



Research Article

SUSPENSION CULTURE AND FLOCCULATION OF *CHLORELLA VULGARIS* FOR IMMOBILISED CULTIVATION IN ANGLED BIOFILM PHOTOBIOREACTORS

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ABSTRACT

Cultivation technology of *Chlorella vulgaris* shows great potential in wastewater treatment along with biomass collection for biofuel production. Suspended, autotrophic cultivation at various scales is still the main method to increase algal biomass in different stages of algae technology. In this study, *C. vulgaris* was cultured to proliferate in a 2 l suspension system to prepare biomass for immobilised cultivation in a biofilm photobioreactor. The initial cell density and light intensity suitable for suspension culture were determined to be 5.10^5 cells/ml and $120 \mu\text{mol photons/m}^2/\text{s}$. After 20 days of culture, the density of dry algal biomass in the 2 l system reached 0.315 g/l. To concentrate the algal suspension for immobilisation into biofilm, alum ($\text{KAl}(\text{SO}_4)_2$) was used at concentrations from 0.2 to 2 g/l to form flocculation. The concentration of alum 0.2 g/l was most efficient (95% of the algae in the suspension was flocculated), and the obtained algae had a strong proliferative ability when being immobilised in the angled biofilm photobioreactor.

Keywords: alum; biofilm; *Chlorella vulgaris*; flocculation; $\text{KAl}(\text{SO}_4)_2$

1. Introduction

Chlorella vulgaris is one of the microalgae species with great potential in wastewater treatment because of the ability to remove heavy metals, organic, and inorganic substances. In addition, the biomass of *C. vulgaris* has the potential to become a new source of biomaterials in the future (Nguyen et al., 2019; Shi et al., 2007). It contains many times more lipids than crops, and the lipid composition has many characteristics suitable for biodiesel production (Suparmaniam et al., 2020).

At a large scale, *C. vulgaris* is usually cultured in suspension in open circular or ditch ponds, using natural light from the sun, or in closed photobioreactors (PBRs) with natural or artificial light source (Liu & Hu, 2013). Algal cultivation in open ponds, with lower

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construction and operating costs, often achieves low yields of less than 1g of dry biomass (DB)/l because the culture is easily contaminated with other microorganisms, and it is difficult to control environmental conditions (Liu & Hu, 2013). The closed PBRs have many advantages over the open systems in controlling the culture environment, preventing infection, and having higher productivity. However, high cost and the difficulty of sterilising complex closed PBRs are the main obstacles (Liu & Hu, 2013). After suspension culture, the density of algal biomass is very low, so the harvesting of biomass needs to be done by different methods such as high-speed centrifugation, chemical flocculation (with chitosan or pH or alum), or membrane filtration. Recently, vertical or angled biofilm PBRs have been successfully applied to culture many species of microalgae including *C. vulgaris* (Do et al., 2019; Naumann et al., 2013; Shi et al., 2007). Immobilised microalgae in biofilms have been used to treat wastewater simultaneously with the harvesting of algal biomass easily because the algal biomass is isolated from the media (Cheng et al., 2017; Shi et al., 2007).

However, initial biomass (inoculum) for biofilm immobilisation needs to be supplied from concentrated suspension culture. The inoculum preparation was usually carried out by high-speed centrifugation (Kiperstok et al., 2017; Tran et al., 2019). Meanwhile, *C. vulgaris* cells are very small in size (only from 2 to 10 μm), so centrifugation is difficult, consuming a lot of time and energy (Demir et al., 2020). In addition, the centrifugation process also increases the risk of microbial contamination into the inoculum and the loss of biomass and reduces the viability of the algal cells. The method of concentrating *C. vulgaris* cells in suspension by flocculation shows great potential compared to other methods (Chua et al., 2020; Matter et al., 2019). However, at present, the methods of flocculating *C. vulgaris* are mainly used to collect biomass for products, not for further culture and proliferation. Furthermore, each chemical used in flocculation has its advantages and disadvantages in terms of cost as well as efficiency of algae biomass recovery (Suparmaniam et al., 2020; Zhu et al., 2018). In particular, the use of alum ($\text{KAl}(\text{SO}_4)_2$) is more efficient in creating algae flocculation, low cost, and simple usage (Mohseni & Zenooz, 2021).

In this study, at a laboratory scale, a strain of *C. vulgaris* was grown with suitable light intensity and initial cell density in 2 l culture systems with white light emitting diodes (LEDs). This algal biomass was the inoculum for culture in angled biofilm PBRs. The flocculation efficiency of different concentrations of alum was also investigated in this study to concentrate the algal suspension for immobilising into angled biofilm PBRs.

2. Materials and methods

2.1. Microalgae strain and breeding

A strain of *C. vulgaris* was provided by Research Institute for Aquaculture No.2, Vietnam. The algae are maintained in 25 ml flasks containing 10 ml of SFM medium with HEPES (1.0 mM), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.21 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 mM), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (13.2 μM), Na_2CO_3 (0.19 mM), NaNO_3 (0.5 mM), vitamins, and trace elements (Ekelhof,

2016). The culture room temperature is 25°C, the light intensity is as low as 30 $\mu\text{mol photons/m}^2/\text{s}$ from the fluorescent lamp and the light/dark cycle is 12/12 h (Shi et al., 2007). The algal strain was cultured to increase biomass in preparation for further experiments under sterile conditions.

2.2. Experiments on the growth of *C. vulgaris* in 2 l culture systems with white LEDs

In these experiments, the algal strain was inoculated into 2 l culture systems containing autoclaved SFM medium to obtain a final culture volume of 1200 ml. The room temperature is about 28 ± 2 °C, and the light/dark cycle is 24/0 h (Yusof et al., 2021). The light source is from a white LED system (HC220V, Vietnam) with the spectrum shown in Figure 1, the maximum light intensity is 140 $\mu\text{mol photons/m}^2/\text{s}$. The energy efficiency of using white LEDs in *Chlorella* culture has been demonstrated because LEDs have high energy conversion efficiency and low heat generation (Yuan et al., 2020). The algal suspension was aerated with clean air supplemented with 0.5% CO₂ continuously, the aeration rate was 5 ml/min (Figure 2). The growth of *C. vulgaris* was monitored over a 20-day culture period.

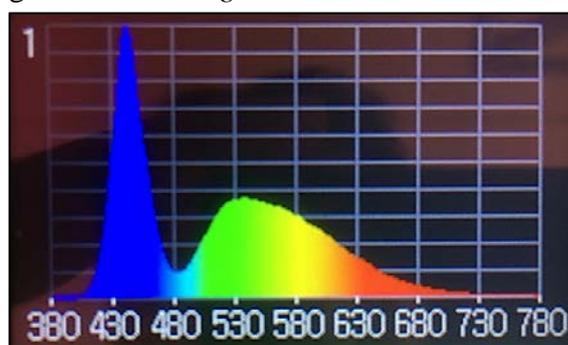


Figure 1. Light spectrum from white LED measured with UPRtek PG100N (Taiwan)

a. Experimental design

- *Experiment to choose the optimal light intensity for the growth of *C. vulgaris* in the 2 l culture system:* In photoautotrophic cultivation, light is the decisive factor affecting the growth of algae. Green algae usually require the light intensity of 115-161 $\mu\text{mol photons/m}^2/\text{s}$. Photosynthesis is inhibited when light intensity exceeds 230 $\mu\text{mol photons/m}^2/\text{s}$ (Wong, 2016). Therefore, this experiment was performed to investigate the effect of light intensities on the growth of *C. vulgaris* cultured 2 l suspension system. Four different illumination intensities were investigated including 80, 100, 120, and 140 $\mu\text{mol photons/m}^2/\text{s}$. White LEDs were installed above the culture systems of the treatments (Figure 2). Different light intensity for each treatment was ensured by increasing or decreasing the distance from the light to the location of the culture system. Photosynthetic photon flux density was determined using a PG100N Handheld Spectral PAR meter (Taiwan). The initial algal density was 2×10^5 cells/ml, which was similar between treatments. The experiment was repeated three times.



Figure 2. The layout of white LEDs above the 2 l suspension culture system

- *Experiment to select the optimal initial density of *C. vulgaris* in the 2 l culture system:* Based on the results by Tran *et al.*, (2017), this experiment was performed with initial densities of 2×10^5 cells/ml, 5×10^5 cells/ml, 10×10^5 cells/ml và 20×10^5 cells/ml. The initial suspension was density determined with a Neubauer chamber and inoculated into 2 l culture systems to achieve the desired density. The manipulations were performed in a sterile cabinet, each treatment was repeated three times. Then, the culture systems were illuminated with an intensity of $120 \mu\text{mol photons/m}^2/\text{s}$, based on the experimental results of choosing the optimal light intensity.

b. Method for determining algal growth in the 2 l culture system: Microalgae samples (12 ml of suspension) were collected to determine the density of dry biomass (g/l) on days 5, 10, 15, and 20 of cultivation. Algal suspensions were measured for optical density (OD) at 682 nm (Nguyen *et al.*, 2019). Each treatment was sampled and measured OD₆₈₂ three times.

The DB of microalgae was calculated from the OD₆₈₂ value based on the pre-established correlation equation between DB and OD₆₈₂: $y = 0.2048x - 0.0015$, where y is DB density (g/l), x is the OD₆₈₂ value (Figure 3). This equation was established by taking samples of suspensions of different concentrations, measuring OD₆₈₂ along with determining the density of DB in the sample. The dry biomass was determined by preparing filter paper, drying at 105°C for 60 min. The filter paper was then weighed and the mass (m_1) recorded. The algal suspension (50 ml) was centrifuged at 8,000 rpm for 5 min. The supernatant was discarded and distilled water was added and further centrifuged to wash away the salts in the residue. The washing procedure was repeated three times. All fresh algal biomass in the centrifuge tube was transferred to the filter paper and dried at 105°C for 120 min. Paper and biomass were dried and weighed until a constant weight (m_2) was obtained. The DB density (g/l) of the algal suspension was calculated by the formula: $M_{\text{DB}} = (m_2 - m_1) \times 20$.

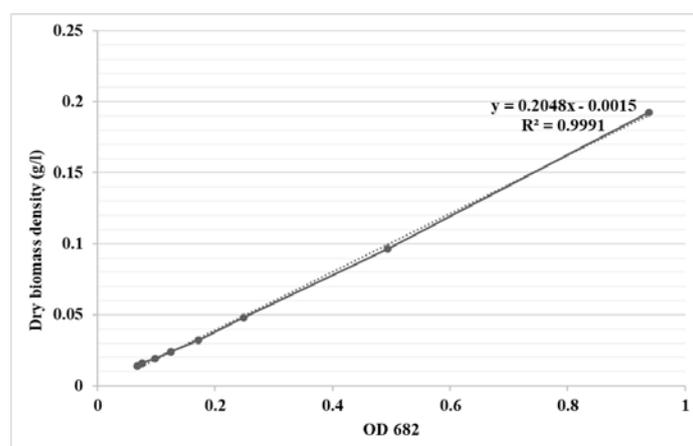


Figure 3. Correlation between OD682 value and DB density of *C. vulgaris* in suspension

2.3. Experiment for choosing the concentration of $KAl(SO_4)_2$ to create flocculation and concentrate the algae biomass in suspension

Our initial tests showed that the minimum concentration of alum to create flocculation of this *C. vulgaris* strain was 0.2 g/l. In addition, based on previous studies (Mohseni & Moosavi Zenooz, 2021), the concentrations of alum investigated in this experiment were 0.2, 0.3, 0.4, 0.5, 1.0, and 2.0 g/l of algal suspension.

This experiment was performed by preparing 250 ml flasks containing 100 ml of *C. vulgaris* suspensions (taken from a 2 l culture system). Algal cell density in all flasks was taken equally, the initial OD682 value was determined to be 0.776. Then, alum was added in definite amounts to give the desired concentrations. The mixtures were transferred into 1,000 ml separatory funnels and allowed to settle for 30 min. Algae-containing flocculation at the bottom of the funnel was collected with a volume of 10 ml. The remaining 90 ml supernatant was mixed and measured for OD682 to determine the number of residual algae. This is the main criterion to choose the optimal alum concentration in the flocculation experiment, the lower the OD682 value, the smaller the number of microalgae remaining in the supernatant, and the higher the flocculation efficiency. Efficiency is calculated by the formula: $H=100\% \times (DB_b - DB_a)/DB_b$, where DB_b is dry biomass (g/l) in the initial suspension and DB_a is dry biomass in the supernatant after flocculating, converted from the OD682 values based on the equation in Figure 3.

2.4. Effect of biomass concentration method on the growth of *C. vulgaris* in angled biofilm PBR

In this experiment, algal concentrates obtained from two different methods, algae collected by centrifugation (control) and alum flocculation, were immobilised into two chambers of a small angled biofilm PBR (Tran et al., 2019) to compare proliferation ability. The methods used in this experiment include:

a. Centrifugal method to obtain concentrated algae suspension: The *C. vulgaris* suspension cultured in a 2 l system was concentrated by centrifugation for 5 min at 4,000

rpm with a large centrifuge ROTANTA 460 RC (Hettich, Germany). The supernatant was discarded, and the algal concentrate below the centrifuge tube was collected (Shi et al., 2007). The procedure was repeated until the required amount of algae was collected for the experiment.

b. Flocculation method to obtain concentrated algae suspension: The *C. vulgaris* suspension was flocculated with $KAl(SO_4)_2$ with a concentration of 0.2 g/l. After 30 minutes in the separatory funnel, the condensed algal suspension at the bottom of the funnel was collected.

c. Immobilisation of microalgae into the biofilm PBR: The components of the biofilm PBR and the devices for immobilising the algae into biofilm were cleaned with Javel solution or autoclaved before use. Source layers (non-woven glass fiber) prepared 0.1×0.5 m in size were immersed in water and spread onto the inner surface of the chamber (Tran et al., 2019). The nonwoven polypropylene fabric used as the substrate layer was soaked and autoclaved before being spread over the source layer. The culture medium was pumped to wet the source and substrate layers before immobilising the microalgae into the biofilm. The concentrated algae suspension was painted with a sterile soft brush onto the substrate to create biofilms.

The concentrated algal suspension was sampled to determine the amount of biomass per ml in preparation for immobilisation into biofilms with an initial density of 5 g DB/m². A filter paper was dried at 105 °C for 60 min and weighed. Then, 0.1 ml of the algae concentrate was transferred to the filter paper, dried, and weighed until the mass was constant. Sampling was repeated three times to calculate the mean. The average amount of DB in each ml of algae concentrate (m_a , g/ml) is calculated by the formula: $m_a = (m_{a2} - m_{a1}) \times 10$, where m_{a2} (g) is the average mass of algae and filter paper, and m_{a1} (g) is the average mass of the filter paper.

Concentrated suspensions of *C. vulgaris*, obtained by centrifugation or flocculation, were immobilised into circular biofilms of 1 cm radius (with an area of 3.14 cm²) to facilitate sampling to monitor the growth of microalgae at different times in the culture process (Figure 4) (Shi et al., 2007). The amount of suspension (V , ml) required for a circular biofilm is determined by the formula: $V = (3.14 \times 5) / (10,000 \times m_a)$.



Figure 4. Microalgae *C. vulgaris* after immobilisation in biofilm PBR

d. Cultivation of *C. vulgaris* in biofilm PBR: In this experiment, to determine the growth ability of algae after being concentrated by different methods, the time of culture in biofilm was shortened to five days. Nutrients were provided for microalgae by 5 l of culture medium. The medium was supplemented with CO₂ to ensure pH 6.5-7.5. The culture room temperature is 28±2 °C, the light/dark cycle is 12/12 h (Shi et al., 2007), the light intensity is about 80 μmol photons/m²/s from the white LEDs.

e. Method for growth determination of *C. vulgaris* cultured in biofilm PBR: After five days of cultivation, the substrate layer and the algal biofilm were collected, dried at 105°C, and weighed until the weight remained constant. Dry biomass (B, g) is calculated by the formula: $B = W_t - W_s$, where W_t is the total dry mass of the substrate layer and algae, and W_s is the dry weight of the substrate layer.

2.5. Data analysis

The data from the experiments were collected and entered into Microsoft excel 365 for preliminary processing. Statistical and graphical analyses were performed using R version 3.4.2. Tukey's HSD analysis was used to determine the difference between treatments.

3. Results and discussion

3.1. Results of experiments on the growth of *C. vulgaris* in 2 l culture system with white LEDs

a. Results of choosing the optimal light intensity for the growth of *C. vulgaris*

The illumination intensities included 80, 100, 120, and 140 μmol photons/m²/s from the white LEDs. Each treatment consisted of three 2 l culture systems (3 replicates). The algal suspension was sampled to determine morphology, total dry biomass at 5, 10, 15, and 20 days after starting cultivation. In general, the DB density of microalgae in the suspension

increased gradually over the culture period (Figure 5). In which, the DB increased rapidly from the starting to day 15. From day 15 to 20, the growth rate of algae slowed down. On day 10, the DB density achieved was similar in all treatments.

After 20 days of culture, the DB density reached the highest 0.298 ± 0.018 g/l at a light intensity of $120 \mu\text{mol photons/m}^2/\text{s}$, the difference was statistically significant compared with the other treatments ($p < 0.05$, $n=6$). At lower light intensities (80 and $100 \mu\text{mol photons/m}^2/\text{s}$), the photosynthetic efficiency is lower, resulting in lower DB densities. However, with higher light intensities ($140 \mu\text{mol photons/m}^2/\text{s}$), photosynthesis can be inhibited, resulting in reduced DB yield. The results of this study are consistent with previous studies, in which, *C. vulgaris* was mainly cultured in suspension under low light intensity (60 - $100 \mu\text{mol photons/m}^2/\text{s}$) (J. Liu & Hu, 2013; Yuan et al., 2020).

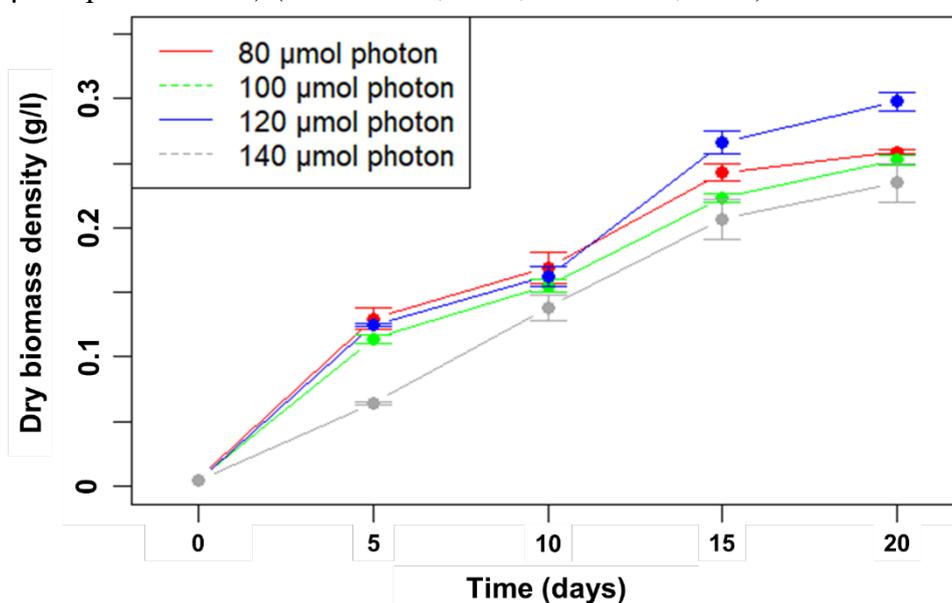


Figure 5. DB density of microalgae at different light intensities in 2 l culture systems

Therefore, in order to prepare the algae biomass that is growing strongly for culture in angled biofilm PBRs, the light intensity of about $120 \mu\text{mol photons/m}^2/\text{s}$ was applied for the next experiments.

b. Results of choosing the optimal initial density of *C. vulgaris* in the 2 l culture system

In all treatments, the DB density of microalgae increased gradually over time from the start of culture to day 20 (Figure 6). In the early days, when the cell density was low, growth occurred rapidly. However, when the cell density increased higher, the growth rate tended to slow down from day 15 to day 20. In suspension culture, when the density of algal cells increases, the mutual shading between the cells reduces the efficiency of photosynthesis, a process required for the synthesis of organics for the growth of algal cells (Liu & Hu, 2013).

The experimental results showed that the highest DB density after 20 days of culture was 0.315 ± 0.005 g/l in the treatment of 5×10^5 cells/ml. The difference was statistically

significant compared with the other treatments ($p < 0.05$, $n = 6$). At the initial densities of 2×10^5 , 10×10^5 and 20×10^5 cells/ml, the DB was similar, 0.272 – 0.282 g/l ($p > 0.05$, $n = 6$).

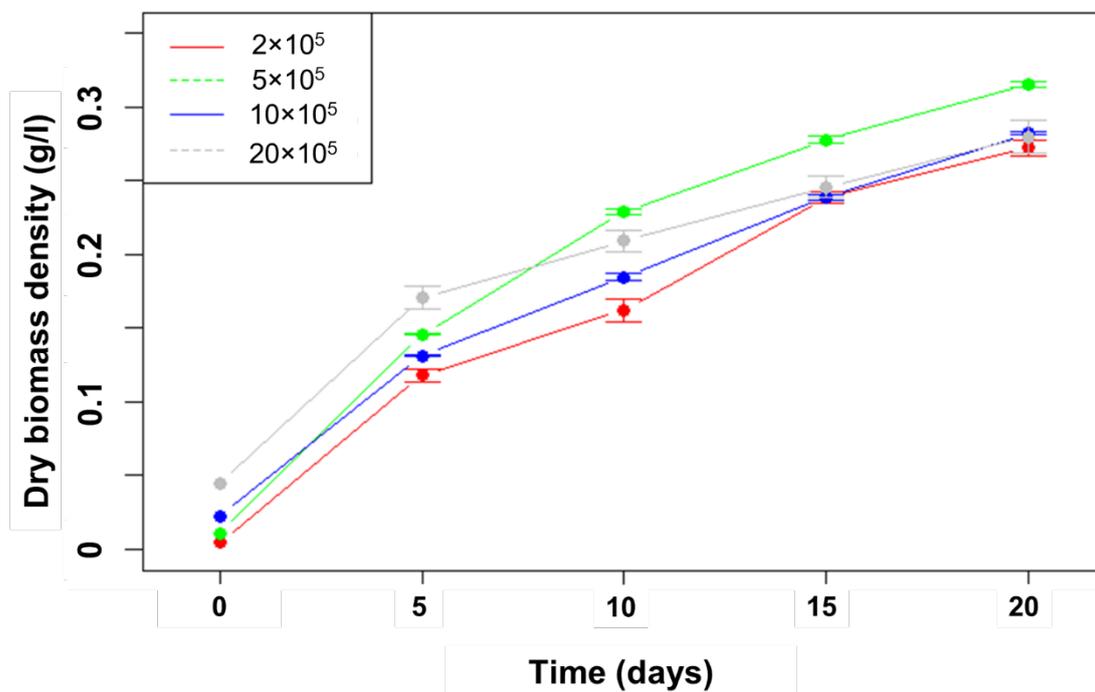


Figure 6. The DB densities of *C. vulgaris* of four initial cell density treatments in 2 l culture systems

Compared with previous studies, the DB obtained in this study is still low (J. Liu & Hu, 2013; Yeh et al., 2010). However, due to the purpose of collecting algal cells that are in a state of strong proliferation for immobilization in biofilm, the culture time was shortened to 20 days and the algae biomass did not reach the maximum. In addition, the photoautotrophic culture of *C. vulgaris* also shows that the productivity is often lower than that of the photoheterotrophic or heterotrophic cultures (Tran et al., 2017). Therefore, the appropriate initial cell density for suspension culture in the 2 l system is 5×10^5 cells/ml (equivalent DB density is 0.0112 g/l).

3.2. Results of choosing the concentration of $KAl(SO_4)_2$ to concentrate the algal biomass in suspension

After adding alum with different concentrations to the suspension, the algal cells began to flocculate with each other and settle to the bottom of the flask (Figure 7). However, at high concentrations of alum (1 and 2 g/l), microalgal cells appeared to adhere to the surface of the erlen and funnel walls, making it difficult to collect biomass. This resulted in a loss in the amount of algal biomass obtained.

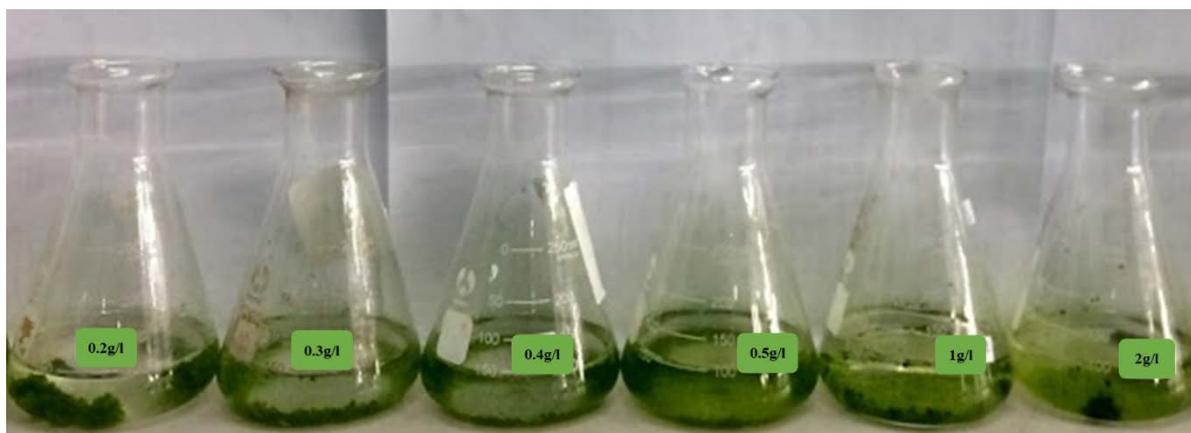


Figure 7. Algae flocculation formation after adding alum at different concentrations

To determine the optimal alum concentration, the OD682 value of 90 ml supernatant was determined and shown in Figure 8. The results show that the OD682 value tends to increase gradually with increasing alum concentration. In which, the lowest OD682 value is 0.045 ± 0.003 and 0.050 ± 0.002 , corresponding to the concentration of alum used of 0.2 and 0.3 g/l, respectively ($p > 0.05$, $n = 3$). However, the difference is statistically significant when compared with the OD682 value of the remaining concentrations ($p < 0.05$, $n = 3$). The efficiency of flocculation to collect algal biomass reached 95% at alum concentration of 0.2 g/l.

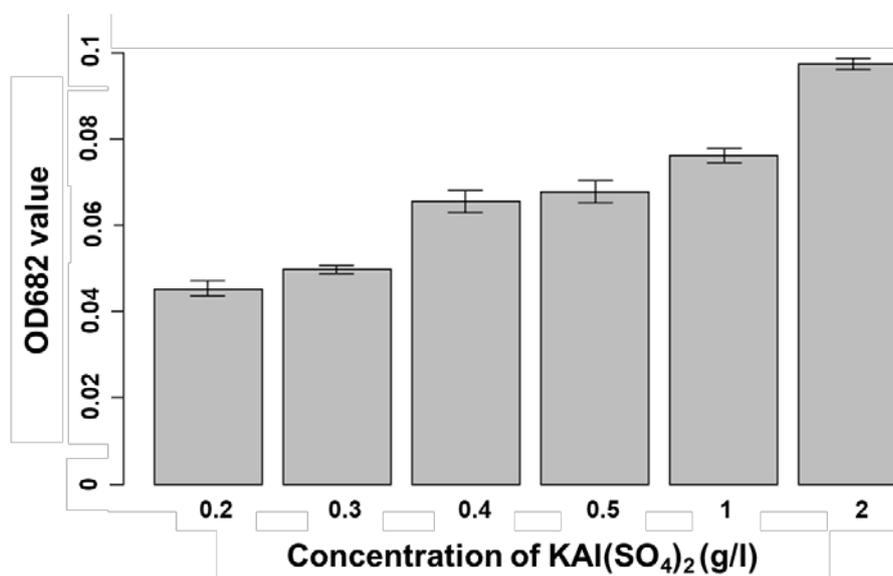


Figure 8. OD682 values of supernatants after flocculating algae with alum at different concentrations

This experimental result is consistent with previous studies, a high concentration of flocculants can reduce the efficiency of algae biomass collection (Mohseni & Moosavi Zenooz, 2021). Furthermore, the algal biomass obtained by flocculation is mainly used for production and not for further cultivation (Zhu et al., 2018). In this study, the concentrated

algal suspension was then cultured to investigate the proliferation ability in the angled biofilm PBRs. Therefore, the lower the concentration of alum used, the less effect on cell viability and reproduction. As a result, a concentration of 0.2 g/l alum was used to concentrate the algal suspension for the next experiment.

3.3. The effect biomass concentration method on the growth of *C. vulgaris* in angled biofilm PBR

After five days of immobilised culture in the angled biofilm PBR, *C. vulgaris* collected by flocculation had better growth than algae collected by centrifugation, biofilm thicknesses were significantly different (Figure 9).

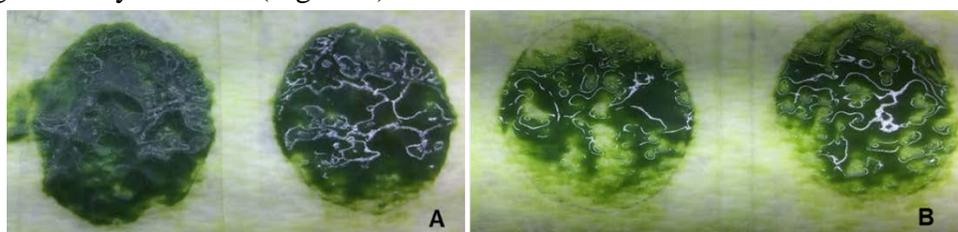


Figure 9. *C. vulgaris* biofilm surfaces after 5 days of cultivation in the angled biofilm PBR: Algae were collected by flocculation with $KAl(SO_4)_2$ (A) and centrifugation (B)

The total DB of microalgae obtained after five days of culture also showed a statistically significant difference between the two sources of algae collected by the methods ($p < 0.05$, $n = 3$). In which, the highest DB was 31.9 ± 5.1 g/m² for flocculated algae (Figure 10).

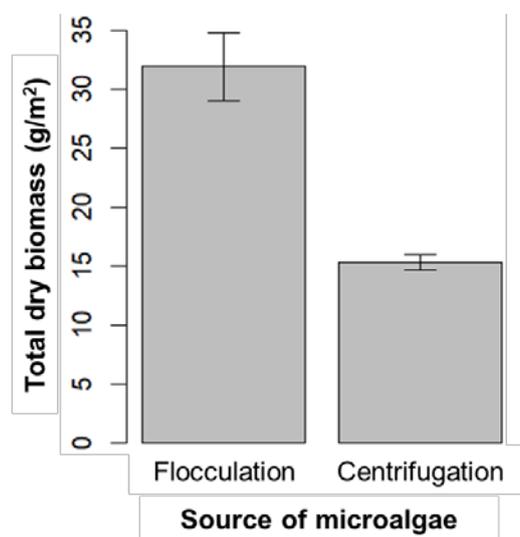


Figure 10. Total DB of *C. vulgaris* obtained after 5 days of culture in biofilm with algae sources collected by 2 different methods

The centrifugation method was used in previous studies to concentrate the algal suspension prepared for immobilisation in biofilm and the algae were still able to proliferate (T. Liu et al., 2013; Melo et al., 2018). In this study, microalgae collected by

centrifugation were still able to grow in biofilms. Compared with similar culture systems, the DB of *C. vulgaris* from the flocculation source is significantly higher than that of Shi et al. (2007) with a source of algae collected by centrifugation. Thus, the high-speed centrifugation can affect the cells, reducing the proliferation ability. Meanwhile, the flocculation of algal suspension with a low concentration of $KAl(SO_4)_2$ minimises labor, energy, and the impact on physiology and proliferation of algal cells.

Based on the results presented above, the angled biofilm PBRs show potential in the cultivation of *C. vulgaris* for biomass production. This opens up other potential applications when growing *C. vulgaris* in biofilm such as wastewater treatment (Shi et al., 2007, 2014). In particular, algal biomass is located separately from the culture medium. This helps achieve the dual goal of both treating wastewater and obtaining biomass for other purposes such as biofuel production. This is one of the outstanding advantages of the biofilm PBRs (Naumann et al., 2013; Shi et al., 2014). However, the optimization of *C. vulgaris* cultivation needs to be performed in further studies before evaluating the cost-effectiveness of angled biofilm PBRs in comparison with other algae culture technologies.

4. Conclusions

The suitable white LED light intensity for the growth of *C. vulgaris* in 2 l suspension culture systems is about $120 \mu\text{mol photons/m}^2/\text{s}$. The initial cell density for suspension culture in the 2 l system is 5×10^5 cells/ml (equivalent to an initial DB density of 0.0112 g/l). Algal biomass that was concentrated by flocculation with alum 0.2 g/l showed high proliferation capacity in angled biofilm PBR.

❖ **Conflict of Interest:** Authors have no conflict of interest to declare.

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NUÔI CÂY HUYỀN PHÙ VÀ TẠO KEO TỤ LẮNG TẢO *CHLORELLA VULGARIS* CHO NUÔI CÂY CỐ ĐỊNH TRONG BIOFILM PHOTOBIOREACTOR PHƯƠNG NGHIÊNG

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TÓM TẮT

Công nghệ nuôi vi tảo *Chlorella vulgaris* cho thấy có nhiều tiềm năng trong việc xử lý nước thải song song với thu sinh khối để sản xuất nhiên liệu sinh học. Việc nuôi tảo kiểu huyền phù, tự dưỡng ở nhiều quy mô khác nhau vẫn là phương thức chủ yếu để tăng sinh khối trong các giai đoạn của công nghệ nuôi tảo. Trong nghiên cứu này, tảo *C. vulgaris* được nuôi tăng sinh trong hệ thống huyền phù 2 l để chuẩn bị sinh khối cho nuôi tảo kiểu cố định trong hệ thống biofilm photobioreactor phương nghiêng. Mật độ tế bào ban đầu và cường độ ánh sáng thích hợp cho nuôi huyền phù được xác định là 5.10^5 tế bào/ml và $120 \mu\text{mol photon/m}^2/\text{s}$. Sau 20 ngày nuôi, mật độ sinh khối khô vi tảo trong hệ thống 2 l đạt 0,315 g/l. Để cô đặc huyền phù tảo chuẩn bị sinh khối cho cố định thành biofilm, phèn chua ($\text{KAl}(\text{SO}_4)_2$) được sử dụng ở các nồng độ từ 0,2 đến 2 g/l để tạo keo tụ lắng. Nồng độ phèn chua 0,2 g/l cho thấy hiệu quả thu tảo đến 95% và tảo thu được có khả năng tăng sinh mạnh khi được chuyển nuôi cố định trong biofilm photobioreactor phương nghiêng.

Từ khóa: phèn chua; biofilm; *Chlorella vulgaris*; keo tụ lắng; $\text{KAl}(\text{SO}_4)_2$