

Characterisation of a simple ‘hanging bag’ photobioreactor for low-cost cultivation of microalgae

Jing Cui,^{a,b}  Saul Purton^b and Frank Baganz^{a*} 

Abstract

BACKGROUND: Microalgae are a diverse group of photosynthetic microorganisms of significant interest to the biotechnology industry, either as a sustainable source of natural compounds, or as light-driven cell factories to produce recombinant metabolites and proteins. Their ability to utilise light, CO₂, and basic nutrients leads to a simple and low-cost phototrophic cultivation process. This is particularly relevant to low- and middle-income countries, all of which require a cultivation system that is cheap, technically simple to operate, readily scalable, and can meet basic Good Manufacturing Practice requirements. A disposable ‘hanging bag’-type photobioreactor operated as a bubble column fits these criteria.

RESULTS: In this study, the characterisation and design modifications to improve the performance of a 15 L hanging bag is reported. The bubble behaviour using different sparger designs was investigated together with gas hold-up, mixing time, and mass transfer coefficient of CO₂. A gas flow rate of 5 L min⁻¹ using a new sparger design and a modified height-to-diameter ratio of the bag led to a two-fold improvement in algal biomass productivity when culturing the green microalga *Chlorella sorokiniana*. The cultivation of a luciferase-expressing *Chlamydomonas reinhardtii* strain in the modified hanging bag also demonstrated an 11% increase in luciferase content.

CONCLUSION: This is the first attempt to characterise this simple hanging bag system that brings the industry-favoured single-use bag concept into the research field of photobioreactor technology. The hanging bag with modified sparger and dimensions improves microalgal biomass productivity and demonstrates the potential of simple and low-cost systems for microalgal cultivation.

© 2021 The Authors. *Journal of Chemical Technology and Biotechnology* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry (SCI).

Keywords: microalgae; single-use photobioreactor; hanging bag; bubble column; phototrophic cultivation

ABBREVIATIONS

| | |
|----------------|--|
| PBR | photobioreactor |
| HB | hanging bag |
| TAP | Tris-Acetate-Phosphate medium |
| OD | optical density |
| UTR | untranslated region |
| DCW | dry cell weight |
| RO | reverse osmosis |
| DO | dissolved oxygen |
| Q _Y | quantum yield |
| PSII | photosystem II |
| D _b | doubling time |
| P _t | the biomass produced per day in t days |

NOMENCLATURE

| | |
|------------------|---|
| μ | specific growth rate |
| k _L a | gas–liquid volumetric mass transfer coefficient |
| ε _G | gas hold-up |

INTRODUCTION

Microalgae represent a large, polyphyletic group of microorganisms encompassing cyanobacteria and many diverse groups of protists.¹ The majority of microalgae are photosynthetic and are found in a wide range of aquatic environments – from fresh water to oceans and hypersaline lakes.² The diverse biology of different microalgal species and their ability to thrive as primary producers in many different habitats has created a rich treasure trove of metabolites that could be exploited commercially in a wide range of biotechnological sectors, such as pharmaceuticals,

* Correspondence to: F Baganz, Department of Biochemical Engineering, University College London, Gordon Street, London, WC1E 6BT, UK. E-mail: f.baganz@ucl.ac.uk

a Department of Biochemical Engineering, University College London, London, UK

b Institute of Structural and Molecular Biology, University College London, London, UK

nutraceuticals, and cosmetics. In addition, recent advances in strain engineering and domestication (including genetic engineering technologies for both the nuclear and chloroplast genomes) now make possible the use of microalgal species as light-driven cell factories for synthesis of high-value recombinant products, such as therapeutic proteins and bioactive compounds.

Although the history of culturing microalgae can be traced back over 2000 years, developments in cultivation technologies for commercial production are still at an early stage compared with well-established fermentation systems for heterotrophic cell platforms, such as bacteria, yeast, or mammalian cells. In the field of microalgal culture, photobioreactor (PBR) technology is a major area of research and development boasting many different designs of PBRs, including tubular systems, flat panel reactors, and bubble columns.³ Increasingly sophisticated PBRs have improved volumetric productivities and light conversion efficiency, but they inevitably incur higher capital costs as the designs involve glass vessels, artificial lighting, pumps, and control units.⁴ However, few studies have focused on simpler reactor designs.

One of the simplest PBR designs is based on single-use 'hanging bags' (HB) made from sterile polythene tubing, and it represents a very low-cost and operationally simple system.⁵ Typically, such HB systems are suspended as single columns or folded to form a hanging 'V' shape. They are illuminated externally, with either natural or artificial lighting, and operated as bubble columns with CO₂ gassing from the base of the bag. The use of sterile, disposable polythene tubing to create a unit of a specific volume allows for a modular operation that is readily scalable, with short set-up and turnaround times and with low capital investment. Despite the technological benefits, the environmental impact of a single-use technology should also be considered in bioprocess design. Solid materials (bag and sub-assemblies) are usually disposed via incineration. Conversely, cleaning and sterilisation are energy-intensive processes that also need a large amount of water and detergents. Gottschalk⁶ estimated that the reduced energy consumption of single-use systems could outweigh the increased solid waste from device disposal. However, the economic and environmental impacts of the single-use cultivation process require further assessment.

The HB-type PBR has shown promise as an inexpensive cultivation system, such that microalgae can commercially compete with

more established production technologies. To cite an instance, the cost of each HB used in this study is less than one US dollar. Additionally, no specific skillset is required for operation, so the system would benefit low- and middle-income countries. Such a bag system (i.e., a vertical installed bubble column-type reactor) has been used in the industry for cultivations of *Nannochloropsis*, *Porphyridium*, and *Chaetoceros calcium* for abalone feeding,⁷ *Haematococcus pluvialis* for astaxanthin production,⁸ and marine algae for oil production.^{9,10} In industrial applications, the working volume per bag ranges from 30 to 500 L, and a scale-out methodology is generally used to enlarge the production capacity.¹¹

In contrast to the other PBR designs mentioned above, most research on bubble column-type PBRs (including the HB) has focused on demonstrating microalgal growth performance.^{12,13} Therefore, there is limited information about the bag system itself, even though they have been accepted in the industry for decades. Technology from extensively studied bubble columns cannot be transferred to the HB without a basic understanding of the bag system. Furthermore, a well-characterised HB is the prerequisite for scale-up/down studies and for further optimising operating conditions and reactor designs. Herein, the present work aims to characterise and optimise key parameters of the HB that will help determine suitable operating conditions for microalgal cultivation. Mixing, mass transfer, gas hold-up, and bubble behaviour were evaluated in the study. We also demonstrate a simple way to modify the sparger and dimensions of the HB to improve the biomass volumetric productivity. The freshwater chlorophyte species *Chlorella sorokiniana* and a *Chlamydomonas reinhardtii* strain (expressing a recombinant luciferase) were cultured as model microalgae of industrial and research interest in the original and modified HBs to test the impact of the modifications on growth and recombinant protein accumulation.

MATERIALS AND METHODS

Microalgal cultivation and media composition

Chlorella sorokiniana UTEX 1230 and *Chlamydomonas reinhardtii* CC-1690 were obtained from the Culture Collection of Algae at the University of Texas at Austin and the Chlamydomonas Resource Center. Seed cultures for inoculating the HB were grown under constant illumination of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in an illuminated shaking incubator (Innova 4340, New Brunswick Scientific, USA) at 25 °C and 120 rpm. The seed was grown in Tris-Acetate-Phosphate (TAP) medium^{14,15} until the late-log phase and then it was used to inoculate the HB to an initial optical density (OD) reading at 750 nm of 0.05. For phototrophic growth, Tris-minimal medium (which has the same composition as TAP medium) was used where acetate was replaced with chloride.¹⁴

Generation of the *C. reinhardtii* chloroplast transformant

Plasmid JC109 (Fig. 1) cloned from pUC19 vector was designed for transforming the *C. reinhardtii* chloroplast, such that the codon-optimised *lucCP* gene encoding firefly luciferase¹⁶ was fused to the *C. reinhardtii* *rrnS* promoter, *psaA* 5' untranslated region (UTR), and *atpB* 3' UTR. The *aadA* cassette¹⁷ conferring spectinomycin resistance was included as a selectable marker and the two genes were targeted to the *psbH-trnE2* intergenic region by incorporating chloroplast flanking regions for homologous recombination, as described by Wannathong *et al.*¹⁷ A Golden Gate-based assembly system was employed for DNA cloning.¹⁸ Cells were transformed using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, USA), and spectinomycin resistant

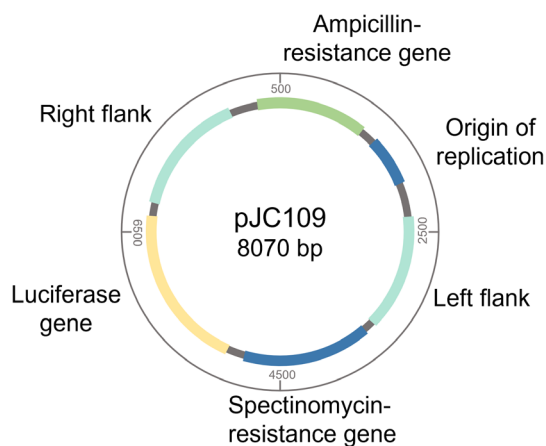


Figure 1. Map of the plasmid JC109 used for transforming the chloroplast genome of *Chlamydomonas reinhardtii*.

colonies were restreaked several times to obtain single colonies on TAP agar plates containing 300 µg mL⁻¹ spectinomycin. To confirm gene insertion and homoplasmy, DNA was extracted by the Chelex 100 method as described by Werner and Mergenhagen,¹⁹ and used in PCR reactions with a forward primer GTCATTGCGAAAATACTG (P1) that binds to the chloroplast genome outside of the *psbH* recombination region and two reverse primers ATGCAAATTAATAAAGGTAAATG (P2) and ACTGGCCTTCCGTTA (P3). A homoplasmic line was selected and used in subsequent experiments.

Luciferase assay

The luminescence of luciferase-expressing *C. reinhardtii* cells was measured by mixing 100 µL cultures with 100 µL Steady-Glo reagents (Promega, USA). Relative light units of samples were measured in a 96-well white plate (Thermo Fisher Scientific, USA) on a microplate reader (FLUOstar Omega, BMG LABTECH, Germany). The gain value was set to 50% of the maximal reading in the preliminary experiment, then fixed at 2400 for each plate reading. Luminescent signals were read each minute for 45 min, and the maximal value over time was recorded as the luminescence of each sample.

Microalgal growth standard curve and kinetics

Microalgal culture concentrations were indirectly determined by measuring OD using a spectrophotometer (UV/Vis Spectrometer UV2, Unicam, UK). Calibration curves ($y = 0.173x$ for *C. sorokiniana* and $y = 0.4939x$ for *C. reinhardtii*, $R^2 > 0.99$) were established for OD₇₅₀ readings versus dry cell weight (DCW) per litre. Microalgal cultures were centrifuged at 4000 *g* for 10 min, and cell pellets were snap-frozen with liquid nitrogen (BOC, UK), then lyophilised at -20 °C and 4 mbar in a freeze-dryer (Edwards, UK). Lyophilised pellets were weighed periodically until there was no further weight reduction.

Cell growth kinetics at exponential phase follow the first order,²⁰ and the specific growth rate (μ) and doubling time (D_t) were determined as follows:

$$dX/dt = \mu X_0 \quad (1)$$

$$D_t = \ln 2 / \mu \quad (2)$$

where X is biomass concentration (g DCW/L), X_0 is biomass concentration at t_0 (g DCW/L), and μ is specific growth rate (h⁻¹).

Biomass productivity is defined as the produced biomass per unit volume per unit time, expressed as follows:

$$P_t = (X_1 - X_0) / (t_1 - t_0) \quad (3)$$

where X_0 and X_1 are the biomass concentrations (g DCW/L) at time 0 (t_0) and time 1 (t_1), and P_t is the biomass produced per day in t days (g DCW/L/d).

Mixing time quantification

A pH tracer method was applied to measure the mixing times of the HBs, and experiments were carried out in reverse osmosis (RO) water with air flow rates from 2 to 10 L min⁻¹. 0.5% (v/v) of 2 M HCl or 2 M NaOH was added onto the liquid surface of the aerating HB. A micro pH probe (Mettler Toledo, USA) was fixed 1–2 cm below the sparger and connected to a pH meter (S220 SevenCompact pH Meter, Mettler Toledo, USA) to record pH

changes. The time required to reach 95% homogeneity was recorded as the mixing time.

Mass transfer coefficient of CO₂

A gassing in/out technique, as described by Garcia-Ochoa and Gomez,²¹ was used to determine the gas–liquid volumetric mass transfer coefficient ($k_L a$) of O₂. Experiments were conducted in an air–water system in HBs at room temperature. A needle-type optical dissolved oxygen (DO) probe (NTH-PSt1 PreSens, Germany) was fixed at 1–2 cm below the liquid level to monitor the DO, and the data was collected and recorded via a transmitter (Microx TX3 trace, PreSens, Germany) and accessory software.

$k_L a$ of CO₂ was estimated by converting $k_L a$ (O₂) based on diffusion coefficients:²²

$$k_L a(\text{CO}_2) = k_L a(\text{O}_2) X (D_{\text{O}_2} / D_{\text{CO}_2})^{1/2} \quad (4)$$

where $k_L a$ (CO₂) and $k_L a$ (O₂) are CO₂ and O₂ mass transfer coefficients (s⁻¹), D_{CO_2} and D_{O_2} are CO₂ and O₂ diffusion coefficients (m² s⁻¹).

Average gas hold-up determination

A manometric method described by Rollbusch *et al.*²³ was employed to determine average gas hold-up values. Axial pressures at different heights of the HB were measured using a wet-to-wet differential pressure transducer (Omega, USA). Neglecting internal effects (fractional drag on the wall and acceleration of phases in the vertical direction) and given that the air density is much less than the density of water, the average gas hold-up in a gas–liquid system is described by the following equation:

$$\varepsilon_G = 1 - \Delta P / (\rho_L g \Delta h) \quad (5)$$

where ΔP is a pressure difference between a distance (Δh), g is the gravitational acceleration (9.8 m s⁻²), ρ_L is a liquid density, and ε_G is a gas hold-up.

Visualisation of bubble behaviour

A high-speed camera (Photron FASTCAM, MC1, Japan) with AF NIKKOR 24–85 mm 1:2.8–4 D lens (Nikon, Japan) was used to capture bubble behaviour. The high-speed camera was mounted on an adjustable tripod 15 cm away. Images of 512 × 512 pixels were captured at 2000 fps.

Microalgal cultivation in the hanging bags

The temperature was set at 25 °C using a closed temperature-controlled room. Fluorescent light strips provided constant illumination of the HBs from one side with a light path of 20 cm and an average light intensity of 100 µmol m⁻² s⁻¹ (Fig. 2(A)). Air was introduced into the HBs at a constant volumetric flow rate of 5 L min⁻¹ and was monitored by a rotameter (Cole-Parmer, USA). Pure CO₂ (vapour withdrawal, BOC, UK) was added into the air flow at 100 mL min⁻¹ (and was monitored by another rotameter) to obtain a 2% CO₂-enriched air. The CO₂-air mixture passed through a 0.22 µm filter (Sartorius, Germany) and was then supplied to phototrophic and mixotrophic cultures.

The working volume of HBs used for microalgal cultures in this study was 15 L. Figure 2(B) illustrates the experimental set-up. The OD, pH, and Quantum yield (Q_y) of photosystem II (PSII) were measured off-line using a spectrophotometer (UV/Vis Spectrometer UV2, Unicam, UK), a pH meter (HANNA, USA), and a fluorometer (AquaPen-C, Photon Systems Instruments, Czech Republic),

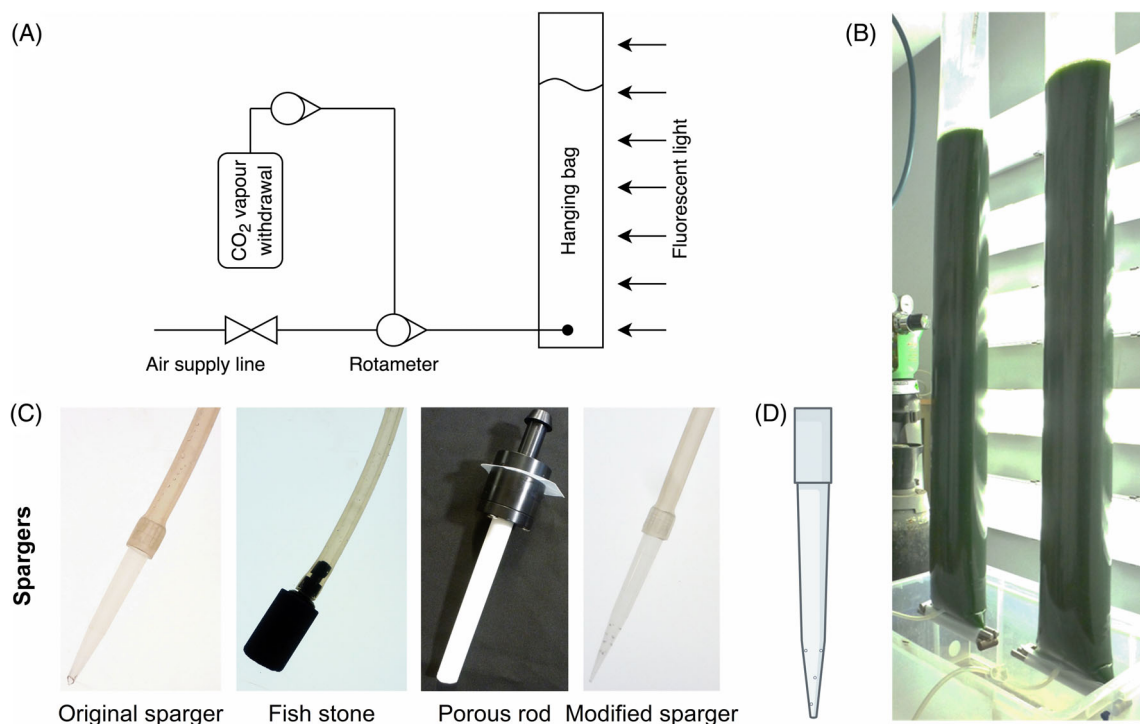


Figure 2. (A) Schematic diagram and (B) image of the hanging bag (HB) cultivation system set-up. (C) Images of different spargers tested with the HB. (D) Schematic diagram of the modified sparger.

respectively. A 30% flash pulse was set for measuring fluorescence Q_f (Fv/fm) of dark-adapted samples. Ammonium and phosphate concentrations in the culture medium were measured by colorimetric test kits (Quantofix, Sigma-Aldrich, USA).

Hanging bag design and dimensions

The HB is a single-use PBR (operated as a bubble column) that is made of a polythene layflat tubing (1000-gauge, UK Packing, UK) and sealed using a heat sealer (Star MK VI, Star Universal, UK). The flat width of the original HB was 22.9 cm, so the corresponding diameter and circumference were 14.6 and 45.7 cm. In the original HB, gas was introduced via a 1 mL pipette tip. The pipette tip was cut to create a sharp end for piercing the bag (Fig. 2(C)). The internal diameter of the pipette tip end was approximately 4 mm.

The modified bag width was 20.3 cm, with a corresponding diameter and circumference of 12.9 and 40.6 cm. A pipette tip with 12 holes of 0.5 mm in diameter was used as the modified sparger (Fig. 2(C)). Throughout this paper, the 'original HB' refers to the bag of 22.9 cm in width aerated via the original sparger, whereas the 'modified HB' refers to the 20.3 cm width bag with the improved sparger.

RESULTS AND DISCUSSION

Sparger choice and visualisation of bubble behaviour in hanging bag

The HB has emerged as a simple microalgal cultivation system but it requires gas sparging to achieve sufficient mixing, mass transfer, and ultimately optimal growth. Here, we assessed several sparger candidates to identify the most efficient, whilst keeping the design simple and low-cost. In the original HB system, the gas phase is introduced using a sharpened pipette tip so that bubbles

are generated from a single orifice at the base of the bag. This straightforward sparger makes the HB system easy to set up and operate but it is under-developed. We tested several sparger designs in preliminary experiments, including a fish stone, a porous rod, and a modified plastic tip (Fig. 2(C)). The fish stone (connected to an air tubing) was inserted from the top of the HB to avoid leakage and this caused the sparger to float to different levels depending on applied liquid volumes and gas flow rates. The porous rod sparger was customised and equipped at the bottom of the HB. However, an air flow of 6 L min^{-1} still could not pass through the porous rod because of the small pore size (20 μm in diameter) and the static pressure in the 10 L HB. This resistance could potentially be addressed by increasing either the pore size or by using compressed air. However, with a view to the simplicity and cost-effectiveness of the HB system, the fish stone and porous rod were not examined further as candidate spargers, and the focus returned to the plastic tip design. Pipette tips are appealing because of their exceptionally low cost, their ease-of-fitting (with the soft polythene of the HB by immediately forming a water-tight seal when pierced with the tip), and the efficient mixing generated by the gas flow. An orifice size of 0.5 mm has been reported to generate a bubbly flow regime with high gas hold-up,²⁴ so 12 holes each with a diameter of 0.5 mm were pierced along the tip wall (Fig. 2(C)). From the perspective of bubble column design, the number of holes on a sparger would affect the bubble regime and, ultimately, the gas-liquid mass transfer.²⁵ Therefore, $k_L a$ is a critical parameter to indicate the effect of the modified sparger. The number of holes was chosen to achieve an even distribution on the conical surface of the sparger (Fig. 2 (D)). This multi-hole pipette tip is referred to hereafter as the modified sparger.

The effect of the sparger design on bubble behaviour under different operating conditions was analysed visually using a

high-speed camera. Figure 3(A) shows the bubble generation and distribution at the sparger region and in the middle of the bag. Generally, bubbles were distributed more radially as the gas flow was increased from 2 to 10 L min⁻¹. As illustrated in Fig. 3(A), the

modified sparger introduced more bubbles with more uniform and smaller sizes, compared to bubbles from the original sparger. From the original sparger, large bubbles with a diameter of approximately 60 mm (in the shape of ellipsoids) floated

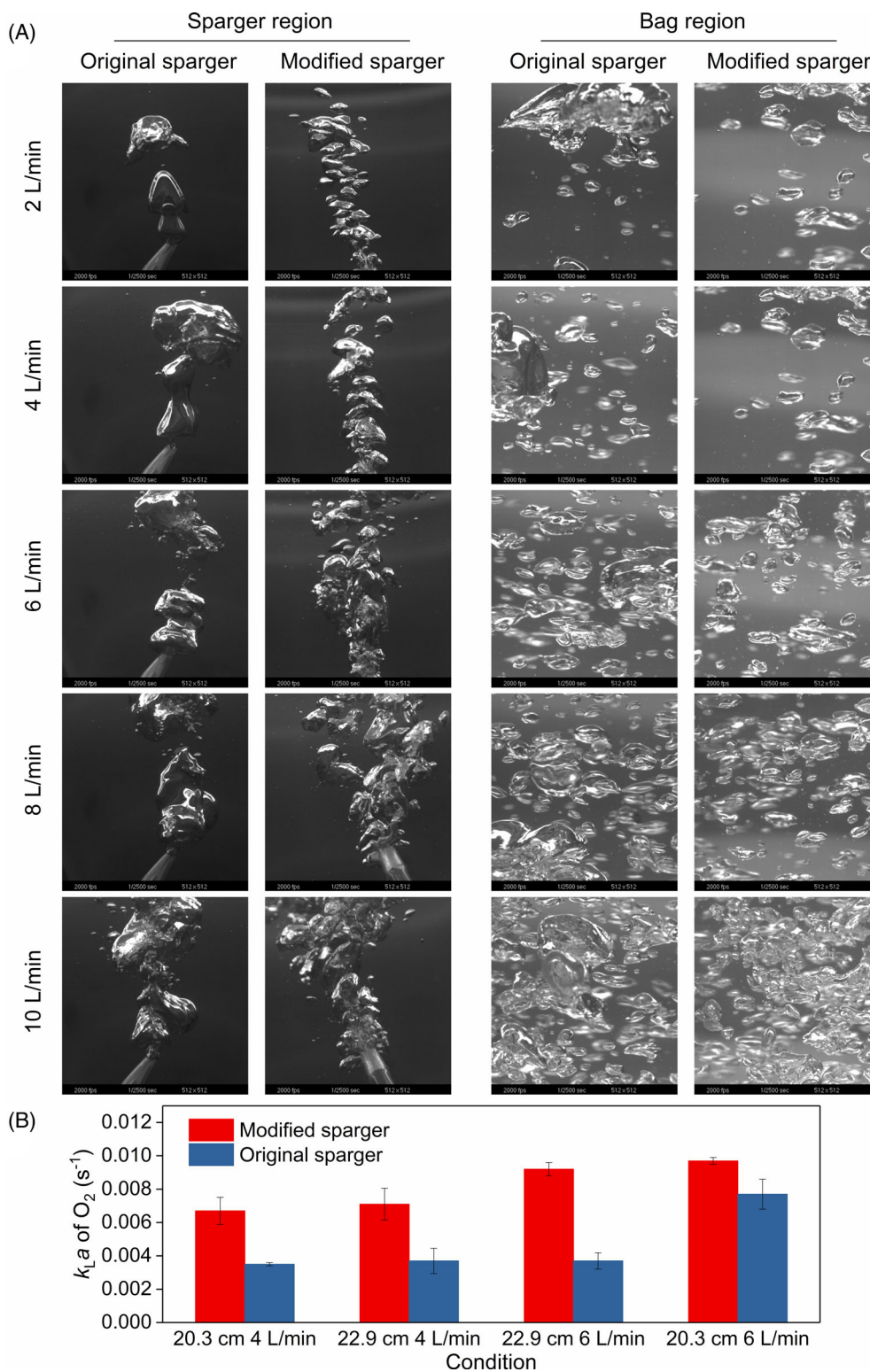


Figure 3. (A) Photographic images of bubbles generated under different air flow rates from the original and modified spargers at the sparger regions and in the middle of the hanging bags. (B) Volumetric mass transfer coefficient ($k_L a$) of O₂ for 10 L bags with 20.3 or 22.9 cm in width, and gas flow rates of 4 or 6 L min⁻¹. Error bars represent sample standard deviations (N ≥ 3).

intermittently, as opposed to swarms of small bubbles rising continuously. Bubble coalescence frequently occurred at the tip of the original sparger (Fig. 3(A)), and this is in line with early studies suggesting that the bubble coalescence mainly occurs around orifices.²⁶ Then, bubbles with a stabilised size rose, indicating that a dynamic equilibrium was reached between bubble coalescence and break-up.²⁷ This bubble behaviour was also observed in other bubble columns with different bubble generators and with vibration.^{28,29} Large bubbles are associated with inefficient mass transfer.³⁰ Subsequently, $k_L a$ values of O_2 for both spargers were investigated under 4 and 6 $L\ min^{-1}$ air volumetric velocities. Also, an HB with a different diameter was tested to see how this change impacted the $k_L a$: the original HB with a flat width of 22.9 cm and a new HB with a narrower width of 20.3 cm. As a result, the corresponding height-to-diameter ratio increased from 8:1 to 12:1 with 20 L of liquid in the bag, allowing for increased surface illumination. Aspect ratios of bubble columns are often in the range 1:1–15:1 and 2:1–5:1 for biochemical applications.^{31,32} However, a large surface-to-volume ratio is preferred in PBR designs since light is essential in microalgal cultivation. The aspect ratio of the HB could be further increased using even narrower bags, but the bag height is restrained by the ceiling height for indoor cultivation.

As shown in Fig. 3(B), HBs with the modified sparger showed greater $k_L a$ s than those with the original sparger under all investigated conditions. For the HB of 22.9 cm in width with the modified sparger, the $k_L a$ (O_2) reached $0.0092\ s^{-1}$ under $6\ L\ min^{-1}$. This value was more than twice the $k_L a$ obtained using the original sparger. The $k_L a$ for the original sparger did not show a marked change under three of the four conditions; the exception occurred under a flow rate of $6\ L\ min^{-1}$ in the narrower HB, where the $k_L a$ was $0.0077\ s^{-1}$. This may result from more vigorous mixing, as seen in Fig. 3(A). This simple analysis reveals that larger bubbles prevail under the flow rate of $4\ L\ min^{-1}$, whereas the coalescence and break-up of bubbles occur more frequently at $6\ L\ min^{-1}$ because of vigorous mixing and the narrower width of the bag.

Gas hold-up

Gas hold-up (ϵ_G) is a critical parameter to indicate transport phenomena in pneumatic bioreactor designs, as it represents the gas proportion in a gas–liquid system. Liquid circulation in a bubble column is introduced by gas dispersion, so ϵ_G is relevant to mixing, heat, and mass transfer. The average ϵ_G of HBs was estimated by measuring pressure differences. The filling volume of the HBs was restricted by the lower limit of the pressure transmitter; hence, only 20 L HBs were studied.

As shown in Fig. 4(A) and (B), the ϵ_G values increased linearly with increasing u_G . Under u_G of $0.5\ cm\ s^{-1}$, the ϵ_G in the modified HB was greater than that in the original HB by 33%. Since the effect of column sizes on ϵ_G is negligible for bubble columns with an aspect ratio greater than 5:1,³² the increase in ϵ_G of the modified HB was likely to result from the improved sparger design. When u_G was below $0.5\ cm\ s^{-1}$, the modified HB showed greater ϵ_G values than the original design, whereas the difference of ϵ_G values between the original and modified HBs gradually reduced when u_G raised to above $0.5\ cm\ s^{-1}$ (Fig. 4(B)). This result indicates a possible transition point of the flow patterns. Further study of the axial and radial ϵ_G of the HB could interpret the phenomenon more completely, and this can be experimentally determined by the Electrical Resistance Tomography technique or simulated by Computational Fluid Dynamics.^{33,34}

Mass transfer coefficient of CO_2

CO_2 molecules diffuse from gas bubbles to the liquid phase and then into cells. Meanwhile, bicarbonate can also be taken up by microalgal cells as an inorganic carbon source.³⁵ Sufficient CO_2 supply and efficient removal of O_2 are essential in the phototrophic cultivation of microalgae. Since the overall mass transfer coefficient is governed by transferring gas through a liquid film on the liquid side,²¹ $k_L a$ was measured to indicate the effectiveness of gas transfer in the HB.

The $k_L a$ values increased linearly with increased air flow rates within the investigated range (Fig. 4(C) and (D)), and predominantly resulted from the corresponding gas proportion in HBs. Accordingly, the $k_L a$ values showed the same trend as the average ϵ_G ; both increasing with u_G . In the original HB, the greater $k_L a$ s were achieved in the HB with a larger liquid volume (Fig. 4(C)), where the $k_L a$ of CO_2 in the 20 L HB was $0.012\ s^{-1}$ when the gas velocity was $10\ L\ min^{-1}$, which was 1.5 times greater than that of the 10 L HB. As the liquid height of the 20 L HB was approximately twice that of the 10 L HB, the residence time for the bubbles in the liquid phase was longer, resulting in higher $k_L a$ s. However, the tendency changed in the modified HB (Fig. 4(D)). The $k_L a$ s increased in the modified HB when the fill volume increased from 15 to 20 L, but the greatest $k_L a$ was found in the 10 L HB. This result signifies a potential change in the flow pattern because continuous bubble break-up and coalescence in a heterogeneous regime can cause a significant rise in $k_L a$.³⁶ The u_G and reactor diameter, the former is related to the Reynold number, are used to determine the fluid regime of bubble columns, but the method does not take sparger designs into consideration.³⁷ Therefore, the conventional method is not appropriate to define the flow pattern of the modified HB because of the non-standard sparger.

In summary, the $k_L a$ s of the modified HB were greater than that of the original HB under the same volumetric velocity and filling volume.

Mixing time

The HB mixing system was assessed by the mixing time, which is defined as the time needed to achieve 95% homogeneity. In the original HB filled with 10, 15, and 20 L water, the average mixing times were 25, 40, and 48 s, respectively, when the flow rate was $2\ L\ min^{-1}$. The mixing time decreased almost linearly with increasing air velocity, and all mixing times were in the range of 10–48 s (Fig. 4(E)). The modified HB demonstrated a similar trend (Fig. 4(F)). The mixing times of both HBs are comparable to that reported for a bubble column PBR with a similar height-to-diameter ratio.³⁸ Large flow rates increase the shear force at the interphase of the gas and liquid, and this can cause cell damage in microalgal cultures.³⁹ Therefore, flow rates greater than $10\ L\ min^{-1}$ were not evaluated in this study. Generally, the mixing times of the modified HB were longer than those of the original HB. The longer mixing time in the modified HB suggested that small bubbles achieved less effective mixing than larger bubbles. This conclusion is in line with that by Kück *et al.*,⁴⁰ who found that the mixing time was dependant on bubble size under certain flow conditions.

Cultivations of *Chlorella sorokiniana* in hanging bags

From preliminary experiments, we observed severe foaming during the cultivation of *C. sorokiniana* when using air flow rates greater than $5\ L\ min^{-1}$. Thus, a flow rate of $5\ L\ min^{-1}$ was selected as the operating condition for microalgal cultivation. Based on the

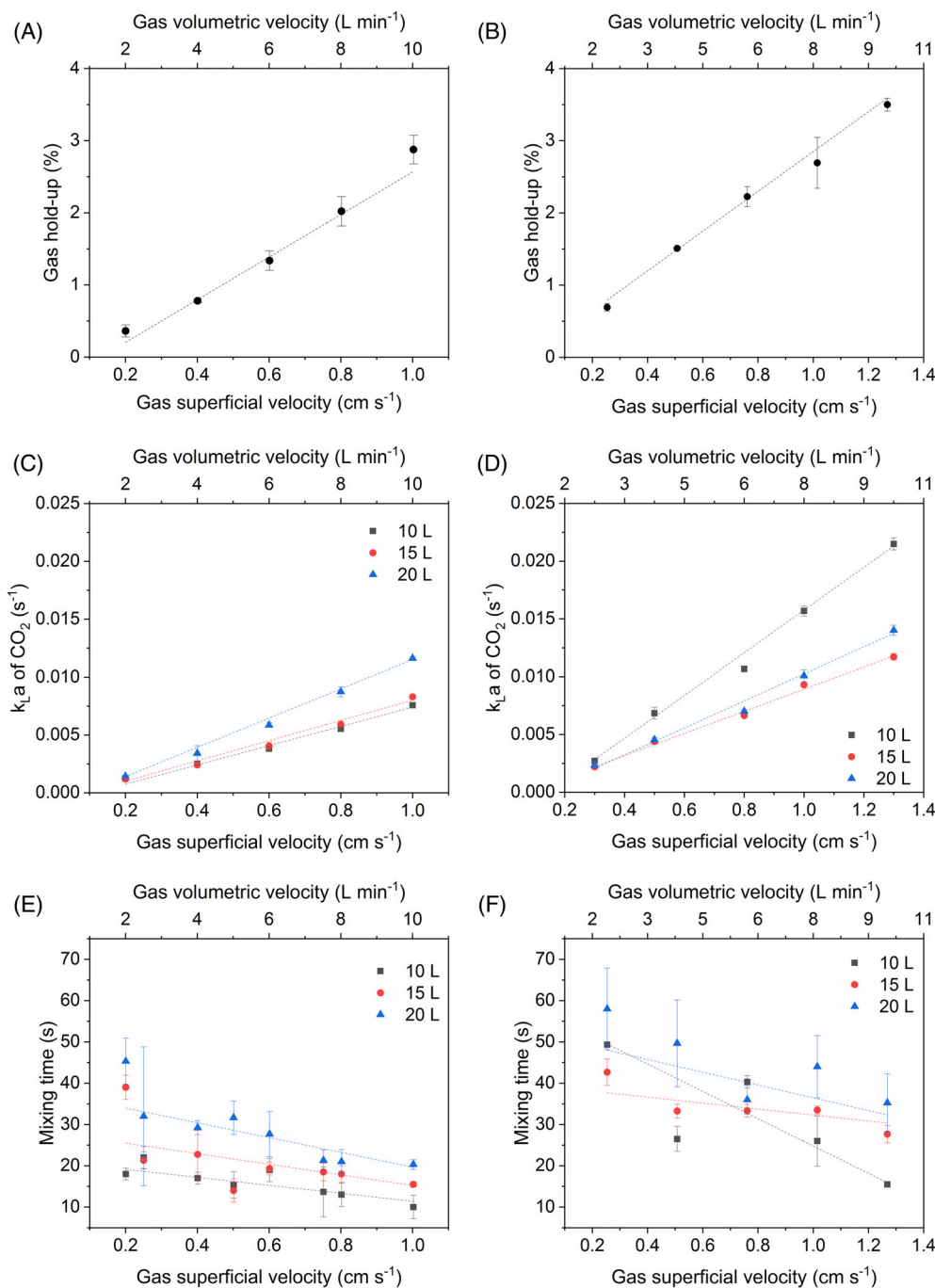


Figure 4. Engineering parameters of hanging bags (HBs). Gas hold-ups of the (A) original and (B) modified HBs filled with 20 L; volumetric mass transfer coefficients (k_{La}) of CO₂ of the (C) original and (D) modified HBs filled with 10, 15, and 20 L; mixing times of the (E) original and (F) modified HBs filled with 10, 15, and 20 L of water under different air velocities. Experiments were conducted in an air-water system. Error bars represent sample standard deviations ($N \geq 3$).

engineering parameters, the modified HB showed a 1.5-fold increase in k_{La} under 5 L min⁻¹ with a working volume of 15 L. Previously, *Chlorella* species have been cultured in bubble columns under aeration conditions ranging from 0.16 to 1 vvm (L/L/min),^{41–43} where 0.33 vvm corresponds to a gas flow rate of 5 L min⁻¹ in the 15 L HB. Hence, our selection of 5 L min⁻¹ falls within the established range.

Chlorella sorokiniana was cultured under phototrophic conditions to evaluate biomass production in the original and modified

HBs filled with 15 L of Tris-minimal medium. The biomass concentration in the modified HB reached 0.35 g DCW/L after 56 h and declined afterwards, which was caused by a pH drop (Fig. 5(B)). The pH gradually decreased from 7.2 to 6.5 over the first 30 h and then dropped to 3.2. The likely reason for this drop in pH is that when the phototrophic growth slowed, the excess dissolved CO₂ could not be utilised for photosynthesis, so instead, it carbonated the medium. Furthermore, ammonium assimilation by *Chlorella* was associated with acidifying of the surrounding

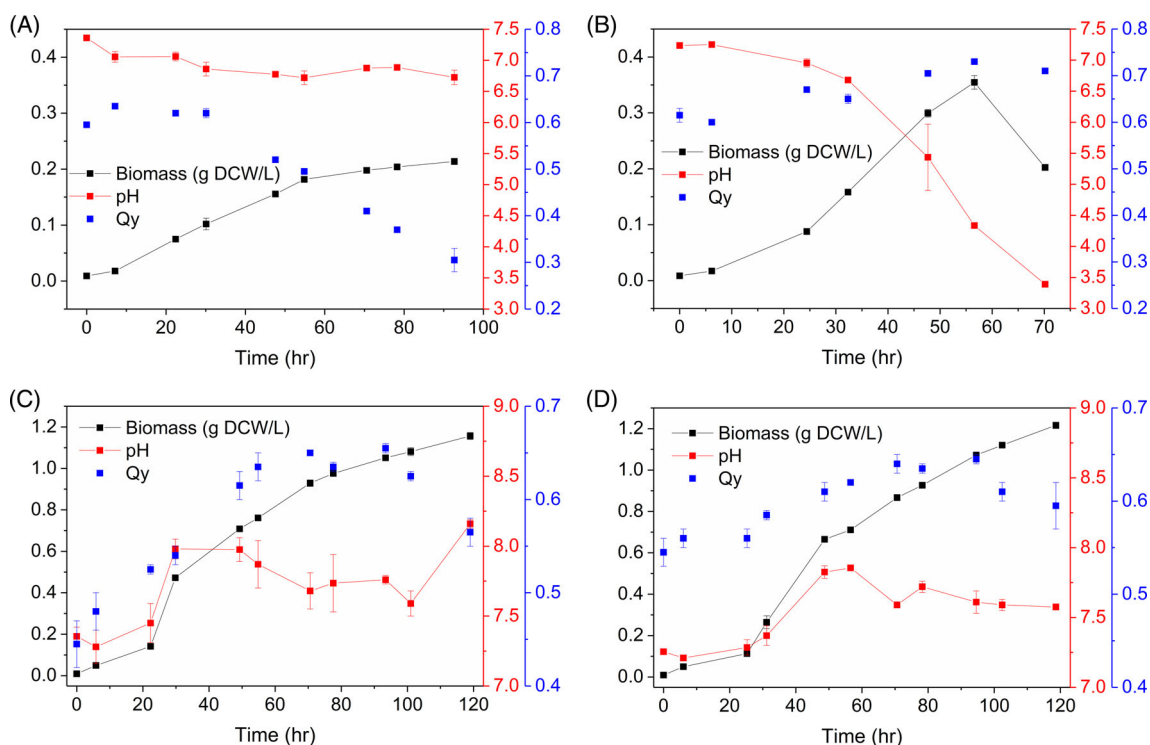


Figure 5. Phototrophic cultivations of *Chlorella sorokiniana* in the (A) original and (B) modified hanging bags (HBs); mixotrophic cultivations of *C. sorokiniana* in the (C) original and (D) modified HBs. All the experiments used a working volume of 15 L and a gas flow rate of 5 L min⁻¹. Q_y represents quantum yield of photosystem II. Error bars represent ranges of two biological replicates.

environment.⁴⁴ In contrast, no evident pH decrease was observed in the original HB cultivation, and pH readings varied between 7.4 and 6.7 (Fig. 5(A)). The difference in pH changes could result from the reduced mass transfer of CO₂ in the original HB. The final biomass achieved in the original HB was 0.21 g DCW/L (which is 40% less than that obtained in the modified HB), and the culture in the modified HB showed an improved growth rate and a two-fold increase in biomass productivity (Table 1).

Changes to Q_y , also known as PSII quantum efficiency, were monitored to indicate microalgal photoactivity. Q_y values of *C. sorokiniana* grown under phototrophic and mixotrophic conditions were reported to be approximately 0.6.⁴⁵ For the culture in the original HB, the Q_y dropped from 0.6 to 0.3 (Fig. 5(A)), which may be a consequence of CO₂ limitation as this can lead to excess reductant in the chloroplast and PSII reaction centre closure. In

contrast, the Q_y values of the culture grown in the modified HB were in the range of 0.6–0.73, suggesting that the cells remained photoactive throughout the cultivation.

At the end of the cultivations, the remaining ammonium and phosphate levels in the medium were measured via colorimetric semi-quantitative methods. The residual concentrations of ammonium in the modified and original HBs were around 50 mg L⁻¹ and 100 mg L⁻¹, respectively. In addition, 75 mg L⁻¹ of phosphate remained in both the modified and original HBs. Ammonium concentrations were reduced by 64% and 26% in the modified and original HBs, respectively, and the phosphate concentration declined by 22% compared to the initial concentrations in the medium. Therefore, the nitrogen and phosphorus concentrations were considered as not limiting throughout the cultivations. The differences in growth rate and biomass

Table 1. Summary of *Chlorella sorokiniana* and luciferase-expressing *Chlamydomonas reinhardtii* growth kinetics using the HB cultivation systems

| Species | Conditions | Growth rate (h ⁻¹) | Doubling time (h) | Productivity (g L ⁻¹ d ⁻¹) | |
|---|--------------|--------------------------------|-------------------|---|----------------|
| <i>C. sorokiniana</i> | Phototrophic | Original HB | 0.083 ± 0.001 | 8.336 ± 0.084 | 0.031 ± 0.001 |
| | | Modified HB | 0.090 ± 0.001 | 7.685 ± 0.117 | 0.066 ± 0.0001 |
| | Mixotrophic | Original HB | 0.143 ± 0.007 | 4.837 ± 0.210 | 0.292 ± 0.001 |
| | | Modified HB | 0.161 ± 0.0003 | 4.307 ± 0.007 | 0.313 ± 0.005 |
| Luciferase-expressing <i>C. reinhardtii</i> | Phototrophic | Original HB | 0.024 ± 0.001 | 28.723 ± 0.773 | 0.076 ± 0.004 |
| | | Modified HB | 0.028 ± 0.001 | 24.90 ± 0.402 | 0.080 ± 0.008 |
| | Mixotrophic | Original HB | 0.057 ± 0.0001 | 12.108 ± 0.032 | 0.369 ± 0.010 |
| | | Modified HB | 0.063 ± 0.0004 | 11.047 ± 0.079 | 0.329 ± 0.012 |

Productivity represents dried biomass produced per day in three days. Data presented are the means of biological replicates, and error bars refer to ranges.

production were probably associated with the availability of CO₂ in the HBs. The productivities of three-day batch cultivations are summarised in Table 1. Compared to cells grown in the original HB, the culture in the modified HB under phototrophic conditions showed an increase of 0.035 g L⁻¹ d⁻¹ in productivity. This finding indicates that the modified HB can provide CO₂ more efficiently, as expected from the *k_La* data (Fig. 5(C) and (D)). The productivity was comparable to that obtained for another *Chlorella* species (*Chlorella ellipsoidea*) when cultured photosynthetically in a 20 L bubble column.⁴⁶

We also explored the cell growth in the HB under mixotrophic conditions, which is the commonly used cultivation mode in the laboratory during process development. *C. sorokiniana* cells were cultured mixotrophically in 15 L of TAP medium. The biomass concentrations in the original and modified HBs reached 1.16 and 1.22 g DCW/L, respectively (Fig. 5(C) and (D)), and the growth kinetics were similar in the two HBs, reflecting a predominant use of acetate (over CO₂) as the carbon source under mixotrophic growth (Table 1). The pH increased during the cultivation because of acetate consumption. The pH in the modified HB was more stable than that in the original HB and can be ascribed to better CO₂ transfer and distribution. The biomass concentrations achieved in both HBs were comparable to those attained by *C. sorokiniana* grown under mixotrophic conditions in a 1 L Duran bottle.⁴⁷

Growth and recombinant protein production in the hanging bags of a *Chlamydomonas reinhardtii* strain engineered to produce luciferase

Whilst cultivated *Chlorella* species represent an attractive food and feed ingredient for the future,⁴⁸ we also wanted to explore the performance of the HB system for recombinant protein production in microalgae. *C. reinhardtii* is a well-studied model microalga with a suite of molecular tools that enable routine genetic engineering and reflect an increasing potential as an industrial platform.⁴⁹ As a recombinant protein we chose the luciferase enzyme from firefly because it allows a sensitive and fast assay with a low background signal in *C. reinhardtii*.⁵⁰ To create a luciferase-expressing *C. reinhardtii* strain, we transformed the chloroplast genome with a gene construct (Fig. 6(A), lower figure)

in which the codon-optimised *lucCP* gene was driven by the endogenous *rrnS* promoter and *psaA* 5' UTR, and targeted to the *psbH-trnE2* intergenic region (Fig. 6(A), the top construct). Since the *C. reinhardtii* chloroplast contains, on average, 80 copies of the circular genome,⁵¹ a three-primer PCR was conducted to confirm the chloroplast DNA homoplasmy (Fig. 6(B)).

Growth of the transformant line under phototrophic conditions was compared in the original and modified HBs. A biomass yield of 0.69 g DCW/L was achieved in the modified HB, whereas the original HB reached 0.62 g DCW/L (Fig. 7(A) and (B)). Despite this modest improvement in biomass accumulation, luciferase assays normalised to the cell density also showed an increase of 11.1%. There were no clear differences in pH and Q_Y changes for the *C. reinhardtii* strain grown in the original and modified HBs. Interestingly, in contrast to *C. sorokiniana* cultures (where Q_Y dropped gradually), the Q_Y of *C. reinhardtii* cultures on day 2 was as low as 0.15 (on average) and slowly rose during cultivation, reaching final values of 0.6 and 0.62 in the original and modified HBs, respectively (Fig. 7(A) and (B)). A typical *C. reinhardtii* Q_Y value was reported as approximately 0.6,^{52,53} implying a favoured growth environment. The Q_Y trend observed in this study could have resulted from the fluorescent lighting used, since fluorescent strips emit ultraviolet radiation that may induce a certain degree of photoinhibition to *C. reinhardtii*.^{54,55} Similar to the findings for *C. sorokiniana* cultures, cell growth under the mixotrophic conditions was similar in the original and modified HBs (Fig. 7(C) and (D)) and achieved 1.23 and 1.18 g DCW/L, respectively, as the highest biomass concentrations during cultivations. As indicated in Fig. 7(D), the culture grown in the modified HB showed the highest content of luciferase at exponential phase (55 h), indicating an optimal harvest point for recombinant protein recovery. Much of the research on *C. reinhardtii* employs cell counts as the unit for culture density; the biomass concentrations achieved in this study were of the same magnitude as several commercial-associated studies.^{56,57} Table 1 summarises the growth kinetics of the transformant grown under all investigated conditions, and the phototrophic and mixotrophic growth rates achieved in the modified HB were improved compared to those of the original HB (*P* < 0.05, one-tailed *t*-test). However, the biomass productivity

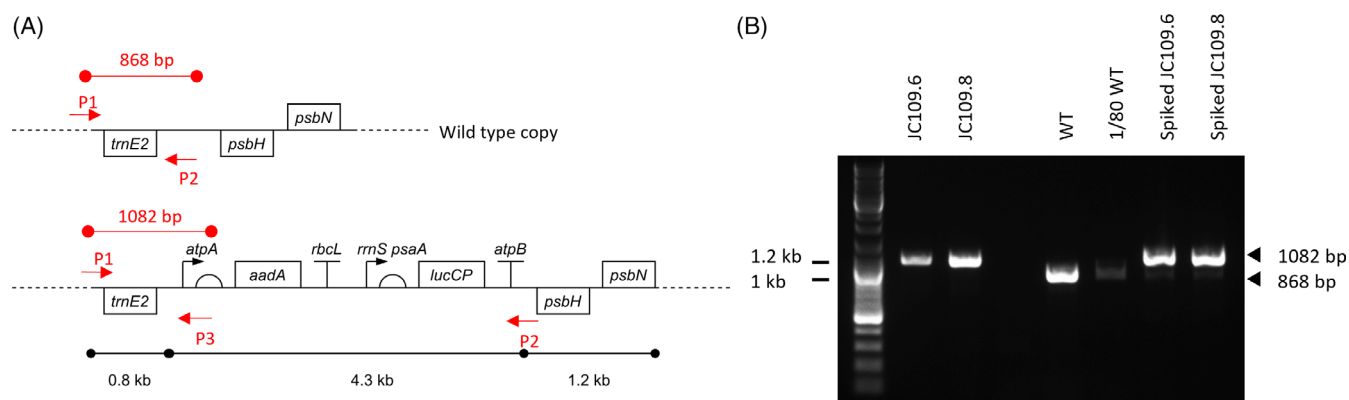


Figure 6. (A) Schematic diagrams of the *psbH-trnE2* region of the wild-type chloroplast DNA (WT cpDNA, upper figure) and recombinant chloroplast genome copy (JC109) containing expression cassettes of spectinomycin resistance (*aadA*) and firefly luciferase (*lucCP*) (lower figure). (B) PCR screening of putative transformants for the insertion of transgenes and homoplasmy of the chloroplast genome by using a set of three primers, including P1, P2, and P3. The WT cpDNA yields a 0.8 kb PCR product with primers P1 and P2, whereas the transformant copy generates a 1.1 kb product with primers P1 and P3. The absence of a WT 0.8 kb product for lines JC109.6 and JC109.8 indicates homoplasmy in the polyplid plastome (i.e., all ~80 copies in the chloroplast contain the transgene). The three-primer PCR method is sensitive enough to identify any residual heteroplasmy as demonstrated with the 1/80 diluted WT sample and the transformant cpDNA sample spiked with 1/80 WT copies.

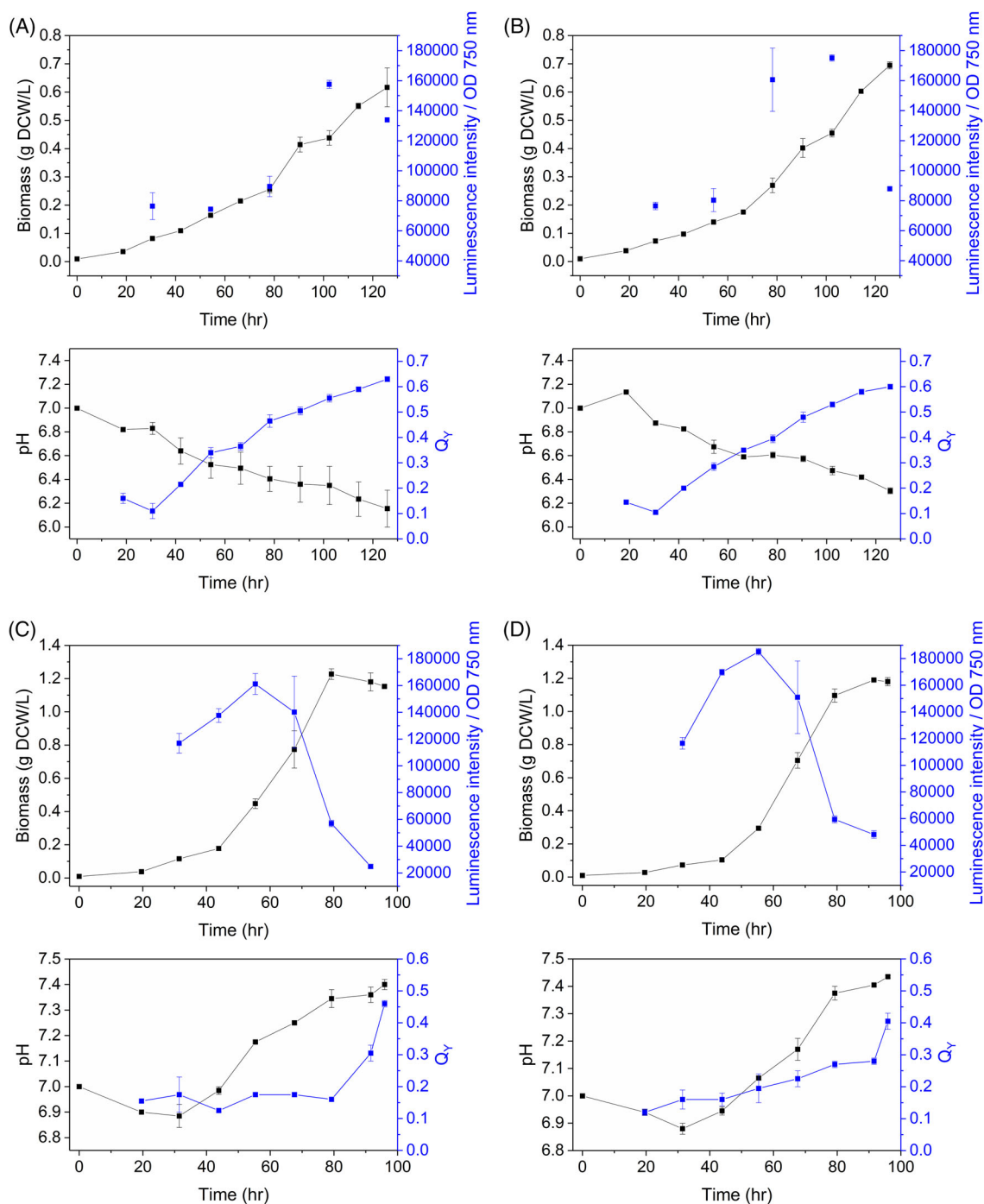


Figure 7. Phototrophic cultivations of the luciferase-expressing *Chlamydomonas reinhardtii* in the (A) original and (B) modified hanging bags (HBs); the mixotrophic cultivations in the (C) original and (D) modified HBs. 15 L of corresponding growth media and 5 L min⁻¹ gas flow rate were used in the experiments. Q_y is quantum yield of photosystem II. Error bars are ranges of two biological replicates.

did not show a significant increase ($P < 0.05$, one-tailed t -test), indicating that operating conditions (such as gas flow rate, CO₂ concentration, and light regime) can be further optimised.

CONCLUSIONS

In this study, improvements to the design and operation of an HB PBR system resulted in increases in ε_G and $k_L a$. Using a gas flow rate of 5 L min⁻¹ and a new sparger device, *C. sorokiniana* and

C. reinhardtii showed improved biomass and recombinant protein production, respectively, under phototrophic conditions. This simple and low-cost HB cultivation system is comparable in performance to other bubble columns, and further optimisation would allow for its use in producing different microalgae of commercial value. Overall, the investigated physical parameters are integral in understanding the engineering environment of the HB system, and this is essential for future optimisation and scale translation studies. For this HB PBR, a scale-out approach would

be suitable. These findings strengthen the concept of using a simple PBR for process development or for the commercial production of microalgal-derived products.

ACKNOWLEDGEMENTS

The work carried out was supported in part by grant BB/R016534/1 from the UK's Biotechnology and Biological Sciences Research Council and grant H2020 REA 774078 from the European Commission.

REFERENCES

- Bhattacharya D and Medlin AL, Algal phylogeny and the origin of land plants. *Plant Physiol* **116**:9–15 (1998). <https://doi.org/10.1104/pp.116.1.9>.
- DeWreede RE, Algal ecology, in *Encyclopedia of Life Sciences*. John Wiley & Sons Ltd, Chichester (2001). <https://doi.org/10.1038/ngp.els.0000317>.
- Ación FG, Molina E, Reis A, Torzillo G, Zittelli GC, Sepúlveda C *et al*, Photobioreactors for the production of microalgae, in *Microalgae-Based Biofuels and Bioproducts: From Feedstock Cultivation to End-Products*, ed. by Gonzalez-Fernandez C and Muñoz R. Cambridge: Woodhead Publishing, pp. 1–44 (2018). <https://doi.org/10.1016/B978-0-08-101023-5.00001-7>.
- Chisti Y, Raceways-based production of algal crude oil. *Green* **3**:195–216 (2013). <https://doi.org/10.1515/green-2013-0018>.
- Martínez-Jerónimo F and Espinosa-Chávez F, A laboratory-scale system for mass culture of freshwater microalgae in polyethylene bags. *J Appl Phycol* **6**:423–425 (1994). <https://doi.org/10.1007/BF02182159>.
- Gottschalk U, Disposables in downstream processing. *Adv Biochem Eng Biotechnol* **115**:171–183 (2010). https://doi.org/10.1007/10_2008_22.
- Brown MR and Blackburn SI, Live microalgae as feeds in aquaculture hatcheries, in *Advances in Aquaculture Hatchery Technology*. Cambridge: Woodhead Publishing, pp. 117–158 (2013). <https://doi.org/10.1533/9780857097460.1.117>.
- Taunt HN, Stoffels L and Purton S, Green biologics: the algal chloroplast as a platform for making biopharmaceuticals. *Bioengineered* **9**:48–54 (2017). <https://doi.org/10.1080/21655979.2017.1377867>.
- Narala RR, Garg S, Sharma KK, Thomas-Hall SR, Deme M, Li Y *et al*, Comparison of microalgae cultivation in photobioreactor, open raceway pond, and a two-stage hybrid system. *Front Energy Res* **4**:29 (2016). <https://doi.org/10.3389/fenrg.2016.00029>.
- Zhu Y, Jones SB and Anderson DB, *Algae Farm Cost Model: Considerations for Photobioreactors*. Richland, WA. U.S. Department of Energy, Office of Scientific and Technical Information; (2018). <https://doi.org/10.2172/1485133>.
- Richmond A and Hu Q, *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, 2nd edn. Hoboken: John Wiley & Sons, (2013). <https://doi.org/10.1002/9781118567166>.
- Vu MTT, Jepsen PM, Jørgensen NOG, Hansen BW and Nielsen SL, Testing the yield of a pilot-scale bubble column photobioreactor for cultivation of the microalga *Chlorella salina* as feed for intensive calanoid copepod cultures. *Aquacult Res* **50**:63–71 (2019). <https://doi.org/10.1111/are.13868>.
- Naira VR, Das D and Maiti SK, Real time light intensity based carbon dioxide feeding for high cell-density microalgae cultivation and biodiesel production in a bubble column photobioreactor under outdoor natural sunlight. *Bioresour Technol* **284**:43–55 (2019). <https://doi.org/10.1016/j.biortech.2019.03.102>.
- Gorman DS and Levine RP, Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci U S A* **54**:1665–1669 (1965). <https://doi.org/10.1073/pnas.54.6.1665>.
- Kropat J, Hong-Hermesdorf A, Casero D, Ent P, Castruita M, Pellegrini M *et al*, A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. *Plant J* **66**:770–780 (2011). <https://doi.org/10.1111/j.1365-3113.2011.04537.x>.
- Matsuo T, Onai K, Okamoto K, Minagawa J and Ishiura M, Real-time monitoring of chloroplast gene expression by a luciferase reporter: evidence for nuclear regulation of chloroplast circadian period real-time monitoring of chloroplast gene expression by a luciferase reporter: evidence for nuclear regulation O. *Mol Cell Biol* **26**:863–870 (2006). <https://doi.org/10.1128/MCB.26.3.863>.
- Wannathong T, Waterhouse JC, Young REB, Economou CK and Purton S, New tools for chloroplast genetic engineering allow the synthesis of human growth hormone in the green alga *Chlamydomonas reinhardtii*. *Appl Microbiol Biotechnol* **100**:5467–5477 (2016). <https://doi.org/10.1007/s00253-016-7354-6>.
- Taylor GM, Mordaka PM and Heap JT, Start-stop assembly: a functionally scarless DNA assembly system optimized for metabolic engineering. *Nucleic Acids Res* **47**:e17 (2019). <https://doi.org/10.1093/nar/gky1182>.
- Werner R and Mergenhagen D, Mating type determination of *Chlamydomonas reinhardtii* by PCR. *Plant Mol Biol Rep* **16**:295–299 (1998). <https://doi.org/10.1023/A:1007583508766>.
- Doran PM, *Bioprocess Engineering Principles*. 2nd, Oxford: Academic Press, (2013). <https://doi.org/10.1016/C2009-0-22348-8>.
- García-Ochoa F and Gomez E, Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. *Biotechnol Adv* **27**:153–176 (2009). <https://doi.org/10.1016/j.biotechadv.2008.10.006>.
- Baquerisse D, Nouals S, Isambert A, dos Santos PF and Durand G, Modelling of a continuous pilot photobioreactor for microalgae production. *J Biotechnol* **70**:335–342 (1999). [https://doi.org/10.1016/S0168-1656\(99\)00085-1](https://doi.org/10.1016/S0168-1656(99)00085-1).
- Rollbusch P, Becker M, Ludwig M, Bieberle A, Grünewald M, Hampel U *et al*, Experimental investigation of the influence of column scale, gas density and liquid properties on gas holdup in bubble columns. *Int J Multiph Flow* **75**:88–106 (2015). <https://doi.org/10.1016/j.ijmultiphaseflow.2015.05.009>.
- Yunos MASM, Halim NKA, Hussain SA, Yusoff HM and Sipaun S, Investigations of bubble size, gas hold-up, and bubble rise velocity in quadrilateral bubble column using high-speed camera. *Am J Eng Technol Soc* **4**:5–15 (2017).
- Clark DS and Blanch HW, *Biochemical Engineering*, 2nd edn. Boca Raton: CRC Press, (1997). <https://doi.org/10.1201/9780429258732>.
- Kyriakides NK, Kastrinakis EG, Nychas SG and Coulas A, Bubbling from nozzles submerged in water: transitions between bubbling regimes. *Can J Chem Eng* **75**:684–691 (1997). <https://doi.org/10.1002/cjce.5450750405>.
- Liu C, Liang B, Tang S and Min E, Effects of orifice orientation and gas-liquid flow pattern on initial bubble size. *Chin J Chem Eng* **21**:1206–1215 (2013). [https://doi.org/10.1016/S1004-9541\(13\)60630-8](https://doi.org/10.1016/S1004-9541(13)60630-8).
- Mohagheghian S, Still AL, Elbing BR and Ghajar AJ, Study of bubble size, void fraction, and mass transport in a bubble column under high amplitude vibration. *ChemEngineering* **2**:16 (2018). <https://doi.org/10.3390/chemengineering2020016>.
- Wang Z, Guo K, Liu H, Liu C, Geng Y, Lu Z *et al*, Effects of bubble size on the gas-liquid mass transfer of bubble swarms with sauter mean diameters of 0.38–4.88 mm in a co-current upflow bubble column. *J Chem Technol Biotechnol* **95**:2853–2867 (2020). <https://doi.org/10.1002/jctb.6445>.
- Kulkarni AA, Mass transfer in bubble column reactors: effect of bubble size distribution. *Ind Eng Chem Res* **46**:2205–2211 (2007). <https://doi.org/10.1021/ie061015u>.
- Besagni G, di Pasquali A, Gallazzini L, Gottardi E, Colombo LPM and Inzoli F, The effect of aspect ratio in counter-current gas-liquid bubble columns: experimental results and gas holdup correlations. *Int J Multiph Flow* **94**:53–78 (2017). <https://doi.org/10.1016/j.ijmultiphaseflow.2017.04.015>.
- Kantarci N, Borak F and Ulgen KO, Bubble column reactors. *Process Biochem* **40**:2263–2283 (2005). <https://doi.org/10.1016/j.procbio.2004.10.004>.
- Sardeshpande MV, Gupta S and Ranade VV, Electrical resistance tomography for gas holdup in a gas-liquid stirred tank reactor. *Chem Eng Sci* **170**:476–490 (2017). <https://doi.org/10.1016/j.ces.2017.04.025>.
- Jiang X, Yang N and Yang B, Computational fluid dynamics simulation of hydrodynamics in the riser of an external loop airlift reactor. *Particulate* **27**:95–101 (2016). <https://doi.org/10.1016/j.partic.2015.05.011>.
- Singh SK, Sundaram S, Sinha S, Rahman MA and Kapur S, Recent advances in CO₂ uptake and fixation mechanism of cyanobacteria and microalgae. *Crit Rev Environ Sci Technol* **46**:1297–1323 (2016). <https://doi.org/10.1080/10643389.2016.1217911>.

- 36 Krishna R and van Baten JM, Mass transfer in bubble columns. *Catal Today* **79–80**:67–75 (2003). [https://doi.org/10.1016/S0920-5861\(03\)00046-4](https://doi.org/10.1016/S0920-5861(03)00046-4).
- 37 Besagni G, Inzoli F, De Guido G and Pellegrini LA, The dual effect of viscosity on bubble column hydrodynamics. *Chem Eng Sci* **158**:509–538 (2017). <https://doi.org/10.1016/j.ces.2016.11.003>.
- 38 Sánchez Mirón A, Cerón García MC, García Camacho F, Molina Grima E and Chisti Y, Mixing in bubble column and airlift reactors. *Chem Eng Res Des* **82**:1367–1374 (2004). <https://doi.org/10.1205/cerd.82.10.1367.46742>.
- 39 Wang C and Lan CQ, Effects of shear stress on microalgae – a review. *Biotechnol Adv* **36**:986–1002 (2018). <https://doi.org/10.1016/j.biotechadv.2018.03.001>.
- 40 Kück UD, Mießner U, Aydin M and Thöming J, Mixing time and mass transfer of rising bubbles in swarm turbulence. *Chem Eng Sci* **187**:367–376 (2018). <https://doi.org/10.1016/j.ces.2018.04.031>.
- 41 Khoo CG, Lam MK and Lee KT, Pilot-scale semi-continuous cultivation of microalgae *Chlorella vulgaris* in bubble column photobioreactor (BC-PBR): hydrodynamics and gas–liquid mass transfer study. *Algal Res* **15**:65–76 (2016). <https://doi.org/10.1016/j.algal.2016.02.001>.
- 42 Guo Z, Phooi WBA, Lim ZJ and Tong YW, Control of CO₂ input conditions during outdoor culture of *Chlorella vulgaris* in bubble column photobioreactors. *Bioresour Technol* **186**:238–245 (2015). <https://doi.org/10.1016/j.biortech.2015.03.065>.
- 43 Vo H-N-P, Bui X-T, Nguyen T-T, Nguyen DD, Dao T-S, Cao N-D-T *et al.*, Effects of nutrient ratios and carbon dioxide bio-sequestration on biomass growth of *Chlorella* sp. in bubble column photobioreactor. *J Environ Manage* **219**:1–8 (2018). <https://doi.org/10.1016/j.jenvman.2018.04.109>.
- 44 Wang J, Zhou W, Chen H, Zhan J, He C and Wang Q, Ammonium nitrogen tolerant *Chlorella* strain screening and its damaging effects on photosynthesis. *Front Microbiol* **9**:3250 (2019). <https://doi.org/10.3389/fmicb.2018.03250>.
- 45 Cecchin M, Benfatto S, Griggio F, Mori A, Cazzaniga S, Vitulo N *et al.*, Molecular basis of autotrophic vs mixotrophic growth in *Chlorella sorokiniana*. *Sci Rep* **8**:6465 (2018). <https://doi.org/10.1038/s41598-018-24979-8>.
- 46 Wang S-K, Hu Y-R, Wang F, Stiles AR and Liu C-Z, Scale-up cultivation of *Chlorella ellipsoidea* from indoor to outdoor in bubble column bioreactors. *Bioresour Technol* **156**:117–122 (2014). <https://doi.org/10.1016/j.biortech.2014.01.023>.
- 47 Lizzul A, Lekuona-Amundarain A, Purton S and Campos L, Characterization of *Chlorella sorokiniana*, UTEX 1230. *Biology (Basel)* **7**:25 (2018). <https://doi.org/10.3390/biology7020025>.
- 48 Amorim ML, Soares J, dos Coimbra JSR, de Leite MO, Albino LFT and Martins MA, Microalgae proteins: production, separation, isolation, quantification, and application in food and feed. *Crit Rev Food Sci Nutr* **61**:1976–2002 (2021). <https://doi.org/10.1080/10408398.2020.1768046>.
- 49 Vavitsas K, Crozet P, Vinde MH, Davies F, Lemaire SD and Vickers CE, The synthetic biology toolkit for photosynthetic microorganisms [OPEN]. *Plant Physiol* **181**:14–27 (2019). <https://doi.org/10.1104/pp.19.00345>.
- 50 Esland L, Larrea-Alvarez M and Purton S, Selectable markers and reporter genes for engineering the chloroplast of *Chlamydomonas reinhardtii*. *Biology (Basel)* **7**:46 (2018). <https://doi.org/10.3390/biology7040046>.
- 51 Purton S, Tools and techniques for chloroplast transformation of chlamydomonas, in *Transgenic Microalgae as Green Cell Factories*, Vol. **616**. Springer, New York, NY, pp. 34–45 (2007). https://doi.org/10.1007/978-0-387-75532-8_4.
- 52 Zheng Y, Xue C, Chen H, He C and Wang Q, Low-temperature adaptation of the snow alga *Chlamydomonas nivalis* is associated with the photosynthetic system regulatory process. *Front Microbiol* **11**:1233 (2020). <https://doi.org/10.3389/fmicb.2020.01233>.
- 53 Wei L, Fan B, Yi J, Xie T, Liu K and Ma W, Mechanistic insights into PH-dependent H₂ photoproduction in bisulfite-treated chlamydomonas cells. *Biotechnol Biofuels* **13**:64 (2020). <https://doi.org/10.1186/s13068-020-01704-0>.
- 54 Allore G, Lefebvre-Legendre L, Chappuis R, Kuntz M, Truong TB, Niyogi KK *et al.*, UV-B photoreceptor-mediated protection of the photosynthetic machinery in *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci U S A* **113**:14864–14869 (2016). <https://doi.org/10.1073/pnas.1607695114>.
- 55 Tilbrook K, Dubois M, Crocco CD, Yin R, Chappuis R, Allore G *et al.*, UV-B perception and acclimation in *Chlamydomonas reinhardtii*. *Plant Cell* **28**:966–983 (2016). <https://doi.org/10.1105/tpc.15.00287>.
- 56 Xie Y, Lu K, Zhao X, Ma R, Chen J and Ho SH, Manipulating nutritional conditions and salinity-gradient stress for enhanced lutein production in marine microalga *Chlamydomonas* sp. *Biotechnol J* **14**:e1800380 (2019). <https://doi.org/10.1002/biot.201800380>.
- 57 Freudenberg RA, Baier T, Einhaus A, Wobbe L and Kruse O, High cell density cultivation enables efficient and sustainable recombinant polyamine production in the microalga *Chlamydomonas reinhardtii*. *Bioresour Technol* **323**:124542 (2021). <https://doi.org/10.1016/j.biortech.2020.124542>.