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Optimization of inoculum cell concentration for enhanced lipid production in laboratory-scale cultivation of the marine microalga *Chlorella* sp. for biofuel applications

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Microalgae are considered valuable bioresources due to their ability to produce high lipid content and grow under a variety of environmental conditions, making them strong candidates for sustainable biofuel production. However, the economic feasibility of microalgae-based biofuels depends on optimizing growth conditions in large-scale cultivation systems. This study investigates the effects of varying inoculum cell concentrations on the growth, lipid yield, and fatty acid composition of the locally isolated microalga *Chlorella* sp. SW5 in 2 L and 5 L cultivation systems. The results indicate that higher inoculum concentrations generally enhance biomass accumulation, with the 2 L system achieving the highest growth rate of 0.42 $\pm$ 0.01 day^{-1} at an inoculum concentration of 10^6 cells/mL. Interestingly, while higher inoculum concentrations reduced lipid production in the 2 L system, the 5 L system showed the highest lipid yield (51.23% $\pm$ 4.71% dry weight) at the highest inoculum concentration (10^7 cells/mL). Despite its moderate growth rate, the 5 L culture with a starting inoculum concentration of 10^7 cells/mL was selected for fatty acid profiling due to its superior lipid yield and productivity. Gas chromatography-mass spectrometry (GC-MS) analysis revealed that the culture produced a total of 93.18% C14-C18 fatty acids, with a profile dominated by saturated (56.33%) and monounsaturated (16.85%) fatty acids, which are essential for biodiesel quality. These findings provide valuable insights into the potential for scaling up microalgal systems for commercial biofuel production, highlighting strategies to optimize productivity.

KEYWORDS

biofuel scalability, fatty acid profile, inoculum concentration, lipid production, microalgae

1 Introduction

The increasing scarcity of fossil fuel resources and rising levels of greenhouse gas emissions from fossil fuel combustion have driven research toward alternative, biomass-derived energy sources. Biomass, including agricultural crops such as corn, soybeans,

sugarcane, switchgrass, and woody plants, as well as algae and waste materials like agricultural residues, has become a focal point in the quest for sustainable biofuel production (Antar et al., 2021). According to the International Energy Agency (IEA), global biodiesel production has experienced remarkable growth, expanding from 806 million liters in 2000 to 21,400 million liters in 2011, and reaching approximately 80,000 million liters in 2020 (Hossain et al., 2020). The yield of biodiesel varies significantly depending on the feedstock used, with microalgae standing out for their exceptional productivity. Microalgae have the potential to produce between 5,000 and 15,000 gallons of biodiesel per acre annually, far surpassing the biodiesel output of other feedstocks (Griffiths et al., 2021).

Microalgae present numerous advantages as a biofuel feedstock, including high lipid content, rapid growth, and efficient carbon dioxide capture. Their cultivation can be carried out on non-arable land, particularly in rural areas where costs are lower, making them a viable and sustainable solution (Chin et al., 2023). As photosynthetic organisms, microalgae thrive in both marine and freshwater environments, and their photosynthetic process is more efficient than that of traditional biofuel feedstocks due to their simple cellular structure, high surface-to-volume ratio, and aquatic habitat. This combination enables microalgae to convert greater quantities of water, carbon dioxide, and nutrients into biomass (Chhandama et al., 2021). Microalgae can double their biomass every two to 5 days due to their rapid growth, and their lipid content ranges from 5% to 68% of their dry mass, depending on the species. As a third-generation biofuel feedstock, microalgae offer a significant advantage over other sources since they do not contain lignin, eliminating the need for the costly delignification processes commonly required in biodiesel production from other biomass feedstocks (Khoo et al., 2023).

Several species of microalgae, including *Botryococcus braunii* (Thurakit et al., 2022), *Nannochloropsis* sp (Palanisamy et al., 2023), *Dunaliella salina* (de Souza Celente et al., 2022), *Chlorella vulgaris* (Carino and Vital, 2022), and *Scenedesmus obliquus* (Trivedi et al., 2022), are known for their high production of hydrocarbons and lipids, making them excellent candidates for biodiesel production. Among them, members of the genus *Chlorella*, particularly *C. vulgaris*, are highly valued for biodiesel production and biomass recovery due to their rapid growth rate, high photosynthetic efficiency, and significant oil content (Abreu et al., 2023). One of the distinctive features of *C. vulgaris* is its ability to thrive in both freshwater and seawater environments. The use of marine *Chlorella* strains is especially appealing given the growing demand for fresh water, which is projected to increase by approximately 30% by 2050 (Gu et al., 2023). Marine *Chlorella* strains offer a unique advantage as they can be cultivated on non-arable land using saline water supplies. A study by Rautenberger et al. (2024) demonstrated that *C. vulgaris* cultivated in a medium with 50% seawater and 50% freshwater under mixotrophic conditions achieved optimal growth and lipid content, with lipids comprising 49% of its dry weight. The lipids were predominantly composed of palmitoleic and oleic acids, highlighting the potential for more sustainable biomass and lipid production. These attributes make marine *Chlorella* sp. an ideal candidate for large-scale biofuel production, further enhancing its commercial potential across various industries.

In previous studies, the primary focus of optimizing microalgal cultures has been to enhance both the quantity and quality of lipid production (Chin et al., 2023; Justine et al., 2023; Andrew et al., 2022; Ibrahim et al., 2022; Sani et al., 2021; Andrew et al., 2013). In this study, the optimization of growth conditions aims to maximize the overall productivity and efficiency of microalgae cultivation in larger laboratory-scale cultivation systems, with inoculum concentration being a key factor. Inoculum concentration plays a critical role in the cultivation of microalgae, influencing various aspects of growth and productivity (Bohutskyi et al., 2016). The concentration of inoculum directly affects the duration of the lag phase, growth rate, final biomass yield, and overall metabolite production. Lower inoculum concentrations can lead to extended lag phases and reduced growth rates due to the limited number of cells available for reproduction and nutrient metabolism. Conversely, higher inoculum concentrations typically shorten the lag phase and accelerate growth by providing a larger initial population of cells for reproduction and nutrient uptake (Zhang et al., 2017). However, excessively high inoculum concentrations can result in competition for light and nutrients, ultimately limiting cell growth and productivity (Bohutskyi et al., 2016). Therefore, optimizing inoculum concentration is essential for achieving maximum biomass yield and efficient resource utilization during microalgae cultivation.

Expanding microalgae cultivation from laboratory settings to commercial scale presents significant challenges, particularly in adapting operational conditions to larger systems (Mutanda et al., 2020). A gradual transition from small-scale to larger volumes is crucial to mitigate these challenges. Typically, scaling up is carried out in increments of 10, progressing from 10 mL to 100 mL, then to 1 L, 10 L, and beyond (Borowitzka and Vonshak, 2017). Producing the inoculum for large-scale commercial cultures is a critical and complex step that directly impacts the success of the cultivation process. This study aims to determine the optimal inoculum concentration for maximizing biomass growth and lipid production in 2 L and 5 L cultures of the marine microalga *Chlorella* sp., a locally isolated strain with significant potential for biofuel applications. The findings from this study are expected to provide valuable insights for enhancing microalgal biofuel production on an industrial scale.

2 Materials and methods

2.1 Microalga isolation and culture conditions

The marine microalga, *Chlorella* sp. SW5, was isolated from a seawater sample collected at the Borneo Marine Research Institute Fish Hatchery, Universiti Malaysia Sabah (6.03916, 116.11284). The microalga was successfully established in Walne's medium (Walne, 1970) and identified as *Chlorella* sp. based on the 18S ribosomal RNA gene using the primer pair, 18SCOMF1 (5′-GCTTGTCTCAAA GATTAAGCCATGC-3′) and 18SCOMR1 (5′- CACCTACGG AAACCTTGTTACGAC-3′) (Zhang et al., 2005). The DNA sequence was subsequently deposited in the NCBI GenBank (Acc. no.: PQ579654). The microscopic image of the microalga *Chlorella* sp. is shown in Figure 1. The *Chlorella* sp. cultures were maintained

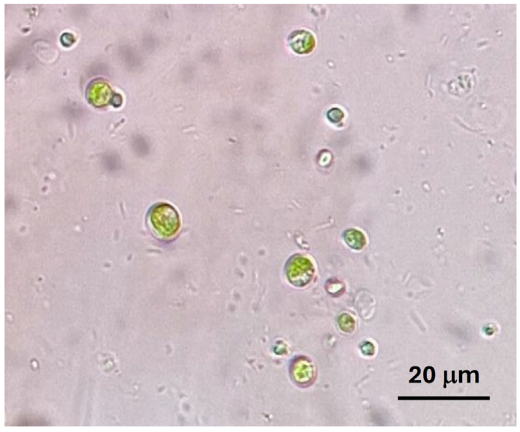

FIGURE 1
Microscopic image of the microalga *Chlorella* sp, showing its characteristic round shape and green pigmentation.

at a temperature range of 23°C–25°C, a 16:8 light/dark photoperiod, and a light intensity of 135 μmol/m^2/s (Chin et al., 2023) provided by 40W cool-white fluorescent light (AQUA-GLO, Japan). All experiments were conducted in triplicate (n = 3) in 2 L and 5 L culture volumes.

2.2 Inoculum concentrations in 2-liter and 5-liter culture medium

The initial inoculum concentrations were set at five levels (10^3, 10^4, 10^5, 10^6, and 10^7 cells/mL) for the 2 L and 5 L laboratory-scale culture systems. Inoculum concentrations were determined by cell counting using a hemocytometer. The microalgal cell inoculum were added into the media in order to achieve the desired five initial cell concentrations (10^3, 10^4, 10^5, 10^6, and 10^7 cells/mL). The culture conditions for this experiment were similar with the maintenance conditions, with each culture flask was aerated using an air pump to promote cell growth. In addition, the nitrate concentration in the Walne's medium was maintained at 5 g/L for this experiment (Chin et al., 2023).

2.3 Growth profile determination

The growth profile of the isolated *Chlorella* sp. was monitored by cell counting every 3 days until cell death was observed, using a hemocytometer (Sambrook and Russell, 2001). For cell suspension preparation, 100 μL of microalgal culture were diluted with 400 μL of Trypan Blue. Approximately 10 μL of the diluted culture were carefully dispensed onto both chambers of the hemocytometer under the coverslip. After a 5-minute incubation, cells were counted on both sides of the hemocytometer under a light microscope. The cell density (cells/mL) was calculated using Equation 1 and used to generate a growth curve. Additionally, the specific growth rate (μ) was determined using Equation 2.

$$\text{Density of cells (cells/ml)} = \text{Average count per square x dilution factor} \times 10^4 \quad (1)$$

$$\text{Growth rate}\,(\mu) = \text{In}(N_2/N_1)/(t_2 - t_1) \quad (2)$$

Where N_1 and N_2 represent the biomass at time t_1 (start point of cultivation) and t_2 (end point of cultivation), respectively.

2.4 Lipid content and lipid productivity determination

Lipid content was determined using a method described by Bligh and Dyer (1959). Microalgal cultures were harvested weekly for lipid analysis. A total of 50 mL of microalgal culture was collected into a pre-weighed tube and centrifuged. The resulting pellet was dried in an oven until a constant weight was achieved, then ground into a fine powder. A mixture of chloroform, methanol, and distilled water (1:1:1 ratio) was added to the tube, with each solvent measuring 1 mL. The mixture was vortexed and left to stand at room temperature for 1 h. The sample was then centrifuged at 5,500 ×g for 5 min, resulting in the formation of two layers. The top layer, containing methanol and water, was discarded, while the bottom layer, containing chloroform and lipids, was transferred to a pre-weighed microcentrifuge tube. Methanol was completely removed using a vacuum rotary evaporator (V-AQ mode) for 1.5 h. The extracted lipid was then weighed, and the lipid yield and lipid productivity were calculated using Equation 3 (Cordeiro et al., 2017) and Equation 4 (Nascimento et al., 2013), respectively.

$$\text{Lipid content (\%)} = [\text{extracted lipid extract (g)} / \text{dry biomass (g)}] \times 100\% \quad (3)$$

$$\text{Lipid productivity (mg/L/day)} = \text{lipid content (\%)} \times \text{biomass productivity} \quad (4)$$

2.5 Fatty acid methyl esters (FAMEs) composition determination

Only *Chlorella* sp. culture with the inoculum concentration that produced the highest lipid yield was selected for FAMEs composition analysis. Triplicate samples were sent to the UNIPEQ Holdings Sdn. Bhd., National University of Malaysia, for detailed FAME analysis. Total fat extraction was performed using a Soxhlet apparatus according to the AOAC 20th Edition (method 991.36). Fatty acid analysis was carried out using Gas Chromatography with Flame Ionization Detection (GC-FID) based on the AOAC 20th Edition: method 996.06.

2.6 Data analysis

All the experiments were conducted in triplicate, and the results were presented as means with standard deviations. Statistical analyses were conducted using SPSS software (version 21.0). Analysis of variance (ANOVA) was employed to assess the

experiments' data, and correlation analyses were used to determine the relationships between specific growth rate, lipid content, and lipid productivity with the different inoculum concentrations in the 2-liter and 5-liter cultivation systems. A p-value of <0.05 was considered statistically significant for all tests.

3 Results and discussion

Scaling up microalgae cultivation from small-scale to mass production presents significant challenges, as microalgae naturally thrive in low-density environments (Qin et al., 2023). Several factors, including the species of microalgae, culture conditions, types of cultivation, metabolic pathways, and the type of culture vessels, play critical roles in successful large-scale cultivation (Novoveská et al., 2023). In this study, the culture volumes of *Chlorella* sp. were increased from 250 mL to 2,000 and 5,000 mL, utilizing optimized culture conditions from previous research (Chin et al., 2023; Andrew et al., 2022). The microalgae's responses in terms of growth rate, lipid content, lipid productivity, and fatty acid profile were comprehensively analyzed to assess the effects of this volume expansion.

The growth curves and growth rates of *Chlorella* sp. in 2 L and 5 L culture media with different inoculum concentrations are depicted in Figure 2A, B and summarized in Table 1, Table 2, respectively. The effect of inoculum concentrations on specific growth rate was statistically significant in both 2 L ($F = 155.90$, $p < 0.05$) and 5 L ($F = 17.01$, $p < 0.05$) culture volumes. The results indicate that higher inoculum concentrations generally enhanced biomass accumulation in both culture volumes. Notably, the growth rates were higher in the 2 L culture system, ranging from 0.23 to 0.42 day^{-1} (Table 1), compared to the 5 L system, which exhibited growth rates between 0.11 and 0.21 day^{-1} (Table 2). The highest growth rate (0.42 day^{-1}) was observed in the 2 L culture system with an inoculum concentration of 10^6 cells/mL, while the lowest growth rate (0.11 day^{-1}) occurred in the 5 L system with a 10^4 cells/mL inoculum concentration. According to Figures 2A,B, all cultures transitioned almost immediately into the exponential growth phase, experiencing a very short lag phase of only one to 2 days, due to the high starting inoculum. The exponential phase lasted approximately 40–45 days for all cultures before entering the stationary phase for about 10 days. However, the 2 L culture with a 10^3 cells/mL inoculum concentration continued its exponential growth phase until day 60. Subsequently, all cultures transitioned into death phase.

Generally, inoculum size directly influences the number of microalgal cells available for replication, thereby affecting overall biomass production. A well-chosen inoculum size enables microalgae to rapidly establish a competitive advantage and adapt to their environment (Bohutskyi et al., 2016). However, excessively large inoculum sizes can introduce growth-limiting stresses due to nutrient depletion and light limitations. Cheng et al. (2018) observed that excessive inoculum sizes may lead to rapid cell growth, increased culture viscosity, and reduced dissolved oxygen levels, which can negatively impact metabolite synthesis. Therefore, selecting an optimal inoculum concentration range is crucial for minimizing retention time, enhancing nutrient recovery, and improving daily biomass productivity (Zhang et al., 2017).

The weekly lipid content and productivity of *Chlorella* sp. cultivated in 2 L and 5 L culture media with varying inoculum concentrations are presented in Figures 2C,D and summarized in Table 1, Table 2. Statistical analysis revealed that the effect of inoculum concentrations on lipid productivity was significant in both the 2 L ($F = 7.03$, $p < 0.05$) and 5 L ($F = 22.67$, $p < 0.05$) cultivation systems. However, there were no significant differences in lipid content across inoculum sizes in either the 2 L ($F = 0.835$, $p > 0.05$) or 5 L ($F = 0.581$, $p > 0.05$) systems. The results indicate that the *Chlorella* sp. isolate accumulated lipids in concentrations ranging from 42.27% to 51.23% dry weight (DW) and achieved lipid productivity between 73.91 and 137.81 mg/L/day at week 6 of cultivation (Table 1, 2). The highest lipid content (51.23% DW) and lipid productivity (137.81 mg/L/day) were observed in the 5 L culture system with an inoculum concentration of 10^7 cells/mL. In contrast, the lowest lipid content (42.27% DW) was recorded in the 2 L system with inoculum concentration of 10^6 cells/mL, while the lowest lipid productivity (73.91 mg/L/day) occurred in the 5 L system with an inoculum concentration of 10^5 cells/mL. As shown in Figures 2C,D, lipid accumulation peaked between weeks 6 and 7 (42.27–51.23 %DW) in both cultivation systems. These findings suggest that the optimal harvest period is between weeks 6 and 7, coinciding with the late exponential growth phase, where biomass reaches its maximum.

The lipid content of *Chlorella* sp. strains generally vary between 5% and 58%, depending on the specific strain and environmental conditions (Ru et al., 2020). In this study, *Chlorella* sp. exhibited lipid contents ranging from 42.27% to 51.23% DW in both the 2 L (Table 1) and 5 L (Table 2) cultivation systems. The elevated lipid content observed in this study can be attributed to a combination of nutrient stress, specifically limited nitrogen availability, and higher light intensity (135 $\mu mol/m^2/s$). The optimized culture conditions of nitrogen-limited media and high light intensity were adopted from a previous study by Chin et al. (2023), where increased lipid yield was observed in four microalgae species cultured in 5 L media. These conditions, including reduced nitrogen and high light intensity, have been shown to promote lipid accumulation by inducing metabolic shifts in the microalgae.

The nitrogen content in the media was reduced by preparing a nutrient stock with 5 g/L of $NaNO_3$, as described by Chin et al. (2023), in contrast to the original $NaNO_3$ concentration of 100 g/L. Under nitrogen-deficient conditions, algae undergo metabolic shifts, which promote the accumulation of lipids and starch in their biomass (Yaakob et al., 2021). Similarly, a study by Ratomski and Hawrot-Paw (2021) observed a two-fold increase in lipid content in *C. vulgaris* when cultured in nitrogen-deficient aquaculture waste. Griffiths et al. (2014) also highlighted the strong correlation between nitrogen limitation and increased lipid accumulation in *C. vulgaris*. Under nitrogen-replete conditions, the lipid content remained stable at 10%-12% throughout the growth period. However, when nitrogen availability was limited, *C. vulgaris* cultures exhibited a substantial rise in lipid content, reaching 50%–65% dry weight, depending on the initial nitrate concentration and the severity of nitrogen depletion (Griffiths et al., 2014).

In this study, *Chlorella* sp. was cultivated under a higher light intensity of 135 $\mu mol/m^2/s$ (Chin et al., 2023), compared to the typical 68 $\mu mol/m^2/s$ used in previous studies (Justine et al., 2023; Andrew et al., 2022; Ibrahim et al., 2022). Light intensity plays a crucial role in photosynthesis and lipid production, with higher intensities favoring the accumulation of neutral storage lipids, particularly triacylglycerols, while lower intensities promote the formation of polar lipids associated with chloroplast membranes (Wacker et al., 2016). Studies on microalgae such as *Scenedesmus*

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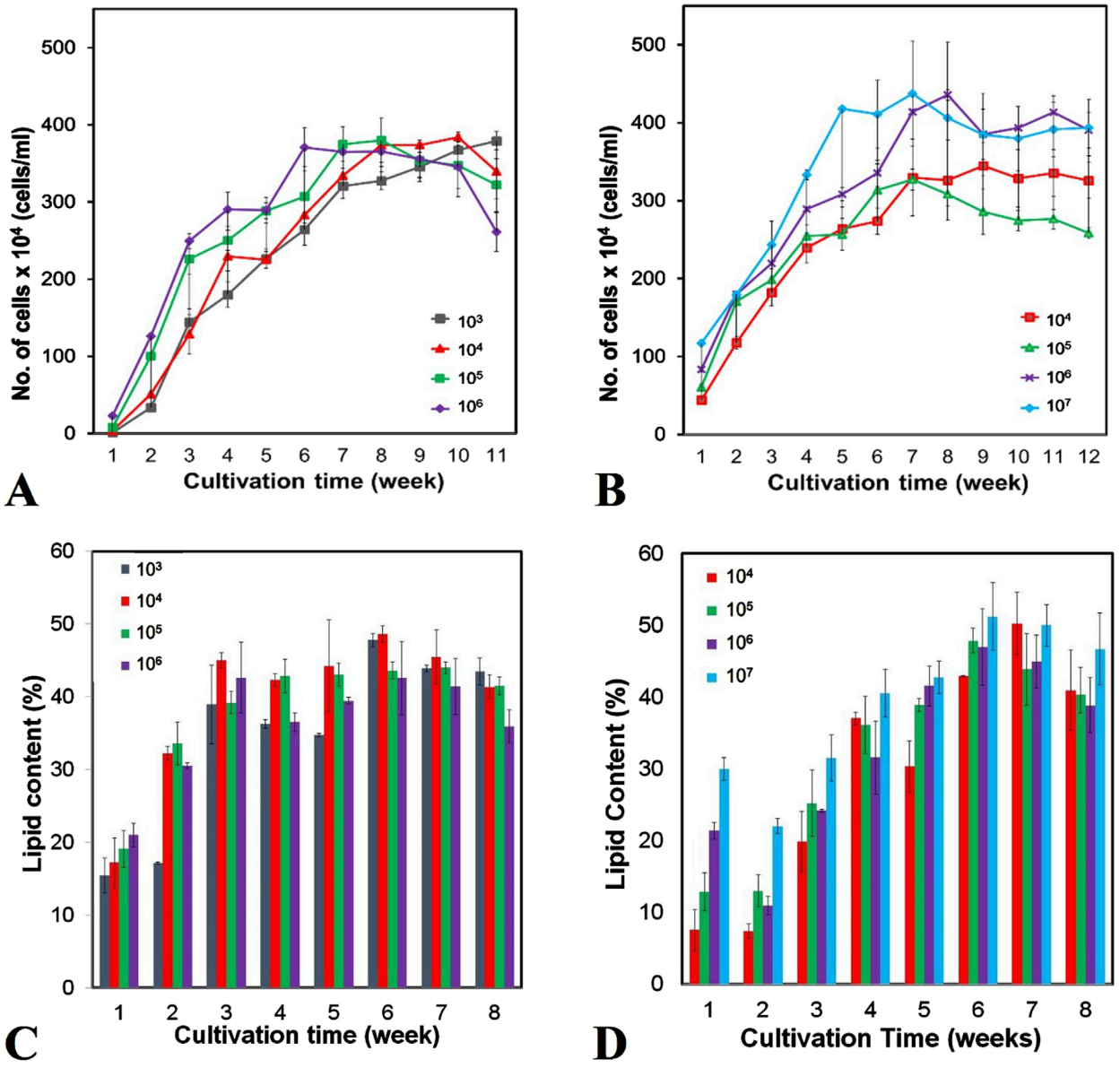

FIGURE 2
Growth curves **(A, B)** and weekly lipid content **(C, D)** of *Chlorella* sp cultured at different initial inoculum concentrations in 2-liter **(A, C)** and 5-liter **(B, D)** systems. Error bars represent the standard deviation of the mean (n = 3).

TABLE 1 Specific growth rate, lipid content, and lipid productivity of *Chlorella* sp. cultures at different inoculum concentrations in 2-liter cultivation system. Means (±SD) with different superscripts in the same column are significantly different.

Inoculum cell concentrations (cells/mL)	Specific growth rate, μ (day^{-1})	Dry biomass weight[a] (mg)	Lipid weight[a] (mg)	Lipid content[a] (% DW)	Lipid productivity[a] (mg/L/day)
10^3	0.23 ± 0.03[a]	90.00 ± 0.00	43.00 ± 0.82	47.78 ± 0.90[a]	102.39 ± 1.94[a]
10^4	0.35 ± 0.02[b]	105.00 ± 4.08	51.00 ± 0.82	48.64 ± 1.11[a]	121.43 ± 1.94[ab]
10^5	0.36 ± 0.01[b]	95.00 ± 4.08	41.50 ± 2.86	43.61 ± 1.13[a]	98.81 ± 6.80[ab]
10^6	0.42 ± 0.01[c]	95.00 ± 12.25	39.50 ± 0.41	42.27 ± 5.05[a]	94.03 ± 0.96[b]

[a]Values were recorded at week 6 of cultivation.

TABLE 2 Specific growth rate, lipid content, and lipid productivity of *Chlorella* sp. cultures at different inoculum concentrations in 5-liter cultivation system. Means (±SD) with different superscripts in the same column are significantly different.

Inoculum cell concentrations (cells/mL)	Specific growth rate, μ (day^{-1})	Dry biomass weight[a] (mg)	Lipid weight[a] (mg)	Lipid content[a] (% DW)	Lipid productivity[a] (mg/L/day)
10^4	0.11 ± 0.01[a]	85.00 ± 12.24	35.50 ± 5.30	42.93 ± 0.05[a]	127.81 ± 4.39[a]
10^5	0.16 ± 0.01[a]	65.00 ± 4.08	31.00 ± 0.81	47.86 ± 1.75[a]	73.91 ± 1.97[b]
10^6	0.13 ± 0.00[a]	113.50 ± 10.20	52.50 ± 1.22	46.98 ± 5.30[a]	122.46 ± 4.79[b]
10^7	0.21 ± 0.00[b]	105.00 ± 4.08	53.50 ± 2.85	51.23 ± 4.71[a]	137.14 ± 1.97[b]

[a]Values were recorded at week 6 of cultivation.

sp. have shown that increasing light intensity not only enhances lipid content but also boosts the production of key fatty acids, particularly C16 and C18 (He et al., 2015). Furthermore, a recent study by Liao et al. (2018) demonstrated that optimizing light and nutrient conditions during different growth phases of *C. vulgaris* can significantly increase lipid productivity, achieving a maximum of 163.42 mg/L/day in the stationary phase under a light intensity of 180 μmol/m^2/s and nutrient-starvation conditions.

The results in Table 1, Table 2 reveal that the cultivation systems with the highest growth rates did not correspond to the highest lipid yields or productivities. Notably, the 5 L system with an initial inoculum of 10^7 cells/mL achieved the highest lipid content (51.23% DW) and productivity (137.81 mg/L/day), despite exhibiting only a moderate growth rate (0.21 day^{-1}). Previous studies have shown an inverse relationship between biomass and lipid yield, highlighting the impact of specific culture conditions and nutrient availability on this relationship. This inverse correlation has been observed in species such as *Chlorophyta sorokiniana* (Griffiths and Harrison, 2009) and *Chlorella* sp. (Cho et al., 2020). A high specific growth rate in microalgae does not necessarily correlate with enhanced lipid production, as lipid accumulation primarily occurs during the stationary phase when biosynthesis shifts towards lipid storage. Therefore, lipid productivity and qualitative lipid composition are more critical parameters than growth rate for selecting species suitable for biodiesel production (Nascimento et al., 2013). The cultivation system with the highest lipid productivity in this study was the 5 L system with the starting inoculum of 10^7 cells/mL (137.81 mg/L/day), coinciding also producing the highest lipid content (51.23% DW). Given its superior lipid productivity and content, the 5 L system with 10^7 cells/mL inoculum was selected for further fatty acid methyl esters (FAMEs) analysis.

Table 3 provides a detailed analysis of the fatty acid compositions of *Chlorella* sp. cultivated in a 5-liter system with an initial inoculum concentration of 10^7 cells/mL. A total of 37 fatty acids (FAs) were analyzed, comprising saturated fatty acids (butyric, caproic, caprylic, capric, undecanoic, lauric, tridecanoic, myristic, pentadecanoic, palmitic, heptadecanoic, stearic, arachidic, henicosanoic, behenic, tricosanoic, and lignoceric acids), monounsaturated fatty acids (myristoleic, cis-10-pentadecenoic, palmitoleic, cis-10-heptadecanoic, elaidic, oleic, cis-11-eicosenoic, erucic, and nervonic acids), and polyunsaturated fatty acids (linolelaidic, linoleic, γ-linolenic, α-linolenic, cis-11,14-eicosadienoic, cis-8,11,14-eicosatrienoic, cis-11,14,17-eicosatrienoic, arachidonic, cis-5,8,11,14,17-eicosapentaenoic, cis-13,16-docosadienoic, and cis-4,7,10,13,16,19-docosahexaenoic acids). The FAs that significantly influence biofuel quality include C_{16} and C_{18} FAs, particularly palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) (Bharti et al., 2024). As detailed in Table 3, *Chlorella* sp. predominantly produced these candidate fatty acids, with palmitic acid (C16:0) comprising 39.46%, linoleic acid (C18:2) at 23.24%, oleic acid (C18:1) at 14.01%, stearic acid (C18:0) at 9.68%, and α-linolenic acid (C18:3) at 3.26%. These FAs are particularly preferred for biofuel production due to their favorable properties, such as oxidative stability and energy content (Khethiwe et al., 2020).

Another fatty acid worth noting for biofuel feedstocks is oleic acid (C18:1). This omega-9 monounsaturated fatty acid is a crucial component due to its multiple applications across various industries, including food, health, and chemicals. High oleic acid content is particularly beneficial for biofuel production, as it enhances the cold-flow properties of biodiesel and is advantageous for hydro-conversion processes in bio-jet fuel production (Choi et al., 2023). The presence of oleic acid in microbial oils contributes significantly to improving the quality and efficiency of biofuels. In this study, *Chlorella* sp. produced a moderate level of oleic acid at 14.01%. A similar finding was reported by Kreft et al. (2020), where *C. vulgaris* cultured in Bold's Basal Medium (BBM) produced 18% oleic acid under both autotrophic and heterotrophic conditions. There is ongoing research focused on enhancing oleic acid production in microalgae, particularly with the goal of achieving more than 50% oleic acid content. This is especially relevant in the development of algae-based edible oils, where higher oleic acid levels can significantly improve the quality and commercial viability of the oils (Kona et al., 2022).

Additionally, *Chlorella* sp. demonstrated a fatty acid profile highly favorable for biodiesel production, with 93.18% of its fatty acids falling within the C_{14}-C_{18} range, which is considered ideal for biofuel. The fatty acid composition included a relatively low percentage of polyunsaturated fatty acids (PUFAs) at 26.82%, balanced by an optimal concentration of saturated fatty acids (SFAs) at 56.33% and monounsaturated fatty acids (MUFAs) at 16.85%. This balance is crucial, as SFAs contribute to good oxidation resistance for biofuel (Longanesi et al., 2022), while the reduced PUFA content and moderate MUFA levels provide the necessary fuel properties, such as oxidative stability and cold flow characteristics, for efficient biodiesel performance (Kumbhar et al., 2022). When compared to previous studies on locally isolated microalgae *Chaetoceros muelleri* and *Isochrysis galbana* cultured under similar conditions (Chin et al.,

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TABLE 3 Fatty acid profile of *Chlorella* sp. in 5-liter cultivation system, with starting inoculum concentration of 10^7 cells/mL.

Structure	Type of fatty acids	Percentages of fatty acid (%)
C 4	Butryic	n.d
C 6	Caproic	n.d
C 8	Caprylic	0.02 ± 0.002
C 10	Capric	0.044 ± 0.003
C 11	Undecanoic	n.d
C 12	Lauric	0.35 ± 0.03
C 13	Tridecanoic	0.03 ± 0.02
C 14	**Myristic**	**1.20 ± 0.14**
C 15	Pentadecanoic	0.43 ± 0.02
C 16	**Palmitic**	**39.46 ± 5.85**
C 17	Heptadecanoic	1.43 ± 0.21
C 18	**Stearic**	**9.68 ± 1.46**
C 20	Arachidic	3.19 ± 0.53
C21	Henicosanoic	n.d
C 22	Behenic	0.17 ± 0.02
C 23	Tricosanoic	0.22 ± 0.23
C 24	Lignoceric	0.12 ± 0.20
C14:1	Myristoleic	0.07 ± 0.02
C 15:1	Cis-10-Pentadecenoic	0.02 ± 0.02
C 16:1	**Palmitoleic**	**2.33 ± 0.34**
C 17:1	Cis-10-Heptadecanoic	0.30 ± 0.08
C 18:1n9t	Elaidic (Trans)	n.d
C 18:1n9c	**Oleic**	**14.01 ± 2.31**
C 20:1n9	Cis- 11-Eicosenoic	n.d
C 22:1n9	Erucic	0.11 ± 0.19
C 24:1	Nervonic	n.d
C 18:2n6t	Linolelaidic (Trans)	n.d
C 18:2n6c	**Linoleic (Cis)**	**23.24 ± 2.25**
C 18:3n6	γ-Linolenic	0.32 ± 0.38
C 18:3n3	**α-Linolenic**	**3.26 ± 0.46**
C 20:2	Cis-11,14-Eicosadienoic	n.d

(Continued on the following page)

TABLE 3 (*Continued*) Fatty acid profile of *Chlorella* sp. in 5-liter cultivation system, with starting inoculum concentration of 10^7 cells/mL.

Structure	Type of fatty acids	Percentages of fatty acid (%)
C 20:3n6	Cis-8,11,14-Eicosatrienoic	n.d
C 20:3n3	Cis-11,14,17-Eicosatrienoic	n.d
C 20:4n6	Arachidonic	n.d
C 20: 5n3	Cis-5,8,11,14,17- eicosapentaenoic	n.d
C 22:2	Cis-13, 16-Docosadienoic	n.d
C 22:6n3	Cis-4,7,10,13,16,19-Docosahexaenoic	n.d
Total SFAs		56.33
Total MUFAs		16.85
Total PUFAs		26.82
Total FAs (C_{14}-C_{18})		93.18

Values are reported as mean percentage (± standard deviation) of fatty acids (n = 3).
Fatty acids highlighted in bold indicating the desired fatty acids for biodiesel (C_{14}-C_{18} FAs).
n.d. = not detected.

2023), *Chlorella* sp. exhibited higher levels of SFAs and PUFAs, although MUFA levels were lower. Despite this, the overall FA composition remains desirable for biofuel production, ensuring the robustness of the biodiesel derived from *Chlorella* sp.

4 Conclusion

This study successfully evaluated the growth, lipid content, and lipid productivity of *Chlorella* sp. in 2-liter and 5-liter cultures, using a locally isolated strain previously identified for its biodiesel potential. The study identified optimal inoculum concentrations, which resulted in satisfactory lipid yields, with growth rates ranging from 0.11 to 0.42 day^{-1}, lipid content between 42.27% and 51.23%, and lipid productivity from 73.91 to 137.81 mg/L/day. The 5-liter cultivation system, with an initial inoculum concentration of 10^7 cells/mL, emerged as particularly promising for biodiesel production, exhibiting the ideal fatty acid profile and balance of SFAs, MUFAs, and PUFAs for biofuel feedstock.

Data availability statement

This manuscript does not involve any research on animals, and therefore, no ethical approval was required for the study.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

EA-S: Formal Analysis, Investigation, Methodology, Writing–original draft, Writing–review and editing. GC: Conceptualization, Formal Analysis, Investigation, Supervision, Writing–original draft, Writing–review and editing. WY: Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Validation, Writing–review and editing. MM: Conceptualization, Data curation, Supervision, Validation, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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