High cell density cultivation of microalgae in novel thin-layer algal reactor

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Abstract

Microalgae are unicellular or simple multicellular photosynthetic microorganisms of rapid growth rate and high photosynthesis efficiency. They have been established as efficient cellular photoreactors with diverse applications in biomanufacturing and environment engineering. However, high algal farming costs have so far impeded large-scale growth of algal biomass and cell density culture (HCDC) of microalgae has shown great potential in reducing algal farming costs.

This thesis presents an in-depth study on the HCDC of microalgae using a novel horizontal thin-layer algal reactor with green alga *Neochloris oleoabundans* as the model species. Systematic analyses on the effects of culture medium composition including nitrogen (N) and dissolved inorganic carbon (DIC) concentration as well as cultivation conditions, e.g., culture pH, light intensity, culture thickness, and intermittent mixing, were conducted. Biomass concentration of up to 35 g/L and areal productivities of up to 16.32 g/m^2/day were achieved. About 98% savings in stirring energy was achieved by adopting a 10-min on and 2-hour off intermittent mixing strategy while obtaining algal growth kinetics similar to that of continuous mixing. It was demonstrated that an incident light intensity of 750 μmol photons/m^2/s and DIC of 80 mM or above were inhibitive to cell division while promoting enlargement of individual cells. A simple equation was proposed and validated to correlate the extinction coefficient with the biomass concentration, allowing more accurate modelling of the light attenuation of algal culture using the Beer-Lambert equation. It was also demonstrated that, when the experimental kinetic data were generated using the correctly, the Monod equation, which has been widely applied to modelling the growth kinetics of heterotrophic microorganism, could be used to model the photoautotrophic growth of microalgae with satisfactory accuracy, resolving a long-standing challenge in this field. It was also demonstrated that the conversion factor in the spectrometric determination of biomass concentration, which has been wide applied to suspended cultures of microbial, plant, and animal cells, was dependent on cell size and cell composition and should therefore be experimentally determined carefully.

Résumé

Les microalgues sont des micro-organismes photosynthétiques unicellulaires ou multicellulaires simples, caractérisés par un taux de croissance rapide et une efficacité photosynthétique élevée. Elles ont été établies comme des photoréacteurs cellulaires efficaces avec diverses applications dans la bioproduction et l'ingénierie environnementale. Cependant, les coûts élevés de la culture des algues ont jusqu'à présent freiné la production à grande échelle de biomasse algale. La culture à haute densité cellulaire (HCDC) des microalgues a montré un grand potentiel pour réduire les coûts de production des algues.

Cette thèse présente une étude approfondie sur la HCDC des microalgues en utilisant un nouveau réacteur algal horizontal à couche mince, avec l'algue verte *Neochloris oleoabundans* comme espèce modèle. Des analyses systématiques ont été menées sur les effets de la composition du milieu de culture, y compris la concentration en azote (N) et en carbone inorganique dissous (CID), ainsi que sur les conditions de culture, par exemple le pH du milieu, l'intensité lumineuse, l'épaisseur de la culture, et le mélange intermittent. Une concentration de biomasse allant jusqu'à 35 g/L et des productivités surfaciques allant jusqu'à 16,32 g/m²/jour ont été obtenues. Environ 98 % d'économies d'énergie de brassage ont été réalisées en adoptant une stratégie de mélange intermittent de 10 minutes activé et 2 heures désactivé, tout en obtenant une cinétique de croissance algale similaire à celle du mélange continu. Il a été démontré qu'une intensité lumineuse incidente de 750 µmol photons/m²/s et une concentration en CID de 80 mM ou plus étaient inhibitrices pour la division cellulaire tout en favorisant l'agrandissement des cellules individuelles. Une équation simple a été proposée et validée pour corréler le coefficient d'extinction avec la concentration en biomasse, permettant de modéliser plus précisément l'atténuation de la lumière dans la culture algale en utilisant l'équation de Beer-Lambert. Il a également été démontré que, lorsque les données cinétiques expérimentales étaient générées correctement, l'équation de Monod, largement appliquée à la modélisation de la cinétique de croissance des micro-organismes hétérotrophes, pouvait être utilisée pour modéliser la croissance

photoautotrophe des microalgues avec une précision satisfaisante, résolvant ainsi un défi de longue date dans ce domaine. Il a également été démontré que le facteur de conversion dans la détermination spectrométrique de la concentration en biomasse, qui a été largement appliqué aux cultures en suspension de cellules microbiennes, végétales et animales, dépend de la taille des cellules et de leur composition, et devrait donc être déterminé expérimentalement avec soin.

Copyright Statement

All materials in this thesis that have previously been published in journals allow their use in graduate thesis. Full references for the original publication are provided where required.

Statement of Contributions of Collaborators

I hereby declare that I am the sole author of this thesis.

I designed the methodologies of the project and performed all the experiments and data analysis. The scientific guidance and editorial comments for the written work were provided by my supervisors, Dr. Lan and Dr. Zhang.

Other sources of assistance in academic discussion and comments will be listed as co-authors in relevant journal papers.

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While obtaining my PhD marks a significant milestone, it is not the end but the beginning of an exciting path ahead. I am eager to apply what I have learned and to continue making impactful contributions to the world.

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Nomenclature, Abbreviations and Symbols

Nomenclature and abbreviations

ABRs	Attached biofilm reactors
C	Carbon
CA	Carbonic anhydrase
CCM	Carbon concentrating mechanism
CDI	Cell density increments
CF	Conversion factor
Chl-a	Chlorophyll a
Chl-b	Chlorophyll b
CO_2	Carbon dioxide
CO_3^-	Carbonate
CS	CO_2 saving
DCW	Dry cell weight
DIC	Dissolved inorganic carbon
dO_2	Dissolved oxygen
EPA	Eicosatetraenoic acid
Fe	Iron
FP-PBR	Flat Panel photobioreactor
GHG	Greenhouse gas
HCDC	High cell density culture
HCO_3^-	Bicarbonate
HFM	Hollow fiber membrane
HTLAR	Horizontal thin-layer algal
reactor	
HTLAR-HFMS	Horizontal thin-layer algal
reactor with hollow fiber membrane sparger	
LCA	Life-cycle assessment
LCDC	Low cell density culture

MBM	Modified Bristol Medium
Mg	Magnesium
N	Nitrogen
$NaHCO_3$	Sodium bicarbonate
O_2	Oxygen
P	Phosphorus
OD	Optical density
PAR	Photosynthetic active radiation
PBRs	Photobioreactors
PTFE	Polytetrafluoroethylene
PSII	Photosystem II
PUFA	Polyunsaturated fatty acid
RAP	Race Algal Ponds
ROS	Reactive oxygen species
RWP	Raceway Pond
S	Sulfur
SD	Standard deviation
S/V	Surface to volume ratio
TTLP	Tilted thin-layer PBR
TLC	Thin-layer culture
TLCR	Thin-layer cascade reactor
TLR	Thin-layer reactor
TPBR	Tubular photobioreactor
VC-PBR	Vertical column photobioreactor

Symbols

cells/mL	Cells per milliliter
g/L	Grams per liter
$g/m^2/day$	Grams per square meter per day
g/L/day	Grams per liter per day
h	Hour
LPM	Liters per minute
min	Minute
vvm	Volumes of gas per volume of liquid per minute
Y_r	the ratio of yield obtained from related thickness alteration strategy compared to that from continuous mixing
°C	Degree Celsius
$\mu mol/m^2/s$	Micromoles of photons per square meter per second

List of Publications

Peer-reviewed articles

1. **Hongying Zhou**, Zitong Xu, Liyuan Zhou, Zisheng Zhang, Ju Wang, Christopher Q. Lan*, "High cell density culture of *Neochloris oleoabundans* in novel horizontal thin-layer algal reactor: effects of localized aeration, nitrate concentration and mixing frequency" Biochemical Engineering Journal, 2023, published, (doi: https://doi.org/10.1016/j.bej.2023.108839).
2. **Hongying Zhou**, Ju Wang , Zitong Xu, Xinyue Wang, Zisheng Zhang, Christopher Q. Lan*, "Two-step cultivation of *Neochloris oleoabundans* in a novel horizontal thin-layer algal reactor: Interplay of pH and dissolved inorganic carbon", Biochemical Engineering Journal, 2024, published, (doi: https://doi.org/10.1016/j.bej.2024.109244).
3. **Hongying Zhou**, Ju Wang, Zisheng Zhang, Christopher Q. Lan*, "High cell density culture of microalgae in horizontal thin-layer algal reactor: modeling of light attenuation and cell growth kinetics" Chemical Engineering Journal, 2024, published, (https://doi.org/10.1016/j.cej.2024.154175).
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Conference presentations

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2. **Hongying Zhou**, Zisheng Zhang, Christopher Q. Lan*, Effects of pH and dissolved inorganic carbon on *Neochloris oleoabundans*: division, cell size, and biochemical composition of cells and a two-step cultivation strategy, the 2023 Canadian Chemical Engineering Conference (CSChE), oral presentation, October 2023, Calgary
3. **Hongying Zhou**, Zisheng Zhang, Christopher Q. Lan*, High cell density culture of microalgae in novel horizontal thin-layer algal reactor, 2024 Engineering Research Celebration Day Poster Competition, poster presentation, March 2024, Ottawa

Chapter 1 Introduction

1.1 Problem statement

Microalgae, microscopic photosynthetic organisms found in aquatic environments, have garnered significant interest due to their ability to produce a wide range of valuable compounds, including lipids [1], proteins [2], pigments [3] and carbohydrates [4]. These compounds have potential applications across various industries, such as biofuels [5], food [6], pharmaceuticals [7], and nutraceuticals [8], making microalgae an attractive subject of study [9]. However, the large-scale application of microalgae is limited by high production costs, a significant portion of which arises from the cultivation process [10]. Traditional cultivation methods, such as open ponds [11], often struggle with issues like contamination, while both open ponds and photobioreactors [12] face challenges with suboptimal light distribution and inefficient nutrient utilization [13]. These limitations significantly affect the yield and economic feasibility of microalgae production [14]. Therefore, there is a pressing need for more efficient cultivation techniques to fully realize the potential of microalgae.

One promising approach to overcoming these challenges is high cell density cultivation (HCDC). This method involves increasing the concentration of microalgae cells within a given volume, thereby enhancing light and nutrient utilization efficiency [6]. HCDC can also reduce contamination risks and offer more controlled environmental conditions. Consequently, these improvements not only boost biomass production but also lower the overall costs associated with large-scale cultivation. To advance HCDC, innovative reactor designs and cultivation strategies are essential. Exploring and optimizing these advanced techniques will be crucial in unlocking the full potential of microalgae.

Thin-layer culture (TLC) is an advanced technique for cultivating microalgae that involves growing the cells in a very shallow layer of culture medium [15]. This method has garnered attention due to its potential to maximize light utilization. In traditional deep cultures, light penetration is often a limiting factor, as light intensity diminishes with depth, preventing the cells at certain depth below the surface from receiving

sufficient light. By contrast, in TLC systems, the shallow depth ensures that all microalgae cells are exposed to adequate light, promoting more uniform and efficient photosynthesis [16]. This setup can lead to increased growth rates and higher overall productivity. One of the most common and typical reactors used in thin-layer culture is the thin-layer cascade reactor (TLCR), which operates as an open system [17]. This type of reactor allows for a continuous flow of culture medium over a series of shallow, inclined surfaces, ensuring that the microalgae receive consistent light exposure and nutrient availability [18].

However, thin-layer culture also faces several significant challenges. One major issue is the need for continuous stirring or circulation to maintain uniform light exposure and nutrient distribution. This process is energy-intensive, significantly increasing the operational costs. Additionally, TLC require continuous circulation and mixing of culture to ensure optimal conditions, which drives up labor and maintenance costs. Currently, HCDC faces several challenges:

1. **Inadequacies in reactor design**: Achieving HCDC requires innovative reactor designs that minimize energy input and reduce costs. Current reactor designs suffer from several issues, such as dependence on continuous stirring, high operational costs, and significant manual maintenance requirements. These drawbacks highlight the need for more efficient and cost-effective solutions.

2. **Complex nutritional and environmental control requirements**: Optimizing the cultivation conditions, including light, pH, temperature, and the supply and ratio of various essential nutrients, is crucial. This is especially important in continuous growth systems where timely replenishment of depleted nutrients is necessary to maintain sustained growth.

1.2 Research objectives

The overarching goal of this research is to achieve autotrophic HCDC of microalgae while minimizing energy input. To accomplish this, the following specific objectives have been identified:

1. **Innovative reactor design**: Develop and optimize open TLC systems to improve energy efficiency and operational feasibility in open systems. This includes designing reactors that reduce the need for continuous stirring and manual maintenance, thereby lowering overall costs.
2. **Medium optimization**: Refine the composition of the culture medium by optimizing key nutrients such as carbon and nitrogen sources. This aims to enhance the growth rate and biomass yield of microalgae under HCDC conditions.
3. **Cultivation Conditions**: Adjust and control critical cultivation parameters including culture thickness, light intensity, and stirring frequency. The goal is to create an environment that supports optimal microalgae growth and maximizes productivity.
4. **Modelling**: Develop predictive models for light distribution and nutrient uptake within the cultivation system. These models will help in refining cultivation strategies to ensure uniform growth and efficient resource utilization.
5. **Analysis and summary**: Summarize the pathways and methodologies developed to achieve HCDC. Conduct comprehensive economic and technical analyses to evaluate the feasibility and scalability of the proposed solutions. This includes assessing the cost-effectiveness and potential industrial applications of the optimized HCDC system.

The microalgae strain used in this research is *Neochloris oleoabundans*, known for its high oil content. For experimental consistency, all aspects of the project will utilize this strain.

1.3 Thesis structure

This thesis is organized into seven chapters. Chapter 1 introduces the background and significance of the research, outlines the project theme, and provides an overview of the thesis structure. Chapter 2 presents a comprehensive review of the technologies

and methodologies for achieving HCDC of microalgae and examines the key cultivation conditions and their impacts on microalgal growth. Chapters 3 to 6 detail the experimental work conducted to achieve HCDC using newly developed thin-layer reactors, each focusing on different factors influencing HCDC. Specifically, Chapter 3 describes the design of a novel open thin-layer reactor and validates the feasibility of intermittent stirring, while also exploring the effects of key nutrients, particularly nitrogen, on microalgal growth. Chapter 4 investigates the effects of pH and dissolved inorganic carbon (DIC) on microalgal growth and includes the design of a two-step cultivation method to reduce the dependence of microalgae on pH and CO_2 supply. Chapter 5 examines the distribution of light within TLC, optimizing light intensity to achieve high cell density microalgal growth, and includes the development and refinement of light distribution models and the exploration of microalgal growth models. Chapter 6 explores the impact of culture thickness on microalgal growth and investigates strategies for dynamically adjusting thickness during cultivation to maximize production. Finally, Chapter 7 summarizes the key findings of the research and discusses future pathways for achieving and optimizing HCDC.

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Chapter 2 Literature Review: High cell density cultivation of microalgae under autotrophic conditions: strategies, challenges, and future directions

Abstract

Microalgae hold significant commercial potential due to their diverse applications, yet the economic feasibility of their commercial-scale production is constrained by relatively high cultivation costs. Enhancing algal productivity while concurrently reducing these costs remains a pivotal challenge, with high cell density cultivation (HCDC) identified as a critical strategy. Achieving HCDC in autotrophic conditions presents unique challenges compared to heterotrophic methods, primarily due to the complexities of light and nutrient management. This paper systematically reviews the strategies and inherent challenges of implementing HCDC in the cultivation of microalgae. It begins with a critical evaluation of bioreactors currently capable of supporting HCDC, identifying shared features that contribute to their efficacy. Subsequent sections delve into the key factors that influence HCDC outcomes, including light distribution and nutrient management, highlighting both their pivotal roles and the operational difficulties they present. Through an integrated analysis, the paper outlines strategic approaches to optimize these factors, emphasizing the necessity of minimizing energy inputs to enhance sustainability. The paper suggests that leveraging innovative technologies and refining HCDC protocols under stringent sustainability criteria represents the most promising avenue for scaling up microalgae production to meet global demands.

Key words: High cell density culture, microalgae, bioreactor design, culture systems, key nutrients

2.1 Introduction

Microalgae, representing an incredibly diverse group of unicellular organisms, stand as a testament to nature's ingenuity and have sparked considerable interest as a sustainable resource. These microscopic powerhouses are capable of performing photosynthesis more efficiently than terrestrial plants, converting sunlight, carbon dioxide, and nutrients into valuable biomass with remarkable rapidity and efficiency [1,2]. This process not only positions microalgae as a potential cornerstone in the transition to renewable energy via biofuel production but also plays a vital player in carbon sequestration efforts, contributing to the mitigation of climate change by absorbing significant amounts of CO_2 from the atmosphere [3]. The versatility of microalgae extends to a multitude of sectors that benefit from their rapid growth and the rich variety of compounds they can produce. In the energy sector, they are seen as a promising solution for the sustainable production of biofuels, offering an alternative to fossil fuels that is both renewable and capable of reducing greenhouse gas emissions [4]. In the realm of nutrition, microalgae are a source of high quality proteins, essential fatty acids like omega-3s, and various vitamins, making them a superfood that can address malnutrition and support food security [5,6]. Moreover, the pharmaceutical industry looks towards microalgae as a biofactory for the production of bioactive compounds, including antioxidants, pigments, and other molecules with therapeutic properties [7–9]. Their ecological role is equally significant, as they contribute to the health and balance of aquatic ecosystems, and their extensive use in wastewater treatment showcases their ability to remediate environmental contamination. By integrating microalgae into these systems, we can harness their natural propensity for growth and nutrient uptake, turning waste into value-added products and encouraging in a true circular economy fashion [4,10].

The rising interest in microalgae is matched by the pursuit of high cell density cultivation (HCDC) methods, aiming for substantial biomass yield and productivity. The collective drive stems from the pursuit of a sustainable and scalable production model that is responsive to the increasing demands of a growing population [11]. Such

a shift towards intensive cultivation, if coupled with precise bioprocessing and environmental condition optimization, promises to make large scale commercial applications more attainable, particularly in land-limited contexts. Many researchers focus on heterotrophic or mixotrophic approaches to achieve HCDC. However, autotrophic cultivation remains the preferred method due to its sustainability and efficiency in utilizing light as an energy source, thereby harnessing the natural photosynthetic capabilities of microalgae for biomass production. Despite its advantages, few studies have systematically summarized the strategies and outcomes of HCDC under autotrophic conditions.

Achieving HCDC through autotrophic methods presents a greater challenge compared to heterotrophic cultivation. Heterotrophic cultivation allows for higher cell densities more easily because microalgae can utilize organic carbon sources directly for growth, independent of light, enabling dense and rapid biomass accumulation, which can be costly and less environmentally friendly compared to using sunlight and atmospheric CO_2. In heterotrophic cultivation, a cell concentration ranging from 50 to 100 g/L is considered HCDC [12] whereas no such established benchmark exists for autotrophic cultivation. Some studies define high cell density as a cell concentration in a range of 10g/L to 20g/L [13], while the highest reported concentration for autotrophic cultivation is 50 g/L [14]. However, such intensive cultivation systems are face challenges. As the cell density in these systems increases, so too does the shading effect, where algae at the surface absorb most of the light, leaving the cells below deprived and thus limiting overall photosynthetic efficiency [15]. This can necessitate the implementation of sophisticated light management strategies to ensure even light distribution and prevent the loss of productivity [16]. Nutrient limitations also become a significant hurdle in HCDC setups. The swift uptake of available nutrients by densely populated algal cultures can lead to deficiencies, stunting growth and affecting biomass quality [17]. Precise nutrient dosing, potentially coupled with real-time monitoring, is essential to maintain optimal growth rates and desired biochemical compositions. Furthermore, the need for advanced harvesting techniques becomes more pronounced

as the cell density rises. HCDC requires more efficient separation processes to recover the algal biomass from the culture medium, which is often complicated by the small size and low settling velocity of microalgae cells [18,19]. Developing cost-effective and scalable harvesting methods is crucial to ensure the economic viability of high algal biomass production [16]. Addressing these challenges is central to the advancement of microalgae as a sustainable source of biofuels and high-value compounds. It demands a multidisciplinary approach, harnessing insights from biology, engineering, and economics to innovate and refine HCDC systems that can sustainably meet global demands [20].

Acknowledging the multifaceted challenges inherent in scaling up microalgal production, this review intentionally focuses on autotrophic HCDC. Such systems harness the natural photosynthetic process of microalgae to generate biomass, emphasizing sustainability and scalability for commercial viability. Central to this discussion are the key factors that drive the efficiency of HCDC systems. Effective light management is crucial in overcoming the self-shading effect that hampers photosynthesis in dense cultures. Optimal nutrient strategies are also paramount, as they ensure the continuous growth and health of the algal biomass. Additionally, the review will examine the innovations in bioreactor design that have emerged to support HCDC. To conclude, the paper will synthesize the contemporary landscape of microalgae HCDC and project into the future, pinpointing possible avenues for research and innovation. The spotlight will be on emergent technologies with the potential to bolster productivity and expand the scale of operations, highlighting the integral role of microalgae in the progression towards a sustainable future.

2.2 Algal bioreactor for HCDC

Bioreactors, serving as the engineered environment for microalgae growth, are fundamental in determining the productivity and feasibility of cultivation systems. As the demand for algal products increases, optimizing bioreactor design for HCDC has become imperative. This involves not just maximizing biomass yield but also ensuring sustainability and economic viability. The evolution of bioreactor technology has been

driven by the need to overcome challenges inherent in microalgae cultivation, such as achieving uniform light distribution, efficient nutrient supply, and effective gas exchange, especially in dense cultures where these factors become critically limiting. This section of the review delves into the technological strides made in bioreactor designs, specifically tailored for HCDC. It encompasses an overview of the different types of bioreactors employed, discusses those particularly suited for HCDC, and addresses the key design considerations and challenges faced in optimizing these systems.

2.2.1 Overview of bioreactor types

Bioreactors play a crucial role in microalgae cultivation, with the type of system significantly impacting the growth, yield, and quality of the produced biomass. Broadly, these systems are classified into open and closed types, each having distinct features suited for different cultivation conditions**.**

2.2.1.1 Open systems

The practice of cultivating microalgae in open systems is a method steeped in history, combining simplicity with effectiveness. Recognized for their cost-efficiency and low maintenance demands, open culture systems have become a staple in large-scale algal production [21,22]. Open systems are a cornerstone in microalgae cultivation, marked distinctly by their direct interaction with the natural environment. Contrasting with the isolated and meticulously controlled conditions of closed systems, open bioreactors operate in harmony with external conditions, making their operation and design quite unique.

One of the defining characteristics of open systems is their utilization of natural conditions. By leveraging sunlight and ambient CO_2, they significantly reduce the dependence on artificial light sources and external carbon sources. This natural approach not only aligns closely with sustainable practices but also makes open systems particularly cost-effective. They typically necessitate lower capital investments and operational costs in comparison to their closed counterparts, as has been concisely summarized in Table 2-1. However, they also face challenges in control over conditions

and risk of contamination. Furthermore, the simplicity inherent in the design and operation of open systems renders them more manageable, especially on a large scale. This simplicity extends to their maintenance, often requiring less technical expertise and resources. Additionally, the scalability of these systems is a notable advantage. Their straightforward design allows for relatively easy expansion, adapting to increasing demands for microalgae production. The combination of natural integration, cost-effectiveness, ease of maintenance, and scalability makes open bioreactor systems a valuable method in the realm of microalgae cultivation.

Predominantly, the industry utilizes various configurations of open systems, including circular pond [23–25], raceway ponds [26], and thin-layer cascades reactor (TLCR) [27,28], each presenting unique advantages in terms of operational cost and productivity. Circular ponds, often favored for their straightforward design, offer a viable solution for moderate-scale algae farming. On the other hand, raceway ponds, characterized by their looped race track-like design, have emerged as a preferred choice in many large-scale operations due to their optimal balance between cost-efficiency and productivity [29–31].These ponds facilitate better control over algal growth conditions compared to circular ponds, yet remain relatively simple to construct and operate. However, in recent years, thin layer cascades have gained attention for their notably higher yield [32]. Despite their higher setup costs compared to traditional raceway ponds, the increased productivity of TLCR systems renders them an attractive option for high-volume algae production.

2.2.1.2 Closed photobioreactors (PBRs)

In recent decades, the development of advanced closed culture systems, commonly referred to as photobioreactors (PBRs), has been a focus of extensive research. PBRs are designed to be isolated from the atmosphere, typically enclosed with transparent materials or contained within transparent tubing [33]. This design significantly reduces the risks of external contamination and offers superior control over growth conditions. In comparison to open culture systems, PBRs boast several advantages: they are more efficient in terms of culture productivity, offer less environmental pollution, have a

large specific surface area, prevent evaporation, and facilitate effective gas-liquid mass transfer [34]. Despite their advantages in controlled cultivation, PBRs often entail higher costs in construction and operation, as compared to open systems, which is also highlighted in Table 2-1.

The most prevalent PBR designs include vertical columns (VC-PBRs), tubular PBRs (T-PBRs), and flat panel PBRs (FP-PBRs) [35]. However, the higher costs associated with constructing and operating these closed systems mean that their commercial application has been largely confined to small-scale operations. These operations typically focus on producing high-value algal products, such as those used in cosmetics and nutraceuticals, including labeled compounds and polyunsaturated fatty acids (PUFAs) for pharmaceutical purposes [21,36]. The construction of a PBR is guided by several fundamental criteria: efficient light utilization, circulation, mass transfer capabilities, construction materials, and temperature control [37]. Overcoming the challenges associated with these factors is crucial for enabling commercial-scale production and expanding the range of microalgal species that can be cultivated in various locations [33]. Recently, the diversity of closed culture systems has expanded, encompassing various types such as tubular PBRs [38], vertical column PBRs [39], flat panel PBRs [40], floating PBRs [41], membrane PBRs [42], and plastic bag PBRs [43], among others. Each of these systems offers unique features and advantages, catering to specific cultivation requirements and applications.

Table 2-1. Comparative analysis of open system and closed photobioreactor in microalgae cultivation

	Open Systems	Closed Systems (PBRs)
Exposure to environment	Exposed to natural conditions	Isolated from the atmosphere
Control over conditions	Limited control	Highly controlled
Risk of contamination	Higher risk	Reduced risk
Cost	Lower capital and operational cost	Higher capital and operational cost
Scalability	Easier to scale up	Challenging to scale up
Suitability for high-value products	Less suitable	More suitable
Energy efficiency	Varies, generally lower	Higher due to controlled environment

2.2.2 Bioreactors suitable for high cell density culture

The success of HCDC hinges on the choice of bioreactor, as the design and operational features of these systems play a crucial role in determining the overall efficiency of microalgae cultivation. In this context, various bioreactor designs have been developed and optimized to meet the specific demands of HCDC. These bioreactors are engineered to provide optimal conditions for microalgae growth, including efficient light distribution, adequate gas exchange, and precise environmental control. Up to this point, among the reactors extensively employed for microalgae cultivation, four types have been identified as particularly effective in facilitating HCDC: Tubular PBRs, Flat panel PBRs (FP-PBRs), Biofilm reactor and Thin-layer cascade reactor (TLCR). Table 2-2 provides a detailed summary of the performance of these four reactors in microalgae cultivation. A shared key characteristic among them is their relatively low culture thickness, which is crucial for ensuring sufficient light exposure to the microalgae. This reduced culture thickness often results in achieving higher biomass concentrations. Nevertheless, each of these four reactors possesses its unique set of attributes and limitations. When embarking on HCDC, selecting an

appropriate reactor that aligns with the specific environmental and experimental conditions is crucial. The following sections delve into the four types of bioreactors that have been effectively employed in HCDC, highlighting their design principles, operational characteristics, and the productivity they have achieved in practical applications.

Table 2- 2. Performance metrics of four types of reactors in microalgae cultivation

Algal species	Culture thickness (mm)	Nutrients supply mode	DCW(g/L)	P_{Amax}(g/m^2/d)	Culture system	Reactor Type	Refs
Nannochloropsis salina	5.6	Fed-batch	50	25	Open	TLC	(A. C. Apel et al., 2017)
Chlorella vulgaris strain R117	6	Batch	18	27	Open	TLC	(Rearte et al., 2021)
Arthrospira platensis	10	Fed-batch	/	> 20	Open	TLC	(Silva Benavides et al., 2017)
Microchloropsis salina	5.5 ± 1.4	Batch	15.4	10	Open	TLC	(Schädler et al., 2019)
Scenedesmus obliquus	5	Fed-batch	20.14	12.1	Open	TLC	(Venancio et al., 2020)
Scenedesmus obliquus	10	Fed-batch	14.6	18.5	Open	TLC	(Venancio et al., 2020)
Scenedesmus sp	20	Semi-continuous	6.5	28.3-47.3	Open	TLC	(Sánchez Zurano et al., 2020)
Neochloris oleoabundans	6	Batch	8	21.42	Open	HTLAR	(Zhou et al., 2023)
Nannochloropsis salina	6	Fed-batch	33	17	Open	TLC	(Pfaffinger et al., 2019)
Nannochloropsis	14	Batch	4.7	7.14	Closed	FP-PBR	(Hulatt et al., 2017)
Nannochloropsis gaditana	20	Batch	5.27	14.8	Closed	FP-PBR	(Pfaffinger et al., 2016)
Scenedesmus acutus	38	Semi-continuous	/	19.0±0.6	Closed	FP-PBR	(Eustance et al., 2016)

Scenedesmus obtusiusculus	22	Fed-batch	13.5	19	Closed	FP-PBR	(Koller et al., 2018)
Scenedesmus ovalternus	22	Fed-batch	19.5	14	Closed	FP-PBR	(Koller et al., 2018)
Dunaliella salina	80	Semi-continuous	/	17.85 ± 0.55	Closed	FP-PBR	(Khadim et al., 2018)
Poterioochromonas malhamensis	50	Batch	15.7	39.25	Closed	FP-PBR	(Hinterholz et al., 2019)
Nannochloropsis gaditana	90	Continuous	/	33.1	Closed	FP-PBR	(San Pedro et al., 2016)
Chlorella sp. GN1	100	Batch	2.12	22.8	Closed	FP-PBR	(Feng et al., 2020)
Spirulina sp.	85	Batch	3.5	18.7	Closed	T-PBR	(de Morais and Costa, 2007)
C. pyrenoidosa	90	Batch	2.1	59.85	Closed	T-PBR	(Tan et al., 2016)
Chlorella PY-ZU1	25	Batch	5.13	/	Closed	HT-PBR	(J. Xu et al., 2020)
Chlorella PY-ZU1	25	Batch	5.5	/	Closed	HT-PBR	(Cheng et al., 2019)
Chlorella PY-ZU2	25	Batch	3.59	/	Closed	HT-PBR	(Xu et al., 2021)
Neochloris oleoabundans	10	Batch	1.6	7.3	Closed	HT-PBR	(Norsker et al., 2021)
Consortium PA6	0.4-0.6	Batch	/	15-20	Closed	ABRs	(Choudhary et al., 2017a)
Consortium PA6	0.364	Batch	24	4	Open	ABRs	(Choudhary et al., 2017b)
Scenedesmus sp. LX1	/	Batch	121.5	10	Open	ABRs	(Xu et al., 2017)
Chlorella vulgaris	0.13-2	Batch	130	7	Open	ABRs	(Boelee et al., 2014)
7 strains	0.01-0.015	Batch	10-35	2.9	Closed	ABRs	(Genin et al., 2015)

2.2.2.1 Tubular PBRs (T-PBRs)

Tubular Photobioreactors (T-PBRs), typically constructed from glass or plastic, are a prominent choice in high cell density culture due to their efficient design. These reactors utilize tubes through which the culture medium is circulated, either by pumps or air streams such as airlift systems) [44]. T-PBRs are diverse in their configuration and can be categorized into three primary groups: serpentine, manifold, and helical. Both serpentine and manifold types offer various arrangements like horizontal, vertical, inclined, or conical, providing flexibility in adapting to different cultivation conditions [45]. While T-PBRs are advantageous in offering a large light surface area and being well-suited for outdoor cultivation of microalgae, they do require significant energy input. The energy is primarily needed for liquid circulation through the tubes and, to a lesser extent, for aeration, harvesting, and water recycling. The estimated energy consumption for biomass production in T-PBRs is about 15 kWh per day per cubic meter of reactor [46]. However, the advancement of T-PBRs is not without its challenges. A notable issue is the tendency of algal cells to adhere to the inner walls of the tubes, a phenomenon that can significantly impede optimal light distribution and nutrient uptake, essential for maintaining HCDC. Additionally, the complexity of accurately analyzing oxygen levels within the reactor presents a technical hurdle [47]. This is particularly critical in T-PBRs, where optimal oxygen concentration is key to sustaining high rates of photosynthesis and growth. Despite these challenges, the ability of T-PBRs to support HCDC underscores their significance in the field of modern microalgae cultivation practices, making them a valuable asset in the pursuit of sustainable and efficient biomass production.

2.2.2.2 Flat panel PBRs (FP-PBRs)

Flat panel reactor is an alternative closed reactor, which can adjust the best light direction according to the change of sunlight intensity and incident direction to increase light transmittance and realize HCDC [35]. The main advantages of this design are the possibility of achieving high area/volume ratios (usually 16 to 80 m^{-1}) and high-volume

biomass yields. Eustance et al. conducted a study comparing the biomass productivity of two *Scenedesmus acutus* strains (*LB 0414 and LB 0424*) in semicontinuous cultivation in outdoor FP-PBRs and raceway ponds. The study found that FP-PBRs showed significantly higher average biomass productivity (19.0±0.6 g/m^2/day) compared to raceways (6.62±2.3 g/m^2/day). Additionally, the photosynthetic efficiency in flat-panel photobioreactors ranged between 1.32 and 2.24%, while in raceways, it was between 0.30 and 0.68% [48]. Moreover, Hulatt et al. found in their study on *Nannochloropsis 211/78* that, when cultivated in optimized FP-PBRs, the microalga achieved a maximum biomass concentration of 4.7 g/L and a productivity rate of 0.51 g/L/day [49]. Nonetheless, FP-PBRs come with distinct drawbacks, most notably their tendency to foster biofilm buildup. The broad, flat surfaces that characterize these reactors offer a conducive setting for the formation of biofilms. This often results in uneven light dispersion across the reactor surface, causing shading areas that can substantially impede the growth and productivity of the algae. Such light obstruction is a critical issue as it directly influences the photosynthetic efficiency of the algae, crucial for their development and yield. Koller et al. discovered on scaling up biomass production with Scenedesmus species that transitioning from lab to pilot scale in a 300 L flat-plate gas-lift photobioreactor system resulted in reduced biomass productivity, mainly due to biofilm formation. However, with nutrient supplementation, *S. ovalternus SAG 52.80* still achieved a biomass concentration of 19.6 g/L and an areal productivity of 197 g/m²/day, highlighting the complexities of microalgal biomass production scalability [50]. The flat design can impede efficient gas exchange and uniform nutrient distribution, especially in HCDC. This limitation is associated with the reduced surface area for gas exchange and potential uneven distribution of essential elements like CO_2 and nutrients [51].

2.2.2.3 Attached biofilm reactors (ABRs)

Attached biofilm reactors (ABRs) represent a significant advancement in the field of microalgae cultivation, offering a novel approach to biomass production. Unlike traditional suspended growth systems, ABRs involve the growth of microalgae on

surfaces or carriers, enabling the formation of dense biofilms [52]. This mode of cultivation leverages the natural tendency of certain microalgae to adhere to surfaces, forming structured communities [53]. The principle of ABRs lies in maximizing the exposure of these biofilms to light and nutrients, which enhances the growth rates and overall biomass productivity [54]. One of the critical benefits of this technology is its potential for HCDC. By optimizing the surface area and ensuring effective distribution of light and nutrients, ABRs can achieve higher cell densities than conventional suspension cultures, making them particularly advantageous for applications like biofuel production. For instant, Zhang et al. demonstrated that attached cultivation significantly enhances biomass productivity of Spirulina platensis, achieving up to 60 $g/m^2/day$ in outdoor bioreactors, while maintaining its nutritional quality [55].Wang et al. also found that cultivating *Arthrospira platensis* in a 10 m^2 experimental system for two months yielded an average biomass productivity of 38 $g/m^2/day$, and an overall CO_2 usage efficiency of 75.1%, underscoring the promising potential of biofilm attached cultivation technology for industrial-scale carbon capture, utilization, and storage application [56].

The advantages of ABRs extend beyond just increased biomass yield. The structure of the biofilms allows for more efficient light penetration and nutrient uptake, thereby improving the overall efficiency of the system. Additionally, the risk of contamination is significantly reduced in ABRs compared to open-pond systems, owing to the contained nature of the biofilm environment [57]. Another notable benefit is the ease of harvesting, as separating microalgae from biofilms can be simpler and more cost-effective than traditional methods used in suspension cultures [58]. However, there are several challenges and limitations associated with ABRs. One of the primary concerns is the risk of biofilm overgrowth, which can hinder light penetration and nutrient distribution within the biofilm, thereby affecting productivity. Operational complexity is another significant issue, as maintaining optimal conditions such as pH, temperature, and nutrient levels in ABRs can be challenging. Additionally, scaling up ABR systems for commercial applications presents technical and economic challenges that are

currently being addressed in ongoing research. The current research in ABR technology is focused on optimizing biofilm formation, enhancing biomass productivity, and developing scalable and efficient systems. Innovations in carrier materials, flow regimes, and lighting strategies are being explored to maximize the efficiency of ABRs. There's also an emerging interest in integrating ABRs with wastewater treatment, where microalgae can play a dual role in biomass production and nutrient removal from effluents [35,59,60].

2.2.2.4 Thin-layer cascade reactor (TLCR)

The thin-layer cascade reactor (TLCR) is a pivotal innovation in the realm of microalgae cultivation, a field that has garnered significant attention for its potential in HCDC. The concept of TLCR originated from the need to address certain limitations inherent in traditional microalgae cultivation systems, such as open ponds and deep-tank PBRs. Initially conceptualized in the late 1950s in Czechoslovakia, TLCR technology has undergone various developmental phases, each contributing to its efficiency and productivity. The initial TLCR designs, characterized by shallow sloped troughs, aimed at maximizing solar irradiation and gas exchange, subsequently evolved to more sophisticated designs by incorporating features to enhance biomass density and productivity [61]. Central to the TLCR's design is its utilization of a series of cascading, shallow channels or layers, through which the algal culture is continuously circulated [62]. It ensures a low depth and fast flow of culture, leading to a high ratio of exposed surface to culture volume, which is beneficial for light absorption and photosynthetic efficiency [63]. The design facilitates rapid light/dark cycling frequencies for cells, crucial for maximizing biomass productivity. Modern TLCR units are known for operating at high biomass densities and achieving high volumetric productivity [28,64,65]. This is complemented by the ability to store culture in retention tanks during unfavorable conditions, thus maintaining continuity in cultivation. TLCRs combine the benefits of open systems, such as direct sun irradiance and simple cleaning, with the advantages of closed systems, including operation at high biomass densities. Silva Benavides and colleagues conducted a comparative study of open circular ponds (OCP)

and TLCR for cultivating *Arthrospira platensis* outdoors [66]. Their findings revealed that cultures grown in TLCR exhibited approximately 20% higher photosynthetic activity compared to those in OCP, achieving the highest biomass productivity of over 20 g/m²/day in TLCR under optimal temperature conditions. Additionally, replicating the outdoor climate in a controlled indoor setting was found to be beneficial for maximizing yield and cell density, enhancing the controllability of the experiment [14,67].

However, the application of TLCR systems has revealed significant challenges. One of the key issues is the need for a powerful circulation system to maintain the culture in a thin layer, which not only increases energy consumption but also poses a risk of mechanical wear. Additionally, maintaining a consistent culture system is challenging, which can lead to uneven light distribution, pH and temperature gradients as well as inadequate mass transfer capacity and impact photosynthesis efficiency [68,69] If the culture is not mixed uniformly, algae can settle at the bottom of the channels, leading to reduced light penetration and inefficiencies in growth. This sedimentation not only affects overall biomass productivity but also complicates maintenance, as settled algae can be difficult to remove and may lead to system blockages. Maintenance and operation of TLCR systems require substantial effort. Regular cleaning and monitoring are crucial to prevent biofouling and maintain optimal conditions.

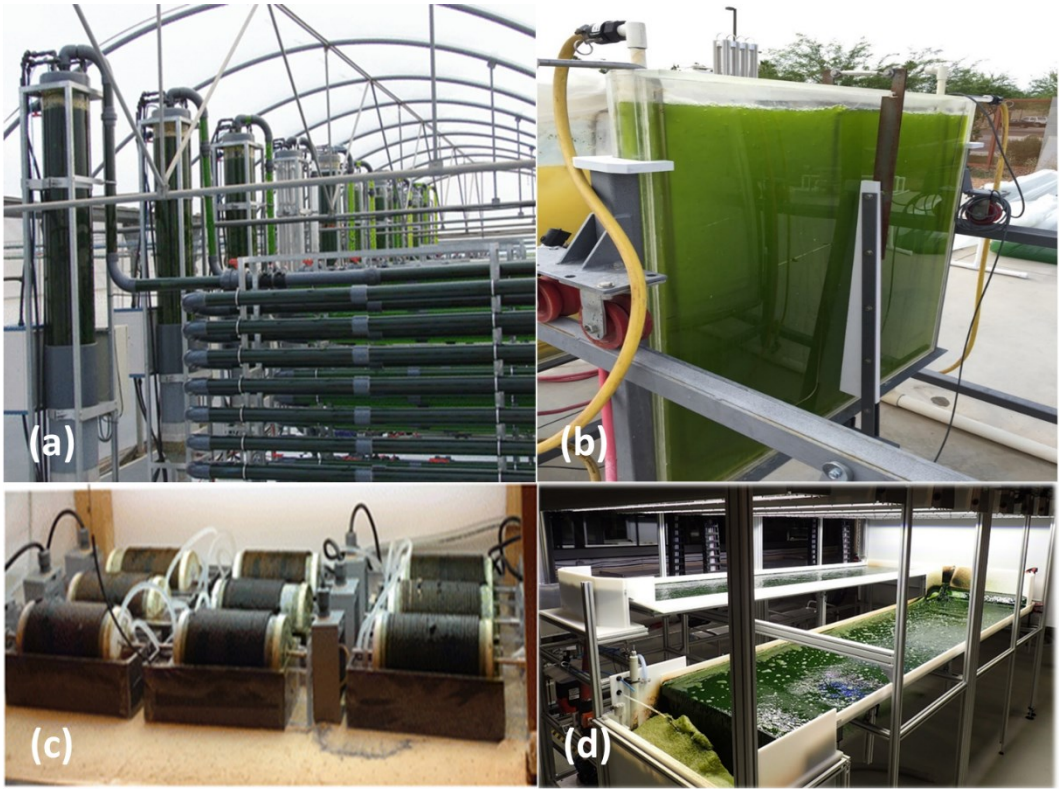

Figure 2-1. Four typical photobioreactors suitable for HCDC: Tubular PBRs [70](Copyright © 2014 American Chemical Society), Flat panel PBRs [71](Copyright © 2016 Elsevier), Attached biofilm reactor [72] (Copyright © 2016, Zachary T. Fica et al) and thin-layer cascade reactor [14] (Copyright © 2017 Elsevier).

2.2.3 Comparing PBR Designs

As depicted in Table 2-3, a comprehensive summary of the distinct features of each reactor type is provided. An obvious commonality is that all four reactors are very thin in thickness. The cultivation thickness typically remains below 5 cm, evident in systems like flat panel photobioreactors, where it ranges from 0.5 to 2.5 cm, TLC at about 6 mm, and biofilm reactors approximately 1 mm thick. Such reduced cultivation thickness is pivotal as it ensures ample and relatively even light distribution, which is essential for the optimal algal growth. Regarding T-PBRs and FP-PBRs, they possess the drawbacks of closed systems, such as high costs and gas exchange issues, which limit their microalgae cultivation density and make large-scale production challenging. Although ABRs can achieve biomass concentrations up to 100 g/L, challenges such as uneven nutrient distribution and complex harvesting processes can limit their practical application in large-scale microalgae cultivation. These limitations stem from the

biofilm structure, which creates microenvironments with variable access to nutrients and light, and the physical nature of biofilms, which makes biomass recovery more labor-intensive than in suspension cultures. TLCR is currently an excellent candidate in terms of either cost or potential of achieving HCDC. However, the manual maintenance input cannot be overlooked. In an open system, the evaporation of water is not favorable for ultra-thin cultivation, requiring constant monitoring of water levels. Additionally, the continuous stirring required for circulation means that energy input may not be less than that in closed systems.

Table 2- 3. Comparison of four typical algal photobioreactors for HCDC

	T-PBRs	**FP-PBRs**	**ABRs**	**TLCR**
System type	Closed	Closed	Open/Closed	Open
Culture thickness (cm)	0.5-5	0.5-2.5	0.01-0.1	0.2-1
Biomass concentration (g/L)	0.5-10 [73]	1-15 [74]	90-150 [75]	15-50 [14]
Scalability	Low	Moderate	Low	Moderate
Cost	High	High	Variable	Low

2.3 Light management in HCDC

In the practice of microalgae HCDC, the role of light emerges as a crucial factor for promoting algal growth. It not only significantly influences the photosynthesis, productivity, cellular composition, and metabolic pathways of algae, but also affects the economic efficiency of the algae cultivation process [76]. This is particularly evident as increasing algal cell density tends to result in a significant reduction in light availability, consequently impeding growth rates. Thus, it becomes clear that in HCDC, managing light is inherently more complex compared to LCDC scenarios. Accordingly, this chapter will delve into an in-depth analysis of light's impact within HCDC environments and will discuss effective strategies to address the issue of light inadequacy.

2.3.1 Light effects on algal culture

Light is pivotal in microalgae cultivation, primarily driving photosynthesis, where microalgae convert light energy into chemical energy. This process, essential for microalgal growth, follows the Eq.1:

$$6CO_2 + 6H_2O + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2 \qquad (1)$$

Effective light capture and utilization are critical for optimizing microalgal biomass production. Current research indicates that light intensity [77], photoperiod [78], light spectral range [79], and flashing light [80] can all affect the growth and cellular composition of microalgae. Among these factors, light intensity is the most important for HCDC, as it is the most likely to become a limiting factor.

2.3.1.1 Light intensity

In the realm of microalgal cultivation, the role of light intensity transcends mere illumination which is a pivotal factor dictating photosynthetic efficiency, cellular metabolism, growth rates, and the composition of cellular nutrients [81–83]. Optimal light intensity catalyzes photosynthesis, enhancing the conversion of light into chemical energy, which is fundamental for microalgal growth and productivity. The majority of microalgae species are adapted to a light intensity range typically between 60 to 700 μmol/m^2/s [84]. This optimal light regime not only influences the rate of energy production but also plays a crucial role in regulating metabolic activities, thereby affecting the biosynthesis of vital macronutrients such as proteins, lipids, and carbohydrates within the cells. It's important to recognize that optimal light intensity for microalgae cultivation varies significantly with the cultivation reactors and species type. Chaiklahan et al. found that the highest productivity of biomass and phycocyanin was achieved under a light intensity of 2300 μmol/m^2/s when investigated the cultivation of *Arthrospira platensis* in photobioreactors under various light intensities and cell concentrations [85]. However, in the case of *Amphiprora sp.*, it was observed that cultivation at an optimal light intensity of 24 μmol/m^2/s resulted in the attainment of the highest biomass concentration, measuring 0.2567 ± 0.003 g/L, along with a maximal lipid content constituting 52.94% of the dry biomass [77]. This phenomenon is attributed to the variance in cultivation reactor depth and cell density, which result in

a heterogeneous distribution of light intensity within the microalgae culture. Despite being the same algal species, these disparities necessitate different optimal light intensities. While pinpointing the best initial light intensity is important, examining the average light intensity received during algal growth might offer deeper insights. Consequently, determining the optimal light intensity for each species should be tailored to the cultivation method, incorporating an understanding of both specific and average light intensities.

The advantageous effects of illumination in microalgal cultivation are subject to certain thresholds, beyond which light can become deleterious. Excessively high light intensity may precipitate photoinhibition, a state characterized by the impairment of the photosynthetic machinery, notably diminishing light energy conversion efficiency [86,87]. This impairment predominantly manifests as damage to the photosystem II (PSII) reaction centers, a critical component of the electron transport chain in photosynthesis. Moreover, excessive illumination can provoke oxidative stress, culminating in the production of reactive oxygen species (ROS) that inflict damage on cellular structures and impede growth [88]. In contrast, light intensity that falls below the optimal threshold poses distinct challenges. Insufficient illumination fails to fulfill the energetic requisites for efficient photosynthesis, resulting in inhibited growth rates. This energy deficit directly impacts metabolic processes, curtailing the biosynthesis of vital macronutrients and decelerating overall cellular proliferation and development.

Hence, achieving an equilibrium in light intensity is pivotal in microalgal cultivation. This necessitates a comprehensive understanding of the interaction between light and the photosynthetic apparatus, as well as the wider metabolic pathways within the cells. The primary objective is to adjust the light conditions to optimize photosynthesis and growth, whilst avoiding the adverse effects of photoinhibition and oxidative stress. Such optimization paves the path for effective and sustainable practices in microalgal cultivation.

2.3.1.2 Photoperiod

The photoperiod, a cycle of alternating light and dark, is crucial for microalgae growth. In the light phase, microalgae perform photosynthesis, absorbing carbon dioxide and releasing oxygen, which facilitates energy accumulation and biomass growth. The dark phase, in contrast, often leads to the consumption of some stored energy. The challenge is to identify an ideal photoperiod for each microalgae species, as this can significantly enhance growth and efficiency. In a study investigating the influence of photoperiods on *Dunaliella salina* (CCAP 19/30), it was found that longer light exposure, including cycles like 4/20, 8/16, 12/12, 16/8, 20/4 hours of light/dark, and continuous light, significantly enhanced growth rates and cell densities, suggesting that nearly continuous light might be most beneficial for this microalgae's growth [78]. However, longer light exposure is not always better, and it also depends on the algae specious. Sirisuk and his team discovered that different light/dark cycles significantly affected the growth of microalgae species, with continuous 24-hour light being most effective for *Nannochloropsis salina* and *Phaeodactylum tricornutum*, while an 18:6 hour light/dark cycle was optimal for *Isochrysis galbana* [89]. Similar study was found that *Chlorella kessleri* achieved maximum biomass under a 20L:4D light/dark cycle, surpassing continuous light exposure, because a dark phase was necessary for synthesizing essential metabolic molecules from ATP and NADPH, which continuous light did not facilitate [90]. Additionally, the photoperiod influences the production of valuable microalgal compounds like fatty acids [91] and pigments [92]. Therefore, mastering photoperiod management not only boosts microalgae biomass but also optimizes the production of high-value by-products, making it a key factor in microalgae cultivation.

2.3.1.3 Light spectral range

Microalgae primarily utilize the visible light spectrum, ranging from 400 to 700 nm, for their growth and metabolic processes [93]. This preference is rooted in the absorption characteristics of their photosynthetic pigments, which are most responsive to light within this range. Visible light provides the energy necessary for photosynthesis, the key process driving their growth and nutrient synthesis. Beyond this spectrum,

ultraviolet (UV) light, with wavelengths shorter than 400 nm, can be harmful to microalgae, potentially causing cellular damage and inhibiting growth due to its high energy [94]. Infrared light, with wavelengths longer than 700 nm, generally has minimal direct effect on microalgae as it lies beyond their photosynthetic absorption spectrum and contributes more to heating the water than to photosynthesis [95]. Within the visible spectrum, different wavelengths have distinct effects on microalgae: blue light (400-500 nm) is highly efficient for photosynthesis and encourages rapid growth and the production of certain secondary metabolites [96]. Red light (600-700 nm) also enhances photosynthesis and is particularly effective in increasing pigment concentration, like chlorophyll, which is essential for energy capture [97]. In microalgal cultivation practices, it is common to implement an interplay of red and blue light spectra, either in a synchronized blend [98] or through a methodical alternation [99], thereby optimizing growth conditions by leveraging the distinct photosynthetic efficiencies and biological reactions these specific wavelengths induce in the algae. Green light (500-600 nm), while absorbed less efficiently, can still positively influence the growth of some algae species under specific conditions. It was reported that green light stress was imposed to trigger increased accumulation of lipids [100]. The varied response to different wavelengths underscores the importance of the light spectrum in microalgal biology.

Adjusting the spectral range for optimal microalgae growth involves several innovative techniques. In the realm of artificial lighting, a variety of sources such as LED lights [101], fluorescent bulbs [102], and halogen lamps [103] are employed to cultivate microalgae [104]. LEDs, in particular, stand out for their ability to provide targeted wavelengths, such as specific blues and reds that are most beneficial for microalgal growth [99,105]. The spectrum of LEDs can be easily adjusted to optimize the light quality and intensity, making them ideal for indoor microalgae cultivation. Fluorescent and halogen lamps, while less efficient in spectral tuning, can still be used in combination with filters to modify the light spectrum. When utilizing sunlight in outdoor cultivation, the challenge lies in managing the broad spectrum it offers.

Utilizing spectral filters [106] and shading nets [107], which selectively attenuate or block wavelengths such as ultraviolet or infrared light, proves to be an effective strategy in microalgae cultivation for modulating the light spectrum. This approach, by filtering out wavelengths that are either suboptimal or deleterious to microalgal growth, facilitates the creation of an enhanced photic environment [108]. Additionally, integrating emerging technologies like selectively wavelength-permeable photovoltaic cells presents a revolutionary approach in the field of microalgae cultivation [79]. It optimizes light exposure for growth while converting unused solar wavelengths into electricity, effectively combining efficient cultivation with sustainable energy production. Such advancements underscore the potential for more efficient and environmentally friendly microalgae farming practices.

2.3.1.4 Flashing light

Flashing light, distinct from the concept of light/dark cycles, is characterized by its rapid and periodic on-and-off illumination, and it plays a crucial role in microalgal cultivation. Unlike constant light exposure, flashing light involves alternating periods of light and darkness, which can enhance photosynthetic efficiency in microalgae [109]. This enhancement is primarily due to the reduced risk of photoinhibition and the optimized use of absorbed light energy [80,110]. During the light phases, microalgae absorb light and generate energy-rich compounds like ATP and NADPH through the light reactions of photosynthesis. Then, in the dark phases, these compounds are utilized more effectively in the Calvin cycle, or dark reactions, for carbon fixation. This intermittent exposure not only prevents the oversaturation of photosynthetic machinery but also ensures a more balanced energy distribution within the cells. It was found that in cultivation of *Chlamydomonas reinhardtii*, light/dark cycles shorter than 12 seconds led to an increase in biomass growth rate, while cycles longer than 40 seconds showed negligible effects on growth compared to continuous light, indicating that shorter L/D cycles positively affect photosynthetic efficiency [111].

The impact of flashing light on microalgae also extends to the improved distribution of light in dense cultures and reduced overall energy consumption in large-

scale cultivation systems. One study on *Chlorella vulgaris* revealed that the microalgae could thrive under super high light intensity (e.g. 7000 μmol/m^2/s) when exposed to specific frequencies of flashing light, without experiencing the detrimental effects usually associated with such high light conditions [112]. Based on these findings, it can be suggested that combining flashing light with high light intensities could be an effective strategy for HCDC of microalgae, as it mitigates the risk of photoinhibition typically encountered under intense illumination. By adjusting the frequency, duration, and intensity of flashing light, it is possible to significantly optimize microalgal growth and productivity, making it a key consideration in algal biotechnology.

2.3.2 Light distribution and limitation in HCDC

In microalgae HCDC, understanding light distribution is essential for optimal light use and biomass production. As the cell density increases, the resultant reduction in light penetration necessitates a strategic and precise management of light exposure. Thus, studying light distribution is vital for advancing microalgal cultivation, particularly in large scale, commercial contexts, by enhancing productivity and ensuring sustainability.

Empirical observations [113–115] from numerous studies have consistently demonstrated an exponential decay of light intensity with increasing depth and algal density, a phenomenon that is critical for understanding and optimizing light utilization in algal cultivation systems. The attenuation of light within microalgal suspensions is governed by the optical properties of the medium, which are in turn influenced by factors such as the thickness of the photobioreactor and the biomass concentration of microalgae. The Beer-Lambert model [116] is the most commonly employed framework to describe light attenuation in microalgae cultures, indicating an exponential decline in light intensity as a function of culture thickness and cellular density. With increasing algal cell density and culture deepness, the light attenuation exacerbates, markedly diminishing the light availability for the lower strata of the culture, thus presenting light limitation as a critical impediment to achieving HCDC. However, the original Beer-Lambert model often struggles to conform to the actual

light propagation scenarios, leading to numerous modifications of the model [117,118] This is because the Beer-Lambert model assumes a homogeneous medium and linear absorption, which may not accurately represent the complex interactions between light and microalgae in a dense culture. Factors such as scattering, refraction, and non-linear absorption can significantly affect light distribution. Additionally, the spatial distribution of cells and the presence of other particulates can further complicate the light penetration, necessitating adjustments to the model for a more accurate depiction of the light attenuation in microalgae cultures. However, most of these modifications are targeted at LCDC with thicknesses of a few centimeters, whereas in reality, at HCDC, even thicknesses of a few millimeters can result in no light penetration. This leads to a lower applicability of their models for HCDC scenarios. While existing models like the Cornet model [119] and fuzzy-logic-based models [120] consider a range of factors including microalgal pigments, reactor geometry, and light incidence angles, they often tend towards complexity.

2.3.3 Light management strategy

Fixed light intensity is commonly used in laboratory-scale algal illumination due to its convenience and ease of operation [121,122]. However, this approach presents several challenges throughout the cultivation process. For instance, in the later stages of cultivation, as the cell density increases, light may not sufficiently penetrate to the bottom of the culture, resulting in inadequate illumination. Conversely, in the early stages of cultivation, the light intensity may be excessively high, leading to photoinhibition. To overcome these challenges, the method of using variable light intensity has been introduced and applied. Wahal et al. found that batch cultures of *Neochloris oleoabundans* grown under sequentially increasing light intensities tailored to culture density achieved up to double the biomass concentration compared to those grown under constant light, maintaining growth rates while using photons more efficiently [123]. This approach requires the light intensity to be adjusted in accordance with cell growth, which can be calculated using the Beer-Lambert model. While effective, this method faces limitations in outdoor cultivation settings, where natural

light fluctuates and cannot be controlled. In a study on *Synechocystis sp.*, it was observed that the optimal growth was achieved at an average irradiance (I_{av}) of 930 μmol/m^2/s, highlighting its high tolerance to light and suitability for outdoor cultivation. However, exposure to higher I_{av} levels resulted in photoinhibition, suggesting that adapting to lower light conditions could potentially enhance productivity in dense cultures where light availability is a limiting factor because cells become more efficient in the use of light [124].

Outdoor cultivation of microalgae using natural sunlight is an effective and sustainable approach that harnesses the abundant energy of the sun. Unlike indoor systems that require artificial lighting, outdoor cultivation systems can significantly reduce energy costs and environmental impact [125]. However, managing these systems involves addressing challenges such as fluctuating light intensities due to weather conditions and potential contamination from open environments. A study found that the use of light guide systems in high-rate ponds for outdoor microalgae cultivation to improve light distribution and increase biomass productivity by reducing dark zones where photosynthesis is limited, and by maintaining more of the culture within optimal light levels for photosynthesis [126]. In high light conditions, outdoor microalgae cultivation faces the significant challenge of photoinhibition. A study using solar trackers in photobioreactors revealed that optimizing light exposure effectively mitigates photoinhibition in low-density cultures and enhances photosynthetic activity in denser cultures, thereby balancing light absorption and temperature to maximize growth and productivity [127].

Consequently, the development of new light management strategies is essential. One such strategy involves adjusting the thickness of the culture system to modify light penetration, thereby accommodating the illumination needs of different stages of cultivation. This innovative approach may offer new avenues for HCDC of microalgae, optimizing light conditions to enhance growth and product accumulation.

2.4 Nutrient effects and supply

In HCDC, the effective management of key nutrients is essential for sustaining rapid growth and achieving high biomass yield. This chapter focuses on the critical role of nutrient supply and optimization strategies in dense cultivation environments. It discusses how tailored nutrient delivery can address the unique challenges of rapid consumption and nutrient balancing, essential for the health and productivity of microalgal cultures.

2.4.1 Key nutrients

Sustainable algal culture hinges on providing a diverse range of nutrients, as reflected by their elemental composition in biomass (dry weight), typically encompassing carbon (30%–50%), oxygen (30%–50%), hydrogen (3%–7%), nitrogen (4%–9%), phosphorus (1%–3%), and trace amounts of other elements such as sulfur, potassium, magnesium, and calcium [44]. In order to obtain the exactly amount need for the culture to meet the best culture requirement, process operation should be done under nutrient limiting conditions. A large variety of recipes has been proposed as culture media such as f/2, Algal, BBM, BG11, Zarrouk, and Mann & Myers [128]. Regardless of the initial nutrient recipe, it is essential to tailor it according to the specific algae strains and the diverse culture conditions encountered, a process that is commonly referred to as medium optimization. In HCDC of microalgae, particular attention must be given to several key nutrients, distinct from the general nutrient array. As shown in Figure 2-2, the volume of published research highlights carbon (C), nitrogen (N) and phosphorus (P) as focal points in microalgae cultivation studies, followed by iron (Fe), sulfur (S), and magnesium (Mg), indicating their pivotal roles as key nutrients for microalgal growth. The selection of these nutrients should adhere to the following criteria: 1) Nutrients with a profound impact on cell division and proliferation. This involves selecting nutrients that exert significant effects on the microalgal cell division. 2) Rapidly depleted nutrients in HCDC: These nutrients, which are quickly consumed in dense cultures and require frequent replenishment, should be prioritized. 3) Limiting nutrients: In the context of HCDC, certain nutrients can become critical limiting factors

for growth. Ensuring their adequate supply is essential to maintain robust and healthy microalgal growth.

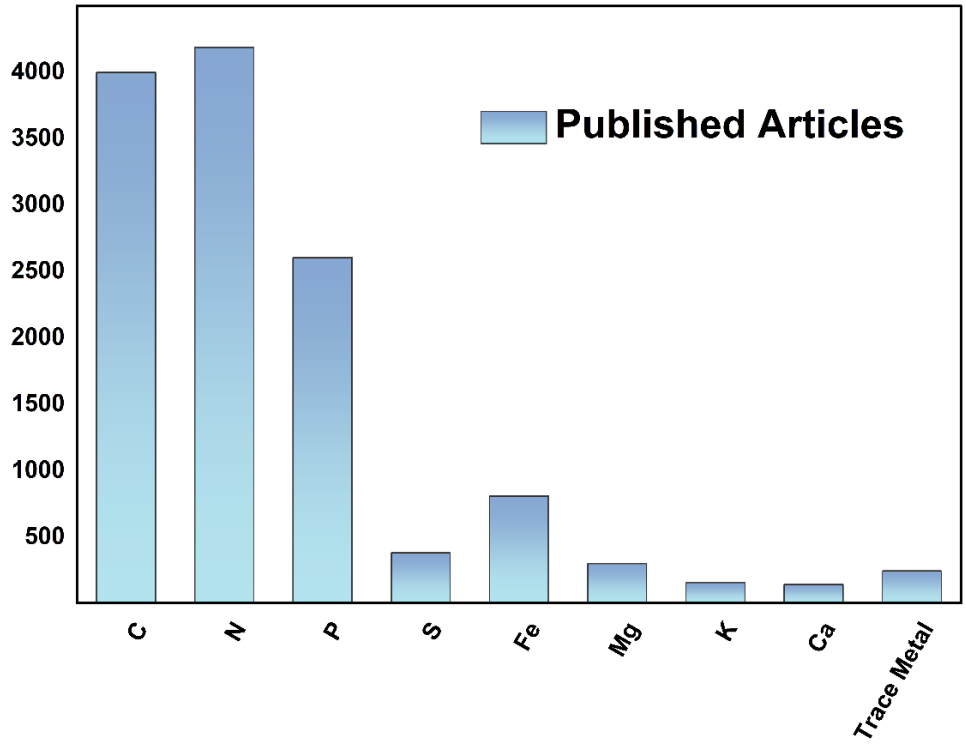

Figure 2-2. Comparison of research volume on different nutrients in microalgae cultivation (2000-2023). (Data information is sourced from Scopus)

2.4.1.1 Carbon

In autotrophic cultivation, inorganic carbon plays a pivotal role in the process of photosynthesis, serving as the primary carbon source for microalgae growth. Through photosynthesis, microalgae convert inorganic carbon, typically in the form of CO_2, into organic compounds that are essential for their cellular structure and energy production. This conversion not only supports the growth but also contributes to the sequestration of CO_2, making microalgae an attractive option for carbon capture and utilization in environmental sustainability efforts. Understanding the dynamics of inorganic carbon in autotrophic microalgae cultures is crucial for optimizing growth conditions and enhancing biomass productivity. In microalgae HCDC, the efficient supply and utilization of inorganic carbon become even more critical, as the increased biomass

demands higher rates of photosynthesis to sustain rapid growth and maintain optimal productivity.

Microalgae can utilize two main forms of inorganic carbon: CO_2 and bicarbonate (HCO_3^-). CO_2 is the most commonly used carbon source in autotrophic cultivation, and its supply must be carefully managed to prevent carbon limitation and inhibition, which can hinder biomass accumulation. Bicarbonate, on the other hand, serves as an alternative carbon source, especially in alkaline conditions where the concentration of dissolved CO_2 is low. Some microalgae species possess carbon concentrating mechanisms (CCMs) that enable them to efficiently utilize HCO^{3-} for photosynthesis.

2.4.1.1.1 CO_2

The influence of CO_2 concentration on microalgae growth is multifaceted and complex [129]. Initially, an elevated CO_2 concentration can lead to a higher rate of carbon fixation, thereby accelerating growth rates in microalgae [130]. The carbon dioxide concentration range for microalgal growth is approximately tested from 0.034% (air content) to about 30%. The optimal concentration of CO_2 for microalgal growth varies depending on the species and growth conditions. There was a study on the effect of CO_2 supply conditions of *Chlorella vulgaris*, a CO_2 concentration of 5% was found to be most suitable with a maximum cell concentration of 5.4 g/L achieved [131]. This is also the concentration of CO_2 used in most microalgae cultures, under constant CO_2 concentration conditions. However, this positive correlation has its limitations. Beyond a certain threshold, the photosynthetic apparatus can become saturated with CO_2, resulting in a plateau in the rate of photosynthesis. Moreover, excessively high concentrations of CO_2 can trigger stress responses in microalgae, leading to reduced growth or even detrimental effects on cellular metabolism. It was found that under high-CO_2 tolerant e.g. 30% would lead to oxidative stress and inhibit the algal growth [132].The interplay between CO_2 concentration and the pH of the culture medium is another critical factor in microalgal cultivation [133]. Excessive dissolved CO_2 in water results in the formation of carbonic acid, which can decrease the pH of the medium, thereby inhibiting algal growth. Consequently, in systems where pH is controlled, the

CO_2 concentration is often dynamically adjusted to counteract the alkalinity produced by photosynthesis, thereby ensuring a stable growth environment. In HCDC, the demand for CO_2 is significantly increased. This is not only to maintain a balanced pH but also to provide sufficient carbon for the enhanced biomass production. As a previous study demonstrated in batch cultivation under open thin-layer conditions, the required carbon dioxide content gradually increased from 5% to 35% as the density of microalgae increased, in order to maintain the pH at 7.5 [134]. Therefore, the efficient supply of CO_2 to each microalgal cell becomes a critical factor in achieving high productivity in HCDC.

2.4.1.1.2 Bicarbonate (HCO_3^-)

The utilization of HCO_3^- as a carbon source for microalgae cultivation has gained significant attention due to its potential for enhancing biomass productivity and carbon capture efficiency. Central to this process is the enzyme carbonic anhydrase (CA), which facilitates the reversible conversion of HCO_3^- to CO_2. Additionally, microalgae employ a CCM that actively transports and accumulates inorganic carbon, including HCO_3^-, within the cell. This ensures a steady supply of CO_2 for the Calvin cycle, even under varying environmental CO_2 concentrations. Collectively, these adaptations enable microalgae to efficiently utilize bicarbonate, offering a versatile strategy for carbon acquisition in aquatic environments.

The use of bicarbonate offers several advantages over gaseous CO_2. Primarily, the enhanced solubility of bicarbonate in water facilitates greater carbon availability in the culture medium, a factor particularly crucial in HCDC, where CO_2 diffusion may be constrained. Furthermore, bicarbonate acts as an effective buffering agent, aiding in the stabilization of the culture medium's pH. Additionally, it is worth noting that a previous study demonstrated that the addition of bicarbonate can to some extent inhibit the contamination of the culture medium by protozoa [135]. Several studies [136–138] have demonstrated the feasibility and benefits of using bicarbonate as a carbon source for microalgae cultivation. For instant, several studies indicated that the judicious addition of bicarbonate can enhance the lipid content within microalgal cells [139,140].

Nevertheless, the utilization of bicarbonate exclusively as a carbon source for microalgal cultivation is fraught with challenges. The progressive elevation of pH accompanying microalgal proliferation can induce alkalization of the culture medium, thus impeding microalgal growth. Moreover, it has been repeatedly demonstrated that an overabundance of bicarbonate can lead to an enlargement of microalgal cell size, a response commonly attributed to stress[134,136]. Current research findings suggest that microalgae exhibit a tolerance range for bicarbonate between 0 and 80 mM [141,142]. At present, there are no established precedents for achieving HCDC of microalgae exclusively with bicarbonate. A potential avenue for exploration might be the concurrent utilization of carbon dioxide and bicarbonate in microalgal cultivation. This strategy could offer effective pH control while providing an ample carbon source to establish a stable buffer system.

2.4.1.2 Nitrogen

Nitrogen is an indispensable nutrient for microalgae, serving as a fundamental building block for proteins, nucleic acids, and chlorophyll [143]. Its availability is a key determinant of microalgal growth and productivity [144,145]. Adequate nitrogen provision is associated with enhanced cell division and biomass accumulation. The form of nitrogen, whether as nitrate, ammonium, or urea, also plays a crucial role in microalgal culture dynamics [146,147]. Nitrate is commonly utilized due to its support for high growth rates and minimal environmental concerns. Numerous microalgae species, including, *Neochloris oleoabundans* [148], *Chlorella vulgaris* [149], *Tetraselmis sp.*[150], et al have been identified as capable of flourishing in environments where nitrate is the primary nitrogen source. In contrast, ammonium can be rapidly assimilated but may inhibit growth or induce toxicity at high concentrations [151]. Urea, while an alternative nitrogen source, requires additional energy for breakdown, potentially impacting biomass accumulation [152]. Furthermore, the metabolic response of microalgae to different nitrogen sources involves adjustments in carbon metabolism for ammonium adaptation and nitrate reduction during photosynthesis [153]. Additionally, the choice of nitrogen source affects the

composition and quality of microalgal biomass, influencing lipid, protein, and secondary metabolite content, which are crucial for applications in biofuel production and nutritional supplementation. For example, it was found that ammonium sources, particularly ammonium bicarbonate, inhibited cell growth but facilitated the highest lipid accumulation of 28.3% in the marine chlorophyte *Tetraselmis sp* [154] . Each nitrogen source has distinct assimilation pathways, influencing not only the growth rate but also the efficiency of nutrient uptake. While, there was also a study that demonstrated the use of a combined nitrogen sources formulation could support the growth of *Chlorella sorokiniana* at similar biomass productivity levels as the widely used BG11 medium, while significantly reducing costs by approximately 95% and enhancing metabolite production [155]. This variability in nitrogen source preference has implications for optimizing microalgal cultivation systems, particularly in applications such as biofuel production, where biomass yield and quality are of paramount importance.

Numerous studies have delved into the optimal nitrogen content for microalgal cultivation, with one notable example being the investigation into *Nannochloropsis oculata*, which established that the optimal nitrate concentration resided below 400 mg/L, as concentrations beyond this threshold result in a noticeable downturn in both growth rate and lipid content [156]. The optimal nitrogen content, however, varies depending on the species of algae and the specific cultivation conditions. Conversely, nitrogen limitation often leads to a slowdown in growth and triggers metabolic shifts, resulting in altered lipid [157], carbohydrate [158], and protein [159] profiles. Research into microalgal growth under nitrogen limitation is both broad and deep. Numerous studies [157,160,161] have shown that microalgae can maintain growth in the absence of sufficient nitrogen, possibly by utilizing an internal "nitrogen pool", such as pigments, which are metabolized into lipids and carbohydrates during nitrogen-deficient conditions. A typical application is the induction of lipid synthesis under nitrogen limitation. For instance, research indicated that the high nitrogen limitation (9.375 mg/L) significantly affected the accumulation of total lipid content (33.87% dry

weight) in the green alga *Picocystis salinarum* [162]. Most studies have focused only on growth under nitrogen limitation which is not conducive to the long-term growth of microalgae. However, in the study by Muthuraj et al., a two-stage cultivation strategy was employed for *Chlorella sp. FC2 IITG*, involving initial high biomass achievement under nutrient-sufficient conditions, followed by a lipid enrichment phase through nitrogen starvation [163]. This approach led to a significant increase in biomass (17.7 g/L) and total lipid productivity (313 mg/L/day), demonstrating its effectiveness in enhancing microalgal biomass production. Moderate enhancement of nitrogen concentrations in the culture medium is advantageous for algal cell division, yet excessive nitrogen levels may inhibit algal growth. For HCDC, the primary concern is not the effect of nitrogen on the cellular components, but rather the determination of the maximum nitrogen concentration the culture can tolerate. This approach is grounded in the objective of ensuring a continuous and sufficient nitrogen supply to support sustained cellular growth. In other words, our aim is not to challenge the algae by limiting nitrogen, but to maintain optimal conditions for their continuous proliferation. In a previous experiment, an ultra-high nitrogen concentration of N-2g/L was used in the cultivation of *N. oleoabundans*, which was found to inhibit algal cell division and cause the algal cells to enlarge, while the optimal nitrogen concentration was 345mg/L resulted in a twofold increase in cell density [164]. Investigating the upper limit of nitrogen concentration is necessary, as it aids in determining the feeding mechanism in the culture, such as the frequency and amount of feedings. Moreover, understanding the sensitivity of microalgae to nitrogen concentration can reduce the need for precise control of nitrogen content.

2.4.1.3 Phosphorus

Phosphorus (P) is a critical nutrient for microalgae, essential for DNA, RNA, and ATP synthesis. It directly impacts cell division, a vital process for microalgal growth and reproduction. Understanding phosphorus's role in microalgal biology is crucial for optimizing cultivation conditions for maximum productivity in HCDC. Adequate phosphorus availability ensures efficient photosynthesis and biomass production.

Phosphorus is usually supplied in the form of phosphates ($H_2PO_4^{1-}$ or HPO_4^{2-}) at concentrations ranging from 10 to 100 mg/L [44]. However, phosphorus limitation can lead to reduced growth rates and hindered cell division [165]. Research found that for *Isochrysis galbana* U4, under phosphorus starvation, cells stored six times the phosphorus for growth and accumulated 50% more lipids while consuming intracellular pigments [166]. Under conditions of nutrient starvation, it is common for cells to accumulate lipids as a survival strategy [167]. However, this lipid accumulation can have a detrimental effect on the normal process of cell division. Moreover, the nitrogen-to-phosphorus (N:P) ratio is a critical factor influencing microalgal growth and metabolism. Imbalances in the N:P ratio can lead to nutrient limitations, affecting growth rates and cellular composition. A commonly referenced N:P ratio for balanced growth in many microalgae is 16:1, based on the Redfield ratio, which is derived from the average elemental composition of marine phytoplankton [168]. However, it was found that the optimum N/P ratio for biomass productivity and nutrient removal from municipal wastewater treatment using microalgae varies from 5 to 30, depending on the ecological conditions in the wastewater [169].

2.4.1.4 Iron

Iron (Fe) is an essential micronutrient for microalgae growth, as it is a key component of the photosynthetic machinery, particularly in the electron transport chain where it is a constituent of cytochromes and iron-sulfur proteins [170,171]. Iron is also involved in chlorophyll synthesis [172] and is a cofactor for many enzymes, including those involved in nitrogen assimilation and DNA synthesis [173]. In HCDC, efficient photosynthesis is essential to support the high biomass productivity, and sufficient iron is necessary to ensure optimal photosynthetic performance. Also, stress factors such as diminished light penetration and nutrient competition can intensify. Iron plays a critical role in the synthesis of stress-response proteins and enzymes, essential for protecting cells from oxidative damage and maintaining cellular integrity, thereby ensuring continued growth [174]. Typically, iron concentrations in microalgal culture media range from 1 to 20 μM, with variations depending on the specific requirements of the

species and cultivation conditions [175]. Excessive iron levels can lead to oxidative stress and cellular damage, negatively impacting growth and overall health [176]. Therefore, maintaining iron within an optimal range is crucial for supporting robust microalgal growth and avoiding the adverse effects of toxicity.

2.4.2 Nutrients supply

The efficiency of microalgal cultivation largely depends on the optimization of nutrient supply, which plays a crucial role in influencing growth rates, biomass productivity, and metabolite accumulation. Various nutrient supply methods, including batch, fed-batch, continuous, semi-continuous and perfusion cultures, have been developed and implemented to meet the specific requirements of different microalgal species and cultivation systems [177–179]. As also shown in Figure 2-3, several methods of supplementation are demonstrated. However, not all nutrient supply methods are suitable for HCDC, making this a topic worthy of discussion.

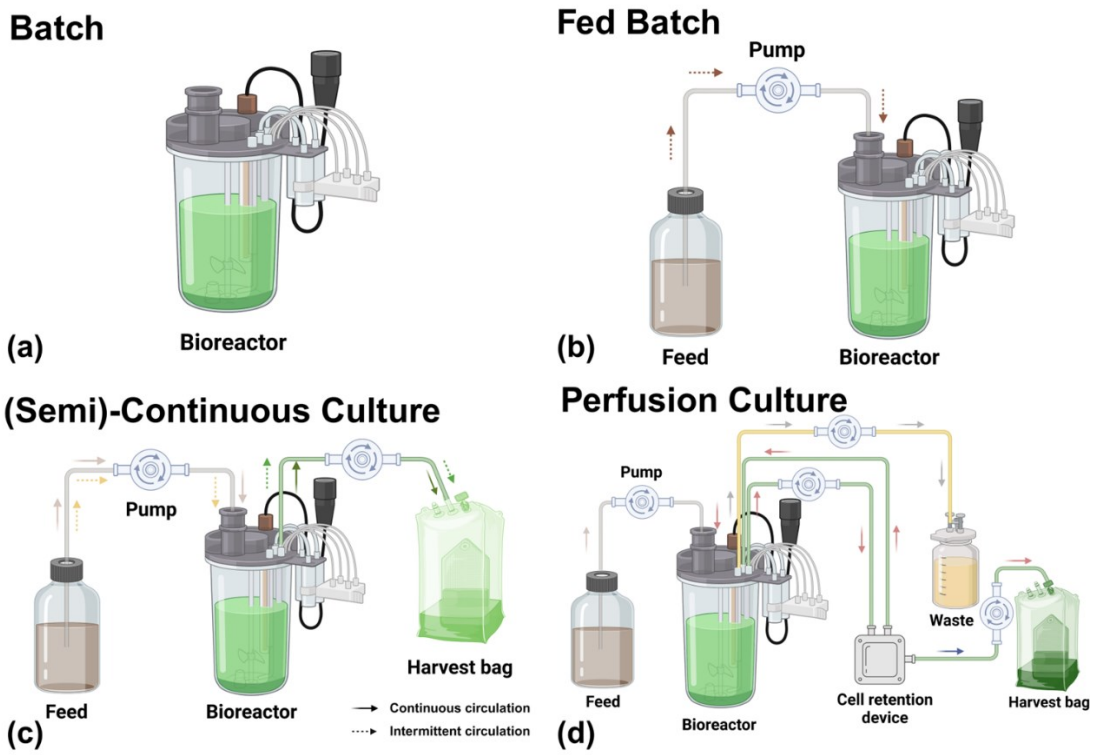

Figure 2-3. Schematic diagram microalgae nutrients supply modes (a) Batch culture, (b) Fed-Batch culture, (c) (Semi)-Continuous cultures, and (d) Perfusion culture. (Created using BioRender.com.)

2.4.2.1 Batch culture

Batch culture is a classical cultivation method where cells are grown in a fixed volume of culture medium until nutrients are depleted or inhibitory by-products accumulate. While simple and cost-effective, batch culture often leads to limited cell densities and is constrained by the finite availability of nutrients and accumulation of waste products. The biomass concentration harvested from batch culture of microalgae cells is generally low, typically ranging between 0.5 g/L to 3 g/L [180,181]. Although optimizing the culture medium, such as adjusting the N/P ratio [182] or increasing nutrient concentration, can to some extent enhance biomass concentration, this improvement is limited. High concentrations of nutrients can create nutrient stress for microalgae, leading to inhibition of growth. For example, a previous study found that when the components of the culture medium were increased to three times their original levels, the biomass concentration increased by 2.5 times which was around 7g/L [164]. However, when the nitrogen concentration was increased to 18 times its original level, microalgal growth was inhibited. Hence, it is apparent that the inherent limitation of batch culture is the inability to add sufficient nutrients at once to ensure the sustained growth of microalgal cells, thus presenting challenges in achieving HCDC.

2.4.2.2 Fed-batch culture

Compared to batch culture, fed batch culture has the potential to achieve HCDC which is a widely applied method of nutrient supplementation in microbial cultivation [183,184]. The primary aim of fed-batch culture is to extend the exponential growth phase, thereby enhancing biomass productivity by preventing nutrient depletion and maintaining optimal growth conditions. This method involves the intermittent or continuous addition of concentrated nutrients to the culture medium, allowing for sustained nutrient availability without significantly altering the culture volume [185]. A study demonstrated that the pulse feeding mode significantly enhanced biomass and lipid production, achieving a maximum lipid-to-dry weight ratio of 58.3% under conditions of 200 μmol photons m^2/s illumination and 10 mg/day nitrate feeding. By carefully regulating the supply of essential nutrients such as N, P, and trace elements, fed-batch culture the chemical composition of microalgal biomass can be modulated

[186,187] and enables the maintenance of a balanced nutrient environment [188], which is crucial for HCDC. Currently, fed-batch culture is primarily utilized for microalgae HCDC under heterotrophic[189] or mixotrophic [190] conditions. A successful example of autotrophic cultivation is the case of Apel et al., who achieved a final biomass concentration of 50 g/L in an open TLC using a fed-batch approach, with 1 L of concentrated feed medium manually added whenever the nitrate concentration dropped below 2 g/L to prevent nutrient depletion [32]. Lu et al. also employed a fed-batch approach utilizing a C/N gradient, resulting in a biomass yield of 9.18 g/L and an astaxanthin productivity of 15.45 mg/L/day [191].

Despite its potential for HCDC, fed-batch culture faces several challenges. One of the primary challenges is the precision required in nutrient supplementation. Nutrient addition must be carefully calibrated based on real-time monitoring or preliminary experiments to prevent both nutrient deficiency, which can limit growth, and nutrient excess, which can lead to waste and potentially inhibit cell growth. Additionally, fed-batch culture is limited by the accumulation of metabolic by-products due to the lack of regular medium exchange. Over time, these by-products can reach inhibitory concentrations, destabilizing the growth environment and negatively impacting microalgal growth. Managing this accumulation is challenging and may require additional interventions to ensure a stable and productive cultivation system.

2.4.2.3 Continuous culture

Continuous culture is a nutrient supply strategy in bioreactor operations, characterized by the simultaneous and constant influx of fresh nutrient medium and efflux of culture fluid, maintaining a stable nutrient environment and culture volume [192]. Due to its continuous operation mode, continuous culture is frequently utilized in wastewater treatment applications, offering a consistent and efficient approach to managing waste streams [193,194]. The underlying principle is the control of the dilution rate (rate of medium flow relative to culture volume) to maintain microbial growth in a steady state, where the growth rate is regulated by the concentration of the limiting nutrient, thereby achieving a balance between nutrient provision and biomass

removal and resulting in uniform cell density and composition. Tang et al. demonstrated that by optimizing the dilution rate, the specific growth rate and microalgal biomass productivity can be effectively controlled, resulting in maximum biomass productivity of *C. minutissima* and *D. tertiolecta* reaching approximately 137 mg/L/day at a dilution rate of 0.33 /day, and around 91 mg/L/day at a dilution rate of 0.42 /day. The continuous culture system offers the advantage of a consistent nutrient supply, ensuring that cells do not experience nutrient deprivation and maintaining a stable growth environment. However, this approach requires a substantial volume of culture medium, increasing the operational costs. Additionally, the open nature of continuous systems makes them more susceptible to contamination, further complicating long-term operation and potentially elevating maintenance expenses [195].

Achieving HCDC in continuous culture can be challenging due to the washout phenomenon, where cells are removed from the system at the same rate as they grow. HCDC can be achieved by optimizing the dilution rate, ensuring sufficient nutrient supply, and maintaining stable operating conditions. A practical solution is to use concentrated nutrient media to provide sufficient nutrition without increasing the dilution rate. However, this method presents challenges in maintaining nutrient balance, as overly concentrated media can cause osmotic stress and nutritional imbalances, emphasizing the complexity of optimizing continuous cultivation for HCDC.

2.4.2.4 Semi-continuous culture

Semi-continuous cultivation [196] is a dynamic microalgae cultivation strategy that combines the advantages of batch and continuous cultures to achieve HCDC and sustained productivity. This approach involves periodically replacing a portion of the culture medium with fresh nutrients while retaining a significant population of cells in the reactor. In a study conducted by Yu et al., the implementation of a semi-continuous cultivation strategy for *Synechocystis sp. PCC 6803* resulted in a maximum total biomass of 3.36 ± 0.09 g, which represented an 86.67% increase compared to the traditional batch cultivation method that yielded 1.80 ± 0.07 g, and furthermore, this approach demonstrated that the maximum total biomass was 289.19% higher than that

obtained without employing the semi-continuous cultivation strategy [197]. By maintaining an optimal balance between nutrient availability and cell density, semi-continuous cultivation can prevent nutrient depletion and toxic metabolite accumulation, enabling microalgae to thrive at higher densities than in traditional batch cultures. Key operational aspects of semi-continuous cultivation include the determination of the optimal replenishment frequency and the concentration of the fresh medium to sustain growth without nutrient limitation. In practice, the microalgal cell density typically follows a cyclical pattern, where it increases following nutrient replenishment, then decreases due to consumption and dilution during medium exchange, and subsequently rises again as cells resume growth. This dynamic equilibrium allows for sustained HCDC, with the specific growth pattern depending on factors such as the replenishment rate, the volume of medium exchanged, and the growth characteristics of the microalgal strain.

Compared with continuous culture, semi-continuous cultivation offers a compelling blend of resource efficiency, simplified control, and operational flexibility, making it more widely used in microalgae cultivation and wastewater treatment [198–200]. By requiring less fresh medium than continuous systems, it achieves cost savings while maintaining high cell densities [201]. The strategy simplifies the control process by reducing the need for constant monitoring and adjustment of nutrient inflow rates. In a pilot-scale study, semi-continuous cultivation of *Scenedesmus* in a TLC over a 10-month period at a constant dilution rate (D = 0.3 $days^{-1}$) demonstrated the capability for long-term pseudo-continuous growth, achieving high biomass productivity ranging from 28.3 g/m^2/day to 47.3 g/m^2/day across different seasons [202]. Moreover, semi-continuous cultivation stands out from fed-batch systems by periodically removing metabolic waste products, preventing their accumulation and potential inhibition of cell growth. The ability to adjust the frequency and volume of medium exchange based on the growth dynamics of the microalgae enhances the operational flexibility of the system, allowing for optimized productivity. However, the very flexibility of semi-continuous cultivation introduces a notable drawback due to its non-continuous nature

of medium replenishment, making the determination of optimal frequency and concentration for medium exchange more complex, as it requires precise alignment with the microalgae's growth dynamics [203]. This complexity introduces greater risks associated with untimely or insufficient replenishment, which can lead to nutrient depletion, suboptimal growth, and reduced productivity.

2.4.2.5 Perfusion culture

Perfusion culture is a nutrient supply strategy grounded in the principles of continuous culture, but it differs by maintaining cells within the bioreactor while continuously supplying fresh medium and removing spent medium. It operates with the addition of a cell retention device, such as a filter or centrifuge, that separates cells from the outgoing stream. Perfusion culture enables HCDC by continuously supplying fresh nutrients and removing inhibitory waste products, allowing cells to grow at high biomass concentrations without being limited by nutrient depletion or toxic metabolite accumulation. In a typical continuous culture, high nutrient supply achieved at a high dilution rate can lead to a decline in cell density due to a high rate of periodic culture outflow, resulting in increased light intensity exposure per cell and significant photoinhibition, as observed in the marine diatom *Chaetoceros gracilis* under high light conditions (1000 μmol /m^2/s) [203]. However, Saki and colleagues found that perfusion culture even under high irradiance (2000 μmol/m^2/s), with a harvesting rate of 20%, led to an increase in dry weight and achieved an areal productivity of 15.6 ± 1.3 g/m^2/d [204].

Similar to continuous culture, perfusion culture typically demands large volumes of culture medium. The incorporation of an additional circulation system also increases maintenance requirements and expenses. A key challenge in perfusion culture is managing the filtration system to prevent clogging and fouling, as these issues can disrupt cell retention and nutrient exchange, necessitating frequent membrane replacement [205]. To address some of these challenges, an alternative approach of periodic circulation can be considered, similar to fed-batch culture but with the added advantage of regularly removing metabolic waste from the culture medium. It could

potentially reduce the need for continuous filtration and lowering medium consumption. However, this strategy requires precise timing and control to maintain optimal growth conditions for the microalgae.

Table 2- 4. Summary of characteristics for different nutrient supply methods.

	Batch	Fed-Batch	Continuous	Semi-Continuous	Perfusion
Operation Simplicity	High	Moderate	Low	Moderate	Low
Nutrient Supply	Fixed	Controlled	Continuous	Periodic	Continuous
Biomass Productivity	Low	High	High	Moderate	High
Risk of Contamination	Low	Moderate	High	High	Moderate
Suitability for Scale-up	Limited	Good	Excellent	Good	Good
Complexity of Setup	Low	Moderate	High	Moderate	High
Continuous Production	No	No	Yes	Yes	Yes
Suitability for HCDC	Limited	Good	Moderate	Moderate	Excellent

2.5 Future directions

The study of HCDC should extend beyond merely attaining ultra-high cell density to incorporate two essential aspects. The first is to deepen the understanding of how to achieve HCDC, which entails exploring the biology of the microalgae, the supply of nutrients, and the optimization of lighting conditions. The second objective is to reduce the costs associated with HCDC, which depends on the design of the reactor structure and culture process. Different from traditional LCDC, HCDC necessitates bioreactor designs that are intricately tailored to meet the sustainability requirements of microalgal growth. This entails a more exacting set of requirements for bioreactors. As delineated in Figure 2-4, such critical parameters should be integrally considered in the reactor's design. The design mandate extends beyond merely fulfilling the fundamental growth prerequisites of microalgae – typically encompassing adequacy in light, nutrient supply, and agitation in an HCDC context – to also encompassing considerations of cost-efficiency and commercial viability. Recommendations for the future direction of HCDC are also summarized in Figure 2-5.

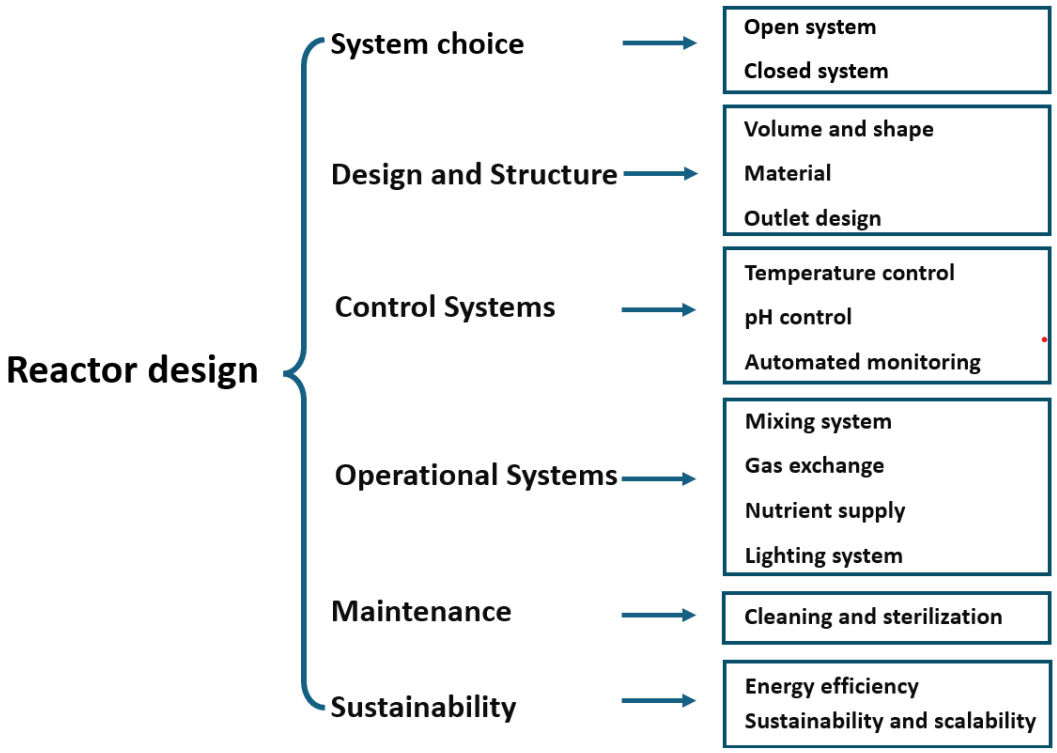

Figure 2-4. Key reactor design considerations for microalgae in HCDC.

2.5.1 Choice of culture system

Open systems are recommended as the preferred choice for microalgae cultivation, not only due to their lower costs but also for their ability to facilitate sufficient gas exchange and support scalability for large-scale operations. While water evaporation remains a concern, strategies such as replenishing water or adding nutrients can maintain a dynamic equilibrium in the culture volume. Additionally, during the exponential growth phase, microalgal cells possess a degree of resilience against native contaminants or predators, particularly in large populations where such threats can be effectively suppressed. Moreover, various preventive measures can be employed to mitigate the risk of contamination, further enhancing the viability of open systems for microalgae cultivation.

2.5.2 Optimizing culture thickness

From the examination of various photobioreactors employed in HCDC, it becomes apparent that there are several universal requirements for these reactors. Foremost is the necessity for a minimal cultivation thickness. Most reactors are engineered with a high surface-to-volume ratio to optimize light distribution. However, this results in a

trade-off with areal productivity as thickness is reduced. Utilizing a constant thickness throughout the cultivation particularly during the initial phase of low cell concentration, is not advisable since light intensity is not a limiting factor at this stage. Therefore, adapting the thickness during the cultivation process emerges as a novel approach to enhance biomass productivity.

2.5.3 Innovative mixing systems

Another pivotal consideration is the mechanism employed for agitating the algal culture. This agitation process is instrumental in achieving a homogeneous distribution of both photonic energy and nutritional elements, alongside facilitating the requisite gas exchange dynamics. The selection of an agitation strategy not only dictates the structural configuration of the bioreactor but also bears implications for the energy consumption associated with the cultivation system. HCDC in suspended systems is more dependent on mixing compared to attached systems. However, continuous mixing is a significant energy input for microalgae cultivation, which needs to be optimized to enhance production efficiency. Exploring strategies such as intermittent stirring or the development of hybrid reactors that incorporate features of both attached and suspended systems could offer pathways to reduce cultivation costs. Fortunately, studies have shown that as the culture thickness decreases, the dependence of microalgae on the frequency of mixing also reduces. Regardless of the approach taken, it is crucial to ensure that the strategies do not compromise the achievement of HCDC.

2.5.4 Dynamic nutrient supply strategies

Another critical design consideration is whether the reactor has an appropriate nutrient replenishment pathway. HCDC critically relies on the provision of nutrients. Perfusion culture is a promising approach that requires further optimization to minimize the excessive use of culture medium. Integrating fed-batch and perfusion culture methods could potentially offer a more efficient and sustainable solution for microalgae cultivation. Also, optimizing the culture medium is also crucial. Depending on the species of algae, the optimal concentration range of nutrients can vary significantly. Tailoring the composition of the culture medium to the specific needs of each algal

species can enhance growth and productivity, Concurrently, the implementation of automatic detection systems is imperative for the timely identification of deficiencies in key nutritional elements. An ingenious aspect of design for open systems is that, despite the disadvantage of water evaporation compared to closed systems, this can be counteracted by regularly supplementing water to replenish nutrients.

2.5.5 Leveraging machine learning for HCDC optimization

Incorporating machine learning technology into the optimization of microalgae cultivation conditions offers a promising avenue for enhancing efficiency and productivity. By leveraging predictive models and algorithms, it is possible to refine parameters such as nutrient composition, pH, temperature, and other environmental factors, which are critical for optimal growth [206]. Given the complexity and time-consuming nature of traditional experimental approaches, machine learning provides a streamlined and effective method for identifying the most favorable conditions for microalgae cultivation. A successful example is that a study where machine learning optimized a semi-continuous outdoor raceway pond, achieving a biomass productivity of 43.3 g/m²/day [207]. Of course, this approach requires extensive experimental data as a prerequisite to support the predictive capabilities of machine learning models.

2.5.6 Sustainability assessments in HCDC systems-LCA

Life Cycle Assessment (LCA) provides a comprehensive evaluation of the environmental impacts from the entire lifecycle of microalgae production, including raw material sourcing, cultivation, processing, and disposal. By identifying energy-intensive steps and optimizing resource use, LCA helps enhance sustainability and reduce ecological footprints [208]. For instance, the existing mature open cultivation systems, such as TLCRs, are capable of achieving HCDC. However, their wider application is limited due to increased labor costs for maintenance, dependency on circulation pumps, and the risk of contamination. Therefore, implementing LCA supports strategic decision-making, ensuring that HCDC systems are both environmentally and economically viable.

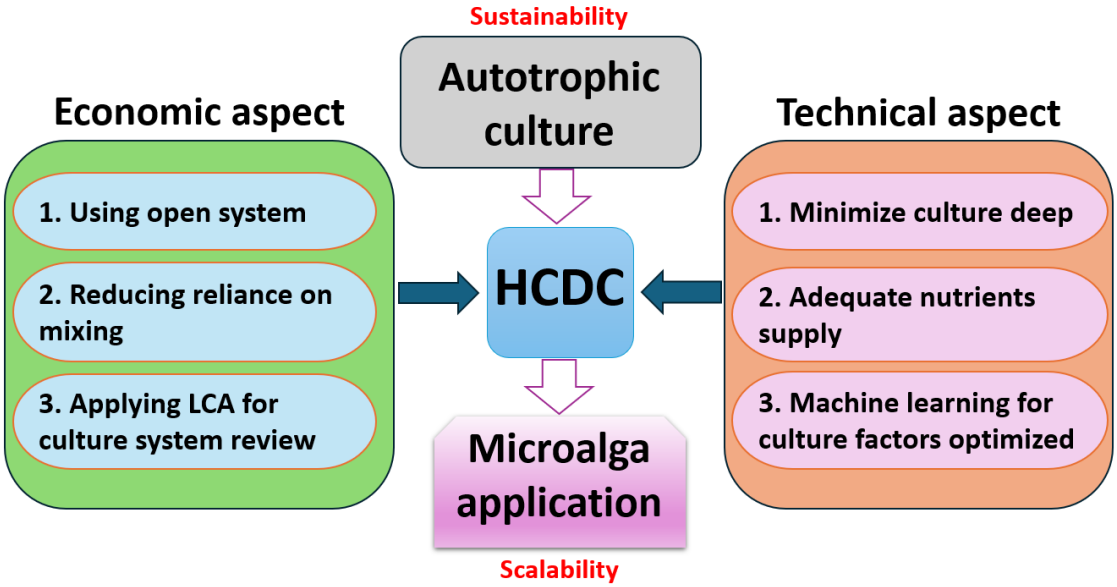

Figure 2-5. Strategic roadmap for the future development and implementation of HCDC.

2.6 Conclusions

HCDC plays a pivotal role in the large-scale application of microalgae, enabling substantial improvements in biomass production. Optimizing cultivation conditions, such as reducing culture depth to ensure adequate light penetration and consistent nutrient supply, is essential for maintaining vigorous algal growth. To achieve sustainable microalgae cultivation, modifications to existing bioreactors and the development of new types of reactors are necessary to support HCDC while minimizing energy inputs. These advancements not only enhance the efficiency and sustainability of microalgae production but also align with environmental goals by reducing the overall carbon footprint. Ultimately, integrating innovative technologies and refining cultivation strategies are crucial for unlocking the full potential of microalgae as a renewable resource in various industries.

CRediT Authorship Contribution Statement

Hongying Zhou: Conceptualization, Investigation, Methodology, Data curation, Visualization, Writing - Original draft. **Zisheng Zhang:** Resources, Supervision,

Writing - Review & Editing. **Christopher Q. Lan:** Conceptualization, Supervision, Project administration, Funding, Resources, Writing - Review & Editing.

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Chapter 3 High cell density culture of *Neochloris oleoabundans* in novel horizontal thin-layer algal reactor: effects of localized aeration, nitrate concentration and mixing frequency

Abstract

A horizontal thin-layer algal reactor was designed, constructed and tested for high cell density culture of an oil-rich green alga, *Neochloris oleoabundans*, at a thickness of 6 mm. A hollow fiber membrane sparger was used for localized aeration with CO_2-enriched air, which allowed control of culture pH at a narrow range of pH 7.0-7.5. The optimal nitrate concentration under the investigated conditions was 345 mg-N/L with a cell density of ~6.6×10^8 cells/mL and biomass concentration of ~8 g/L. The volumetric and areal biomass productivities were 1.81 g/L/day and 10.85 g/m^2/day, respectively and the maximum volumetric and areal productivities 3.57 g/L/day and 21.42 g/m^2/day, respectively. Intermittent mixing at a frequency of once every two hours (five min mixing each time) produced a cell growth profile similar to that of continuous mixing. Such intermittent mixing resulted in 98.38% saving on mixing energy and 33.9% reduction in water evaporation. It was observed that metabolic stress such as high pH and high nitrate concentration could inhibit cell division while cause cell size to enlarge abnormally. The results indicate that it is technically feasible to have high cell density culture of photoautotrophic microalgae with intermittent mixing and no culture circulation, opening the door towards the development of open algal farming systems that are of low capital, operational, and maintenance costs.

Keywords: high cell density culture; microalgae; thin-layer algal reactor; energy-saving

3.1 Introduction

The rapid growth of the global population [1] and industrialization [2] have intensified the demand for energy, especially the dependence on fossil fuel energy [3]. The limitation and depletion of fossil fuels [4] have prompted researchers to explore renewable energy sources such as biofuels [5]. Eukaryotic microalgae and prokaryotic cyanobacteria (collectively algae hereafter) are aquatic primary producers of superb photosynthetic efficiency as unicellular or simple multicellular photoautotrophs. They can grow 20-30 times faster than the best land crops [6] and can dedicate the entire biomass to the production of target products, comparing very advantageously to differentiate plants which can use only part its biomass, e.g., seeds, leaves, stems, and/or roots, for accumulation of desired products. In addition, algal farming does not require arable land and can use wastewater[7] or seawater[8] for algal cultivation. These features make them a likely solution to the three most pressing crises facing the humanity, i.e., energy crisis [5,9], food crisis [10], and climate change.

The green algae *Neochloris oleoabundans* has been established as an ideal feedstock of biodiesel production for its high lipid content which is in the range of 20% to 55% of the algal dry weight [11,12]. Research has confirmed that under nitrogen starvation [13,14] cells tend to accumulate more lipids, with total lipid productivity of up to 0.133 kg m^{-2} day^{-1}. It has also demonstrated great potential for CO_2 mitigation and wastewater treatment [15], especially for nutrient removal [16] and heavy metal removal [17]. A large number of studies [18,19] have focused on the use of *N. oleoabundans* to produce bio-oil. However, the commercial application of *N. oleoabudans*, similar to that of other algae, has been largely impeded by the high cost of algal cultivation and downstream processing in terms of both dollars and energy expenditures.

Commercial algal farming[20] has so far been limited to production of a few high value products using algal species adapted to extreme pH and/or salinity. The high cost of algal farming in both dollar sense and energy expenditures has been the primary bottleneck impeding mass algal farming for food and biofuels[21]. As an illustrative

example, the areal productivity of algal oils is estimated to be ~30 times of the best land oil crops, whereas their costs of production are >5 times of the latter[22]. The engineering inefficiencies of current algal farming systems apparently surpasses the biological efficiency of algae in a significant way. Using Race Algal Ponds (RAP), which is so far the most widely adopted commercial algal farming system[23,24], as an example, it has the following major inefficiencies: 1) biomass concentration is around 0.5 g/L or in the best case scenario 1.0 g/L, leading to inherited low volumetric productivity and therefore high capital and operational costs; 2) continuous circulation is required to facilitate gas exchange of culture and light absorbance by cells, resulting in high energy intensity and operational costs; 3) dewatering dilute algal cultures and drying wet biomass are energy intensive; 4) like other conventional open system, RAP is easy victim of contaminants such as wild algae and protozoa, necessitating the use of algae adapted to extreme conditions such as high salinity, e.g., *D. salina*, and extreme pH, e.g., *Spirulina platensis*.

Extensive studies have been carried out in the past decades, leading to some innovative technologies that partially succeeded in overcoming these inefficiencies. Closed photobioreactors (PBRs)[25], e.g., tubular[26], flat-panel[27], and airlift PBR[28], offer relatively tight control over cultivation conditions and can sustain mildly higher biomass concentration and volumetric productivity than RAP. However, they demand tremendously large capital, operational, and energy costs and have proven to be less competitive than RAP in overall cost-effectiveness[29]. An open floating horizontal PBR[30] was demonstrated to harness wave energy for mixing and therefore eliminate the need of pumping (for culture circulation and mixing) and aeration. It produced a maximum *S. platensis* biomass concentration of 4.3 g L^{-1} and an average areal biomass productivity of 18.2 g m^{-2} d^{-1}. Given the limited scale of individual floating platforms, however, the capital costs could be prohibitive for large-scale algal farming, which would require a vast number of such platforms for commercial production.

One of the most important advances in developing technologies for cost-effective algal farming is probably the conception and demonstration of high cell density culture (HCDC) of algae in an open system, i.e., tilted thin-layer PBR (TTLP)[31], which is called as thin-layer cascades (TLCs) by other researchers[32]. Outdoor fed-batch cultivation of *Chlorella sp.* using a TTLP achieved an average areal biomass productivity of 38.2 g m^{-2} day^{-1} (which was later corrected to be ~29 g $m^{-2}d^{-1}$) and biomass density of 40–50 g L^{-1} [33]. It is evident that the high biomass concentration and high volumetric productivity of TTLP would result in substantial energy and cost savings in both algal farming and biomass dewatering. The high cell density would also make the culture much less sensitive to contamination of wild algae and predators in comparison to conventional open algal farming facilities such as RAP. A recent life-cycle assessment (LCA) on the greenhouse gas (GHG) emissions of aviation fuel produced using algal biomass indicates that TLCs (GHG 81 g CO_2e per MJ) do have a clear advantage over RAP (94 g CO_2e per MJ) [34]. However, it also concludes neither of them achieved sufficient GHG savings for compliance with the Renewable Energy Directive II. Apparently, the superb biomass concentration and volumetric productivity sustained in TTLP was not sufficient to compensate the high cost of it.

Two features of TTLP that incur significant energy consumption and complexity are its requirement of continuous circulation and use of separate vessel for mixing, aeration and supplementation of fresh medium. To this end, we designed and tested a novel horizontal thin-layer algal reactor with hollow fiber membrane sparger (HTLAR-HFMS) for HCDC of an oil-rich green alga, *Neochloris oleoabundans*. The HTLAR-HFMS does not require culture circulation, can operate with intermittent mixing, and supplement CO_2 via localized aeration. The effects of localized aeration using CO_2-enriched air, concentration of nitrogen source sodium nitrate, and frequency of intermittent mixing on cell growth, cell division, and cell morphology were studied systematically.

3.2 Materials and methods

3.2.1 Microalgal strain and media

The seed of *N. oleoabundans*, (UTEX# 1185) was purchased from UTEX Culture Collection of Algae, the University of Texas at Austin. Algae were pre-cultured in 300ml flasks containing 150ml fresh Modified Bristol Medium (MBM) with the recipe of (per liter): 0.7 g $NaNO_3$, 0.138 g K_2HPO_4, 0.2469 g $MgSO_4$, 0.025 g $CaCl_2$, 0.322 g KH_2PO_4, 0.025 g NaCl, 0.0068 g $FeCl_3$ and 1 mL A_5 solution which was consisted of (per liter): 1.6423 g EDTA-Fe, 2.86 g H_3BO_3, 1.81 g $MnCl_2{\cdot}4H_2O$, 0.22 g $ZnSO_4{\cdot}7H_2O$, 0.079 g $CuSO_4{\cdot}5H_2O$, and 0.039 g $(NH_4)_6Mo_7O_2{\cdot}4H_2O$. In the open TLC, non-sterile MBM medium was used for all experiments to be closer to the actual application scene. The pH of the medium was adjusted to 7 before further use. Specifically, to determine the influence of nitrogen on cell growth, Nitrate content varied in the range of 115 - 2070 mg-N/L (N concentration) which corresponding to 1N (115mg-N/L), 3N, 9N, 18N, respectively.

3.2.2 Seed culture of microalgae

The inoculum of microalgae was prepared in 500 mL cultivation bottles containing 400 mL fresh medium which was sterilized at 121°C for 20 min. After cooling down to room temperature, 20 mL of the inoculum was inoculated into the sterilized medium. An air stream, after passing through the gas washing bottle containing distilled water, enriched with 5% CO_2 was sparged into the cultures at a flow rate of 1 vvm. The bottle was illuminated with 2 LED lights under the light intensity of 920 μmol/s/m^2 that were programmed to be 12 h on and 12 h off, with a continuous agitation by a magnetic stir placed at the bottom of the bottle. The algae cultivated until the exponential phase was used for further studies.

3.2.3 Cultivation in the thin layer algal reactor

3.2.3.1 Thin-layer algal reactor with hollow fiber membrane sparger

A schematic diagram of HTLAR-HFMS is shown in Fig.3-1a, where several crystallization dishes (Sigma, BRAND 455743) made of soda-lime glass with a height of 50 mm and a diameter of 100 mm were used as the vessels to house the thin-layer culture. Each dish sits on a magnetic agitator with a magnetic stir was located at the

bottom of each dish to provide continuous or periodical mixing as needed. A piece of microporous HFM (PURESEA SPRING Co., Ltd. Tianjin, China) made of hydrophobic polytetrafluoroethylene (PTFE) attached to the bottom of the dish was used as a sparger to introduce air or CO_2-enriched air to the culture at a pre-determined constant flowrate. The HFM was of 0.8 mm ID, a wall thickness of 0.175 mm, and pore size of 0.3 μm, which had been used for localized deoxygenation of algal culture in our previous studies [35]. It did not allow the passage of water but permits efficient passage of air through its pores. On top of each dish, an outlet of water tubing was used to compensate the water loss due to evaporation, which is significant due to the large surface/volume (A/V) ratio of a thin-layer culture and the vigorous agitation and aeration. The assembly accept the gas cylinders was housed in a customer-designed chamber, which is not shown. Two fans installed inside the chamber (not shown) were used to control the temperature, which was monitored, together with the relative humidity inside the chamber, using a Govee Thermometer (H5179). Cultivation without HFMS were carried out without aeration.

Typical profiles of temperature and relative humidity profiles in a dark/light cycle are shown in Fig.3-1b. The temperature was approximately 18 ºC during the dark (night) time, which was substantially lower than the room temperature, which was 23 ºC, and the relative humidity varied between 35-36%. During the light (day) time, the chamber temperature varied between 26 and 28 ºC, substantially higher than the room temperature. The chilling effect during the night-time was caused by water evaporation while the heating effects during daytime was due to the heat released by lights.

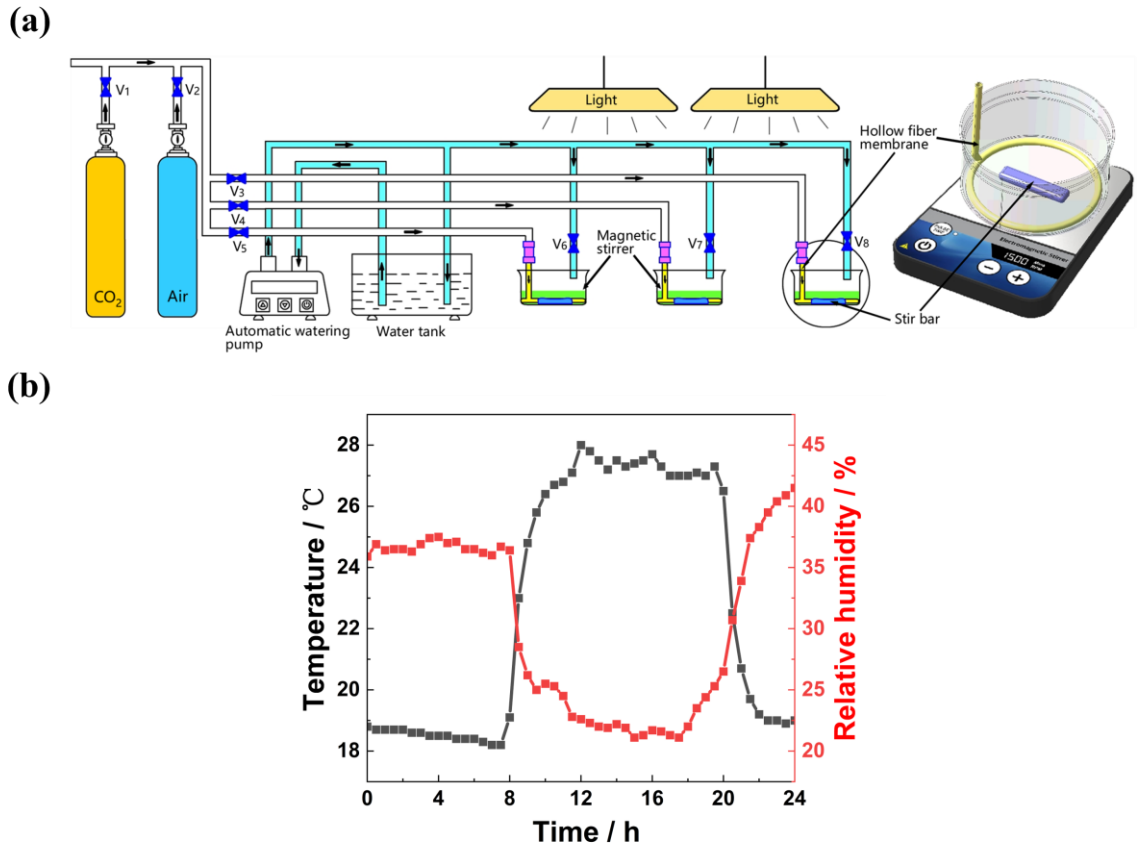

Figure 3-1. Schematic diagram of a HTLAR-HFMS system (a) and typic profiles of the temperature and relative humidity inside the chamber during a full dark/light cycle (b).

3.2.3.2 Cultivation

Seed culture of algae was harvest by centrifugation and then inoculated to the non-sterilized medium in HTLAR with an initial concentration of 0.5 g/L. Cultivation was carried out under continuous stirring through a magnetic stirrer unless otherwise specified. The thickness of the culture was adjusted to 6 mm by adding 48 mL of culture to each vessel (ID 100 mm). The illumination was supplied by two 100 W LED lights and the light intensity was set to be 920 μmol/s/m^2 and were programmed to 12h/12h on/off cycles. During the cultivation, water was replenished every hour to compensate the water evaporation, which was monitored by the balance beneath each magnetic agitator. The water volume of each replenishing was pre-determined and was adjusted manually once or more per day according to the actual water loss, which varied due to the change of relative humidity of the day. The pH of culture was controlled by

adjusting the ratio of the CO_2/air streams at a constant aeration rate and monitored real time by the pH probe of TUYA Water Quality Monitor 6-in-1 device (Voogoo, China). The temperature was controlled at 28±1°C.

3.2.4 Analytical methods

3.2.4.1 Biomass concentration

Biomass concentration (g/L) of algae was determined by measuring the optical density of culture using a GENESYSTM 10S UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., USA) at wavelength 700 nm. Samples were diluted to appropriate ratios to give an OD700 reading between 0.1 and 0.8. The conversion factors were determined by the standard curve related between optical density and biomass concentration which were varied because of the culture condition changes. Basically, the suspended algae were centrifuged at 4200 RCF and washed with deionized water 2 times and then dried for at least 24h in an oven at 80°C until the constant weight was reached.

3.2.4.2 Cell number density and cell morphology of microalgae

Cell number densities of studied microalgae were measured by counting cell number using a hemacytometer (Improved neubauer, Phase Counting Chamber w/2 cover glass, USA) under a phase-contrast microscope (Infinity II BX40, Olympus, Canada) at a magnification of 200 with the assistance of a software (Infinity Capture, Lumenera Scientific, Ottawa, Canada). The cell morphology of microalgae was captured by the same hemacytometer but be observed at a magnification of 600 with the help of software (Infinity Analysis, Lumenera Scientific, Ottawa, Canada). Samples used for counting microalgae cells were diluted to appropriate ratios.

3.2.4.3 Nitrate concentration

Nitrate concentrations were determined by High-Performance Liquid Chromatography (HPLC)[16] with a system (Agilent Technologies Inc., CA, USA) loaded with an Anion IC-PAKTM column (50 x 4.6 mm, 10 µm particle size, Waters, Millipore, USA) under the flow rate of the mobile phase-controlled at 1 mL min^{-1} and

the UV detector set at 220 nm. The mobile phase (pH 8.5) was composed of borate buffer/gluconate concentrate, methanol, acetonitrile, and deionized water at a ratio of 2:12:12:74 (v/v/v/v). The borate buffer/gluconate concentrate contained 0.07 M sodium gluconate, 0.3 M H_3BO_3, 0.1 M $Na_2B_4O_7$ and 3.8 M glycerol in deionized water. The operation temperature was 50°C. The injection volume was set to be 30 μL and the retention time was about 4.2 mins. Samples were diluted to the appropriate interval according to a pre-determined standard curve.

3.2.4.4 Cell content of photosynthetic pigments

The cellular contents of pigments including chlorophyll a (Chl-a), chlorophyll b (Chl-b) and carotenoids were determined by following the methods described by Lichtenthaler [36]. Briefly, the samples were diluted to a proper biomass concentration and then 2 mL of the mixture was centrifuged at 4200 RCF for 5 min. The cell pellets were then suspended in 2 mL methanol and stored in the fridge at 4 °C overnight. The extracted samples are measured under wavelengths of 470, 665 and 652 nm, and calculated by Eqs. 3-1– 3-4:

$$\text{Chlorophyll a } \left(\frac{\text{mg}}{\text{L}}\right) = (16.72\text{A}_{665} - 9.16\text{A}_{652}) \times \text{dilution ratio} \quad (3\text{-}1)$$

$$\text{Chlorophyll b } \left(\frac{\text{mg}}{\text{L}}\right) = (34.09\text{A}_{652} - 15.28\text{A}_{665}) \times \text{dilution ratio} \quad (3\text{-}2)$$

$$\text{C}_{\text{carotenoid}} \left(\frac{\text{mg}}{\text{L}}\right) = \frac{1000\text{A}_{470} \times \text{dilution ratio} - 1.63\text{C}_\text{a} - 104.9\text{C}_\text{b}}{221} \quad (3\text{-}3)$$

$$\text{Pigments} = \text{Chlorophyll a} + \text{Chlorophyll b} + \text{C}_{\text{carotenoid}} \quad (3\text{-}4)$$

The cellular pigments content (with the unit of mg/g) was calculated by dividing the volumetric concentration of pigments (unit: mg/L) by biomass concentration (g/L).

3.2.4.5 Lipid extraction and measurement

The microalgal biomass was harvested from the culture suspension at the end of batch cultivation. Microalgal lipids were extracted using a modified method described by Bligh and Dyer [37]. Generally, about 100 mg dry biomass (dried in an oven at 80°C) was first grounded to powder and then placed into the lipid extraction process in a 9

mL mixture of chloroform: methanol (2:1 v/v) and 1.5 miu deionized water. The solvent mixture containing microalgal biomass was then left on a shaker for 24 h at 220 rpm. After the extraction was completed, the mixture was centrifuged for 10 min at 4200 RCF and three phases were formed, methanol and water in the upper phase, biomass in the middle phase, chloroform and lipid in the lower phase. The chloroform/lipid phase was collected and transferred into a pre-weighed aluminum plate. Another 6 mL of chloroform was added to the remaining and mixed thoroughly followed by centrifuge and the chloroform phase was collected and added to the plate. Nitrogen gas was used to evaporate the chloroform, and then the plate was transferred to a 65 °C oven until constant weight. The lipid content (C_L, mg/g) of dried biomass was calculated accordingly.

3.2.5 Calculations

3.2.5.1 Biomass and lipid productivity

To obtain the volumetric biomass productivity (P_V) and areal biomass productivity (P_A) of biomass, Eq. 3-5 and Eq. 3-6 were used.

$$P_V = (X_2 - X_1)/t \qquad (3\text{-}5)$$

$$P_A = P_V/h \qquad (3\text{-}6)$$

where P_V (g L^{-1} day^{-1}) is volumetric biomass productivity, P_A (g m^{-2} day^{-1}) areal biomass productivity, X_1 and X_2 the biomass concentration at the beginning and end of a period of cultivation t (day), and h (mm) the thickness of culture.

3.2.5.2 Energy saving assessment

The energy savings in HTLAR-HFMS using intermittent stirring is estimated using Eq. 3-7:

$$E_S = \left(1 - \frac{T_M}{T}\right) \times 100\% \qquad (3\text{-}7)$$

where E_S (%) is the energy saving for mixing, T_M (mins) mixing time of each mixing on/off cycle, and T (mins) the time of each on/off cycle of mixing.

The water saving using intermittent mixing is estimated using Eq.3-8:

$$W_S = \left(1 - \frac{W_I}{W_{Con}}\right) \times 100\% \qquad (3\text{-}8)$$

W_S was the water saving (%), W_I (g) the total water evaporation of intermittent mixing in a day and W_{Con} (g) the total water evaporation of continuous mixing in a day. Water evaporation was measured by a balance periodically.

3.3 Results and discussion

3.3.1 Cultivation of *N. oleoabundans* in HTLAR with or without aeration

3.3.1.1 Localized aeration using hollow fiber membrane sparger

Batch cultivation was carried out in HTLAR with or without HFMS and the pH change were monitored in real time. As shown in Fig.3-2a, pH increased rapidly in the first 12 hours and was stabilized at high pH level above 11 during the remaining cultivation time. On the contrary, for the culture with HFMS, the culture pH was controlled well in the range of 7-7.5 by keeping constant aeration rate while enriching the air stream with pure CO_2.

In a microalgal culture, the pH is dominated by the dissolved CO_2 (dCO_2) concentration due to the following balance [38].

$$H_2CO_3(H_2O + CO_2) \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \qquad (3\text{-}9)$$

In the HTLAR without HFMS, CO_2 was supplied by the absorbance of CO_2 from the atmosphere through the culture surface, which was limited by the low CO_2 concentration in the gas phase (~0.04%) and the small gas-liquid interface. The rapid pH increase in the first 12 hours indicates that the rate of CO_2 supply was not sufficient consumption for the growth of algae during that period.

The dCO_2 in culture is determined by the following mass balance:

$$dC_{CO_2}/dt = K_L a(C^* - C_{CO_2}) - q_{CO_2}X \qquad (3\text{-}10)$$

where C_{CO2} is the dCO_2 concentration, K_La the volumetric mass transfer coefficient of CO_2, C^* the CO_2 solubility in the culture, q_{CO2} specific rate of CO_2 consumption by algal cells, and X the biomass concentration of algae in culture.

In the first 12 hr, the rapid increase of pH, i.e., decrease of proton concentration, indicates the reaction described by Eq. 3-9 shifted toward the left to consume protons, which corresponds to a scenario of decrease of C_{CO2} with time, i.e., $dC_{CO2}/dt<0$. According to Eq. 13, when $dC_{CO2}/dt<0$, $K_La(C^* - C_{CO_2}) < q_{CO_2}X$. In other words, the rate of CO_2 adsorption by culture was smaller than the rate of CO_2 consumption by algal cells. This is reasonable because 1) in the HTLAR without HFMS, the surface area/volume ratio for CO_2 adsorption (a=A/V), is limited due to the lack of air bubbles in the system; and 2) the value of C^* is very small due to the low CO_2 in air, which is ~0.047% (47 ppm).

The culture pH plateaued after 12 hours of cultivation, indicating that the CO_2 absorbance and consumption was at balance, which was likely due to the following two factors: 1) the slowdown of cell growth at the unfavorably high pH of 11 or above reduced the specific rate of CO_2 consumption (q_{CO2}) and therefore the rate of CO_2 consumption ($q_{CO2}X$); and 2) the high culture pH increased the solubility of CO_2 (C^*) and therefore the driving force of CO_2 absorbance (C^*- C_{CO2}), which led to increase of the rate of CO_2 absorbance, i.e., $K_La(C^*- C_{CO2})$.

The conventional HTLAR as described by Doucha and Lívanský [39] supplies CO_2 and controls culture pH through vigorous circulation while sparging CO_2 into the culture in a reservoir that is separated from the culture lane. This design necessitates the consumption of large quantities of energy for pumping. In the contrary, the current design continuously sparges CO_2 to the culture locally through the HFMS located at the bottom of culture vessel and therefore eliminates the need of continuous circulation. The effectiveness of this approach for control of culture pH, which also serves as an indicator of the sufficiency of CO_2 supplementation, is shown in Fig. 3-2a.

3.3.1.2 Cell division and cell size of *N. oleoabundans*

As shown in Fig.3-2b, the maximal biomass concentrations that could be reached in cultures with and without HFMS aeration were similar, i.e., 5.72 g/L and 5.12 g/L, respectively. However, the growth rate was much faster with HFMS aeration, with biomass concentration peaked at the 4^{th} day of cultivation, than that without, which peaked on the 7^{th} day. It is apparent that the continued increase of biomass concentration without cell division after the 4^{th} day in the culture without aeration (Figs. 3-2b & 3-2c) was due to the enlargement of individual cells (Fig.3-2d). In other words, the high culture pH inhibited cell division but forced individual cells to increase in size (as shown in Fig. A-1). Similarly, a previous study of our group [38] also found that the mean cell size of *N. oleoabundans* increased significantly in cultures at pH 9.5 compared with that at pH 7.5. It is reasonable to conclude that, even at pH 11, *N. oleoabundans* cells still have the ability to utilize dissolved inorganic carbon (DIC), which exists predominately in the forms of carbonate ions (CO_3^-) (>80%), at a much less extent (<20%) bicarbonate ions (HCO_3^-), and almost zero dCO_2[38] as the carbon source for photosynthesis. This is a strong evidence that *N. oleoabundans* has the carbon concentrating mechanism (CCM) that allows them to utilize HCO_3^- as carbon source[40].

As shown in Fig.3-2c, the cell density of culture increased rapidly from 5.7×10^7 cells/mL after inoculation to 4.1×10^8 cells/mL at the 4^{th} day, corresponding to cell numbers increase of 8 times or cell division of ~3 generations in the culture with HFMS aeration. On the contrary, the cell density of the culture without HFMS aeration increased only slightly from 5.7×10^7 cells/ml upon inoculation to a plateau at ~1.2×10^8 cells/mL on the 4^{th} day of cultivation, corresponding to only one generation of cell division. In other words, cell division was severely inhibited in the culture without HFMS aeration in comparison to that in the culture with. Fig. 3-2d shows that the cell size was practically constant in the culture with aeration while continuously increased until the 7^{th} day in the culture without.

Culture pH could affect cell growth in many different ways including 1) causing multivalent metal ions such as Fe^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} to precipitate and

therefore reducing the availability of these important factors to cells; 2) reducing the concentration of dissolved CO_2 and bicarbonate (HCO_3^-), which could be utilized by cells as carbon source for photosynthesis [40]; and 3) increasing the bioenergetic cost of cells to pump protons from outside of cells to inside the cells against the proton concentration gradient to maintain the intracellular pH in a favorable range.

It was reported that some multivalent metal ions, e.g., manganese, was required for the cell division cycle CDC1 gene of yeast to function [41]. Although not reported to our knowledge, the same or similar dependency on manganese and/or other metal ions may exist in the cell division of *N. oleoabundans* and other microalgal species. It is worth noting that the solubility of $Mn(OH)_2$, the most likely Mn(II) compounds at culture pH 11, is approximately 36 μM at 25ºC, which is almost 4 times of the Mn(II) concentration in the medium (9.14 μM). Therefore, deprivation of Mn(II) or any other trace elements for cells by means of precipitation seems unlikely. One possibility is that the transportation of Mn(II) or other key polyvalent ions through ion channels across cytoplasmic membrane, which is essential for cell division, was inhibited at pH 11. However, we have no direct evidence to support the hypothesis at this point although there are plenty of research results indicating transportation of ions through ion channels are pH-dependent[42,43]. Another possible explanation for the inhibition of cell division by high pH is carbon-source deficiency because the proportion of the DIC species that are available as carbon source for the photosynthesis of *N. oleoabundans*, i.e., dCO_2 and HCO_3^-, would decrease rapidly with the increase of culture pH. However, the fact that the size of individual cells continued to increase after cell division stopped on the 4^{th} day until the 7^{th} day in the culture without aeration suggests that significant metabolite flux was still generated. Therefore, the inhibition of cell division must have another mechanism.

The striping effects of the aeration could help deoxygenation of the culture and therefore maintain a relatively low dO_2 condition in the culture with aeration than that without. High level of dissolved oxygen (dO_2) may enhance the photorespiration of microalgae, which reduces photosynthesis efficiency. Photorespiration and the first step

of CO_2 fixation in photosynthesis are catalyzed by the same enzyme, i.e., Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO). While photorespiration requires the oxygenase function of RuBisCO, CO_2 fixation utilizes the carboxylase function of the enzyme and the ratio of dCO_2/dO_2 in culture determines the relative activity of the carboxylase and oxygenase functions of RuBisCO. Furthermore, high dO_2 combining with high temperature and/or high radiance intensity may cause the formation of reactive oxygen species, e.g., $H_2O\cdot$, and $O\cdot$, and $\cdot OH$, which may oxidized unsaturated biomolecules such as unsaturated fatty acids in cell membranes, pigments, and DNA, therefore cause strong stress to microalgal cells[44]. High level of dO_2 was found to have a negative effect on microalgal growth in previous studies. For instance, a dO_2 of 30 g/m^3 was found to induce a 30% loss in biomass productivity on *Chlorella vulgaris*[45].

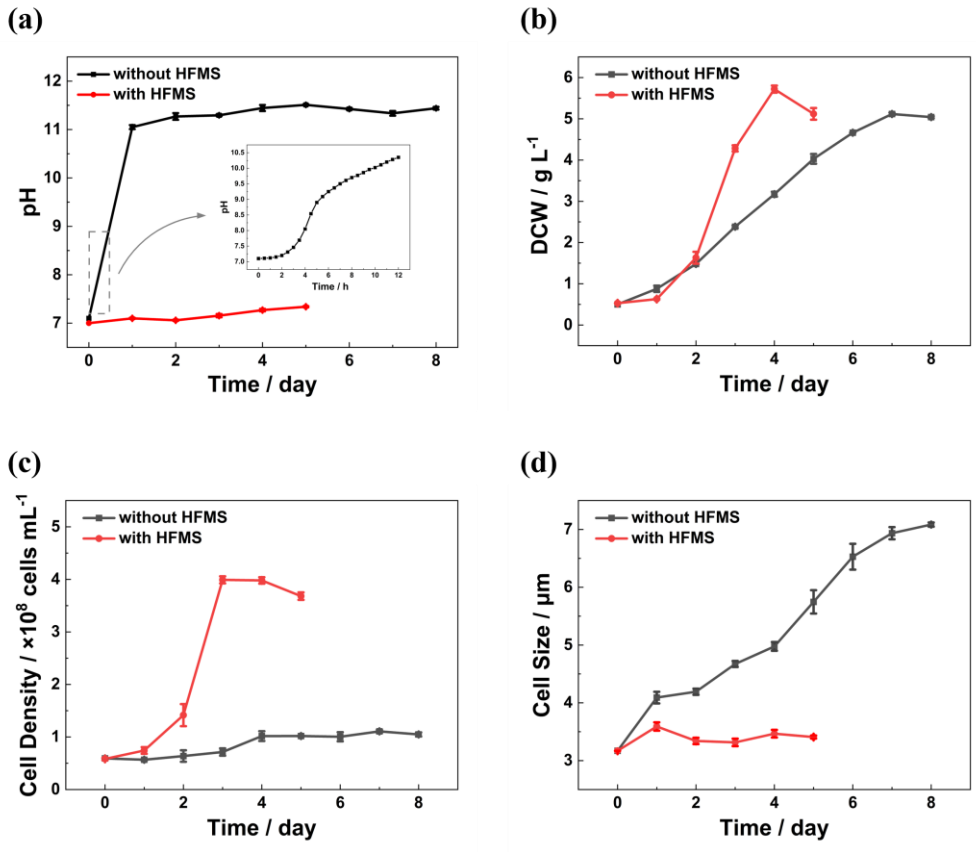

Figure 3-2. pH profiles of algal culture (a), time course profiles of biomass concentration (g/L) (b), cell number density (x10^8 cells/mL) (c), and cell size (μm) (d) in HTLAR with or without aeration. Data are mean values of triplets.

3.3.2 Effect of nitrate concentration on cell growth and biochemical composition of *N. oleoabundans*

3.3.2.1 Effect on cell growth: cell size and cell division

The effect of nitrate contraction in the range of Nitrate content varied in the range of 1N-18N, with 1N being the nitrate concentration in MBM (115 mg-N/L) and 18N with nitrate concentration 18-fold of 1N, on algal cell growth in HTLAR-HFMS with culture pH controlled between 7.0-7.5 by varying CO_2 content in the aeration stream, was investigated and the results are shown in Fig.3-3. As shown in Fig. 3-3a, the highest biomass concentration was obtained at 7.96 g/L in 18N medium, followed by that in 3N, 9N and 1N media. The cell growth in terms of biomass concentration increase is affected by both cell number density and cell size. As shown in Fig. 3-3b, the maximal cell number density of *N. oleoabundans* increased significantly when $NaNO_3$ increased from 1N to 3N, which however, decreased significantly when $NaNO_3$ further increased to 9N and 18N. These results indicate that the N-source in MBM was not sufficient to support the cell division, which is confirmed by the depletion of nitrate in the 2^{nd} day of cultivation with 1N (Fig.3-3d). The data also indicate that cell division was negatively impacted when nitrate concentration further increased from 3N to 9N and 18N. The maximum cell number density, which was 6.86 x 10^8 cells/mL, was obtained on the 4th day at 3N. As shown in Fig.3-3c, cell size was constant in cultures of 1N and 3N with an average of 3.41μm but increased over time in cultures of 9N and 18N. The average cell size of 18N reached 5.63 μm on the last day of culture. Once again, a correlation between hindered cell division and increase of cell size was observed while the mechanism of inhibition of cell division by high nitrate concentration remains to be elucidated in future studies. The MBM containing sodium nitrate 3 folds of its original concentration (3N) was chosen for studies hereafter.

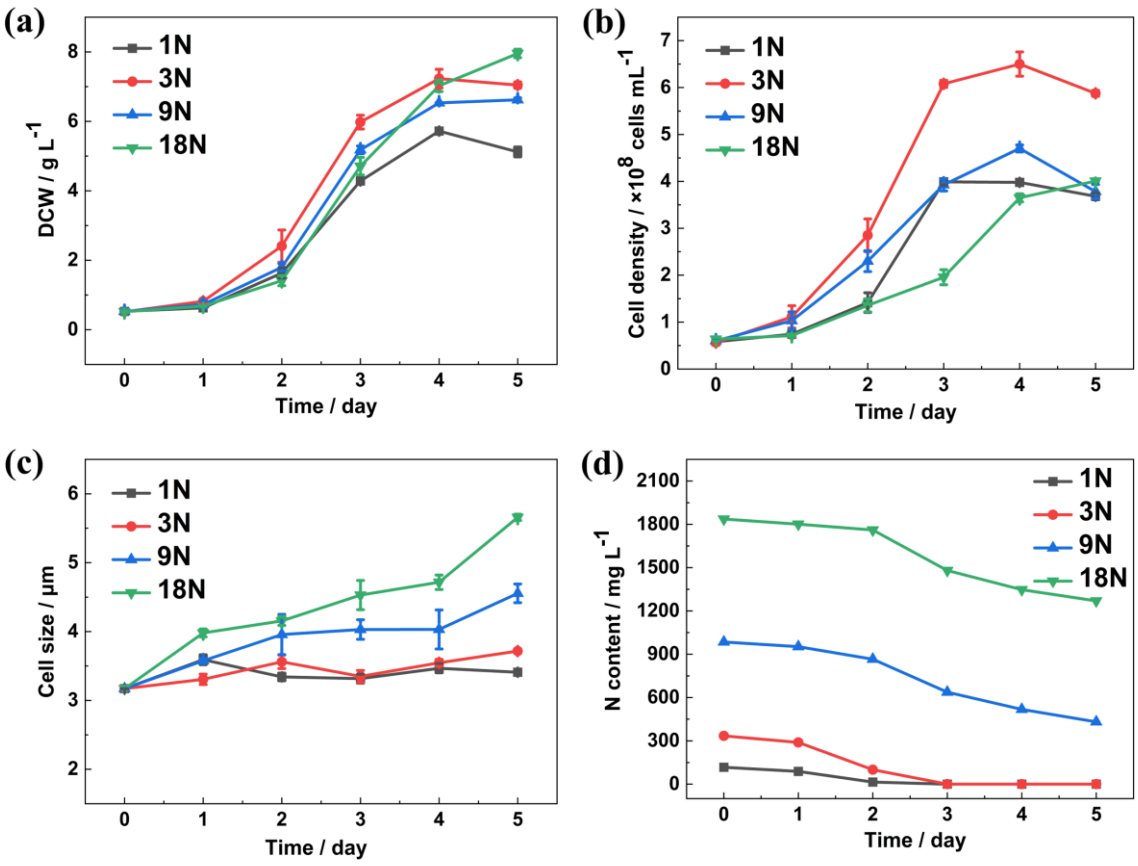

Figure 3-3. Nitrate concentration in medium on cell growth of *N. oleoabundans* (a) biomass concentration (g/L); (b) Cell number density (x10^8 cells/mL); (c) cell size (μm); and (d) residual nitrate concentration. Data are mean values of triplets.

According to the nitrogen consumption curve shown in Fig. 3-3d, for 1N and 3N, nitrate was depleted in 2nd and 3rd day, although cells growth continued until the 3rd and 4th day, respectively. In the meantime, significant residual nitrate remained at the end of the cultivation, i.e., the 5th day, for 9N and 18N, indicating excessive nitrogen source at 9N or above. Furthermore, the cell density decreased when high sodium nitrate increased from 3N to 9N and then to 18N, suggesting that high sodium nitrate might constitute a metabolic stress what would inhibit cell division. In the meantime, as shown in Fig. 3-3c and Fig.3-4, the cell sizes were constant and similar in the media containing 1N and 3N but enlarged at 9N and enlarged even more at 18N. Once again, the abnormal cell enlargement is correlated to the inhibition of cell division. Inhibition of algal cell growth at high nitrate concentration was also reported by other researchers. For instance,

Feng et al.[46] found that high concentration of sodium nitrate inhibited the growth of *Chlorella sp.* GN1 with the highest sodium nitrate concentration being 2 g/L, which corresponded to 329 mg-N/L, i.e., 2.9N in this study.

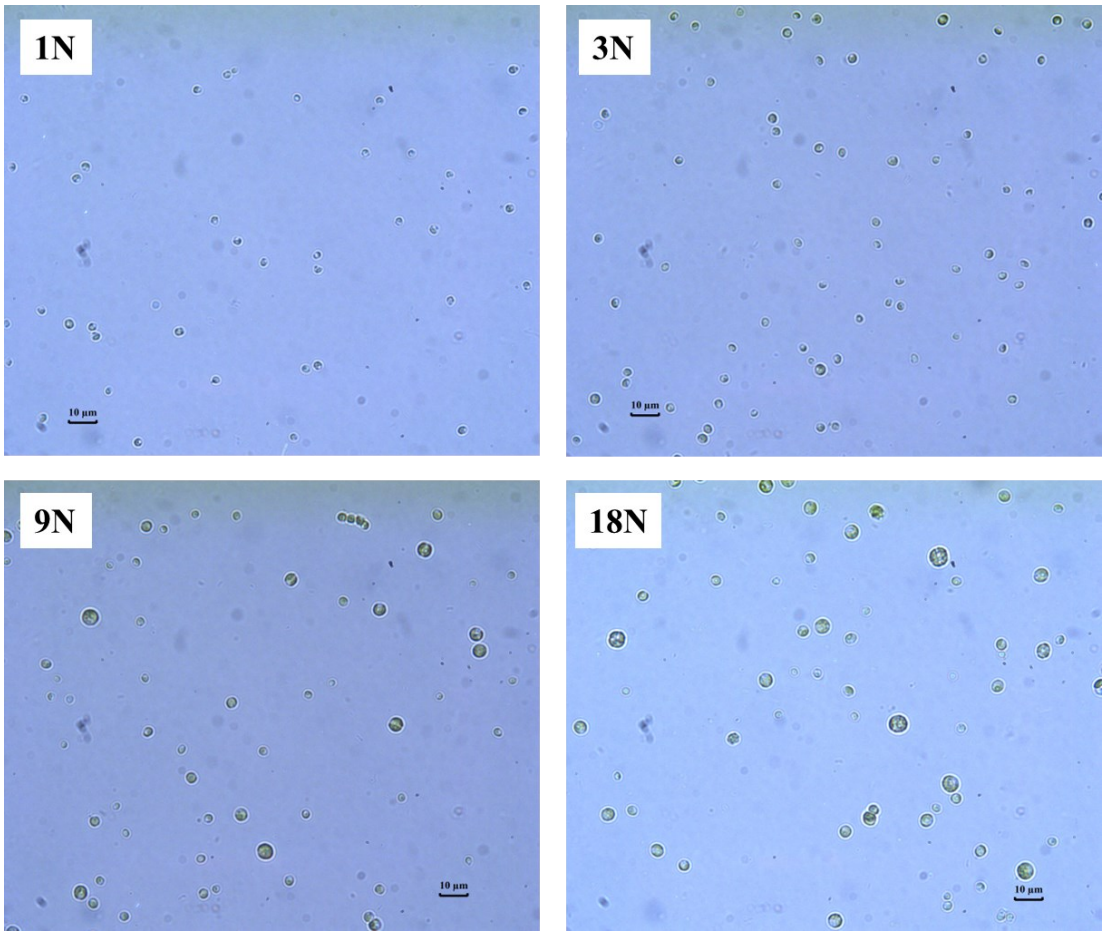

Figure 3-4. The cell morphology of *N. oleoabundans* in the media of different sodium nitrate contents.

3.3.2.2 Photosynthetic pigments and lipid contents

As shown in Fig.3-5a, the cellular contents of Chl-a and Chl-b both decreased in the first three days and then were stabilized in the last 2 days. For the content of carotenoids, it increased in the first day and then went downward until being stabilized on the 3rd day. This trend is different from that observed in our previous study on the cultivation of *N. oleoabundans* in cultivation bottles [13] or a BCPBR [35]. This phenomenon might be explained as the response of algal cell to high irradiance condition. In this study, the seed culture was prepared in cultivation bottles with an

inner diameter of 7.62 cm while the culture thickness in the HTLAR was 0.6 cm. Since the cultivation bottles and the HTLAR were in the same illuminated chamber and therefore share the same light source, the average light intensity received by cells in the HTLAR would be magnitudes higher than that in the cultivation bottles according to the Beer–Lambert law. The first points in Fig.3-5a represent the cellular pigment contents in the seed culture while the rest the change of the cellular contents of different pigments with time in HTLAR. Apparently, transfer of cells from low radiance environment in cultivation bottles to high light radiance in HTLAR caused the cellular contents of chlorophylls a and b to decrease significantly with time until being stabilized on the 3rd day. This is compatible with the results of Conceição et al. [47], which showed strong correlation between the light radiance and pigment cellular contents and the modulation of related genes in marine alga *Phaeodactylum tricornutum.* For *P. tricornutum* exposed up for 72 h to radiance of 150 and 750 μmol photons m^{-2} s^{-1}, no statistically significant difference was observed in biomass production. However, significantly higher cellular contents of photosynthetic pigments, including chlorophylls and carotenoids, were observed at the low radiance than the high radiance. One possible explanation is that under high radiance condition in the HTLAR, cell growth was limited by other factors other than radiance. Therefore, cells did not need high pigment content to maximize light capture, opposite to that at low radiance.

Fig.3-5a also shows that in the stable stage, i.e., after the 3rd day of cultivation, pigment contents of cells increased with the concentration of sodium nitrate in medium. While this correlation could be tentatively explained for the chlorophylls since both Chlorophylls a and b are N-containing molecules, it remains to be elucidated for carotenoids, which have no N in their molecules. As shown in Fig.3-5b, with an increase in medium nitrate concentration, lipids accumulation decreased except for the 18N. The highest lipid content was 377.8 mg/g obtained in the 1N culture, which was slightly higher than the 373.0 mg/g obtained from 3N. These results, i.e., relatively high lipid content when nitrate concentration was 3N and 1N in comparison to that at 9N, suggest that nitrogen deficient likely occurred at the relative low nitrate concentration

of 3N or less under the HCDC conditions. It was found in our previous studies that nitrogen deficiency could induce the accumulation of lipids in microalgae cells as algal cells may direct the metabolite flux generated by photosynthesis to the synthesis of lipids as the storage of C-source and energy source [13]. The increase of cell lipid content at 18 N corresponds to the decrease of cell division (Fig.3-3b) and increase of the abnormal enlargement of cell size (Fig.3-3c), indicating that the high nitrate concentration likely constituted a metabolic stress to algal cells, even though the mechanism is unknown at present. Other studies [48,49] also reported positive effects of nitrogen limitation on lipid accumulation. For example, the oil content of *Chlorella zofingiens* [50] was 33.5% in nitrogen sufficient medium while achieved 65.1% in nitrogen deficient medium. Under extreme environment, such as nutrient deficiency or sufficiency, the biological composition would be changed.

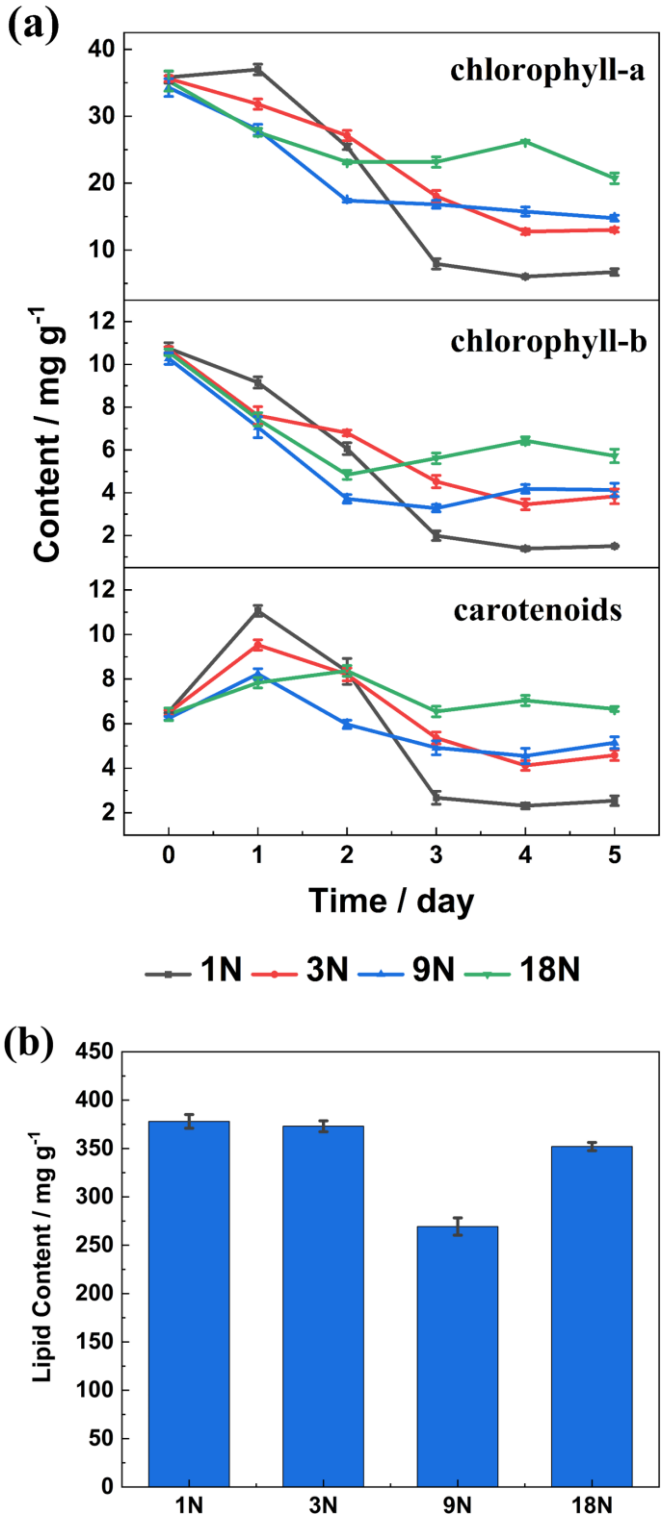

Figure 3-5. Cellular contents of (a) pigments (mg/g) and (b) lipid (mg/g) of *N. oleoabundans* in media of different nitrate concentrations. Data are mean values of triplets

3.3.3 Effect of mixing frequency on cell growth of *N. oleoabundans*

Strong, continuous, and therefore energy intensive mixing is essential in conventional microalgal cultivation systems to homogenize culture, which helps ensure all cells to be evenly exposed to light and nutrients and prevent algal cell settling down. Mixing also promotes heat transfer and helps avoid thermal stratification and improves gas exchange between the liquid phase and the gas phase in culture [51]. Unlike cultivation in bottles with continuous mixing, which failed to achieve high cell density and biomass concentration under the same conditions (as shown in Fig.A-2), HTLAR would hypothetically be less dependent on mixing because of the small culture thickness, which limits the light path distance as well as the distance of mass transfer and heat transfer. Nonetheless, periodic mixing would still be beneficial.

Different mixing frequencies were tested for their effect on the growth of *N. oleoabundans* in HTLAR-HFMS and the results are shown in Fig.3-6. The mixing duration of each mixing period was five minutes. When reducing the mixing frequency from continuous mixing to once every 6 hours during the light period, both growth rate and cell number density of algal cells were reduced remarkably. However, intermittent mixing of once every two hours showed comparable biomass concentration and slightly higher cell number density compared to that of continuous mixing. The related parameters are summarized in Table 3-1.

Comparing the mixing energy saving (E_S) and water evaporation reduction (W_R) of the once every two hours mixing frequency with that of continuous mixing, it is clear that the mixing energy demand was saved by 95.8% while the water loss by evaporation was reduced by 33.9%. When the algal cells were not being stirred, they were in a state of slow settling.

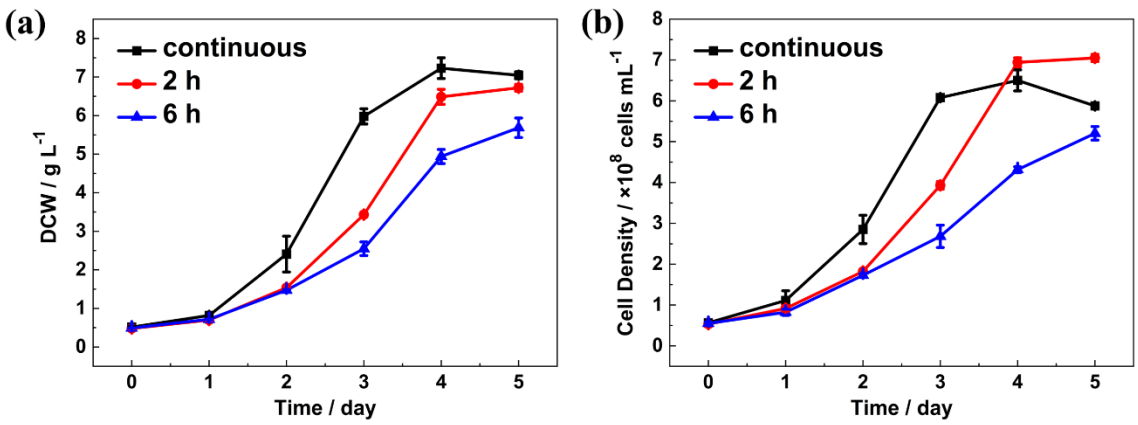

Figure 3-6. Effect of mixing frequency on cell growth of *N. oleoabundans* in 3N medium (a) biomass concentration (g/L) and (b) cell number density (x10^8 cells/mL). Data are mean values of triplets.

Table 3- 1. Summary of algal growth parameters and energy evaluation under different mixing frequencies in HTLAR-HFMS.

	continuous	**2 h**	**6 h**
Cell number density(max)	68.64	71.135	53.21
Biomass concentration (g/L)	7.04	6.72	5.68
P_v (g/L/d)	1.81	1.34	1.14
P_A (g/m^2/d)	10.85	8.07	6.82
P_{Vmax} (g/L/d)*	3.57	3.05	2.39
P_{Amax} (g/m^2/d)*	21.42	18.32	14.35
E_S	0	95.83%	98.61%
W_S	0	33.90%	38.90%

* $P_{A,max}$ and $P_{V,max}$ are the maximum daily areal and volumetric productivity of algal biomass, respectively.

3.4 Conclusions

A one of its kind algal reactor, i.e., horizontal thin-layer algal reactor with localized aeration was constructed and tested for high cell density culture of oil-rich green alga *N. oleoabundans*. Substantial cell growth was observed at pH 11, at which dCO_2 approaches zero, indicating that *N. oleoabundans* has CCM, the metabolic pathway enabling the utilization of bicarbonate ions as carbon source for photosynthesis. Biomass concentration of ~8 g/L was achieved with HTLAR, which is much higher than what could be achieved with conventional suspended photoautotrophic cultures of microalgae using closed or open systems. The results indicate that high cell density culture of photoautotrophic cultivation of microalgae with intermittent mixing and no culture circulation is technically feasible, opening the door towards the development of open algal farming systems that is of low capital, operational, and maintenance costs in terms of both dollars and energy. The biomass attained with the HTLAR is, however, much lower than that reported of TTLP. Further optimization of cultivation conditions such light intensity, cultivation temperature, and the employment and optimization of a fed-batch process aiming at bringing up the biomass concentration and productivity are warranted.

CRediT authorship contribution statement

Hongying Zhou: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft. **Zitong Xu:** Investigation, Methodology. **Liyuan Zhou:** Methodology, Data Curation. **Zisheng Zhang:** Resources, Supervision. **Ju Wang:** Data Curation. **Christopher Q. Lan:** Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition, Resources.

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Chapter 4 Two-step cultivation of *Neochloris oleoabundans* in a novel horizontal thin-layer algal reactor: interplay of pH and dissolved inorganic carbon

Abstract

This study explored the impacts of culture pH and addition of $NaHCO_3$ on the growth of an oil-rich green alga *Neochloris oleoabundans.* It was observed that in the absence of aeration, the culture pH fluctuated over wide ranges during the day/night cycles without additional DIC, which was flatted significantly by adding DIC in culture, demonstrating the effectiveness of DIC as a pH buffer. Addition of DIC in the range of 80-320 mM led to an increase in cell size and inhibition of cell division, which became more severe with the increase of DIC concentration in the tested range. Under 160 mM DIC, pH's effects on algal growth were studied at constant levels (7.5, 8.5, and 9.5). Lower pH increased cell division inhibition, linked to rising dissolved CO_2. Based on these findings, a two-step cultivation strategy was devised and explored. By growing algae in the first stage in cultures without additional DIC and with culture pH controlled at 7.5, followed by cessation of aeration and addition of 160 mM $NaHCO_3$ on the fourth day of cultivation, similar cell growth rate and lipid productivity were obtained as that in cultures with continuous aeration throughout the 8-day cultivation, with lower cell density but larger cell size. By discontinuing aeration, a reduction of 76.73% in aeration CO_2 and 50% of aeration energy were achieved.

Keywords: microalgae cultivation; pH and DIC effects; two-step cultivation; CO_2 saving

4.1 Introduction

Microalgae, a diverse group of photosynthetic microorganisms, hold unique ecological roles with far-reaching implications for the global climate, ecological balance, and biodiversity [1,2]. Notably, they possess an extraordinary capacity to convert carbon dioxide into valuable biomass and valuable compounds, such as pigments, antioxidants, starch, proteins, and lipids [3]. They have attracted increasing attention as a sustainable source of biomass and bioenergy due to their fast growth rate, high biomass yield, and ability to grow in a wide range of environmental conditions [4]. Over the years, they have been exploited for numerous applications in various fields including food and feed [5], pharmaceuticals [6], biofuels [7], and environmental remediation [8].

In particular, the pursuit of microalgal strains possessing high lipid content, coupled with efforts to augment the concentration of premium constituents within microalgal cells, and the enhancement of overall microalgal biomass yield, have all contributed to bolstering microalgae's standing as a promising feedstock for biofuel production [9]. However, it is noteworthy that despite the considerable strides made in harnessing microalgae for biofuel production and other applications, the commercial production of algal biofuels as a replacement of conventional fossil fuels remains an elusive goal. This is primarily attributed to the inherent challenges associated with the production and purification of biofuels, which tend to incur higher operational expenses [10]. As an illustrative example the tremendous potentials, it was estimated that microalgae could produce 50% transportation fuels for the USA using only 1.1-2.5% of the existing cropping lands of the country [11]. The commercialization of microalga-based products, however, has so far been limited by the high costs of microalga cultivation in terms of both dollars and energy [2,12]. Consequently, the realization of microalgae's potential in the biofuel sector hinges heavily upon the imperative of cost reduction strategies and the pursuit of innovative process control methodologies. The sustainable integration of microalgae into the biofuel supply chain necessitates a concerted effort to optimize production processes, streamline purification techniques,

and explore economically viable cultivation strategies.

Recently, many studies [13,14] have focused on the cost-down of cultivation of algae. Compared with closed photobioreactors [15,16], open culture systems have low capital, operational, and maintenance costs and therefore have been the choice for most large-scale commercial alga cultivation [17]. As one of the most recent developments aiming at cost-reduction in microalgal farming, a novel horizontal thin-layer algal reactor (HTLAR) was designed and tested, which could achieve high cell density (HCDC) of under intermittent stirring with the algal cell density of ~ 7×10^8 cells/mL and 98% energy saved [18]. Moreover, HCDC in HTLAR not only offers high volumetric and areal biomass cell growth rate but also advantages for the subsequent algae harvesting process, resulting in the treatment of a tremendously reduced culture volume. Nevertheless, this technology is still in its embryo and extensive research efforts are needed to address different challenges, among which is the escape of large amounts of unused CO_2 into the atmosphere because of the short residence time of air bubbles in the culture, causing difficulties in culture pH control and adding to the overall cost.

CO_2 plays a pivotal role in autotrophic microalgae cultivation, serving as an indispensable element that not only provides a vital carbon source for the growth of algal cells but also functions as a crucial factor in maintaining the requisite environmental pH levels throughout the cultivation process [19,20]. Culture pH is a crucial factor in microalgal metabolism as it affects the availability and uptake of nutrients, enzymatic activity, photosynthetic efficiency, and thereby the productivities of both biomass and bioproducts of microalgae [21,22]. In addition to affecting the microalgae themselves, pH may also determine the solubility of minerals and the proportions of different dissolved inorganic carbon DIC species, i.e., dCO_2 (H_2CO_3), HCO_3^-, and CO_3^-, in the culture [23]. Excessively high or low pH affects the protonation and deprotonation of ionizable molecules in culture and therefore the availability of effective nutrients such as DIC and salts containing nitrogen and phosphorus [24]. The optimal pH for microalgal growth varies depending on the species and environmental

conditions. Generally, microalgae prefer slightly alkaline conditions, with pH values ranging from 7.5 to 9.0 [25]. Maintaining appropriate pH in microalgal cultivation is challenging due to the dynamic nature of microalgal metabolism and the dependency of culture pH on dissolved CO_2 (dCO_2) balance, which is dependent on the interplay between CO_2 metabolism of microalgae and the supplementation of CO_2 [26,27]. In recent years, bioprocess automation that involves using sensors and control systems to monitor and adjust the pH of the culture in real-time by varying the flowrate of CO_2 stream has emerged as the predominant approach in lab scale and pilot scale algal reactors [28]. However, using such a sophistic control system would inevitably add cost to the process, countering the efforts of cost reduction, that has been a major concern in large scale algal farming. Most importantly, such a controlling mechanism requires continuous circulation and mixing of culture, which is the number one contributor to energy intensity in microalgal farming. Thus, it is imperative to conduct further research to investigate the influence of pH on microalgae growth and explore alternative carbon sources, such as dissolved inorganic carbon (DIC), to reduce the reliance on pH control and mitigate CO_2 wastage. The resolution of this issue holds paramount importance in enhancing the efficiency and sustainability of open systems.

In this study, we first systematically investigated the dynamics of culture pH and cell growth in an HTLAR without aeration and then the interactions between culture pH and DIC in a stirred-tank photobioreactor in terms of their impacts on cell division, cell size, and cell biochemical composition of *Neochloris oleoabundans*, an oil-rich green alga that is a favorable candidate for algal oil production and other applications. This green alga is highlighted as a promising candidate for algal oil production, mainly due to its high content of C16 and C18 fatty acids (~90% of its total fatty acids), along with significant amounts of saturated (~30%) and monounsaturated fatty acids (~33%), making it well-suited for biodiesel synthesis [29,30]. The stirred-tank photobioreactor allowed precise control of culture pH. Based on these results, a two-stage cultivation strategy was proposed and tested in an HTLAR with periodical aeration, which was demonstrated to have the potential of significant savings in aeration energy and CO_2

supplementation.

4.2 Materials and methods

4.2.1 Microalgal strain and media

N. oleoabundans (UTEX# 1185) was purchased from UTEX Culture Collection of Algae, the University of Texas at Austin. Microalgae were pre-cultured in 250 ml flasks containing 100 ml fresh Modified Bristol Medium (MBM) with the recipe of (per liter): 0.7 g $NaNO_3$, 0.138 g K_2HPO_4, 0.0823 g $MgSO_4$, 0.025 g $CaCl_2$, 0.322 g KH_2PO_4, 0.025 g NaCl, 0.0023 g $FeCl_3$ and 1 mL A_5 solution which was consisted of (per liter): 1.6423 g EDTA-Fe, 2.86 g H_3BO_3, 1.81 g $MnCl_2{\cdot}4H_2O$, 0.22 g $ZnSO_4{\cdot}7H_2O$, 0.079 g $CuSO_4{\cdot}5H_2O$, and 0.039 g $(NH_4)_6Mo_7O_2{\cdot}4H_2O$. Specifically, the pH of medium was varied within the range of 7 to 11 to investigate its impact on cell growth, with adjustments made using 5 M KOH (aq). Also, to investigate the effect of DIC on algae growth, different concentrations of DIC ranging from 0 to 320 mM was added to the culture medium.

4.2.2 Seed culture

To prepare the inoculum, i.e., seed culture, 400 mL of fresh medium was sterilized at 121°C for 30 minutes in 500 mL cultivation bottles. Once cooled to room temperature, 20 mL of inoculum was added to the sterile medium. A stream of air, which was enriched with 5% CO_2 and passed through distilled water in a bottle for moisturization, was sparged into the cultures at a flow rate of 0.5 vvm. The bottle was illuminated with 2 LED lights, providing a light intensity of 5000 lux, programmed for 12 hours on and 12 hours off cycles, and continuously agitated by a magnetic stir located at the bottom of the bottle. The microalgae were cultivated until reaching the exponential phase before being used as inoculum. The algal cells were pre-cultured for four generations under respective conditions to allow for acclimation to the conditions, e.g., pH and DIC concentration, of a particular culture.

4.2.3 Microalga cultivation

4.2.3.1 Algal cultivation in HTLAR

An HTLAR with or without aeration was used in the cultivation to investigate the

effects of DIC concentrations effects under the uncontrolled pH condition on algal growth. The reactor's design, as presented in Fig.3-1, was elaborated upon in our previous study. Briefly, the HTLAR consisted of several glass dishes, with each dish equipped with a piece of hollow fiber membrane (HFM), a magnetic stirrer, and a water replenishing system. Experiments were carried out with continuous stirring. To compensate for the water loss due to evaporation, which was substantial due to the large surface/volume (A/V) ratio of the HTLAR culture, periodical addition of deionized water was conducted. The hollow fiber membrane was anchored onto the bottom of the dishes to provide aeration with CO_2-enriched air to maintain the culture pH at relatively stable value in the range of 7-7.5 by using constant air flow of 4 vvm air stream containing 5-35% CO_2 accordingly, when applicable. For experiments without aeration and therefore pH control, gas supply was discontinued.

The acclimated seed culture of algae was inoculated to sterile medium in HTLAR with an initial biomass concentration of 0.05 g/L. The thickness of the culture was controlled at 6 mm by adding 48 mL of culture to each dush (ID 100 mm). The illumination was supplied by two LED lights and the light intensity was set to be 5000 lux at culture surface and were programmed to 12h/12h on/off cycles. The water volume for each replenishment during cultivation was predetermined and adjusted manually at least once a day to account for actual water loss, which varied based on changes in daily the relative humidity in the cultivation chamber where the HTLAR was located. Real-time pH monitoring was conducted using a TUYA Water Quality Monitor 6-in-1 device (Voogoo, China), while temperature was maintained at 28±1°C.

Two types of cultivation tests were carried out with the HTLAR, i.e., single step batch culture and two-step cultivation. For the single step cultures, different concentrations of sodium bicarbonate (0 - 320 mM) were added in medium for cultivation under the non-aeration mode and the initial medium pH was not adjusted. The cultures containing 0 mM $NaHCO_3$ was treated as the control and their initial pH was adjusted to 10.5 while the initial pH of cultures with added $NaHCO_3$ was not adjusted since bicarbonate has buffering capacity. For the two-step cultivation, the

initial medium pH was adjusted to 7.5 were specified in the test. Then appropriate amounts of $NaHCO_3$ were added at times as specified in the text.

4.2.3.2 Algal cultivation in 3-L photobioreactor

The studies on the effects of pH at 160 mM bicarbonate were carried out in 3-L stirred-tank photobioreactors, which were converted from BioFlow 110 bioreactors (New Brunswick Scientific GMI Inc., Canada) by equipping each of them with 12 full-spectrum fluoresce lamps with a light intensity of 1500 μmol/(m^2·s) (Philips Plant & Aquarium T18/15, Philips Electronics Ltd. ON, Canada). The bioreactors were equipped with evenly arranged lamps programmed to operate in 12 hours on/12 hours off light/dark cycles. Prior to inoculation, 2 L of fresh medium containing 160 mM bicarbonate in the bioreactor was sterilized at 121℃ for 30 minutes. After cooling to room temperature, the medium was inoculated with 30 mL seed culture and then cultivated at 28℃, 200 RPM with aeration using constant air flow of 1 LPM (i.e., 0.5 vvm). The culture pH was controlled at a specific value (e.g., 7.5, 8.5, and 9.5) by automatically adjusting the flowrate of pure CO_2 to the air stream through a gas mixer and pumping alkaline solution (KOH 10M) when necessary. The air stream passed through a bottle containing distilled water for moisturization, which was then filtered through a 0.45 um microfilter before being sparged at the bottom of the bioreactor into the culture.

4.2.4 Analytical methods

4.2.4.1 Biomass concentration

To determine the concentration of algae biomass (in g/L), the optical density of the culture was measured at a wavelength of 700 nm (OD_{700}) using a GENESYSTM 10S UV-Vis spectrophotometer from Thermo Fisher Scientific Inc. Samples were appropriately diluted to achieve an OD_{700} reading between 0.1 and 0.8. Conversion factors were established based on a standard curve that correlated optical density with biomass concentration. These factors varied depending on changes in the culture conditions. Specifically, to prepare the samples, the suspended algae were first centrifuged at 6000 RCF and washed twice with deionized water. The resulting pellets

were then dried in an oven at 60°C until a constant weight was achieved, which usually took at least 24 hours.

4.2.4.2 Cell number density and cell morphology of microalgae

The cell number densities of the microalgae under investigation were determined by counting cells with a hemacytometer (Improved Neubauer, Phase Counting Chamber w/2 cover glass, USA) under a phase-contrast microscope (Infinity II BX40, Olympus, Canada) at 200× magnification. A software, Infinity Capture (Lumenera Scientific, Ottawa, Canada), was used to assist in the cell counting process. Cell morphology was observed using the same microscope at a magnification of 600×, aided by software Infinity Analysis (Lumenera Scientific, Ottawa, Canada). Samples used for cell counting were appropriately diluted to ensure precise measurements,

4.2.4.3 Photosynthetic pigments content

The cellular contents of pigments, including that of chlorophyll a (Chl-a), chlorophyll b (Chl-b), and carotenoids, were quantified using the methods developed by Lichtenthaler [31]. In brief, the samples were diluted to a suitable biomass concentration, and 2 mL of the mixture was centrifuged at 6000 RCF for 10 minutes. The resulting cell pellets were resuspended in 2 mL of methanol and then stored in a refrigerator at 4 °C for 24 hours to ensure complete extraction of the pigments. The extracted samples were then analyzed for pigment content using a spectrophotometer that measured absorbance at wavelengths of 470 nm, 665 nm, and 652 nm. The pigment concentrations were calculated using equations below, where the specific equations used depended on the pigment being analyzed. The volumetric pigment concentration was determined using the following equations:

$$\text{Chlorophyll a (mg / L)} = (16.72A_{665} - 9.16A_{652}) \times \text{dilution ratio} \quad (4\text{-}1)$$

$$\text{Chlorophyll b (mg / L)} = (34.09A_{652} - 15.28A_{665}) \times \text{dilution ratio} \quad (4\text{-}2)$$

$$\text{Carotenoid (mg / L)} = (1000A_{470} \times \text{dilution ratio} - 1.63C_a - 104.9C_b) / 221 \quad (4\text{-}3)$$

$$\text{Pigments} = \text{Chlorophyll a} + \text{Chlorophyll b} + \text{Carotenoid} \quad (4\text{-}4)$$

The cellular pigments content (mg/g) was calculated by dividing the volumetric concentration of pigments (mg/L) by biomass concentration (g/L).

4.2.4.4 Lipid extraction and measurement

At the end of batch cultivation, microalgal biomass in the culture was harvested and washed three times with centrifugation before being subjected to oven drying at 60 ^{o}C. To extract microalgal lipids, a modified Bligh and Dyer method [32] was employed. Approximately 100 mg of oven-dried (60°C) biomass was ground to fine powders and then subjected to lipid extraction in a 9 mL mixture of chloroform: methanol (2:1 v/v) and 1.5 mL of deionized water. The mixture was left on a shaker for 24 hours at 200 RPM to allow for solvent penetration and complete extraction of lipids from the microalgal biomass.

Once the extraction was completed, the mixture was centrifuged at 6000 RCF for 10 minutes, leading to the formation of three phases: methanol and water in the upper phase, biomass in the middle phase, and chloroform and lipid in the lower phase. The chloroform/lipid phase was carefully collected and transferred to a pre-weighed aluminum plate. The remaining mixture was supplemented with 6 mL of chloroform, mixed thoroughly and centrifuged again, and the chloroform phase was then collected and added to the plate. To remove any remaining solvent, nitrogen gas was used to evaporate the chloroform, and the plate was then transferred to a 60°C oven until constant weight. The lipid content (C_L, mg/g) of dried biomass was calculated accordingly, providing valuable information on the lipid production potential of the microalgae.

4.2.4.5 Determination of carbohydrates

The carbohydrate content in the microalgal samples was determined using the phenol-sulfuric acid method [33]. This method is based on the reaction between carbohydrates and sulfuric acid, which forms furfural derivatives that subsequently react with phenol to produce a colored compound. To quantify the total carbohydrates, 10 mg of dried microalgal biomass was used as the starting material. This biomass underwent sulfuric acid hydrolysis to break down complex carbohydrates and extract

simpler sugars. Following hydrolysis and subsequent neutralization, the filtrate was then subjected to the colorimetric assay.

For the colorimetric assay, 0.2 mL of the filtrate was mixed with 0.2 mL 5% phenol. Then, 1 mL concentrated sulfuric acid (~ 95.5%) was carefully added. The mixture was left undisturbed for 10 minutes and then agitated in a shaking bath for an additional 20 minutes to achieve thorough mixing. Subsequently, the absorbance of the sample was determined at 490 nm using a GENESYSTM 10S UV-Vis spectrophotometer (Thermo Fisher Scientific Inc.) A predetermined standard curve was used for the extrapolation of total carbohydrate concentrations (C_C, mg/g) in the tested samples.

4.2.4.6 Zeta potential

To measure the zeta potential of cells, a Zetasizer nanometer (Malvern Panalytical, UK) were used. Briefly, 1 mL culture sample diluted to an appropriate ratio was transferred a cuvette, which was placed in a cuvette holder. Using the "Zeta Potential" measurement mode of the Zetasizer to measure the electrophoretic mobility of the particles in the sample. The results were used to calculate the zeta potential.

4.2.5 Calculations

4.2.5.1 Apparent specific growth rate and cell growth rate

The maximum specific growth rate (μ_{max}) was determined by calculating the slope of the Ln(X) vs t curve within the linear range. This range corresponded to the exponential phase, where the apparent specific growth rate remained constant.

To obtain the cell growth rate (P) and lipid productivity (P_l), Eqs. 4-5 and 4-6 were used.

$$P = (X_2 - X_1) / t \qquad (4\text{-}5)$$

$$P_l = PC_l \qquad (4\text{-}6)$$

where P is the volumetric biomass growth rate, X_1 and X_2 the biomass concentration at the beginning and end of a period of cultivation t (day) and C_l the cellular lipid content at the end of cultivation.

4.2.5.2 CO_2 saving assessment in thin-layer algal reactor

For two steps cultivation of microalgae in the thin-layer reactor, gas aeration was different depending on the time of $NaHCO_3$ addition. Fig. 4-1 shows the variation of CO_2 content in the aeration air stream with time when the culture pH was controlled in the range of 7-7.5.

CO_2 saving (CS) could be calculated by Eqs. 4-7 - 4-10 as follows:

$$DCC_i = Q \times V \times 60 \text{ mins} \times 12 \text{ h} \times C_i \qquad (4\text{-}7)$$

$$CC_A = \sum DCC_i \qquad (4\text{-}8)$$

$$CC_T = DCC_{S1} + DCC_{S2} + \ldots DCC_{S8} \qquad (4\text{-}9)$$

$$CS\ (\%) = (1 - CC_A / CC_T) \times 100\% \qquad (4\text{-}10)$$

Where DCC_i is the daily CO_2 consumption at Day *i* of cultivation (SCM/day), *Q* the aeration rate, which was kept constant at 4 vvm (volume per volume per minute), *V* the culture volume (m^3), *Ci* (%) the CO_2 concentration in the aeration stream in day *i*, 60 mins the aeration rate from minutes to hours, and 12 h the total daily aeration duration in our experiments. CC_A (SCM) the total CO_2 consumption during an 8-day cultivation batch, and CC_T (SCM) the maximum CO_2 consumption during an 8-day course of cultivation, and CS (%) the CO_2 saving.

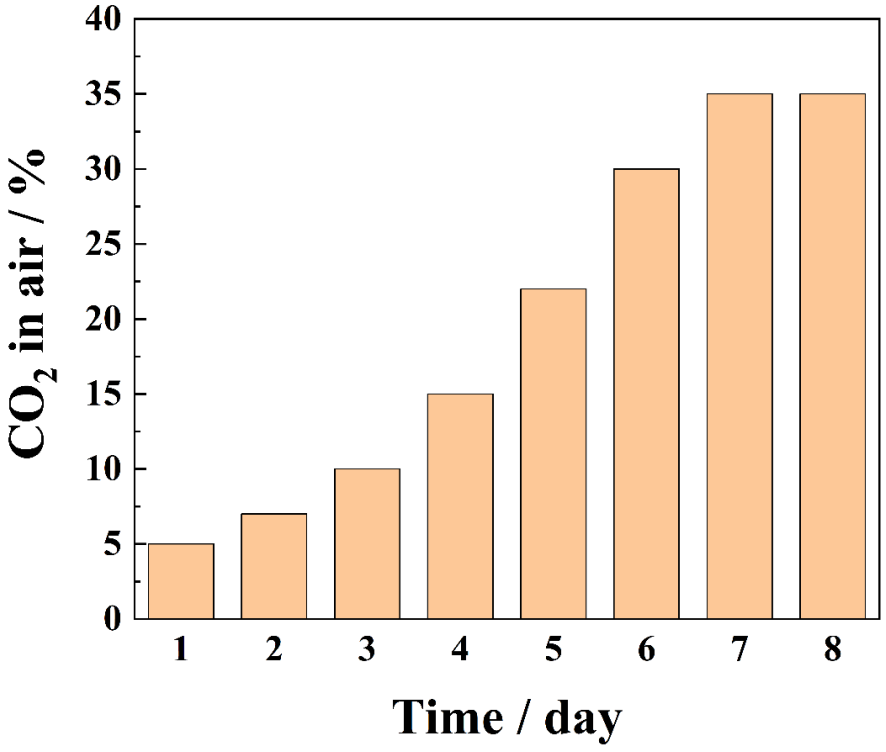

Figure 4-1. CO_2 ratio of the total gas inlet in the thin-layer cultivation at pH 7.5.

4.2.5.3 Calculation of the mole fraction of different DIC species at given pH

To determine the distribution and concentration of dissolved carbon sources in aqueous solutions with the addition of 160 mM of $NaHCO_3$ at pH 7.5, 8.5, and 9.5, the following equations are used:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \qquad (4\text{-}11)$$

$$HCO_3^- + H^+ \rightleftharpoons 2H^+ + CO_3^{2-} \qquad (4\text{-}12)$$

Here, H_2CO_3, HCO_3^-, and CO_3^{2-} represent dissolved carbon dioxide, bicarbonate, and carbonate ions, respectively, while the equilibrium constants for the reactions are determined by the first and second acid dissociation constants, i.e., pKa_1 and pKa_2, of H_2CO_3, which are 6.35 and 10.33, respectively. To calculate the concentrations of the different species of DIC in solution, the Henderson-Hasselbalch equation is employed:

$$pH = pKa + \log([A^-] / [HA]) \qquad (4\text{-}13)$$

where [HA] and $[A^-]$ represent the concentrations of the acid and its conjugate base,

respectively. For bivalent acid H_2CO_3, which is equivalent to dCO_2,

$$Ka_1 = [H^+] \times [HCO_3^-] / [H_2CO_3] = [H^+] \times [HCO_3^-] / dCO_2 \quad (4\text{-}14)$$

$$Ka_2 = [H^+] \times [CO_3^{2-}] / [HCO_3^-] \quad (4\text{-}15)$$

The total concentration of dissolved inorganic carbon ([CT]) in the culture can be expressed as:

$$[DIC] = dCO_2 + [HCO_3^-] + [CO_3^{2-}] \quad (4\text{-}16)$$

where pKa_1 is 6.37 and pKa_2 is 10.33 at 28 °C, H^+ is the hydrogen ion concentration, CO_2 total is the total dissolved CO_2 concentration.

For comparison, the hypothetic partial pressure of CO_2 (pCO_2) in the gas (atm), assuming dissolved CO_2 in pure water, was calculated using the Henry's law as follows corresponding to the dCO_2 levels in culture under different DIC and pH combinations:

$$CO_2\% = K_{CO2} \times dCO_2 / P \times 100\% \quad (4\text{-}17)$$

where K_{CO2} is the Henry's constant of CO_2 at 25 °C with pure water (29.41 L atm mol^{-1}) and P the pressure of the aeration stream (1 atm).

4.2.6 Statistical analysis

All data were processed using Excel 2016 and all figures were drawn by Origin 2021. Reported are the mean values of three independent replicates with standard deviations. Error bars in the figures are the standard deviation (SD) from the mean values, providing a visual representation of the data spread.

4.3 Results and discussion

4.3.1 Effects of DIC on cultivation of *N. oleoabundans* in HTLAR without aeration

4.3.1.1 Effectiveness of DIC for pH buffering in algal culture

Batch cultivation of *N. oleoabundans* was carried out in HTLAR without aeration and the pH change were monitored in real time to investigate the effectiveness of DIC in the range of 0-320 mM added $NaHCO_3$ for culture pH buffering. The termination of cultivation was based on the completion of cell growth stopped, leading to different

durations of cultivation under different conditions. As presented in Fig. 4-2a, in the absence of added sodium bicarbonate, the pH displayed a diurnal pattern with pH increase during the days and decrease during the nights, forming a wave-like curve over the entire course of cultivation. The valley pHs of these profiles, which occurred at nights, increased from approximately pH 8.84 on the first night (1.25^{th} day) to pH 9.78 on the last night (9.25^{th} day) while the peak pHs recorded during the day times was largely constant, fluctuating between pH 10.71 and pH 11.17. The cultures contained added DIC exhibited much more stable pH profiles with minor diurnal fluctuations while both the valley and peak pHs increased gradually but steady over the course of cultivation with the increase being more rapid in the first three days. Comparing the pH of cultures containing different concentrations of DIC, i.e., 80, 160 mM, and 320 mM $NaHCO_3$, at the same time, the pH of cultures follows the order of 80 mM>160 mM>320 mM. These results suggest that the added DIC acted quite effectively as a pH buffer in the HTLAR without aeration. The observed ups and downs of culture pH during the light and dark periods could be attributed to the CO_2 consumption by photosynthesis in the light periods, i.e., the daytime, and CO_2 production in the respiration during the dark periods, i.e., the nighttime, respectively. The addition of DIC, particularly at higher concentrations, compensated to certain extent the CO_2 consumption by microalgae during photosynthesis, leading to a reduction of pH rising during the light periods. The mechanism behind the observed pH changes can be explained by the following equations:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-} \qquad (4\text{-}18)$$

The consumption of CO_2 by microalgae for photosynthesis during daytime would drive the reaction as depicted in Eq. 18 to shift left, consuming H^+ ions and therefore causing culture pH to increase. At nighttime, the consumption of O_2 by the respiration of microalgae for bioenergetic metabolism would be associated with the release of CO_2, which in combination with the absorbance of CO_2 from air, would push the balance of the reaction towards the right side, i.e., dissociation of protons, leading to an increase of $[H^+]$ and therefore decrease of culture pH.

The added DIC acted as a buffer agent to prevent drastic pH changes, resulting in a more stable culture pH during both light and dark periods in the cycles and it is not surprising that higher DIC concentration offered larger pH buffering capacity and therefore stabler culture pH profiles. The incrementation of daily pH valley values with time can be attributed to the increase of biomass concentration during the cultivation (Fig. 4-2a), which would increase the rate of CO_2 consumption (r_{CO2}) as shown in the following equation.

$$r_{CO2} = q_{CO2} \times X \qquad (4\text{-}19)$$

where q_{CO2} (mg/h.g) is the specific rate of CO_2 consumption by microalgae at a biomass concentration of X (g/L), which can be treated as a constant within certain constraints. The increase in r_{CO2} would cause the decrease of dCO_2 in culture when it was larger than the rate of CO_2 absorbance by culture.

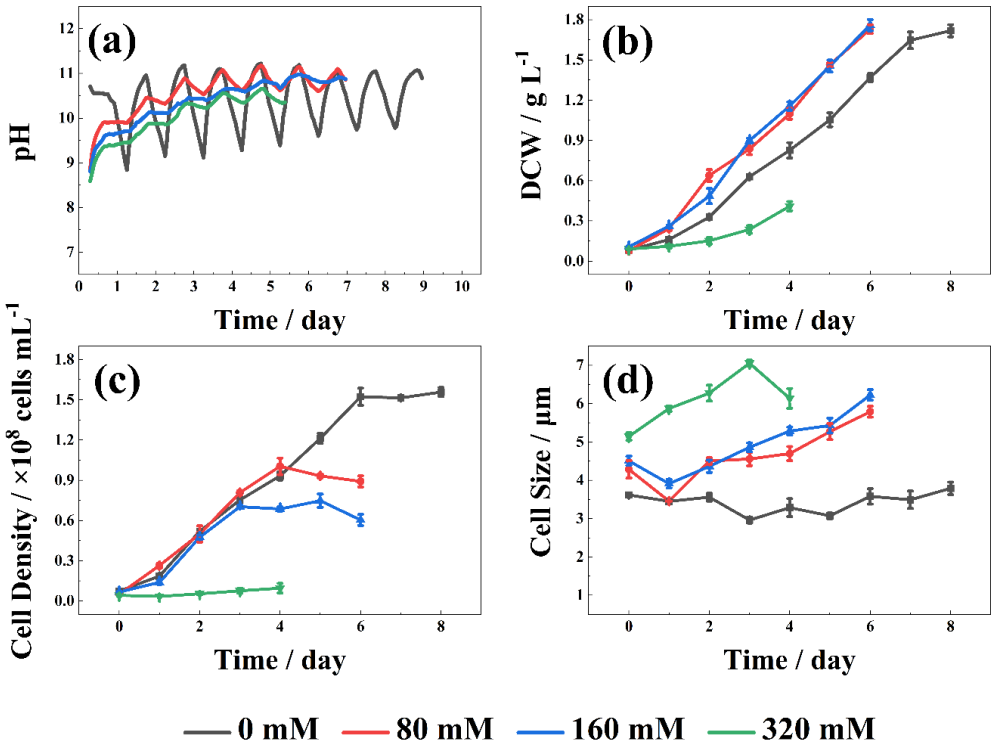

Figure 4-2. Effect of additional $NaHCO_3$ concentration on pH variations (a), biomass concentration (g/L) (b), cell density (cells/mL) (c) and cell size (μm) (d) of *N. oleoabundans* without pH control. The data are mean values of three replicates and the error bars are standard deviations.

4.3.1.2 Effects of $NaHCO_3$ concentrations on cell growth of *N. oleoabundans*

As shown in Fig. 4-2b, the cultures with the addition of 80 mM and 160 mM $NaHCO_3$ had similar biomass concentration profiles, reaching a maximum biomass concentration of 1.73 g/L and 1.76 g/L, respectively, after 6 days of cultivation, corresponding to biomass productivities of 0.288 and 0.293 g/L/day during this period, respectively. In contrast, the algae without $NaHCO_3$ addition reached a biomass concentration of 1.38 g/L on the 6^{th} day (growth rate 0.23 g/L/day) and a maximum biomass concentration of 1.72 g/L on the 8^{th} day of cultivation (growth rate 0.17 g/L/day). While algal growth rates were larger in cultures with added DIC in the range of 80-160 mM when compared with that in cultures without it, the latter was able to maintain cell growth for two more days to achieve much higher maximum biomass concentration at the end of cultivation. The algae in cultures containing 320 mM added $NaHCO_3$ exhibited the slowest growth, reaching a maximum biomass concentration of only 0.41 g/L after 4 days of cultivation (0.103 g/L/day), demonstrating a clear inhibitive effect of high DIC on cell growth of *N. oleoabundans*.

Fig. 4-2c depicts the effect of DIC concentration on the cell density profile, and therefore the cell division, and Fig. 4-2d on the cell size profile, in the course of the 8-day batch cultivation. For the control group, which had no added $NaHCO_3$, the cell density reached a plateau of approximately 1.58×10^8 cells/mL on the 6^{th} day, corresponding to a little more than 6 generations of division over the initial cell density (4.2×10^6 cells/mL). For the cultures with 80, 160, and 320 mM added DIC, the cell density reached the maximum values at 1×10^8, 7×10^7, 1×10^7 cells/mL, corresponding to approximately 4, 4.5, and 1 generations of division, respectively. The inhibition of added DIC to cell division is significant, indicating the added DIC in the tested range was a strong metabolic stress to cells, which increased with DIC concentration. Furthermore, considering the fact that the culture pH followed the order of 80 mM>160 mM>320 mM, it seems that the repression of cell division was primarily due to the high DIC in culture.

Fig. 4-2d shows the cell size profiles of algae in cultures of different DIC

concentrations. The initial cell sizes of algae in different cultures were 3.62, 4.29, 4.51, and 5.14 μm for culture containing 0, 80, 160, and 320 mM added $NaHCO_3$, respectively. This was due to the adaptation of algae in the seed cultures, which contained the same amount of $NaHCO_3$ as in the corresponding culture media. While algae in the culture without added $NaHCO_3$ maintained a constant average cell size of approximately 3.5 μm throughout the cultivation period, that in cultures with added $NaHCO_3$ had further increase in cell size with time and greater increases was observed with higher DIC concentrations. The most drastic cell size increase occurred with algae in the cultures with 320 mM added DIC, which had an increase in average cell size from an initial value of 5.1 μm to a maximum value of approximately 7.0 μm at the 3rd day of cultivation. Since in each culture the DIC concentration would not change dramatically other than that caused by the balance between DIC utilization by cells and CO_2 absorbance from air, these observations suggest pH also had synergistic effects that enhanced the metabolic stress that caused the abnormal enlargement of cell size.

Table 4-1. Summary of key parameters of *N. oleoabundans* growth in the media with different $NaHCO_3$ concentrations without aeration.

	$NaHCO_3$ concentration (mM)			
	0	**80**	**160**	**320**
DCW_{max} (g/L)	1.72	1.73	1.76	0.41
Cell density (10^8 cells/mL)	1.58	1.00	0.75	0.10
Cell size (μm)	3.52	5.81	6.23	7.03
μ_m (d^{-1})	0.68	1.06	0.70	0.32
Growth rate (g/L/d)	0.215	0.288	0.293	0.102

Fig. 4-3 presents the micrographs of algal cells at the end of cultivation of the same magnification of 600×, which shows the visually visible difference between the size of

different cells grown in cultures of different DIC concentrations. There is a strong correlation between the increase of average cell size and the inhibition of cell division in association with added DIC concentration.

These phenomena suggest that the added DIC inhibited factor(s) that was (were) directly related to cell division while allowed photosynthesis to continue, resulted in abnormal increase of the size of individual cells at reduced or ceased cell division. The key parameters of *N. oleoabundans* growth in the media with different $NaHCO_3$ concentrations without aeration has been summarized in Table 4-1. Notably, without the addition of $NaHCO_3$, no change was observed in the size of algae cells, although culture pH did fluctuate significantly. It is worth noting that, in our previous studies [18], an increase in cell size from approximately 3 to 7 μm was observed when the same algae were cultured in the same reactor without pH control. This may be attributed to the utilization of unadopted seed cultures in prior studies [18] while in this study all seed cultures were prepared using the same media and cultivation conditions for the corresponding cultivation tests for adaptation. For this particular test, the seed culture was prepared in MBM medium without additional $NaHCO_3$ or pH control for 4 consecutive culture transfers until the cell size was stabilized. Systematic investigation on the ability of *N. oleoabundans* and other microalgae to adapt to different metabolic stresses, including culture pH fluctuation and high DIC concentration, is under way.

Above results revealed that DIC concentration had a significant impact on algal growth, particularly in terms of cell size and cell division. The findings suggest that high DIC concentrations can lead to an increase in cell size and inhibition of cell division, ultimately affecting algal growth. These results have important implications for the cultivation of algae and provide insight into the factors that regulate algal growth.

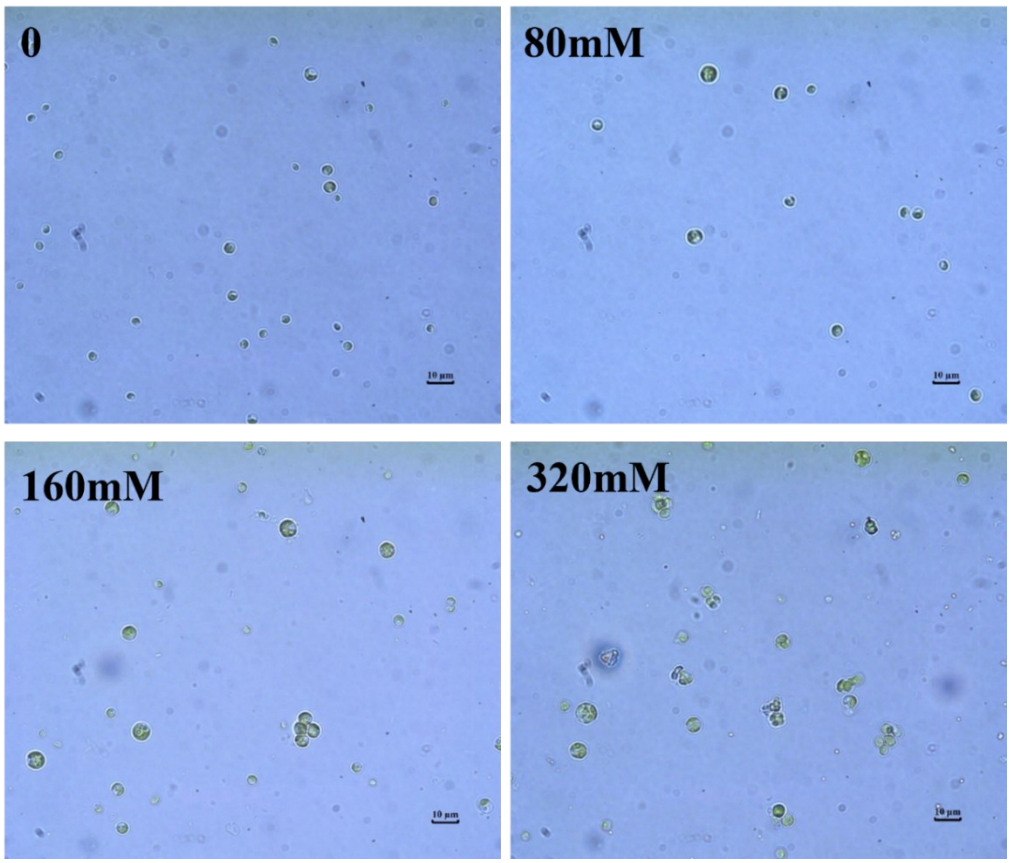

Figure 4-3. The cell morphology of *N. oleoabundans* at the last day in the media of different $NaHCO_3$ concentrations (600× magnification).

4.3.1.3 Zeta potential and cellular lipid contents

Fig.4-4a shows the zeta potential of algal cells on the final day of cultivation under different $NaHCO_3$ concentrations. When compared at the same pH, the zeta potential of cells decreased with increasing culture DIC concentration and was consistently negative, suggesting that the surface of the algal cells became more negatively charged as culture DIC concentrations increased. This may be due to the presence of more carboxyl and hydroxyl groups on the cell surface. These findings are in line with previous studies that have reported an increase in surface negative charge density with increasing pH [34] and may have implications for the electrostatic interactions between algae cells and other particles in the aquatic environment [35]. For instance, large negative charges at cell surface may help prevent the aggregation of cells and therefore maintain more homogeneous cultures [36–38].

Fig.4-4b presents the lipid contents of the algae grown in cultures with different

DIC concentrations, which were 298±0.86, 279±3.94, 314±2.41, and 336±3.01 mg/g for DIC concentrations of 0, 80, 160, and 320 mM, respectively. These data suggest a trend of increasing lipid cell content with increasing DIC concentration, with that at 80 mM a likely outlier. The variation in lipid content observed in the study can be explained through the utilization of carbon sources and environmental stress experienced by algal cells. Irrespective of DIC supplementation, the pH of algal growth consistently remained above 10.5 during the light period when cells were actively carrying out photosynthesis. This necessitated algal cells to rely on Carbon Concentrating Mechanisms (CCMs) for effective carbon acquisition in high pH conditions, primarily due to the predominance of bicarbonate and carbonate at elevated pH levels [39]. Algal cells were compelled to convert bicarbonate into usable CO_2 via CCMs. As the DIC concentration increased, the total environmental carbon content rose, leading to a higher carbon/nitrogen (C/N) ratio. As indicated by earlier research, it was observed that when nitrate-N served as the nitrogen source, an increase in the C/N ratio led to a significant reduction in lipid content and TAG production of *Chlorella sp. HQ*, with total lipid content dropping by 17.82% to 57.43% and TAG yields decreasing by 25.86% to 82.67% [40][41]. This is quite understandable since high C/N ratio meant more abundance of carbon sources but relatively less nitrogen sources. Therefore, the microalgal cells would tend to synthesize more lipids and/or carbohydrates as storage materials of carbon/energy source.

Under conditions up to 80 mM DIC, algal cells appeared to prioritize cell proliferation and growth over lipid accumulation, likely because the abundant carbon supply facilitated their growth processes even the pH were above 10. However, when the DIC concentration exceeded 80 mM, algal cells seemed to experience environmental stress caused by high carbonate concentrations [42], which forced the slowdown of cell growth (Fig.4-2b) and in particular cell division (Fig.4-2c). Consequently, an increased portion of the metabolic flux generated in photosynthesis was directed to the biosynthesis of triglycerides as storage materials. The increase in lipid content beyond the 80 mM DIC threshold reflected algal cells' adaptive response

to the high DIC environment, showcasing their dynamic nature in adjusting to varying environmental carbon levels.

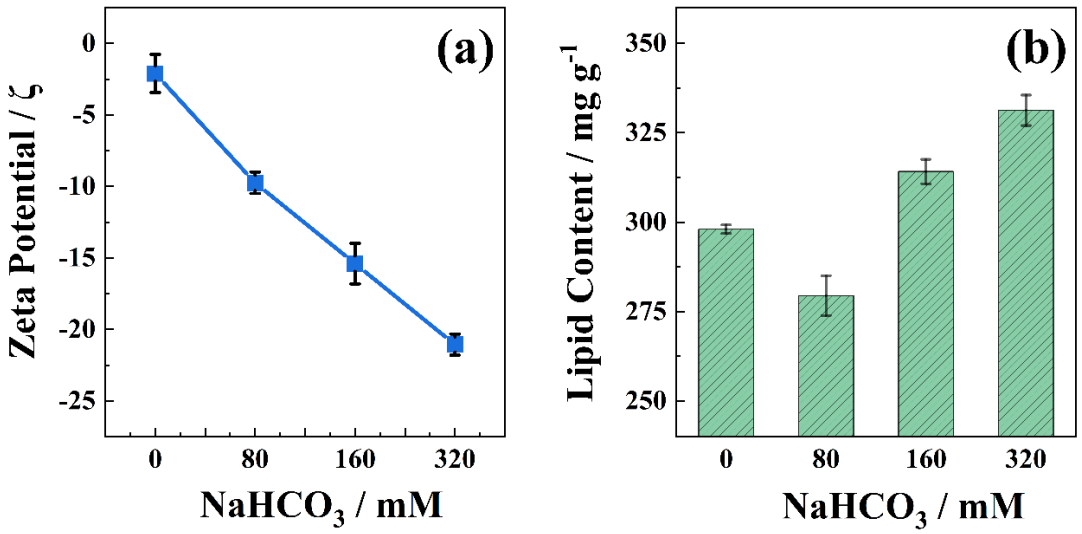

Figure 4-4. Zeta potential (a) and lipid content (b) of *N. oleoabundans* under different culture $NaHCO_3$ concentrations measured under pH 10.5. The data are mean values obtained from three replicates and are presented with error bars.

4.3.2 Interplay between DIC and culture pH

The above study revealed that DIC concentration had a significant impact on algal growth, particularly in terms of the strong inhibition on cell division and the abnormal enlargement of cell size. Results also indicated possible interaction between DIC concentration and culture pH, a well-established parameter that has strong impacts on cells. The effect of pH on cell growth of *N. oleoabundans* at the presence of 160 mM added DIC was therefore studied using a photobioreactor converted from a Bioflo 110 stirred-tank bioreactor, which had precise pH control via adjustment of the CO_2 stream flowrate at a fixed air stream flowrate for aeration, which is lacked by the HTLAR. A control culture at pH 7.5 and without added $NaHCO_3$ was included for comparison.

4.3.2.1 Concentration of different DIC species in cultures

Table 4-2 lists the concentrations of individual DIC species, i.e., dCO_2, $[HCO_3^-]$, and $[CO_3^-]$, in pure water containing 160 mM $NaHCO_3$ at three pH levels, i.e., 7.5, 8.5,

and 9.5. In algal cultivation in the 3 L stirred-tank PBR, aeration with air streams enriched by pure CO_2 stream at varied rate was used to maintain the culture at setting points, including the culture without added $NaHCO_3$ at pH 7.5, i.e., the control, and cultures with 160 mM added $NaHCO_3$ at three different pH levels, i.e., 7.5, 8.5 and 9.5. For the control, the sole source of DIC the CO_2 in the CO_2-enrich air. Whereas DIC sources of cultures with 160 mM $NaHCO_3$ included both the added $NaHCO_3$ and CO_2 in the aeration stream. Therefore, the data shown in Table 4-2 represent the approximate concentrations of individual DIC species above that of the control. Furthermore, the CO_2% in Table 4-2 corresponds to the required additional CO_2 in the aeration stream at equilibrium with the culture if the same additional dCO_2 in culture derived from the added $NaHCO_3$ were to be achieved without by CO_2 enrichment instead of adding $NaHCO_3$ to the medium.

Table 4-2 Concentration of DIC species of *N. oleoabundans* due to the addition of 160 mM NaHCO3 in media at varied pH

pH	CO_2%	dCO_2 (mM)	$[HCO_3^-]$ (mM)	$[CO_3^{2-}]$ (mM)
7.5	32	11.03	148.75	0.22
8.5	3.4	1.16	156.52	2.32
9.5	0.3	0.10	139.29	20.61

As shown in Table 4-2, it is evident that under the conditions of pH 7.5 and 160 mM $NaHCO_3$, the dCO_2 level was 11.03 mM, which was equivalent to the dCO_2 in pure water when it was at equilibrium with a gas phase having a CO_2% in the environment accounted for 32% which greatly exceeded the CO_2 concentration required for microalgae growth. It was reported that dCO_2 became inhibitive to the photosynthesis and growth of microalgae [43] and plants [44] under high atmospheric CO_2 content. Huang et al. [43] reported that marine green alga *Dunaliella salina* could tolerate up to 30% CO_2 in the aeration stream to obtain a biomass growth much improved in comparison to aeration with air without CO_2 enrichment but much worse than other levels of CO_2 enrichment (1-20% CO_2). The best growth was achieved with 1% CO_2

enrichment. The carbon concentration mechanism significantly influences algae growth and CO_2 biofixation by inhibiting carbonic anhydrase (CA) activity under high CO_2 concentrations due to increased bicarbonate and carbonate ions and decreased pH in the culture medium [45]. It is worth noting that, at culture pH 8.5 and 9.5, the dCO_2 concentration in the water was 1.16 and 0.10 mM, corresponding to 3.4% and 0.3% CO_2 in pure water. However, the vast difference in dCO_2 levels only led to insignificant different biomass growth. It has been established that *N. oleoabundans* may have carbon concentration mechanism that enables microalgae to utilize bicarbonate for photosynthesis [20]. The fact that bicarbonate had a relatively constant concentration across different pH levels in this study suggest that bicarbonate, which had concentration magnitudes higher than that of dCO_2, might have served as the primary carbon source for photosynthesis under the investigated condition with the inhibitive effects of dCO_2 relieved at pH 8.5 and 9.5, in comparison to that at pH 7.5.

4.3.2.2 Effects on cell growth: cell size and cell division

As shown in Fig. 4-5a, the control group exhibited the highest biomass concentration among all the tests, which was 2.26 g/L after 12 days of cultivation with ongoing growth and the addition of 160 mM DIC in the pH 7.5 culture led to a significant reduction in cell growth, reaching 0.69 g/L at Day 12 of cultivation, which was only 30% of that with the control group. Furthermore, an increase in pH in the cultures containing 160 mM $NaHCO_3$ was found to be positively correlated with the increase in biomass concentration with the biomass concentration at the 12^{th} day to be 0.71 and 1.18 g/L in cultures at pH 8.5 and 9.5, respectively. Fig.4-5b depict the effects of pH in cultures with 160 mM DIC on the cell size of algae in comparison with that of the control group. In the control group the average cell size fluctuated between 3-4 μm and remained relatively stable throughout the 12-day culture period. However, the addition of DIC caused the enlargement of algal cells, with the average cell size of algae grown at pH 7.5, 8.5, and 9.5 to be 6.36, 5.92, and 5.61 μm, respectively. Fig.4-5c reveals that the cell division of the control group continued until the last day of cultivation, i.e., the 12^{th} day, achieving a maximum cell density of 2.42×10^8 cells/mL.

However, adding 160 mM to culture at pH 7.5 almost completely inhibited cell division with the cell density of culture fluctuated within the margin of experimental error throughout the course of the 12-day cultivation, with a maximum cell density of only 1.83×10^7 cells/mL. The cell density of algae grown at pH 8.5 and 9.5 with 160 mM DIC supplementation increased slightly to around 3×10^7 cells/mL, representing approximately a one-generation division. As shown in Fig.4-5d, the conversion factor in general increased with cell size when compared within the same batch culture, which observed cell size enlargement with time except for the control group (Fig. 4-5c). More detailed discussion about the relationship between the CF and cell size can be found in Section 4.3.2.3. The key algal growth information has also been shown in **Table 4-3**.

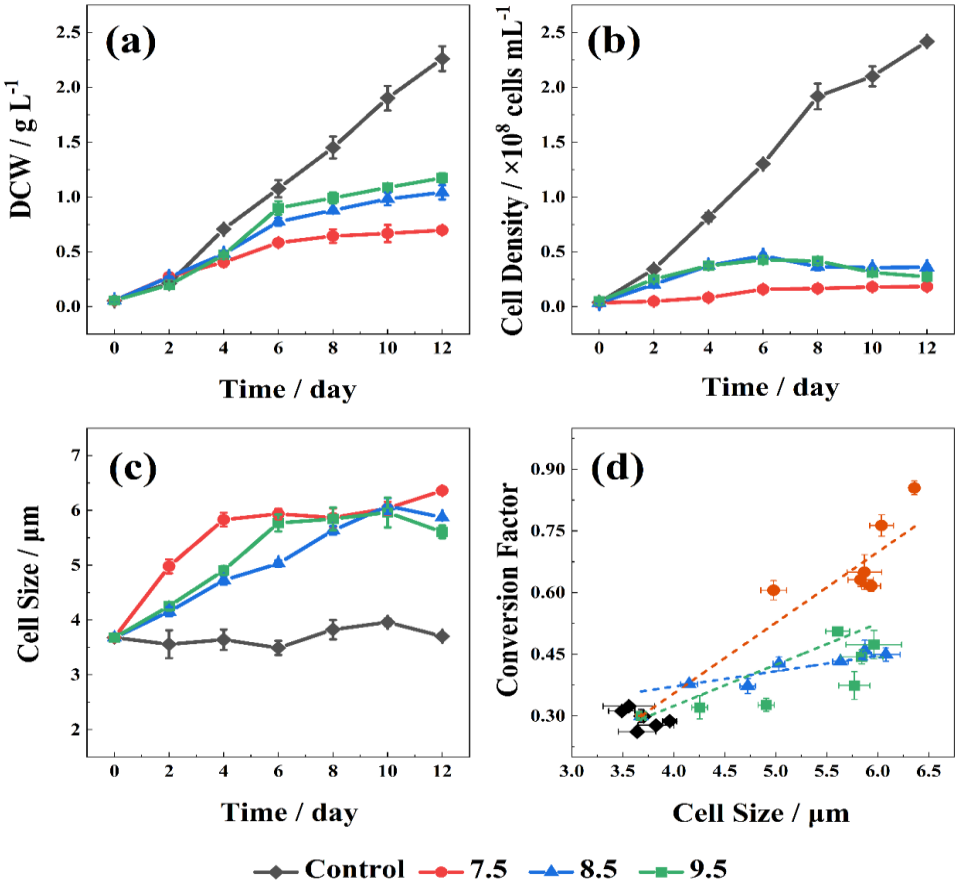

Figure 4-5. Cell growth of *N. oleoabundans* in media containing 160 mM $NaHCO_3$ at pH 7.5, 8.5 or 9.5 (the control was a culture at pH 7.5 without added $NaHCO_3$): biomass concentration (a), cell density (b), cell size (c) and conversion factor (d). The data are mean values obtained from three replicates and are presented with error bars.

Table 4-3. Summary of key parameters of *N. oleoabundans* growth in media containing 160 mM NaHCO3 at pH 7.5, 8.5 or 9.5

	Control	pH 7.5	pH 8.5	pH 9.5
DCW_{max}(g/L)	2.26	0.70	1.04	1.18
Cell density (10^8 cells/mL)	2.42	0.18	0.36	0.27
Cell size (μm)	3.70	6.36	5.92	5.61
μ_m (d^{-1})	1.10	0.74	0.85	0.91
Cell growth rate (g/L/d)	0.188	0.058	0.087	0.098
Lipid productivity (mg/L/d)	54.43	16.04	24.96	30.09

The above results indicate that algal growth was inhibited when DIC presented in the culture at concentration of 80 mM or higher primarily due to the inhibition of cell divisions, and the extent of this inhibition varied depending on the culture pH. The most severe inhibition in terms of both cell division inhibition and abnormal enlargement of cell size was observed at pH7.5, which was counterintuitive since pH7.5 was considered to be a pH that favours cells growth of *N. oleoabundans* in comparison to the higher culture pH of 8.5 and 9.5, when no added DIC was involved [46]. In the meantime, cell growth continued for a substantial period after the cessation of cell division by means of enlargement of individual cells at the presence of 160 mM at all tested culture pH. These data suggest that photosynthesis and other metabolism pathways pertaining to the growth of individual cells were still active. In other words, one or more metabolic activities directly related to cell division were inhibited by the addition of 160 mM. It is somewhat unexpected that the growth of algae was most severely inhibited at pH 7.5 with 160 mM added DIC, at which the cell division was the lowest while the cell enlargement was the largest. The difference of cell sizes in different cultures can be observed virtually in Fig.4-6.

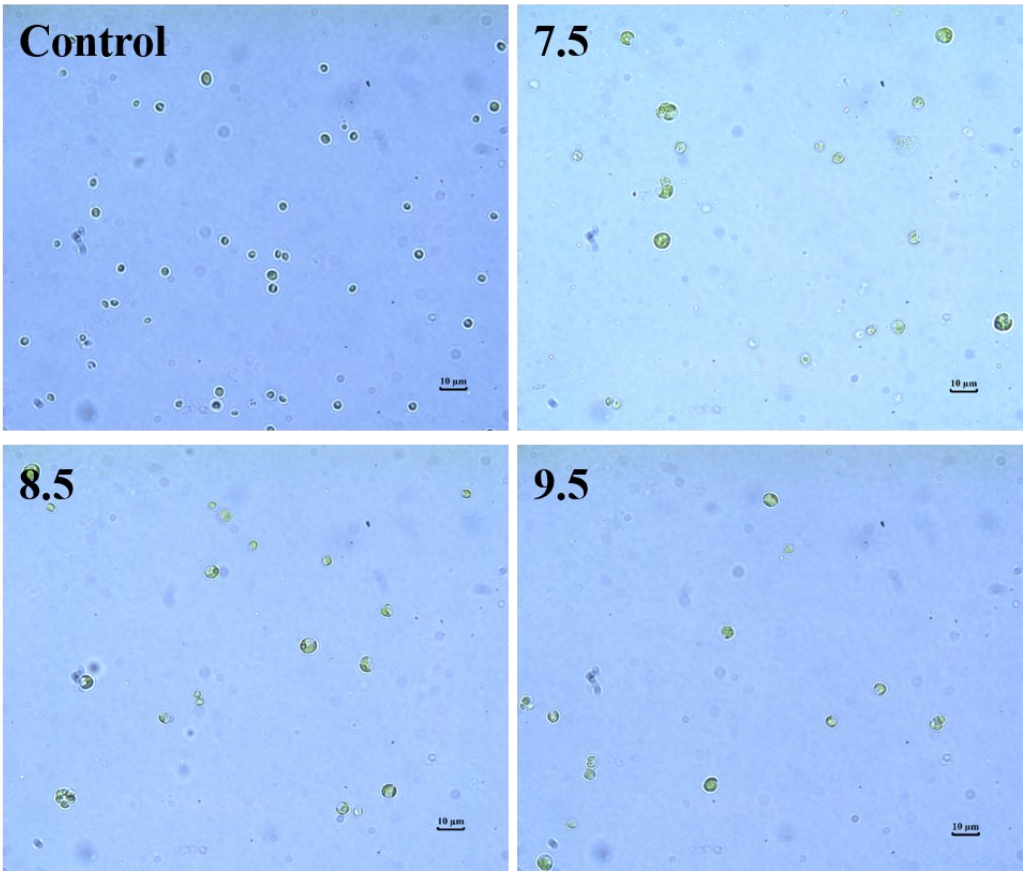

Figure 4-6. The cell morphology of *N. oleoabundans* at the end of cultivation in the media containing 160 mM $NaHCO_3$ at pH 7.5, 8.5 or 9.5 (600X magnification).

4.3.2.3 Optical density/biomass concentration conversion factor: dependency on cell size and other factors

Spectrometry has been widely used in determining biomass concentration in cultures of both heterotrophic and autotrophic cells. It is simple, fast, and reliable in most cases with a conversion factor (CF), i.e., the ratio of optical density (OD) measured at a certain wavelength and the dry cell weight (DCW) biomass concentration established in a range that the OD and DCW concentration had a linear relationship. However, in our studies where cell size changed under metabolic stresses, it was observed that the CF was not constant. Instead, it depended primarily on cell size.

As shown in Fig. 4-5d, when all data points are considered, the CF values in general increased with cell size but in a scattered manner. For the control, i.e., the algal cultures without added $NaHCO_3$ at pH 7.5, there was minimal variation in cell size, and the CF

also largely remained constant. The scattering pattern of these point could be attributed to the deviation of the method, i.e., the optical measurement of cell culture samples. Comparing the data of the three cultures containing 160 mM added $NaHCO_3$ at pH 7.5, 8.5, and 9.5 separately, three linear curves could be obtained reflecting the increase of CF with cell size. Once again, significant scattering existed with all three sets of data, which could be attributed to sample measurement. Notably, the pH 7.5 culture exhibited the largest slope while the pH 8.5 culture had the smallest. In other word, the CF was also influenced by culture pH, which may affect the composition and structure of macromolecules on cell surface [35] and therefore the CF of cells. The variation of CF of the same microbial strain due to medium and cultivation condition, such as culture pH has so far been overlooked and a fixed CF value was commonly used to calculate biomass concentration from OD value. This approach relies on the assumption of a linear relationship between biomass concentration and light absorbance at specific wavelengths within certain range. While this might be acceptable in many cases, e.g., the control in this set of tests, it is evident that CF may vary under other scenarios, such as in those cultures containing 160 mM $NaHCO_3$. More systematic studies on the interplay between CF and various factors affecting light absorbance of algal cells, such as cell morphology and cell surface properties are warranted to insurance accuracy and reliability of biomass concentration estimations using spectrometric approaches.

4.3.2.4 Photosynthetic pigments, lipids and carbohydrates contents

Fig. 4-7 shows the time-course profiles of pigment contents and the final lipid content in algal cells at different pH levels in cultures with 160 mM DIC and the control (pH 7.5 without added $NaHCO_3$). Consider the chlorophyll a and chlorophyll b profiles in Fig. 4-7 and the dCO_2 and CO_2% data in **Table 4-2**, the chlorophyll contents decreased with the increase of dCO_2 level in culture. This is consistent with the trend reported by Huang et al. [39] with *D. salina*, which also exhibited decrease of chlorophyll cell content with increase of CO_2% in aeration stream. On the other hand, while decrease of carotenoid cell content was observed in *N. oleoabundans* decrease of pH with cultures containing 160 mM added $NaHCO_3$, as was the case of chlorophyll

profiles, the control, which had a culture pH of 7.5 and the lowest dCO_2 due to its zero added $NaHCO_3$ had carotenoid cell content in the stable stage that was only slightly higher than the 160 mM pH 7.5 culture. Apparently, the DIC level of culture had very limited impact on the cellular carotenoid content whereas pH appeared to the determining factor. This phenomenon could hypothetically be attributed to the photoprotective role played by carotenoids in photosynthesis, where they are responsible for scavenging excessive reactive oxygen species (ROS). At high culture pH levels, there might be an elevated production of ROS, which may cause oxidative damage to the photosynthetic apparatus and other cellular materials [47,48]. Therefore, the observed increase in carotenoid content in in high pH culture might reflect the alga's adaptation to cope with the enhanced oxidative stress through the upregulation of carotenoid biosynthesis. It was reported that excessive dCO_2 may lead to a saturation of carbonic anhydrase, an enzyme critical for CO_2 assimilation in algae. This saturation may decrease the availability of CO_2 for photosynthesis, which can reduce the efficiency of light utilization and energy conversion, resulting in a decreased chlorophyll content [49]. It was reported that the chlorophyll concentration and biomass of *Chlorella vulgaris* at 16% CO_2 level were similar to those at ambient CO_2 concentration, but further increases in CO_2 concentration decreased both parameters [50].

The lipid content of algal cells without DIC at pH 7.5 was 289±1.70 mg/g. Upon addition of 160 mM DIC, the lipid content decreased to 275±2.5 mg/g at the same culture pH of 7.5. However, comparing between the cultures containing 160 mM at different pH, the lipid content increased with culture pH, i.e., 288±2.3 at pH 8.5, and 306±1.6 mg/g at pH 9.5. The observed increase of cellular lipid content in cultures containing 160 mM added DIC with the increase of culture pH was consistent with our previous observations with another green algae, *Chlorella vulgaris* [42] and seems to affirm the hypothesis that high $[CO_3^-]$ is likely a stimulator of lipid accumulation of algal cells [42]. On the other hand, the decrease of lipid content at the addition of 160 mM $NaHCO_3$ at pH 7.5 is somewhat counterinuitive since it must had a higher $[CO_3^-]$

than the culture without added $NaHCO_3$ at the same pH, i.e., the control. As shown in Table 4-2, the [CO_3^-] of cultures with 160 mM $NaHCO_3$ would have only 0.22 mM more [CO_3^-] than the control, which probably was not sufficient to stimulate lipid synthesis of cells. On the other hand, the dCO_2 at pH 7.5 is 11.03 mM with 160 mM added $NaHCO_3$ than that without at the same pH, corresponding to saturating the cultures with 30% CO_2 in the aeration stream than the control. This seems to indicate that while high [CO_3^-] likely is stimulating, high dCO_2 is probably inhibiting to lipid biosynthesis in algal cells.

It not entirely surprising but nonetheless interesting to observe that the carbohydrates content of cells exhibited an opposite trend as that of the lipid. It increased from 309.55 ±3.47 mg/g in cultures at pH 7.5 without added $NaHCO_3$ to 381.95±5.72 mg/g in cultures of pH7.5 with 160 mM added $NaHCO_3$. Furthermore, there was a notable decrease in carbohydrate content as the pH level increased with cultures containing 160 mM. This phenomenon could be tentatively explained by the fact that both carbohydrates and lipids could be used as storage of carbon source and energy source by *N. oleoabundans* cell. When the pathways for biosynthesis of lipids were stimulated, less metabolic flux would be directed to carbohydrates and vice versa.

There are reports indicating that algal cells tend to accumulate more carbohydrates rather than lipids, likely as an adaptive response to the high CO_2 environment [49,50]. Under these conditions, algae adjust their metabolic pathways to more effectively utilize the surplus carbon source, converting it into carbohydrates rather than for lipid production. It was reported that excessive levels of CO_2 (greater than 15%) were unfavorable for lipid accumulation in green alga *Nannochloropsis oculata* [51]. These data reflect the algae's flexibility in responding to varying environmental conditions, illustrating their ability to adjust energy storage strategies and metabolic pathways in response to changes in carbon availability and environmental stress.

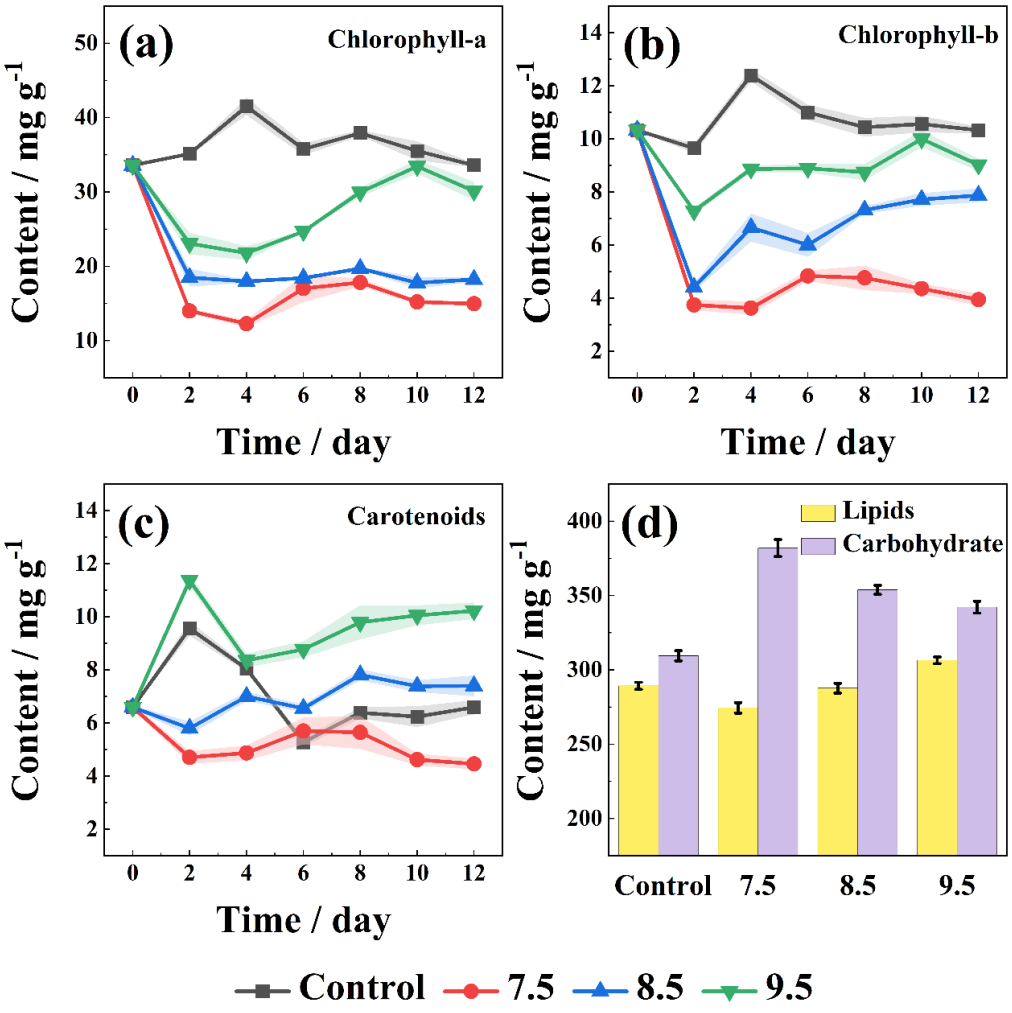

Figure 4-7. Cellular contents of pigments including chlorophyll-a (a), chlorophyll-b (b) and carotenoids (c) and lipid and carbohydrate (mg/g) (d) of *N. oleoabundans* in media of 160 mM $NaHCO_3$ at different pH. The data are mean values obtained from three replicates and are presented with shaded regions and error bars.

4.3.3 Two-step cultivation of *N. oleoabundans* in HTLAR

A two-stage cultivation method was tested, where algae were initially grown in medium with zero $NaHCO_3$ with culture pH controlled at around 7.5 using aeration with CO_2 enriched air, and then supplemented with 160 mM $NaHCO_3$ at a pre-determined day of cultivation, when the aeration was stopped as well. The pH was monitored throughout the entire process. Fig. 4-8a illustrates the pH changes during the two-step culture with the addition of $NaHCO_3$ at different time points. It can be

observed that after stopping aeration and adding $NaHCO_3$, the pH gradually increased to above 10.5.

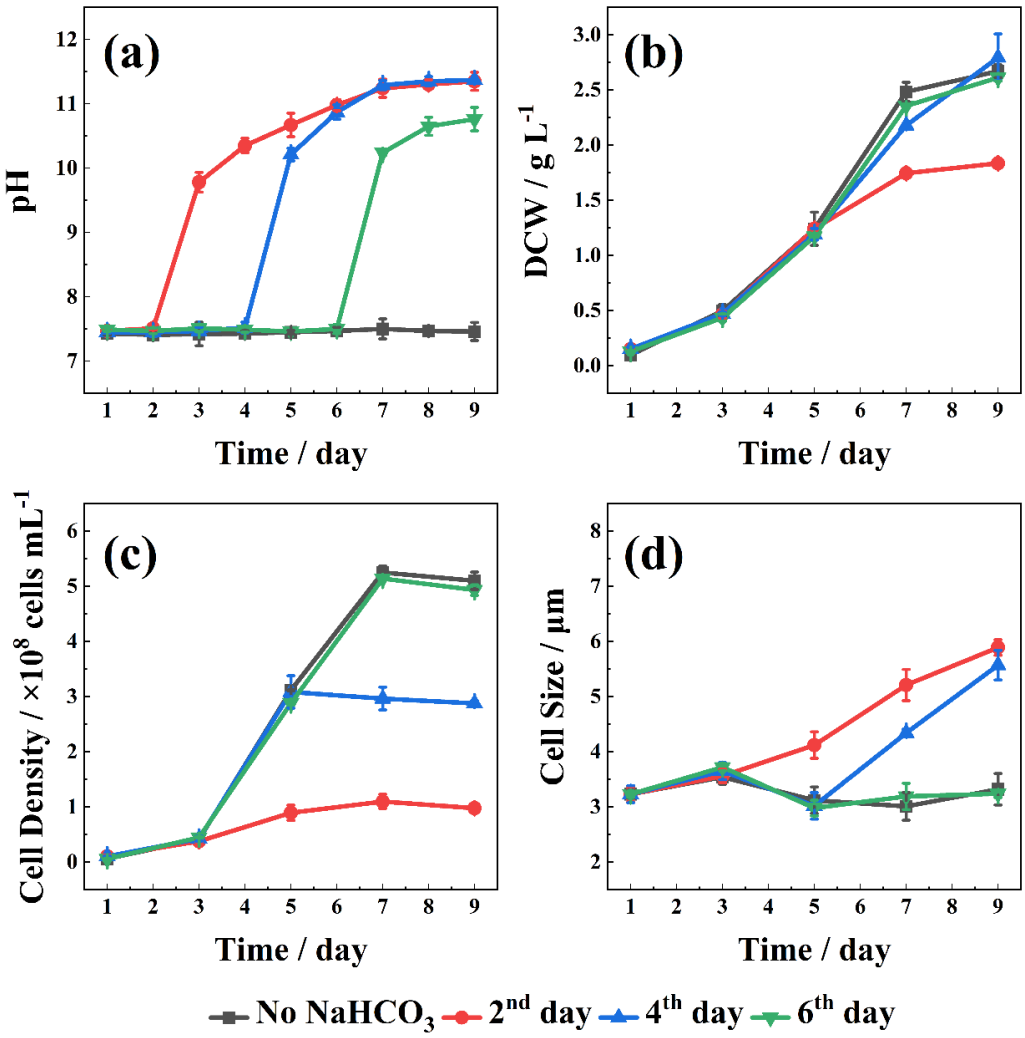

Figure 4-8. Two-stage cultivation of *N. oleoabundans* with the combination of CO_2 input and 160 mM $NaHCO_3$ as carbon sources without pH control pH change (a); Biomass concentration g/L(b); Cell density x10^8cells/mL (c); Cell size μm (d). "No $NaHCO_3$" refers to a cultivation condition where pH was maintained at 7.5 and carbon source was provided solely through aeration with air stream enriched by CO_2, without the addition of $NaHCO_3$. 2, 4, and 6 represent the time points at which aeration was ceased and 160 mM sodium bicarbonate was introduced into the culture without pH control. The data are mean values obtained from three replicates and are presented with error bars.

Figures 4-8b, 4-8c, and 4-8d illustrate the effects of adding $NaHCO_3$ at different time points on algal biomass concentration, cell number, and cell size, respectively. Regarding biomass concentration, except for the algae that received DIC on the second day, which had a lower biomass concentration of only 1.84 g/L after eight days of cultivation, the addition of 160 mM $NaHCO_3$ did not have a significant impact on biomass concentration, which was around 2.7 g/L. As shown in Fig.4-8c, the addition of $NaHCO_3$ on the second day significantly inhibited the cell density of algae, with no visible cell density increase after the fourth day and a maximum cell density of 10^8 cells/mL. The culture that was supplemented with $NaHCO_3$ on the fourth day had only a slight inhibition of cell division, with a maximum cell density of 3×10^8 cells/mL. However, adding DIC on the sixth day did not significantly affect algal growth, as the final cell density reached 5×10^8 cells/mL. Regarding changes in cell size, Fig. 4-8d shows that the addition of $NaHCO_3$ led to enlargement of algal cells as soon as $NaHCO_3$ was added.

The results are consistent with previous sections. $NaHCO_3$ addition inhibited algal cell division and increased cell size, attributed to carbon source insufficiency and altered algal metabolism under high pH and DIC conditions. It can be seen from **Table 4-4** that adding 160 mM on the 4^{th} day of cultivation was the best among the tested conditions, which resulted in slightly larger cell growth rate and lipid productivity, 76.73 % saving on aeration CO_2 and 50% saving on aeration time and energy in comparison to that of the control, i.e., culture without $NaHCO_3$ but with culture pH controlled at 7.5 with CO_2 enriched air. Elevated pH and DIC concentrations tend to promote the accumulation of more lipids in algal cells. Moreover, the two-stage cultivation method would also help reduce the costs associated with the monitoring of culture in the later stage of cultivation.

Table 4-4. Summary of key parameters of *N. oleoabundans* growth, lipid production, and aeration in HTLAR.

	pH controlled at 7.5	no pH control	0 day ($NaHCO_3$*)	2nd day ($NaHCO_3$)	4th day ($NaHCO_3$)	6th day ($NaHCO_3$)
Biomass (g/L)	2.75	1.72	1.76	1.84	2.79	2.61
Cell density (10^8 cells/mL)	5.10	1.56	0.61	0.98	2.88	4.93
Cell size (μm)	3.43	3.79	6.23	5.89	5.57	3.24
Lipids content (mg/g)	285	298	314	319	309	289
Lipid productivity (mg/L/d)	97.97	64.07	69.08	73.37	107.76	94.29
Aeration saving	0	100%	100%	75%	50%	25%
CO_2 saving	0	100%	100%	92.45%	76.73%	44.03%

* $NaHCO_3$: 160 mM $NaHCO_3$ added in target day without pH control.

4.4 Conclusions

The influences of pH and DIC concentration on the growth of *N. oleoabundans* were examined in this study. The addition of $NaHCO_3$ was found to be inhibitive cell division but stimulative to the growth of individual cells by means of cell enlargement, indicating that high DIC concentration could be inhibitive to factor(s) related to cell division but unrelated to photosynthesis and other metabolic pathways related to the synthesis of cell materials for the growth of individual cells. Based on this finding, a novel two-step cultivation approach was devised to mitigate aeration and reduce carbon dioxide wastage. By introducing $NaHCO_3$ on the fourth day of cultivation and ceasing aeration, it was possible to achieve comparable biomass concentration with lower cell

density but larger cell size while saving a remarkable 76.73 % of aeration, which is costly in terms of energy, labour, and other costs. This innovative method holds great promise for optimizing resource utilization and enhancing the efficiency of algal cultivation processes. In addition, systematic studies on the effects of culture pH with or without 160 mM added $NaHCO_3$ indicate that dCO_2 was likely the inhibitor to cell division while the bicarbonate ions were likely the primary carbon source for photosynthesis, confirming the existence of the carbon concentration mechanism in *N. oleoabundans.* It was further found that the optical density and biomass concentration ratio was dependent cell size while other factors such as the structure of cell surface might also played important roles, highlighting the necessity of precautions while using the conventional spectrometric method for determination of biomass concentration.

CRediT authorship contribution statement

Hongying Zhou: Conceptualization, Investigation, Methodology, Data curation, Visualization, Writing - Original draft. **Ju Wang:** Investigation, Data curation, Visualization. **Zitong Xu:** Methodology, Data Curation. **Xinyue Wang:** Data Curation. **Zisheng Zhang:** Resources, Supervision. **Christopher Q. Lan:** Conceptualization, Supervision, Project administration, Funding, Resources, Writing - Review & Editing.

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Chapter 5 High cell density culture of microalgae in horizontal thin-layer algal reactor: modeling of light attenuation and cell growth kinetics

Abstract

This study delved into the light intensity effects and light attenuation modeling in high cell density culture (HCDC) of green alga *Neochloris oleoabundans*. The research primarily focused on how different light intensities influence cell growth in terms of cell division and cell mass, and the biochemical composition within the cells. Recognizing the need to avoid overestimating light path by excluding data point in the data zone where the light intensity was zero, we proposed and verified a novel modified Beer-Lambert model, which was superb in fitting experimental data and predicting light attenuation in both low and high cell density cultures. Taking advantage of the prediction power of the modified Beer-Lambert model, we devised an approach to maintain constant mean light intensity (I_{mean}) and by adjusting the incident light intensity (I_0). The results indicate that at an I_{mean} of approximately 66.67 μmol/m²/s and 6 mm culture thickness, maximum volumetric and areal biomass productivities of 2.72 g/L/day and 16.32 g/m^2/day, respectively, were achieved. Whereas the highest biomass concentration of 28.20 g/L was produced in 20 days at I_{mean} 50 μmol/m²/s. Photoinhibition to cell division become evident at I_0 750 μmol/m²/s and above. Using the data generated at constant I_{mean} in the constant specific growth rate range, superb fitting to the Monod model with a R^2 of 0.9910 was demonstrated, highlighting the importance of generating reliable data for the modelling of the kinetics of photoautotrophic growth of microalgae, which had considered to be challenging.

Keywords: light attenuation, high cell density culture of microalgae, growth kinetics, Monod model, modified Beer-Lambert model

5.1 Introduction

Microalgae, as unicellular or simple multicellular photosynthetic microorganisms, have garnered immense interest due to their vast potential in applications such as wastewater treatment [1], CO_2 bio-mitigation [2] and production of an array of value-added products including biofuels [3], food additives [4], animal feed [5], biofertilizers [6], and nutraceuticals and pharmaceuticals [7]. Their notable attributes include rapid growth rates, adaptability to varied environments, and vast capacity for carbon dioxide sequestration [8,9]. Despite the tremendous potential of microalgae, there are still technical bottlenecks impeding their cost-effective mass production. These include high-cost and low-efficiency algal cultivation systems and barriers in system scalability [10]. This issue is compounded by inefficient techniques for harvesting [11] and subsequent processing [12], which further strain the economic feasibility. Therefore, to unlock the full industrial promise of microalgae, there is an imperative need for a holistic re-examination and innovation in cultivation methodologies.

Among the conventional cultivation systems, open culture systems have gotten popularity for their simplicity, good scalability, and cost-effectiveness in comparison to closed systems [13,14]. However, these systems often grapple with extremely low sustainable biomass concentration, which is typically below 1.0 g/L for lab scale systems and 0.5 g/L in commercial systems [15,16], and therefore low volumetric productivity that leads to high operational costs. To this end, high cell density culture (HCDC) of microalgae has emerged as a promising alternative [17,18] and we have developed a novel horizontal open thin-layer algal reactor (HTLAR) for such purpose [19]. One distinctive attribute of this design is its reduced culture depth, which enhances the surface/volume ratio and therefore light penetration. Moreover, the incorporation of hollow fiber membranes in this design presents an innovative mechanism for CO_2 delivery, which augments CO_2 absorbance by culture, and therefore culture pH control and photosynthetic efficiency. The design of this reactor is inspired by traditional thin-layer cascade (TLC) reactors [20], with modifications aimed at achieving intermittent stirring to reduce energy consumption for mixing. According to a Life Cycle

Assessment study, the greenhouse gas emissions of aviation fuel produced using the algal biomass from TLC (81 g CO_2e per MJ) were lower than those from open raceway ponds (94 g CO_2e per MJ) but still not sufficient to meet the Renewable Energy Directive II requirements due to significant impacts from factors such as mixing power [21]. Previous studies have demonstrated that the HTLAR can achieve approximately 92% energy savings in stirring through optimized stirring frequency. This offers potential benefits over traditional TLC systems, which requires continuous circulation by pumping.

Apart from the optimization of reactor design, the optimization of cultivation parameters is of paramount importance. While numerous studies have been reported on the effects of cultivation parameters such as pH [22], temperature [23], light intensity [24], and nutrients [25], most of them were done for conventional low cell density cultures. Light serves as the energy source for the photoautotrophic growth of microalgae and is therefore fundamental to algal growth and metabolism, including the synthesis of biomolecules of commercial interests [26,27]. Insufficient light can plunge the microalgae into a state of photo-limitation, curtailing its growth potential [28]. Conversely, excessive light exposure risks pushing the culture into a regime of photo-inhibition [29,30], where the high radiance intensity becomes counterproductive, potentially hampering cell growth or causing damage to cells. Thus, striking the right balance in light intensity becomes essential in maximizing microalgal productivity. While we have no control over the intensity of solar radiance in a typical outdoor algal farming system, some measures would allow us to maximize light harvest, e.g., by adjusting orientation of solar panels [31,32] or minimize light inhibition, e.g., by partial shielding [33,34]. A thorough understanding of the light penetration, attenuation, and distribution in an algal culture would provide valuable insights.

Currently, there are several studies on light distribution models including the Lambert-Beer model [35], the Cornet model [36], and the Fuzzy-logic model [37]. However, most of these models are developed based on data collected from conventional low cell density cultures that have low biomass concentrations (0.1g/L-

3g/L) and large cultivation depths (1-20 cm) and are primarily intended for closed photobioreactors [38–41]. While some pioneering works focus on HCDC of microalgae, there is much confusion and ambiguity in such works. For instance, Doucha and Lívanský had to coin a concept, i.e., mean (specific) extinction coefficient (ε_{mean}), to account for the complexity of light attenuation in HCDC [42]. The value of ε_{mean} was dependent on culture thickness and biomass concentration of the culture, which contradicts the nature of specific extinction coefficient as an intrinsic property of the material of concern, i.e., the algal cells in this case since the effects of both the culture thickness and biomass concentration were accounted for explicitly in related equations. Furthermore, researchers have been struggling to develop satisfactory kinetic models to correlate the dependency of the photoautotrophic growth of microalgae with light intensity with little success. For instance, in a recent study, Esteves et al. [43,44] assessed nine kinetic models using both experimental results and literature values concerning the growth of *Chlorella vulgaris*, using either the Beer-Lambert or the Cornet model for light attenuation. The fitting the experimental data with these models were all very poor, as suggested by the R^2 values ranging from 0.055 to 0.805.

In this study, the horizontal thin-layer algal reactors [45] were utilized to achieve HCDC of *Neochloris oleoabundans*, which is distinguished by its high lipid cell content [45]. The initial phase of the investigation focused on growth patterns under constant incident light intensity I_0 at various starting biomass concentrations. Then, a modified Beer-Lambert Model was proposed and verified using experimental data, which better reflects the constraints imposed by HCDC and confirms the specific extinction coefficient as an intrinsic property of algal culture independent of culture thickness and biomass concentration. It was also found that the Monod kinetic model, as example of well-established models found wide application in modelling the kinetics of the growth of heterotrophic microorganisms, to be applicable to the photoautotrophic growth of microalgae, provided that the experimental data were generated using the appropriate approach, solving a long standing challenge in the field.

5.2 Materials and methods

5.2.1 Microalgal strain and media

The *N. oleoabundans* (UTEX# 1185) strain was acquired from the UTEX Culture Collection of Algae at the University of Texas at Austin. Before experimentation, microalgae were cultivated in 250mL flasks filled with 80 ml of freshly prepared Modified Bristol Medium (MBM) following the specified recipe (per liter): 0.7 g $NaNO_3$, 0.138 g K_2HPO_4, 0.0823 g $MgSO_4$, 0.025 g $CaCl_2$, 0.322 g KH_2PO_4, 0.025 g NaCl, 0.0023 g $FeCl_3$ and 1 mL A_5 solution which was consisted of (per liter): 1.6423 g EDTA-Fe, 2.86 g H_3BO_3, 1.81 g $MnCl_2{\cdot}4H_2O$, 0.22 g $ZnSO_4{\cdot}7H_2O$, 0.079 g $CuSO_4{\cdot}5H_2O$, and 0.039 g $(NH_4)_6Mo_7O_2{\cdot}4H_2O$.

5.2.2 Seed culture

To prepare the microalgae inoculum, which serves as the seed culture, aseptic techniques were employed. Fresh medium, totaling 400 mL, was subjected to sterilization at 121°C for 20 minutes within 500 mL cultivation bottles. After cooling to room temperature, 20 mL of the inoculum was introduced into the sterilized medium. A continuous stream of air, enriched with 5% CO_2 and purified through a gas washing bottle containing distilled water, was introduced into the cultures at a flow rate of 0.5 vvm to maintain the pH of the cultivation at 7.5. The cultivation bottle was illuminated using two LED lights, providing a light intensity of 160 μmol/m^2/s, programmed for 12 hours of light followed by 12 hours of darkness. Additionally, the cultures were kept in constant motion by a magnetic stirrer positioned at the bottom of the bottle. The microalgae were cultivated for 6 to 10 days, when they were ready as seed cultures.

5.2.3 Microalga cultivation

5.2.3.1 General cultivation process

In the current research, the horizontal thin-layer algal reactor (HTLAR) was utilized for cultivation purposes. The design specifics of this reactor are illustrated in Fig.3-1, as detailed extensively in our previous publication. The HTLAR was comprised of several glass dishes (having an internal diameter of 100 mm). Each dish was equipped with an intrinsic hollow fiber membrane (HFM), a magnetic stirrer, and a system designated for water replenishment. During the experiments, continuous

stirring was a constant only at daytime. Given the large surface-to-volume (A/V) ratio of the HTLAR, periodic addition of deionized water was deemed necessary to counteract the substantial water loss due to evaporation. The HFM, strategically positioned at the bottom of the dishes, served the purpose of aeration, utilizing CO_2-enriched air. This method ensured that the pH of the culture was consistently maintained within the range of 7 to 7.5, achieved using a constant airflow rate of 4 vvm. To ensure a culture thickness of 6 mm, 48 mL of the culture was meticulously added to each dish. Illumination was facilitated by two LED lights, operating on a structured 12-hour on/off cycle. Light intensity varied and was measured using the Dual-Range Light Meter (Traceable® Calibration Control Company, Canada).

5.2.3.2 Cultivation with varied initial biomass concentrations

To investigate the effect of different starting biomass concentrations on microalgal growth, cultures were inoculated with varying initial concentration (from 1 g/L to 18 g/L). Each concentration was then cultivated under two distinct light environments: low light intensity at 83.3 μmol/m^2/s and high light intensity at 833 μmol/m^2/s. All other experimental parameters and conditions were kept consistent with the general cultivation process as delineated in section 5.2.3.1.

5.2.3.3 Cultivation under different mean light intensities

In this set of experiments, the influence of mean light intensity ($I_{mean)}$ on microalgal growth was assessed. Cultures were initiated with a biomass concentration of 1 g/L. Five different levels of I_{mean} were employed, i.e., 8.33, 16.67, 33.33, 50, and 66.67 μmol/m^2/s. The I_0 was adjusted at the beginning of a light period of a cycle according to the biomass concentration at the end of the last light period using the modified Beer-Lambert model, i.e., Eqs. 12 and 13. It should be noted that the I_{mean} would be decreasing continuously during the light period and become zero in the dark period, although it would be set to the pre-set value at the beginning of the light period. To guarantee abundant nutrient availability, the culture medium was periodically refreshed. Specifically, the culture medium was replaced every time the biomass concentration increased by 2.5 g/L to ensure an adequate nutrient supply for cell growth. All other

cultivation parameters were the same as described in the general cultivation process outlined in section 5.2.3.1.

5.2.4 Measurement and analysis of light attenuation in cultures

To gain insights into the light attenuation characteristics in microalgal cultures, the light intensity at the base of the cultures (denoted as I_h) was determined across various experimental conditions. Cultures were prepared with varying biomass concentrations, spanning a spectrum from a minimal 0.05 g/L up to a significant 60 g/L. Different culture thicknesses were set to simulate various depths of light penetration, ranging from 1 mm up to 16 mm. A spectrum of initial light intensities I_0 was employed, starting from a minimal intensity of 8.33 μmol/m^2/s and extending up to an intensity of 833 μmol/m^2/s. A traceable dual-Range light meter (Fisher Scientific, Canada) was placed at the bottom outside of the dish to sense the light intensity passing through. The precise range and intervals were determined based on experimental requirements. Following the above procedures, measurements of I_h were recorded.

5.2.5 Analytical methods

5.2.5.1 Measurement of biomass concentration

To assess the biomass concentration (X, g/L), optical density readings of the culture were acquired at a 700 nm wavelength (OD_{700}) using a GENESYSTM 10S UV-Vis spectrophotometer from Thermo Fisher Scientific Inc. Samples were adjusted to ensure an OD_{700} range of 0.1 to 0.8. A relationship between optical density and biomass concentration was delineated by a standard curve. Factors from this curve were adjusted in response to shifting culture conditions. In the sample preparation stage, the algae in suspension underwent centrifugation at 6000 RCF, followed by a double rinse with deionized water. The resultant pellets were oven-dried at 60°C until reaching a consistent weight, typically after a 24-hour period.

5.2.5.2 Estimation of cell density and observation of cell morphology

To evaluate the cell densities of the a microalga culture, cell counts were conducted using a hemacytometer (Improved Neubauer, Phase Counting Chamber w/2 cover glass, USA) and visualized under a phase-contrast microscope (Infinity II BX40, Olympus,

Canada) at 200x magnification. The Infinity Capture software (Lumenera Scientific, Ottawa, Canada) assisted in counting cells. Additionally, cell morphology was studied using the same hemacytometer but at a 600x magnification, with assistance from the Infinity Analysis software (Lumenera Scientific, Ottawa, Canada). Prior to counting, samples underwent dilution for accuracy in measurements.

5.2.5.3 Photosynthetic pigments content

The concentrations of pigments, specifically chlorophyll a (Chl-a), chlorophyll b (Chl-b), and carotenoids, were deduced through Lichtenthaler's methodology [46]. Samples were first diluted to an appropriate biomass concentration. Following centrifugation at 6000 RCF for 10 minutes, the cell pellets were reconstituted in 2 mL of methanol and stored at 4 °C overnight. These samples were later assessed for pigment content via a spectrophotometer set at wavelengths of 470 nm, 665 nm, and 652 nm. Using equations 1-4, concentrations of the various pigments were calculated. The pigment concentration on a volumetric basis was defined using the subsequent equations:

$$\text{Chl-a (mg / L)} = (16.72A_{665} - 9.16A_{652}) \times \text{dilution ratio} \quad (5\text{-}1)$$

$$\text{Chl-b (mg / L)} = (34.09A_{652} - 15.28A_{665}) \times \text{dilution ratio} \quad (5\text{-}2)$$

$$\text{Carotenoid (mg/L)} = (1000A_{470} \times \text{dilution ratio} - 1.63C_a - 104.9C_b) / 221 \quad (5\text{-}3)$$

$$\text{Pigments} = \text{Chl-a} + \text{Chl-b} + \text{Carotenoid} \quad (5\text{-}4)$$

Where C_a and C_b are the content Chlorophyll a and Chlorophyll b,respectively. The cellular content of the pigments (mg/g) was ascertained by dividing the volumetric pigment concentration (mg/L) by the respective biomass concentration (g/L).

5.2.5.4 Lipid extraction and quantification

Post-cultivation, the microalgal biomass was harvested, centrifuged, and washed thrice. The biomass was then oven-dried at 60 °C. For lipid extraction, an adaptation of the Bligh and Dyer technique [47] was employed. Around 100 mg of oven-dried biomass was pulverized and subjected to lipid extraction in a solvent mix of chloroform:

methanol (2:1 v/v) and deionized water. This mixture was agitated for 24 hours at 220 rpm to facilitate solvent penetration and thorough lipid extraction. After extraction, centrifugation at 6000 RCF for 10 minutes led to a triphase formation: upper methanol and water phase, middle biomass phase, and lower lipid-infused chloroform phase. The lipid phase was delicately harvested and moved to a pre-weighted aluminum plate. The residual mixture was enhanced with additional chloroform, mixed, centrifuged, and the resulting chloroform phase combined with the previous extract on the plate. To evaporate any lingering solvent, nitrogen gas was employed, after which the plate underwent heating in a 60°C oven until a stable weight was achieved. The lipid content (C_L, mg/g) of the desiccated biomass was then determined, offering insights into the microalgae's lipid production capability.

5.2.5.5 Determination of carbohydrates

The carbohydrate content in the microalgal samples was determined using the phenol-sulfuric acid method [48]. This method is based on the reaction between carbohydrates and sulfuric acid, which forms furfural derivatives that subsequently react with phenol to produce a colored compound. To quantify the total carbohydrates, 10 mg of dried microalgal biomass was used as the starting material. This biomass underwent sulfuric acid hydrolysis to break down complex carbohydrates and extract simpler sugars. Following hydrolysis and subsequent neutralization, the extracted sugars in the filtrate were prepared for the colorimetric assay.

For the colorimetric assay, 0.2 mL of the extracted sugar solution was combined with 0.2 mL of 5% phenol. Following this, 1 mL of concentrated sulfuric acid was cautiously introduced. After combining the reagents, the mixture was left undisturbed for 10 minutes. It was then agitated in a shaking bath for an additional 20 minutes to achieve thorough mixing. Subsequently, the absorbance values of the samples were recorded. A predetermined standard curve facilitated the extrapolation of total carbohydrate concentrations (C_C, mg/g) in the tested samples.

5.2.6 Calculations and Modeling

5.2.6.1 Productivity

To obtain the volumetric biomass productivity (P_V) and areal biomass productivity (P_A) of algae, Eq. 5-5 and Eq. 5-6 were used.

$$P_V = (X_2 - X_1)/t \qquad (5\text{-}5)$$

$$P_A = P_V/h \qquad (5\text{-}6)$$

where P_V (g L^{-1} day^{-1}) is volumetric biomass productivity, P_A (g m^{-2} day^{-1}) areal biomass productivity, X_1 and X_2 the biomass concentration at the beginning and end of cultivation t (day), and h (mm) the culture thickness.

To obtain the cell lipid productivity (P_l) and carbohydrates productivity (P_C). Eqs. 5-7 and 5-8 were used.

$$P_l = P_V C_l \qquad (5\text{-}7)$$

$$P_C = P_V C_C \qquad (5\text{-}8)$$

where C_l and C_C indicate the cellular lipid content and carbohydrates content at the end of cultivation, respectively.

5.2.6.2 Light attenuation determination and model construction

The light intensity I_h (μmol/m^2/s) at distance h (mm) from the algal culture surface can be expressed in accordance with the Lambert-Beer law as:

$$I_h = I_0 \times \exp(-A(X)h) \qquad (5\text{-}9)$$

where I_0 (μmol/m^2/s) is the photosynthetic active radiance (PAR) at the surface of algal culture, $A(X)$ the extinction coefficient, which could be related to X and ε by the Beer-Lambert law:

$$A(X) = \varepsilon X \qquad (5\text{-}10)$$

where ε (m^2/g) is the mass specific extinction coefficient, X (g/L). Furthermore, the following modified Beer-Lambert law with an exponent n was proposed and verified in this study.

$$A(X) = \varepsilon X^n \qquad (5\text{-}11)$$

The original Beer-Lambert law linearly relates $A(X)$ to X but the modified Beer Lambert law relates $A(X)$ to X in an exponential relationship. Therefore, the I_h was calculated using the following equation when the modified Beer Lambert law was used:

$$I_{\hbar} = I_0 \times \exp(-\varepsilon X^n \hbar) \qquad (5\text{-}12)$$

where I_h presents the calculated light intensity at distance h (mm) from the algal culture surface by using the original Beer-Lambert law and I_{hM} the calculated light intensity at distance h (mm) from the algal culture surface by using the modified Beer-Lambert law. For a mean light intensity I_{mean} (μmol/m^2/s) in the algal culture layer of thickness h (mm), it follows that:

$$I_{mean} = \frac{1}{\hbar}\int_0^{\hbar} I dz = \frac{I_0 - I_{\hbar}}{-\ln(\frac{I_h}{I_0})} \qquad (5\text{-}13)$$

Considering Eqs. (5-10) and (5-11), it follows that for calculating I_{mean} using the Beer Lambert law,

$$I_{mean} = \frac{1}{\hbar}\int_0^{\hbar} I dz = \frac{I_0}{\hbar}\int_0^{\hbar} \exp(-\varepsilon X Z) dz = I_0 \frac{1-\exp(-\varepsilon x h)}{\varepsilon X \hbar} \qquad (5\text{-}14)$$

Using the modified Beer Lambert law,

$$I_{mean} = \frac{1}{\hbar}\int_0^{\hbar} I dz = \frac{I_0}{\hbar}\int_0^{\hbar} \exp(-\varepsilon X^n Z) dz = I_0 X^{-n} \frac{1-\exp(-\varepsilon X^n \hbar)}{\hbar \varepsilon} \qquad (5\text{-}15)$$

5.2.6.3 Specific growth rate prediction by Monod Model

To obtain the net specific growth rate (μ), the equation below has been used:

$$\mu_{net} = \frac{LN(X_2) - LN(X_1)}{t_2 - t_1} \qquad (5\text{-}16)$$

where t_2 and t_1 are the corresponding time points (day), and X_2 and X_1 are the corresponding biomass concentrations (g/L).

Monod equation was used to fit the experimental data as follow:

$$\mu = \frac{\mu_m \times I_{mean}}{K_I + I_{mean}} \qquad (5\text{-}17)$$

where μ is the total growth rate (day^{-1}), μ_m the maximum specific growth rate (day^{-1}) and K_I the half-saturation constant. Assume the endogenous metabolism for cell maintenance was negligible, then $\mu = \mu_{net}$.

5.2.7 Statistical analysis

All data were processed using Excel 2016 and all figures were drawn by Origin 2021. Reported are the mean values of three independent replicates with standard deviations. Error bars in the figures are the standard deviation from the mean values, providing a visual representation of the data spread. Regression fitting was performed using Python, with NumPy being employed for numerical computations and data

handling. The fitted model was then used to generate predictions and assess the goodness of fit through statistical metrics like the coefficient of determination (R^2).

5.3 Results and Discussion

5.3.1 Effect of initial biomass concentration and light intensity on cell growth of *N. oleoabundans*

5.3.1.1 Effect of initial biomass concentration and light intensity on algal cell growth

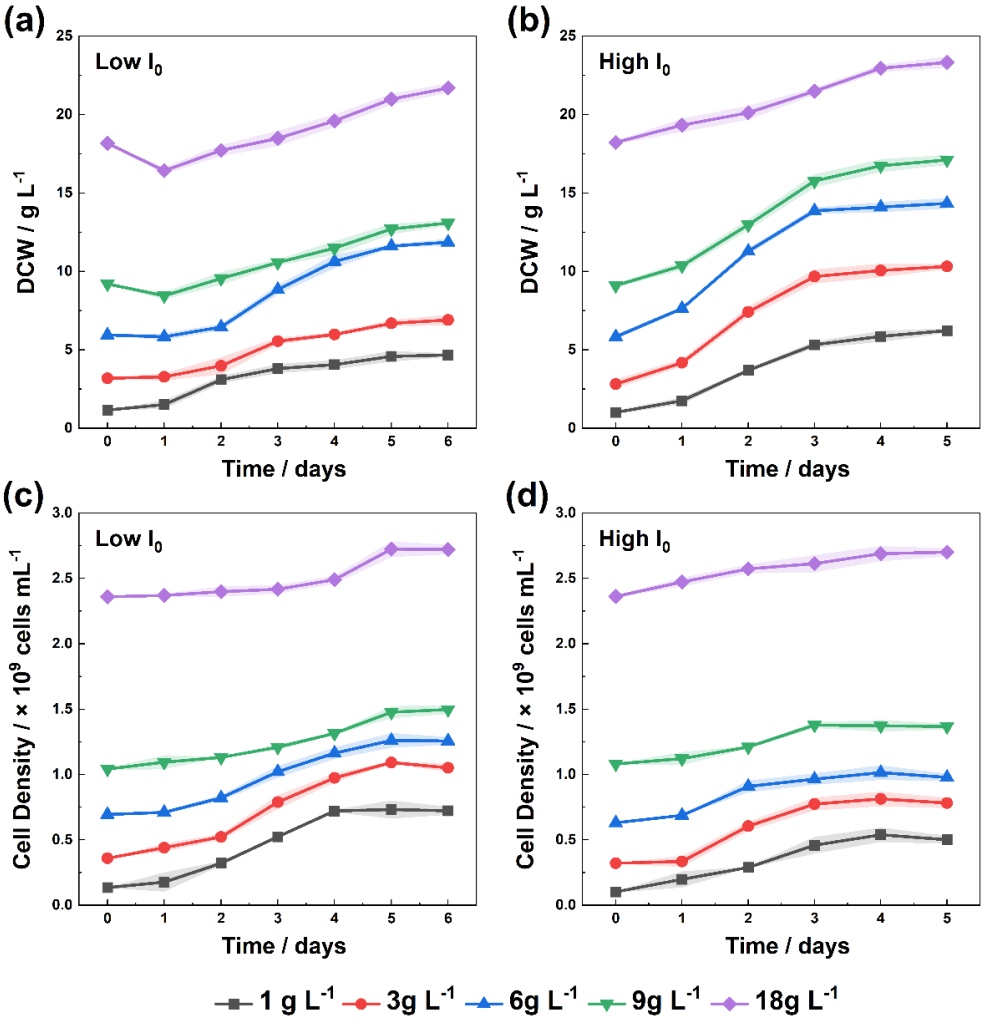

Figure 5-1. Effects of initial biomasss concentration on algal cell growth of *N. oleoabundans* in terms of biomass concentration (g/L) and cell desnity (cells/mL) at incident light intensity of 83.3 μmol/m^2/s (low I_0) and 833 μmol/m^2/s (high I_0).

Figure 5-1 displays the growth of algae at various initial biomass concentrations (X_0) (from 1 to 18 g/L) under two different constant incident light intensities: 83.3

μmol/m^2/s (low I_0) and 833 μmol/m^2/s (high I_0). As shown in Figure 5-1a, algae at low I_0 showed a noticeable lag phase for all X_0 except for the case of X_0 =1 g/L when a slight increase of biomass concentration was observed in the first day of cultivation. In the meantime, no visible change of biomass concentration was observed for X_0 of 3 or 6 g/L and a slight decrease was observed at X_0 9 or 18 g/L. On the other hand, as shown in Figure 5-1b, there was no visible decrease in biomass concentration on the first day of cultivation with all initial biomass concentrations under high I_0. These data indicate that the light intensity received by individual cells, which was dependent on both the incident light intensity and biomass concentration for a culture of constant thickness, was the limiting factor that caused the lag phase at low I_0. Figures 5-1c and 5-1d indicate that cell division stopped on the 4^{th} or 5^{th} day of cultivation for all the tested X_0 and I_0, even though the final biomass concentrations of some low X_0 cultures were lower than the initial biomass concentration of the high X_0 culture.

As shown in Figure 5-2, the maximum areal productivity (P_A) was observed at an initial biomass concentration of 6 g/L, which was 5.9 and 8.5 g/m^2/d at low and high I_0, respectively. For algae that were exposed to the high I_0, the P_A was typically in a range of 5 - 8.5 g/m^2/d, which was more than the 3-6 g/m^2/d observed for algae at the low I_0. As shown in Figures 5-1c and 1d, as well as Figure 5-2, the cell density of cultures increased with time in all cases, however, the extent of increase varied. Under the selected light intensity conditions, even when subjected to an initial inoculation concentration as high as 18 g/L, cell division still happened. Nonetheless, it is noteworthy that under the high I_0, the cell division of algae with an initial biomass concentration of 18 g/L was similar to that under low I_0. When the x_0 increased from 3 g/L to 9 g/L, there was a smaller increment in cell density (CDI) under low I_0. In contrast, the CDI was more significant when x_0 rose from 9 g/L to 18 g/L The zenith cell density increase was found at an initial biomass concentration of 3 g/L, with the cell density increased by 0.69×10^9 cells/mL and 0.46×10^9 cells/mL corresponding to 83.3 μmol/m^2/s and 833 μmol/m^2/s, respectively. As also depicted in Figure 5-2, cells exposed to high I_0 exhibited a greater average size relative to their counterparts grown

under low I_0 at low X_0 of 1 g/Lwith the mean diameter of algal cells at 83.3 and 833 μmol/m^2/s being 3.52 and 3.98 μm, respectively. Furthermore, the increment in cell size with high I_0 was mitigated as X_0 was elevated. For example, at 9 g/L, the mean cell diameters were 3.66 and 3.68 μm, respectively. These data indicate that the cells experienced certain extent of light inhibition at the high I_0, i.e., 833 μmol/m^2/s, which was more severe at low X_0 than at high X_0.

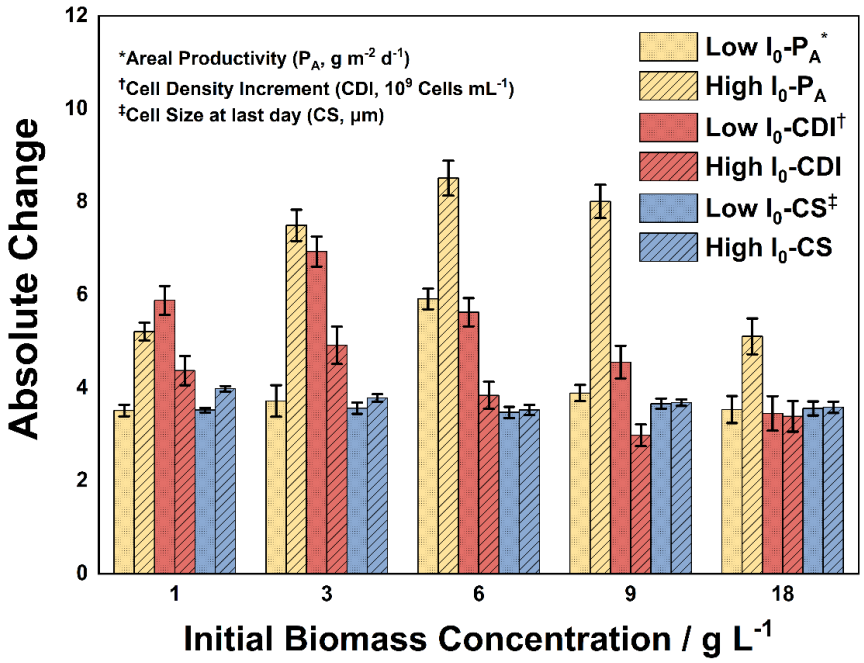

Figure 5-2. Comparison in the areal productivity (P_A), cell density increments (CDI) and cell size (CS) at the end of the cultivation under varies initial biomass concentration at incident light density of 83.3 μmol/m^2/s (low I_0) and 833 μmol/m^2/s (high I_0).

HCDC of algae can be achieved in thin layer algal reactor which was been proven. Within the selected range of initial biomass concentrations, the algal cells were capable of undergoing cell division, which further confirmed the advantages of thin-layer culture mode. This was observed even under the lower light intensity of 83.3 μmol/m^2/s, where cell growth was still achievable. The data indicated that algal cells, when cultivated under higher light intensities, achieved greater biomass concentrations, yet their cell division did not rival those observed under lower light conditions.

Interestingly, even with this increased biomass, there was a clear decline in cell division. This boost in biomass concentration was mainly due to an increase in cell size and shifts in the cellular biomolecular content, which will be discussed in the following section. This pattern was further exemplified by the growth outcomes of algae with varying initial inoculation concentrations. With an increase in algal cell density, the mean light intensity available to each cell was reduced, subsequently leading to a less pronounced increase in biomass concentration. Given that the cell division was still lower than in algae initiated with a 3 g/L inoculation at both light intensity conditions, it could be deduced that factors other than photoinhibition, possibly including suboptimal average light intensity, metabolic byproduct toxicity, or elevated dissolved oxygen levels, might have been at play. It should be noted that at a light intensity of 833 μmol/m^2/s, increasing the initial biomass concentration of algae to 18 g/L did not improve cell division. Nonetheless, the cell division at this concentration was still better than at a 9 g/L inoculation concentration. This indicated that 833 μmol/m^2/s inhibited the division of algae at lower biomass concentrations, while algae with higher biomass concentrations required a higher light intensity to sustain their growth. Although 833 μmol/m^2/s did not enhance cell division in algae at 18 g/L, the optimal light intensity might have been less than 833 μmol/m^2/s. However, the ability to withstand high light intensity was clearly superior in the cultures of higher density than those at lower concentrations. Furthermore, the enhanced cell division rates at 83.3 μmol/m^2/s suggested that the light intensity of 833 μmol/m^2/s might have surpassed the saturation threshold for the algae growth. Due to the experiment being conducted only under light intensities of 83.3 μmol/m^2/s and 833 μmol/m^2/s, the light saturation point for algal cell growth could not be identified. It is well-known that in algal cultures, the required light intensity increases as the concentration of algae rises. Therefore, algae grown under variable light conditions benefited from more sustained growth.

5.3.1.2 Effect of initial biomass concentrations on biochemical composition of algal cells

Figure 5-3 depicts the changes in pigment, lipid, and carbohydrate content of algal

cells under two different light intensities. Figures 5-3a and 3b present the variations in algal pigment content with time, clearly illustrating the distinct differences in total pigment content of algal cells at different x_0 under two distinct incident light intensities, i.e., high I_0 and low I_0. The cellular content of pigments decreased with algae cultivation time both under low and high I_0 with any of the tested initial biomass concentrations. However, the decrease was faster at high I_0 than at low I_0 when compared at the same x_0. When compared between different x_0 at the same I_0, then this decrease was less pronounced at higher x_0. At high I_0, the cellular pigment content did not exhibit a significant difference at different x_0, showing a consistent decline with cultivation time. It is very interesting to notice that, as shown in Figure 5-3c, the pigment contents of cells were positively correlated to biomass concentration when compared at the same I_0. Furthermore, the cells grown at high I_0 had much lower pigment cellular content than at low I_0. Since cells in cultures of higher biomass concentration are exposed to lower light intensity at the same I_0, it is logical to hypothesize that cells would synthesize more pigments for more efficient light capture when the local light intensity (I_h) is low. Figure 5-3d illustrates the relationship between the content of lipids and carbohydrates within algal cells and the initial inoculation concentration on the final day of cultivation under two light intensities. It is evident that, compared to the high I_0, algae under the low I_0 possessed a higher lipid content and a lower carbohydrate content. For low I_0, as the initial biomass concentration increased, there was a slight downward trend in lipid content, while for high I_0, the correlation was less apparent. The carbohydrate content at high I_0 exhibited a negative correlation with the initial biomass concentration.

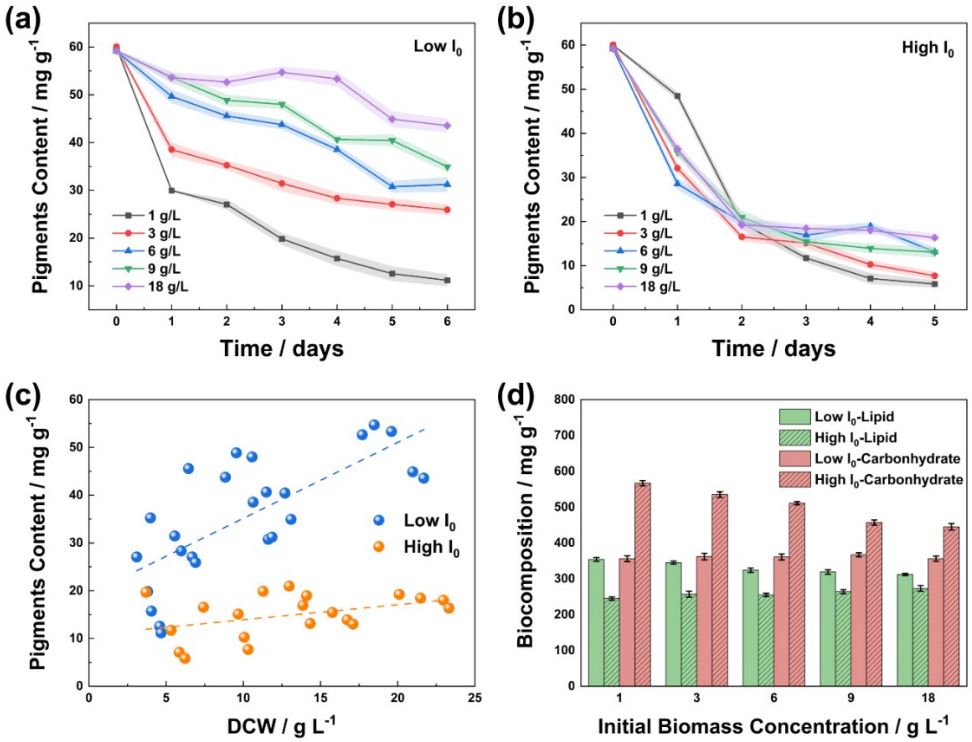

Figure 5-3. Algal cell biochemical composition: cellular pigment contents of *N. oleoabundans* under various initial biomass concentrations at incident light intensity 83.3 μmol/m^2/s, i.e., low I_0 (a) and 833 μmol/m^2/s, i.e., high I_0 (b); the relationship between pigment content and biomass concentration (c) and cellular lipids and carbohydrates contents of *N. oleoabundans* under various initial biomass concentrations at low and high I_0 (d).

Experimental data show that algal cells that were exposed to high light intensities underwent metabolic adjustments that led to an increase in carbohydrate and decreases in lipid and pigment synthesis. Such changes might be linked to photoinhibition caused by excessive light [49]. In these conditions, algae could face stress, potentially harming essential photosynthetic proteins and thus affecting cell division and metabolism [29]. This process is often linked to oxidative stress within the cells, where the overabundance of light radiance surpasses the photosynthetic capacity of algal cells, leading to the production of reactive oxygen species (ROS) and potential damage to the photosynthetic machinery, particularly to Photosystem II (PSII). The genesis of ROS and consequently oxidative stress can be exacerbated by environmental factors, such as

the presence of algicidal bacteria. For instance, *Bacillus sp. LP-10* has been shown to induce oxidative stress in *P. globosa*, serving as a biotic stressor that can amplify the effects of photoinhibition [50]. As a response, algae might dial down pigment production to reduce light absorption to mitigate the effects of photoinhibition [51]. Moreover, under bright light, algal cells might grow in size, leading to an increase in protective compounds within the cell. There was in an increase in light intensity led to a decrease in the concentration of photosynthetic pigments *Parachlorella kessleri*, suggesting a shift in cellular response to manage the light stress [52]. These compounds, like carotenoids, are designed to absorb and disperse excess light energy, providing a shield against potential light-induced damage [53]. In parallel, lipid synthesis, an energy-intensive process, was downregulated, conserving resources for the cells' immediate stress responses. Moreover, the cells' pigment levels decreased, a strategy likely adopted to reduce light absorption and minimize the risk of further stress due to the potential for photodamage under intense light conditions. This metabolic shift indicated the cells' attempt to balance their immediate survival against the detrimental effects of excess light energy. The difference in pigment content trends under high and low light conditions suggests that microalgae at higher cell density could be better equipped to cope with the harmful effects of high light intensity. This is likely because as biomass concentration increases, the mean light intensity that each cell receives decreases, leading to a reduction in light-induced stress. However, in this study, the microalgal cells were exposed to extremely high light intensities e.g. 833 μmol/m^2/s. Even at an inoculation of 18 g/L, the biomass concentration was not sufficient to offset the negative effects of such high light intensity, resulting in no significant increase in cellular pigment content.

5.3.2 Validation of the modified Beer-Lambert model for light attenuation

5.3.2.1 Effects of incident light intensity, biomass concentration, and culture thickness on light attenuation: experimental results

Experiments were conducted to investigate the changes in light intensity with culture thicknesses, i.e., light path distance, at different algal biomass concentrations

(X), and incident light intensities (I_0). Figures 5-4a and 4b depicted the variations in light intensity at the bottom (I_h) and the mean light intensity (I_{mean}) within the algal culture, respectively. I_{mean} was calculated using Eq.5-14. Observations from Figures 5-4a and 4b indicate an exponential decay in light intensity (I_h) with the increase of culture thickness in relatively thin cultures and low biomass concentration. Furthermore, I_h was observed to reach zero beyond certain boundaries of culture thickness and cell density, an outcome that is in agreement with the expected absorption and scattering effects of light as it travels through the culture. The data reveal that with a high I_0 such as 833 μmol/m^2/s, even at relatively large thickness, the average light intensity could be maintained at a relatively high level, suggesting that incident light intensity can somewhat counterbalance the effects of increased depth and cell concentration. Despite this, the compensatory capability of high I_0 was limited and complete dark layer of culture with zero I_h may exist in deep HCDC cultures.

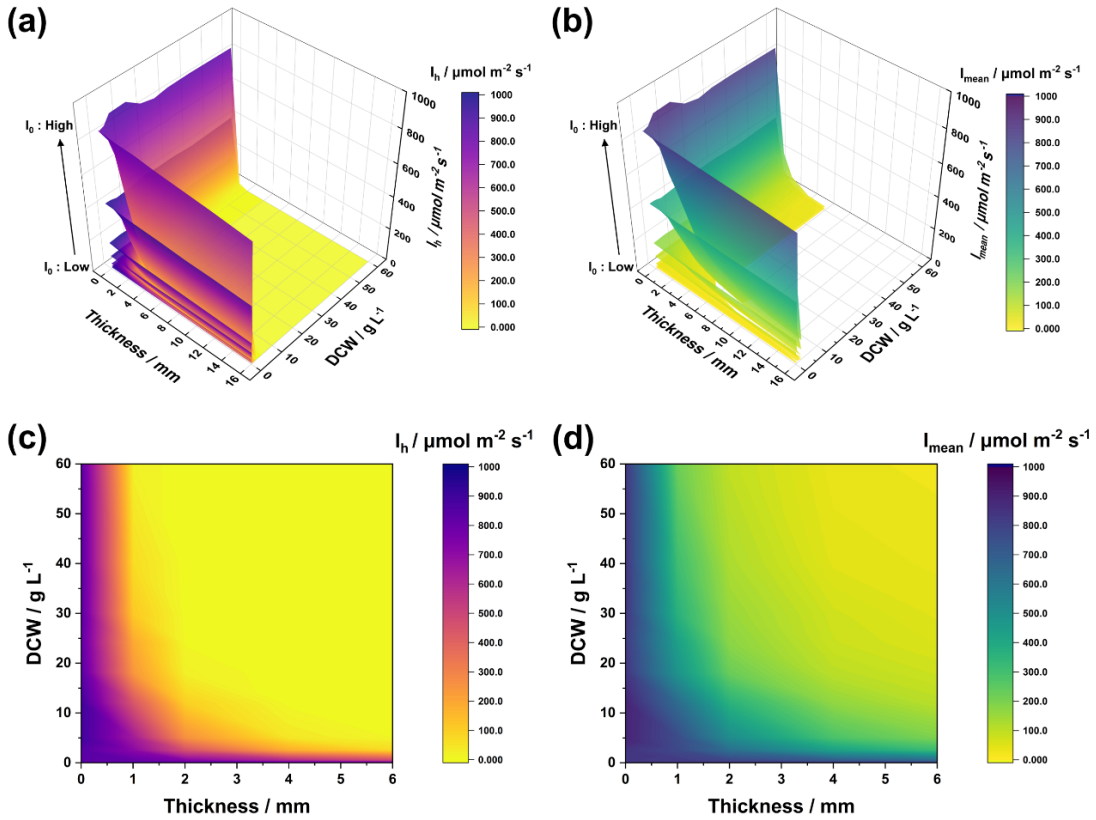

Figure 5-4. Light attenuation in culture of *N. oleoabundans*: effects of culture thickness, incident light intensity (I_0), and biomass concentration (DCW) on I_h (a) and I_{mean} (b);

2D heat maps of I_h (c) and I_{mean} (d) at incident light intensity (I_0) 833 μmol/m^2/s (d).

In the culture of photosynthetic microorganisms, the attenuation of light intensity was governed by the inherent light absorption and scattering of algal cells. While the photopigment molecules such as chlorophylls and carotenoids within the algal cells exhibited a strong absorption capacity for particular wavelengths of light [54], the scattering effects are strongly impacted by cell morphology and size [55]. Biomass concentration is another parameter that showed strong effects on light attenuation. At a given culture thickness, a self-shading effect became pronounced with the increase of biomass concentration, making it increasingly difficult for light to penetrate and reach the bottom of the culture system. This effect was particularly prominent in HCDC of algae, which results in more frequent absorption/scattering events before the light could reach the bottom [56].

5.3.2.2 Validation of the modified Beer-Lambert model for light attenuation in algal culture

Table 5-1. Regressed parameters of the original and modified Beer-Lambert model

	Beer-Lambert Model	Modified Beer-Lambert Model
ε	0.1205	0.1373
n	/	0.8058
R^2	0.9772	0.9910

Table 5-1 lists equation parameters obtained by nonlinear fitting of the experimental data presented in Figures 5-4a to the original and modified Beer-Lambert models, i.e., Eqs. 10 and 11 excluding the data points when I_h= 0. The modified model, characterized by its non-linear relationship between the extinction coefficient and cell concentration, had an R^2 value of 0.9910 when fit with experimental data, representing a remarkable increase from that of the original model (R^2=0.9772). This improvement signifies a more accurate correlation of light intensity with light path and biomass concentration. The introduction of the empirical exponent *n* allowed a more precise correlation describing the dependency of $A(X)$ and I_h on X. The exponent *n* was less than 1, i.e., 0.8058, indicating an overestimation of the effect of biomass concentration

on light attenuation by the original Beer Lambert law. Furthermore, the original Beer Lambert law underestimated the specific extinction coefficient (0.1205) since the modified Beer-Lambert model, which was the more accurate among the two, gave a larger ε value of 0.1373. These observations could be hypothetically explained by the fact that increasing the biomass concentration and hence the cell density would reduce the light intensity individual cells are exposed to and therefore the scattering and absorption of light by these cells. It is also worth noting that the specific extinction coefficient ε was a constant in both the original and the modified Beer-Lambert model, confirming that it is indeed an intrinsic property of the culture.

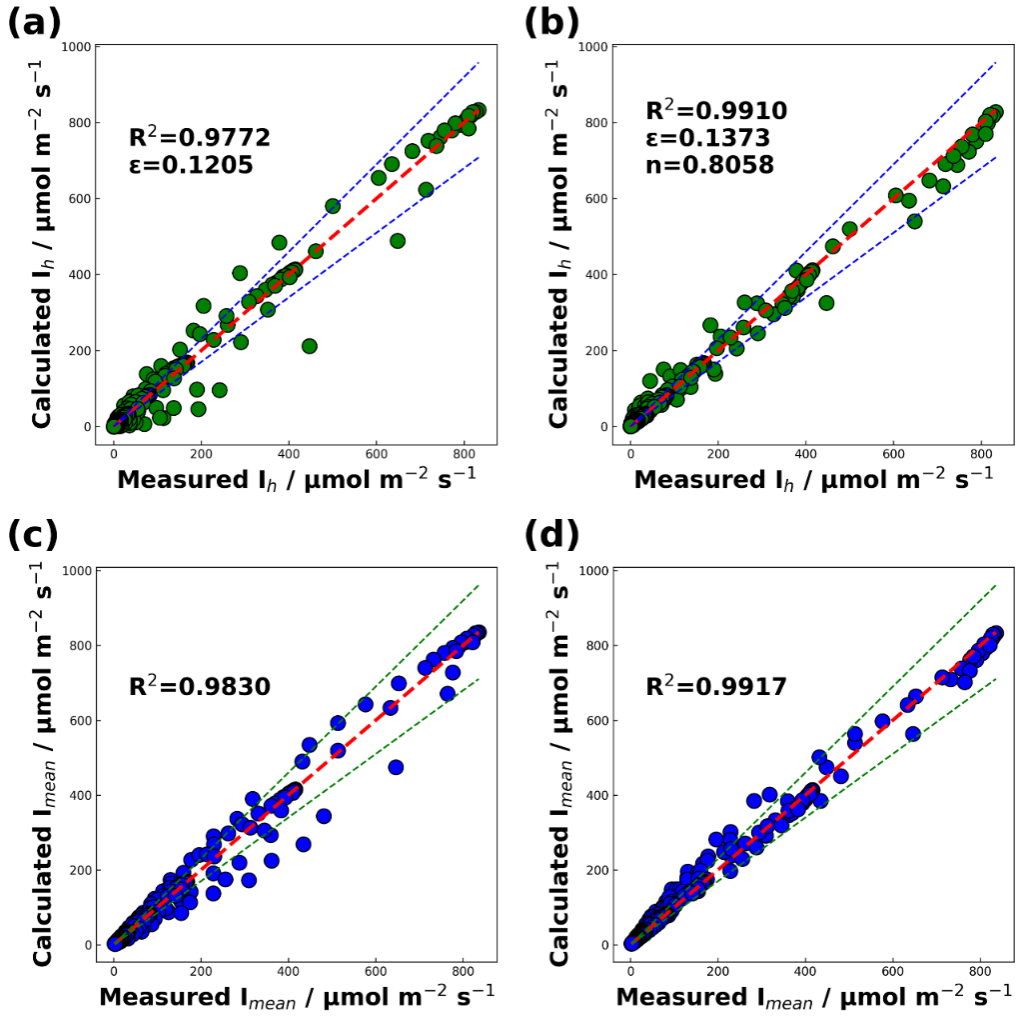

Figure 5-5. Predicted versus experimental: I_h by Beer-Lambert model (a), I_h by modified Beer-Lambert model (b), I_{mean} by original Beer-Lambert model (c), and I_{mean} by modified Beer-Lambert model (d). The broken lines are boundaries of ±15% error.

Figure 5-5 compares values of I_h and I_{mean} predicted by the modified and original Beer-Lambert model with the experimentally determined values, which underscores the modified model's enhanced accuracy. The advantage of the modified model was especially pronounced under relatively high biomass concentrations, where the original model proved inadequate. It is worth mentioning that some researchers [42] introduced the concept of mean (specific) extinction coefficient, i.e., ε_{mean}, which was dependent on the biomass concentration and culture thickness, when using the original Beer-Lambert model for light attenuation. This was likely because they did not exclude the data points when the light intensity was already zero (I_h=0).

At relatively low *X* and high I_0, light can penetrate the entire culture and *h* equals the culture thickness. However, when *X* increases, light attenuation increases exponentially and the threshold of I_h=0 may be reached at certain depth of the culture. The culture would be therefore separated in to two zone, i.e., the light zone and the dark zone. The light zone is above the critical culture depth, where the cells were exposed to light with the intensity decreasing with culture depth. Whereas the dark zone is below the critical culture depth, where the cells are all deprived of exposure to light. In such a scenario, *h* is the distance from the culture surface to the point where I_h=0, which is the difference between the culture thickness and the dark zone thickness. Since I_h was determined experimentally at the bottom of a culture, a practical approach of determining the *h* would be to exclude all the data points with I_h = 0. Failing to do that, using the culture thickness rather than the actual *h* in Eq. 5-9 or Eq. 5-10 would cause the underestimation of the extinction coefficient, i.e., *A(X)* and consequently the underestimation of ε. At constant *X* and I_0, increasing culture thickness would increase the dark zone thickness, resulting in an elevated overestimation of *h* and therefore underestimation of ε, projecting a false dependency of ε on culture thickness. At constant I_0 and culture thickness, the increase of *X* would lead to the decrease of the light zone thickness, resulting in an elevated overestimation of *h* and therefore underestimation of ε, projecting a false dependency of ε on *X*.

Table 5-2. Comparative overview of light attenuation models

Model name	Formula	R^2	ref
Beer-Lambert Model	$I(L,X) = I_0\, exp(-K_a \times L \times X)$	/	[58]
Modified Beer-Lambert Model	$I(L,X) = I_0\, exp(-(K_a \times X + b) \times L)$	0.9799	[59]
Modified Beer-Lambert Model	$I(L,\mathrm{X}) = I_0\, exp(-K_a \times L \times \mathrm{X} \times \frac{L^q}{P_k + L^q})$	0.9920	[56]
Modified Beer-Lambert Model	$I = I_0 exp(-\frac{A_{max} \times X}{K_{at} + X} \times L)$	/	[35]
Modified Beer-Lambert Model	$I = I_0 \times \exp(-(k_l + \alpha \times X) \times z)$	/	[60]
Cornet model	$\frac{I}{I_0} = \frac{4\alpha_1}{(1+\alpha_1)^2 \times e^{\alpha_1} - (1-\alpha_1)^2 \times e^{-\alpha_1}}$ $\alpha_1 = (\frac{E_a}{E^a + E_s})^{\frac{1}{2}}$ $\alpha_2 = (E^a + E_s) \times \alpha \times X \times L$	/	[57]
Cornet model	$\frac{I}{I_0} = \frac{4\alpha_1}{(1+\alpha_1)^2 \times e^{\alpha_1} - (1-\alpha_1)^2 \times e^{-\alpha_1}}$ $\alpha_1 = (\frac{E_a}{E^a + E_s})^{\frac{1}{2}}$ $\alpha_2 = (E^a + E_s) \times \alpha \times X \times L$	0.9929	[59]
Modified Cornet model	$\frac{I}{I_0} = \frac{4\alpha_1}{(1+\alpha_1)^2 \times e^{\alpha_1} - (1-\alpha_1)^2 \times e^{-\alpha_1}}$ $\alpha_1 = (\frac{E_{a,a}X_a + E_{a,b}X_b + E_{a,c}X_c}{(E_{a,a}X_a + E_{a,b}X_b + E_{a,c}X_c) + E_sX})^{\frac{1}{2}}$ $\alpha_2 = (E_{a,a}X_a + E_{a,b}X_b + E_{a,c}X_c + E_sX) \times \alpha \times L$	0.967	[36]
Modified Cornet model	$\frac{I}{I_0} = \frac{4\alpha_1}{(1+\alpha_1)^2 \times e^{\alpha_{21}} - (1-\alpha_1)^2 \times e^{-\alpha_{21}}} + \frac{4\alpha_1}{(1+\alpha_2)^2 \times e^{\alpha_{22}} - (1-\alpha_2)^2 \times e^{-\alpha_{22}}}$	/	[61]
Fuzzy-logic model	$I = \frac{\sum_n \emptyset_{A_n}(I_0) \times f_n \times (I_0, r, C_X)}{\sum_n \emptyset_{A_n}(I_0)}$ With n encompassing (Beer-Lambert; Reynolds–Pacala; Reynolds–Pacala modified) models	/	[37]
Modified Beer-Lambert Model	$I_{hN} = I_0 \times \exp(-\varepsilon X^n h)$	0.9910	This study

Table 5-2 lists several models for light attenuation in cultures of microalgae. While some of them suffer from low accuracy as reflected by their small R^2 values, some others such as the Cornet model [57] and fuzzy-logic-based models [37] consider a range of factors including microalgal pigments, reactor geometry, and light incidence angles. These models are compromised by their complexity, which have obfuscated their practical application since the quantification of model parameters has proven to be challenging in many scenarios. In this context, the modified Beer Lambert model introduced in this research represents a meaningful improvement, distinguished for its simplicity and practical approach while still respecting the basic principles of the Beer model. Through the inclusion of extra exponential terms and adjustable coefficients, this model addressed the challenges posed by HCDC systems, offering a simple and yet reliable correlation dipitcing the dependency of light distribution on light path and cell density, adding a valuable tool for light distribution studies.

5.3.3 Modelling cell growth: dependence of specific growth rate of cells on I_{mean}

5.3.3.1 HCDC of microalgae at stable I_{mean}

Due to the exponential attenuation of light upon the increase of culture thickness and biomass concentration, I_{mean} is more representative of the light intensity the cells in culture are exposed to than I_0. Therefore, to better understand the influence of light intensity on cell growth, experiments were carried out at a variety of different pre-set I_{mean} values by adjusting I_0 daily according to the biomass concentration at the end of the day before with a culture thickness of 6 mm throughout the cultivation period. The culture medium was replaced after each increment of 3 g/L in biomass concentration to guarantee sufficient nutrition.

As depicted in Figure 5-6a, significant increase in I_0 was required to maintain a pre-set I_{mean} in parallel with the growth of the algal cells and this increase in I_0 was more evident at higher I_{mean} levels. At I_{mean} levels of 100 and 150 μmol/m^2/s, there was a notable exponential growth in I_0 corresponding to the algal growth. As shown in Figures 5-6b and 6c, increasing the I_{mean} resulted in enhanced growth of algal cells in terms of biomass concentration and cell density. The maximum biomass concentration and cell

density were achieved at an I_{mean} of 50 μmol/m^2/s. After 20 days of cultivation, the algal biomass concentration had grown from 1 g/L to 28.20 g/L, and the cell density reached 2.56 x 10^9 cells/mL. Within a certain range, the growth rate of algal cells accelerated with the increase in I_{mean}. As shown in Table 5-3, when I_{mean} was increased from 8.33 μmol/m^2/s to 66.67 μmol/m^2/s, the volume productivity of algal cells had increased from 0.32 g/L/day to 1.36 g/L/day. However, beyond 50 μmol/m^2/s, further increasing the I_{mean} gradually inhibited algal growth, more evidently in terms of cell density. Continuously increasing I_{mean} did not further accelerate algal cell growth; instead, cell division was inhibited in the later stages of cultivation. In conjunction with Figure 5-6a, it was evident that when I_o exceeded 833 μmol/m^2/s, cell division in the algae ceased. For example, algal cells at average light intensities of 66.67, 100, and 150 μmol/m^2/s experienced slowed or halted division on the 5^{th}, 9^{th}, and 13^{th} days, respectively, with the highest cell densities only being 2.04 x 10^9, 1.57 x 10^9, and 0.68 x 10^9 cells/mL. When I_{mean} exceeded 66.67 μmol/m^2/s, the increase in algal biomass concentration did not diminish despite the slowing down or cessation of cell division, which can be attributed to changes in the intracellular biological components of the cells. Figure 5-6d illustrated the lipid, carbohydrate, and pigment content within the algal cells at the end of cultivation under varying I_{mean} levels. It was observed that the pigment content progressively diminished as the I_{mean} increased. In contrast, the lipid content initially rose before declining. The peak lipid concentration, at 378.23 mg/g, was noted when I_{mean} reached 33.33 μmol/m^2/s. Regarding the carbohydrate content, there was a notable ascending trend with the escalation of I_{mean}. Elevated light intensities appeared to prompt the algal cells to amass a greater quantity of carbohydrates, culminating in a maximum carbohydrate concentration of 567.99 mg/g. As shown in Table 5-3, variations in the cellular biochemical composition resulted in a shift in the conversion factor, which increased in value with the rising levels of I_{mean}.

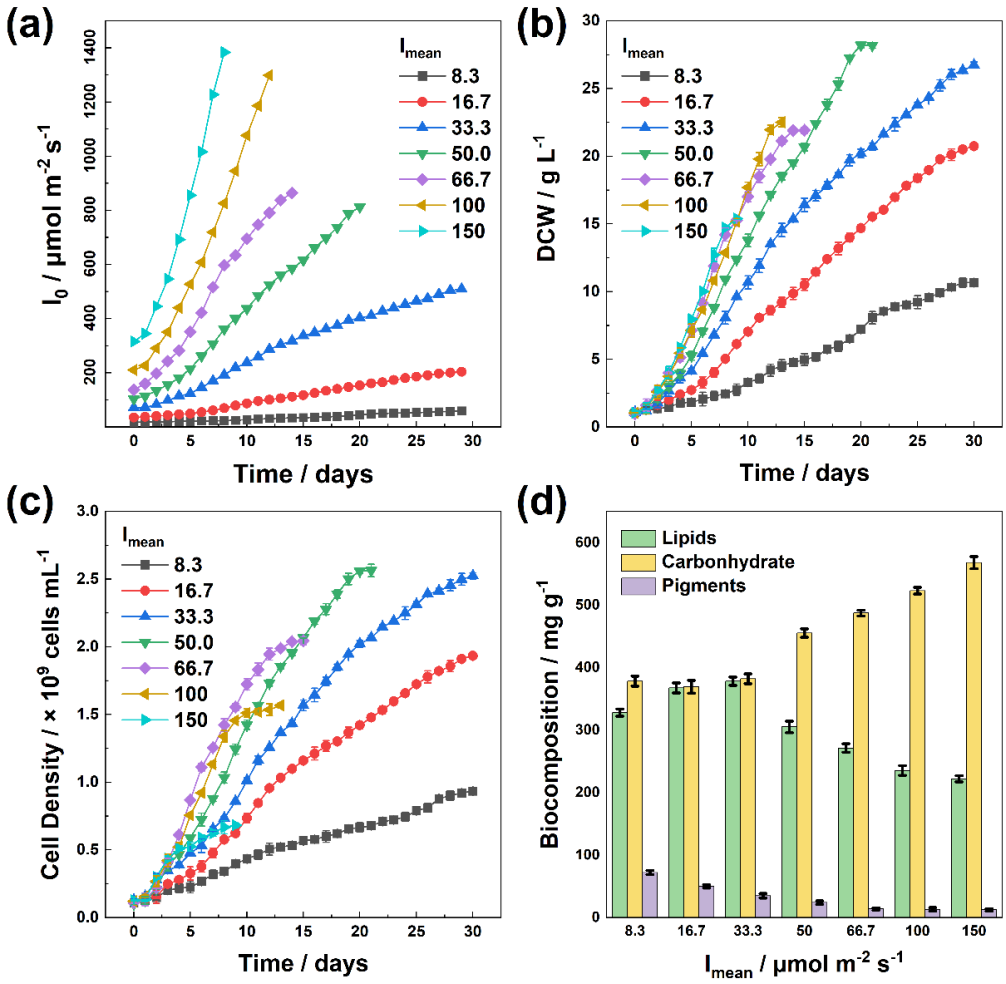

Figure 5-6. Cultivation of *N. oleoabundans* based on the light management mechanism of I_{mean} (ranging from 8.33 µmol/m^2/s to 150 µmol/m^2/s): Changes in I_0 (a), effects on algal cell biomass concentration (b), effects on algal cell density (c) and effects on cellular biocompositions

Based on the results, the optimal average light intensity was 50 µmol/m^2/s in terms of the maximum biomass concentration and cell density. However, the optimal volumetric productivity (P_v), at 1.65 g/L/day, corresponding to an areal productivity (P_A) of 9.91 g/m^2/day, was attained at 100 µmol/m^2/s. This phenomenon was attributed to the fact that algal cell growth seemed influenced not only by the factor of I_{mean} but also significantly by I_0. The levelling of the cell density can be used to infer the approximate onset of photoinhibition. When cell division is inhibited, it indicates that

the light intensity has surpassed the maximum threshold that the microalgae can endure. Close exam of Figure 6c indicates that photoinhibition using the slowing down of cell division as the criterion, occurred at the 4^{th}, 9^{th}, and 12^{th} day of cultivation at I_{mean} of 150, 100, and 66.7 μmol/m^2/s, respectively. It can be found from Figure 6a that these days all correspond to I_0 values of approximately 750 μmol/m^2/s. We can therefore tentatively consider this as the threshold of light intensity that would trigger photoinhibition in *N. oleoabundans*. As the algal biomass concentration increased, maintaining a specific I_{mean} necessitated an exponential increase in I_0. This escalation often resulted in I_0 surpassing the maximum light intensity threshold tolerable by the algae which aligned with the results of Li et al. [35], who observed that maintaining an average light intensity of approximately 50 μmol/m²/s, coupled with an incident light intensity below 540 μmol/m²/s, was conducive to *Porphyridium purpureum* culture. Furthermore, the observed decrease in pigment content within the algal cells as I_{mean} increased suggests that under low light intensities, algae tended to synthesize more pigments to enhance the efficiency of light capturing.

The impact of light intensity on microalgae growth was twofold, affecting the increase in biomass concentration and the rise in cell density. Previously, studies had rarely explored these aspects concurrently. A singular focus on either biomass concentration or cell density often led to inaccurate assessments. Results of this project indicate that the biomass concentration could continue to rise after the suppression of cell division. In other words, cell division is more sensitive to photoinhibition than some other metabolisms such as the photobiosynthesis and the carbohydrate biosynthesis in these cells. Furthermore, the study adopted intermittent replacement of the culture medium to ensure that the microalgae grew in a nutrient-rich environment to maintain nutrient sufficiency for cells growth. In the context of adequate nutrition, it was determined that an I_{mean} of 66.67 μmol/m^2/s was the most conducive lighting condition for algal cell growth at a culture thickness of 6 mm at which maximum volumetric productivity (2.72 g/L/day) and areal productivity (16.32 g/m^2/day) were obtained.

Table 5-3. Summary of algal growth parameters under different I_{mean}

	Daily Circle I_{mean} (μmol/m²/s)						
	8.33	**16.67**	**33.33**	**50**	**66.67**	**100**	**150**
Cell number density(max)	9.34	19.34	25.23	25.63	20.44	15.67	6.82
Biomass concentration (g/L)	10.66	20.74	26.73	28.20	21.91	22.51	15.39
P_v (g/L/d)	0.32	0.66	0.89	1.36	1.40	1.65	1.59
P_A (g/m²/d)	1.92	3.94	5.35	8.16	8.37	9.91	9.56
P_{Vmax} (g/L/d)*	0.85	1.09	1.61	2.10	2.72	2.53	2.69
P_{Amax} (g/m²/d)*	5.10	6.54	9.66	12.6	16.32	15.18	16.14
I_0 (max)	58	203	508	812	863	1298	1383
Conversion factor	0.34	0.40	0.45	0.51	0.53	0.56	0.64

* $P_{A,max}$ and $P_{V,max}$ are the maximum daily areal and volumetric productivity of algal biomass, respectively.

The impact of light intensity on microalgae growth was twofold, affecting the increase in biomass concentration and the rise in cell density. Previously, studies had rarely explored these aspects concurrently. A singular focus on either biomass concentration or cell density often led to inaccurate assessments. In this project, for example, while the biomass concentration continued to rise, cell division had already been suppressed. This highlighted the onset of light inhibition under high light intensities, which impeded the division of algal cells. Furthermore, the study adopted intermittent replacement of the culture medium to ensure that the microalgae grew in a nutrient-rich environment. This approach was crucial to prevent alterations in cell composition due to deficiencies in certain elements, as deficiencies in N, P, and Mg are known to affect photosynthesis. Given that microalgae consume nutrients at varying rates under different light intensities, maintaining nutrient sufficiency rendered the experiment more scientifically robust. In the context of adequate nutrition, it was

determined that an average light intensity of 66.67 μmol/m^2/s, while keeping the maximum input light intensity I_0 under 750 μmol/m^2/s, was seemingly the most conducive lighting condition for algal cell growth at a cultivation depth of 6 mm. This was attributed to its facilitation of optimal cell division, coupled with its impressive maximum volumetric productivity and areal productivity, which were recorded at 2.72 g/L/day and 16.32 g/m^2/day, respectively. Although an I_{mean} of 100 μmol/m^2/s achieved the best productivity, if cell division was not considered, its cultivation process consumed more light energy, reaching a maximum illumination of 1298 μmol/m^2/s, which was not economically efficient.

5.3.3.2 Verification of Monod model for cell growth of *N. oleabundans*

Based on the observed growth behavior of microalgae under various mean light intensities (I_{mean}), the specific growth rate has been modeled using the Monod model which has been shown in Figure 5-7. Two approaches were employed for model fitting. In the first approach, all available data were used, providing a general overview of the microalgae's growth response across a wide range of I_{mean}. In the second approach, the data were selectively filtered to include only the linear regression of ln(x) versus t was satisfactory (R^2>0.99), and the slope of this linear segment was used as the specific growth rate of microalgae for fitting with the Monad model. As seen in Table 5-4, the fitting results are better when using the selected data with a constant specific growth rate. This is reasonable since I_{mean} was kept constant during the cultivation and a decline of specific growth rate, which occurred only in the later stage of high I_{mean} cultivations, when cell growth was inhibited. As presented in Table 5-4, *N. oleoabundans* under the tested conditions had a maximum specific growth rate (μ_{max}) of 0.5807 day^{-1} and a half-saturation constant (K_I) of 38.9591 μmol/m^2/s, with an excellent fit indicated by an R^2 value of 0.9951. This μ_{max} value suggests that, under optimal light conditions, the microalgae could double their biomass approximately every 1.19 days, highlighting their potential for rapid growth and efficient light utilization in biomass production. The relatively low K_I value indicates a good affinity for light, enabling effective growth even at moderate light intensities, which is beneficial for optimizing light availability

in cultivation systems.

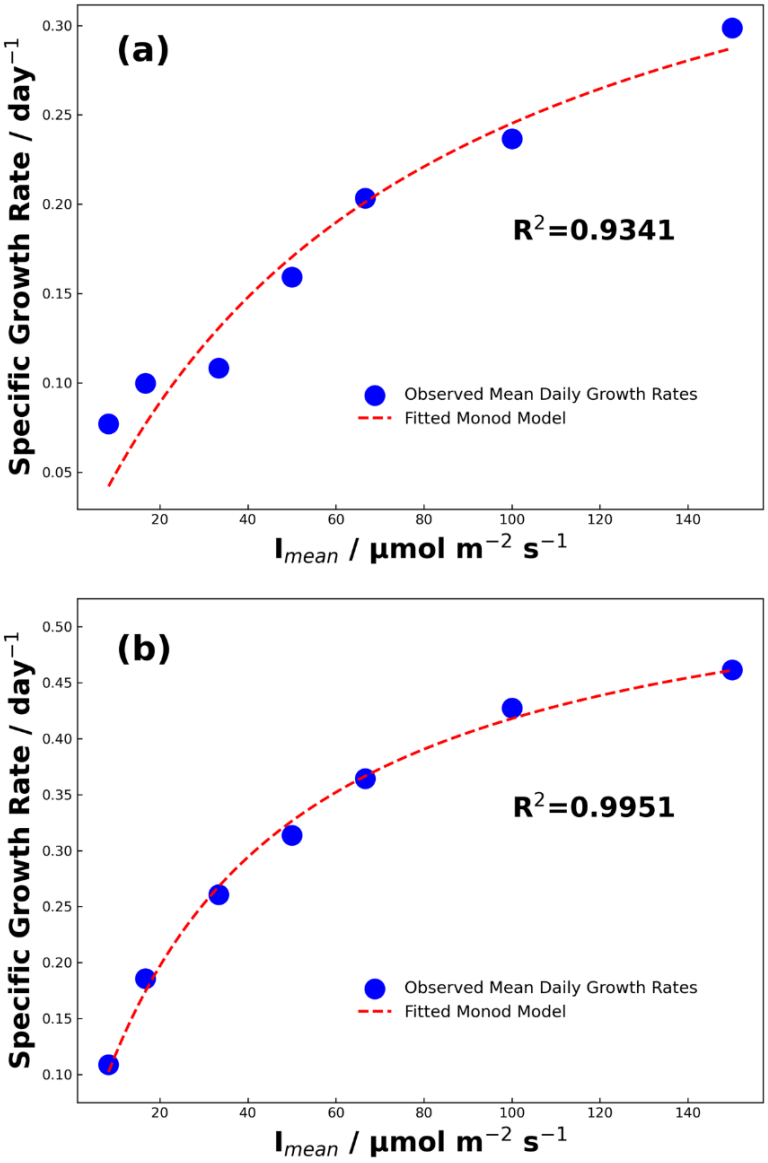

Figure 5-7. Monod model fitting curve for microalgal specific growth rate with all data applied (a) and selected data applied (b)

The Monod model is a simple kinetic model that has been widely used for the cell growth of heterotrophic microorganisms. However, its application to photoautotrophic growth microalgae, like almost all existing kinetic models, has been shown to be far from satisfactory. For instance, in a recent article, Esteves et al. [43] assessed nine kinetic models including the Monod model using both experimental results and literature values concerning the growth of *Chlorella vulgaris*. The R^2 value of fitting the experimental data with these models was between 0.055 and 0.805. More

specifically, the R^2 of Monod model fitting was 0.793. It should be noted that all these data sets were generated following the conventional approach of maintaining a constant I_0 and therefore decreasing I_{mean} during the course of cultivation. It seems to be quite evident that a major contributor to the poor fitting performance of these models was the use of experimental data that were of poor quality and, at least for the Monod model, using the correct data as did in this study would lead to excellent fitting. This is because using cultures at constant I_0 for kinetic studies would cause the continuous decrease of I_{mean} and therefore specific growth rate with time due to the increase of biomass concentration in the course. Furthermore, the afore discussed dark zone with I_h=0 may be developed when biomass increases beyond certain threshold, introducing unnoticed viables to the growth kinetics that divert the experimental kinetic data away from the course predicted by the kinetic models.

Table 5-4. Monod model parameters

	All data applied	Selected data applied
μ_{max} (day^{-1})	0.4369	0.5807
K_I (μmol/m²/s)	78.1584	38.9591
R^2	0.9341	0.9951

5.4 Conclusions

This study investigated the impact of light on the growth of *N. oleoabundans* alga, including effects on biomass concentration, cell density, and intracellular biochemical composition using the mean light intensity as the controlled variable. A modified Beer-Lambert model was proposed and verified with experimental data, which provides improved fitting in light penetration of HCDC. Using the modified model, we were able to adjust the incident light intensity to maintain the mean light intensity at preset values for two purposes, 1) to investigate the effects of light intensity on cell growth and cellular biochemical composition; and 2) to verify the applicability of the Monod model for the modeling of the dependence of specific growth rate on $I_{mean.}$ Experimental results indicated that the optimal I_{mean} for cultivation is estimated to be around 66.67 μmol/m^2/s.

Notably, the highest biomass concentration achieved was 28.20 g/L in 20 days, observed at the light intensity of 50 μmol/m^2/s. On the other hand, incident light intensity of 750 μmol/m^2/s or above was shown to be inhibitive to cell division. It should be noted that a thinner culture thickness can significantly minimize light attenuation, thus enhance photosynthetic efficiency and push up the maximum biomass concentration attainable in a suspended microalgal culture. However, a thinner culture will have smaller areal productivity for a given biomass concentration. To this end, countermeasures such as starting a culture with relatively large thickness and gradually reduce the thickness at a rate that matches the increase of biomass concentration to maximize the productivity of a cultivation system is underway.

While it has been a longstanding challenge to find suitable kinetic model for the correlation of cell growth (specific growth rate) and mean light intensity, our results show that using experimental data generated at stable mean light intensity with small range cyclic variation with which the specific growth rate, which more accurately was actually the average specific growth of an entire light/dark cycle, was constant led to satisfactory fitting of the Monod model with R^2 of 0.9951. The results also help dispel a mistaken approach of introducing a mean specific extinction coefficient that is dependent on both biomass concentration and light path distance, confirming that the specific extinction coefficient is an intrinsic property of an algal culture that is independent of biomass concentration and light path distance.

CRediT authorship contribution statement

Hongying Zhou: Conceptualization, Investigation, Methodology, Data curation, Visualization, Writing - Original draft. **Ju Wang:** Data curation, Visualization. **Zisheng Zhang:** Resources, Supervision. **Christopher Q. Lan:** Conceptualization, Supervision, Project administration, Funding, Resources, Writing - Review & Editing.

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Chapter 6 Optimizing microalgae cultivation: Evaluating the impact of culture thickness, intermittent stirring, and dynamic thickness adjustment strategies

Abstract

High cell density cultivation (HCDC) of microalgae is pivotal for their large-scale application, yet optimizing this process remains challenging. While previous studies have demonstrated that reducing cultivation thickness can achieve HCDC, thinner layers often encounter reduced areal productivity. This study investigates the effects of cultivation thickness on thin-layer microalgae culture and the optimization of variable thickness cultivation strategies. Firstly, the study examined the effects of cultivation thickness on both HCDC and low cell density cultures (LCDC) of microalgae, revealing that increasing culture thickness was beneficial for LCDC, as it enhanced total yield; conversely, in HCDC, increasing the thickness led to a decline in areal productivity. To delve deeper, we conducted experiments with an initial biomass of 0.3g, exploring the impact of varying cultivation thickness on final yield, and found that under continuous stirring, a 36mm thickness resulted in the highest microalgae production, reaching 1.10g, while under intermittent stirring, a 20mm thickness proved optimal, yielding 0.91g. Furthermore, by employing a variable thickness cultivation method, we were able to effectively balance areal productivity across different thicknesses and biomass concentrations, particularly under intermittent stirring conditions. The 36-20 strategy emerged as the most efficient, achieving a maximum net yield of 1.01g and a cell number of 105.22 x 10^9 cells. This approach also significantly reduced the overall costs associated with microalgae cultivation, including medium consumption, labor, and maintenance expenses, providing a novel framework for advancing thin-layer cultivation techniques, and thereby paving the way for more sustainable and scalable microalgae production.

6.1 Introduction

Microalgae, efficient photo cellular factories, are increasingly recognized for their role in addressing global challenges in environment protection as well as food and energy production [1,2]. As versatile photosynthetic microorganisms, microalgae are capable of converting carbon dioxide into valuable biomass, making them integral to sustainable strategies in carbon capture and sequestration [3,4]. Beyond their environmental benefits, microalgae serve as a vital source of bioactive compounds [5], with applications including biofuels [6], human foods, animal feeds, nutraceuticals, and pharmaceuticals [7,8], offering renewable alternatives to fossil fuels and contributing to food security. The optimization of microalgae cultivation presents a critical avenue for enhancing biomass production efficiency [9]. Achieving higher biomass yields with reduced resource input not only aligns with the principles of sustainable development but also holds the key to unlocking the economic viability of microalgae-based products [10]. Despite the potential, optimizing cultivation practices to maximize productivity while minimizing environmental footprint remains a complex challenge [11]. This underscores the significance of innovative research in microalgae cultivation, aiming to refine and adapt cultivation conditions to harness the full potential of these microorganisms.

Achieving high cell density cultivation (HCDC) of microalgae presents a practical approach to reducing the costs associated with their growth processes [12]. Traditional approaches to microalgae cultivation, characterized by a fixed culture thickness [13], encounter inherent challenges that impede achieving HCDC. For systems employing deeper culture volumes, such as tanks or vertical columns [14,15], the challenge of sustaining HCDC intensifies in the cultivation's later stages [16]. This difficulty arises from diminished light penetration as algal cell density increases, which in turn compromises growth conditions and caps the biomass potential at suboptimal levels [17,18]. Thin-layer cultivation (TLC) has emerged as a viable solution for realizing HCDC, offering a method to mitigate the risk of insufficient light—a common limitation in denser cultures [19,20]. This approach enables microalgae to be cultured

more effectively by reducing the depth of the culture medium, thereby enhancing light penetration and potentially increasing biomass concentration. However, this approach unveils its shortcomings in the early growth phases, where light is not a scarce resource. The reliance on minimal culture depth for improved light penetration, while theoretically advantageous, inadvertently detracts from areal productivity, leading to a paradox where the overall yield does not meet expectations. Moreover, the necessity for larger surface areas to accommodate thin-layer systems introduces additional challenges, including increased land use and potentially higher operational costs. Consequently, adopting a cultivation strategy that allows for the adjustment of culture thickness is imperative. As microalgae proliferate, tailoring the culture depth to match their growth stages becomes a cornerstone for maintaining stable productivity. This dynamic approach to managing culture thickness is not just about ensuring optimal light penetration but also about optimizing the entire cultivation ecosystem for enhanced microalgal productivity. It encompasses a holistic consideration of nutrient utilization efficiency, as well as the energy and labor costs associated with subsequent harvesting processes. Scientifically exploring the optimal timing and degree of thickness reduction thus becomes a critical area of research.

On the other hand, mixing or stirring represents one of the primary energy inputs within the system [21,22], playing a crucial role in not only ensuring uniform light intensity and nutrient distribution but also preventing potential harm to algal cells caused by excessive dissolved oxygen content, maintaining an optimal environment for efficient cultivation [23,24]. However, the energy demand for stirring constitutes a significant portion of the total cost associated with microalgae production [25]. With the ever-increasing energy prices, finding strategies to reduce stirring frequency emerges as a vital approach to achieving cost-effective cultivation. Finding the optimal balance between minimizing energy inputs and maintaining culture quality is essential for enhancing the economic and sustainable aspects of microalgae cultivation. Previous research [26] has shown that, even with intermittent stirring, achieving HCDC (~7g/L) comparable to that of continuous stirring is possible even in one-batch cultivation mode.

However, intermittent stirring under conditions of varying culture thickness or different cell densities remains an understudied area, which is crucial for determining the design of the cultivation strategy.

In this study, an oil-rich green alga, *Neochloris oleoabundans* [27], was selected and cultivated within a novel thin-layer reactor. Firstly, the impact of different culture thicknesses on the growth of microalgae at both low and high densities were explored. Progressing from these foundational observations, under the control of initial inoculum biomass, the effects of varying culture thicknesses on microalgae growth and final yield were examined. Building on this, the study investigated the changes in microalgae yield under conditions of intermittent stirring. Ultimately, a cultivation mechanism involving thickness adjustment was designed and examined with the aim of optimizing microalgal growth and maintaining productivity. Aimed at enriching the collective understanding of HCDC cultivation, this research integrates a detailed economic feasibility analysis, offering valuable insights to inform strategic planning for future microalgal production endeavors.

6.2 Materials and methods

6.2.1 Microalgal strain and media

The strain of *N. oleoabundans* (UTEX# 1185) was sourced from the UTEX Culture Collection of Algae at the University of Texas, Austin. Before initiating the experiments, the microalgae were pre-cultivated in 250 mL flasks, each containing 80 mL of a freshly prepared Modified Bristol Medium (MBM). The composition of MBM (per liter) was as follows: 0.7 g $NaNO_3$, 0.138 g K_2HPO_4, 0.0823 g $MgSO_4$, 0.025 g $CaCl_2$, 0.322 g KH_2PO_4, 0.025 g NaCl, 0.0023 g $FeCl_3$ and 1 mL A_5 solution which was consisted of (per liter): 1.6423 g EDTA-Fe, 2.86 g H_3BO_3, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, and 0.039 g $(NH_4)_6Mo_7O_2 \cdot 4H_2O$.

6.2.2 Seed culture

For the preparation of the microalgae seed culture, sterile techniques were utilized. A volume of 400 mL of fresh medium was sterilized by autoclaving at 121°C for 20 minutes in 500 mL cultivation flasks. Once cooled to ambient temperature, 20 mL of

pre-cultured inoculum was transferred into this sterile environment. To ensure optimal pH levels of 7.5, the cultures were aerated with a continuous flow of air mixed with 5% CO_2, which was purified by passing through a gas washing bottle filled with distilled water, at a rate of 0.5 vvm. Illumination was provided by two LED lamps set to deliver a light intensity of 160 μmol/m^2/s under a photoperiod of 12 hours light and 12 hours darkness. A magnetic stirrer, placed at the base of each flask, ensured the cultures remained uniformly mixed. The microalgae cultures were grown to the exponential phase before being utilized in further experimental setups.

6.2.3 Microalga cultivation

6.2.3.1 General cultivation process

In this study, the HTLAR was employed for the experiments. The design features of this reactor are depicted in Fig.3-1, which have been thoroughly described in our earlier work. The HTLAR is comprised of several glass dishes, each featuring an internal diameter of 100 mm. Essential components integrated into each dish include a built-in hollow fiber membrane (HFM) for aeration, a magnetic stirrer to facilitate mixing, and an automated water replenishment system designed to offset the water loss due to evaporation. Stirring was maintained continuously during daylight hours to enhance mixing and gas exchange. The HFMs, strategically positioned at the base of each dish, provided aeration with CO_2-enriched air, ensuring the pH of the culture remained stable between 7 and 7.5 by regulating the airflow to 4 vvm. Lighting was provided by two LED lamps, set to a 12-hour light/dark cycle, with light intensity adjustments as measured by a Dual-Range Light Meter from Traceable® Calibration Control Company, Canada. To maintain sufficient nutrient levels during the cultivation period, medium replacement was conducted systematically via centrifugation. This was specifically implemented each time the biomass concentration increased by 3g/L, with the exact volume of medium replaced being determined by the parameters of the experimental design. Variables such as light intensity, culture thickness, mixing frequency and initial inoculation biomass concentration were adjusted based on the requirements of each experiment and are further explicated in the sections that follow.

6.2.3.2 Experiments on the effect of culture thickness

In the experiments designed to explore the influence of culture thickness on microalgal growth, initial biomass concentrations were set at 1g/L and 12g/L to simulate low and high density inoculation scenarios in one batch cultivation mode, respectively. For the 1g/L cultures, a light intensity of 150 μmol/m^2/s was chosen to circumvent light limitation, while the 12g/L cultures were subjected to a higher light intensity of 550 μmol/m^2/s to prevent photoinhibition, aligning with insights from prior studies. Continuous stirring was implemented throughout the daylight hours to promote uniformity in growth conditions. The variation in culture thickness, ranging from 3mm to 36mm, was systematically adjusted by altering the volume of the culture medium to explore its effect on microalgal development.

6.2.3.3 Experiments on constant inoculation biomass

For the experimental design aimed at investigating the effects under a constant inoculation biomass of microalgae, an initial inoculum biomass of 0.3g was introduced into culture mediums with culture thicknesses spanning from 3mm to 36mm, resulting in biomass concentrations ranging from 1.11g/L to 13.33g/L. The light intensity was maintained at a constant 550 μmol/m^2/s. Depending on the specific needs of the experiment, the stirring frequency was adjusted, alternating between continuous stirring and periodic stirring—implemented every two hours for a span of 10 minutes each session.

6.2.3.4 Experiments on investigating cultivation modes with adjustable culture thickness

For the experimental exploration of adjusting culture medium thickness during cultivation, modifications to the thickness were made at strategic points, often coinciding with the replacement of the medium. For instance, an adjustment from an initial 36mm thickness to 20 mm was executed on the fourth day, as dictated by the experimental parameters. Conducted under conditions of intermittent stirring, these trials started with an algal inoculation biomass of 0.3g, where the starting concentration varied with the medium's thickness. The illumination was consistently set at 550

μmol/m^2/s throughout the experiment.

6.2.4 Analytical methods

6.2.4.1 Measurement of biomass concentration

To evaluate algae biomass concentration (g/L), optical density measurements were taken at a wavelength of 700 nm (OD700) utilizing a Thermo Fisher Scientific Inc. GENESYS™ 10S UV-Vis spectrophotometer. The optical density of samples was regulated to maintain values between 0.1 and 0.8 for precision. A standard curve was used to map the relationship between optical density and biomass concentration, which was periodically adjusted to reflect changes in the cultivation environment. For sample processing, algae were first centrifuged at 6000 RCF and subsequently rinsed twice with deionized water. The collected algal pellets were then subjected to drying at 60°C until a consistent weight was achieved, generally over a duration of 24 hours.

6.2.4.2 Cell density determination

Cell densities of the microalgae under investigation were assessed by performing cell counts using an Improved Neubauer hemacytometer (Phase Counting Chamber w/2 cover glass, USA), with observations made via a phase-contrast microscope (Infinity II BX40, Olympus, Canada) at 200x magnification. The process of counting cells was supported by Infinity Capture software (Lumenera Scientific, Ottawa, Canada).

6.2.5 Calculations

6.2.5.1 Cell growth rate and productivity

To calculate the volumetric biomass productivity (P_V) and areal biomass productivity (P_A) of the algae, the following equations were utilized:

$$P_V = \frac{(X_2 - X_1)}{t} \qquad (6\text{-}1)$$

$$P_A = P_V h \qquad (6\text{-}2)$$

Here, P_V is defined as the volumetric biomass productivity (g L^{-1} day^{-1}), P_A as the areal biomass productivity (g/m^2/day), and X_1 and X_2 represent the biomass concentration (g/L) at the start and finish of the cultivation period t (in days), respectively, and h (in mm) denotes the culture thickness.

The maximal specific growth rate (μ_{max}) was determined from the slope of the

natural logarithm of biomass concentration, i.e., $Ln(X)$, versus time (t), which was shown by using the equation below, within the exponential growth phase where the apparent specific growth rate was constant.

$$\mu_{max} = \frac{d(Ln(X))}{dt} \qquad (6\text{-}3)$$

6.2.5.2 Net yield

To calculate the net yield and the net increase in cell density of microalgae, the following formulas were employed:

$$Y_N = X_1V_1 - X_0V_0 \qquad (6\text{-}4)$$

$$CD_N = CD_1V_1 - CD_0V_0 \qquad (6\text{-}5)$$

where Y_N the net yield (g), X_0 and X_1 the biomass concentration at the beginning and end of the cultivation period, respectively. CD_N is the net increase in cell density (x10^9 cells), and CD_0 and CD_1 are the biomass concentration at the beginning and end of the cultivation period, respectively. V_1 (mL) is the volume of the culture on the final day of cultivation, and V_0 (mL) is the volume on the first day.

6.2.5.3 Energy saving

The energy saving achieved in HTLAR-HFMS with intermittent stirring is calculated with the following equation (Eq. 6-6):

$$Es = \left(1 - \frac{T_M}{T}\right) \times 100\% \qquad (6\text{-}6)$$

where E_S (%) represents the percentage of mixing energy saving, T_M (mins), the duration of mixing in each on/off cycle, and T (mins) the total duration of each mixing cycle, encompassing both on and off periods. In the intermittent stirring experiments, stirring was conducted once every two hours for a period of 10 minutes each time. As a result, Es were calculated to be 91.67%.

The efficiency of culture medium usage, defined as the net yield of microalgae produced per unit volume of the culture medium, was calculated using the following formula:

$$M_E = \frac{Y_{Ni}}{M_{Ti}} \qquad (6\text{-}7)$$

where M_E represented the medium usage efficiency (g/L), Y_{Ni} the biomass of algae (g)

at a specific culture thickness, and M_{Ti} the total volume of culture medium (mL) utilized throughout the cultivation process at that specific thickness.

The demand for centrifugation during the cultivation and harvest process is determined by the volume required for centrifugation. In this study, a centrifuge unit is defined as 22.5 mL. Thus, the formula for calculating the centrifuge demand is as follows:

$$Centrifuge\ Unit = \frac{M_{Ti}}{22.5} \qquad (6\text{-}8)$$

The manual maintenance parameter, *Hm*, is determined by the number of times the culture medium is replaced, serving as a measure of the required human intervention in microalgae cultivation which could be described as follow:

$$H_M = \sum Medium\ change\ frequency \qquad (6\text{-}9)$$

6.3 Results and Discussion

6.3.1 Effect of culture thickness on the growth of *N. oleoabundans* at low and high initial biomass concentration

N. oleoabundans was cultivated in culture mediums of varying thicknesses: 6, 12, 20, and 36 mm in one batch mode, corresponding to the culture volumes of 45, 90, 150, and 270 mL, respectively. Two initial inoculation concentrations, 1 and 12 g/L were chosen to represent scenarios of low and high cell density, respectively. This experiment was designed to investigate the effects of varying culture thicknesses on microalgal growth across distinct scenarios of low and high cell density conditions, aiming to uncover the specific influences of cultivation depth under diverse biomass concentrations.

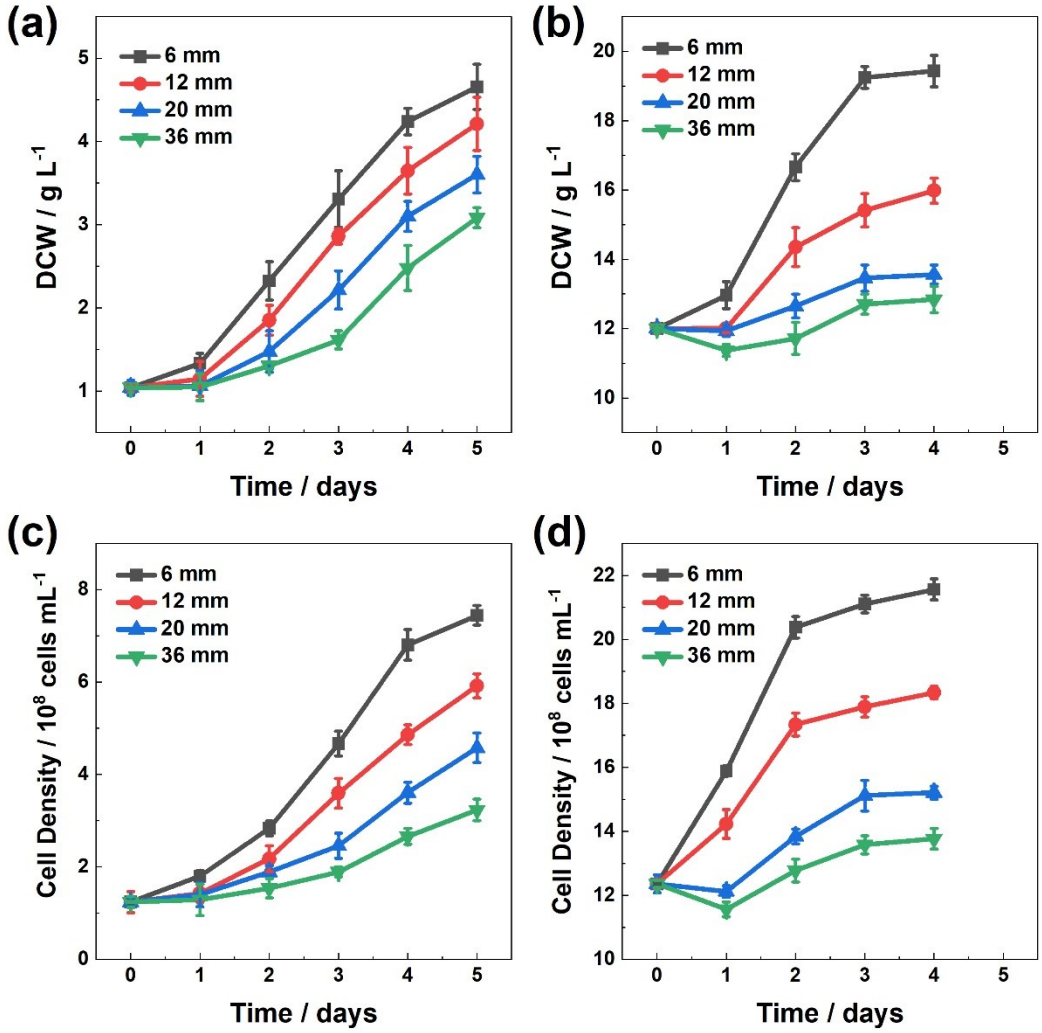

Figure 6-1. Effect of culture thickness on *N. oleoabundans* growth: Biomass concentration of cultures of different inoculums: (a) 1 g/L, (b), 12 g/L; and cell density of cultures of different inoculum: (c) 1 g/L; and (d) 12 g/L

Figure 6-1 illustrates the impact of culture thickness on biomass concentration and cell density in microalgal cultivation, with data showcasing a consistent suppression of microalgal growth as culture thickness increased, observed across both low and high initial concentration settings. In scenarios with lower initial biomass concentrations, the maximum biomass observed decreased from 4.66 g/L at a thickness of 6mm to 3.08 g/L at 36mm. Conversely, for cultures initiated with higher biomass concentrations, the maximum biomass concentration observed diminished from 19.43 g/L at a 6mm

thickness to 12.34 g/L at 36mm. Furthermore, an initial lag phase of was noted across all conditions, becoming more pronounced and extending in duration with higher inoculation cell densities and increased culture thicknesses. Notably, cultures at 20 mm and 36mm thicknesses with a 12 g/L inoculation density experienced a decline in biomass concentration on the first day. Figures 6-1c and 1d depicte the changes in microalgal cell density. Particularly under high cell density inoculation scenarios, a marked acceleration in cell division was observed, reaching a peak as early as the third day. This rapid division showed the vigorous growth capacity of microalgae at a culture thickness of 6 mm, where cell density impressively climbed to 2.16 $x10^9$ cells/mL. However, for algae cultivated at thicknesses of 20 mm or 36 mm, the increase in cell division was less marked, amounting to an approximate rise of only about $1.5x10^8$ cells/mL. In contrast, algae inoculated at lower densities did not seem to attain their peak cell density within the initial five days of cultivation, continuing to undergo division, particularly in cultures with thicknesses exceeding 12 mm.

Table 6-1. Summaries of key parameters of microalgal growth under different culture thicknesses and inoculum concentrations

	Low concentration				**High concentration**			
Culture thickness (mm)	6	12	20	36	6	12	20	36
μ_{max} **(d^{-1})**	0.56	0.46	0.36	0.28	0.25	0.18	0.06	0.06
P_V **(g/L/d)**	0.72	0.63	0.51	0.41	1.86	0.99	0.39	0.21
P_A**($g/m^2/d$)**	4.34	7.61	10.25	14.71	11.14	11.93	7.78	7.52
Y_N **(g)**	0.16	0.29	0.38	0.55	0.33	0.36	0.23	0.23
CD_N **(x 10^8 cells)**	279.3	421.3	500.4	538.4	414.0	538.2	427.5	380.7

Table 6-1 summarized the pivotal parameters of microalgal growth, illustrating that, across algae inoculated at consistent concentrations, there was a discernible decrease in growth rate as culture thickness augmented—a trend largely mirrored in volumetric productivity as well. The maximum volumetric productivity was achieved at a thickness of 6mm, with values of 0.72g/L/d and 1.89g/L/d for low and high initial inoculation

densities, respectively. However, the areal productivity exhibited a different pattern. For algae inoculated at low biomass concentrations, areal productivity increased with culture thickness, from 4.34 g/m^2/d at 6mm to 14.71 g/m^2/d at 36mm, with the corresponding highest net yield of 0.55g observed at 36mm. In contrast, for algae with higher initial inoculum concentrations, their areal productivity experienced an initial rise with an increase in thickness, reaching an apex of 11.93 g/m^2/d at 12 mm, before subsequently declining. For a given culture thickness, exemplified by a 6mm depth, the areal productivity at an initial inoculation density of 1 g/L was markedly inferior compared to that observed at 12 g/L, recording a modest 4.34 g/m^2/d. On the flip side, when considering a thicker culture medium of 36mm, inoculations at lower concentrations showcased a pronounced enhancement in areal productivity. Conversely, algae initiated with a higher concentration demonstrated suboptimal growth outcomes at this thickness, as indicated by the minimal areal productivity of merely 7.52 g/m^2/d, alongside diminished net yields and less pronounced increases in cell density.

The experimental findings demonstrated that culture thickness had varying impacts on microalgal growth. A commonality in the growth behavior of algae cultured at both high and low densities was that the cell division rate decreased as the culture thickness increases. This was attributed to the reduction in average light intensity experienced by the algae with an increase in culture thickness, which undoubtedly diminishes the efficiency of photosynthesis. In the case of algae grown at low cell densities, while the biomass concentration diminished with an increase in culture thickness, the thicker cultures showcased enhanced areal productivity, which proved beneficial for the final yield. Despite the light intensity being merely 150 μmol/m^2/s, augmenting the thickness did not have a negative impact on the net yield levels for algae at low densities. In contrast, for algae at high cell densities, even with a light intensity of 550 μmol/m^2/s which was selected under the premise of avoiding light inhibition, an increase in culture thickness resulted in decreased areal and volumetric productivities, which ultimately affected the net yield adversely. The increase in culture volume was unable to counterbalance the decline in productivity, which is consistent with expectations;

particularly in HCDC, where enhanced culture volume led to significantly reduced light penetration through the medium, impeding algal growth due to insufficient illumination [28]. With increased depth, algae at the bottom layers were unable to access enough light, a phenomenon previously confirmed by previous research. Additionally, in denser cultures with greater culture thickness, algae cells faced more prone to the negative effects impacts of elevated dissolved oxygen (DO) concentrations [29]. In HCDC, photosynthesis can cause DO to accumulate to levels [30] that may inhibit algal growth, with thicker cultures exacerbating this effect by restricting oxygen diffusion.

In summary, it becomes evident that microalgae cultures exhibit differing levels of sensitivity to culture thickness between low and high cell density conditions. This underlines the reason why successful HCDC is predominantly achieved in environments with reduced culture thickness. Beyond affirming the critical role of thin-layer cultivation for attaining HCDC, our findings introduce a strategic hypothesis: initiating cultivation with a larger volume at lower algal cell densities and judiciously decreasing culture thickness in response to increasing algal cell density could optimize growth. Despite the observation that lower-density cultures achieve higher net yields with increased thickness, they also necessitate a larger input of biomass. Thus, subsequent discussions will focus on a more detailed investigation, specifically under conditions of uniform net biomass input.

6.3.2 Cultivation with constant algal inoculation under continuous mixing

In this experiment, the inoculated biomass was kept constant at 0.3g, distributed into culture mediums of varying thicknesses—3, 6, 12, 20, and 36 mm—to examine the influence of culture thickness on microalgal growth and the eventual net yield. The experiment was conducted under continuous mixing conditions, with timely medium replacement to ensure an adequate supply of nutrients. The cultivation period lasted for 8 days, with all processes carried out under continuous stirring and a fixed light intensity of 550 μmol/m^2/s.

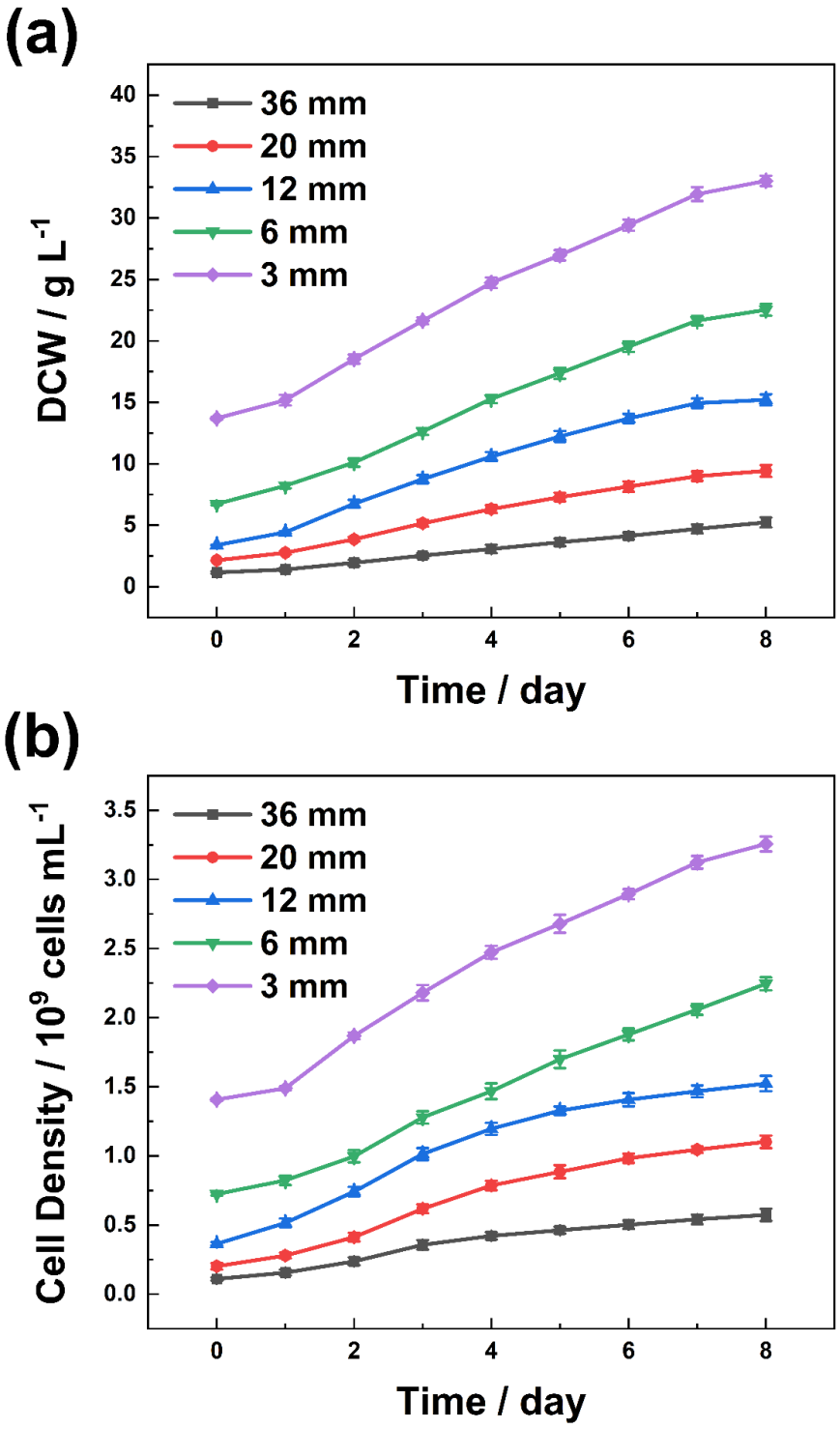

Figure 6-2. The effect of different culture thicknesses on *N.oleoabundans* growth under continuous stirring with a fixed inoculation biomass

Figure 6-2 illustrated the growth of algae at different culture thicknesses under a fixed inoculation biomass. Given the initial inoculation biomass of 0.3g, the

corresponding initial inoculation concentrations varied as 1.11, 2, 3.33, 6.66, and 13.32 g/L with decreasing culture thickness. On the first day of cultivation, a lag phase was observed across all conditions, which became more pronounced with an increase in culture thickness. It was observed that, despite higher culture cell densities, a relatively rapid growth rate was maintained with reduced culture thickness. For instance, at a 3mm culture thickness, microalgae grew from an initial biomass concentration of 13.32 g/L to 33.02 g/L after 8 days of cultivation, with cell density increasing from 1.41 to 3.26 x10^9 cells/mL. Conversely, at a 36mm culture thickness, the biomass concentration only grew from 1.11 g/L to 5.23 g/L after 8 days. By the third day of cultivation, algae in cultures thicker than 12mm began to exhibit a slowdown in cell division rate, as depicted in Figure 6-2b. Detailed calculations were performed and summarized in Table 6-2. Detailed calculations were carried out and summarized in Table 6-2, facilitating a more comprehensive analysis.

Table 6-2. Key parameters of microalgal growth at various culture thicknesses under continuous stirring

	Culture thickness (mm)				
	36	**20**	**12**	**6**	**3**
Culture volume (mL)	270	150	90	45	22.5
M_T **(mL)**	540	450	360	270	180
Centrifuge (unit)	24	20	16	12	8
H_M **(unit)**	2	3	4	6	8
M_E **(g/L)**	2.04	2.42	2.96	2.63	2.42
Y_N **(g)**	1.10	1.09	1.06	0.71	0.44
CD_N **(x 10^9 cells)**	125.17	135.06	104.32	68.45	41.60
P_V **(g/L/d)**	0.51	0.91	1.48	1.97	2.42
P_A **(g/m^2/d)**	18.35	18.17	17.73	11.85	7.25

Table 6-2 summarized the key growth parameters and cultivation conditions for the microalgae. It was found that P_V increased with a decrease in culture thickness, rising

from 0.51 g/L/d to 2.42 g/L/d. Conversely, P_A showed an opposite trend, decreasing from 18.35 g/m^2/d to 7.25 g/m^2/d. Meanwhile, the final Y_N decreased as culture thickness reduced, with the highest net yield of 1.10 g achieved at a thickness of 36 mm. Despite this, the cultures at 12, 20, and 36mm exhibited similar net yields. Nonetheless, a thickness of 12 mm distinguished itself with superior medium utilization efficiency and a more economical use of centrifuge units. Compared to the 36mm thickness, which consumed 540 mL of medium, the 12 mm thickness only used 360 mL, achieving the highest medium use efficiency of 2.96 g/mL among all tested thicknesses. The total consumption of culture medium (M_T) decreased with the reduction in culture thickness, leading to a corresponding decrease in the number of centrifuge units required. For instance, at a culture thickness of 3 mm, M_T was only 180 mL, necessitating daily medium changes, and resulted in a total of only 8 centrifuge units. However, the number of H_M increased as culture thickness decreased. This was attributed to the observation that, as the microalgae cultivation progressed, lower culture thicknesses, which corresponded to higher P_V, required more frequent medium changes.

Across different culture thicknesses, with the same inoculation biomass of 0.3g, the net yield decreased as the thickness reduced, indicating that despite the higher volumetric productivity of thin-layer cultures, their lower areal productivity led to an inability to achieve higher net yields. The reasons are multifaceted, primarily due to the varying initial inoculation concentrations, which influenced how light affected the algae at each culture thickness differently. For example, a potential reason for the reduced cell division rate in later stages at a 12 mm thickness could be insufficient light. Indeed, balancing volumetric and areal productivity to optimize microalgae production and reduce costs has always been a challenge in thin-layer cultivation. However, it was evident that lower culture thicknesses achieved relatively higher M_E and reduce the need for centrifugation. Encouragingly, thicknesses of 3mm or lower were capable of reaching biomass concentrations of at least 30 g/L, supporting the viability of thin-layer HCDC. Experiment 6.3.1 highlighted that HCDC in thicker setups, like at 36mm with an inoculation concentration of 1.11g/L, does not favor algal growth due to the inability

to achieve HCDC, despite an abundance of nutrients.

It's critical to recognize that the experiment's observations are influenced by the specific light intensities and initial biomass used, indicating that alternate conditions might yield different results. The phenomena observed in this experiment were specific to the conditions of an inoculation biomass of 0.3g and a light intensity of 550 μmol/m^2/s, aiming to showcase a methodology for analyzing issues in microalgae cultivation. Addressing the areal productivity challenges inherent in thin-layer cultivation requires extensive optimization, such as employing cultivation models with adjustable light intensities or enhancing the culture medium composition. The manual estimation for medium replacement also introduces a level of imprecision in medium utilization. Traditionally, cultivation approaches have sought to improve areal growth rates by increasing the specific surface area, exemplified by the use of Thin-layer cascade (TLC) reactors. These reactors, despite having the same volume, offer a higher surface area with a minimal culture thickness of 6mm. Differently from this study, TLC reactors depend on pumps for their circulation system, a factor of energy consumption that invites comparison with the energy demands of conventional tank reactors, potentially necessitating a higher energy input for suspension. This study was conducted under the premise of consistent surface area, ensuring that the energy expended on mixing remained similar across all tested culture thicknesses.

The objective of this experiment was to establish a foundational hypothesis for experimental design and, from a production standpoint, provide insights into balancing output with resource utilization. The challenge for thin-layer cultivation, focusing solely on net yield, lies in how to overcome the barrier of lower areal productivity and enhance competitiveness against traditional thicker cultivation mode.

6.3.3 Cultivation in fixed algal input under intermittent mixing conditions

In this study, a consistent initial inoculation biomass was maintained, with a shift in methodology to intermittent mixing. A strategy of stirring once every two hours, with each stirring session lasting 10 minutes, was employed to explore the variations in microalgal growth under reduced mixing dependency across different culture

thicknesses.

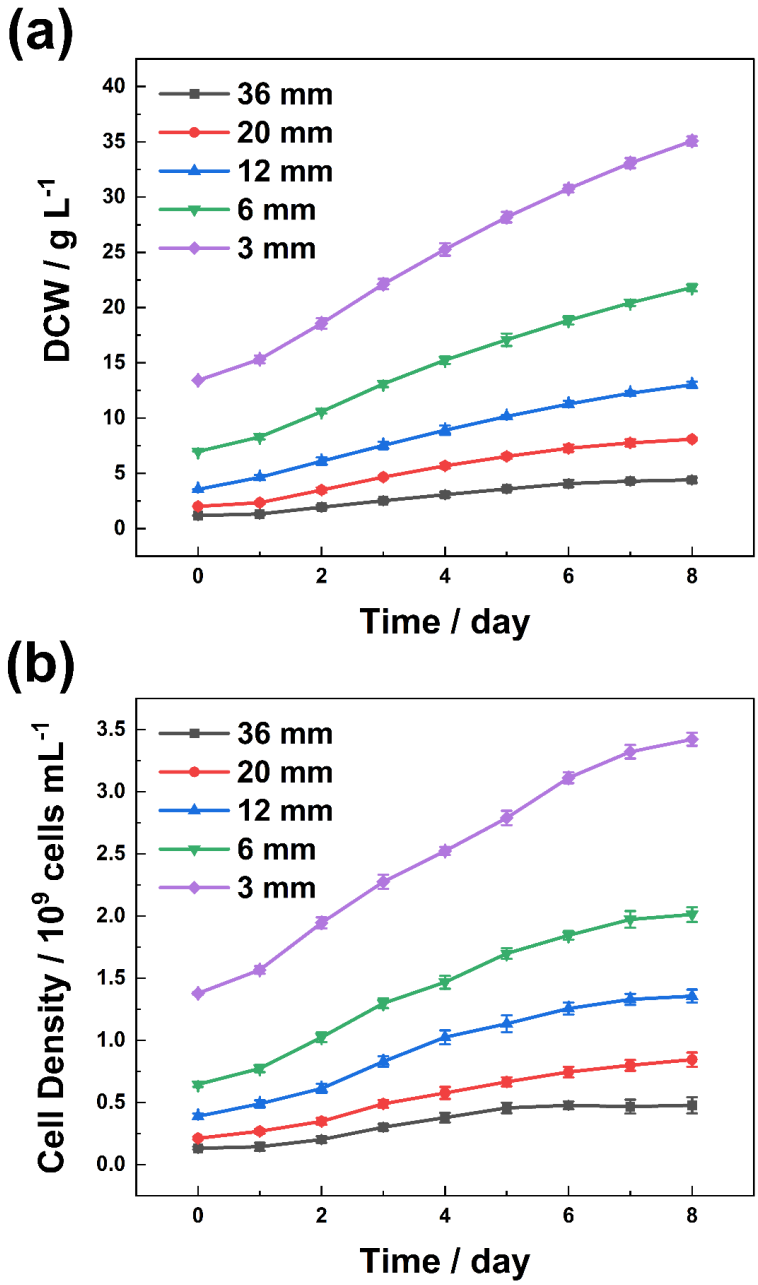

Figure 6-3. The effect of different culture thicknesses on *N.oleoabundans* growth under intermittent mixing with a fixed inoculation biomass

Figure 6-3 depicted the effects of various culture thicknesses on microalgal growth under intermittent stirring conditions, while maintaining the initial inoculation biomass at 0.3g. It was observed that algal cells managed to divide and grow normally across all culture thicknesses during the early phases of cultivation. However, for cultures with

thicknesses exceeding 12 mm, cell growth began to be inhibited after the third day, in stark contrast to conditions of continuous stirring. For instance, algae cultivated for 8 days at thicknesses of 20 mm and 36 mm reached biomass concentrations of merely 8.09 g/L and 4.40 g/L, respectively, with their cell densities reaching only 4.78 x 10^8 cells/mL and 8.45 x 10^8 cells/mL. On the other hand, the growth inhibition effect observed with intermittent stirring became less significant in cultures thinner than 6mm. Specifically, at a thickness of 6mm, the final biomass concentration achieved was 21.81g/L, mirroring the outcomes seen with continuous stirring. Remarkably, at a thickness of 3mm, algae not only matched but exceeded the biomass concentration observed with continuous stirring, achieving a high of 35g/L, accompanied by a superior cell density of 3.42 x 10^9 cells/mL.

Table 6-3. Key parameters of microalgal growth at various culture thicknesses with intermittent stirring

	Culture thickness (mm)				
	36	**20**	**12**	**6**	**3**
Culture volume (mL)	270	150	90	45	22.5
M_T **(mL)**	540	450	360	270	180
Centrifuge (unit)	24	20	16	12	8
H_M **(unit)**	2	3	4	6	8
M_E **(g/L)**	1.61	2.03	2.37	2.47	2.71
Y_N **(g)**	0.87	0.91	0.85	0.67	0.49
CD_N **(x 10^9 cells)**	93.42	94.95	87.03	61.52	45.99
P_V **(g/L/d)**	0.40	0.76	1.18	1.85	3.05
P_A **(g/m^2/d)**	14.51	15.20	14.20	11.12	9.16
Y_r	79%	84%	80%	94%	112%

*Y_r refers to the ratio of yield obtained from intermittent mixing compared to that from continuous mixing.

Table 6-3 presented a detailed summary of critical growth and cultivation

parameters observed under conditions of intermittent stirring. Notably, intermittent stirring exhibited a pronounced influence on net yields, particularly at higher culture thicknesses. At a thickness of 36mm, the net yield was merely 0.87g, constituting 79% of the yield compared to continuous stirring conditions. Conversely, algae cultivated at a 6mm thickness achieved a net yield of 0.67 g, reaching 94% of the continuous stirring yield. Despite the decrease in culture thickness, P_V showed an upward trend. However, in comparison to continuous stirring, P_A at a 36mm thickness did not demonstrate an advantage, registering at only 14.51 g/m^2/d. Still, larger culture thicknesses managed to retain higher areal productivity than their thinner counterparts. When considering medium utilization efficiency, an increase was observed with escalating culture thickness. Relative to continuous stirring, M_E declined at higher culture thicknesses, yet improved at lower thicknesses. For example, at a 20mm thickness, M_E dropped from 2.42 g/L to 2.03 g/L, whereas at 3mm, it increased from 2.42 g/L to 2.71 g/L. Other metrics, such as centrifuge units and H_M, aligned with findings from continuous stirring scenarios.

In comparison to the continuous stirring approach, intermittent mixing has been validated as more effective for thin-layer cultivation by reducing energy consumption. Throughout this study, the dependency of algae on stirring was found to decrease with diminishing culture thickness. This reduction in dependence is linked to enhanced light availability and more efficient gas exchange, particularly in terms of dissolved oxygen expulsion, in thinner cultures. Despite employing a maximum thickness of 36 mm in the experiments—a thickness considered lower relative to traditional tank bioreactors—algal growth was nonetheless observed to be compromised. Specifically, during the period without mixing, algal cells tended to settle, creating a sedimentation effect that obstructed light penetration to the deeper layers, thereby inhibiting photosynthesis and adversely affecting cell division and proliferation. Algae cultured in thinner environments appeared better suited to intermittent stirring, with a notable observation that reducing stirring frequency further augmented the net yield. This phenomenon can be attributed to the fact that the positive impact of mixing on algal

suspension becomes negligible in ultra-thin conditions; rather, excessive mixing could result in a disproportionate distribution of algae, especially evident in the extreme thinness of 3 mm cultures. The observation that the reduction in culture thickness has a diminished effect on net yield under intermittent stirring conditions is beneficial for HCDC. It not only facilitates considerable energy savings from reduced mixing requirements but also provides valuable insights for the design of cultivation strategies.

6.3.4 Cultivation optimization through variable culture thickness mode

The results from the experiments above indicated that cultivating algae at a consistent thickness throughout the process inevitably leads to a decline in algal productivity, thereby reducing the economic viability of algae production. In response, this investigation introduced an innovative cultivation strategy, characterized by the periodic adjustment of culture thickness throughout the cultivation under intermittent stirring conditions. To ensure consistency in comparative analysis, the initial inoculation biomass was kept constant at 0.3g.

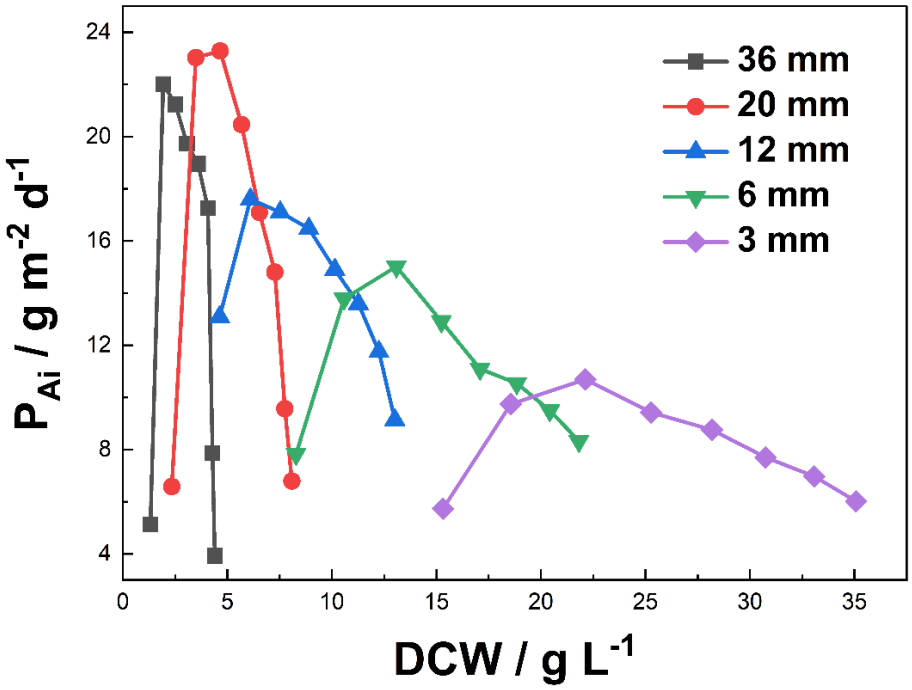

Figure 6-4. Variations in daily areal productivity (P_{Ai}) with algal biomass concentration across various culture thicknesses under intermittent stirring conditions

Before devising the strategy for varying culture thickness, it was crucial to investigate the relationship between daily areal productivity (P_{Ai}) and biomass concentration to pinpoint the optimal moment for thickness adjustment. Figure 6-4 depicted this relationship under intermittent stirring across various culture thicknesses. It became clear that the trend of P_{Ai}, regardless of thickness, followed a pattern of initial increase followed by a decrease. As culture thickness was reduced, the peak values of P_{Ai} systematically decreased, a finding that was consistent with data outlined in section 6.3.3. Considering the first day marked the lag phase, there was a significant surge in P_{Ai} during the early phases, which then declined following the exponential growth phase. It was particularly observed that algae cultivated at higher thicknesses exhibited the most dramatic declines in P_{Ai} during the later stages. For example, at 36mm thickness, when the biomass concentration was below 5g/L, P_{Ai} fell to just 3.93 g/m^2/d. Although algae in thinner cultures showed generally lower P_{Ai}, their rate of decline in the later stages was more gradual; for instance, with a biomass concentration of 20.43g/L at 6mm thickness, P_{Ai} remained at 9.50 g/m^2/d. This observation led to the proposition that utilizing the relationship between P_{Ai} and biomass concentration across different thicknesses as a guide for adjusting culture thickness could offer a practical method for algae cultivation. More specifically, when P_{Ai} was observed to decrease or sustain at a lower range, the culture thickness was reduced to the subsequent level to preserve a higher level of P_{Ai}.

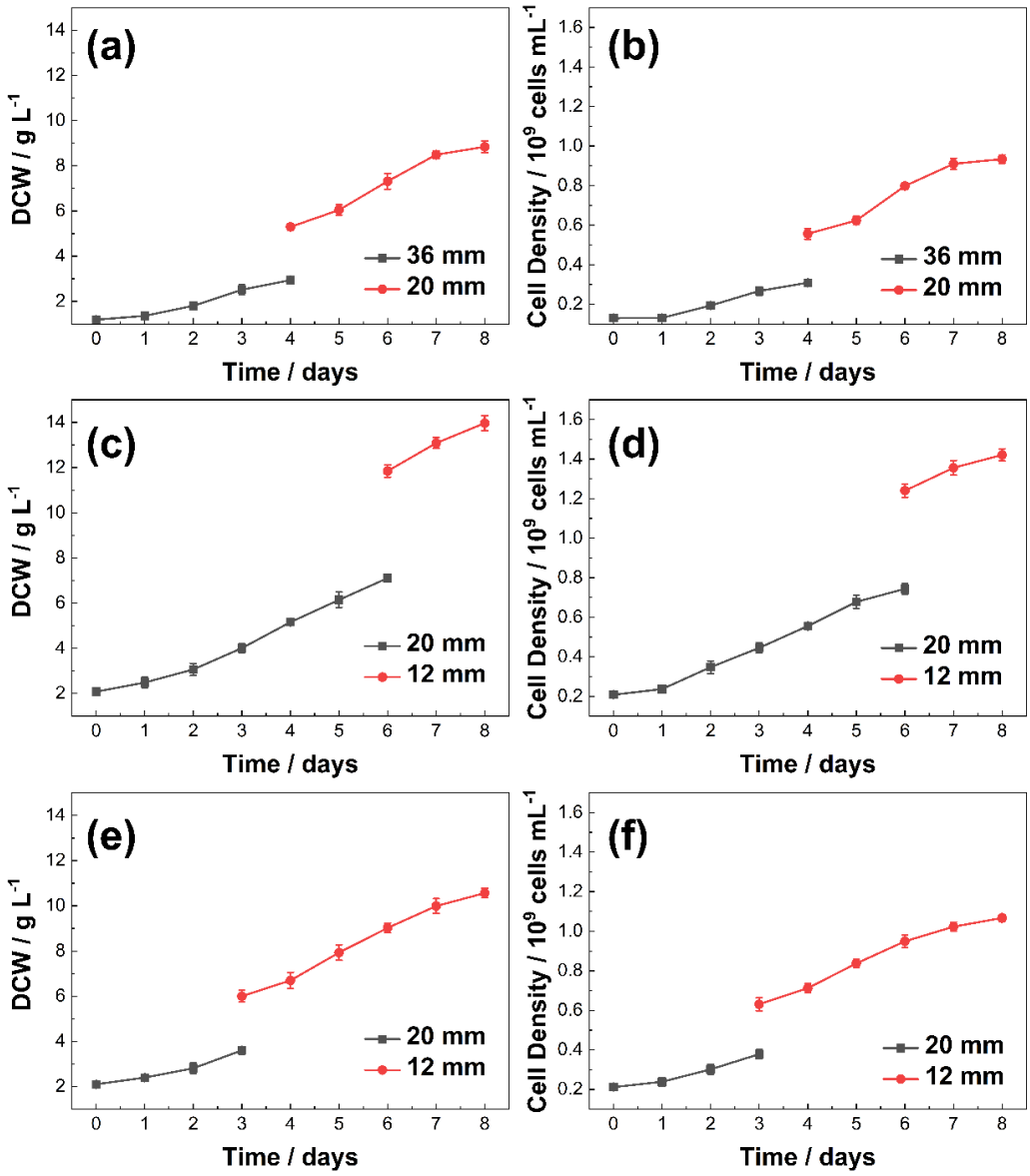

Figure 6-5. Microalgal growth patterns in culture thickness adjustment cultivation modes under intermittent stirring: (a) ”36-20” initial cultivation at a 36mm thickness followed by a switch to 20mm at day 4th ; (b) the "20-12(6)" strategy starts with cultivation at 20mm followed by a switch to 12 mm at day 6th and (c) the "20-12(3)" approach begins with cultivation at 20mm, followed by a switch to 12 mm at day 3rd .

Thus, three distinct cultivation schemes for adjusting thickness were conceptualized, as shown in Figure 6-5, which presents the variations in biomass and cell concentrations. The results indicated that upon modifying the culture thickness, there was a notable adjustment in biomass concentration as well. Specifically, algae

subjected to an initial 36-20 mm thickness alteration strategy achieved a biomass concentration of 8.85g/L by the end of the cultivation period, corresponding to a cell density of 0.93 x 10^9 cells/mL. When comparing the 20-12(6) and 20-12(3) thickness adjustment strategies, it became evident that the 20-12(6) strategy yielded the highest final biomass concentration at 13.97 g/L, surpassing the 10.57 g/L achieved with the 20-12(3) strategy.

Table 6-4. Key parameters of microalgal growth under culture thickness adjustable mode with intermittent stirring

	36-20	**20-12(6)**	**20-12(3)**
M_T **(mL)**	420	390	330
Centrifuge (unit)	18.67	14.67	17.33
H_M **(unit)**	2	3	3
M_E **(g/L)**	2.40	2.42	1.93
Y_N **(g)**	1.01	0.95	0.64
CD_N **(x 10^9 cells)**	105.22	96.71	64.35
Y_r	91.73%	86.70%	58.35%
M_{Tr}	77.78%	86.67%	73.33%
E_S	91.70%	91.70%	91.70%

*Y_r refers to the ratio of yield obtained from related thickness alteration strategy compared to that from continuous mixing. M_{Tr} refers to the ratio of total medium consumption obtained from related thickness alteration strategy compared to that from continuous mixing.

Table 6-4 illustrates the critical growth parameters for microalgae cultivated under three distinct thickness-varying mechanisms. Notably, the 36-20 strategy emerged as the most effective, securing the highest Y_N and CD_N at 1.01g and 105.22 x 10^9 cells, respectively. Meanwhile, the 20-12(6) approach yielded marginally lower results than the 36-20, with a Y_N of 0.95g and a CD_N of 96.71 x 10^9 cells. However, these outcomes were superior to those achieved with a consistent thickness under intermittent stirring, notably requiring a smaller M_T due to the late-stage reduction in culture thickness,

which effectively decreased medium usage. In contrast, the 20-12(3) strategy's Y_N was substantially lower, only reaching 0.64g, which significantly underperformed compared to the net yields of 0.91g and 0.85g observed with a constant thickness of 20 mm or 12 mm under intermittent stirring conditions which indicated that the modification in thickness for this strategy inadvertently led to a decrease in culture efficiency. What merits particular attention is the impact of this innovative method on production yields compared to the traditional continuous stirring approach. Observations revealed that for the 36-20 and 20-12(6) cultivation strategies, the net yields achieved were respectively 91.73% and 86.70% of those under continuous stirring at culture thicknesses of 36 mm and 20 mm. Conversely, the 20-12(3) approach significantly lagged, capturing only 58.35% of the yield observed with continuous stirring at a 20mm thickness. On the other hand, due to the adoption of intermittent stirring, there was a significant energy savings of 91.7% in mixing energy input compared to continuous stirring.

The approach of managing culture thickness based on areal productivity proved feasible. Optimizing for the best areal productivity indices at each thickness level, corresponding to certain biomass concentrations, facilitated an overall increase in yield. The 20-12(3) strategy served as a cautionary example, demonstrating how premature thickness adjustments could significantly diminish final yields. Moreover, it was observed that, within the context of continuous stirring for this experiment's specific thickness and initial inoculation setups, altering thickness was unnecessary. The most favorable yields for fixed-thickness cultivation were achieved within a range of 36mm to 12 mm. This was attributed to the fact that the cultivation period of 8 days did not reach a point of growth limitation, or in other words, its high areal growth rate did not necessitate a reduction in thickness. However, when contemplating intermittent stirring for production, the diminished areal productivity observed in later stages at higher thicknesses emerged as a constraining factor. Consequently, lowering the culture thickness acted to counterbalance this limitation, leading to an increase in net yield. Furthermore, this study reaffirmed the reduced stirring needs of thin-layer cultivation, while also highlighting that adopting a singular thin-layer approach, such as cultivation

at 6mm or even 3 mm thickness, results in lower areal productivity, particularly at lower initial inoculation densities, as revisited in section 3.1. This inadequacy in areal productivity often necessitated an expansion of surface area, thereby increasing both land usage and stirring efforts. In summary, integrating strategies of intermittent stirring with thickness adjustment can achieve economically viable algae cultivation, enhancing net yield and offering a novel perspective for thin-layer cultivation.

6.4 Conclusions

This study explored the effects of culture thickness and intermittent stirring on achieving microalgae HCDC. It was found that culture thickness has a more significant negative impact on microalgae at high cell densities compared to low densities. This approach not only improves the microalgae production efficiency but also reduces input costs in terms of energy consuming and medium requirements. Intermittent stirring, while saving approximately 91% of the energy consumption compared to continuous stirring, negatively impacts the yield of microalgae in thicker cultures. However, by employing a variable thickness cultivation method, the areal productivity across different thicknesses and biomass concentrations can be effectively utilized to alleviate the decline in microalgae yield under intermittent stirring. This method also significantly reduces the overall cost of microalgae cultivation, including medium consumption, labor maintenance, and other associated expenses. However, the success of the variable thickness cultivation method relies on precise control and assessment of the cultivation process. The timing of reducing culture thickness is crucial. Therefore, detailed experimental designs and predictive methods such as machine learning are recommended for future research.

CRediT authorship contribution statement

Hongying Zhou: Conceptualization, Data curation, Investigation, Methodology, Visualization, Writing – original draft. **Zisheng Zhang:** Resources, Supervision. **Christopher Q. Lan:** Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition, Resources.

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Chapter 7 Conclusions and future directions

This thesis focuses on the HCDC of microalgae under autotrophic conditions. HCDC is crucial for the large-scale application of microalgae as it can reduce costs and energy demands. Achieving HCDC of microalgae under photoautotrophic conditions has been challenging due to the exponential light attenuation along the increase of the thickness and the cell density of the culture. A thorough understanding of the principles governing the interactions between microalgal cells and the environment is paramount towards the design and optimization of algal farming systems for cost-effective algal biomass production. While such understanding has largely been established via intensive studies over the past decades for conventional low cell density suspended cultures of a few commercially important microalgal species, data on HCDC of photoautotrophic microalgae are scarce.

7.1 Summary of research

In this research, a novel thin-layer reactor was successfully designed to achieve HCDC while reducing the dependency on mechanical stirring, thus lowering energy consumption. The results demonstrated that with optimized culture media, biomass concentration could reach approximately 8 g/L in batch cultures. Furthermore, intermittent stirring reduced energy consumption by about 92% while maintaining similar biomass productivity. The results of this novel approach underscore its potential for energy-efficient and high-yield microalgae cultivation.

The study investigated the effects of carbon sources (CO_2 and dissolved inorganic carbon, DIC) and pH on microalgae growth. Results indicate that DIC concentration above a certain threshold could inhibit cell division while leading to abnormal enlargement of algal cells. These results indicate that high DIC concentration repressed the metabolic pathways required by cell division but not the growth of individual cells, e.g., the photosynthesis pathways. Based on these observations, a two-step cultivation method was proposed and tested, which was characterized by supplementation of carbon source by aeration using CO_2-enriched air streams for cell growth in the first stage of cultivation to promote cell division and the second stage with the addition of

$NaHCO_3$ of appropriate concentration at the absence of aeration to promote the enlargement of individual cells. The optimal condition of the two-step cultivation approach could achieve similar biomass yields while saving around 77% CO_2 and therefore the corresponding costs and energy required for aeration to supply CO_2. These findings are crucial for improving the sustainability and cost-effectiveness of microalgae cultivation, demonstrating a feasible method to reduce dependence on CO_2, a major input in traditional cultivation processes.

Since light is a limiting factor in HCDC, it is important to be able to accurately model light attenuation as a function of culture thickness and cell density. To this end, we proposed and verified a modified Beer-Lambert model to account for internal scattering and shading effects, allowing accurate modeling of local light intensity and mean light intensity in cultures of various thicknesses and biomass concentrations. Using the modified Beer model, we generated data on cell growth at controlled mean light intensities to verify the applicability of the Monod model for cell growth kinetics under HCDC conditions. A maximum biomass concentration of approximately 28 g/L was achieved.

Lastly, the impact of culture thickness on microalgae growth was investigated. One limitation of thin-layer cultivation is that, despite achieving higher cell densities, the final yield per unit volume is often lower than that of conventional LCDC due to reduced thickness. Thus, increasing culture thickness emerged as a worthwhile area of study. The results indicated that dynamically adjusting the culture thickness in response to microalgae growth could enhance overall yield while still achieving high final cell density by continuously decreasing the culture thickness as the microalgae grow.

Here are the key findings of this research:

1. The novel thin-layer reactor design significantly reduced energy consumption for mechanical stirring while achieving HCDC.
2. Utilizing DIC as a carbon source in a two-step cultivation method can conserve CO_2 and achieve similar biomass yields to CO_2-based cultivation.

3. Exploring and optimizing the light model helps in understanding microalgae HCDC.
4. Dynamically adjusting culture thickness enhances overall yield, addressing a key limitation of thin-layer cultivation.

7.2 Contributions to the field

This research contributes significantly to the field of photoautotrophic HCDC of microalgae in several key aspects. First, through extensive exploration, we successfully achieved HCDC of microalgae under autotrophic conditions, demonstrating that HCDC is no longer limited to heterotrophic or mixotrophic systems. Second, we provided new design insights for cultivation reactors, focusing on reducing energy consumption related to stirring and gas supply. These innovations lay the groundwork for the large-scale application of microalgae by minimizing the energy footprint of cultivation processes. Theoretically, we elucidated the propagation of light in HCDC and refined the Beer-Lambert model to accurately fit this scenario. Moreover, we proposed a novel cultivation strategy that dynamically adjusts culture thickness to enhance yield, a pioneering approach aimed at optimizing both productivity and cost-effectiveness. All these investigations were driven by the pursuit of higher yields and lower costs, making significant strides in both the optimization of cultivation processes and theoretical model development.

7.3 Unresolved issues and future directions

Despite the significant advancements made in this study, several issues remain unresolved:

First, the novel thin-layer reactor currently faces challenges in scaling up for industrial applications. It remains a conceptual or laboratory-scale solution due to several reasons: the implementation of intermittent stirring requires further exploration; the use of hollow fibers for aeration in thin layers is difficult to maintain and service; and controlling the culture thickness on a large scale may prove challenging.

Second, the supply of nutrients needs detailed investigation. In continuous cultivation, nutrients must be replenished periodically. Developing methods to detect

and timely supplement key nutrients is crucial. In this study, nutrient supplementation was achieved by periodically replacing the entire culture medium, which is neither cost-effective nor labor-efficient.

Third, while the approach of dynamically adjusting culture thickness shows promise, determining the optimal timing and magnitude of thickness adjustments requires extensive experimentation and deeper exploration. Identifying the precise conditions under which these adjustments should be made will be essential for maximizing yield and efficiency in microalgae HCDC.

Future research should focus on several key areas to build upon the advancements made in this study. First, continued innovation in reactor design is essential. Exploring open systems and finding more efficient cultivation methods, such as integrating nutrient and water supplementation systems and improving intermittent stirring techniques, will be crucial. Second, while pursuing high cell density, it is also important to maximize overall biomass yield. The cultivation method that involves dynamically adjusting culture thickness should be further investigated to determine the optimal conditions for maximum yield. Third, optimizing the culture medium is vital, as nutrient consumption in HCDC differs significantly from low-density cultures. Tailoring the nutrient composition to meet the specific needs of high cell density cultures will enhance growth efficiency. Fourth, applying machine learning techniques to optimize cultivation conditions, such as culture thickness and key nutrient levels, can provide predictive insights and improve overall process efficiency. Lastly, leveraging genetic engineering to reduce the sensitivity of microalgae to environmental conditions can facilitate rapid growth and make cultivation more robust.

By addressing these areas, future research can further advance the commercial viability of HCDC microalgae production systems, making them more efficient and scalable for industrial applications.

Appendix

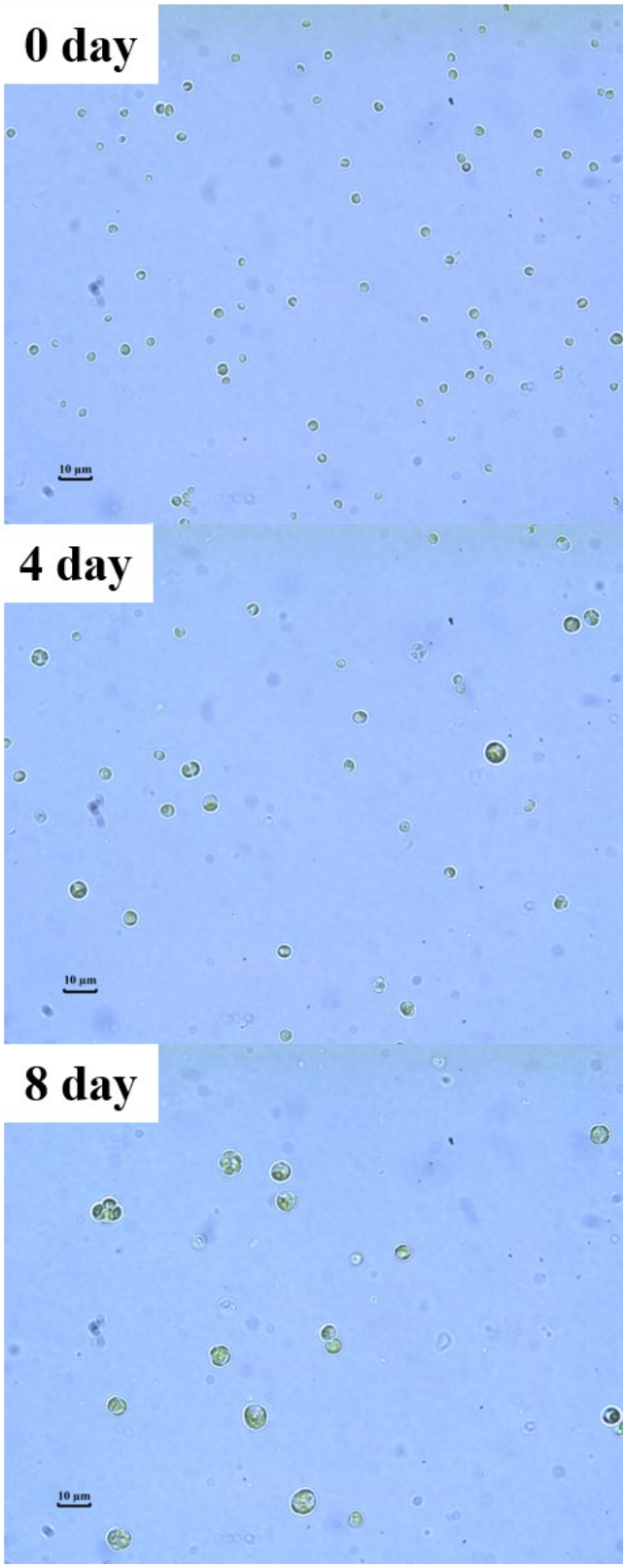

Figure A-1. The cell morphology of *N. oleoabundans* without HFMS at the day of 0, 4 and 8.

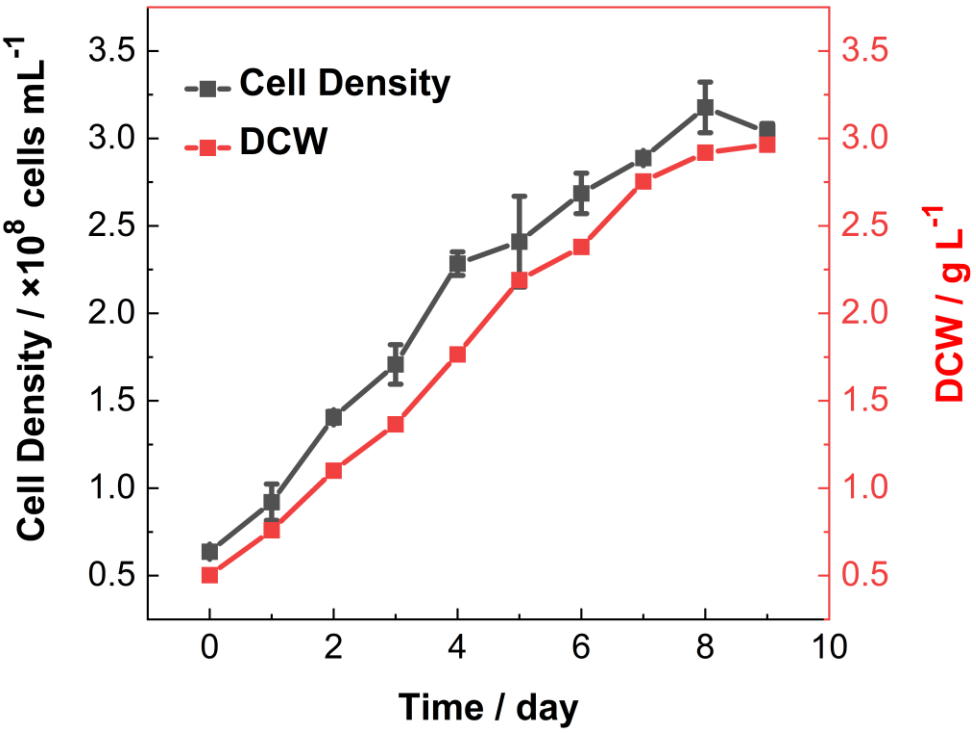

Figure A-2. Time course profiles of cell number density and biomass concentration in bottle (working volume is 400 mL). Data are mean values of triplets.