Cultivation of *Chlorella vulgaris* in Sequential Flow Photobioreactor System: Influence of Recycled Culture Medium on Growth, Lipid and Protein Content

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**Abstract.** In the present work, the reusability of culture medium to grow *Chlorella vulgaris* was examined in an effort to reduce the freshwater footprint of microalgae cultivation. The microalagae was cultivated in a sequential-flow system equipped with five photobioreactors connected in series. Furthermore, the effect of recycled culture medium without additional nutrients on the growth, lipid and protein content of microalgae was evaluated under optimized cultivation conditions. Experimental results showed that biomass concentration and growth rate reduced when the recycled culture medium was used. The maximum biomass concentration was achieved in the fresh medium with a total biomass yield of 1.42 g/L. Meanwhile, lipid content was found to increase when the microalgae was cultivated in the recycled medium, in which the highest lipid content (58.01 wt.%) was attained from photobioreactor 5 (cycle 2). On the contrary, the protein content (11.98 wt.%) of the microalgae in the recycled medium was considerably lower than the fresh medium (49.20 wt.%). These results suggested that the reuse of culture medium to grow microalgae is possible, however, it has a significant impact on the biochemical compositions of *Chlorella vulgaris*. Therefore, it is important to determine the desired product prior to reusing the medium for subsequent cultivation process.

1. **Background**

Microalgae biomass is a promising source of alternative energy as it composed of valuable compounds including carbohydrate (12–17%), lipid (14–22%), and protein (51–58%), which can be used for production of value-added products (e.g. biofuels, nutritional supplements, and health [1]. Besides, it is also able to produce a wide range of biofuels including hydrocarbons, methane, syngas, kerosene, diesel, and gasoline [2]. Microalgae lipid has always known to be one of the sustainable sources of biodiesel production. In fact, microalgae-derived biodiesel has the potential to replace non-renewable
transport fuels without negatively influencing the supply of food and other crop products [3]. Besides, the lipid extracted residual biomass contains a high amount of carbohydrates and protein that can be converted into useful biomaterials such as bioethanol [4], animal feed [5], and biogas [6], which can greatly improve the economics of green biorefinery.

However, one of the major technical limitations of microalgal biorefinery is the excessive use of freshwater for microalgae cultivation, thus reduces the sustainability of the system due to an increase in production cost [7]. Therefore, recycling spent culture medium was considered as a viable option in order to reduce the huge water demand required for microalgae biomass production [8]. Moreover, the spent medium still contains valuable nutrients to support the growth of microalgae biomass [7]. Besides, the presence of extracellular organic matters excreted by microalgae in the recycled medium could act as an essential nutrient for subsequent cultivation system [9]. Nevertheless, nutrient stress can alter the biochemical composition of microalgal biomass cultivated in recycled medium [10]. Although it is a well-known fact that the imposition of external stress (e.g. nutrient starvation, pH and salinity) may induce lipid accumulation in microalgae, yet their involvement on the synthesis of other organic metabolites (e.g. protein) are still unclear [9, 11, 12]. In view of this, *Chlorella vulgaris* considered as one of the promising candidate owing to their robustness, high growth rate and tolerance to high CO₂ concentration [13].

Therefore, in the present study, freshwater microalgae *Chlorella vulgaris* was grown in a sequential flow photobioreactor system with recycled culture medium in order to investigate the effect of nutrient limitation on growth behaviour and biochemical composition (e.g. lipid and protein) of microalgae cell.

2. Material and methods

2.1. *Chlorella vulgaris* strain and culture medium

The *Chlorella vulgaris* strain was obtained from the Centre for Biofuel and Biochemical Research, Universiti Teknologi PETRONAS and the stock culture was preserved in Bold’s Basal Medium (BBM) at 25 °C– 28°C with an initial pH value of 6.8 [14]. The seed culture was continuously illuminated with cool-white fluorescent light (Philip TL-D 36 W/965) with a light intensity ranging from 60 to 70 μmol m⁻² s⁻¹ [15].

2.2. Microalgae cultivation in a sequential flow system: Effect of the fresh and recycled medium

In this study, an organic fertilizer (TANI) was prepared according to the method described by Tan et al. [15] as the nutrient source. The chemical analysis of TANI organic fertilizer show that it is composed of various nutrients including nitrogen (5.9 %w/w), phosphorus (1.2 %w/w), potassium (11 %w/w), calcium (15.1 %w/w), magnesium (2.9 %w/w), chloride (12.9 %w/w), boron (0.3 %w/w), iron (0.3 %w/w), zinc (218.3 mg/kg), copper (76.8 mg/kg), and manganese (368.6 mg/kg) to support the microalgae growth. *Chlorella vulgaris* was cultivated in sequential-flow photobioreactor system (SF-PBR) that were made up of five Duran bottles connected in series with a working volume of 5 L each (335 mm in height, and 182 mm in diameter) for continuous flow of air from PBR1 to PBR5 (Figure 1). The PBRs was labelled consecutively as PBR1, PBR2, PBR3, PBR4 and PBR5. The cultivation medium (FM) was supplied with 8 %v/v of TANI organic fertilizer nutrients and 8 %v/v of seed culture with an initial cell density of 1.26 g/L at pH value 3-3.5 with a 9 L/min air flow rate. The culture medium pH value was adjusted back to 3-3.5 by using 1 M of sulphuric acid (H₂SO₄). The photobioreactors were continuously illuminated with cool-white fluorescent light (Philip TL-D 36W/865, the light intensity of 60–70 μmol m⁻² s⁻¹) at a temperature of 25 ± 5 °C. The microalgae biomass was harvested via gravitational sedimentation method after 14 days of cultivation period.

The spent medium was collected after the microalgae harvesting process for subsequent cultivation. The spent medium was re-used up to three times to cultivate microalgae in order to study the effect of nutrient deficiency on microalgae growth and biochemical composition. The recycled mediums are labelled as R1, R2, R3 attributed to the number of cycles.
2.3. Lipid extraction
Harvested microalgae biomass was oven-dried at 105 °C for 24 hours. The dried biomass (0.2 g) was then ground to a powder prior to the extraction process. Microalgae lipid extraction was conducted in a Soxhlet extractor using a 2:1:0.25 v/v mixture of methanol, chloroform and water, respectively at 75 °C for 8 h. The resulting crude lipid upon solvent recover was oven-dried for 24 h to remove the moisture content. The total lipid content was determined gravimetrically.

2.4. Analysis procedure
2.4.1. Microalgae growth measurements. The Chlorella vulgaris growth behaviour in fresh and spent mediums was measured based on the optical density of the culture at 688 nm using UV-Vis Spectrophotometer (UV-2600 Shimadzu). The relationship between optical density and the biomass concentration \( N \), g/L of Chlorella vulgaris was established by linear regression as per Eq. (1) below:

\[
N = 0.532(OD_{688}) + 0.0333, R^2 = 0.972
\]

Meanwhile, the biomass productivity \( P_{\text{max}}, \text{g/L/day} \) and specific growth rate \( \mu, \text{day}^{-1} \) were calculated according to the Eq. (2) and (3) below, respectively:

\[
\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1)
\]

\[
P_{\text{max}} = (N_2 - N_1) / (t_2 - t_1)
\]

where \( N_1 \) and \( N_2 \) are the microalgae biomass concentration (g/L) at the beginning \( t_1 \) and end \( t_2 \) of the logarithmic growth phase (day).

2.4.2. Quantification of lipid and protein. The lipid content \( Y, \% \) and productivity \( P_{\text{lipid}}, \text{g/L/day} \) of microalgae was determined based on Eq. (4) and (5) below, respectively:

\[
Y(\%) = \frac{W_L}{W_{DA}} \times 100 \%
\]

\[
P_{\text{lipid}}(gL^{-1}\text{day}^{-1}) = \frac{Y(\%) \times N_f}{\text{cultivation time (day)}}
\]

where \( W_L \), \( W_{DA} \) and \( N_f \) are the weights of the extracted lipids (g), the initial weight of dry microalgae biomass (g) and final biomass concentration (g/L), respectively. The protein content in Chlorella vulgaris was estimated based on Eq. (6) below [16]:

\[
\text{Protein Content (\%)} = N(\%) \times 6.25
\]
where $N$ (%) is the total nitrogen content determined using CHNS analyser (Perkin-Elmer Model 2400).

3. Results and discussion

3.1. Microalgae growth in the fresh and recycled medium

*Chlorella vulgaris* growth in fresh (FM) and recycled (R1, R2, and R3) culture medium was examined and presented in Figure 2a and b. As shown in Figure 2a, biomass concentration was largely affected by the medium recycling operation where the total biomass generated in the first cycle (R1) was reduced by 37.79% as against the fresh medium (1.42 g/L). The subsequent cultivations using recycled medium R2 and R3 had further demonstrated a significant growth reduction with total biomass production of 0.68 g/L and 0.43 g/L, respectively. Similar trend of reduction in biomass productivity (Figure 2b) was observed for microalgae grown in recycled medium, where the highest productivity of 0.83 to 0.86 g/L/day was attained by FM. Furthermore, it was observed that the *Chlorella vulgaris* in R1 and R2 medium reached the stationary phase 6 days earlier than the FM, whereas the biomass concentration in R3 started to decline after day 10 of the cultivation period, indicating growth inhibition. The decreasing trend of growth in R1 to R3 was mainly due to the nutrient deficiency in the culture medium [8]. Moreover, accumulation of organic metabolites (e.g. free fatty acids and polysaccharides) excreted by microalgae during the cultivation process could be inhibitory for cell growth [17]. Besides, the presence of these organic matters may affect the rheological properties of culture medium, thus influences the efficient nutrient uptake and gas exchange between microalgae and medium [18, 19]. At the same time, some of these extracellular products released during cultivation (e.g. protein, nucleic acids and peptides) may also promote the microalgae growth [7, 9, 17]. This might be the reason for *Chlorella vulgaris* to re-grow in repeated recycled water without any additional nutrients.

![Figure 2. Chlorella vulgaris growth (a) and biomass productivity (b) in fresh and recycled culture medium.](image)

3.2. Effect of culture medium recycling on lipid and protein composition of *Chlorella vulgaris*

The lipid contents and lipid productivity of *Chlorella vulgaris* in fresh and recycled mediums are shown in Table 1. Interestingly, the average lipid content was observed to increase from FM to R1 and R2 with the highest lipid content of 58.01 wt.% recorded for SF-R2-PBR5. Although the lipid content of biomass in recycled medium (R1 and R2) is considerably high, however, it displayed a negative correlation with the lipid productivities due to low biomass productivities (Figure 1) in the recycled medium. It has been reported that microalgae cultivation with nutrient stress factor tends to accumulate more lipid [20]. This is because under nutrient-limited environment (e.g. nitrogen) microalgae cell growth halt due to reduction in synthesis of protein for cell division. Hence, the cell uses an alternative metabolic pathway to fix inorganic carbon sources in order to accumulate energy.
storage components (e.g. lipid and starch) to survive in these adverse environmental conditions [21, 22]. On the other hand, at the 3rd cycle (R3) the lipid content of microalgae reduced by 24.46 % as compared to the one obtained in R2. The similar trend of lipid accumulation was observed by Farooq et al. However, the exact mechanism behind the reduction of lipid in the repeated recycling water was not clearly explained so far [9].

### Table 1. Lipid content and productivity of microalgae biomass cultivated using fresh (FM) and recycled (R1, R2, and R3) culture medium

| Photobioreactors | FM    | R1    | R2    | R3    |
|------------------|-------|-------|-------|-------|
|                  | LC    | LP    | LC    | LP    | LC    | LP    | LC    | LP    |
| SF-PBR1          | 47.75 | 0.041 | 50.40 | 0.024 | 38.78 | 0.014 | 39.17 | 0.009 |
| SF-PBR2          | 42.11 | 0.035 | 43.85 | 0.022 | 42.06 | 0.014 | 33.56 | 0.008 |
| SF-PBR3          | 40.02 | 0.034 | 42.12 | 0.020 | 50.72 | 0.016 | 35.46 | 0.005 |
| SF-PBR4          | 40.15 | 0.034 | 41.10 | 0.018 | 42.03 | 0.013 | 26.61 | 0.003 |
| SF-PBR5          | 39.65 | 0.033 | 41.50 | 0.016 | 50.72 | 0.016 | 35.46 | 0.005 |
| **Average**      | **41.91** | **43.77** | **46.32** | **34.99** |

*LC- lipid content (% of DCW); LP- lipid productivity (g/L/day)

Meanwhile, the opposite trend was perceived for protein accumulation in *Chlorella vulgaris*. Figure 3 demonstrates the protein accumulation trend for microalgae cultivated in the fresh and recycled medium. The obtained results indicate that the protein content decreased under the nutrient-limited condition of recycled medium. The highest protein content (41.38 wt.%) was observed for microalgae cultivated in the first PBR of fresh medium, and the values started to decrease as it progresses from PBR1 to PBR5. Although a similar trend was observed for recycled medium (R1 to R3), however, the average protein content for R2 and R3 is significantly lower than those of FM and R1. Since nitrogen is the major component of amino acids, which is essential for the synthesis of protein molecules, thus nitrogen-limited environment could severely affect the protein content in microalgae cell [16]. Moreover, the reduction in protein content of biomass from PBR1 to PBR5 may be attributed to the depletion in carbon source (e.g. carbon dioxide) [23].

![Figure 3](image-url) **Figure 3.** Protein accumulation in *Chlorella vulgaris* cell cultivated using fresh (FM) and recycle (R1, R2 and R3) culture medium.

### 4. Conclusion

The present work evaluates the feasibility of growing *Chlorella vulgaris* in nutrient limited recycled culture medium and their effect on the biochemical composition of microalgae biomass. Obtained results demonstrate that nutrient deficiency had greatly affected microalgae growth as well as protein content in the recycled medium. On the other hand, lipid content was elevated under nutrient stress
environment. Hence, this study suggested that recycled culture medium can be a viable option to re-grow the microalgae, however, additional nutrients are required in order to support the growth of microalgae. Therefore, future works should be focused on evaluating biochemical response of microalgae in nutrient replenished recycled medium. Furthermore, it is important to determine the targeted product prior to the application of the recycled medium. The re-use of culture medium can reduce cost as well as the water footprint of the cultivation process, thus improves the economic feasibly of microalgae biofinery.

5. References
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