capable of generating nearly 550 μm microbubbles in comparison with conventional PBR which generates around 3 mm bubbles for *H. pluvialis* culture. The gas flow rates were set to be 0.05 L min\(^{-1}\) and 0.25 L min\(^{-1}\) for the former and later, respectively. The results were shown in Figure 5. Generally, the dry weight of *H. pluvialis* cultured in the MDPBR increased by nearly 30 times at the end of a week, with an overall specific growth rate was relatively close to the optimal value 0.49 d\(^{-1}\) as expected and the exponential growth was maintained up to the seventh day. Almost all the cells were confirmed to be vegetative at the final day of cultivation via microscope observation (Figure S6). It was therefore confirmed that our MDPBR can maintain the optimal growth of *H. pluvialis* under proper light intensity. Besides, the experimental data were found to be almost consistent with the simulated data, which confirmed the reliability of the experimental data as well as the validity of the kinetic model for *H. pluvialis* growth in Table 1. In comparison, the *H. pluvialis* in the conventional PBR grew linearly instead of exponentially and achieved 0.21 d\(^{-1}\) of specific growth rate with only about 0.06 g L\(^{-1}\) of final biomass concentration, whereas the MDPBR achieved approximately 5 times higher biomass concentration (0.3 g L\(^{-1}\)), with only 1/5 of gas flow rate. The results suggest that the MDPBR could be 25 times more efficient than the conventional PBR, in terms of output-input ratio.

**CONCLUSION**

In this study, the CO\(_2\) demand of maintaining *H. pluvialis* exponential growth was profiled, implying insufficient carbon supply through the traditional bubble system. To meet the demand, a novel MDPBR was proposed to facilitate mass transfer through minimizing bubble size. Our hypothesis of CO\(_2\) supply and *H. pluvialis* growth has been validated via a cultivation trial in MDPBR, in which a significant improvement (5 times higher) of the *H. pluvialis* biomass productivity has been achieved with reduction in power consumption. Furthermore, our previous work (Wu et al., 2020) which applied higher light intensity suggested that our MDPBR has a potential of reaching higher biomass than that has been achieved so far. This technology offers an efficient alternative for *H. pluvialis* mass production and could further serve as a useful tool for CO\(_2\) mitigation and bioproduct production.

**Figure 5. The biomass growth of *H. pluvialis* in both the conventional PBR and the MDPBR**

The orange and gray dotted line represent the theoretical growth of *H. pluvialis* in the 5 L MDPBR and in the 5 L conventional PBR, respectively. The solid circle and triangle curves demonstrate the experimental data of *H. pluvialis* growth in the 5 L MDPBR and in the 5 L conventional PBR, respectively. Data points are presented as the mean ± standard deviation for triplicate measurements (n = 3).
Limitations of the study

This study designed MDPBR for *H. pluvialis* massive growth, and the CO₂ capture ability of *H. pluvialis* has been proved. However, the light intensity in this study may not be optimal for maximum photosynthesis, in terms of CO₂ supply. The stabilities and efficiency of MDPBR should be especially followed with interests and improved for the practical applications. The CO₂ supply can maintain a 7-day log growth so far; nevertheless, MDPBR can be adapted to exploit higher algal productivity in industrialized production. For the astaxanthin induction stage, whether acetate addition or increasing CO₂ supply will benefit astaxanthin accumulation can be further explored.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Growth and carbon consumption kinetics
  - Mass transfer tests
  - Microbubble-driven photobioreactor culture
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103461.

ACKNOWLEDGMENTS

This work was financially supported by the S&T Projects of the Economic, Trade and Information Commission of Shenzhen (No. 20180124085935704), Guangdong Basic and Applied Basic Research Foundation (2020B1515120012), as well as the S&T Projects of Shenzhen Science and Technology Innovation Committee (JCYJ20200109142822787, JCYJ20200109142818589, and RCJC2020071411433069).

AUTHOR CONTRIBUTIONS

Kebi Wu, Kezhen Ying, Jin Zhou, Yi Tao and Zhonghua Cai designed the research. Kezhen Ying, Kebi Wu and Dai Liu, Lu Liu conducted the experiments. Kebi Wu and Kezhen Ying drafted the manuscript and analyzed the data. Jin Zhou, Yi Tao, Xiaoshan Zhu and Zhonghua Cai contributed to the critical revision of the article. James Hanotu provided an editorial service.

DECLARATION OF INTERESTS

There is no conflict of interest, informed consent, human, animal rights applicable or Chinese patent (ZL201810168432.1). All authors agreed to the authorship and the submission of the manuscript for peer review.

Received: April 24, 2021
Received Revised: September 26, 2021
Accepted: November 11, 2021
Published: December 17, 2021

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental models: Organisms/strains** | | |
| H. pluvialis: FACHB-712 | Culture Collection of Algae at Chinese Academy of Sciences | FACHB-712 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Bold’s basal medium | Culture Collection of Algal and Protozoa, Scottish Marine Institute, UK | https://www.ccap.ac.uk/wp-content/uploads/MR_BB.pdf |
| **Software and algorithms** | | |
| CO₂ mass transfer efficiency | This paper (Equation 4) | NA |
| Total carbon concentration | This paper (Equation S7) | NA |
| ImageJ | Version 1.8.0 | https://imagej.nih.gov/ij/download.html |
| Prism - GraphPad | Version 7.0 | https://www.graphpad.com/scientific-software/prism/ |
| SPSS | version 20.0 | https://www.ibm.com/support/pages/downloading-ibm-spss-statistics-20 |
| **Other** | | |
| ZEISS primovert light microscopes | Carl Zeiss AG | https://www.zeiss.com/microscopy/us/products/light-microscopes.html |
| pH meter | Mettler Toledo | FiveEasy Plus 28 |
| Ceramic diffusers | Zibo Wastewater Treatment Technology Co. Ltd. | NA |
| Luminometer | Onset Computer Corporation | HOBO-Temp/Light MX2202 |

RESOURCE AVAILABILITY

**Lead contact**
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Prof. Zhonghua Cai (caizh@sz.tsinghua.edu.cn).

**Materials availability**
Not applicable.

**Data and code availability**
Cell growth and bioreactor parameter data reported in this paper will be shared by the lead contact upon request. This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

**H. pluvialis FACHB-712** was obtained from the Culture Collection of Algae at Chinese Academy of Sciences and maintained in the BBM medium suggested by CCAP (Culture Collection of Algal and Protozoa, Scottish Marine Institute, UK). Culture temperature was maintained at 25–26°C.

METHOD DETAILS

**Growth and carbon consumption kinetics**
To investigate the impact of carbon concentration on H. pluvialis growth (referring particularly to the growth at vegetative phase) as well as the kinetics of growth and carbon consumption, 250 mL-Duran bottles containing 140 mL of BBM medium each were prepared for the experiment (Figure S1).
The primarily inorganic carbon in the atmosphere is CO₂, while in water body, inorganic carbon concentration is defined as the sum of all different forms (Dodds and Whiles, 2010):

Under experimental condition, gaseous and bicarbonate (HCO₃⁻) is the major form of CO₂ in the medium the pH of which is around 7–8 (Dodds, 2002). To avoid the gas exchange of atmospheric CO₂, and ensure the carbon supply, a bicarbonate solution was used to elaborate the consumption kinetics.

For every three Duran bottles, different amount of NaHCO₃ were added to make the final carbon concentration at 2 mM, 4 mM, and 8 mM, respectively. 10 mL of H. pluvialis at exponential growth phase (with concentration of about 7.5 × 10⁵ cells mL⁻¹) was inoculated to each bottle.

According to previous studies, as a light sensitive species the optimal lighting has been reported as 20 μmol photo m⁻² s⁻¹ (Kaewpintong et al., 2007) and for the first stage cultivation, studies has been carried on under illumination of 20–25 μmol m⁻² s⁻¹ (Ding et al., 2019; Huang et al., 2021). Hence, it is suitable to use 20 μmol photo m⁻² s⁻¹ cultivation condition for energy saving concerns. Continuous illumination was provided by the LED white light at the intensity of 20 μmol photo m⁻² s⁻¹. Culture temperature was maintained at the room temperature (25–26°C). pH value at 7–8 was widely considered to favor the vegetative cultivation (Borowiak et al., 2021; Choi et al., 2017). The pH value was sustained at 7–8 with HCO₃⁻/CO₂ buffer system.

To ensure that the carbon concentration was the only limiting factor, the cultivation was only lasted for 36 h to minimize the effects of light attenuation and nutrients depletion when the culture became denser. The experiment was run in triplicate. The biomass and carbon concentrations were measured every 12 h. Except for the sampling period, each Duran bottle was sealed tight for almost the entire culture period to maximally prevent the CO₂ loss to the atmosphere. Besides, a separate set of Duran bottles containing 2 mM, 4 mM, and 8 mM of total carbon in the same BBM medium but without inoculating H. pluvialis was applied as the blank control to offset the CO₂ loss during the sampling period. The cell counts were conducted through microscopy. A standard curve between cell counts and dry weight was established via a preliminary experiment. The average dry weight per cell was found to be 1 × 10⁻⁹ g, which was identical to the value reported by others (Olaizola, 2000). Therefore, the dry biomass in this study was estimated based on the cell counts. The total inorganic carbon concentration (C₅) was measured by the Elemental Analyzer (Flash 2000 HT). To be noted, the ‘carbon concentration’ mentioned all through the text refers to the total inorganic carbon concentration, including dissolved CO₂, HCO₃⁻ and CO₃²⁻.

Regarding the kinetics, the overall specific growth rate (μ', d⁻¹) for a certain period was calculated through Equation S1,

\[
μ' = \frac{(\ln W_2 - \ln W_1)}{(t_2 - t_1)} \quad \text{Equation S1}
\]

where W₂ and W₁ are the biomass dry weight (g L⁻¹) at time t₂ and t₁, separately.

The rate of biomass production is represented by Equation S2, of which the integration form is presented as Equation S3,

\[
\frac{dW}{dt} = μ \cdot W \quad \text{Equation S2}
\]

\[
W = W_0 e^{μt} \quad \text{Equation S3}
\]

where dW/dt is the instantaneous growth rate (g L⁻¹ day⁻¹), μ is the instantaneous specific growth rate (d⁻¹), W and W₀ mean the instantaneous and initial biomass concentration (g L⁻¹), separately. t is the time of cultivation. While the instantaneous specific growth rate μ (d⁻¹) can be estimated using Monod equation (Equation 1).

Based on substrate consumption model (Zhang et al., 1999), the consumption rate of carbon (dC/C/ dt, mol L⁻¹ day⁻¹) is described as Equation S4, its integration form Equation S5 represents the total amount of carbon (ΔC, mol L⁻¹) consumed by microalgae within a certain period,

\[
\frac{dC}{dt} = \frac{μ \cdot W}{Y_{W/C}} = \frac{μ \cdot W_0 e^{μt}}{Y_{W/C}} \quad \text{Equation S4}
\]
where \( Y_{W/C} \) is the yield coefficients based on carbon (g mol\(^{-1}\)).

The residual concentrations of carbon (\( C_C, \) mol L\(^{-1}\)) is calculated via Equation S6,

\[
C_C = C_C^0 - \Delta C_C = C_C^0 - \frac{W_0}{Y_{W/C}} (e^{t_1} - 1)
\]

Equation S6

The key coefficients/constants for the kinetics model including the maximum growth rate (\( \mu_{\text{max}} \)), yield coefficient on carbon (\( Y_{W/C} \)), the half saturation constant of carbon (\( K_C \)) were obtained via graphic methods, which were demonstrated by our previous work (Liu et al., 2017).

Mass transfer tests

To study the CO\(_2\) mass transfer property of microbubbles as well as the impacts of the relevant parameters on it, two practical volumes of reactor (\( V_{\text{vol}} \)), three ratios of height to diameter (\( H/D \)), three average microbubble sizes (\( d_{\text{av}} \)) and three flowrates (\( Q \)) were selected for KLa (mass transfer coefficient) tests (Figure S2). Six airlift bioreactors with two working volumes (5 L and 10 L) and three \( H/D \) ratios (1:1, 3:1 and 5:1), made of PMMA, were customized from Hongxing Machinery Co. Ltd. (Ningbo, Zhejiang, China). For each reactor, there was a replaceable diffuser housing fixed at the bottom to allow switching the diffusers. Microbubbles with average sizes (\( d_{32} \)) of 333 \( \mu \)m, 464 \( \mu \)m, and 554 \( \mu \)m were generated from three types of ceramic diffusers specifically customized by Zibo Wastewater Treatment Technology Co. Ltd. (Zibo, Shandong, China). Here, the average bubble diameters were characterized using a high-speed camera via a separate experiment. Bubbles were generated by injecting 5% CO\(_2\) mixture gas through the ceramic diffuser placing at the bottom of a transparent fish tank. The gas flowrate was set to be 0.1 L min\(^{-1}\). A tripod halogen flood-light was adjusted to a proper position to illuminate the bubbles. A high-speed camera connected to a computer was placed in front of the fish tank to visualize the bubbles, and a black paper was attached to the back of the fish tank, making the bubbles easier to be visualized. The captured bubble images were then analyzed by a software "ImageJ".

For each mass transfer test, 5% CO\(_2\) mixture gas (balanced with N\(_2\)) provided by a gas cylinder was injected into the reactor containing a certain volume of deionized water. The flowrate was measured by a flow meter fixed in between the inlet of the diffuser and the outlet of the gas cylinder. Three gas flowrates (0.15, 0.3 and 0.5 L min\(^{-1}\)) were applied in this experiment. The pH value was recorded every 30 s, measured by a pH meter (FiveEasy Plus 2B). The total carbon concentration (\( C_T \)) was calculated based on the pH value by using Equation S8 reported in Ying et al. (Ying et al., 2013a, 2013b),

\[
\frac{C_T}{C_T^0} = \left(1 + 10^{\frac{pH-6.381}{10}} + 10^{\frac{pH-16.758}{10}}\right) \left(10^{-pH-14} + Na^+\right) \frac{10^{-2pH}}{10^{-6.381}-pH+2 \times 10^{-16.758}}
\]

Equation S7

where Na\(^+\) specifically means the concentration of sodium (mol L\(^{-1}\)) for the sodium bicarbonate added.

The CO\(_2\) mass transfer coefficient KLa for each test was determined via the graphic method interpreted by Chisti (Erickson, 1990; Ying et al., 2013a). The mass transfer rate (dC/dt) can be described in Equation S9, where C represents the concentration of total dissolved carbon while \( C^* \) is the equilibrium/saturation concentration. Its integration form can be written as Equation S9, where t stands for time, \( C_t \) and \( C_0 \) are the instantaneous concentration and initial concentration of total dissolved carbon, respectively. By plotting \( \ln \left(\frac{C^* - C_0}{C^* - C_t}\right) \) versus t, a linear regression is easily tractable, where the slope value equals to the KLa.

\[
\frac{dC}{dt} = K_{La} (C^* - C)
\]

Equation S8

\[
\ln \frac{C^* - C_0}{C^* - C_t} = K_{La} \cdot t
\]

Equation S9

Microbubble-driven photobioreactor culture

The cultivation was conducted adopting typical two-stage mode. At the vegetative growth stage, \( H. \) pluvialis were cultured in an MDPBR with suitable illumination for one week (Figure S3). At the second stage, the algal broth was transferred into several flasks for astaxanthin induction under higher irradiance. Since this study has mainly focused on \( H. \) pluvialis vegetative growth, so the detailed information about
astaxanthin accumulation at second stage was not mentioned. **Figure S4** showed the set-up for a 5 L lab trial. 200 mL of *H. pluvialis* growing at exponential growth phase was added to a customized MDPBR containing 5 L BBM medium. The H/D ratio of the MDPBR was 5:1. 1% CO₂ mixture gas was continuously dosed into the MDPBR at 0.05 L min⁻¹, with the microbubbles generated at an average size of 550 μm. The key parameters here regarding to reactor geometry and microbubbles, including height, diameter, bubble size and gas flow rate, were specifically determined. An additional 5 mM of sodium bicarbonate was included in the BBM medium, maintaining the pH at around 7.5 for the entire culture period under constant CO₂ bubbling. Two of fluorescence lamps providing continuous illumination were fixed at the side of MDPBR using iron stands. As *H. pluvialis* started to grow, the light attenuation occurred simultaneously, so the number of fluorescence lamps was doubled after 3 days to maintain a sufficient illumination. As a result, the light intensity at the reactor center was kept at around 16–20 μmol photo m⁻² s⁻¹ during the whole culture period, measured by a luminometer (HOBO-Temp/Light MX2202, Onset Computer Corporation) immersing into the algal broth. 5 mL of sample was taken each day for the measurement of growth. A control experiment was also conducted in a 5 L conventional PBR with the same geometry settings. The gas sparger generating bubbles with approximately 3 mm in diameter was employed at the bottom of the PBR. To maintain a sufficient mixing, the flowrate of 5% V/V (i.e. 0.25 L min⁻¹) was applied in the control experiment. The other parameters were set as the same as for the MDPBR.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The data were processed by one-way analysis of variance using SPSS version 20.0 (SPSS, USA) and Prism-GraphPad version 7.0. The average value of three replicate samples was expressed as mean ± standard deviation. A value of P < 0.05 was considered statistically significant.