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Research Article

Optimization of *Chaetoceros calcitrans* (Paulsen) Takano 1968 Biomass Production via Fed-Batch Culture Mode

Kumari Geetha Muniandy,[1] **Murni Karim**,[2,3] **M. Shariff**,[1] **Fatimah Md. Yusoff**,[2] **A. Christianus**,[2] **and Keng Chin Lim**[2]

[1]*Laboratory of Aquatic Animal Health and Therapeutics, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[2]*Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[3]*International Institute of Aquaculture and Aquatic Sciences, Universiti Putra Malaysia, 70150, Port Dickson, Negeri Sembilan, Malaysia*

Correspondence should be addressed to Murni Karim; murnimarlina@upm.edu.my

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This study investigated the effectiveness of adopting a fed-batch culture mode with intermittent feeding of culture media to enhance the biomass production of *Chaetoceros calcitrans*. Three experimental runs were undertaken, each comprising four cycles. The nutritional composition of the *C. calcitrans* biomass produced was evaluated. The experiment yielded a consistent production of *C. calcitrans* dry biomass, with outputs of 257.01, 251.88, and 252.41 g obtained in a cultivation period of 28 days per cycle. The initial inoculum concentrations for each run were high, measuring 4.2×10^7, 4.2×10^7, and 4.4×10^7 cells mL^{-1}, respectively. A consistent increase in cell density and dry biomass was observed across all cycles. The cell density at the harvesting point was between 7.3×10^7 and 1.10×10^8 cells mL^{-1}. The nutritional analysis of the *C. calcitrans* biomass revealed that protein comprised the largest proportion of the total biomass at $41.8 \pm 0.7\%$, followed by carbohydrates at $27.2 \pm 0.1\%$ and lipids at $11.3 \pm 0.6\%$. The biomass contained a total of 17 amino acids, comprising 43.2% essential amino acids and 56.7% non-essential amino acids, with the highest concentration observed for glutamic acid ($17.6 \pm 0.1\%$). The fatty acid composition of *C. calcitrans* was recorded with predominant polyunsaturated fatty acids (PUFAs) ($44.6 \pm 0.2\%$). The results suggest a possible cost-saving mechanism, as the culture mode adopted yielded higher microalgal biomass production while retaining a good range of nutritional profiles of *C. calcitrans* biomass.

1. Introduction

The demand for microalgal biomass in the bioeconomy as a source of blue biotechnology is increasing, with diverse applications. The application of microalgae biomass in the pharmaceutical, veterinary, nutraceutical, biomedical, bioenergetics, food supplements, and public health sectors is likewise growing in line with the growth of research and advanced technologies. Furthermore, microalgal biomass is regarded as the best source of feedstock for ensuring food security while minimizing adverse environmental effects [1]. Thus, the measurable economic value of microalgae has been generated in several sectors and applications, including pharmaceuticals, cosmetics, food, and feed [2]. In particular, Taiwan, Japan, the United States, China, Spain, Brazil, and Germany are among the leading producers of microalgae biomass and derivatives [3], with an annual biomass production of approximately 19,000 tonnes [4]. China is estimated to produce about 9,600 tonnes of microalgae biomass in the form of dried powders annually, with a retail value exceeding USD 650 million [5]. In Japan, the microalgal biofuel market is valued at approximately 128 billion yen (1,170 million USD) [6]. This gives the food and pharmaceutical industries a competitive edge [3] with numerous applications, such as *Spirulina* and *Chlorella*'s protein supplements, pigment from *Dunaliella* sp. and *Haematococcus pluvialis*, food colourings made from *Schizochytrium* sp., and EPA and DHA from *Crypthecodinium cohnii* [7]. This resulted in the economic value of microalgae being estimated to be USD 3.4 billion by 2020 and is expected to increase to USD 4.6 billion by 2027 [8].

To date, *Spirulina* sp. and *Chlorella* sp. are two of the most commercialized microalgae species on a global scale [9]. This is due to the fact that commercial-scale production of diatoms remains limited to the production of live feed for shrimp and clam mariculture [10], hindering the exploitation of their potential biomass benefits for various applications. Bacillariophyceae microalgae (diatoms), such as *C. calcitrans*, contain a significant metabolic diversity and bioactive substances that have been utilized as dietary supplements, medicinal ingredients for human ailments, and to promote animal survival in mariculture [11, 12]. *Chaetoceros calcitrans* is widely used as a live feed in marine mollusc crustacean, shrimp, and fish larvae [13, 14] and is considered one of the predominant microalgae species in aquaculture [15]. In fact, *C. calcitrans* is a high-value diatom species and is considered the best choice for larval stage feeding due to its balanced contents of PUFAs and vitamins [16, 17]. A recent study by Azizan et al. [12] also reported 54 metabolites belonging to the classes of carotenoids, chlorophylls, fatty acids, glycerolipids, glycerolphospholipids, sterol lipids (C20 isoprenoids), diterpenes, and N-acyl amino acids, as well as their derivatives, including benzoic acid esters, fatty acid esters, fatty amides, and fatty aldehydes, were discovered from the crude extract of *C. calcitrans*.

Several challenges impede large-scale microalgal biomass production of diatoms, for instance, *C. calcitrans*, including low cell density, reduced light penetration in dense cultures, self-shading, and the small size of microalgal cells [1]. The reduction of microalgal cell size with culture age is also considered one of the limiting factors. Optimizing the *Chaetoceros calcitrans* by employing several culture methods and enhancing growth conditions such as temperature [14], composition of the nutrient medium [18], and light penetration [19] have been intensively investigated to optimize the production of *C. calcitrans*. Prior studies also indicated other approaches to optimize productivity, such as higher temperatures and stronger light intensities for rapid growth and high productivity of *C. calcitrans* [14]. Besides, it was found that the growth rates of *C. calcitrans* varied when glass and plastic jars were used, with glass jars allowing for higher light penetration [20]. This is supported by Lai et al. [21], which reported that the production of *C. calcitrans* in plastic bags was 10 times lower compared to glass annular photobioreactors. Furthermore, the initial cell density of the inoculum is also strongly influenced, which is crucial to maximizing yield. It has been reported that the growth profile of *C. calcitrans* may vary depending on the initial inoculum cell density, resulting in variable durations of the lag and log phases [22]. Besides enhancing the gas–liquid mass transfer of CO_2, it is expected to improve the productivity of microalgae culture in photobioreactors, regardless of the adopted culture system [23]. However, the optimization of cultivation conditions, particularly culture modes, which are crucial for increasing biomass production, failed to entice enough attention. The optimization of culture mode is deemed the most straightforward and efficient way to achieve high biomass, which improves the productivity of microalgae cultivation [24]. Culture modes such as batch, fed-batch, continuous, or semi-continuous, which determine the nutrient availability throughout the cultivation process, heavily impact the productivity of large-scale microalgae cultures. Fed-batch culture mode is an approach to sustaining productivity with high cell densities throughout the culture period [25]. In the fed-batch cultivation mode, the cultured biomass is harvested either fully or partially, and the remaining culture serves as the inoculum for the next cycle. Besides, the culture volume increases during operation until the culture reaches the predetermined volume or the bioreactor capacity with constant or increasing cell densities. The fed-batch process involves the feeding of concentrated nutrient media, which allows for the retention of biomass [25]. The fed-batch culture mode for microalgal cultivation might benefit in achieving high productivity in terms of biomass and biocomponent content, such as lipids and triglycerides. In addition to these benefits, this approach offers advantages such as reduced substrate and end-product inhibition, as well as increased lipid production [26, 27].

The batch culture mode is widely used when cultivating *C. calcitrans* [28]. Besides, there are studies on the cultivation of *C. calcitrans* by continuous culture mode, which reported lasted for 125 days with an average of 42 days without contamination and no significant differences in the proximate composition of biomass [28]. The authors also reported the continuous culture using a 40-L bag with a culture volume ranging from 16 to 29 L, producing cells between 7 and 13×10^6 cells mL^{-1} d^{-1}. Another study recorded a growth rate of 1.32×10^6 cells mL^{-1} day^{-1} for *C. calcitrans* in a flat-panel photobioreactor through 30 days of continuous culture [29]. In comparison, the semi-continuous culture of *C. calcitrans* conducted in a 17-L airlift photobioreactor resulted in a growth rate of 9.45×10^6 cells mL^{-1} day^{-1} [30]. It has been observed that in the semi-continuous culture mode of *C. calcitrans*, despite increases in total lipid of biomass recorded, a lower growth rate was observed [18]. There were also minor variations in *C. calcitrans*'s fatty acid content between batch and continuous cultures [28]. Furthermore, with increasing cell density, the amount of nitrogen per cell of *C. calcitrans* grown in batch cultures decreased, suggesting a possible lower protein content in batch cultures; however, even with the fluctuating growth rate of *C. calcitrans* in the semi-continuous culture method, the amount of carbohydrates in the biomass was rather consistent [18]. Conversely, in a comparison of *C. calcitrans* cultures produced indoors and outdoors, the outdoor cultures grew faster, reaching a biomass of 2.33×10^6 cells mL^{-1} on day 27 for the indoor culture and a maximum of 2.53×10^6 cells mL^{-1} on day 25 for the outdoor culture [31]. This prompted the impetus to seek a possible solution to optimize the biomass production of *C. calcitrans* with a suitable large-scale cultivation method. Besides, no investigation has been performed on the effectiveness of *C. calcitrans* biomass production in fed-batch mode with intermittent feeding of culture medium. The fed-batch culture mode might benefit in achieving high cell density and productivity in terms of biomass and biocomponent content such as lipids and triglycerides. Therefore, this study is aimed to determine the feasibility of adopting a fed-batch culture system with intermittent feeding of culture medium to

optimize the biomass productivity of *C. calcitrans* while investigating the nutritional quality of the produced biomass, which can be adopted on an industrial scale as a novel culture model.

2. Materials and Methods

2.1. Microalgae Culture. The experiment was conducted at the Aquatic Animal Health Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The *C. calcitrans* (UPMC-A0010) was obtained from the microalgae culture collection of the Aquatic Animal Health and Therapeutics Laboratory, Institute of Bioscience, Universiti Putra Malaysia. To avoid any form of contamination, a proactive action was executed by sterilizing all containers used throughout the production process. The borosilicate glassware was submerged in Decon washing detergent (without phosphate) for 24 hr. It was then rinsed with tap water that had been filtered and subsequently immersed in a solution of hydrochloric acid with a concentration of 10% for a period of 15 min. After being submerged in hydrochloric acid, the glassware was cleansed with tap water, subsequently rinsed with deionized water, and finally dried in an oven at a temperature of 180°C for 30 min. The glassware and other laboratory consumables, including pipette tips and cotton plugs, underwent sterilization by autoclaving at a temperature of 121°C for a period of 15 min. The larger culture containers, such as a 10 L initial column and a 120 L annular photobioreactor (Series F and M-TI 50/40, Germany), were sterilized after each run to avoid cross-contamination by immersing them in a 10 mg L^{-1} solution of sodium hypochlorite for 24 hr. Subsequently, the containers were rinsed with filtered tap water. The Conway medium used for all experimental cultures was sterilized by autoclaving at a temperature of 121°C for a period of 15 min and then cooled to room temperature before use.

Experimental designs comprised two phases: a first small-scale culture of 50 mL to 10 L to provide the initial inoculum for the second phase of large-scale culture. This starter inoculum was conducted in batch mode, as described by Lai et al. [21]. The *C. calcitrans* cells were transferred with a sterile glass pipette into a 50-mL conical flask containing 20 mL of medium and closed with a cotton plug. Then, the 20 mL of culture, where the growth was indicated by a brownish red colouration (early stationary phase) during the 5th day, was subsequently scaled up to 100 mL, 250 mL, 500 mL, and 1.5 L of cultures. After that, 3 L culture was used to initiate the culture in the starter column (10 L) with 7 L culture medium and cultivated to the early stationary phase. The second phase of large-scale culture was designed to deploy fed-batch culture mode, with intermittent feedings of culture medium in the annular photobioreactor (Series F and M-TI 50/40, Germany), as improvised from large-scale batch culture conducted by Lai et al. [21]. In order to maximize production and overcome potential limitations on the growth of diatoms, a stable cultural environment was maintained throughout both stages of the experimental culture. The cultures were consistently aerated to ensure adequate mixing and to maintain the microalgae in suspension. An ideal growing environment was achieved by maintaining the temperature between 21 and 25°C [32]. To address the decrease in cell growth rate caused by limited light in the fed-batch culture approach utilized in this experiment, a constant source of light illumination is provided at an intensity of 80 μmol photon m^{-2} s^{-1} [33]. Briefly, 20-L cultures from starter columns were introduced into the annular photobioreactor containing 20 L of Conway medium, thus producing a total of 40 L of initial inoculum (day 1). Subsequent medium feeding was carried out on the 3rd day (40 L culture + 20 L medium), the 4th day (60 L culture + 20 L medium), and the 5th day (80 L culture + 20 L medium) and allowed to grow for another 2 days before harvest. Then, 80 L of culture was harvested with a tubular separator (Hanil-J-1250, Korea) at 10,000 rpm. The second cycle was initiated by adding 20 L of culture medium to the remaining 20 L of culture. Four cycles were initiated, yielding 340 L of culture per run. The reproducibility of the developed fed-batch mode was tested with a culture run two more times. Upon harvesting, the biomass was frozen at −20°C in a freezer (Samsung, China) and then pooled to freeze-dry (Heto LyoPro 3000, Taiwan).

2.2. Growth Monitoring. Microalgal growth patterns throughout the experimental culture were monitored to determine cell density and dry biomass estimation. The cell densities were obtained by cell counting using a Neubauer Hemocytometer (GmbH, Wertheim, Germany), according to Banerjee et al. [34]. The samples were transferred to the haemocytometer chamber using a sterile pipette and allowed to settle for approximately 2–3 min. Cells were counted in five small squares in the centre block, and cell densities were determined using the following formula:

$$\text{Cell density}\,(\text{cells mL}^{-1}) = \frac{\text{Total number of cells counted}}{10 \times 4 \times 10^6}, \quad (1)$$

where 10 = total squared counted in two chambers and 4×10^6 = sample volume ($0.2 \times 0.2 \times 0.1 = 0.004\,\text{mm}^3$) expressed in cm^3 (mL).

The dry biomass estimation was expressed as a dry weight obtained from a known volume. Briefly, the analysis was performed by filtering a 2 mL sample through a pre-weighed, pre-combusted (105°C, overnight) glass fibre filter paper (0.45 μm pore size) (Whatman, UK). The filters were dried overnight at 60°C and then cooled in desiccators for 30 min before being weighed on a balance. The dry weight was determined by obtaining the weight difference between pre-weighed and dried filter paper (after and before filtration) [35]. Specific growth rates of culture were determined according to Guillard [36] as per the following formula:

$$\mu\,(\text{day}^{-1}) = \frac{\ln(F_1/F_0)}{t_1 - t_0}, \quad (2)$$

where F_1 = biomass at the time of harvest (t_1) and F_0 = biomass at times of day one, t_0.

The volumetric productivity (VP) of all culture runs was determined by the formula as follows:

$$VP\,(g/\mathrm{L/day}) = \frac{(X_1 - X_0)}{n}, \quad (3)$$

where X_1 = final biomass and X_0 = initial biomass and n =days of cultivation.

2.3. Nutrient Analysis. The nutrient composition of *C. calcitrans* was analyzed from a random sampling of total freeze-dried biomass produced by a fed-batch culture system with intermittent feeding of culture medium. Freeze-dried microalgal biomass was pooled and subjected to nutrient analysis. The total protein and crude lipid contents were determined according to Lowry et al. [37] and Folch et al. [38], respectively. Meanwhile, the total carbohydrate content was determined by the phenol-sulphuric method following Dubois et al. [39]. The moisture content was determined using an automatic moisture analyser (Mettler Toledo, HC103). Briefly, readings were taken after 1 g of the sample was dried at 120°C. The ash content was determined according to AOAC [40].

2.4. Fatty Acid Methyl Esther (FAME). FAME analysis was conducted according to Salimon et al. [41]. Briefly, the direct methylation was conducted by adding 1 mL of hexane to the lipid sample, followed subsequently by the addition of 1 mL of sodium methoxide. The solution was vigorously stirred before being allowed to stand for 10 min (FAME separation). The FAMEs produced were analysed by gas chromatography (GC) equipped with the non-polar stationer phase BPX70 capillary column (SGE forte™). The isothermal method is used for fatty acid composition analysis with the following temperature settings: an oven temperature of 30–250°C and a detector temperature of 240°C. Helium is used as a carrier gas at a flow rate of 1.3 mL min^{-1}. Peaks obtained from the chromatogram were used to quantify the size of each peak, with each peak representing a fatty acid compound identified according to the IUPAC 2.301 standard with a known internal standard (Sigma).

2.5. Amino Acid Profile. The amino acid profile of the samples was determined according to Sánchez-Machado et al. [42] by the HPLC method with fluorescence detection. The mobile phase was maintained with AccQ Tag Eluent A (WICOM Germany GmbH) and AccQ Tag Eluent B (WICOM Germany GmbH) at a flow rate of 1 mL min^{-1} and a column temperature of 36°C. The Pierce Amino Acid Standard H (Thermo Scientific™) was used as the calibration standard for protein hydrolysates. Briefly, the sample was prepared by adding 5 mL of 6N hydrochloric acid (HCL) to a 0.2 g sample and subsequently heated at 110°C for 24 hr. Then, 4 mL of stock alpha-aminobutyric acid (AABA) (2.5 mM) was added and made up to 100 mL. The solutions were filtered through a filter paper (0.45 μm cellulose membrane filter) and a syringe filter (0.2 μm). Then, 10 μL of 2-amino-4-bis (aryloxybenzyl) aminobutanoic acids (AABA) (equal to 1 nmole of AABA) was added for derivatization. Samples of 10 μL were aliquots into vials and dried under vacuum conditions for 30 min. Samples were mixed with 100 μL sample diluents (AccQ Tag Eluent A) and vortexed before injecting 5 μL into the Water HPLC 501 (Milipore, USA). Then amino acids derived from samples were separated by reversed-phase high-performance liquid chromatography (HPLC) with UV detection at 250 nm.

2.6. Statistical Analysis. Statistical analysis was performed using SPSS 26.0 for Windows (SPSS Inc., Chicago., IL, USA). All the data obtained were analysed using one-way analysis of variance (ANOVA). Duncan's multiple range test was applied for pairwise comparison of the means. Data were expressed as mean ± standard error of the mean (SEM). All differences were regarded as statistically significant at $P < 0.05$.

3. Results

3.1. Growth Analysis. The production process of *C. calcitrans* has been shown in the first phase of batch culture and the following phase of large-scale culture, adopting the fed-batch culture method (Figures 1 and 2).

Throughout the culture period, the pH of the culture medium was recorded in the range of 7.9–8.6. No contamination or culture crashes were recorded as well. The microscopic observation indicated cell size was consistently uniform (Figure 3). Microalgal growth data were presented in two phases. The first phase provides results from 22 days of starter culture (Figures 4 and 5). The growth pattern in the starter culture showed similarity in all culture runs, with a gradual increase on the first 3 days of each upscaling followed by slow growth on the 4th and 5th day. As expected, scaling up the culture resulted in an immediate decrease in the growth parameters studied, followed by an increase in growth. Thus, the starter culture provided inoculum at initial cell densities of 1.34×10^8, 1.27×10^8, and 1.14×10^8 cells mL^{-1} for the 1st, 2nd, and 3rd runs, respectively. This resulted in dry biomass production of 1.17, 1.23, and 1.17 g L^{-1} for the 1st, 2nd, and 3rd runs, respectively. Second-phase growth data were recorded in fed-batch mode by intermittent feeding of culture medium in annular photobioreactors. Data were presented for each complete run consisting of four harvests, totalling 340 L of culture volume per run (Figures 6 and 7). The large-scale culture showed a constant growth curve. A reduction in growth occurred once 80% of the culture was harvested, and the following cycle was initiated. Day 1 readings for cell density were 4.2×10^7, 4.2×10^7, and 4.4×10^7 cells mL^{-1}, respectively.

In subsequent cycles, however, cultures exhibited initial cell density readings in the range of 2.3–3.4 $\times 10^7$ cells mL^{-1}. Lower densities were observed in the subsequent cycles (2nd, 3rd, and 4th) compared to the inoculum in the first cycle. The cell density at the harvesting point ranged from 7.3×10^7 to 1.10×10^8 cells mL^{-1}, with an average cell density of only 10 million cells mL^{-1}. The same pattern was observed when estimating dry biomass as the growth parameter. The highest dry biomass recorded in this large-scale culture system was 0.78 g L^{-1}. A continuous growth pattern in each cycle was observed with intermittent feeding of culture medium. After being grown for 2 days, the intermittent feeding of the culture medium on the 3rd day gradually increased the cell density on the fourth day. This pattern was consistent for medium feeding on the 4th and 5th days.

Besides, using this culture model, 3,737.36 g of biomass was harvested as concentrated paste from a 1,020 L culture volume. This gave an average of 3.66 g L^{-1} of wet weight. The freeze-dried product of the concentrated paste produced 761.3 g (0.75 g L^{-1}) of *C. calcitrans* biomass, approximately

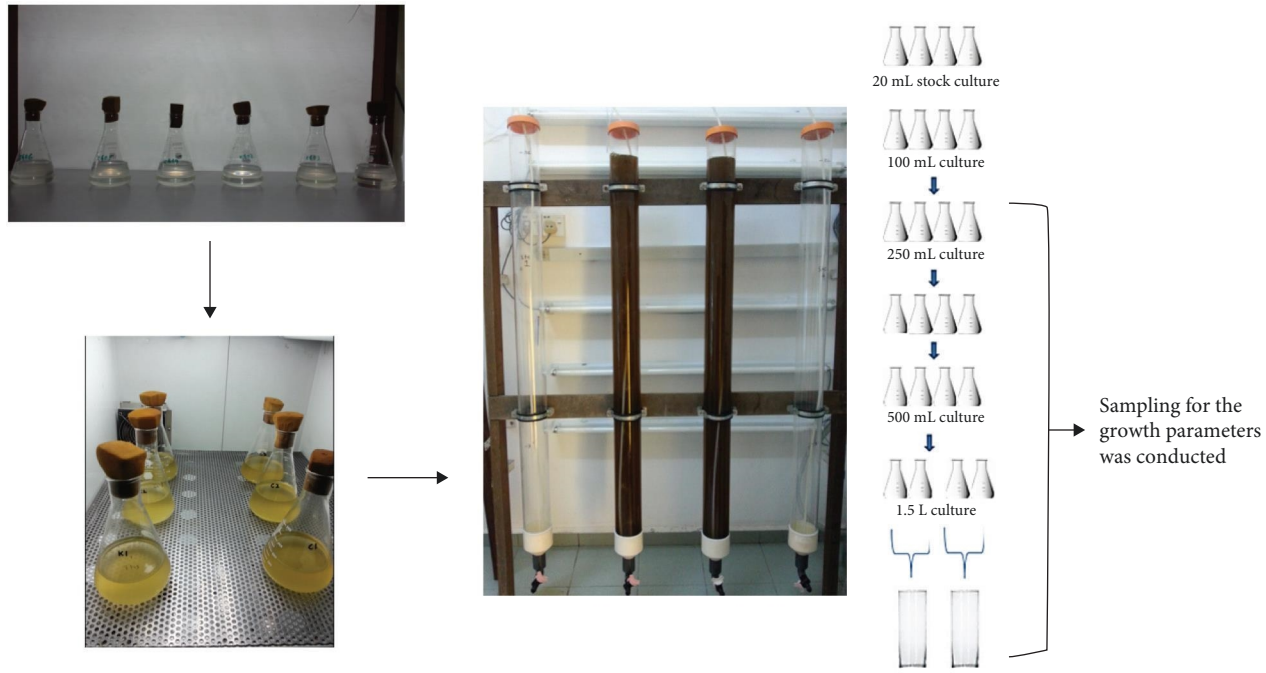

FIGURE 1: Flow chart of the first phase of *Chaetoceros calcitrans* production via batch culture.

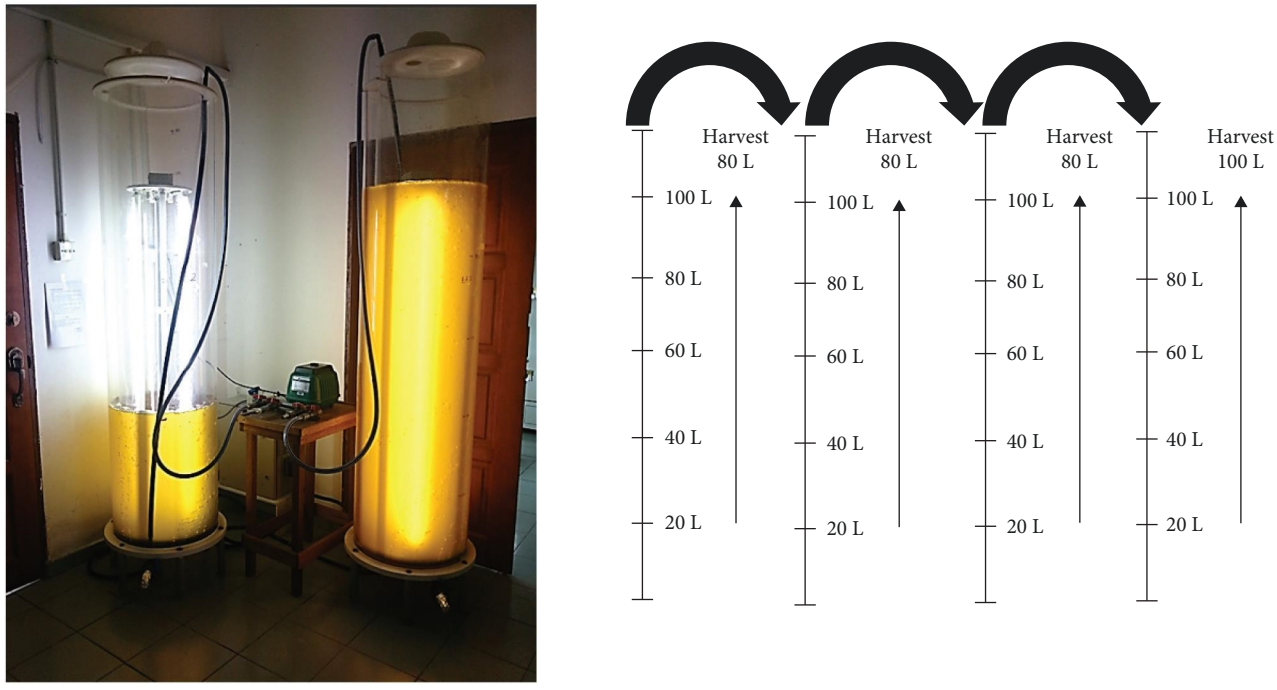

FIGURE 2: Flow chart of the second phase of *Chaetoceros calcitrans* production via a fed-batch culture mode with intermittent feeding of culture medium.

20% of the concentrated paste. The volumetric productivity monitored between each culture runs resulted in no significant differences ($p > 0.05$) as a comparison for the reproducibility of the culture model adopted (Table 1). The determination of the deviation in biomass production between each cycle in the respective culture runs was monitored in comparison with the specific growth rate μ (day^{-1}) of each culture cycle. Specific growth rate μ (day^{-1}) showed an increasing pattern until Cycle 3 and consistently decreased for all culture runs in Cycle 4 (Table 2). However, no significant differences ($P > 0.05$) were indicated among each cycle in all culture runs.

3.2. Nutrient Analysis. Chaetoceros calcitrans showed the highest proportion of protein content with $41.8 \pm 0.7\%$, followed

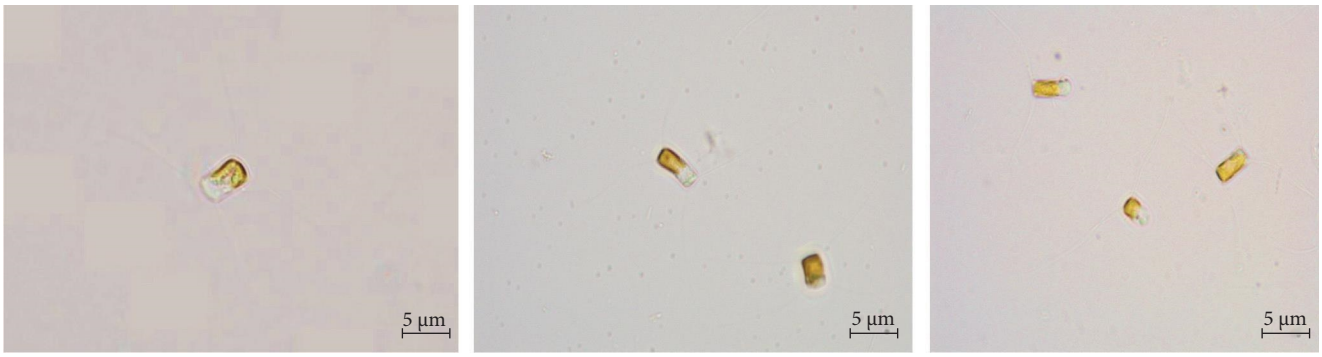

FIGURE 3: Photomicrographs of *Chaetoceros calcitrans* cultured using a fed-batch culture mode with intermittent feeding of culture medium. The bar is equivalent to 5 μm.

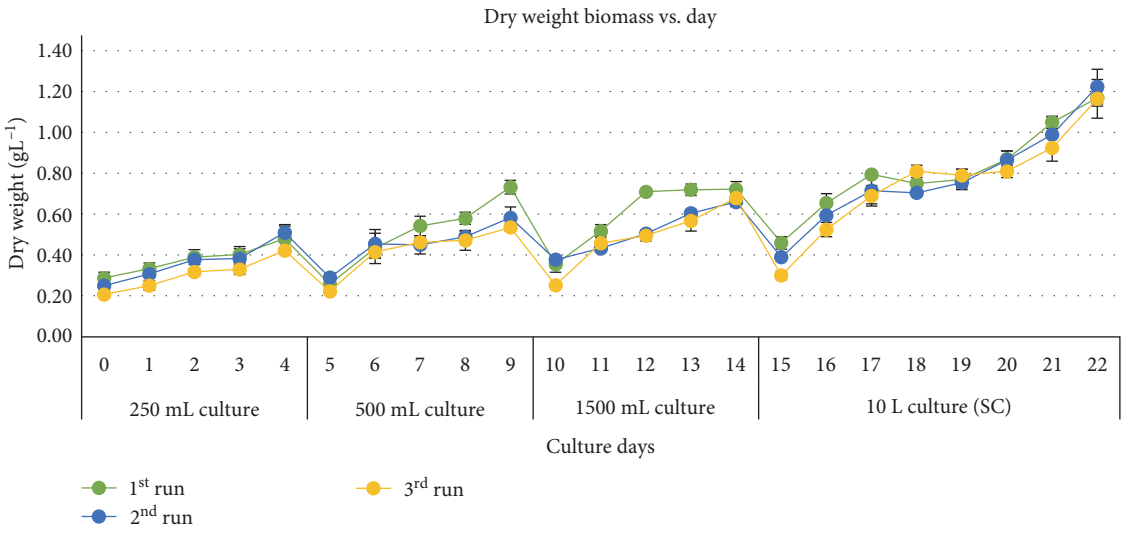

FIGURE 4: Dry weight biomass of *Chaetoceros calcitrans* from 250 mL to 10 L. Values are mean ± SEM ($n = 3$).

by carbohydrate with 27.2 ± 0.1% and lipid with 11.3 ± 0.6% (Figure 8).

The amino acid profile comprised 17 amino acids, including essential amino acids (43.2%) and non-essential amino acids (56.7%) (Figure 9). The highest concentration was glutamic acid (17.6 ± 0.1%), followed by aspartic acid (12.6 ± 0.1%) and arginine acid (8.1 ± 0.1%). Hydroxyproline acid, which is often found in the cell walls of diatoms, was also detected at 0.8 ± 0.2%. Essential amino acids, namely, arginine, isoleucine, leucine, lysine, phenylalanine, valine, and threonine, were found in the range of 4.3%–8.1%, while histidine and methionine acid recorded 1.9% and 0.1%, respectively. The fatty acid composition of *C. calcitrans* showed a predominance of polyunsaturated fatty acids (PUFAs), totalling 44.6 ± 0.2%, followed by saturated fatty acids (SFA) at 38.2 ± 0.4% and monounsaturated fatty acids (MUFAs) at 17.7 ± 0.1% (Figure 10). Omega 3 (n-3) dominated PUFA content with 29.8 ± 0.1%, while omega 6 (n-6) consisted only of 14.7 ± 0.1%.

4. Discussion

In this study, the growth of *C. calcitrans* cultures was monitored in both a starter culture system in a batch culture mode and in a large-scale culture system via a fed-batch culture mode. The starter culture growth of *C. calcitrans* showed higher productivity than the large-scale production in the annular photobioreactor. This is supported by Grobbelaar [43], who stated that higher microalgae productivity at either high volumetric or areal production rates is achievable in small-scale production systems or laboratory setups (<5 m^2). In this study, each phase was cultured using a different culture mode, starting with a batch culture to produce a higher cell density of inoculum for large-scale culture in a fed-batch mode. Nevertheless, volumetric productivity largely depends on the reactor's surface-to-volume ratio [44], resulting variation between the two culture phases. However, it is important to highlight that the high growth rate in the starter culture may facilitate large-scale production since it served as the high initial inoculum for larger-scale culture. Since the ultimate objective of the present study was to optimize biomass production of *C. calcitrans*, the high cell density inoculum from the starter culture for each run (4.2×10^7, 4.2×10^7, and 4.4×10^7 cells mL^{-1}, respectively) was proven to be able to produce the cell density at the harvest point ranging from 7.8×10^7 to 1.1×10^8 cells mL^{-1}. This yielded dry biomass in the range of 0.58–0.78 g L^{-1}.

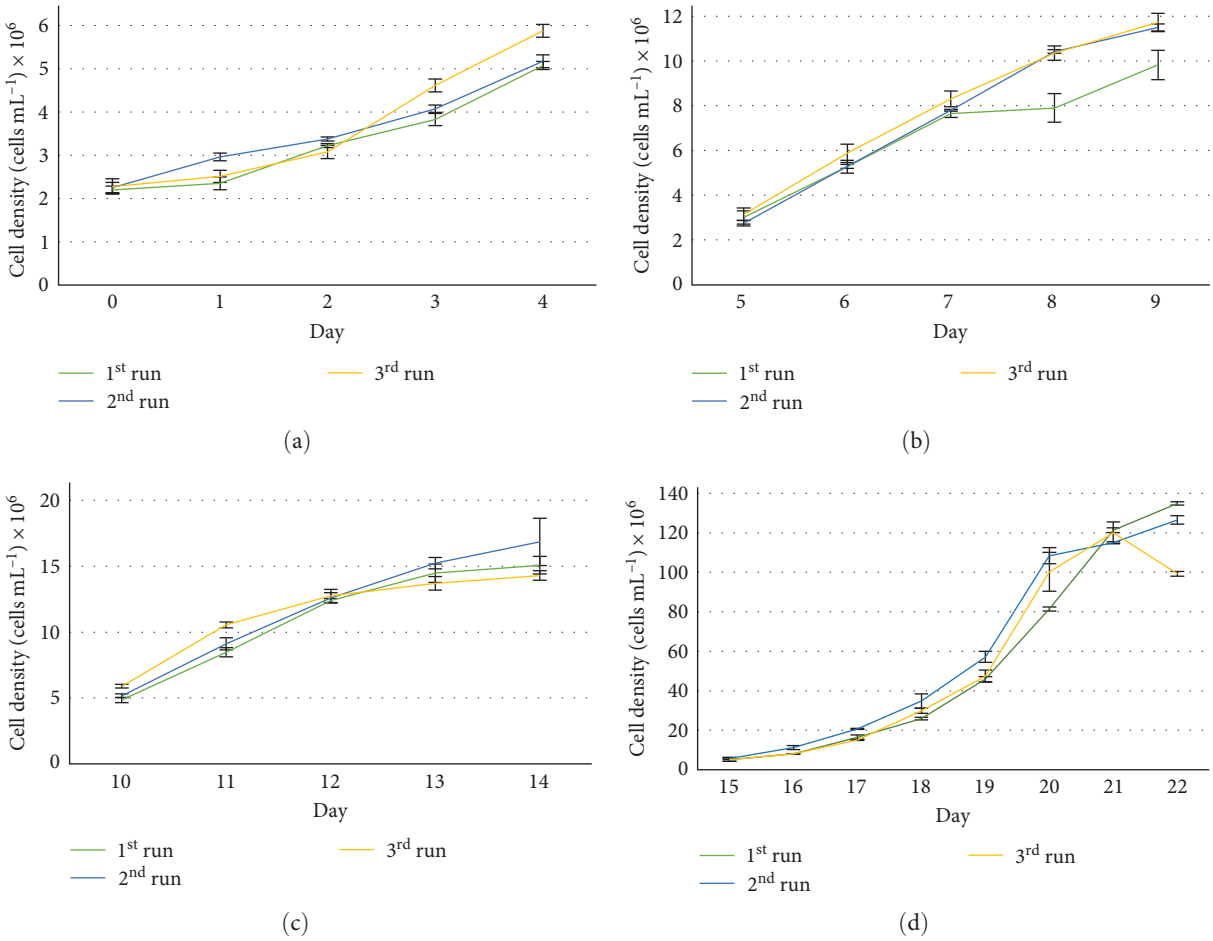

FIGURE 5: Cell density of *Chaetoceros calcitrans*: (a) 250 mL, (b) 500 mL, (c) 1,500 mL, and (d) 10 L. Values are mean ± SEM ($n = 3$).

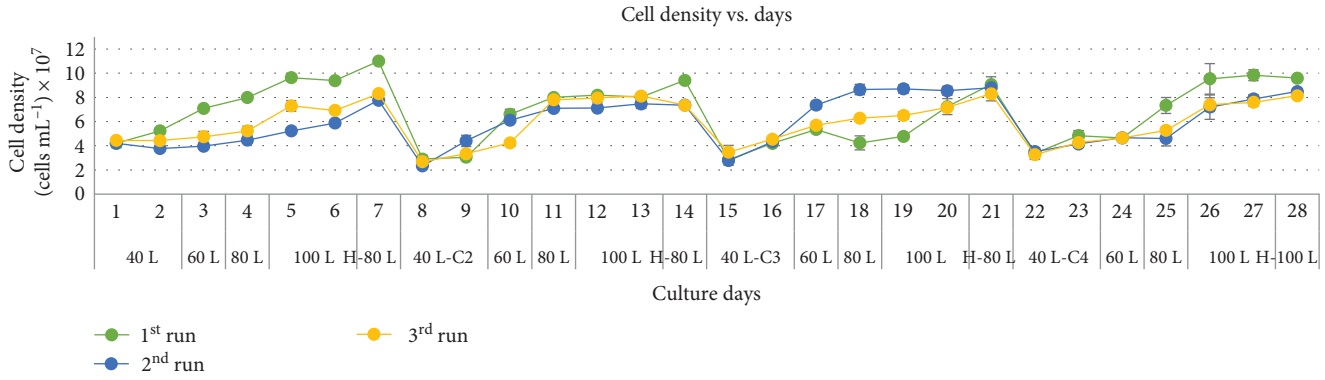

FIGURE 6: The cell density of *Chaetoceros calcitrans* in annular photobioreactors (120 L) using a fed-batch culture mode with intermittent feeding of culture medium. Values are mean ± SEM ($n = 3$).

When dealing with a closed system, it is crucial to take into account the high cell density of the inoculum. This can have a detrimental impact on microalgal culture and biomass production. Beyond a certain density threshold, the cells can no longer grow due to photoinhibition and limitations in nutrient transfer. A photoinhibition was reported to be induced in a 1 cm photobioreactor by the initial cell densities of 8.34×10^6 cells mL^{-1} of *Chaetoceros muelleri* due to the excess light and variation in areal cell population densities [45]. Contrariwise, in the present study, by adapting intermittent feeding of culture medium, the cell densities were maintained high (10 million cells mL^{-1}) throughout the culture, and a stationary phase was avoided. Adding culture medium in the right proportion at the right time to the dense culture allows dilution to permit effective light utilization. Besides, by diluting the medium at an appropriate rate, the thickness of

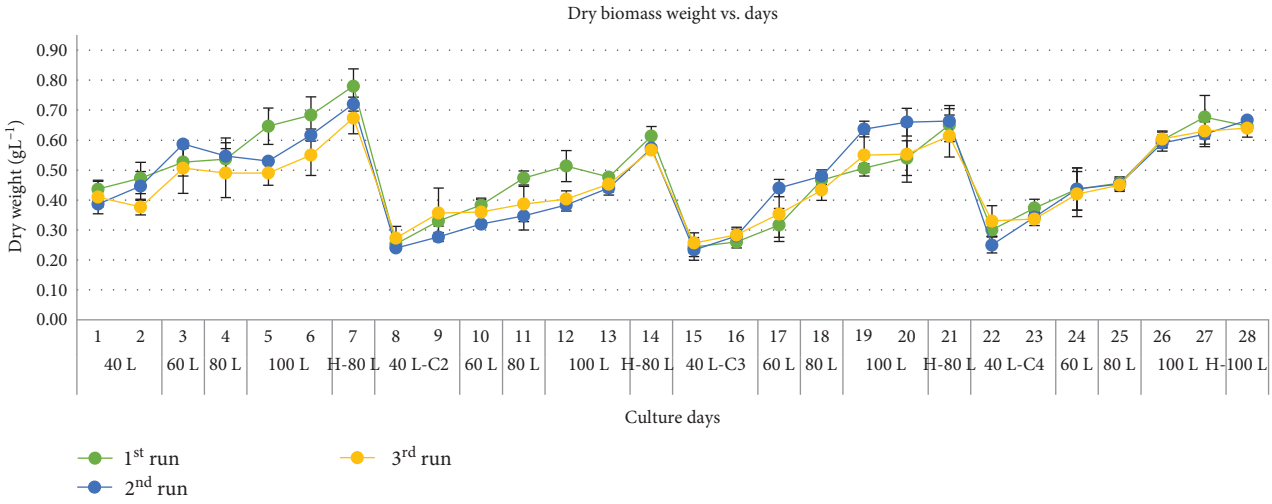

FIGURE 7: Dry weight biomass of *Chaetoceros calcitrans* in annular photobioreactors (120 L) using a fed-batch culture mode with intermittent feeding of culture medium. Values are mean ± SEM ($n = 3$).

TABLE 1: Biomass production and volumetric productivity of *Chaetoceros calcitrans* in annular photobioreactors (120 L) using a fed-batch culture mode with intermittent feeding of culture medium.

Culture	Biomass production (g)		Volumetric productivity (g/L/day)
	Concentrated paste	Freeze-dried	
1st run	1,264.58	257.01	0.21 ± 0.01
2nd run	1,228.75	251.88	0.28 ± 0.04
3rd run	1,244.03	252.41	0.23 ± 0.06
Total	3,737.36	761.30	ns

Values are expressed as the mean ± SEM, $n = 3$; ns = not statistically significant ($P > 0.05$).

TABLE 2: Specific growth rate μ (day^{-1}) of *Chaetoceros calcitrans* biomass production in annular photobioreactors (120 L) using a fed-batch culture mode with intermittent feeding of culture medium.

Culture	Specific growth rate μ (day^{-1})		
	1st run	2nd run	3rd run
Cycle 1	0.083 ± 0.02	0.091 ± 0.02	0.073 ± 0.03
Cycle 2	0.126 ± 0.01	0.124 ± 0.00	0.107 ± 0.02
Cycle 3	0.142 ± 0.03	0.151 ± 0.03	0.125 ± 0.04
Cycle 4	0.110 ± 0.01	0.142 ± 0.02	0.079 ± 0.05
Statistical significance	ns	ns	ns

Values are expressed as the mean ± SEM, $n = 4$; ns = not statistically significant ($P > 0.05$).

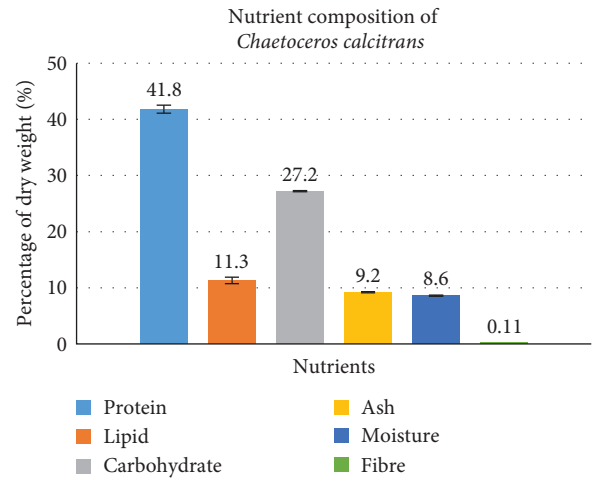

FIGURE 8: Nutrient composition of *Chaetoceros calcitrans* produced in annular photobioreactors (120 L) using a fed-batch culture mode with intermittent feeding of culture medium (% of dry weight). Values are mean ± SEM ($n = 6$).

the culture medium will reduce, and photoinhibition or self-shading can be avoided, while cell division is unaffected [18]. This was held by Ji et al. [46], who reported that the microalga *Pseudochlorococcum* sp., improved both biomass production (0.05–3 g DM m^{-2} of dry weight) and growth rate by increasing the inoculum density. The microalgae strains of *Chlorella vulgaris*, *Chlorella sorokiniana*, *Scenedesmus acutus* f. *alternans*, and *Scenedesmus dimorphus* were reported to have their greatest biomass density when inoculated with an inoculum of 10^6 cells mL^{-1} [47]. However, the implications of the current findings are insufficient, given the impact of operational process factors such as the effect of inoculum density on the microalgal production of biomass and biocompounds [48], especially in the large-scale production of a fed-batch of *C. calcitrans*, which is considered a new approach.

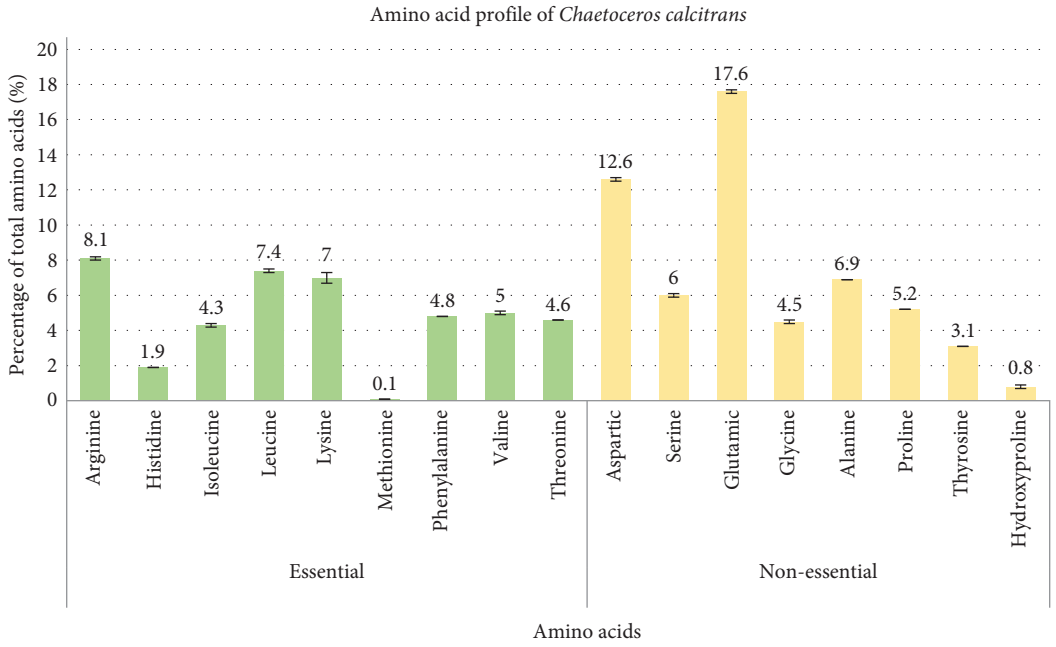

FIGURE 9: Amino acid profile of *Chaetoceros calcitrans* produced in 120 L annular photobioreactor using a fed-batch culture mode with intermittent feeding of culture medium. Values mean ± SEM ($n = 2$).

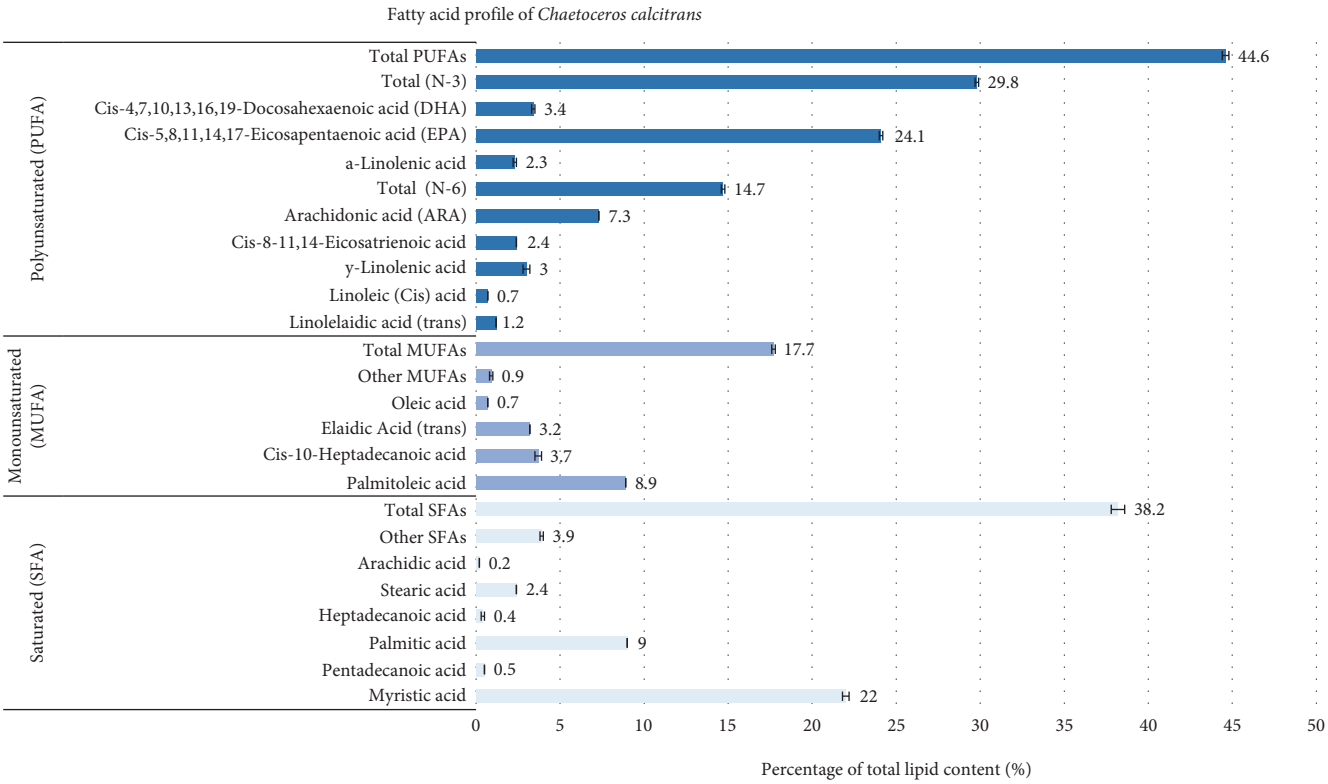

FIGURE 10: The fatty acid profile of *Chaetoceros calcitrans* produced in 120 L annular photobioreactors using a fed-batch culture mode with intermittent feeding of culture medium. Values mean ± SEM ($n = 2$).

To our knowledge, this is the first description of *C. calcitrans* culture in a 120-L annular photobioreactor with intermittent feeding of culture medium in fed-batch mode. Intermittent feeding of the medium adopted in this study supplied nutrients continually to the culture and served as a dilution factor to avoid self-shading conditions. This coincides with [12, 14], where the modification of nutrient supply is a crucial technique to maximize microalgal growth. Thus, in the present study, the nutrient

limitation was avoided by feeding the medium at a short, 2-day interval. Intermittent feeding of medium avoids nutrition limitation in dense culture, and the cells are maintained in a nutrient-replete state. Supplying sufficient nutrients to microalgae culture is crucial for producing a large quantity of high-quality microalgae biomass [49]. In comparison to a previous study of *C. calcitrans* production in the same 120-L annular photobioreactor by Lai et al. [21] without adapting the fed-batch mode, they incorporated the same production facility, system, and cultured species. Lai et al. [21] reported an average of 0.28 g L^{-1} dry biomass, with the maximum dry biomass estimate being 0.49 g L^{-1} with an inoculum cell density of 4.37×10^6 cells mL^{-1}. The cell density at the harvesting point was not reported by the team. It took the team 25 days to scale up the culture from 40 to 100 L by adding 20 L of medium every 5 days when the culture reached the late exponential phase. Nevertheless, the present study only required 7 days to produce 100 and 340 L cultures with relatively higher cell density and dry biomass in 28 days. A total of 761.3 g (0.75 g L^{-1}) from three culture runs was produced in the current study, with each run producing 257.01, 251.88, and 252.41 g of *C. calcitrans* dry biomass, respectively. The three culture runs responded similarly, demonstrating that the cells had been exposed to similar amounts of average light and nutrients with no differences in volumetric productivity. The reproducibility of biomass production at each run serves as prominent evidence of the effectiveness of adopting this culture mode. Although the cell density was maintained at 10 million cells mL^{-1}, a continuous decrease in cell density and dry biomass weight with each subsequent cycle was observed throughout all culture runs, suggesting that the culture is still in the adaptation phase after the harvesting process [29, 30]. Additionally, a semi-continuous culture system was used to culture *C. calcitrans* by Milagros et al. [50] also showed a similar decrement in subsequent cycles. The cell densities were reported higher in Cycle 1 (8.4×10^5 cells mL^{-1}) than in Cycle 2 (6.6×10^5 cells mL^{-1}) and Cycle 3 (4.5×10^5 cells mL^{-1}) and, contrary to our study, the culture collapsed in Cycle 4. This was prominent in our study, where the specific growth rate between all cycles decreased during culture cycle four in all three culture runs. The reduction pattern on subsequent cycles could be due to the increasing culture age and the production and accumulation of spermatogonia, cytoplasm, metabolites, and other cellular debris that can adhere to the cultured cells and form flocs, inhibiting their growth and even causing a culture crash [28]. However, no culture crash or culture collapse occurred in the present study. This demonstrated the potential of limiting culture cycles in the fed-batch culture system to avoid a possible culture crash due to contamination from suspended floc accumulation. It is also important to note that no flocs formed during the whole experimental culture in this study. Kaspar et al. [28] reiterate that floc accumulation signifies that a microalgal culture may be about to crash.

The nutritional content of the produced microalgal biomass in this study indicates the effectiveness of the culture system, apart from its promising growth performance [19]. The growth stage and the physiological composition of the microalgal cells primarily influence the cellular biochemical composition of microalgae. In this study, the culture method adapted provides cells grown under constant light, temperature, and nutrient supply and harvested in the middle of the exponential phase to ensure healthy cells and high-quality biomass. Hu et al. [51] stated that under ideal culture conditions, microalgae produced biomass that is high in protein (30%–50%), carbohydrates (20%–40%), lipids (8%–15%), and 5%–20% dry weight of fatty acids. The present investigation reveals a variety of similar nutritional compositions. The protein content accorded well with previous studies [34, 52–54]. Besides, a nutrient-depleted state results in higher lipid and carbohydrate content [55, 56]. Hence, the present study suggests that the culture was in a nutrient-rich state. Wang et al. [57] indicated that single-stage fed-batch cultures without stress induction improved lipid productivity and content by 1.60- and 1.92-fold.

The optimization of fatty acid content is influenced by culture mode, which is reflected by adequate nutrient supply and appropriate light intensity in the culture system. Previous research has indicated that a reduced dilution rate, increased light intensity, and smaller cell size have a significant effect on the formation of polyunsaturated fatty acids (PUFAs) [58]. The higher n-3 and n-6 percentages detected in the present study were also consistent with Natrah et al. [53]. A high level of cellular energy and the availability of essential nutrients for cellular functioning and growth were both indicated by the accumulation of higher n-3 levels in the current study, as reported by Tiez and Zeiger [59]. The high percentage of EPA shown in the present study agrees with Rivero-Rodriguez et al. [60] and Griffiths and Harrison [61]. Adapting fed-batch culture with intermittent feeding allowed relevant dilution of media and ensured sufficient light supply to the culture, which is crucial for the stable production of a uniform-size cell. However, smaller cell size is a crucial factor for optimizing the production of *Chaetoceros* sp. for a higher PUFA content [58]. This is usually achieved with a lower dilution rate in batch, semi-, or continuous culture systems. The amino acid *C. calcitrans* in the present study was in accordance with Natrah et al. [53]. It has been determined that the composition of amino acids in diatoms is highly responsive to external factors such as nitrogen availability, light intensity, and photoperiod [62]. Additionally, it is also affected by the growth stage of the microalgae culture [63]. The percentage of higher total non-essential amino acids compared to total essential amino acids in the present study showed a common profile of diatoms [63], with a predominance of arginine ($8.1 \pm 0.1\%$) and leucine ($7.4 \pm 0.1\%$) for essential amino acids, and glutamic ($17.6 \pm 0.1\%$) and aspartic ($12.6 \pm 0.1\%$) being the most abundant non-essential amino acids. Higher percentages of essential amino acids could be achieved by adopting the cycle for illuminating and harvesting cells in the late-exponential growth phase [63], which was not applied in the current study.

5. Conclusions

Optimization of culture conditions is important in influencing the physiological growth and cellular biochemical properties

of *C. calcitrans*. To our knowledge, this is the first description of *C. calcitrans* cultured in fed-batch mode with intermittent feeding of culture medium in a 120 L annular photobioreactor. The cultivation technique produced more microalgae biomass faster, indicating a cost-saving on an industrial scale. Since no culture failures occurred throughout the trial, the cultivation system's characteristics promote culture stability and reduce contamination. The obstacle encountered in high-cell density cultures of *C. calcitrans* in large-scale systems can be rectified by adopting the intermittent feeding of culture medium. Furthermore, the culture mode adopted did not negatively affect the nutritional profile of the *C. calcitrans* biomass produced. Further development will depend on a suitable cultivation system that provides efficient utilization of light and nutrition for favourable production output. This can be achieved by adapting suitable growth kinetic studies or feeding essential, specific culture mediums instead of the complete medium. However, careful control conditions should be applied to microalgal culture to obtain homogeneous production in terms of quality and quantity. Finally, the mode in which the microalgae is cultivated is another factor that helps to achieve the objectives of high cell density, high productivity, and good nutrient range at the same time.

Data Availability

The data used to support the findings of this study are included within the article in terms of figures and tables, and raw data are available from the corresponding author upon request.

Disclosure

No interference by any party is encountered in the role of study design, data collection and analysis, or even data interpretation, when writing the manuscript or when deciding to publish the results.

Conflicts of Interest

The authors declare no conflict of interest.

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References

[1] P. Tandon and Q. Jin, "Microalgae culture enhancement through key microbial approaches," *Renewable and Sustainable Energy Reviews*, vol. 80, pp. 1089–1099, 2017.

[2] G. L. Bhalamurugan, O. Valerie, and L. Mark, "Valuable bioproducts obtained from microalgal biomass and their commercial applications: a review," *Environmental Engineering Research*, vol. 23, no. 3, pp. 229–241, 2018.

[3] N. Hosseinkhani, J. I. McCauley, and P. J. Ralph, "Key challenges for the commercial expansion of ingredients from algae into human food products," *Algal Research*, vol. 64, Article ID 102696, 2022.

[4] L. G. Ramírez Mérida and R. A. Rodríguez Padrón, "Application of microalgae in wastewater: opportunity for sustainable development," *Frontiers in Environmental Science*, vol. 11, Article ID 1238640, 2023.

[5] J. Li, Y. Liu, J. J. Cheng, M. Mos, and M. Daroch, "Biological potential of microalgae in China for biorefinery-based production of biofuels and high value compounds," *New Biotechnology*, vol. 32, no. 6, pp. 588–596, 2015.

[6] M. Iijima, "Japan biofuels annual 2017," 2017.

[7] E. Jacob-Lopes, M. M. Maroneze, M. C. Deprá, R. B. Sartori, R. R. Dias, and L. Q. Zepka, "Bioactive food compounds from microalgae: an innovative framework on industrial biorefineries," *Current Opinion in Food Science*, vol. 25, pp. 1–7, 2019.

[8] P. Loke Show, "Global market and economic analysis of microalgae technology: status and perspectives," *Bioresource Technology*, vol. 357, Article ID 127329, 2022.

[9] H. R. Lim, K. S. Khoo, K. W. Chew et al., "Perspective of *Spirulina* culture with wastewater into a sustainable circular bioeconomy," *Environmental Pollution*, vol. 284, Article ID 117492, 2021.

[10] H. Tokushima, N. Inoue-Kashino, Y. Nakazato, A. Masuda, K. Ifuku, and Y. Kashino, "Advantageous characteristics of the diatom *Chaetoceros gracilis* as a sustainable biofuel producer," *Biotechnology for Biofuels*, vol. 9, no. 1, pp. 1–19, 2016.

[11] L. Zanella, P. Pertile, M. Massironi, and E. Caviola, "Extracts of microalgae and their application," U.S. Patent No. 9,974,819, 2018.

[12] A. Azizan, M. Maulidiani, R. Rudiyanto et al., "Mass spectrometry-based metabolomics combined with quantitative analysis of the microalgal diatom (*Chaetoceros calcitrans*)," *Marine Drugs*, vol. 18, no. 8, pp. 3–5, 2020.

[13] I. Sirakov, K. Velichkova, S. Stoyanova, and Y. Staykov, "The importance of microalgae for aquaculture industry," *International Journal of Fisheries and Aquatic Studies*, vol. 2, no. 4, pp. 31–37, 2015.

[14] F. Kong, Z. Ran, J. Zhang et al., "Synergistic effects of temperature and light intensity on growth and physiological performance in *Chaetoceros calcitrans*," *Aquaculture Reports*, vol. 21, Article ID 100805, 2021.

[15] P. Charoonnart, S. Purton, and V. Saksmerprome, "Applications of microalgal biotechnology for disease control in aquaculture," *Biology*, vol. 7, no. 2, Article ID 24, 2018.

[16] A. S. Gomes, S. D. Lourenço, P. M. Santos et al., "High dietary protein, n − 3/n − 6 ratio and *β*-carotene enhances *Paracentrotus lividus* (Lamarck, 1816) larval development," *Aquaculture Research*, vol. 53, no. 15, pp. 5398–5412, 2022.

[17] C. R. V. Bastos, I. A. B. Maia, H. Pereira, J. Navalho, and J. C. S. Varela, "Optimisation of biomass production and nutritional value of two marine diatoms (Bacillariophyceae), *Skeletonema costatum* and *Chaetoceros calcitrans*," *Biology*, vol. 11, no. 4, Article ID 594, 2022.

[18] K. I. Reitan, G. Øie, H. Jørgensen, and X. Wang, "Chemical composition of selected marine microalgae, with emphasis on lipid and carbohydrate production for potential use as feed resources," *Journal of Applied Phycology*, vol. 33, no. 6, pp. 3831–3842, 2021.

[19] M. Akbarnejad, H. R. Islami, M. J. Baboli, M. S. Mehrgan, and Y. Filizadeh, "Effect of light and nitrogen concentration on the growth and lipid content of marine diatom, *Chaetoceros calcitrans*," *Iranian Journal of Fisheries Sciences*, vol. 19, no. 2, pp. 986–993, 2020.

[20] M. Jannah, M. F. Ulkhaq, M. H. Azhar, Suciyono, and dan W. Soemarjati, "Growth performance of laboratory-scale *Chaetoceros calcitrans* in different containers," *IOP Conference Series: Earth and Environmental Science*, vol. 236, no. 1, pp. 3–8, 2019.

[21] J. I. Lai, F. M. Yusoff, and M. Shariff, "Large-scale culture of a tropical marine microalga *Chaetoceros calcitrans* (Paulsen) Takano 1968 at different temperature using annular photobioreactors," *Pakistan Journal of Biological Sciences*, vol. 15, no. 13, pp. 635–640, 2012.

[22] S. Kundu, J. Mukherjee, F. Yeasmin et al., "Growth profile of *Chaetoceros* sp. and its steady state behaviour with change in initial inoculum," *Current Science*, vol. 115, no. 12, pp. 2275–2286, 2018, https://www.jstor.org/stable/26978591.

[23] S. Steven, D. L. Friatnasary, A. K. Wardani, K. Khoiruddin, G. Suantika, and I. G. Wenten, "High cell density submerged membrane photobioreactor (SMPBR) for microalgae cultivation," *IOP Conference Series: Earth and Environmental Science*, vol. 963, no. 1, Article ID 012034, 2022.

[24] F. Han, H. Pei, W. Hu, M. Song, G. Ma, and R. Pei, "Optimization and lipid production enhancement of microalgae culture by efficiently changing the conditions along with the growth-state," *Energy Conversion and Management*, vol. 90, pp. 315–322, 2015.

[25] R. Martínez-Macías, E. Meza-Escalante, D. Serrano-Palacios, P. Gortáres-Moroyoqui, P. E. Ruíz-Ruíz, and G. Ulloa-Mercado, "Effect of fed-batch and semicontinuous regimen on *Nannochloropsis oculata* grown in different culture media to high-value products," *Journal of Chemical Technology and Biotechnology*, vol. 93, no. 2, pp. 585–590, 2018.

[26] N. Liu, B. Guo, Y. Cao et al., "Effects of organic carbon sources on the biomass and lipid production by the novel microalga *Micractinium reisseri* FM1 under batch and fed-batch cultivation," *South African Journal of Botany*, vol. 139, pp. 329–337, 2021.

[27] Y.-H. Chang, K.-S. Chang, C.-L. Hsu et al., "A comparative study on batch and fed-batch cultures of oleaginous yeast *Cryptococcus* sp. in glucose-based media and corncob hydrolysate for microbial oil production," *Fuel*, vol. 105, pp. 711–717, 2013.

[28] H. F. Kaspar, E. F. Keys, N. King, K. F. Smith, A. Kesarcodi-Watson, and M. R. Miller, "Continuous production of *Chaetoceros calcitrans* in a system suitable for commercial hatcheries," *Aquaculture*, vol. 420–421, pp. 1–9, 2013.

[29] S. Krichnavaruk, W. Loataweesup, S. Powtongsook, and P. Pavasant, "Optimal growth conditions and the cultivation of *Chaetoceros calcitrans* in airlift photobioreactor," *Chemical Engineering Journal*, vol. 105, no. 3, pp. 91–98, 2005.

[30] S. Krichnavaruk, S. Powtongsook, and P. Pavasant, "Enhanced productivity of *Chaetoceros calcitrans* in airlift photobioreactors," *Bioresource Technology*, vol. 98, no. 11, pp. 2123–2130, 2007.

[31] P. B. Ajithkumar, S. Joseph, and K. Vidya, "Growth pattern of stock cultures of five selected species of marine microalgae maintained under indoor controlled environment and under outdoor conditions," *Indian Journal of Fisheries*, vol. 66, no. 1, pp. 131–137, 2019.

[32] G. Raghavan, C. K. Haridevi, and C. P. Gopinathan, "Growth and proximate composition of the *Chaetoceros calcitrans* f. *pumilus* under different temperature, salinity and carbon dioxide levels," *Aquaculture Research*, vol. 39, no. 10, pp. 1053–1058, 2008.

[33] C. D. Kang, S. J. Han, S. P. Choi, and S. J. Sim, "Fed-batch culture of astaxanthin-rich *Haematococcus pluvialis* by exponential nutrient feeding and stepwise light supplementation," *Bioprocess and Biosystems Engineering*, vol. 33, no. 1, pp. 133–139, 2010.

[34] S. Banerjee, W. E. Hew, H. Khatoon, M. Shariff, and F. M. Yusoff, "Growth and proximate composition of tropical marine *Chaetoceros calcitrans* and *Nannochloropsis oculata* cultured outdoors and under laboratory conditions," *African Journal of Biotechnology*, vol. 10, no. 8, pp. 1375–1383, 2011.

[35] P. Lavens and P. Sorgeloos, "Manual on the production and use of live food for aquaculture," in *FAO Fisheries Technical Paper*, FAO Rome, 1996.

[36] R. R. L. Guillard, "Division rates," in *Handbook of Phycological Methods*, J. Stein, Ed., p. 2, Cambridge University Press, Cambridge, UK, 1973.

[37] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the folin phenol reagent," *Journal of Biological Chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[38] J. Folch, M. Lees, and G. H. Sloane-Stanley, "A simple method for the isolation and purification of total lipids from animal tissues," *Journal of Biological Chemistry*, vol. 226, no. 1, pp. 497–509, 1957.

[39] M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, "Colorimetric method for determination of sugars and related substances," *Analytical Chemistry*, vol. 28, no. 3, pp. 350–356, 1956.

[40] AOAC, *Official Methods of Analysis of AOAC International*, AOAC International, Maryland, USA, 1990.

[41] J. Salimon, M. Said, S. Ramli, and M. Lazim, *Oil and Fat Analysis*, UKM Publishing, Bangi, Malaysia, 2006.

[42] D. I. Sánchez-Machado, J. López-Cervantes, J. López-Hernández, P. Paseiro-Losada, and J. Simal-Lozano, "High-performance liquid chromatographic analysis of amino acids in edible seaweeds after derivatization with phenyl isothiocyanate," *Chromatographia*, vol. 58, no. 3-4, pp. 159–163, 2003.

[43] J. U. Grobbelaar, "Microalgae mass culture: the constraints of scaling-up," *Journal of Applied Phycology*, vol. 24, no. 3, pp. 315–318, 2012.

[44] M. R. Tredici, N. Biondi, E. Ponis, L. Rodolfi, and G. Chini Zittelli, "Advances in microalgal culture for aquaculture feed and other uses," in *New Technologies in Aquaculture: Improving Production Efficiency, Quality and Environmental Management*, G. Burnell and G. Allan, Eds., pp. 610–676, CRC Press, Boca Raton, 2009.

[45] S. F. Li, A. Fanesi, T. Martin, and F. Lopes, "Biomass production and physiology of *Chlorella vulgaris* during the early stages of immobilized state are affected by light intensity and inoculum cell density," *Algal Research*, vol. 59, Article ID 102453, 2021.

[46] B. Ji, W. Zhang, N. Zhang, J. Wang, G. A. Lutzu, and T. Liu, "Biofilm cultivation of the oleaginous microalgae *Pseudochlorococcum* sp," *Bioprocess and Biosystems Engineering*, vol. 37, no. 7, pp. 1369–1375, 2014.

[47] P. Bohutskyi, D. C. Kligerman, N. Byers et al., "Effects of inoculum size, light intensity, and dose of anaerobic digestion centrate on growth and productivity of *Chlorella* and *Scenedesmus*

microalgae and their poly-culture in primary and secondary wastewater," *Algal Research*, vol. 19, pp. 278–290, 2016.
[48] M. M. El-Dalatony, Y. Zheng, M.-K. Ji, X. Li, and E.-S. Salama, "Metabolic pathways for microalgal biohydrogen production: current progress and future prospectives," *Bioresource Technology*, vol. 318, Article ID 124253, 2020.
[49] L. Xin, H. Hong-ying, G. Ke, and S. Ying-xue, "Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp," *Bioresource Technology*, vol. 101, no. 14, pp. 5494–5500, 2010.
[50] M. R. de la Peña, A. V. Franco, H. P. Igcasan Jr et al., "Microalgal paste production of the diatom *Chaetoceros calcitrans* using electrolytic flocculation method at optimum culture conditions," *Aquaculture International*, vol. 26, no. 4, pp. 1119–1134, 2018.
[51] Q. Hu, M. Sommerfeld, E. Jarvis et al., "Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances," *The Plant Journal*, vol. 54, no. 4, pp. 621–639, 2008.
[52] T. R. Parsons, K. Stephens, and J. D. H. Strickland, "On the chemical composition of eleven species of marine phytoplankters," *Journal of the Fisheries Board of Canada*, vol. 18, no. 6, pp. 1001–1016, 1961.
[53] F. M. I. Natrah, F. M. Yusoff, M. Shariff, F. Abas, and N. S. Mariana, "Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value," *Journal of Applied Phycology*, vol. 19, no. 6, pp. 711–718, 2007.
[54] E. B. Seraspe, S. Gabotero, M. R. De la Peña, I. G. Pahila, and E. C. Amar, "Evaluation of dietary freeze-*dried Chaetoceros calcitrans* supplementation to control *Vibrio harveyi* infection on *Penaeus monodon* juvenile," *Aquaculture*, vol. 432, pp. 212–216, 2014.
[55] M. R. Brown, S. W. Jeffrey, J. K. Volkman, and G. A. Dunstan, "Nutritional properties of microalgae for mariculture," *Aquaculture*, vol. 151, no. 1–4, pp. 315–331, 1997.
[56] T. C. Adarme-Vega, D. K. Y. Lim, M. Timmins, F. Vernen, Y. Li, and P. M. Schenk, "Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production," *Microbial Cell Factories*, vol. 11, no. 1, Article ID 96, 2012.
[57] T. Wang, X. Tian, T. Liu et al., "A two-stage fed-batch heterotrophic culture of *Chlorella protothecoides* that combined nitrogen depletion with hyperosmotic stress strategy enhanced lipid yield and productivity," *Process Biochemistry*, vol. 60, pp. 74–83, 2017.
[58] S. Tachihana, N. Nagao, T. Katayama et al., "High productivity of eicosapentaenoic acid and fucoxanthin by a marine diatom *Chaetoceros gracilis* in a semi-continuous culture," *Frontiers in Bioengineering and Biotechnology*, vol. 8, Article ID 602721, 2020.
[59] L. Tiez and E. Zeiger, *Plant Physiology*, Sinauer Associates Inc. Publishers, Sunderland, 5th edition, 2010.
[60] S. Rivero-Rodríguez, A. R. Beaumont, and M. D. C. Lora-Vilchis, "The effect of microalgal diets on growth, biochemical composition, and fatty acid profile of *Crassostrea corteziensis* (Hertlein) juveniles," *Aquaculture*, vol. 263, no. 1–4, pp. 199–210, 2007.
[61] M. J. Griffiths and S. T. L. Harrison, "Lipid productivity as a key characteristic for choosing algal species for biodiesel production," *Journal of Applied Phycology*, vol. 21, no. 5, pp. 493–507, 2009.
[62] A. A. Kolmakova and V. I. Kolmakov, "Amino acid composition of green microalgae and diatoms, cyanobacteria, and zooplankton (review)," *Inland Water Biology*, vol. 12, no. 4, pp. 452–461, 2019.
[63] E. Granum, S. Kirkvold, and S. M. Myklestad, "Cellular and extracellular production of carbohydrates and amino acids by the marine diatom *Skeletonema costatum*: diel variations and effects of N depletion," *Marine Ecology Progress Series*, vol. 242, pp. 83–94, 2002, https://www.jstor.org/stable/24866134.