**Article**

**Growth of *Haematococcus pluvialis* on a Small-Scale Angled Porous Substrate Photobioreactor for Green Stage Biomass**

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**Abstract:** In the production of astaxanthin from *Haematococcus pluvialis*, the process of growing algal biomass in the vegetative green stage is an indispensable step in both suspended and immobilized cultivations. The green algal biomass is usually cultured in a suspension under a low light intensity. However, for astaxanthin accumulation, the microalgae need to be centrifuged and transferred to a new medium or culture system, a significant difficulty when upscaling astaxanthin production. In this research, a small-scale angled twin-layer porous substrate photobioreactor (TL-PSBR) was used to cultivate green stage biomass of *H. pluvialis*. Under low light intensities of 20–80 µmol photons m$^{-2}$ s$^{-1}$, algae in the biofilm consisted exclusively of non-motile vegetative cells (green palmella cells) after ten days of culturing. The optimal initial biomass density was 6.5 g m$^{-2}$, and the dry biomass productivity at a light intensity of 80 µmol photons m$^{-2}$ s$^{-1}$ was 6.5 g m$^{-2}$ d$^{-1}$. The green stage biomass of *H. pluvialis* created in this small-scale angled TL-PSBR can be easily harvested and directly used as the source of material for the inoculation of a pilot-scale TL-PSBR for the production of astaxanthin.

**Keywords:** astaxanthin; biofilm; Green stage; *Haematococcus pluvialis*; porous substrate photobioreactor

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1. **Introduction**

*Haematococcus pluvialis* is a unicellular green microalga known for its accumulation of astaxanthin to 5% of its dry weight [1,2]. Due to its powerful antioxidant activity, natural astaxanthin from algae is being produced and increasingly applied in the medical and nutraceutical industries [2]. Currently, *H. pluvialis* can be cultivated in two ways to obtain biomass, as well as astaxanthin. The traditional method is cultivation in suspension, often separated into two phases: a first phase comprising the production of green cell biomass and a second, astaxanthin accumulation phase involving red akinetes [3,4]. A disadvantage of the suspension culture is its relatively slow growth and low final cell densities, resulting in high energy requirements for harvesting the biomass [5,6].

The second method for cultivating *H. pluvialis* is immobilized culturing, in which microalgae are fixed to form a biofilm on a layer that serves as a substrate [7–11]. Various types of biofilm-based photobioreactors have been developed, including submerged systems and porous substrate photobioreactors (PSBRs) [12]. In submerged systems, the algal biofilms are submerged in the medium from which they take up nutrients [8,12]. In PSBRs,
the biofilm is separated from the medium by a microporous substrate, and the surface of the biofilm is in contact with the ambient atmosphere [12,13]. In their most popular configuration, the twin-layer PSBR (TL-PSBR), PSBRs have been experimentally tested on a wide variety of algae [12–16], and the dynamics of their biofilms have been characterized and modelled in some detail [16–20]. When applied to *H. pluvialis*, immobilized cultivation is usually a one-phase culture, as the algae increase in biomass and turn red to produce astaxanthin simultaneously with biofilm [15,21].

In Vietnam, angled biofilm-based PSBRs for *H. pluvialis* have been successfully designed and operated [21,22]. This system shows potential for the production of astaxanthin from *H. pluvialis* at the bench- and pilot-scales. To prepare initial green algal biomass, *H. pluvialis* is usually cultured in a suspension with increasing volumes. Algae are then collected after about 20–25 days of cultivation by centrifugation. After centrifugation, the concentrated algal suspension is applied to the substrate layer to grow into the biofilm [2,15,22]. With a usual initial biomass density of 5–7.5 g m$^{-2}$, the volume of algal culture needed to inoculate 1 m$^2$ biofilm is about 15–20 L [15,21,22]. Therefore, the process of growing algae in a suspension to inoculate biofilms in PSBRs is still an issue that needs to be solved when upscaling astaxanthin production to the pilot scale [21].

In *H. pluvialis* cultivation, high light intensities, up to 2000 µmol photons m$^{-2}$·s$^{-1}$ sunlight, have been shown to be the most important single factor for the induction of astaxanthin accumulation [4,23]. In contrast, low light intensities, below 100 µmol photons m$^{-2}$·s$^{-1}$, are often used to maintain and increase the density of microalgae in a suspension in the green phase [24–27]. However, the effects of low-light intensity on the growth and cell divisions of *H. pluvialis* in TL-PSBR biofilms have previously not been studied in detail [15]. Following the above analysis, a small-scale angled PSBR was used to grow green-stage *H. pluvialis* to take advantage of immobilized cultivation to address the problem of inoculation of the PSBR. In this study, we focused on the determination of the light intensity most suitable for the growth and maintenance of the vegetative green stage of the algal cells. Moreover, the initial biomass (inoculum) density of the biofilm was also optimized to achieve the highest dry biomass yield.

2. Materials and Methods

2.1. Culture Maintenance of Algal Strain

The *H. pluvialis* strain CCAC (Culture Collection of Algae at the University of Cologne) 0125 (https://www.uni-du.de/biology/ccac/) was cultured to maintain in 50 mL of a Blue-Green 11 medium with HEPES buffer (BG11-H) [28] in 100 mL flasks with a low-light intensity of 25–30 µmol photons m$^{-2}$·s$^{-1}$ provided by fluorescent lamps (FLs; 18 W cool daylight lamp, Philips Electronics and Lighting, Inc., Hong Kong, China). The culture temperature was controlled at 20–24 °C, and a 14/10 h light/dark cycle was used.

2.2. Experimental Design

The two experiments of this study were carried out sequentially on a small scale (each growth area: 0.1 × 0.5 = 0.05 m$^2$) angled PSBR [21,22].

1. **Effect of low light intensity on pigments and growth of *H. pluvialis* in green stage on PSBR**: With the goal of obtaining algae in the green phase, white light from FLs was used as a light source with intensities of 20–100 µmol photons m$^{-2}$·s$^{-1}$. As a small-angled PSBR has 6 chambers (Figure 1), the distance of each one from the light source was different. Each biofilm area in the chambers was exposed to different low light intensities and was considered as one experiment for one value point of this parameter. In this experiment, an initial algal density of 5.0 g dry weight m$^{-2}$ was applied to the biofilm. The cultivation time on the PSBR was ten days with a 14/10 h light/dark cycle.

2. **The optimal initial algal density of *H. pluvialis* in the green stage on PSBR**: Initial biomass densities of 3.5, 5.0, 6.5, and 8.0 g dry weight m$^{-2}$ were tested. Four chambers were used for each inoculum using the same media source, air pump, and lighting...
system. Algae were immobilized immediately after centrifugation. The white light intensity was set at 80 µmol photons m\(^{-2}\)s\(^{-1}\). The microalgal dry biomass productivity levels in these experiments were compared with a culture in a suspension under identical experimental conditions.

![Figure 1. A chamber of a small-scale angled porous substrate photobioreactor (PSBR) was used in the experiments.](image)

### 2.3. Algae Immobilization on a Small-Scale Angled PSBR

Preparation of microalgal biomass for immobilization: The culture medium was modified BG11-H [28]. The suspended cultivation of H. pluvialis was done in 0.5 and 2 L Erlenmeyer flasks (culture time was about 10 and 14 days, respectively) at 21–25 °C. The green algae were illuminated by FLs at 40 µmol photons m\(^{-2}\)s\(^{-1}\), using a 14/10 h light/dark cycle. Algae (the ratio of cells in the vegetative stage having two flagella was above 80% when examined by light microscopy) were concentrated by centrifugation at 800× g for 5 min. The supernatant was removed.

Determination of dry biomass in the concentrated algal suspension: One milliliter of the concentrated algal suspension was transferred onto filter paper (dried and weighed, \(m_1\)) and dried at 105 °C for 2 h. Filter paper and dried algae were cooled in a desiccator for 30 min and weighed, and the drying process was repeated until the mass was constant, \(m_2\) [28]. The formula calculated the biomass of H. pluvialis in 1 mL of an algal suspension was: \(m_{(g/mL)} = (m_2 - m_1)\); the experiments were performed with three biological replicates to calculate the average dry weight.

The amount of the concentrated algal suspension immobilized onto a surface area was calculated according to the formula: \(V = \frac{M_i}{m}\), where \(V\) (mL) is the volume of the concentrated algal suspension needed to make a 0.05 m\(^2\) biofilm and \(M_i\) is the initial immobilized dry biomass (g·m\(^{-2}\)).

Immobilized cultivation: A nutrient-replete BG11-H medium was applied for ten days by replacing 10 L after 2 or 3 days. The addition of 1% CO\(_2\) (v/v) to the culture media was used as a carbon source and a way to adjust pH (6.5–8). The electrical conductivity of the BG11-H medium was kept in 1800–2000 µS cm\(^{-1}\) by adding distilled water after every day.

### 2.4. Analytical Methods

Measurement of immobilized dry algal biomass: After ten days of immobilized cultivation, the algal and substrate layers were harvested and dried at 105 °C for 2 h [15]. The dried product was cooled in a desiccator for 30 min and weighed. The drying process was repeated until an unchanged total weight of \(m_b\) was obtained. The dry biomass (per m\(^2\) of biofilm) was calculated as \(M_a = (m_a - m_b)/0.05\) (g·m\(^{-2}\)), where \(m_b\) is the
weight of dry substrate layer without algae and 0.05 is the square of a biofilm area in the small-scale PSBR.

Dry algal biomass productivity (g·m\(^{-2}\)·d\(^{-1}\)) was calculated based on equation
\[
M_p = (M_a - M_i)/10
\]
(where \(M_a\) is the dry biomass after ten days and \(M_i\) is the initial immobilized dry biomass).

Measurement of carotenoid/chlorophyll ratio in the dry biomass: The carotenoid/chlorophyll ratio is an essential indicator of the physiological state of \(H.\) pluvialis, whether it is the green stage or the red stage [29]. The ratio of carotenoid to chlorophyll is about 0.2 in the green stage and increases in the red stage by order of magnitude to reach about 2–9 [30,31]. The cells transforming green vegetative motile cells to green vegetative palmella cells have carotenoid/chlorophyll ratios of about 0.2–1.0 [32]. This ratio in the dry biomass was determined based on the method of Strickland et al., (1972). To 0.005 g of dry biomass, 2 mL of acetone 90% were added, and then the mixture was ground with a glass pestle and centrifuged at 800 \(\times\) \(g\) for 5 min, and then the supernatant was collected. We continued adding acetone 90% to the residue, grinding, and repeated the process until the resulting solution was colorless. The volume was made up to precisely 10 mL with 90% acetone, kept in a 15 mL centrifuge tube, and covered tightly to limit the evaporation of acetone. The pigment extract was spectrophotometrically measured at 665, 645, 630, and 480 nm [33].

Pigments were calculated according to formula:
\[
\text{carotenoid/(chlorophyll(a + b))} = 4.0 \times E_{480}/((11.6 \times E_{665} - 1.31 \times E_{645} - 0.14 \times E_{630}) + (20.7 \times E_{645} - 4.34 \times E_{665} - 4.42 \times E_{630})),\]
where \(E_i\) is the extinction value obtained at the respective wavelength \(i\) [33].

Cell morphology determination: Cell samples were collected from the biofilm after ten days of culturing, suspended in an isotonic solution, fixed with 2% formaldehyde, and observed morphologically with a Nikon DS-Fi1 camera microscope (Nikon Corporation, Japan) to analyze different phases of the life history of \(H.\) pluvialis.

2.5. Data Analysis

Statistical analysis and resulting charts were made using the programming language R (version 3.5.2). The presented values are an average of six biological replicates with corresponding standard deviations.

3. Results

3.1. Effect of Low Light Intensity on Pigments and Growth of \(H.\) pluvialis in the Green Stage on a TL-PSBR

Algae in the biofilm were cultured at low light intensities from 20–100 \(\mu\)mol photons m\(^{-2}\)·s\(^{-1}\) to stimulate the growth of \(H.\) pluvialis and were harvested after ten days to determine dry biomass productivity and pigment ratios.

The results of dry biomass determination show that the higher the intensity of light, the higher the dry biomass productivity (Figure 2). The lowest dry biomass productivity was 2.6 g·m\(^{-2}\)·day\(^{-1}\) when illuminated with a light intensity of 20 \(\mu\)mol photons m\(^{-2}\)·s\(^{-1}\). At the light intensities of 80 and 100 \(\mu\)mol photons m\(^{-2}\)·s\(^{-1}\), the dry biomass productivities were 5.9 and 6.4 g·m\(^{-2}\)·day\(^{-1}\), respectively, but the difference was not statistically significant (\(p > 0.05, n = 6\)). However, the dry biomass productivities at these two light intensities were significantly higher than the dry biomass productivities obtained when culturing algae at all lower light intensities (\(p < 0.05, n = 6\)).
Figure 2. Final biomass and biomass growth rate (average of biomass increase per day over ten days) of *Haematococcus pluvialis* under different light intensities (in the range of 20–100 µmol photons m\(^{-2} \cdot \text{s}^{-1}\)).

The ratio of carotenoid/chlorophyll and cell morphology was used to determine the cell stage after harvest. The results clearly showed that the carotenoid/chlorophyll ratio increased gradually with an increment of the light intensity in the range of 20–100 µmol photons m\(^{-2} \cdot \text{s}^{-1}\). Still, it never exceeded the value 1.0 in any case (Figure 3). The difference was statistically significant for this ratio at light intensities from 20 to 80 µmol photons m\(^{-2} \cdot \text{s}^{-1}\) \((p < 0.05, n = 6)\).

In all manipulations and after ten days, cells had no flagella and represented the palmella cell stage (Figure 4). Light intensities of 20, 40, 60, and 80 µmol photons m\(^{-2} \cdot \text{s}^{-1}\) resulted in green cells in all cases. However, at the highest applied light intensity (100 µmol photons m\(^{-2} \cdot \text{s}^{-1}\)), some palmella cells were noticed to accumulate some astaxanthin (Figure 4e). This result was consistent with those of the carotenoid/chlorophyll ratio and observing biofilm’s surface after ten days of culturing.
Consequently, the light intensity of about 80 μmol photons m\(^{-2}\cdot s\(^{-1}\) was found to be the most suitable for the growth of *H. pluvialis* and still maintained cells at the green vegetative stage in biofilm.

### 3.2. Influence of Inoculum on Growth and Pigments of *H. pluvialis* in the Green Stage on a TL-PSBR

The final dry biomass obtained after ten days depended on the amount of microalgae initially applied to the biofilm (Figure 5). The highest final dry biomass values were 71.4 and 71.9 g·m\(^{-2}\) when the initial biomass densities were 6.5 and 8.0 g·m\(^{-2}\), respectively, and the difference was not statistically significant (*p* > 0.05, *n* = 6). With the initial biomass densities of 3.5 and 5.0 g·m\(^{-2}\), the final dry biomass values were significantly lower (*p* < 0.05, *n* = 6) than at the two higher initial densities.

![Figure 4. Cell morphology of *H. pluvialis* under different values of low light intensities of 20 (a), 40 (b), 60 (c), 80 (d), and 100 (e) μmol photons m\(^{-2}\cdot s\(^{-1}\) (red arrow indicates a red palmella cell with accumulated astaxanthin).](image)

![Figure 5. Final biomass and biomass growth rate (average of biomass increase per day over ten days) of *H. pluvialis* at different initial biomass densities after ten days of growth in a PSBR with a light intensity of 80 μmol photons m\(^{-2}\cdot s\(^{-1}\).](image)

The highest biomass growth rate (average of biomass increase per day over ten days) was 6.5 g·m\(^{-2}\cdot d\(^{-1}\) with an initial microalgae density of 6.5 g·m\(^{-2}\), equivalent to the initial
density of 8.0 g·m⁻² (6.4 g·m⁻²·d⁻¹) ($p > 0.05$, $n = 6$) and higher than the biomass growth rates at the lower two initial cell densities ($p < 0.05$, $n = 6$) (Figure 5).

With the same low light intensity (80 µmol photons m⁻²·s⁻¹), the ratio of carotenoid/chlorophyll in the microalgae showed no significant difference at the initial algae densities of 3.5, 5.0, 6.5, and 8.0 g·m⁻² ($p > 0.05$, $n = 6$). These ratios were all less than 1 (Figure 6), indicating that the cells were all in the green palmella cell phase, consistent with the results of the light microscopic observations (Figure 7).

Figure 6. The ratio of carotenoids to chlorophylls in H. pluvialis at different initial biomass densities after 10 days of growth in a PSBR with a low light intensity.

Figure 7. Cell morphology of H. pluvialis at different initial biomass densities—3.5 (a), 5.0 (b), 6.5 (c), and 8.0 (d) g·m⁻²—after 10 days of growth in a twin-layer porous substrate photobioreactor (TL-PSBR) with a light intensity of 80 µmol photons m⁻²·s⁻¹.

4. Discussion

In suspension cultures of H. pluvialis, a low light intensity from white FLs was often used for growing algae in the green phase. However, the green biomass productivity in the suspension cultures was still low after extended cultivation times and decreased with increasing culture volume due to the shading effect (Table 1).
Table 1. Comparison of *H. pluvialis* green phase cultivation results in an angled biofilm-based photobioreactor system (this study) with other cultivation systems based on the surface area.

| System                      | Strain Number | Medium     | CO₂ (%) | Light Condition (µmol Photons m⁻²·s⁻¹) | Cultivation Period (Green Phase) (Days) | Dried Biomass Productivity (g·m⁻²·Day⁻¹) | References |
|-----------------------------|---------------|------------|---------|----------------------------------------|----------------------------------------|------------------------------------------|------------|
| Indoor open pond            | 26            | BG11       | For controlling pH                      | 20–350 14/10 h                         | 12                                      | 2.2 a                              [34]     |
| Flask (250 mL) SAG 34/1d    |               |            |         |                                        |                                        |                                          |            |
| Flask (3 L) CCMA-451        |               | BBM        | 1.5     | 20–30 Continuous                       | 8                                      | 2.88 a                [25]     |
| Immobilized biofilm (0.08 m²) NIES-144 NIES-N |       | None       | 150     | Continuous                             | 10                                     | 3.7                                  | [2]        |
| Immobilized biofilm (0.08 m²) SAG 34-1b |       | BG11       | 1.5     | 20–100 Continuous                      | 7                                      | 2.3–6.6                            [2]        |
| Immobilized biofilm (0.05 m²) CCAC 0125 Modified BG11 |       |            | 1       | 90 (high pressure Na lamp 14/10 h)     | 8                                      | 5.4                                  | [28]       |
| Angled immobilized biofilm (0.05 m²) CCAC 0125 Modified BG11 |       |            |         | For controlling pH                      | 80 (FL) 14/10 h                        | 6.49 This study                      |            |

* The values are converted to ‘per surface area’.

With a multi-stage life history, *H. pluvialis* cells still have a phase shift when cultured over long periods under a low light intensity because of limiting nutrients (primarily nitrogen and phosphorus) [3]. After inoculation from resting stages, algal vegetative cells have two flagella and are thus motile. After a few weeks (depending on the strain used and the environmental conditions), cells shed flagella and transform into immotile green cells (so-called palmella stages). These cells can accumulate astaxanthin upon the introduction of stimuli (such as high light intensity or nutrient limitation), transforming into the red phase (the so-called akinetes). During the red phase transition, astaxanthin accumulation increases more than 10-fold compared to young palmella stages [29]. Recent studies have shown that motile cells (with two flagella) can also accumulate astaxanthin when stressed by high light intensity, thus facilitating subsequent astaxanthin extraction [24,25]. However, astaxanthin’s content accumulated in the motile cell has been found to be significantly lower than in the palmella, non-motile form. Furthermore, motile cells were found to be sensitive to high light intensity, resulting in photobleaching [35]. In a small TL-PSBR system, motile cells shred flagella and immobilize in biofilm, but non-motile cells are more resistant to stresses and more able to accumulate astaxanthin.

At the lab scale, a small-scale vertical TL-PSBR was used to monitor the influence of low light intensities on biofilm growth: the biomass increased linearly throughout 31 days at light intensities from 20 to 90 µmol photons m⁻²·s⁻¹ from a high-pressure sodium lamp [28]. The growth rate reached 5.4 g·m⁻²·d⁻¹ with a full BG 11-H medium, and aeration was supplemented with 5% CO₂ [28]. In this study, when a small-scale angled PSBR was used, the growth rate was about 6.5 g·m⁻²·d⁻¹ at a white light intensity of 80 µmol photons m⁻²·s⁻¹ without CO₂ to the gas phase. The addition of CO₂ to the gas phase in a PSBR does not affect microalgae growth at low light intensities [16,28,36].
However, the addition of CO$_2$ to the BG11-H medium to ensure a stable environmental pH is necessary. An FL led to a significantly higher growth efficiency than a high-pressure sodium lamp. With the same light intensity, the effective light spectrum from the FL for photosynthesis has been found to reach 60%, which is more efficient than light from the high-pressure sodium lamp at 38% [37]. The growth of *H. pluvialis* has been demonstrated to be much faster with the use of light from light-emitting diodes (LEDs), the effective spectrum of which can reach over 90% [38,39]. However, the FL was still shown to be the optimal light source for the green stage. With the same low light intensity (8 µmol photons m$^{-2}$s$^{-1}$), the use of FLs resulted in more proliferation than red or blue LEDs over a 12-day culture period. The carotenoid ratio was significantly lower [38]. However, the combination of red and blue LEDs showed great promise for simultaneous proliferation with astaxanthin accumulation. Red and blue LEDs are currently being used on pilot-scale TL-PSBRs, resulting in high dry biomass and astaxanthin productivity (unpublished data).

Beside dry biomass productivity, the criteria for creating algal biomass in the green phase were the primary purpose of this study. After inoculation on a TL-PSBR, almost all of the cells shed their flagella and rapidly transformed into the green palmella stage [40]. Light from a high-pressure sodium lamp causes algal cells in the biofilm’s top layers to change into the red phase even with light intensities below 90 µmol photons m$^{-2}$s$^{-1}$. This stage enhanced the acclimation of *H. pluvialis* to the environment [15,40]. In a suspension culture, the red phase transition is minimized because the cell changes its position in the suspension so that the light intensity it receives is constantly fluctuating [6]. For an immobilized culture, cells on the surface of biofilm will receive light with a steady intensity. They are easily stimulated to accumulate astaxanthin and transform into the red phase [2,15]. White light from FLs also causes phase transformations when the light intensity is 100 µmol photons m$^{-2}$s$^{-1}$. This study showed that a white light intensity of 80 µmol photons m$^{-2}$s$^{-1}$ from FLs ensured the growth of microalgae while maintaining the cells in the green vegetative stage.

The initial algal stock is usually determined by cell density or dry biomass per unit volume [34,41]. However, the initial amount of algae in an immobilized culture in a PSBR is generally calculated by the weight of dry biomass per unit area [2,15,26,27,42]. Therefore, it is essential to optimize the initial algal density when using a completely different culture system. In an immobilized culture, a lower initial biomass density minimizes the centrifugation step to concentrate the algal suspension, but the dry biomass productivity is usually lower [2,26,27]. The results of this study showed that the initial algal biomass of 6.5 g·m$^{-2}$ yielded the highest dry biomass per day in the range of 5–10 g·m$^{-2}$ commonly applied in previous studies in similar culture systems [2,15,28]. A comparison of algal productivities between immobilized cultivation in TL-PSBRs and suspended cultivation is not appropriate because of their fundamental differences. However, the convenience and effectiveness of astaxanthin production in TL-PSBRs have been previously demonstrated [2,15,21,22]. This study’s results offer one solution to the problem of input algal biomass for astaxanthin production in large-scale PSBRs. Currently, the green palmella biomass obtained from the small-scale TL-PSBRs is used in pilot-scale angled PSBRs to accumulate and accumulate astaxanthin (unpublished data).

5. Conclusions

A low light intensity of 80 µmol photons m$^{-2}$s$^{-1}$ is suitable for *H. pluvialis* proliferation but retains cells in the form of green palmella cells when cultured in a small-scale angled TL-PSBR. An initial biomass density of 6.5 g·m$^{-2}$ is optimal for microalgal growth over a 10-day culture period. The cultivation of green phase *H. pluvialis* in a TL-PSBR under these conditions guarantees a reliable source of initial biomass for application in larger-scale TL-PSBR systems, providing an alternative to the suspended cultivation of the initial biomass.

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