Optimal nutritional modes combination of *Chromochloris zofingiensis* in multiple cultivation process for biomass and astaxanthin productivity

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ABSTRACT

Large scale microalgal production of pigments, lipids, biofuels, and proteins have been widely considered for a variety of industrial demands in the feed, food, nutraceuticals, pharmaceuticals, and cosmetics. Due to some specific characteristics such as ability to grow in multiple trophic modes with high growth rate and productivity, *Chromochloris zofingiensis* has become an emerging species for commercial astaxanthin production. The growing microalgal industry is obtaining these natural antioxidant products by cultivating *C. zofingiensis* on diverse mineral media and organic substrates. However, the astaxanthin productivity is quite lower than *Haematococcus pluvialis*, but improvements in biomass and astaxanthin productivity under different nutritional modes is desirable. It is known that a high biomass can be obtained by culturing microalgae via fermentation. On the other hand, various efforts have been made to increase the astaxanthin production through stress factors including high light, glucose, and nitrogen starvation. While it is known that microalgae can be normally cultured in multiple steps (i.e., stock, preculture, and the production of biomass), the optimum modes for each step to maximize the biomass and astaxanthin productivity has not been examined yet. Thus, the major aim of this Ph.D. thesis was to clarify the optimum nutritional modes for the high biomass productivity and astaxanthin accumulation of *Chromochloris zofingiensis* using various modes of nutrition combinations.

Firstly, in Chapter II, cell proliferation of *C. zofingiensis* in various modes of nutrition in the preculture stage was examined. A set of sequential cultures (autotrophic, mixotrophic and heterotrophic) to the similar mode of nutrition was performed and compared. The growth curves in the cells precultured autotrophically and mixotrophically showed same trends for the first and second precultures. However, the cells precultured heterotrophically showed a decreased specific growth rate in the second preculture. The biological characteristics including

the cell size, intracellular compounds such as starch and lipids and furthermore, nuclei were also examined.

Secondly, in Chapter III, the impact of preculture on the biomass productivity via combination of nutritional modes for optimizing the culture phases (for stock, preculture and biomass production) were evaluated. The production of the biomass in mixotrophic culture showed a greater maximal biomass productivity (1.28 g L^{-1} d^{-1}) than autotrophic (0.065 g L^{-1} d^{-1}) and heterotrophic (0.448 g L^{-1} d^{-1}) modes. The cells precultured heterotrophically showed an increase in the rate of growth and the biomass production when they were cultured in autotrophic and mixotrophic biomass production phase. Furthermore, it was found out that when heterotrophically precultured cells were cultured in the prolonged dark cultures, i.e., heterotrophic biomass production phase, there was a decrease in the growth and the biomass productivity. Based on the mechanism for the impact of combination of light and dark cultures conditions on biomass productivity, which was evaluated, the modes of nutrition for stock, preculture, and biomass production steps that were optimized were autotrophic (to maintain the growth for following dark culture), heterotrophic (for the storage of intracellular components, starch, lipids, and nuclei,), and mixotrophic (to attain high productivity), respectively were suggested.

Lastly, in Chapter IV, this study revealed a combination of nutritional modes as an effective biomass production strategy by accumulation of multinucleated cells in the dark and rapid proliferation of cells in the light. The suggested nutritional modes for stock, preculture and biomass production on the induction phase were evaluated. The mixotrophic fed-batch reached the highest biomass productivity 2.52 g L^{-1} d^{-1} when the cells were precultured heterotrophically. Furthermore, this study evaluated the transition of high biomass cells into the next astaxanthin induction phase using column reactors. The potential of *C. zofingiensis* via transition of nutritional modes for a successful mass cultivation has also been discussed.

1 Chapter 1 GENERAL INTRODUCTION

1.1 Microalgal cultivation, a growing industry

Microalgae are gaining popularity recently, not only due to their high nutritional content but also for the promotion of a heathy diet. They play a significant role in the primary food chain of aquatic ecosystems. Microalgae also play a major role in diminishing the atmospheric carbon and depletion of the greenhouse gases emissions (Mularczyk et al., 2020; Patel et al., 2022). The algal cultivation has become more frequent for the food industry, animal and aquaculture feed purposes (Mularczyk et al., 2020). The higher production cost of algae is also increasing the demands of high-value added microalgal by-products and metabolites (Patel et al., 2022). The high profitability and market demand of microalgal carotenoids are one of the major focuses of the current research. The carotenoid production from the microalgae requires less labor work and can be easily harvested (Ren et al., 2021). The microalgal carotenoid production can be generally categorized as primary carotenoids (some xanthophylls and β-carotenoids) and secondary carotenoids (astaxanthin, lutein, violaxanthin, antheraxanthin and zeaxanthin) (Nisar et al., 2015), where primary carotenoids can directly contribute in the photosynthesis, while the secondary carotenoids are accumulated under stress conditions for protection purposes (Liaqat et al., 2023). The carotenoids such as lutein, β-carotene, astaxanthin, zeaxanthin, canthaxanthin, violaxanthin, Chl *a*, and so on are considered high-value products and have several commercial applications in the food, feed, nutraceutical, pharmaceutical, and cosmetics industries (Bar et al., 1995; Liu et al., 2014; Nisar et al., 2015; Ren et al., 2021; Chen et al., 2022). Of all the carotenoids, astaxanthin, one of the natural red ketocarotenoids, has attracted much attention. The research articles published on astaxanthin production has been increasing since the past 10 years (Fig. I-1) due to its superior antioxidant characteristics which is 10 times higher than other carotenoids such as lutein, canthaxanthin, zeaxanthin, and 1000 times higher than vitamin E (Patel et al., 2022).

Due to its expanding health advantages and numerous uses in the food and pharmaceutical industries, natural astaxanthin is in high demand on a global scale (Basiony et al., 2022). The global astaxanthin market is expected to reach USD 93.66 billion by 2028, from USD 77.66 billion with rate (CAGR) of 2.38% (https://www.verifiedmarketresearch.com/product/astaxanthin-market/) (Fig. I-2). Astaxanthin can be obtained through extraction from either natural resources or chemical synthesis. The chemical production of astaxanthin has been dominating the current market of natural astaxanthin but its biological functions and food safety remains a topic of concern (Li et al., 2011; Kumar et al., 2022). Since synthetic astaxanthin is derived from the petrochemicals, it raises problems in food safety, pollution, and sustainability, thus, the chemical astaxanthin is only allowed to be used in the aquaculture, and not allowed for the human consumption and animal feed other than aquaculture applications (Li et al., 2011; Nguyen, 2013). Therefore, production of astaxanthin through natural resources has been an increasing area of focus lately.

1.2 Green microalga *Chromochloris zofingiensis*

The natural astaxanthin production is in higher demand due to its present $7000/kg global market value (Koller et al., 2014; Chen et al., 2022). The natural astaxanthin in the highest content is mostly found in *Haemotococcus pluvialis* (Hata et al., 2001; Sun et al., 2015) (Fig. I-3a, b); recently, *Chromochloris zofingiensis* (Fig. I-3c, d) another alga has attracted a great interest for the astaxanthin accumulation (Liu et al., 2014; Sun et al., 2019; Mularczyk et al., 2020). The attempts to increase the astaxanthin production in the previous studies using different bioreactors in different nutritional modes have been shown in Table I-1. *C. zofingiensis*, a green microalga, is considered as an alternate astaxanthin producer to *H. pluvialis* and *Xanthophyllomyces dendrorhous*, red yeast (Meyer and du Preez, 1994;

Domínguez-Bocanegra and Torres-Muñoz, 2004) due to its high productivity (Azaman et al., 2017; Zhang et al., 2017a; Sun et al., 2019). Due to the high cost, low productivity, and unpreventable contamination, the productivity of astaxanthin in *Haematococcus pluvialis* is the most challenged part (Liu et al., 2013). The astaxanthin content ranges between 0.06–0.5% of the microalgal dry cell weight (DW) in *C. zofingiensis* (Sun et al., 2008; Zhang et al., 2016), however, it is lower than, in the microalga *H. pluvialis* (5–6% of the dry cell weight) (Yang et al., 2016). But *C. zofingiensis*, possesses several advantages such as high biomass productivity and faster growth with low contamination. It also has an ability to grow in three different nutritional modes, i.e., autotrophic, mixotrophic, and heterotrophic (Fig. I-4) (Chen et al., 2017; Sun et al., 2019; Zhang et al., 2017). The improvement in the growth enhancement and astaxanthin accumulation in *C. zofingiensis* under different modes of nutrition has grown significantly in recent years (Ip and Chen, 2005b; Wang and Peng, 2008; Azaman et al., 2017). Therefore, *C. zofingiensis* has a potential to produce more astaxanthin with high or comparable productivity than *H. pluvialis* by a combination of different nutritional modes.

1.3 Importance of culturing microalgae in multiple phases

The microalgal cultivation process for carotenoids consists of several phases, including stock, preculture, biomass production, and carotenoid induction. The stock culture phase maintains the microalgal strains, whereas the phase of the preculture is the first stage in cultivating the microalgae under suitable cultivation conditions. Following that, the cells precultured are shifted to a large-scale production phase for cell biomass. Finally, during the carotenoid induction phase, the microalgal biomass may be subjected to a range of stresses, including as different light intensities, depletion of nutrients, and high level of salinity, for accumulating substantial quantities of important secondary pigments like astaxanthin

(Chowdhary et al., 2022).

From previous knowledge, it is known that the *C. zofingiensis* cells are usually maintained in a stock phase and then are directly transferred into the next desired cultivation phase (either biomass or carotenoid induction phase). *C. zofingiensis* is known to grow in different nutritional modes, which possibly can affect the biomass productivity and morphological characteristics. A previous study revealed that different modes of nutrition can affect the morphology, biomass, and chemical compositions of *C. zofingiensis* cells (Azaman et al., 2017). The autotrophic cells can accumulate high content of pigments, but the reduction in growth rate caused by self-shading at high cell density conditions is detrimental (Rise et al., 1994; Chen et al., 2017; Benedetti et al., 2018; Metsoviti et al., 2020). The heterotrophic culture under darkness is added with glucose one of the organic carbon source, tends to achieve maximum cell density productivity, however, the cells are deficient in cellular pigments obtained via photosynthesis, such as carotene and chlorophyll *a* and chlorophyll *b* (Fan et al., 2012). The mixotrophic culture combines the merits of both autotrophic and heterotrophic cultivations, leading to a higher cell growth and high carotenoid contents and is more advantageous than both of the cultivation conditions (Ip and Chen, 2005b; Zhang et al., 2017b; Shen et al., 2019; Zhang et al., 2021b). Besides, in the mixotrophic cultivation, the crucial carotenoids that are produced during photosynthetic processes, like β-carotene, can be stored under illuminated conditions (Zhang et al., 2017), although, the carotenoid content is not as high in mixotrophic cultivations (about 2 mg g^{-1}) to that of autotrophic cultivations (about 7 mg g^{-1}) (Ip and Chen, 2005b; Chen et al., 2017). The recent desire to increase the astaxanthin production of *C. zofingiensis* commercially has been in the limelight and various attempts were made to improve the accumulation of astaxanthin in *C. zofingiensis*. While the previous studies have revealed about increasing the astaxanthin content (mg g-dry $weight^{-1}$) via the stress factors such as high light, glucose, nitrogen starvation in *C. zofingiensis* (Bar et al., 1995; Sun

et al., 2008; Zhang et al., 2017a). However, there has been no knowledge about the effect of combination of nutritional modes from a previously cultured phase to the following culture phase regarding the biomass production and astaxanthin accumulation. It is known that the biomass productivity can be effectively increased via fermentation process which not only increases the cell density but the growth rate through glucose fed heterotrophic cultivation (Liu et al., 2014; Zhang et al., 2017a). Therefore, combination of different traits of modes of nutrition for cell biomass and accumulation of astaxanthin can be an efficient strategy.

1.4 Optimization of biomass productivity as well as astaxanthin induction phase

The optimization of each culture phase, particularly the biomass production and product induction stages, has previously been reported. For example, *C. zofingiensis* biomass productivity has been improved for light intensity (Imaizumi et al., 2014; 2016), glucose concentration (Ip and Chen, 2005b; Wang and Peng, 2008), and nitrate concentration (Ip and Chen, 2005b; Chen et al., 2017). With respect to, astaxanthin induction, light intensity (Bar et al., 1995; Chen et al., 2017; Zhang et al., 2017a; Sun et al., 2019), nutrient concentration (Bar et al., 1995; Minyuk et al., 2020), and salinity (Pelah et al., 2004; Kou et al., 2020) have been optimized previously. However, the transitional modes of nutrition in any of the culture phases (such as stock, preculture, or biomass production) to the next induction phase has been barely discussed. Also, lesser knowledge about the cellular growth and morphological fluctuations triggered by the shifts (transition) between different modes of nutrition for cultivation of *C. zofingiensis* on a large-scale for commercial applications usage is known. The process of two-stage for the optimizing the biomass production phase and later the astaxanthin induction phase in different cultivation modes has been attempted in the previous studies (Zhang et al., 2017a; Sun et al., 2019). The cell density can be improved via fed batch fermentation via heterotrophic cultivation to attain the maximum biomass productivity, followed by induction via increased

light intensity for accumulating the astaxanthin content (1–3 mg g^{-1}) and productivity (4–9 mg L^{-1} d^{-1}) using the two-stage strategies. However, optimization of either the biomass production phase or astaxanthin induction phase was the only focus in these studies. The importance of transition of nutritional modes in different culture phases (a previous culture to the following phase) has not been explored and is required to understand the change in morphological and chemical characteristics. Therefore, the combinational effects of preculture and biomass production on astaxanthin induction phase may also convey the best nutritional modes for increasing the astaxanthin productivity. Additionally, the variation in the microalgal growth and morphological characteristics initiated by the shifts of modes of nutrition needs to be clarified.

1.5 Objectives

The aim of this Ph.D. thesis was to develop an effective three-phase cultivation (Fig. I-5) of *Chromochloris zofingiensis* for biomass and astaxanthin productivity through the transition of modes of nutrition namely, autotrophic, mixotrophic and heterotrophic modes by achieving the following objectives:

1) To enhance the growth of *C. zofingiensis* through changes in the modes of nutrition in preculture phase (Chapter II),
2) To suggest and clarify the best conditions for stock, preculture and biomass production phase (Chapter II, III),

Thereafter, based on the results obtained in Chapter II, and III, the feasibility of the suggested nutritional modes for multiple phases (such as preculture, biomass production and carotenoid induction phase) was evaluated in terms of improving the mass-cultivation of *C. zofingiensis* as a part of general discussion (Chapter IV).

Table

Table I-1. Astaxanthin production using different bioreactors in previous studies

Strain	Cultivation mode (biomass)	Reactor (Biomass)	Reactor (astaxanthin)	Astaxanthin content (mg g^{-1})	Astaxanthin productivity (mg L^{-1} d^{-1})	References
Chromochloris zofingiensis ATCC30412	M, batch	Batch	0.25L, Microplate based	6.51	3.24	Chen et al., 2017
Chromochloris zofingiensis ATCC30412	H, Two-step	Fermentation	Rotating floating bioreactor	0.7	5.2	Zhang et al., 2017a
Chromochloris zofingiensis ATCC30412	Two-stage (s)	Fermentation	Thin column reactor	2.69	9.9	Sun et al., 2019
Hamaetococcus pluvialis NIES-114	H, Fed-batch	Max blend fermentor	Glass vessel	16	4.4	Hata et al., 2001
Hamaetococcus pluvialis 712	M, Fed-batch	Fed-batch	5L photobioreactor	49.09	10.20	Sun et al., 2015
Xanthophyllomyces dendrorhous, mutant	H	-	2L glass fermenter, grape juice	1.3	-	Meyer et al., 1994
Xanthophyllomyces dendrorhous, mutant	H	-	0.25L, Raw coconut milk	1.8	-	Domíngue et al., 2004

Notes: M-Mixotrophic culture, H-Heterotrophic culture
Two-step and Two-stage (s) cultivation mode includes the biomass production phase and astaxanthin induction phase.

Figures

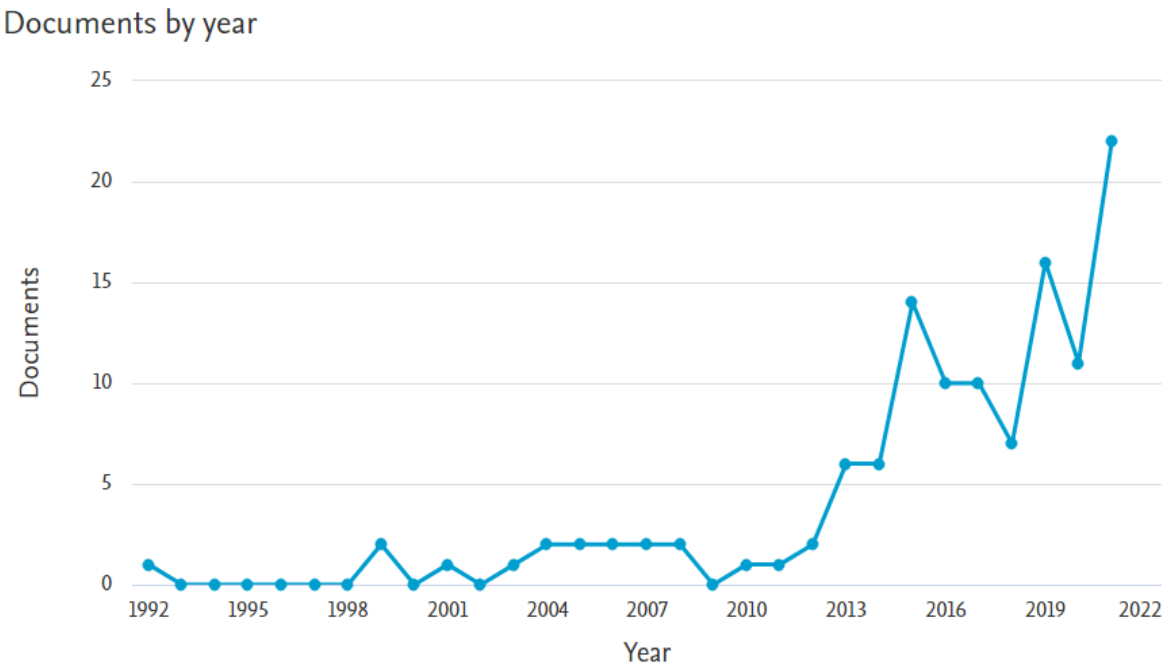


Fig. I-1. Trend of articles published for microalgal astaxanthin production by Scopus. (Scopus.com)

Fig. I-2. Global astaxanthin market
(https://www.verifiedmarketresearch.com/product/astaxanthin-market/)

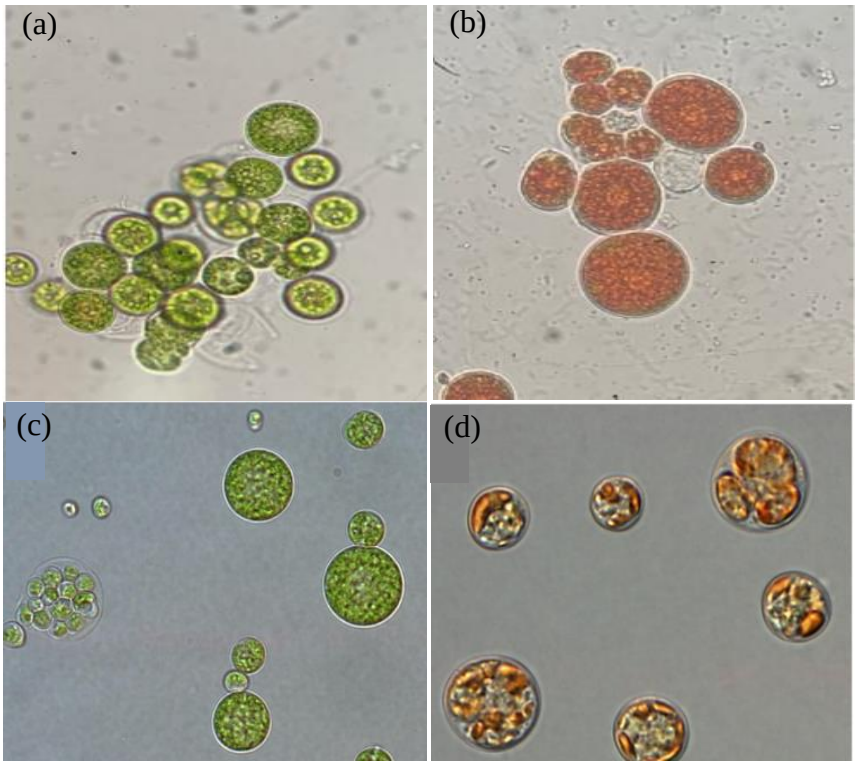


Fig. I-3. *Haematococcus pluvialis* (a) green and (b) red cells, and (c, d) *Chromochloris zofingiensis* cells accumulating astaxanthin

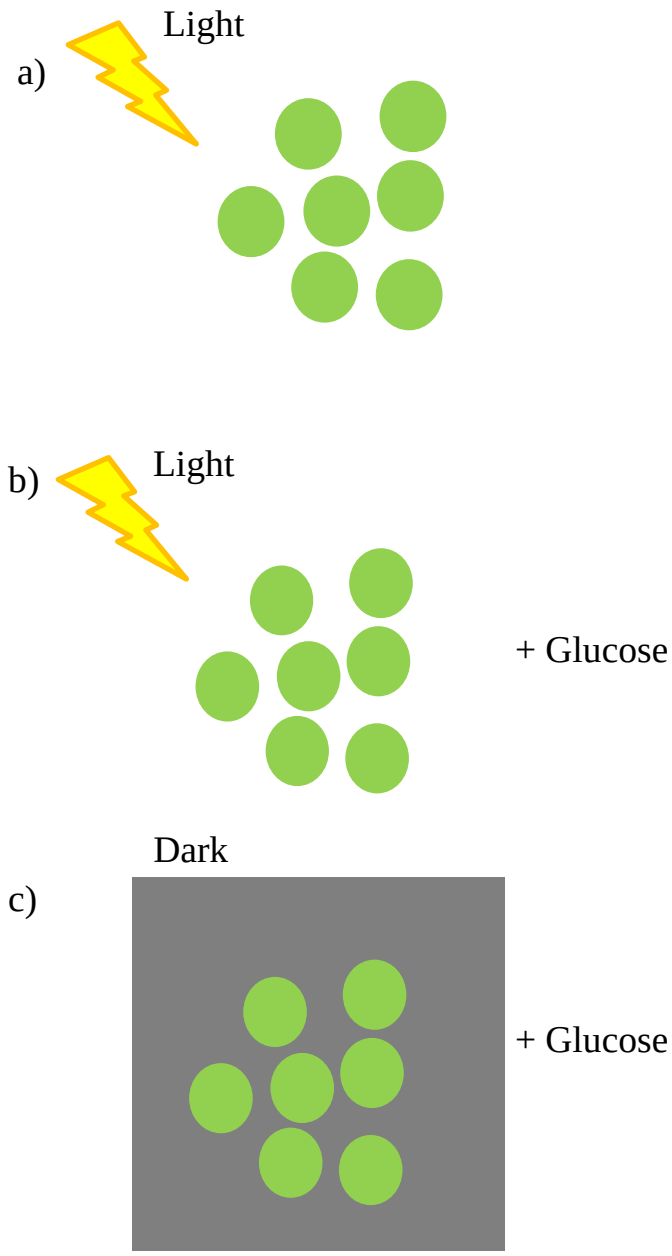


Fig. I-4. Three modes of microalgal cultivation, a) autotrophic, b) mixotrophic, and c) heterotrophic cultivation.

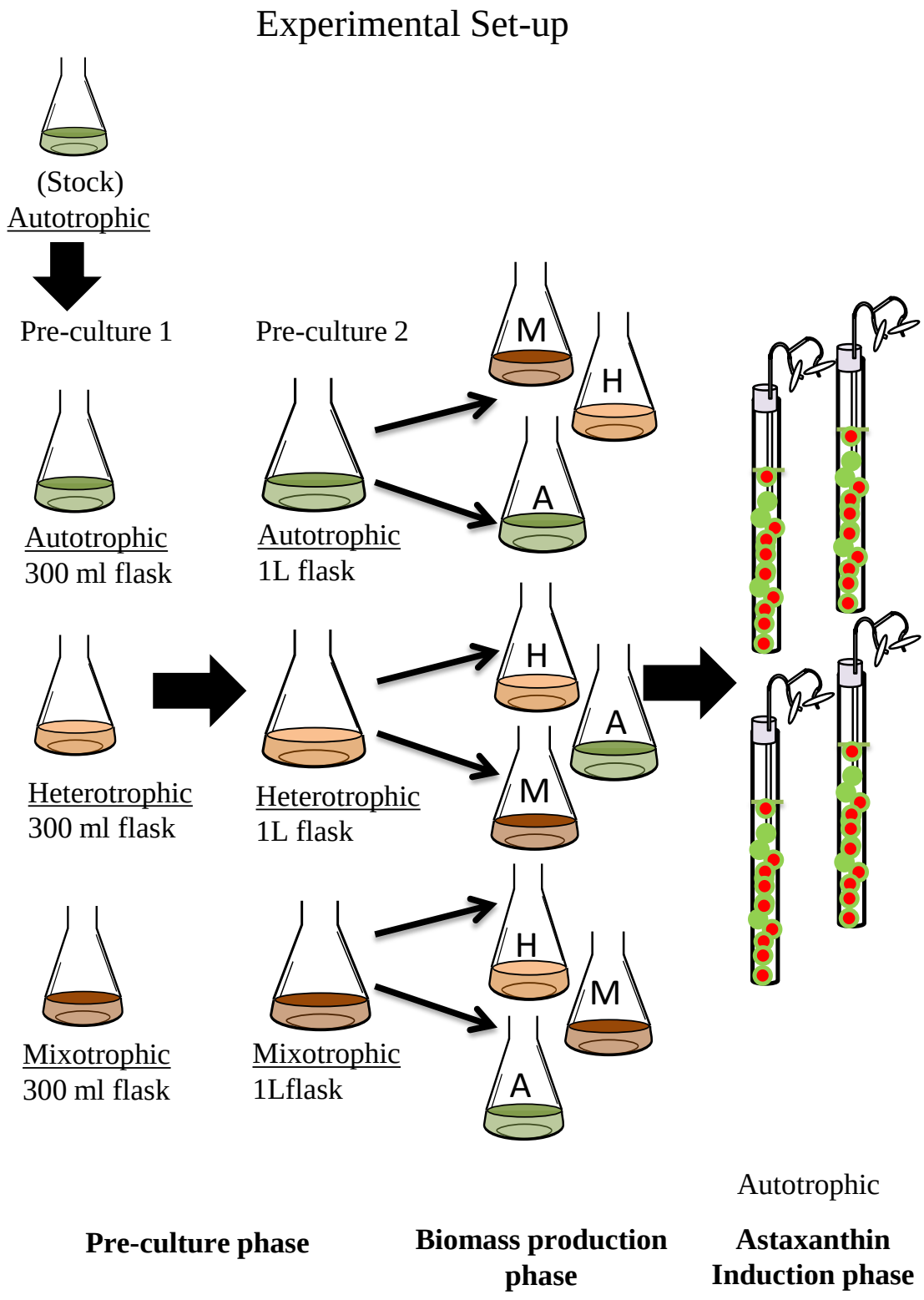


Fig. I-5. Experimental setup for the study (A- Autotrophic, M- Mixotrophic and H- Heterotrophic).

2 Chapter 2 GROWTH OF *CHROMOCHLORIS ZOFINGIENSIS* IN THREE MODES OF NUTRITION

2.1 Introduction

The ability of *Chromochloris zofingiensis* to grow in multiple modes of nutrition such as autotrophic, heterotrophic and mixotrophic has made this species an emerging model for the enhancement of the cell biomass and astaxanthin accumulation. The cultivation of *C. zofingiensis* cells in different modes of nutrition from the inoculum/stock phase directly to the biomass production phase has been known. However, the biochemical and morphological characteristics, and nuclei tend to change when cultured in different nutritional modes (Chowdhary et al., 2022; 2023). The previous studies have mostly focused on biomass production contents using different light intensities and glucose concentrations (Ip and Chen, 2005b; Imaizumi et al., 2014; Chen et al., 2017) and astaxanthin induction phase using high light and nitrogen starvation (Zhang et al., 2017a; Sun et al., 2019; Kou et al., 2020a). Therefore, the optimization of each cultivation phase (stock, preculture and biomass production) in three nutritional modes is necessary to understand the growth pattern of *C. zofingiensis* cells within the same/ transition of nutritional modes. The information regarding the changes in cell growth, morphological (cell size and shape), nuclei and intracellular characteristics induced due to each nutritional mode is minimal. Therefore, after the stock phase, a preculture phase was designed firstly to understand the effect of stock on preculture phase in different nutritional modes. From our previous knowledge, chemical compositions (Chen et al., 2015; Azaman et al., 2017) and differences in gene expressions (Zhang et al., 2017a; Ye and Huang, 2020) were observed in various nutritional modes, however, the effect of preculture in transition of different nutritional modes has never been studied.

Hence, this chapter determined to develop promising modes of nutrition for the stock and preculture phase. The growth of the cells in the preculture with different combinations of

modes of nutrition in *C. zofingiensis* were analyzed. In this chapter, we maintained a subsequence of precultures twice with the similar conditions to let the cells adapt to every nutritional mode. The cell growth, morphology, and nuclei accumulation under various modes of nutrition were investigated.

2.2 Material and Methods

2.2.1 Microalgae and cultivation modes

Chromochloris zofingiensis (Dönz) Fucíková and L.A. Lewis ATCC 30412, a green microalga was attained from American Type Culture Collection (ATCC, Rockville, USA). It was later maintained in MBM known as modified Bristol's medium (Ip and Chen, 2005b), irradiated with 60 μmol m^{-2} s^{-1} intensity at 12-h light:12-h dark cycle in a Erlenmeyer flasks (250 mL) at 25 °C.

The effect of the preculture conditions were determined by performing two sets of preculture. Therefore, for the cells to acclimatize to the similar modes of nutrition, a set of first preculture conditions (500 mL) followed by a set of second preculture conditions (1L) were performed and evaluated. The preculture conditions maintained in the nutritional modes have been mentioned in Table II-1. The cells maintained in the inoculum/stock were transferred into the first preculture in MBM with atmospheric air (aeration at 0.2 vvm) continuously for 6 days at 25 °C. The microalgal cells in the first preculture were further transferred to the second preculture for the following 6 days under the similar mode of nutrition in a series of consecutive precultures. Aseptic sampling was performed every 24 hours.

2.2.2 Analytical Parameters

The growth (optical density) of the cell culture was determined at 750 nm by using

the UV-visible spectrophotometer (UV-2450, Shimadzu, Japan). The culture sample was diluted when the optical density reached 0.4 or higher.

The cell count analyses were conducted depending on the cell density and length by using two separate types of hemacytometer, Thoma (Hirschmann, Germany) and a bacteria counter (SLGC, Koshigaya, Japan). The formalin fixed cell samples were used to measure the cell diameter (μm) by firstly getting pictures of the cells by utilizing an optical microscope (Axioskop-2 Plus Carl Zeiss, Germany) which was attached with a digital camera (EOS Kiss X7i, Canon, Japan). Then, Image J software (National Institute of Health, USA) was used for performing the analyses. The distribution of the size of the cells was examined by measuring 30 cells in all triplicates of the sample, making it to a total of 90 cells for each of the condition.

The nuclei analyses in the cell samples were conducted by staining it with a 10% working SYBR Gold Nucleic Acid Gel Stain solution (Thermo Fisher Scientific, USA; Ex/Em 495 and 300 nm/537 nm) which was diluted (400×) and kept for 15 min. Later, the pictures of the nuclei were used to count the nuclei through an attached digital camera (EOS Kiss X7i, Canon, Japan) on a fluorescent microscope (Axioskop-2 Plus Carl Zeiss, Germany).

A modified methodology was conducted for measuring the starch content (Brányiková et al., 2011). Using an ultrasonic homogenizer (UH-50, SMT, Japan), the harvested cells on GF/F filter were directly blended in 4 ml of 80% ethanol. After being scraped off the filter, the microalgal cells were placed in a 15-ml tube and centrifuged for 10 minutes at 4,000 rpm (revolutions per minute). The pigments were separated three times in ethanol (80%) for 15 minutes at 68°C and then centrifugated for 5 minutes at 2,500 rpm. The remaining pellets without the supernatant were used for the next step. The hydrolysis of the starch was done by addition of 30% of the perchloric acid to the pellets, and the suspension was stirred at 25°C for 15 min to get the supernatant. This process was carried out 3 times. The supernatants then were mixed to make a total volume of 10 ml. Then, for 10 minutes, 5 ml of 98% weight concentrated

sulphuric acid and 1 ml of 6%, w/v phenol were reacted with aliquot part of 2 ml of a starch solution that was solubilized at room temperature. The absorbance at 490 nm was measured with a spectrophotometer (HACH) and measured by comparing it to a standard calibration curve by using glucose and later the measured glucose values were multiplied by 0.9. Then, a standard curve for determining starch was obtained (Brányiková et al., 2011).

The analysis for measuring lipids in the cell was performed by a method known as the sulfo-phospho-vanillin method (Mishra et al., 2014). A phosphovanillin reagent was prepared by dissolving 0.6 g vanillin in 10 ml absolute ethanol and 90 ml deionized water and continuous stirring. Then, to the mixture phosphoric acid which was concentrated (900 ml) was added and later kept in darkness till consumed. The reagent's high activity was ensured by producing it fresh soon before each trial run. The filtered cells were mixed with 2 ml of concentrated sulphuric acid before heating it at 100°C for 10 minutes and ice bath was used to cool it for 5 minutes. Then, 1 ml supernatant was transported to a glass tube (vial) after centrifuging microalgal cells at 4,000 rpm for 5 min. The vial was then filled with 2.5 ml of sulfo-phospho-vanillin and incubated at 37°C for 15 minutes. Finally, the spectrophotometer (HACH) was used for reading the absorbance at 530 nm, and a standard of 200 mg processed canola oil in 100 mL chloroform was utilized to quantify the samples with a comparison to the standard curve.

The data were expressed as mean ± standard deviation (SD) and each of the measurements were performed in triplicates. Tukey-Kramer multiple comparison tests at $P < 0.05$ were used to determine the significance of the results, followed by a one-way analysis of the variance.

2.3 Results

2.3.1 Growth of *Chromochloris zofingiensis* among different precultural modes of nutrition

A variance in the growth curves were obtained in the subsequent precultures to the same mode (autotrophic, mixotrophic, or heterotrophic) (Fig. II-1). The preculture in the mixotrophic mode showed an increase in the growth than the precultures in the autotrophic and heterotrophic mode when compared to the optical density curves of the 1st precultures. The specific growth rates reached 0.867 ± 0.067, 0.586 ± 0.069, and 0.387 ± 0.040 d^{-1} in the 1st precultures i.e., mixotrophic, autotrophic, and heterotrophic, respectively.

When the cells were cultivated in two sequential precultures (first and second), a comparable growth of cells was noticed in the autotrophic and mixotrophic modes (Fig. II-1a, b). But the growth rate of the second heterotrophic preculture (0.239 ± 0.029 d^{-1}) was slower than the first heterotrophic preculture (0.387 ± 0.040 d^{-1}).

2.3.2 Biological characteristics

2.3.2.1 Cell size and intracellular compounds

The cells cultured in different preculture nutritional conditions showed variations in their cell characteristics. The continuous cultivation of *C. zofingiensis* in autotrophic mode in the two precultures led the cells maintain their size between 5-6 µm (Fig. II-2a). The cells precultured in mixotrophic condition remained between 7-9 µm on the day 6 of the preculture period (Fig. II-2b). On the other hand, the heterotrophic cells maintained their size around 15 µm.

The differences in cell size in different nutritional modes also lead to the change in the cellular characteristics, like accumulation of intracellular compounds. The starch accumulation

in autotrophic, mixotrophic, and heterotrophic was 0.032, 0.077, and 0.045 g g^{-1} respectively. In terms of lipid accumulation, 3.12 and 3.89 % (w/w) were observed in autotrophic and mixotrophic mode, respectively. However, the heterotrophic cells accumulated about 6.53 % lipid.

2.3.2.2 Analysis of nuclei

In this study, not only accumulation of starch and lipids was observed but also different nutritional modes showed differences in number of nuclei per cell (Fig. II-3). In autotrophic nutritional mode, an average of 1.29 nuclei $cell^{-1}$ was observed on the last day of preculture 2. However, mixotrophic cells showed the average of 3.17 nuclei $cell^{-1}$. The heterotrophic cells, on the other hand, were not only larger in size but also accumulated about average of 13.48 nuclei $cell^{-1}$ (Fig. II-4).

2.4 Discussions

In the first and second precultures, the precultured cells in the autotrophic and mixotrophic condition showed same growth patterns which suggested that the cells may have acclimated to the similar nutrition conditions. The mixotrophic cultivation showed a high specific growth rate probably because the coordination of both photosynthesis and carbon uptake was synergistic. In a previous study it was demonstrated that microalgae may fix inorganic carbon through photosynthesis (called as autotrophy) and synchronous carbon assimilation (called as heterotrophy) in the mixotrophic cultivation (Zhang et al., 2021b). Therefore, the mixotrophic cultivation possibly increased the conversion of the energy up to twice due to presence of light and glucose.

The autotrophic cultivation, though, was solely depending on illumination for the

conversion of the energy in this study. Also, the reduced CO_2 may also have been a reason for a decreased growth in autotrophic culture. The minimal growth and nuclei number in autotrophic culture can also be elucidated by the *C. zofingiensis* cell properties for the division. A recent study found that in a *C. zofingiensis* culture grown on a 14h-L/10h-D cycle with a 800 μmol m^{-2} s^{-1} intensity of high light, most of the nuclear and division of the cells took place in the darkness (Koren et al., 2021). The reason that the autotrophic and mixotrophic preculture showed a decrease in the rate of growth and nuclei number, which was irradiated with light continuously at 260 μmol m^{-2} s^{-1} intensity, which shows that for continuing nuclear and cell division, the dark cultures are required for the autotrophic *C. zofingiensis*. It was also reported that the growth of *C. zofingiensis* reduced to about 80% when cultured at a high- light with intensity 300 μmol m^{-2} s^{-1} in comparison to the one cultured at a weak-intensity of light continuously (50 μmol photons m^{-2} s^{-1}) (Sun et al., 2020).

However, the heterotrophic culture did not exhibit the same specific growth rate as high as the mixotrophic cultivation, which could be attributed to the use of glucose as the sole carbon source for growth and lack of light. Although the growth rate in the second preculture of the heterotrophic culture was reduced by nearly 40%, the cells remained intact. The reduced cell division was due to the reduced growth rate in the second heterotrophic preculture.

The two major intracellular substances that provide cell energy for the development and cell cycle events are starch and lipids (DNA replication and nuclear/cellular division) (Vítová et al., 2014). For example, *Chlorella* and *Parachlorella* which are known to overproduce starch (Brányiková et al., 2011; Vítová et al., 2014), whereas some types of microalgae like *Chlamydomonas reinhardtii* produce lipid for storage (Wang et al., 2009; Goodenough et al., 2014; Vítová et al., 2014). The digestible starch (ds; up to 60%) and 45% ds from lipid and starch, respectively, can be accumulated by *Chlorella* based on the strains and cultivation conditions (Vítová et al., 2014; Chen et al., 2015). The different cell sizes in

the nutritional modes in this study (Fig. II-2) could be explained by the accumulation of lipids and starch in different nutritional modes. The accumulation of nuclei and intracellular compounds would be explained in detail in Chapter III.

2.5 Conclusion

The present work demonstrated that *C. zofingiensis* in different nutritional modes showed changes in growth rate, cell morphology and accumulation of nuclei. It was also revealed that culturing the cells in heterotrophic cultivation twice can reduce the growth rate of *C. zofingiensis.* The autotrophic as a stock culture was optimized for the different preculture conditions.

Table

Table II-1. Conditions for the preculture and biomass phase.

Conditions	Light intensity ($\mu mol\ m^{-2}\ s^{-1}$)	Glucose ($g\ L^{-1}$)	Temperature (°C)	Aeration rate (vvm)	Agitation (rpm)
Preculture					
Autotrophic	260	0	25	0.2	0
Mixotrophic	260	10	25	0.2	0
Heterotrophic	Dark	10	25	0.2	0
Biomass phase					
Autotrophic	260	0	25	0.2	260
Mixotrophic	260	10	25	0.2	260
Heterotrophic	Dark	10	25	0.2	260

Figures

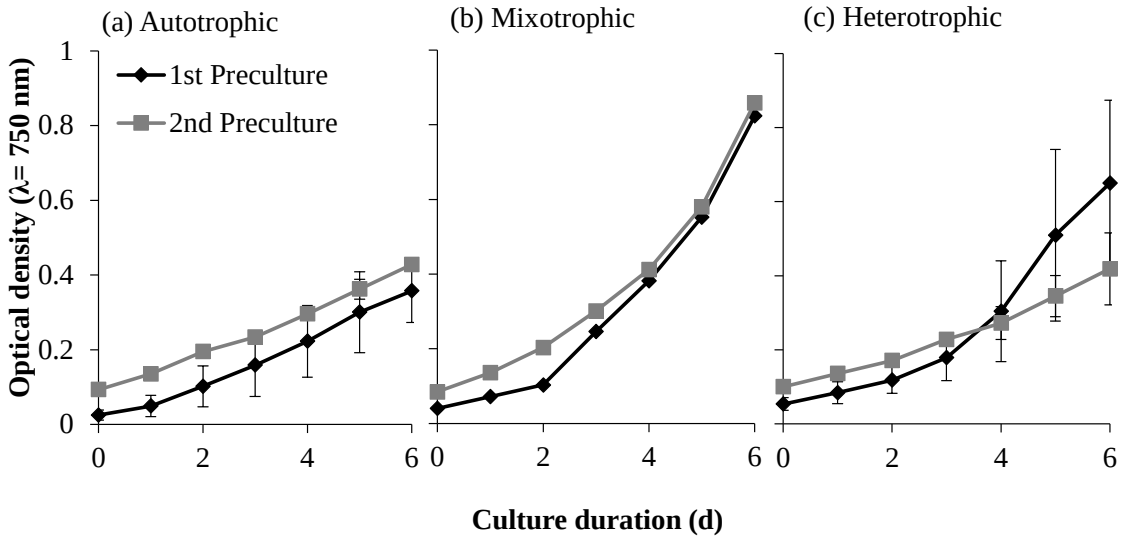


Fig. II-1. Growth curve of *Chromochloris zofingiensis* in three nutritional modes where 1st preculture was followed by 2nd preculture, (a) autotrophic, (b) mixotrophic, (c) heterotrophic culture. Data are expressed as the mean ± standard deviation, $n = 3$

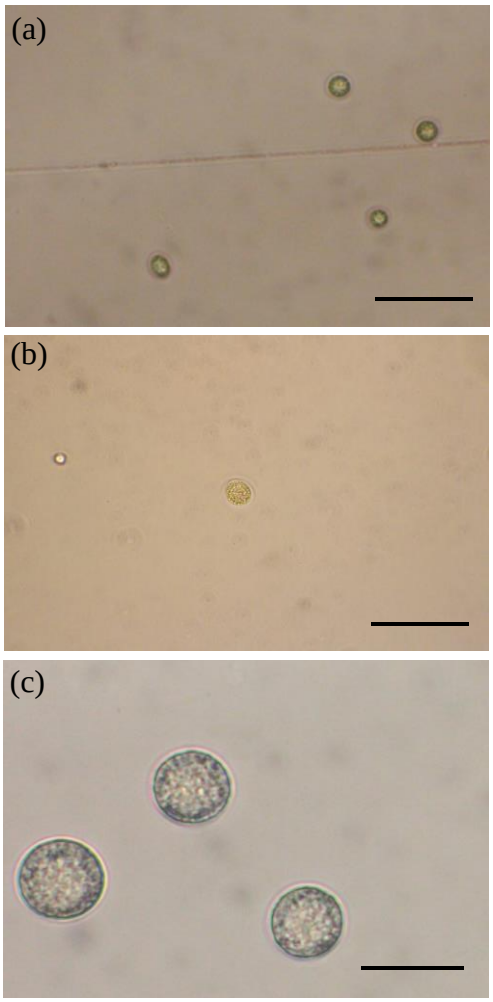


Fig. II-2. Cell pictures of *Chromochloris zofingiensis* in preculture, (a) auto-, (b) mixo-, and (c) heterotrophic on day 6. *Scale bar 20*μm

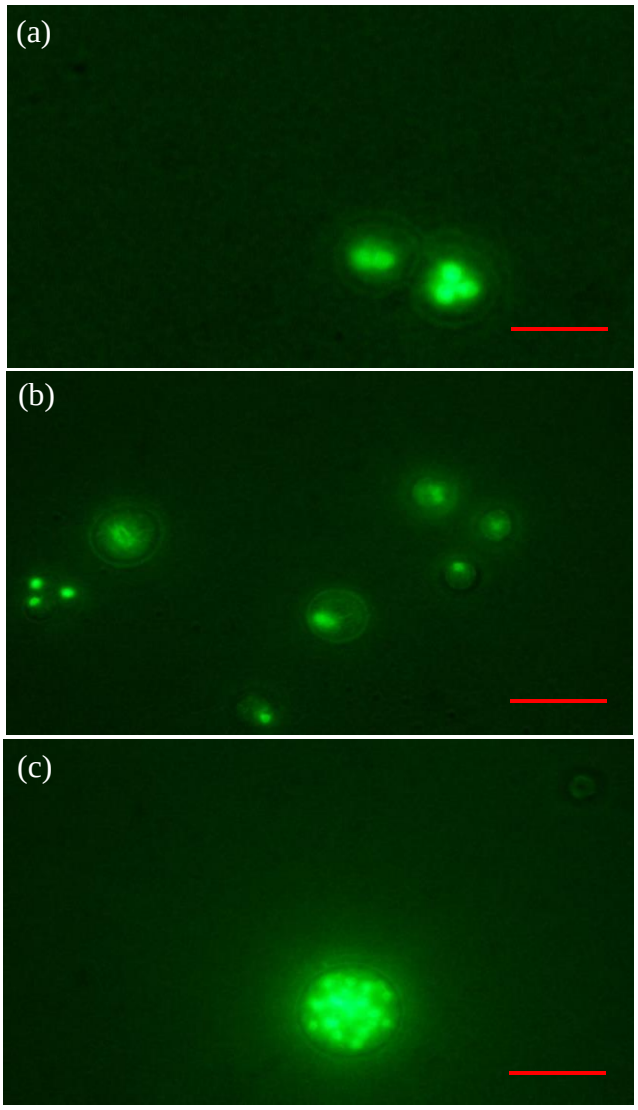


Fig. II-3. Nuclei pictures of *Chromochloris zofingiensis* in preculture, (a) auto-, (b) mixo-, and (c) heterotrophic on day 6. *Scale bar 20*μm

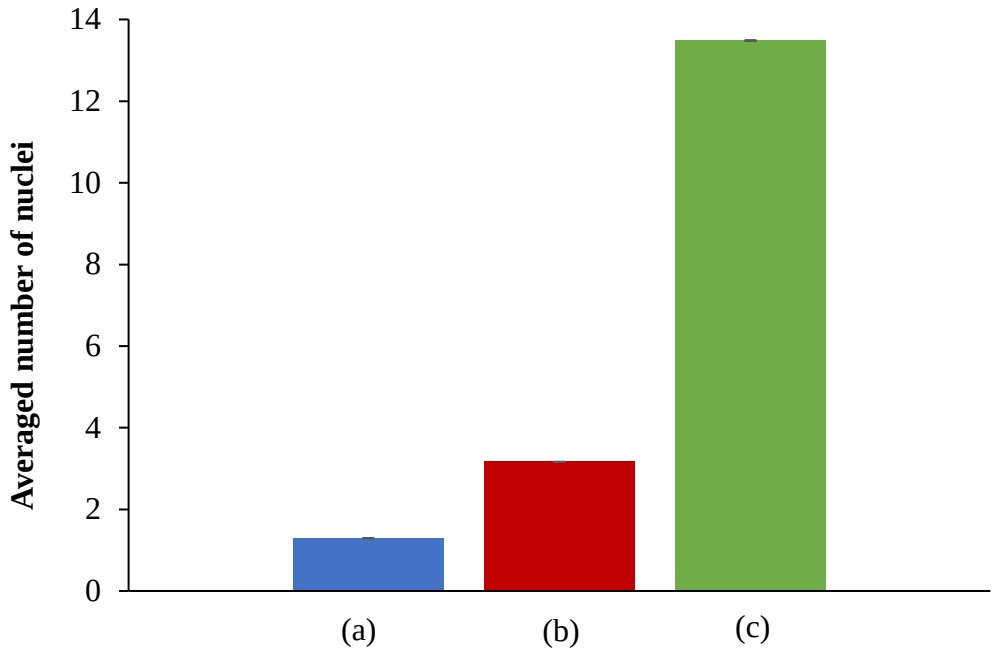


Fig. II-4. Averaged number of nuclei of *Chromochloris zofingiensis* in preculture, (a) auto-, (b) mixo-, and (c) heterotrophic on day 6.

3 Chapter 3 EFFECT OF TRANSITION OF NUTRITIONAL MODES ON CELL GROWTH AND BIOMASS PRODUCTIVITY OF *CHROMOCHLORIS ZOFINGIENSIS*

3.1 Introduction

In Chapter II, the effect of stock on the two set of precultures in different nutritional modes was evaluated. The growth of *Chromochloris zofingiensis* cells of autotrophic and mixotrophic in the same preculture modes was about the same. However, an increase in the growth rate in the preculture 1 (heterotrophic) was observed when transferred from stock (autotrophic) but a reduced growth rate was seen when precultured heterotrophic cells were cultured to the same preculture 2 (heterotrophic) mode.

The next step in this PhD study was to understand the impact of preculture on the biomass production phase. The previous research articles have mentioned about the biomass production of *C. zofingiensis* in every continuous mode of nutrition. For instance one study revealed that nutritional modes tend to affect not only the biomass productivity but also the cell morphology and chemical compositions (Azaman et al., 2017). Few other studies particularly focused on improving the biomass productivities, such as, in the heterotrophic cultivation mode, previous studies focused on using different concentrations of glucose (Ip and Chen, 2005a; Sun et al., 2008; Zhang et al., 2016), in autotrophic mode, using different salinity concentrations and light intensities (Orosa et al., 2001), and different acetate, glycerol, light and glucose concentrations in mixotrophic mode (Ip and Chen, 2005b; Wang et al., 2013; Rahimi and Jazini, 2021). However, the transitional effect of different nutritional modes from an earlier culture phase (stock, preculture) to a following phase (biomass productivity) is not known as mentioned before. The growth pattern of *C. zofingiensis* cells in transition from one nutritional

cultivation phase to another is needed to understand the physiology and adaptation of the cells in different nutritional modes.

In addition to physiological characteristics of *C. zofingiensis*, multinucleation and intracellular energy storage may contribute to enhance the growth in *C. zofingiensis*. Some green algae such as *Chlamydomonas, Chlorella,* and *Scenedesmus* commonly show multinucleation (Spudich and Sager, 1980; Bišová and Zachleder, 2014; Roth et al., 2017; Koren et al., 2021; Zachleder et al., 2021). It was also mentioned that *Chromochloris zofingiensis* cultivated in autotrophic mode at the high light intensity of 800 µmol photons/m^2/s^1 (14-h L/10-h D) also showed multinucleation and multiple fission (Koren et al., 2021). The main reproduction strategy in microalgae such as *Chlorella, Chlamydomonas, Desmodesmus, Scenedesmus* and *Chromochloris* is multiple-fission cell-cycle. *C. zofingiensis* is the mostly investigated species in regards to the biotechnological applications with the production of pigments and oil (Koren et al., 2021). Therefore, *C. zofingiensis* fast cell division mechanisms needs to be understood with an evaluation based on the cell cycle (Spudich and Sager, 1980). *Chromochloris zofingiensis* nuclear dynamics during the transition of modes of nutrition needs to be analyzed which may reveal the causes and effect for nuclear divisions and cytokinesis with the process of increased division of cells.

Additionally, the enhancement of the growth in *C. zofingiensis* might be explained by the energy storage intracellular compounds. It was seen in the previous study that mixotrophic culture of *C. zofingiensis* showed accumulation of starch and lipids and their usage for the division of cells (Chen et al., 2015). Thus, the enhancement of the cell growth via degradation of starch or lipids is highly likely. Thus, to clarify the processes of environmentally triggered prompt growth in *C. zofingiensis*, the growth of the cells requires to be examined with the nuclei number and the intracellular accumulated components. Furthermore, the reason whether the multinucleation triggers will help to promote the mass-cultivation of *C. zofingiensis* needs

to be clarified.

Thus, the aim of this PhD study was to propose the potential modes of nutrition for stock, preculture (Chapter II) and biomass production phase (Chapter III). The growth of the cells, morphology, nuclei number, storage of intracellular components, and the division of cell pattern of *C. zofingiensis* by a combination of the nutritional modes was evaluated to recognize the growth mechanism. Additionally, the clarification of the multinucleation also might enhance the astaxanthin productivity with the suggested nutritional modes.

3.2 Materials and methods

3.2.1 Culture conditions

In this study, the effects of the preculture conditions on the biomass production phase was evaluated. The two sequential precultures as described in Chapter II were conducted for the cells to adapt to the similar nutritional modes. The precultured cells in three modes of nutrition were further cultured to another phase known as biomass production phase under the autotrophic, mixotrophic and heterotrophic cultivation modes.

3.2.1.1 Biomass production phase

The microalgal cells in the second preculture with an optical density of 0.1 were collected, washed, and resuspended in the batch phases of the 3 modes of nutrition (autotrophic, heterotrophic, and mixotrophic) for the biomass production phase (1L medium bottles) in MBM with continuous aeration of 0.2 vvm and stirring at 260 rpm till the stationary phase was reached. The autotrophic and mixotrophic condition were irradiated with continuous light (24 h), and lastly the heterotrophic culture was kept totally dark. The concentrations of glucose and the light intensity are described in Table II-1. The experiments were performed in triplicates

with aseptic sampling every 24 h.

3.2.2 Analysis

The dry cell weight measurement was conducted by filtration of the cell culture by usage of a glass fiber filter (pore size of 0.7 µm) which was pre-weighed (GF/F, Whatman, USA). The dry cell weight was measured by washing the cells 3 times with the distilled water and drying for 8 h at 80 °C and later by using weighing scale (LIBBOR AEG-220, Shimadzu, Japan).

The algal filtrate samples for the concentration of the nutrients (NO_3-N and PO_4-P) analysis were stored at −20°C and were analyzed using a nutrient analyzer which was automatic (FIAlyzer-1000, FIAlab, USA).

The length of cell and the density were determined by a previously described method in section 2.2.2.

The nuclei staining of the cell samples was conducted with similar methodology as mentioned in section 2.2.2.

Lipid and starch content was analyzed by method previously discussed (cf. Section 2.2.2.). The data are expressed as mean ± standard deviation (SD) with the measurement carried out in triplicate.

The pigment compositions were determined by filtered microalgal cells, washing them several times with distilled water, and then extracting them in 5 ml of DMF (N,N-dimethylformamide) and keeping them overnight at −20°C (Furuya et al., 1998). Prior to the analysis, 0.22-µm PTFE filter (Millex-FG, Merck, Japan) was used to filter the extracted samples. The carotenoids in the samples were identified and quantified via ACQUITY UPLC H-Class LCMS system (Waters, USA). 100% acetonitrile (Solvent A), 100% 2-propanol

(solvent B), and 10% methanol (solvent C) were utilized for the mobile phase. 50% of the Solvent A, 40% of solvent B, and 10% of solvent C was reached at the start of the measurement which was followed by a linear gradient to 2% of solvent A, 40% of solvent B, and 58% of solvent C. After 12 minutes, the mixture was switched to solvent A 50%. The authentic pigments standards; astaxanthin, β-carotene, canthaxanthin, lutein, zeaxanthin, violaxanthin, and chlorophyll *a* were bought from Danish Hydraulic Institute (Hørsholm, Denmark) and evaluated using a reverse phase ACQUITY UPLC BEH C18, 1.7µm; 2.1 × 50 mm column (Waters, USA). The identification and quantification of chromatographic peaks of the samples was performed by contrasting the absorbance and mass spectrum to the standards.

3.2.3 Calculations

The calculations for the total nuclei number (N; nuclei/mL) was done using equation (1):

$$\mathrm{N} = \sum_n \left(n \times C \times \frac{\%\ \mathrm{distribution}_n}{100} \right), \quad (1)$$

where C is the cell density of the culture (cells/mL), *n* is the nuclei number in each cells (nuclei/cell), and % distribution$_n$ is the fraction of cells that had the respective nuclei number.

The calculations of the specific growth rate of the cell number μ_c (d^{-1}) was as per equation (2):

$$\mu_c = \frac{lnC_2 - lnC_1}{d_2 - d_1}, \quad (2)$$

where C_1, C_2 and d_1, d_2 are the number of cells at days, respectively (Chen et al., 2017).

The calculations for the specific growth rate of nuclei per cell, μ_n (d^{-1}), was as per equation (3):

$$\mu_n = \frac{lnN_2 - lnN_1}{d_2 - d_1}, \quad (3)$$

where N_1, N_2 and d_1, d_2 are the total nuclei number and days, respectively.

3.3 Results

3.3.1 The growth comparison among different modes of nutrition

The growth rate (d^{-1}) and productivity of cell biomass (g L^{-1} d^{-1}) in the biomass production phase was similar to that of the preculture phase in mixotrophic, autotrophic, and heterotrophic conditions. In comparison, the averaged maximum specific growth rate in the mixotrophic nutritional mode had (0.868 ± 0.281 d^{-1}) was higher than the autotrophic (0.578 ± 0.306 d^{-1}) and heterotrophic (0.447 ± 0.070 d^{-1}) culture (Fig. III-1). In the same way, in biomass production phase, the maximum production was greater in the mixotrophic (1.28 ± 0.033 g L^{-1} d^{-1}) phase than in the heterotrophic (0.448 ± 0.363 g L^{-1} d^{-1}) and autotrophic (0.065 ± 0.053 g L^{-1} d^{-1}) phase (Fig. III-5). The size of the cells in the heterotrophic biomass production phase (13.37 ± 1.19 μm) was substantially larger (n = 9, p < 0.05) than the ones cultured in the mixotrophic (9.21 ± 1.02 μm), and autotrophic (5.72 ± 1.25 μm) (Fig. III-2, 3, 4).

3.3.2 Effect of preculture modes of nutrition on the biomass phase

The influence of modes of nutrition in preculture on the biomass production varied depending on whether the conditions were in irradiation (auto- and mixotrophic) or dark (heterotrophic).

3.3.2.1. Light phase

Specific growth rate in the heterotrophically precultured cells reached 0.931 ± 0.033 d^{-1} and 1.19 ± 0.044 d^{-1} in the phase with irradiation (autotrophic and mixotrophic condition) respectively (Fig. III-1a, b). In the biomass production phase, the productivity for autotrophic reached highest in the heterotrophically (0.122 ± 0.140 g L^{-1} d^{-1}) than autotrophically and mixotrophically precultured cells (0.017 ± 0.006 and 0.057 ± 0.024 g L^{-1} d^{-1}, respectively) (Fig. III-5a). The cell density of heterotrophic preculture showed a rapid increase from 4.21 × 10^6 on day 4 to 2.04 × 10^7 cells mL^{-1} on day 10 when cultivated in the light phase (autotrophic biomass production phase) (Fig. III-6a). Furthermore, the cell density of the heterotrophic preculture kept increasing and reached the highest 5.81 × 10^7 cells mL^{-1} at the end of the cultivation (Fig. III-6a). In mixotrophic biomass production phase, on the other hand, the biomass productivity was almost similar in auto-, mixo-, and heterotrophically precultured cells (1.32 ± 0.368, 1.26 ± 0.502, and 1.27 ± 0.632 g L^{-1} d^{-1}, respectively, Fig. III-5b). The cell density reached about the same on day 10 in all the three precultured modes (Fig. III-6b).

3.3.2.2. Dark phase

The cells precultured heterotrophically depicted a decreased growth rate when cultivated in the dark heterotrophic phase for biomass productivity. As opposed to the light phase, cells precultured heterotrophically showed lesser growth and biomass production in the heterotrophic biomass phase (0.364 ± 0.008 d^{-1}, 0.034 ± 0.077 g L^{-1} d^{-1} respectively, Fig. III-1c, 5c). However, greater specific growth rate (0.504 ± 0.022 and 0.437 ± 0.015 d^{-1}, respectively; Fig.III-1c) and biomass productivity (0.714 ± 0.284 and 0.596 ± 0.108 g L^{-1} d^{-1}, respectively; Fig. III-5c) was seen in the cells precultured autotrophically and mixotrophically. The heterotrophically precultured cells reached 1.40 × 10^7 cells mL^{-1} (Fig. III-6c).

3.3.3 Pigment content

The autotrophic biomass production phase showed significantly ($n = 3, p < 0.05$) higher chlorophyll *a* content (7.54 mg g^{-1}) as compared with the mixotrophic (1.84 mg g^{-1}) and heterotrophic (1.25 mg g^{-1}) biomass production phases (Fig. III-8). The autotrophic biomass production phase showed significantly higher total carotenoids ($n = 3$, $p < 0.05$) than in the mixotrophic and heterotrophic biomass production phase in this study as well (Fig. III-7). The cells precultured heterotrophically not only indicted the highest specific growth rate and biomass production when cultured in light, the astaxanthin content in the autotrophic biomass phase was also higher (0.64 ± 0.005 mg g^{-1}) than autotrophically and mixotrophically precultured cells (0.27 ± 0.068, 0.42 ± 0.117 mg g^{-1} respectively; Fig. III-7).

3.4 Discussions

3.4.1 Cell growth, characteristics, pigments, and biomass production in the light phase

3.4.1.1. Mixotrophic mode for high biomass production

The mixotrophic productivity in the (highest; 1.28 ± 0.033 g L^{-1} d^{-1}) biomass production phase, was higher on average than in the heterotrophic (0.448 ± 0.363 g L^{-1} d^{-1}) and autotrophic (0.065 ± 0.053 g L^{-1} d^{-1}) production phases (Fig. III-5). As seen in earlier researches that *C. zofingiensis* can grow while utilizing both carbon and light (Zhang et al., 2017b; 2021b).

The growth enhancement in *Chromochloris zofingiensis* in a mixotrophic condition was recently discussed with a detailed mechanism (Zhang et al., 2021b). Furthermore, to explain the increased productivity, a collaborative relation between chloroplast (photosynthesis), cytosol, and mitochondria (glucose metabolism) was proposed (Zhang et al., 2021b). The ability to collaborate across cytosol is provided by chloroplast and mitochondria

which have transporters of carbon and energy on their membranes such as triose phosphate or phosphate translocators, glucose or phosphate transporters, and ATP or ADP carriers (Haferkamp et al., 2002; Flü et al., 2011; Dyo and Purton, 2018; Zhang et al., 2021b). The upregulation the genes involved in the transportation of ATP between cytosol and chloroplast (CAAC; chloroplast ATP/ADP carrier) as well as mitochondria and cytosol (NTT; nucleotide translocator) suggested the ATP transport from mitochondria to chloroplast (Zhang et al., 2021b). The ATP and organic compounds supply reduces the need for CO_2 fixation by RuBisCO (rate-limiting phase of photosynthesis). The NPQ (nonphotochemical quenching) and photorespiration—processes in which energy of light is lost in the form heat and chemical—are further reduced as a result of the improved ATP consumption for metabolism of organic carbon in chloroplast, which improves the efficiency of energy utilization for growth of the cell. The NO_3-N in mixotrophic cultivation in this study also got depleted around day 6 of the cultivation period (Fig. III-9). Thus, an elevation in the biomass production of *Chromochloris zofingiensis* cultivated in the mixotrophic cultivation was possibly because of the synergistic coordination between carbon assimilation and photosynthesis.

However, reduction in the activity of the photosynthesis in mixotrophic condition with organic carbon addition was reported in a previous study. For example, irradiated *Chlamydomonas reinhardtii* culture was added with acetate and a significant reduction in the assimilation of inorganic carbon and oxygen evolution was observed (Heifetz et al., 2000). In this PhD thesis, the biomass production of the mixotrophic phase revealed a similar lower chlorophyll *a* content (Fig. III-8), which possibly reduced the activity of photosynthesis. Despite the reduced photosynthetic activity, the total biomass output of mixotrophic cultures was higher than the autotrophic and heterotrophic cultures, due to both organic carbon uptake and the previously noted coordination between chloroplast and mitochondria synergistically.

3.4.1.2. Effect of dark precultured cells in light phase

In this study, it was discovered that the cells precultured heterotrophically had greater specific growth rates and biomass productivities in the irradiation (autotrophic and mixotrophic) biomass production phases than the cells precultured autotrophically and mixotrophically (Fig. III-1,5). In the autotrophic biomass production phase, the maximal specific growth rate in cells precultured heterotrophically was approximately a 2.2 to 2.4-times greater than in cells precultured auto- and mixotrophically ($n = 3$, $p < 0.05$). Correspondingly, in mixotrophic biomass production phase, the cells precultured heterotrophically had a 1.6 to 1.8-fold greater maximal specific growth rate than the cells precultured auto- and mixotrophically ($n = 3$, $p < 0.05$). It was interesting that in the earlier studies with high mixotrophic production, the seed preparation technique in the dark heterotrophic condition was also followed (Zhang et al., 2017b; 2021b) (Table III-1). Therefore, possible productivity enhancements can be achieved by maintaining the inoculums in dark heterotrophic conditions for the culture which is irradiated. In fact, cells precultured heterotrophically resulted in the maximum specific growth rate (1.19 ± 0.044 d^{-1}; Fig. III-1) in the mixotrophic biomass production phase. It was found out that another research adjusted the dark condition for the seed or the inoculum maintenance, and it showed almost same value (1.27 d^{-1};(Zhang et al., 2021b)) which was greater than the cells precultured mixotrophically (0.057 d^{-1};(Chen et al., 2017)) in our study. Thus, two possible mechanisms can be evaluated in the cells precultured heterotrophically for an increased growth rate in this study: (1) storage of growth-enhancing intracellular compounds and (2) synergistic cooperation of carbon uptake as well as photosynthesis.

The cells precultured heterotrophically in the autotrophic and mixotrophic biomass production phases accumulated growth-promoting compounds, which possibly increased the growth rate. There are two kinds of growth-enhancing factors that were accumulated and were

evaluated in this study, first is nuclei and second is intracellular compounds like starch and lipids. The rapid cell division in the heterotrophically precultured cells (Fig. III-6a), led to a reduced cell size and the cellular weight (Fig. III-10) likely indicating cellular changes (carbon reserves). The high specific growth rate of *C. zofingiensis* cells precultured heterotrophically might be because of the multiple fission (Fig. III-12). Larger cells having more than 6–8 nuclei giving rise to several daughter cells led to high growth rates, as mentioned in the previous studies (Bišová and Zachleder, 2014; Koren et al., 2021). However, on day 0 the change of inoculum precultured heterotrophically to autotrophic condition, a larger number of nuclei (8.07; averaged) was viewed than in the cells precultured autotrophically in our study (Fig. III-10a). The averaged nuclei number in heterotrophically precultured multinucleated cells decreased rapidly from 8.07 at the start to 1.26 on 2nd day (Fig. III-10a). The quick reduction in cell size coincided with the reasonably quick decrease in the nuclei number (Fig. III-10c). The multinucleated cells lead to a rapid division through multiple fission processes once the culture was shifted to the autotrophic condition. However, on the other hand, the cells precultured autotrophically preserved a size between 5–6 µm throughout the cultivation time. The autotrophically precultured condition showed a reasonable decrease in the growth of the cell and a smaller number of nuclei through the culture time possibly due to the characteristics of the cellular division in *Chromochloris zofingiensis*. A previous study observed that *C. zofingiensis* cells cultivated in a high light of 800 µmol m^{-2} s^{-1} intensity with a 14-h L/10-h D cycle showed divisions of nuclei and cells in the dark cultivated phase (Koren et al., 2021). The reason for cells precultured autotrophically showing decreased growth rate and a low nuclei number in autotrophic biomass production phase that was irradiated at 260 µmol m^{-2} s^{-1} intensity continuously depicts that the autotrophic cultivation of *C. zofingiensis* requires dark phases for conducting divisions of nuclei and cells.

However, the microalgal cells precultured heterotrophically showed a rise in the cell growth rate from day 0 to 3 under autotrophic culture condition, which persisted despite the nuclei decreasing in quantity, similar to cells precultured autotrophically (Fig. III-1a, 10a). Thus, it is possible that the growth improvement under the heterotrophically precultured cells in the autotrophic biomass phase did not just depend on the multiple rounds of fission. The two predominant intracellular components, starch and lipids, may also be in control of delivering energy for cell cycle events (DNA replication and nuclear/cellular division) and cell growth (Vítová et al., 2014). *Chlorella* can store as much as 60% digestible starch (ds) and 45% from lipid and starch, correspondingly, according to the type of microalgal strain and the condition of the culture (Vítová et al., 2014; Chen et al., 2015). Under the heterotrophically precultured cells in autotrophic biomass phase, it was observed that the revealed amount of cellular lipids decreased over the course of the autotrophic culture from day 1 to the last day (Fig. III-13a). Thus, the continuous growth of cells precultured heterotrophically after the third day might be due to the consumption of the stored lipids. Chen et al., (2015) reported that the energy for fueling up the process of cellular division in *C. zofingiensis* is provided by the utilization of the stored lipids conducted in the conditions fed with glucose. In contrast, increased lipid and starch during the culture cultivation of the cells precultured autotrophically was observed (Fig. III-13a, b). Although the autotrophically precultured condition showed little cell division (Fig. III-6a), the cell dry weight seemingly rose until the end of cultivation time (Fig. III-5a). As discussed above, continuous 24-h light supply under the autotrophically precultured condition may have inhibited the nuclear and cell division. Nevertheless, the intracellular components, lipid and starch, accumulated and the photosynthesis continued.

Another possible mechanism in the dark cultured cells for the high growth rate could be the synergistic coordination between the metabolism of the glucose that occurs in the chloroplast, cytosol, mitochondria, and photosynthesis. Along with the synergistic interactions

present in the mixotrophic condition, a similar cooperative shifting of the dark cultured cells to the autotrophic and mixotrophic biomass production phases may have taken place (Zhang et al., 2021b). As mentioned above, accumulation of nuclei, starch, and also lipids within the cells that were precultured in the heterotrophic cultivation. Thus, the transfer of these heterotrophically precultured cells into irradiation (autotrophic and mixotrophic) conditions may have used up the stored sources of carbon for the synergetic cooperation between the citric acid cycle, photosynthesis, and the glycolysis.

The key genes involved in the *C. zofingiensis* carotenoid biosynthesis pathway are upregulated by light, which may have contributed to the high pigment content in the autotrophic cells (Fig. III-7a) (Zhang et al., 2017a). However, the mixotrophic conditions in this investigation with light and glucose indicated a decreased carotenoid level (Fig. III-7b). It's probable that reduction in light availability and a decline in the pigment concentration were caused by the high biomass during the mixotrophic biomass production phase. In this study, it was observed that comparable pigment contents existed in the autotrophic biomass production phase, even though the earlier heterotrophic and mixotrophic preculture stages had a low carotenoid content (Fig. III-7a). This indicates that high carotenoid production in cells cultured in autotrophic mode is not the exclusive choice. Thus, it can be suggested that the carotenoid concentration in the later phase is not significantly impacted by the previous culture's nutritional phase.

In this study, the impact of the precultural modes of nutrition on the subsequent phase made it evident how crucial optimization is to the improve the mass cultivation of *C. zofingiensis*. It was indicated that *C. zofingiensis* cells can store growth enhancing components, nuclei and intracellular compounds in heterotrophic preculture, which results to maximum biomass yields and growth when cultivated in the irradiation conditions (mixotrophic and autotrophic). Therefore, it is suggested that mixotrophic cultivation in the latter phase and

heterotrophic nutritional mode in the former phase are both viable options for boosting biomass productivity in *C. zofingiensis*.

3.4.2 Biomass production in the dark heterotrophic phase

3.4.2.1. *Chromochloris zofingiensis* cultivation in the dark heterotrophic mode

A reduction in the specific growth rate (0.239 ± 0.029 d^{-1}) in the second preculture (heterotrophic mode) (Fig. II-1) was observed when the cells were transferred to the first preculture (heterotrophic mode) from the stock culture, 0.387 ± 0.040 d^{-1}. However, in the biomass production phase that was heterotrophic, the cells precultured auto- and mixotrophically depicted higher rates of growth (0.504 ± 0.022 and 0.437 ± 0.015 d^{-1}, respectively) and higher biomass production (0.714 ± 0.284 and 0.596 ± 0.108 g L^{-1} d^{-1}, respectively) than in the cells precultured heterotrophically (0.364 ± 0.008 d^{-1}, 0.034 ± 0.062 g L^{-1} d^{-1}) (Fig. III-1c, 5c). A previous study reported highest productivity in heterotrophic cultivation was 7.03 g L^{-1} d^{-1} (Zhang et al., 2017a) which is much higher than the maximum biomass production in heterotrophic mode in this study, 0.448 ± 0.363 g L^{-1} d^{-1}. *Chromochloris zofingiensis* displayed significant variations in biomass productivities, which may be related to the different feeding conditions used in this study and the previous study, which used a fed batch fermentation instead of a one-time batch cultivation. As this study showed maximum biomass production in the mixotrophic condition than in the heterotrophic one, a fed-batch mixotrophic culture may be used to obtain a higher or equivalent biomass productivity with heterotrophic cultivation 7.03 g L^{-1} d^{-1}. The mixotrophic condition with fed-batch mixotrophic cultivation is yet preferable to the dark heterotrophic cultivation in the high cell density culture when irradiation is constrained, thus, high cell biomass is required to verify this.

3.4.2.2. Multinucleation and intracellular compounds accumulation in the dark mode

This study showed that the multinucleated cells precultured heterotrophically increased the growth rate and led to rapid cell division via multiple fission. It was concluded the dark cultivations triggers multinucleation since our study observed only the cells precultured heterotrophic showed multinucleation. The *C. zofingiensis* cells precultured autotrophically in the dark phase showed escalation in the cell density only until day 4 (Fig. III-6c); however, it was depicted that as the cell dry weight increased, glucose uptake and biomass increased without cellular division (Fig. III-5c). Additionally, during the cultivation, there was an increasing tendency in the size of the autotrophically precultured cells (Fig. III-11b), which can partly be attributed to multinucleation. The averaged nuclei number increased by 4.4 times under this condition from the initial day to day 2 (Fig. III-11a) and the cell diameter increased at the same time (Fig. III-11a). Previous research also revealed that multinucleation in *Scenedesmus* corresponds with the enlargement in cell size (Ivanov et al., 2019), *Chlamydomonas* (Cross and Umen, 2015), and *Chromochloris zofingiensis* (Koren et al., 2021). The cell diameter kept increasing even though the multinucleation stopped on day 2, which suggested that intracellular components might have accumulated. Furthermore, lipid and starch contents in the cells precultured autotrophically from day 0 to day 8 showed 10.9- and 7.5-folds increase respectively (Fig. III-13c, d). These findings depict that early multinucleation takes place in the dark heterotrophic cultivations and an increase in cell length could be a sign of multinucleation or the buildup of intracellular substances.

The number of nuclei tend to increase in the heterotrophically precultured cells showed that the number of nuclei increased by approximately 2-folds in than in the autotrophically precultured cells (Fig. III-11a). These findings suggest that an increase in multinucleated cells numbers can be maintained under dark heterotrophic growth conditions.

The number of nuclei on an average in the heterotrophically precultured condition remained similar on day 0 and last day, 8.95 nuclei cell^{-1} and 9.51 nuclei cell^{-1}, respectively, even though some fluctuations were observed (Fig. III-11a). Cell growth in the heterotrophically precultured condition was lesser than cells precultured autotrophically (Fig. III-5c), and furthermore, no clear trend was observed in the cell diameter (Fig. III-11c). These results indicate that the heterotrophically precultured cells showed no occurrence of further multinucleation in the heterotrophic biomass production phase. Although future research is necessary to understand what triggers in the heterotrophic precultured cells to show continue multinucleation in the dark phase, but it can be said that the dark cultivation in preculture can be reduced to less than 12 days (two-times preculture) in order to get more multinucleated cells ready for an increased cell growth and biomass. An increase in the content of intracellular components (lipids and starch) was observed in the heterotrophically precultured cells as well (Fig. III-13c, d). A previous study with dark heterotrophic culture showed escalated the cellular lipid up to ~52% of the cell dry weight in *C. zofingiensis* (Zhang et al. 2021). Therefore, future studies to optimize the duration of the heterotrophic preculture cultivation time for the accumulation of the intracellular compounds and maximal multinucleation for the enhancement of the growth when transferred to the irradiated cultivations.

3.5 Proposed combinations of modes of nutrition

In this chapter, when heterotrophic cells were cultured (Fig. III-5b), in the mixotrophic nutritional cultivation, the maximum biomass productivity was observed. As a result, it was recommended that a mixotrophic biomass production phase when combined with a heterotrophic preculture of *C. zofingiensis* be used to increase biomass productivity. Although, the growth rate in the heterotrophic cultivation experienced reduction in the growth when the cells cultured heterotrophically were cultivated for a longer time (i.e., 2 or 3 subsequential dark

cultures) (Figs. II-1c and III-1c). Thus, it can be depicted that the either auto- or mixotrophic cultures inoculum are needed for preparing the heterotrophic cultures. The mixotrophic mode, however, is unsuitable for the stock culture as the glucose can cause contamination during cultivation, and its utilization and reduction throughout the stock process could make it challenging to maintain the cells in good conditions. Furthermore, a decreased growth rate in the heterotrophic biomass production phase with the cells precultured mixotrophically. Thus, the proposed optimum combinations of modes of nutrition for higher biomass productivity in *C. zofingiensis* (Fig. III-14) are; autotrophic for stock, heterotrophic for preculture, and mixotrophic for the biomass production phase.

3.6 Conclusion

The present study demonstrated that multinucleation of *Chromochloris zofingiensis* cells was observed under dark, followed by rapid growth with high biomass productivity and multiple fission when cells were irradiated, furthermore, accumulating the intracellular components (starch and lipids). It was also observed that in order for stock or preculture phase to effectively grow, light is necessary, for example, the *C. zofingiensis* in heterotrophic cultivation mode. These results demonstrated an effective approach for enhancing biomass productivity by integrating the appropriate modes of nutrition in the stock, preculture, and biomass production phases.

Figures

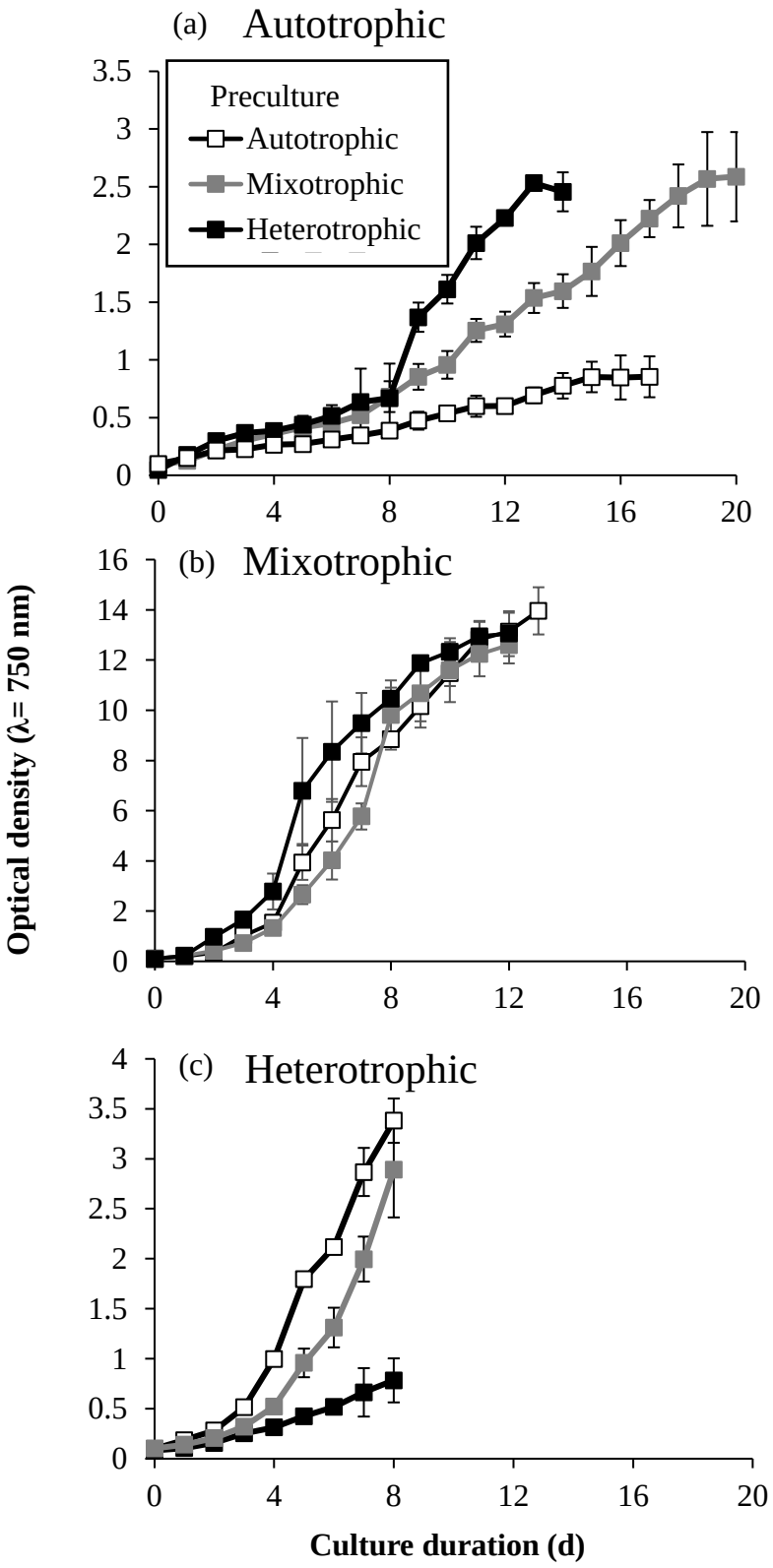


Fig. III-1. Growth curve of *Chromochloris zofingi*ensis in preculture, auto-, mixo-, and heterotrophic to (a) autotrophic, (b) mixotrophic and (c) heterotrophic modes. Data are expressed as the means ± standard deviation (n = 3)

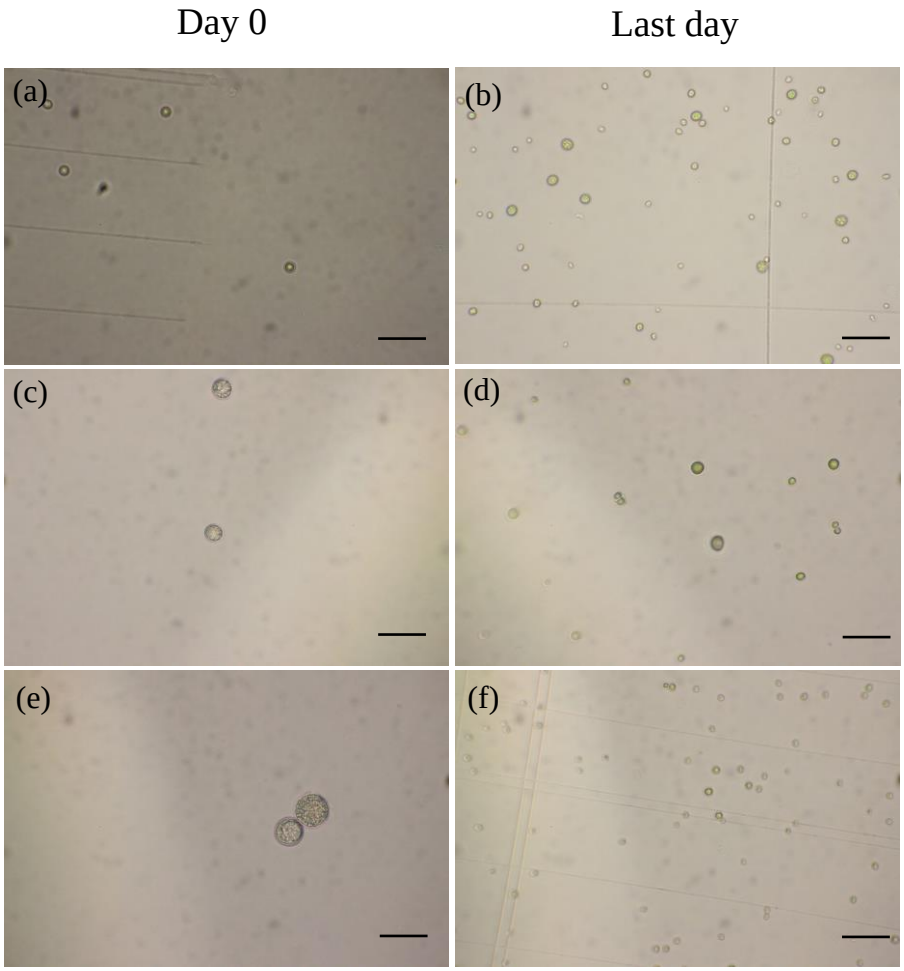


Fig. III-2. Cell pictures of *Chromochloris zofingiensis* in preculture, (a) auto-, (c) mixo-, and (e) heterotrophic on day 0 to autotrophic mode (b, d, and f) on the last day of cultivation. *Scale bar 20*µm

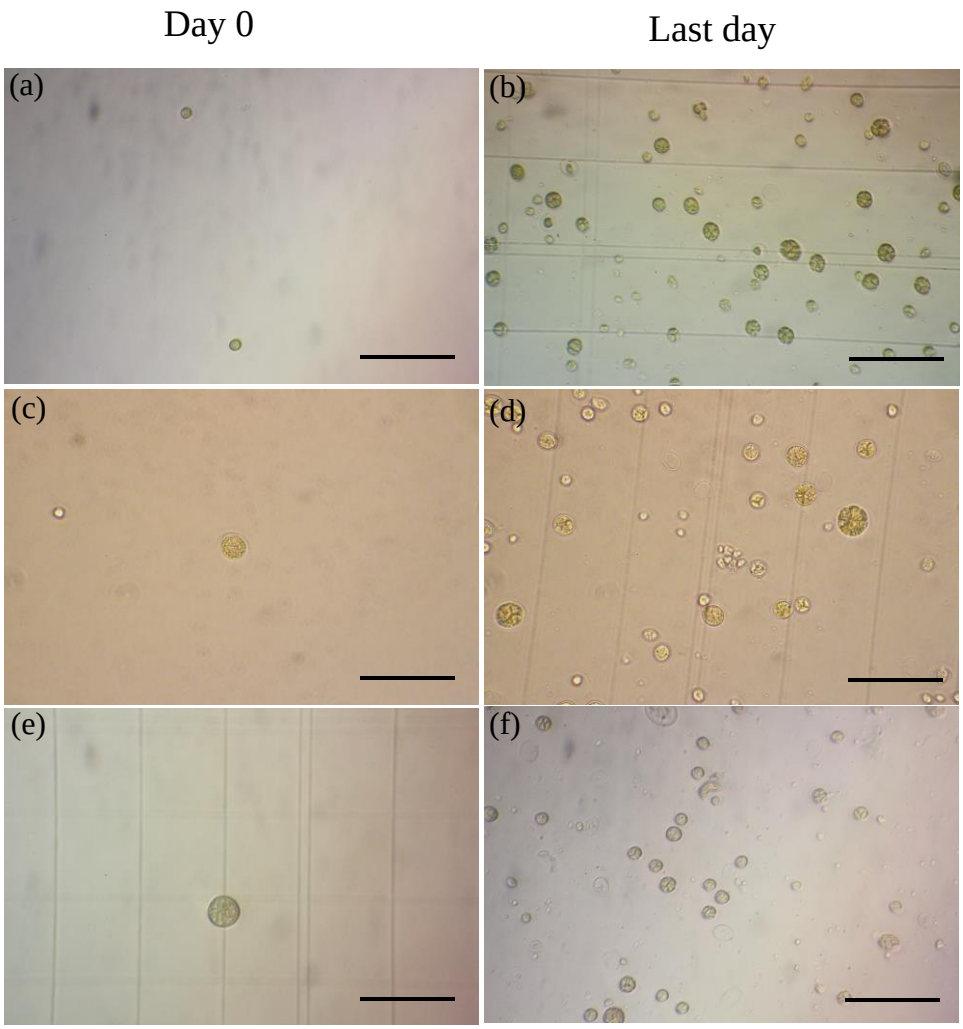


Fig. III-3. Cell pictures of *Chromochloris zofingi*ensis in preculture, (a) auto-, (c) mixo-, and (e) heterotrophic on day 0 to mixotrophic mode (b, d, and f) on the last day of cultivation. *Scale bar* 20 µm

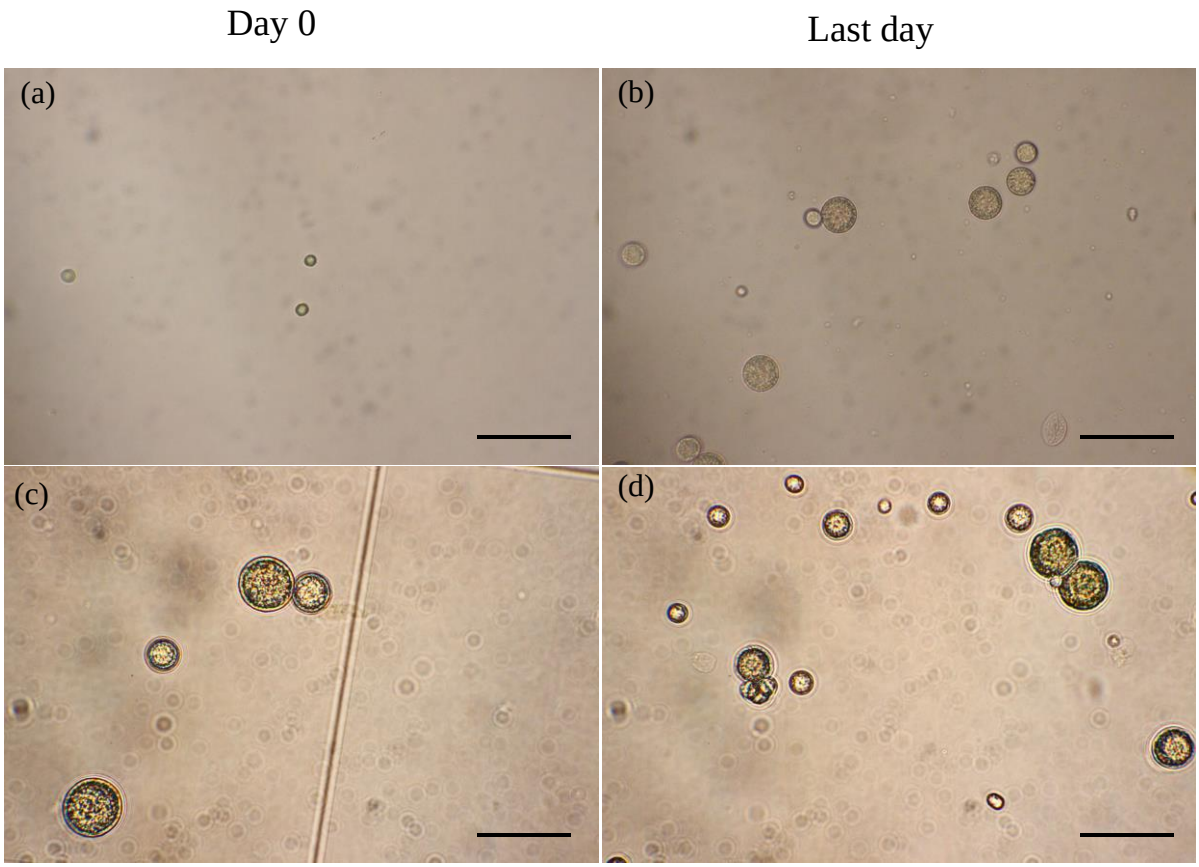


Fig. III-4. Cell pictures of *Chromochloris zofingi*ensis in preculture, a) auto-, and c) heterotrophic on day 0 to heterotrophic mode (b and d) on the last day of cultivation. *Scale bar* 20 µm

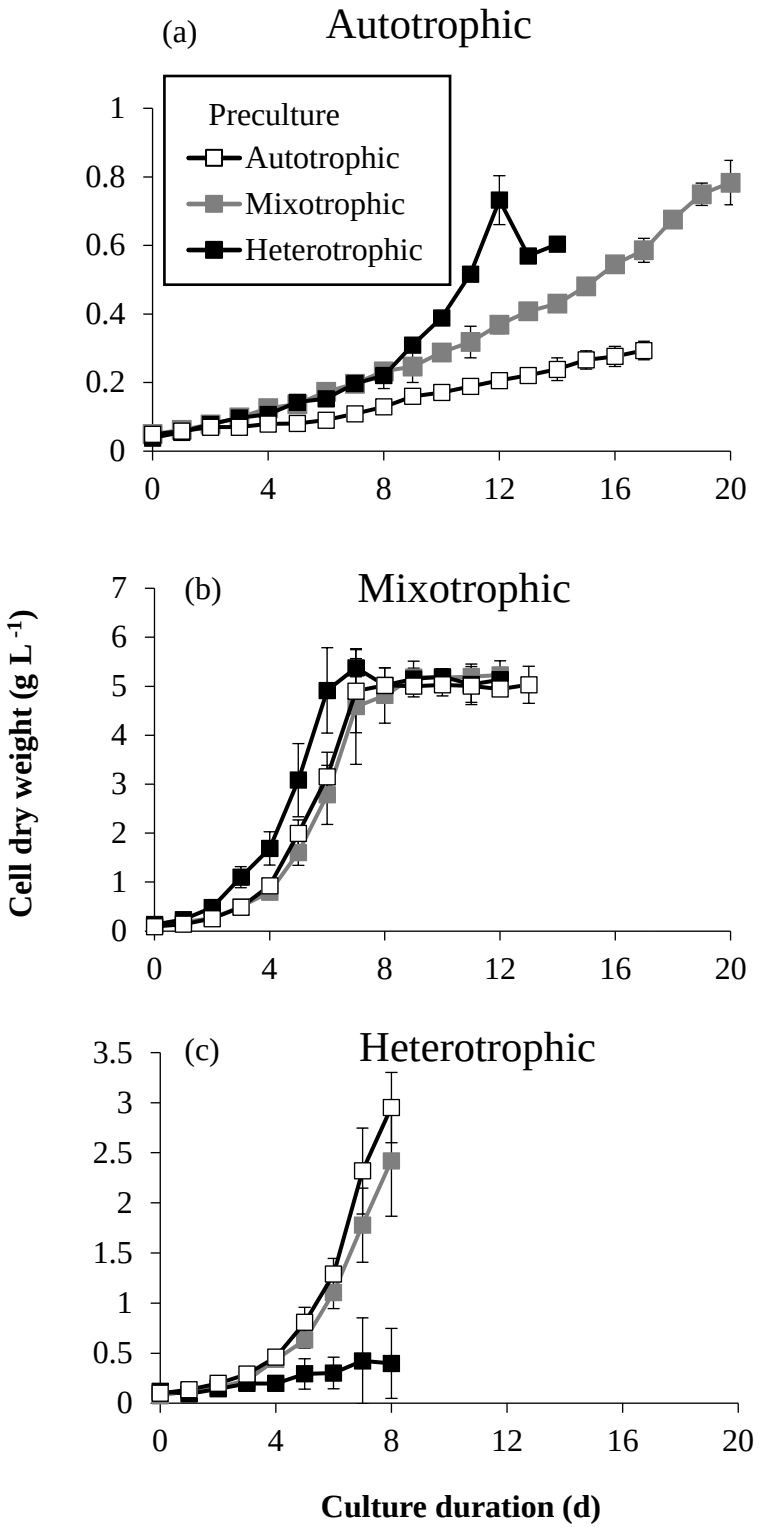


Fig. III-5. Cell dry weight of *Chromochloris zofingi*ensis in preculture, auto-, mixo-, and heterotrophic to (a) autotrophic, (b) mixotrophic and (c) heterotrophic modes. Data are expressed as the means ± standard deviation (n = 3)

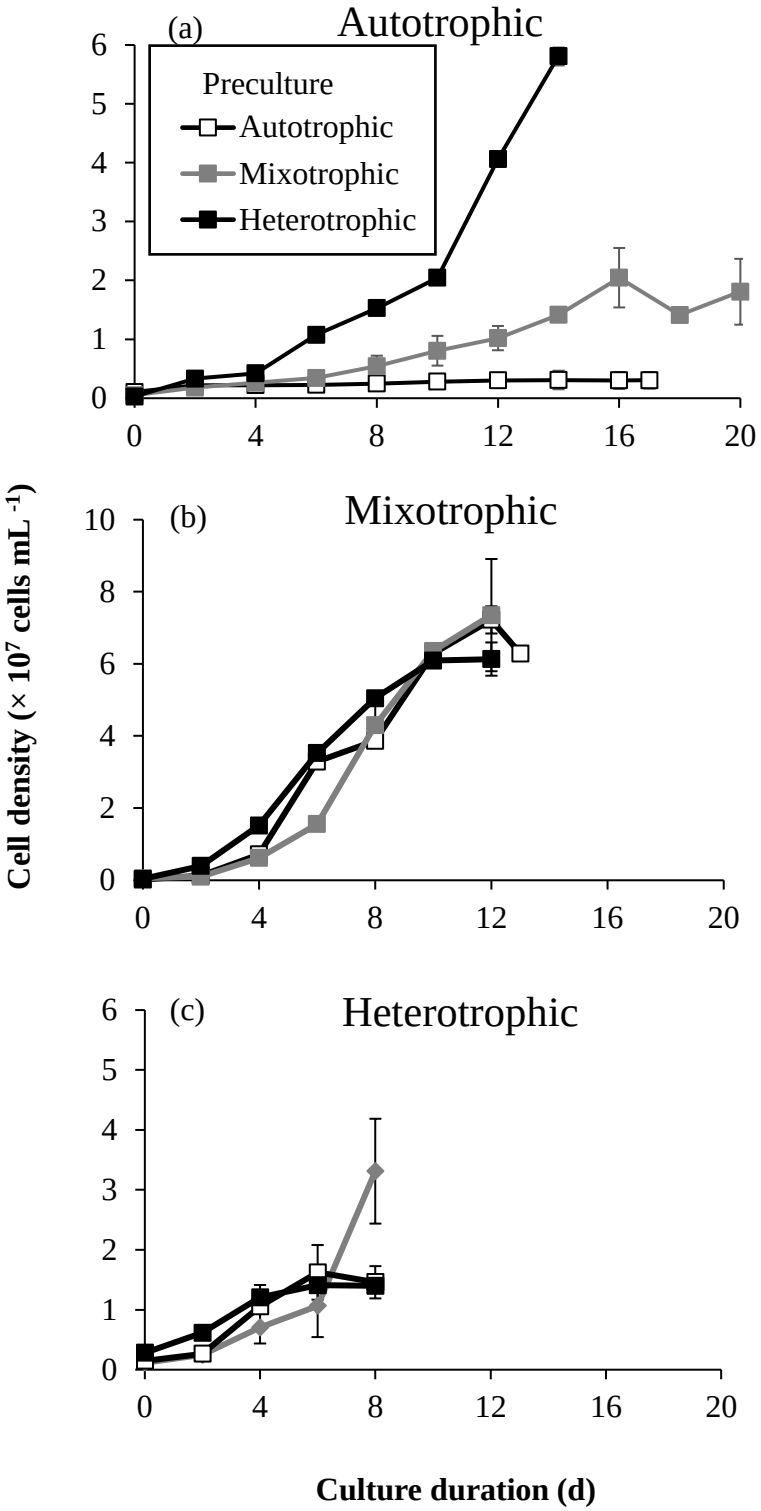


Fig. III-6. Cell density of *Chromochloris zofingi*ensis in preculture, auto-, mixo-, and heterotrophic to (a) autotrophic, (b) mixotrophic and (c) heterotrophic modes. Data are expressed as the means ± standard deviation (n = 3)

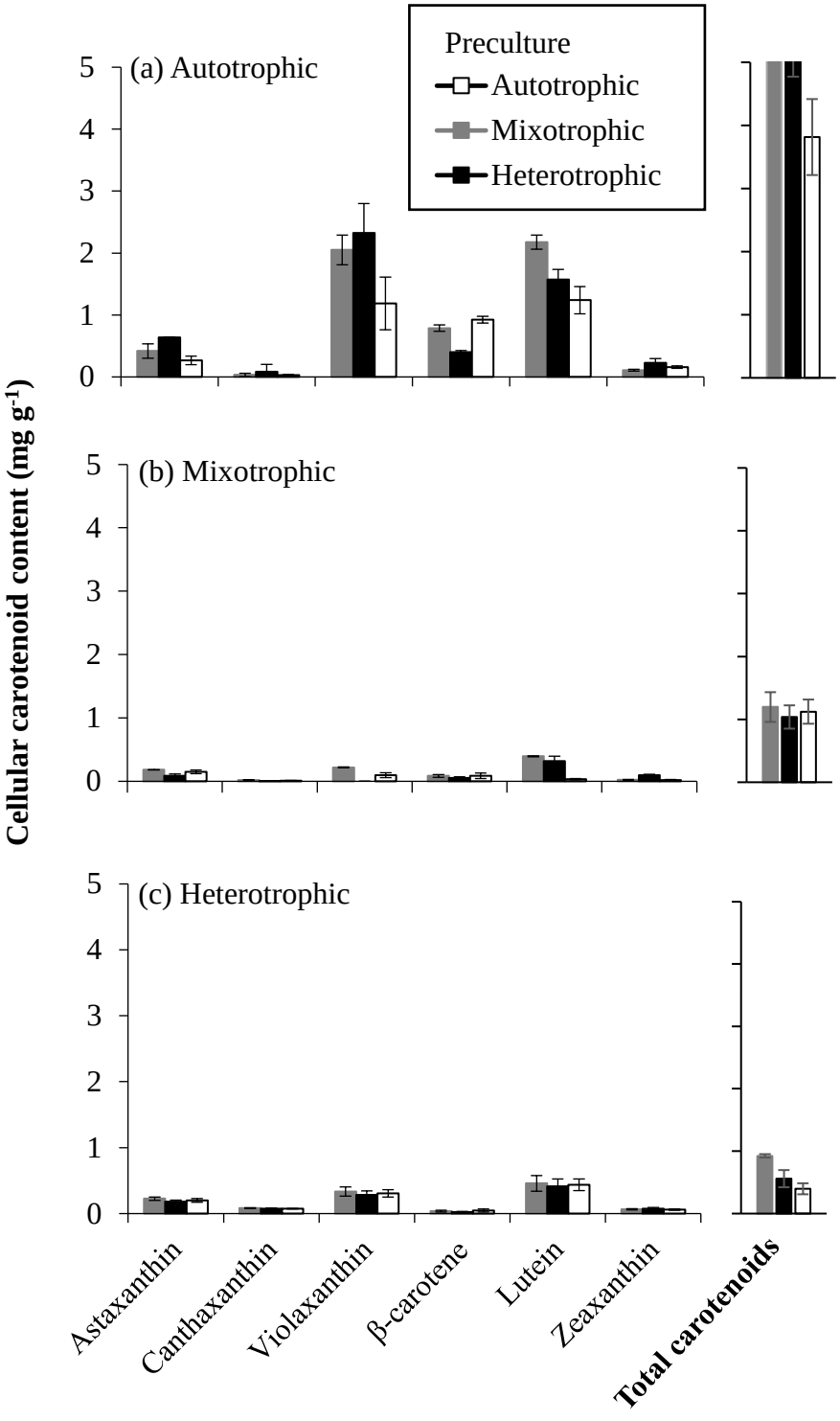


Fig. III-7. Carotenoid content of *Chromochloris zofingi*ensis with three preculture conditions, auto-, mixo-, and heterotrophic to, (a) autotrophic (b) mixotrophic, and (c) heterotrophic conditions. Astaxanthin content shown is the sum of free, mono and di-esters. Data are expressed as the mean ± standard deviation, $n = 3$.

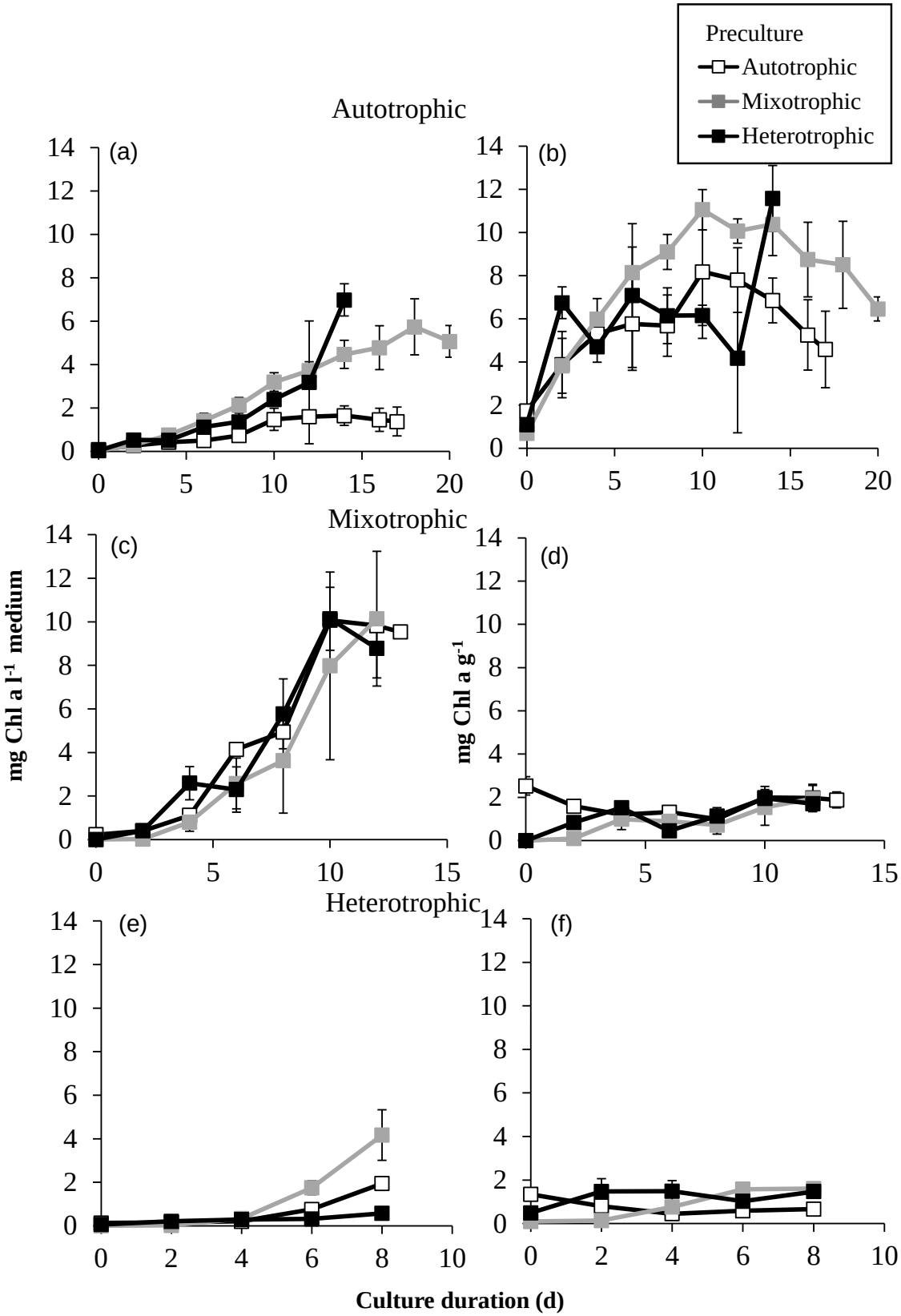


Fig. III-8. Chl *a* concentration (mg L^{-1}) and content (mg g^{-1}) of *Chromochloris zofingi*ensis with three preculture conditions, auto-, mixo-, and heterotrophic to (a, b) autotrophic, (c, d) mixotrophic, and (e, f) heterotrophic conditions. Data are expressed as the mean ± standard deviation, $n = 3$.

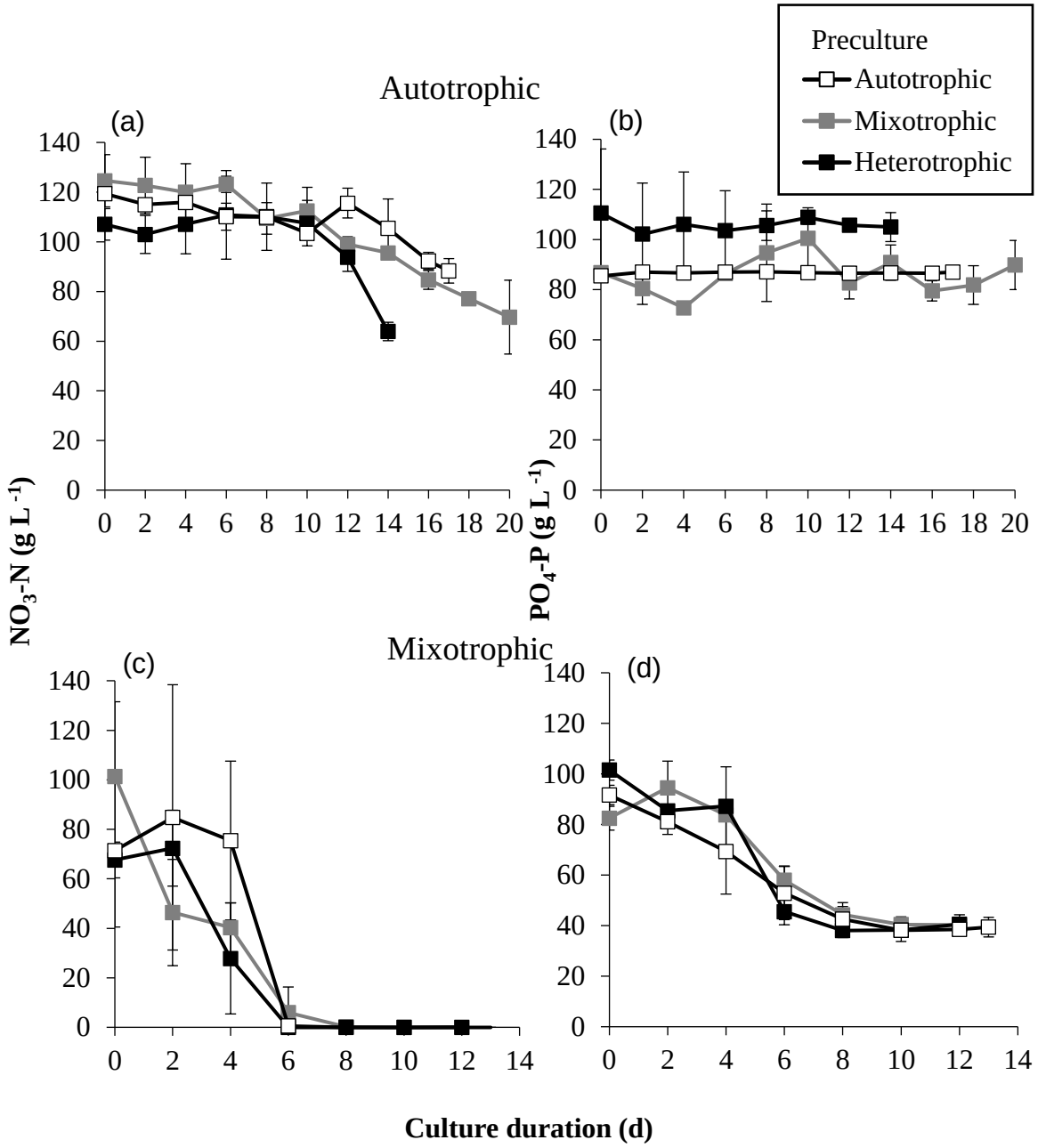


Fig. III-9. Nutrient concentration (NO_3-N and PO_4-P) of *Chromochloris zofingi*ensis with three preculture conditions, auto-, mixo-, and heterotrophic to, (a, b) autotrophic, and (c, d) mixotrophic conditions. Data are expressed as the mean ± standard deviation, $n = 3$.

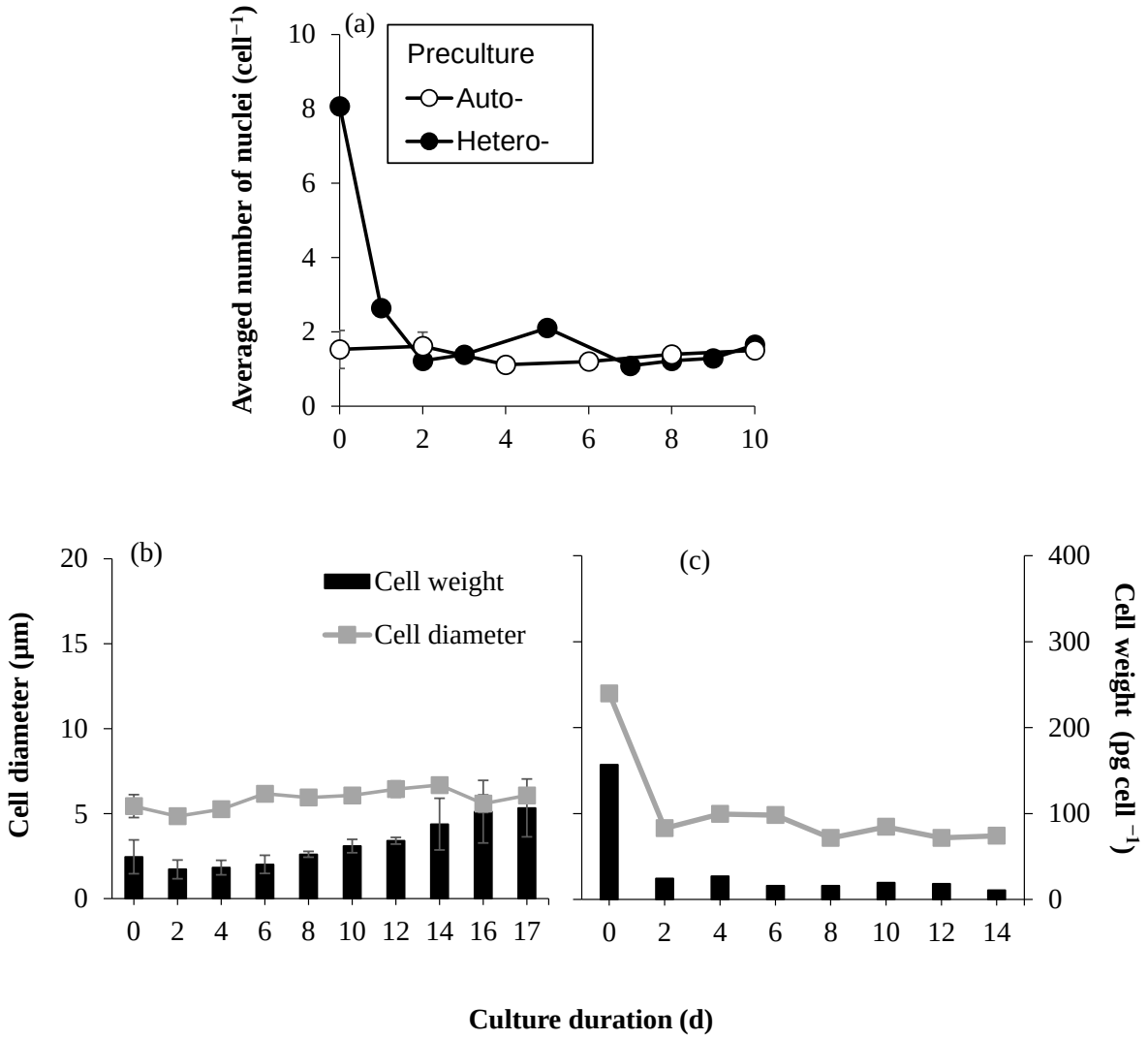


Fig. III-10. Average number of nuclei per cell (a), cell diameter (b) and cell weight (c) in auto-, and heterotrophic preculture to autotrophic nutritional mode. Data are expressed as the mean ± standard deviation, n = 3, except for H-A condition that was conducted in duplicates.

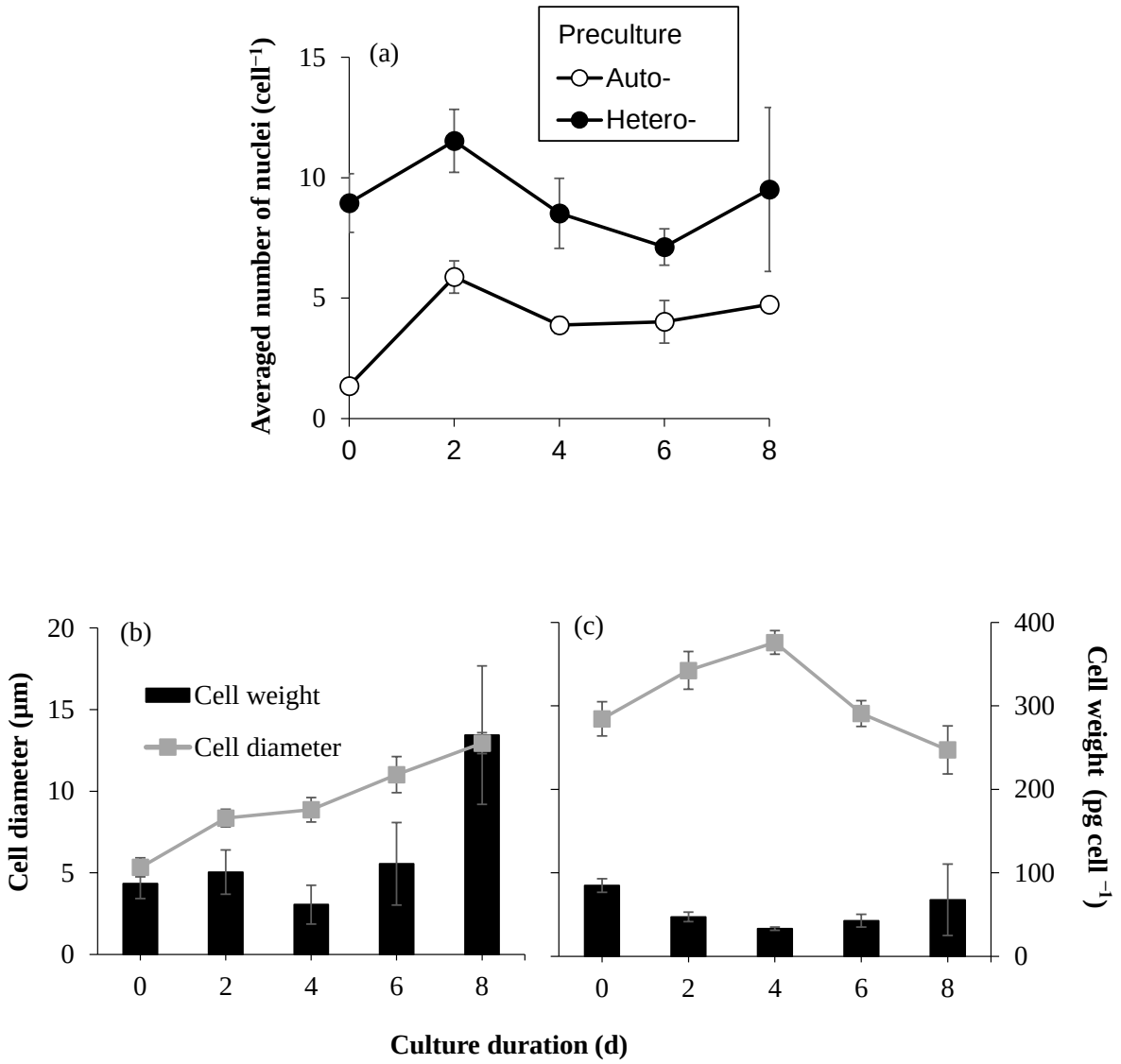


Fig. III-11. Average number of nuclei per cell (a), cell diameter (b) and cell weight (c) in auto-, and heterotrophic preculture to heterotrophic nutritional mode. Data are expressed as the mean ± standard deviation, n = 3, except for H-A condition that was conducted in duplicates.

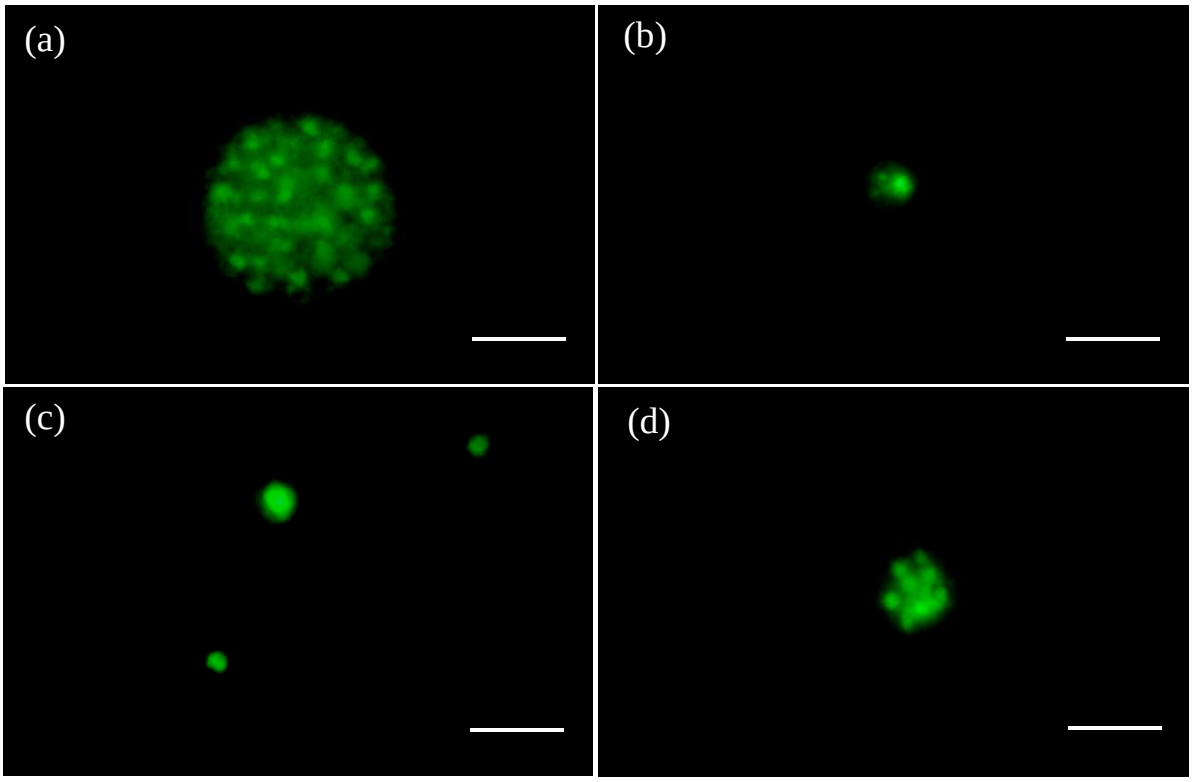


Fig. III-12. Nuclei pictures of heterotrophically precultured cell in autotrophic mode at (a) day 0 and (b) day 1, and autotrophically precultured cells in heterotrophic mode at (c) day 0 and (d) day 1 showing rapid cell division and accumulation of multi nuclei, respectively. *Scale bar* 20 μm

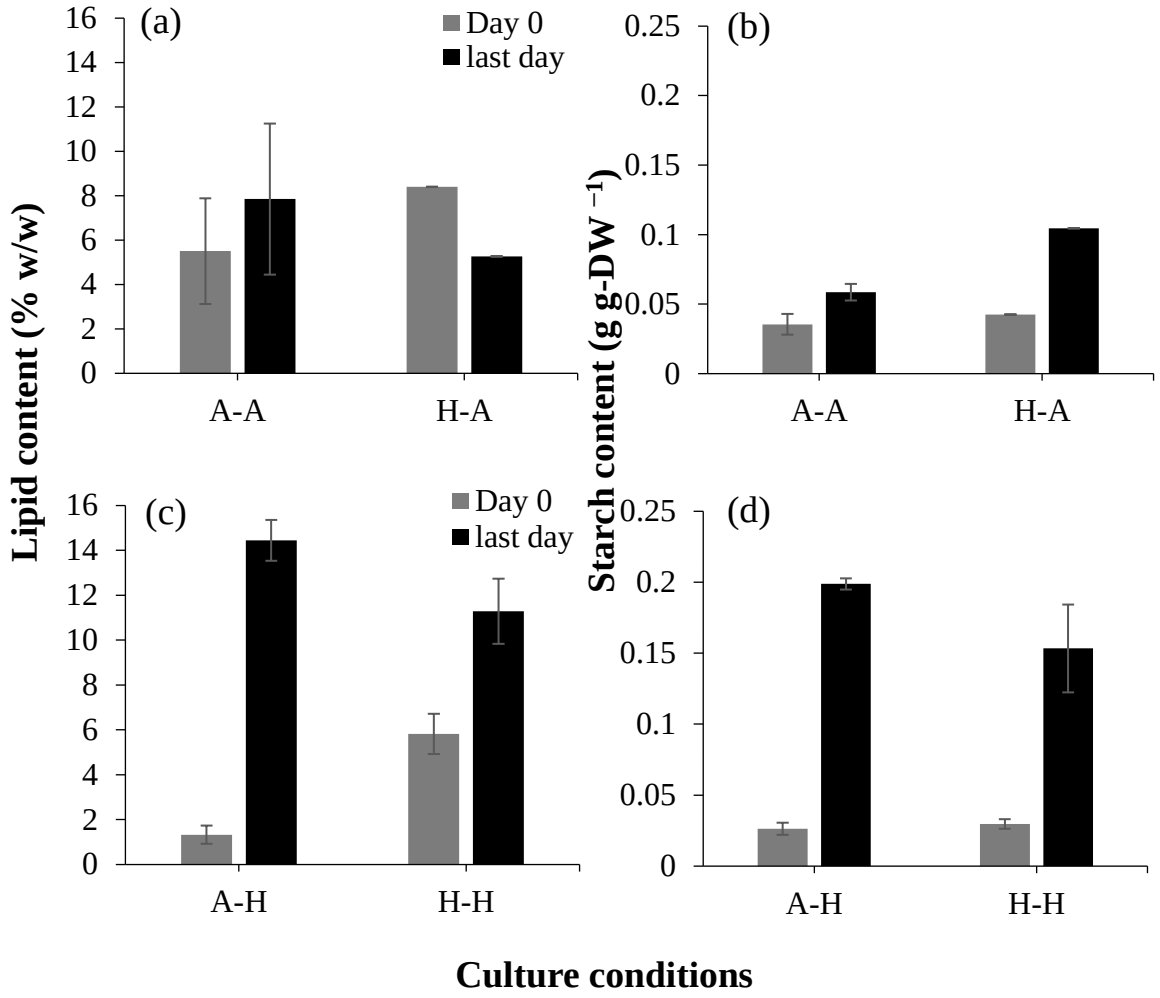


Fig. III-13. Cell composition, (a) lipids, and (b) starch in auto- (A-A), and heterotrophic (H-A) preculture to autotrophic nutritional mode, and (c) lipids, and (d) starch in auto- (A-H), and heterotrophic (H-H) preculture to heterotrophic nutritional mode. Data are expressed as the mean ± standard deviation, $n = 3$.

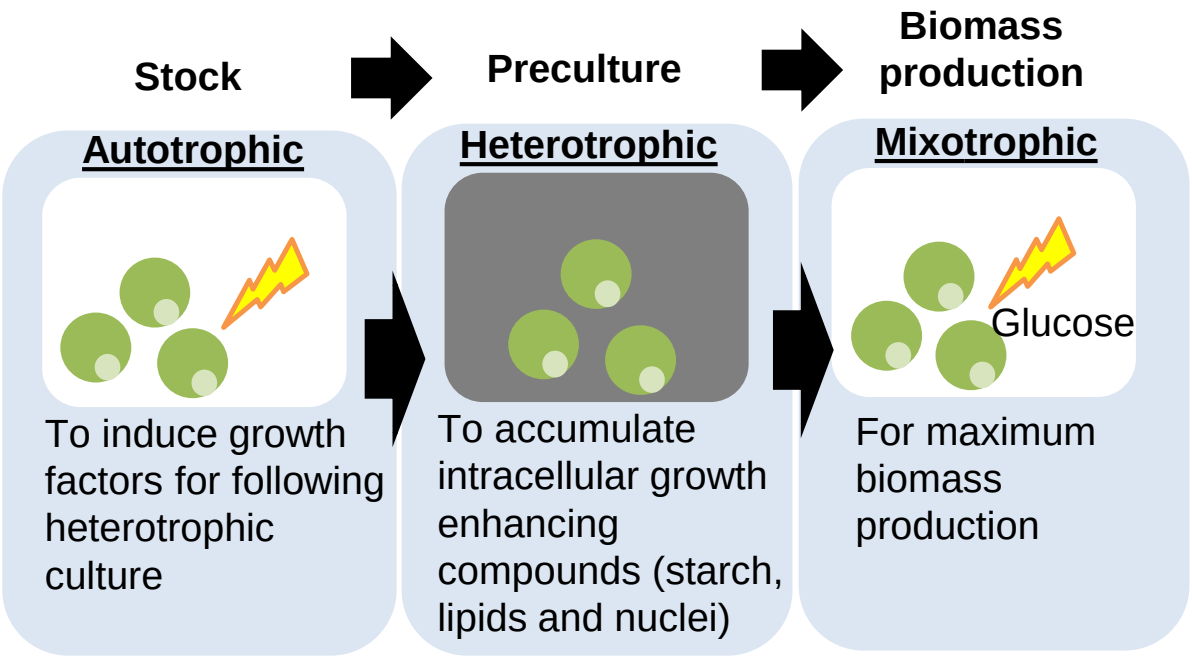


Fig. III-14. Suggested conditions for stock, preculture and biomass production phase are autotrophic, heterotrophic and mixotrophic nutritional modes

4 Chapter 4 A PROPOSED THREE-PHASE CULTURE FOR ASTAXANTHIN ACCUMULATION IN *CHROMOCHLORIS ZOFINGIENSIS*

4.1 Introduction

In Chapter II and III, it was suggested that heterotrophically precultured cells can increase the growth rate and biomass productivities (Chowdhary et al., 2022) because of multinucleation and storage of the intracellular components, lipids and starch upon transition of nutritional modes. The high biomass productivity in the mixotrophic nutritional mode with heterotrophic precultured cells clearly depicted the influence of preculture cultivation phase on the biomass productivity. However, the influence of biomass productivity on the latter phase (carotenoid induction phase) is still required for the improvement of the astaxanthin productivity in *C. zofingiensis*. Therefore, in this PhD study, a three-phase cultivation of *C. zofingiensis* to increase the astaxanthin productivity was proposed and conducted. After the optimization of phases of *C. zofingiensis* such as stock, preculture and biomass production, a three-phase cultivation process with heterotrophic mode in preculture, mixotrophic mode in the biomass production phase, and high light induction in the carotenoid phase were performed (Fig. IV-1).

4.2 Material and Methods

The cells were cultured in preculture phase 1 and 2, similar to Chapter II and III, in autotrophic and heterotrophic mode. Cells at an exponential phase in each mode were re-inoculated and cultured in a mixotrophic fed-batch (260 µmol photon m^{-2} s^{-1} light with 10 g L^{-1} glucose) nutritional mode for biomass production phase. After 3 days of cultivation, the concentration of 15 g L^{-1} glucose was added to the medium bottle for providing sufficient nutrients and maintenance of the algal growth. After reaching the highest biomass productivity

(linear growth phase), the high dense cells from autotrophic and heterotrophic preculture to mixotrophic fed-batch were directly transferred for acclimatization phase in low light intensity (100 µmol photon m^{-2} s^{-1}) for 4 days as 1, 2 and 4. After acclimatizing for days 0, 1, 2 and 4, cells were directly transferred for astaxanthin induction phase in high light intensity (400 µmol photon m^{-2} s^{-1}) in tubular shaped reactors (test tubes were used for this phase).

4.3 Results

As a result, when the cells were transferred from heterotrophically precultured cells into the mixotrophic cultivation, the maximum growth rate 1.07 d^{-1} was observed than in autotrophically precultured cells, 0.74 d^{-1} (Fig. IV-2a). The heterotrophically precultured biomass yield reached 9.06 g L^{-1} in mixotrophic fed-batch cultivation (Fig. IV-2b). The cell dry-weight in autotrophically precultured cells, on the other hand, reached 8.14 g L^{-1}. The biomass productivity in heterotrophically precultured cells reached the highest on day 6 of the cultivation period, 2.51 g L^{-1} d^{-1}. The cells after reaching the highest biomass production (Fig. IV-2b) in the linear growth phase were further transferred directly into the column reactors for the carotenoid induction phase.

The *C. zofingiensis* high dense cells from the mixotrophic fed-batch were directly cultured, without (0 day) and into the low light irradiation (60 µmol m^{-2} s^{-1}) acclimatization phase with 1, 2 and 4 days in test tubes (column reactors) with continuous aeration. Later, the cells after acclimatization were transferred into the high light irradiation (400 µmol m^{-2} s^{-1}). The heterotrophically precultured high dense cells subjected to directly high light induction reached 16.50 ± 1.32 g L^{-1}, while the autotrophically precultured cells reached 18.86 ± 2.50 g L^{-1} cell dry weight on the last day of the cultivation period (Fig. IV-4). The green *C.*

zofingiensis cells turned from green to orange in both heterotrophically (Fig. IV-5) and autotrophically (Fig. IV-6) precultured cells.

The carotenoid accumulation in the induction phase showed an increase in the astaxanthin yield in both conditions. The heterotrophically precultured cells showed an increase from 2.11 on day 0 to 5.80 mg L^{-1} in astaxanthin yield on the last day of cultivation. However, the astaxanthin yield obtained with autotrophically precultured cells was 3.35 mg L^{-1} (Fig. IV-7). The change in the color from day 0 to day 5 and the final day of cultivation period can be seen in heterotrophically and autotrophically precultured cells (Fig. IV-5, 6). It was also found out that other secondary carotenoids also increased in both conditions. However, the mixotrophic fed-batch high dense cells acclimation in the low light for one day showed a higher astaxanthin yield in the control conditions (autotrophically precultured) than the heterotrophically precultured cells (5.01 and 1.94 mg L^{-1}, respectively; Fig. IV-7). Two- days of low light acclimation showed 4.90 and 6.40 mg L^{-1} astaxanthin yield in hetero- and autotrophically precultured cells (Fig. IV-8). Lastly, four days of low light acclimation reached 5.04 and 6.54 mg L^{-1} astaxanthin yield in hetero- and autotrophically precultured cells (Fig. IV-9).

4.4 Discussions

The previous studies have mentioned about the heterotrophy-photoinduction culture strategy, where cells are cultured in heterotrophic condition for higher cell density and are later shifted into irradiation conditions for astaxanthin accumulation (Zhang et al., 2017a; Sun et al., 2019). However, this PhD study investigated the high dense *C. zofingiensis* cells in the mixotrophic fed-batch biomass phase into low light acclimation and then high light intensity for the induction phase. The light acclimation phase in the heterotrophically precultured cells

did not show a significant difference in the astaxanthin productivity between the 4 conditions. However, the transfer of dense cells from mixotrophic fed batch showed the highest astaxanthin yield and productivity when the cells were transferred into high light (400 µmol photon m^{-2} s^{-1}) intensity. This reveals that possibly the acclimatization of low light is not needed for the transfer of heterotrophically precultured cells to the following phase. However, in the autotrophically precultured cells, even though the astaxanthin productivity was low in the other conditions, 4-days of low light acclimation showed 2.73 mg L^{-1} d^{-1} astaxanthin productivity. It is possible that the presence of light throughout, starting from preculture phase to carotenoid acclimation phase and the high light intensity, might have triggered the necessary genes for astaxanthin biosynthesis in the 4-days of low light method. Moreover, the presence/ absence of nitrate could also have led to a difference in the productivity in these experiments. However, further studies are still needed to confirm whether the low light acclimatization step is required which can help trigger the necessary genes for the astaxanthin biosynthesis. Also, the analysis of nutrients to understand at what point (acclimation phase and high light intensity induction period) nitrate was exhausted, to clearly understand the trigger of high light intensity and nitrate, which is known to be the best stress condition (Zhang et al., 2017a; Sun et al., 2019) for astaxanthin biosynthesis in *C. zofingiensis*. Overall, the accumulation of primary carotenoids was found to be less in this study, possibly because of the high light stress triggering the genes for the biosynthesis of the secondary carotenoids. β-Carotene reduction from day 0 to the last day showed an increase in the astaxanthin and other secondary carotenoids in the cell. This possibly means the β-Carotene to astaxanthin conversion was slow and could have been cultivated for a longer duration to achieve an increased astaxanthin production. In a previous study it was mentioned that increase of astaxanthin concentration was supported by the decrease of β-Carotene and canthaxanthin (Basiony et al., 2022). *Chromochloris zofingiensis* has an ability to accumulate a comparable amount of astaxanthin to *H. pluvialis* (Liu et al.,

2014; Zhang et al., 2017a; Sun et al., 2019). Thus, the cultivation of high concentrated *C. zofingiensis* cells into the column tube reactors for a higher astaxanthin production for longer cultivation days can be a suitable option. Additionally, future explorations including genetic engineering tools to further understand the biosynthetic pathway which benefits the improvements in the astaxanthin biosynthesis is required.

4.5 Conclusion

The proposed three-phase culture for improvement of astaxanthin production in *Chromochloris zofingiensis* for stock, preculture, biomass production and astaxanthin induction steps are autotrophic, heterotrophic/ autotrophic, mixotrophic and autotrophic respectively.

Figures

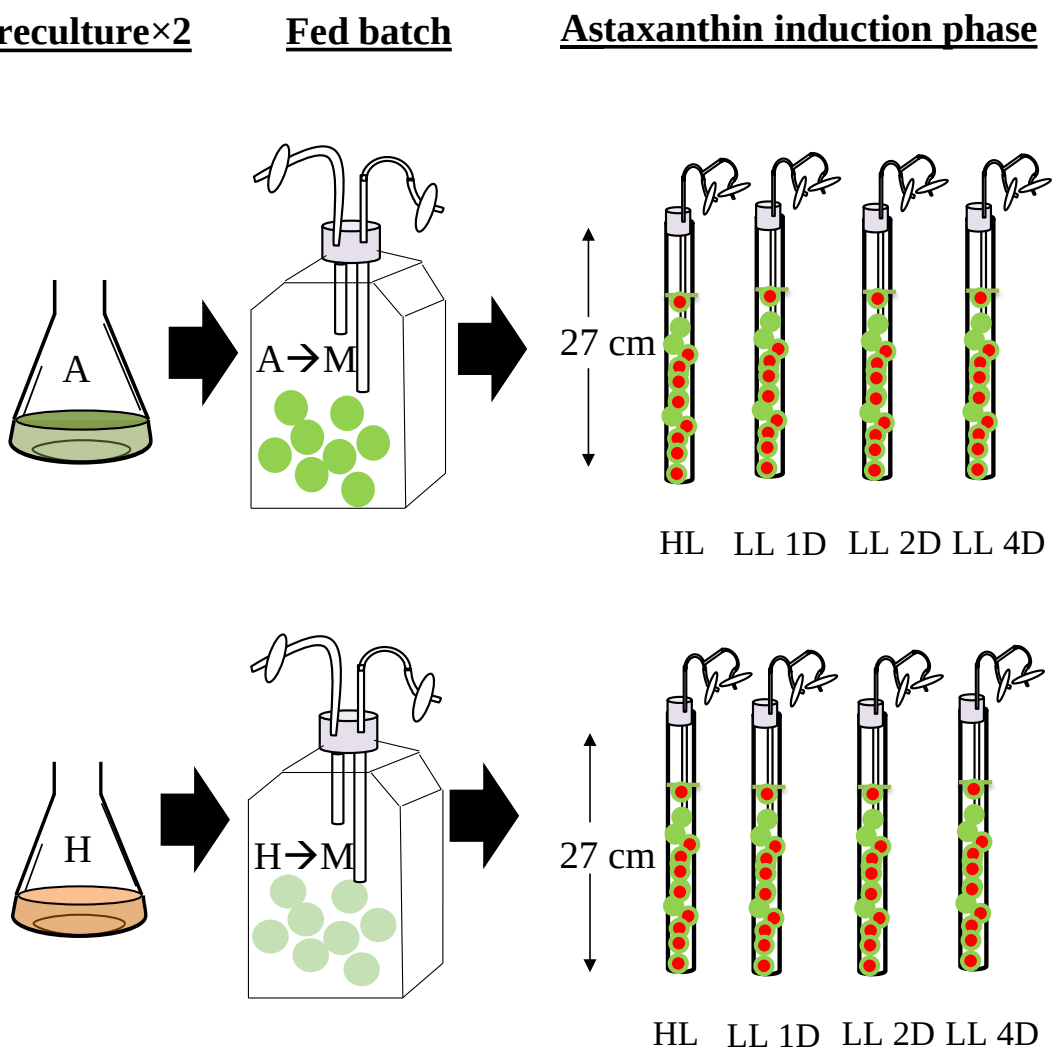


Fig. IV-1. Experimental set up for astaxanthin production in autotrophically (A) and heterotrophically (H) precultured cells in mixotrophic (M) fed-batch into high light intensity (HL) and low light acclimation for day 1, day 2, and day 4 as LL D1, LL D2 and LL D3 respectively. Later, LL D1, LL D2 and LL D4 cells were transferred into high light intensity.

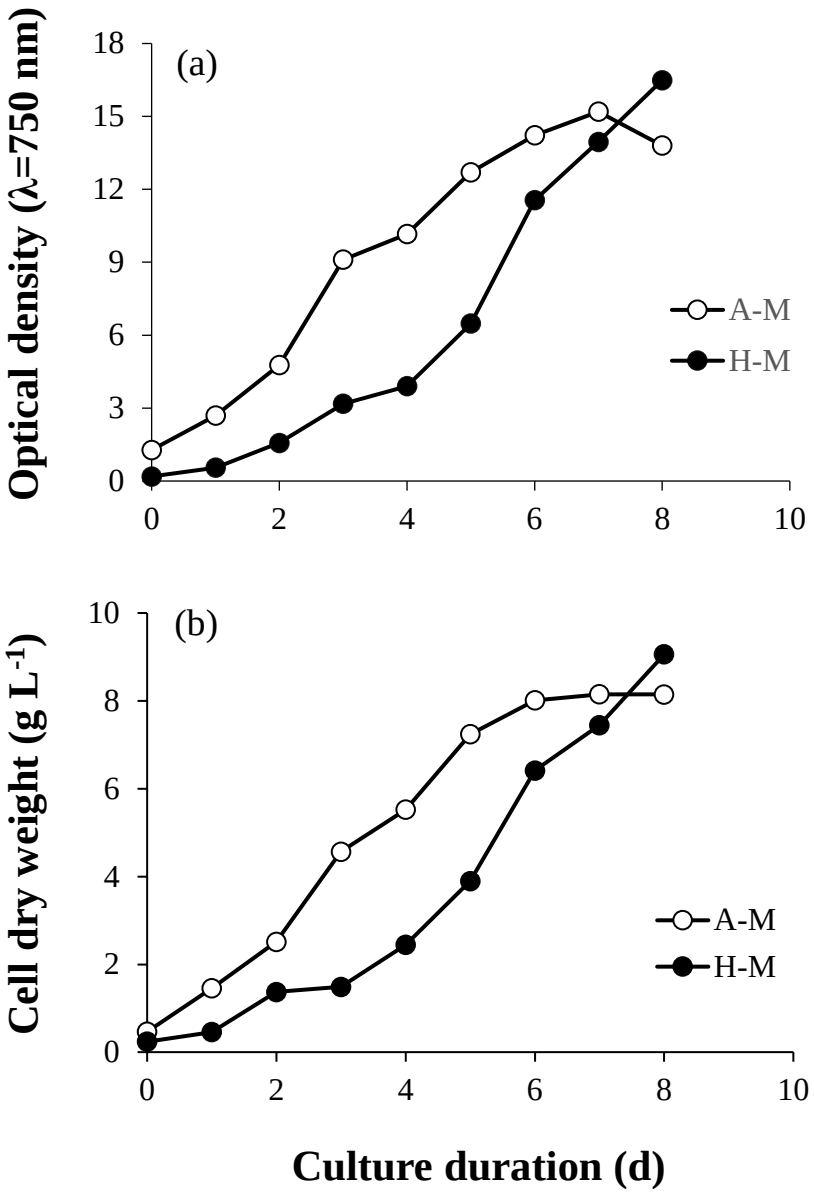
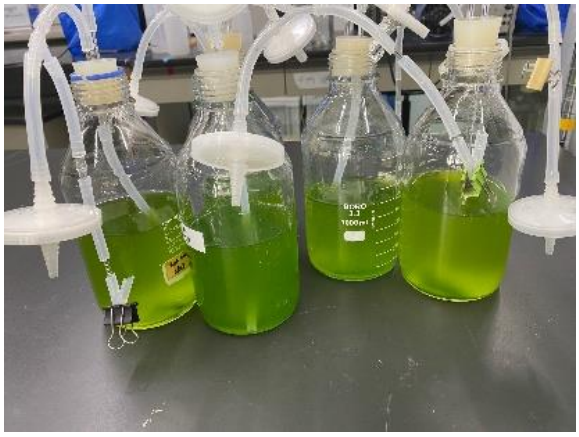


Fig. IV-2 Growth curve of *Chromochloris zofingiensis* (a), and cell dry weight (b) in auto-, and heterotrophic preculture to mixotrophic fed-batch, A-M and H-M, respectively.

(a)



(b)

(c)

Fig. IV-3 Fed-batch cultivation of *Chromochloris zofingiensis* on day 6 of preculture in (a) autotrophic, and (b) heterotrophic to first day of (c) mixotrophic fed-batch.

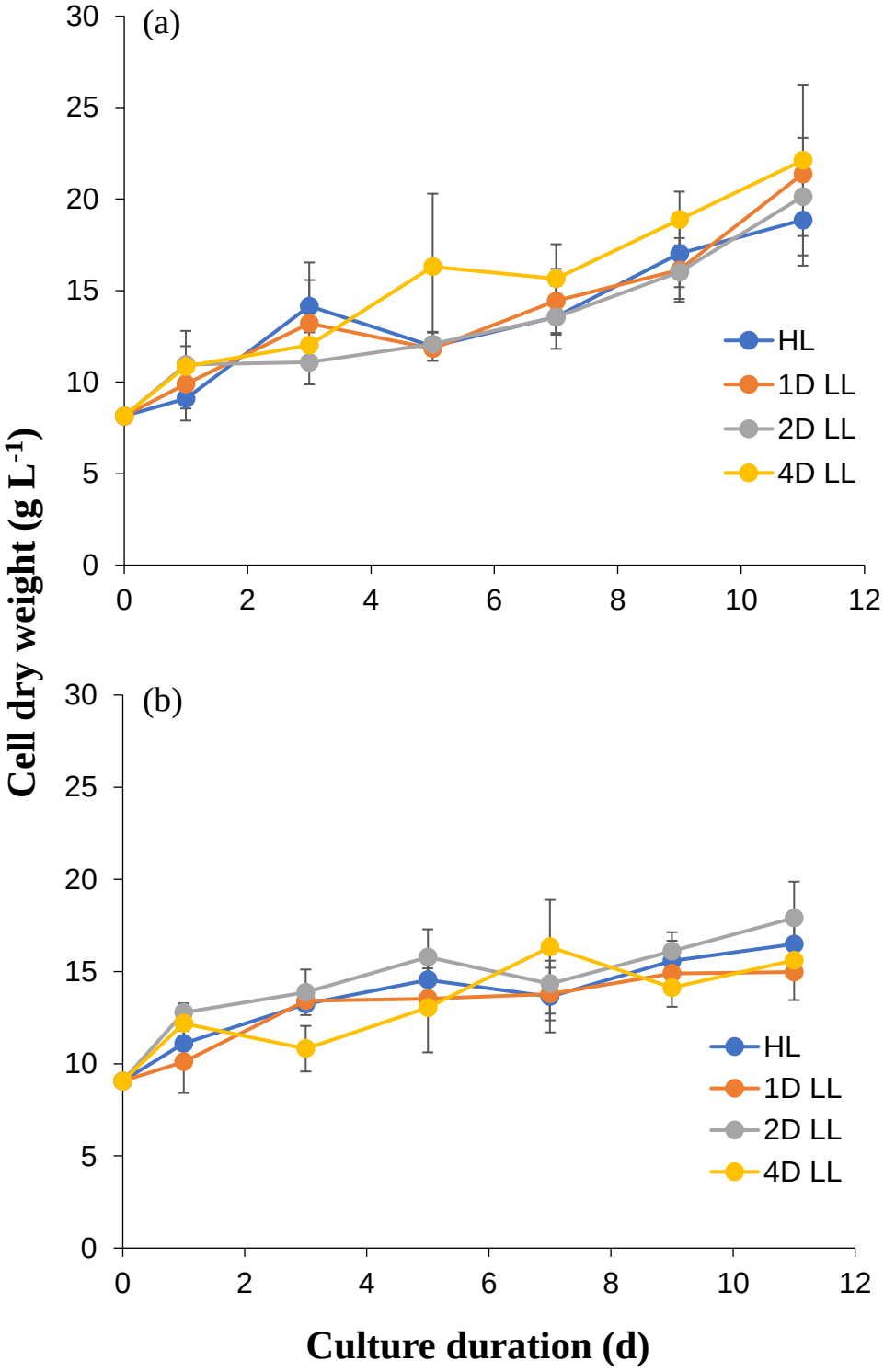


Fig. IV-4. Cell dry weight in the astaxanthin induction phase with autotrophically (a) and heterotrophically (b) precultured cells in high light (HL) and after the low light acclimation in low light for days 1,2 and 4 (LL D1, LL D2, and LL D4 respectively) in high light.

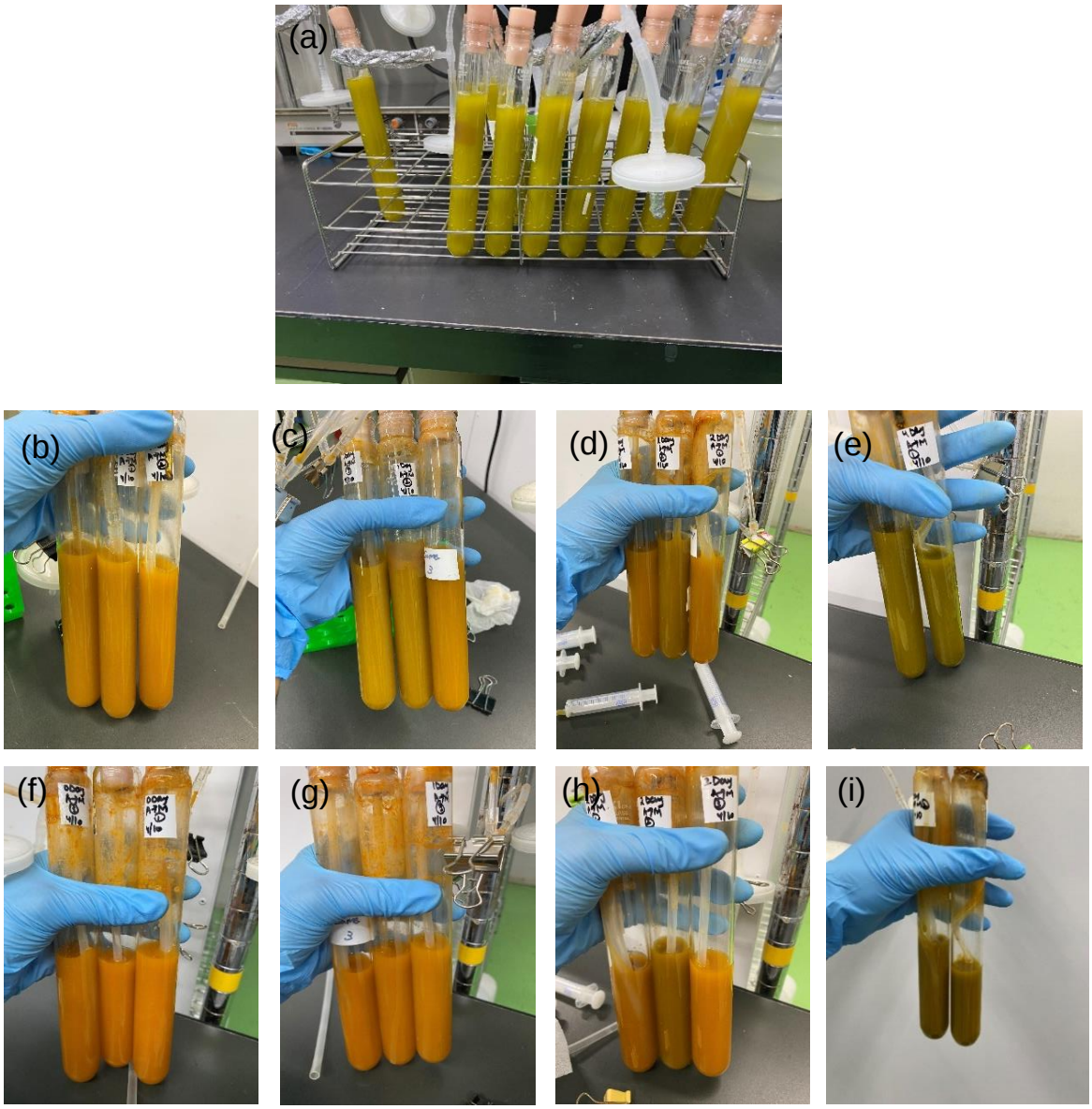


Fig. IV-5 Astaxanthin induction phase of autotrophically precultured *Chromochloris zofingiensis cells* in column reactors on day 0 (a) from mixotrophic fed-batch to day 5 of (b) high light induction, and low light acclimation for (c) day 1, (d) day 2, and (e) day 4 and last day of (f) high light induction, and low light acclimation for (g) day 1, (h) day 2, and (i) day 4.

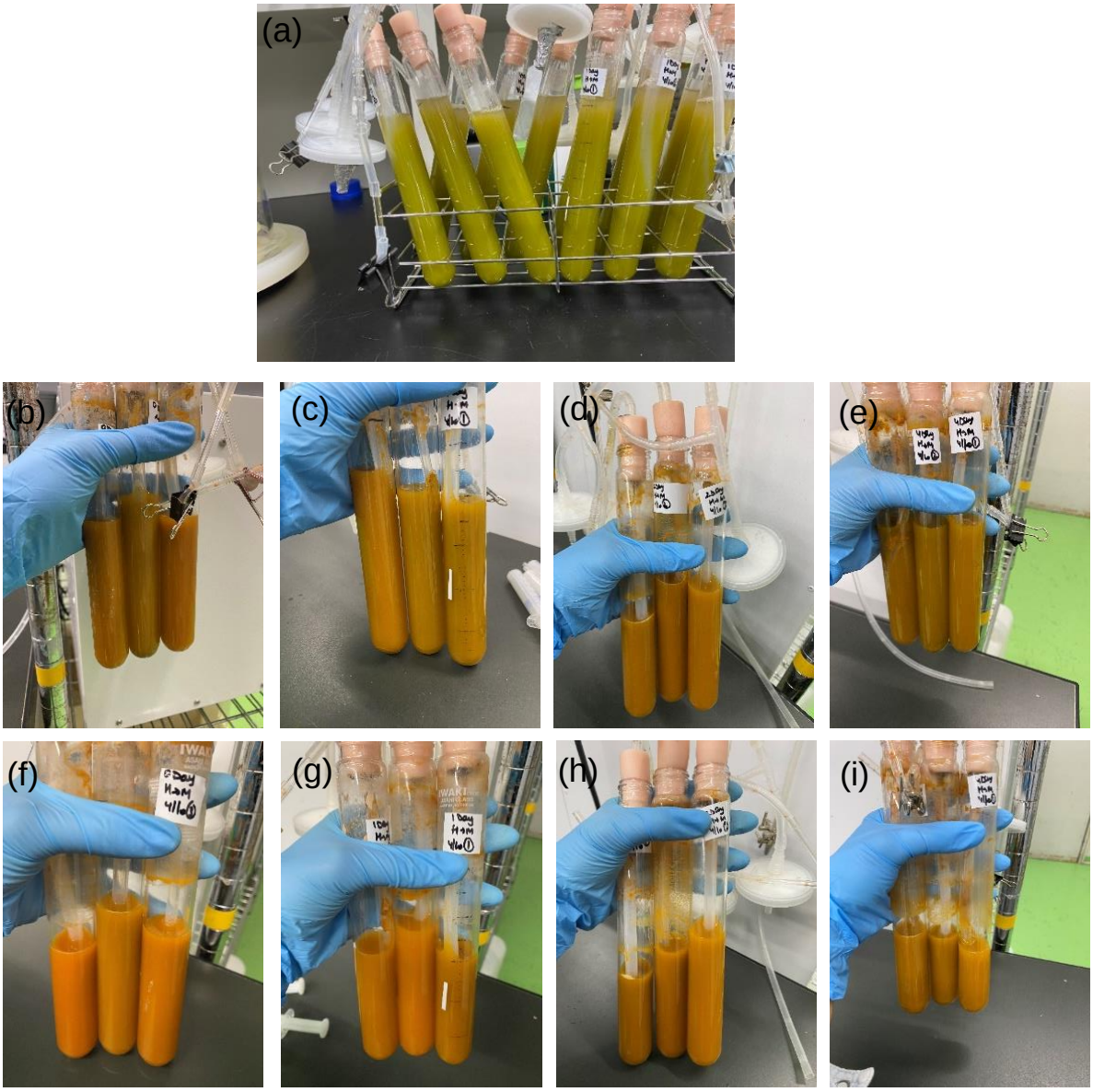


Fig. IV-6 Astaxanthin induction phase of heterotrophically precultured *Chromochloris zofingiensis* cells in column reactors on day 0 (a) from mixotrophic fed-batch to day 5 of (b) high light induction, and low light acclimation for (c) day 1, (d) day 2, and (e) day 4 and last day of (f) high light induction, and low light acclimation for (g) day 1, (h) day 2, and (i) day 4.

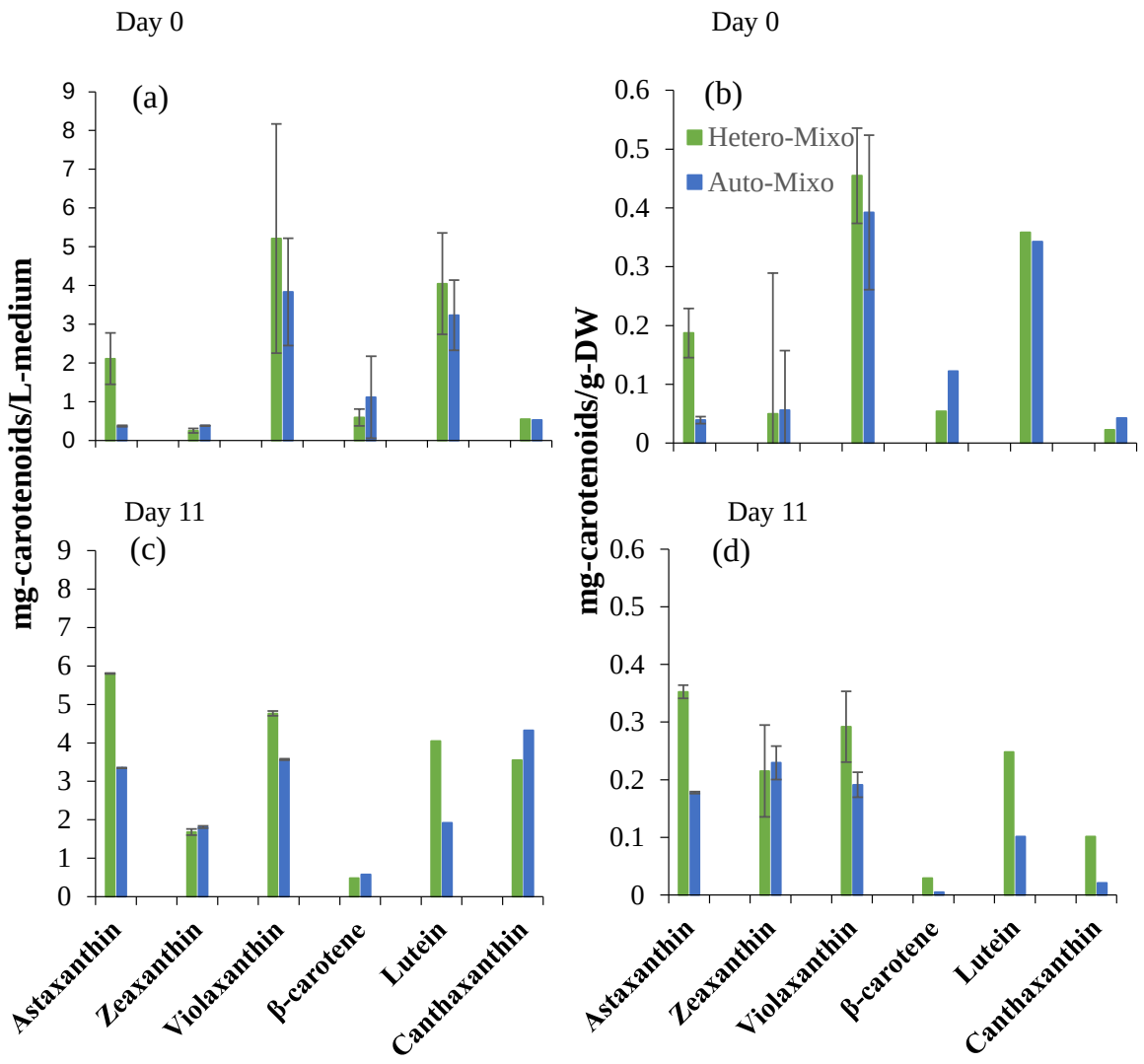


Fig. IV-7. Carotenoid yield (a, c) and content (b, d) on the first and the last day of the cultivation period in the induction phase with heterotrophically and autotrophically precultured cells in directly high light condition.

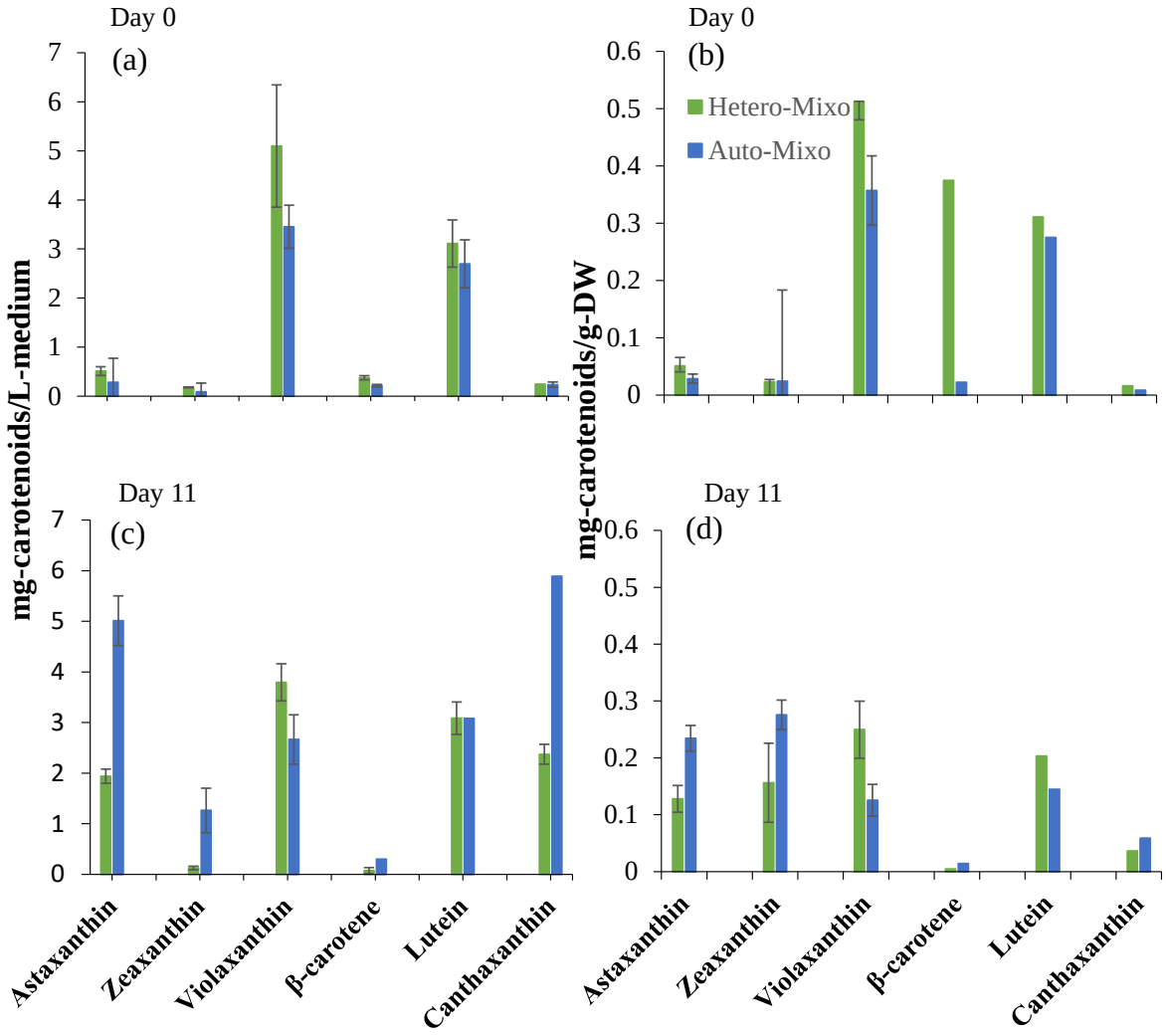


Fig. IV-8. Carotenoid yield (a, c) and content (b, d) on the first and the last day of the cultivation period in the induction phase with heterotrophically and autotrophically precultured cells after one day of low light acclimatization in high light.

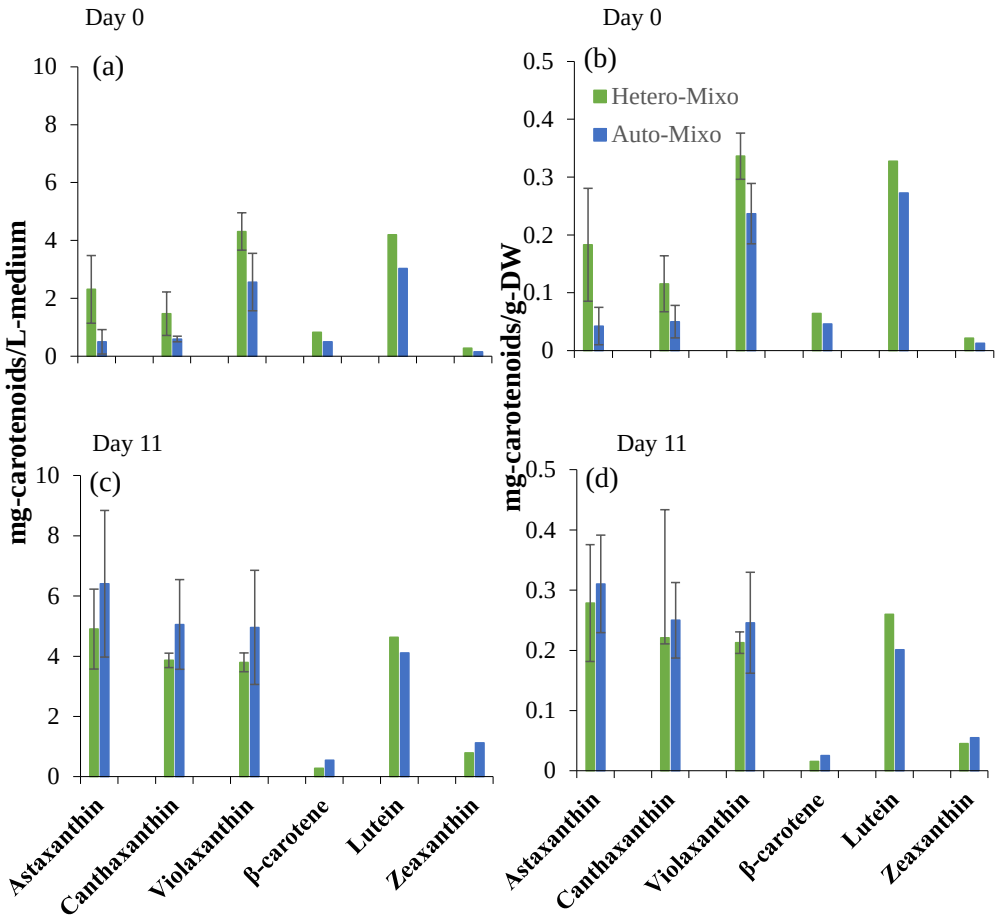


Fig. IV-9. Carotenoid yield (a, c) and content (b, d) on the first and the last day of the cultivation period in the induction phase with heterotrophically and autotrophically precultured cells after two days of low light acclimatization in high light.

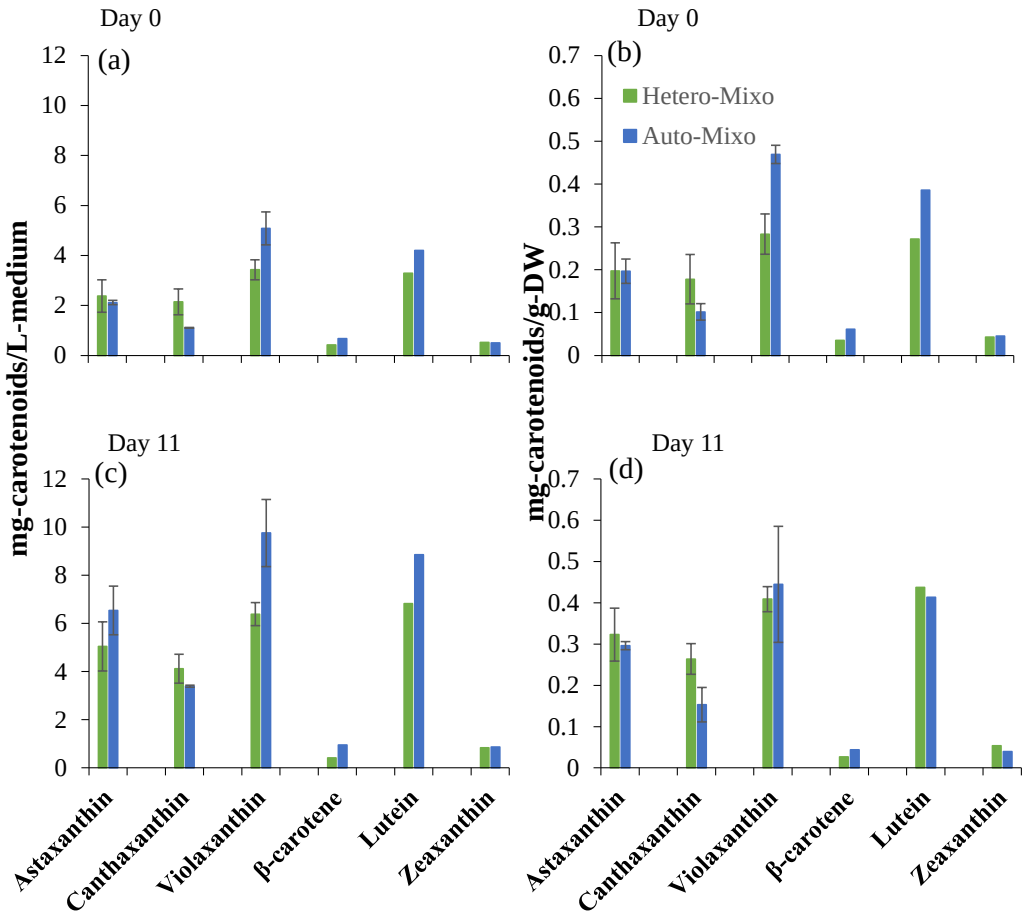


Fig. IV-10. Carotenoid yield (a, c) and content (b, d) on the first and the last day of the cultivation period in the induction phase with heterotrophically and autotrophically precultured cells after four days of low light acclimatization in high light.

5 Chapter 5 GENERAL DISCUSSIONS

5.1 Cell growth and biomass productivity in mixotrophic mode

The mixotrophic nutritional mode precultured with the heterotrophic cells showed the highest biomass productivity. This PhD study obtained maximum biomass production (1.28 g L^{-1} d^{-1}), with cells precultured heterotrophically with *C. zofingiensis* as compared to the other batch mixotrophic cultivation studies, which showed a ranged 0.59-0.72 g L^{-1} d^{-1} (Table V-1) (Oncel et al., 2011; Wang et al., 2013; Chen et al., 2017). Additionally, fed-batch mixotrophic cultivation showed 2.51 g L^{-1} d^{-1} biomass productivity, which is the highest known as compared to the previous studies. The heterotrophic condition of *C. zofingiensis* observed the highest (7.03 g L^{-1} d^{-1} productivity (Zhang et al., 2017)), and several other studies focused on the heterotrophic cultivation of *C. zofingiensis* (Ip, 2005; Sun et al., 2008; Wang and Peng, 2008; Liu et al., 2011; Sun et al., 2019). However, in all of the aforementioned research studies, the stock/inoculum was maintained in the presence of light ranged 20–90 μmol m^{-2} s^{-1}, which might have allowed for the high biomass productivity. However, if a dark cultivation persists beyond a batch, the production might deteriorate. Therefore, prior to the microalgal transition to a dark culture phase, irradiation seems to be crucial for cell proliferation.

Therefore, it can be inferred that mixotrophic cultivation in *C. zofingiensis* can lead to a higher/comparable biomass productivity to heterotrophic cultivation. As previously explained, mixotrophic cultivation combines the synergistic relationship of both photosynthesis and carbon assimilation, thus, we propose mixotrophic cultivation using dark precultured cells for even higher biomass productivities. Further optimization of the fermentation process should be considered which will eventually increase the cell density by promoting the volumetric productivities.

5.2 The potential of *Chromochloris zofingiensis* for mass cultivation and astaxanthin production

The microorganisms-based production of astaxanthin is still a growing industry. The commercial astaxanthin production using *Haematococcus pluvialis* have been reported in the previous studies, because of its maximum astaxanthin accumulation between 19-49 mg g^{-1} (Sun et al., 2015; Wen et al., 2015). However, the lower biomass production in *H. pluvialis* is prone to contamination with microbes, which further reduces the production of astaxanthin commercially. Thus, the attributes of higher cell density and cheaper methods for breakdown of cells are important for improving the astaxanthin accumulation. *Chromochloris zofingiensis* has a potential of achieving high biomass productivities and is a potential natural astaxanthin producer, also its unique cell structure with higher cell wall disruption efficiency makes it valuable species for low-cost natural astaxanthin production. Thus, further improvements in *C. zofingiensis* can make it invaluable.

According to the previous studies in *C. zofingiensis* (Table V-2) which were conducted as a single stage process for astaxanthin accumulation in autotrophic, heterotrophic and mixotrophic inductions achieved productivities ranging 0.8-2.8 mg L^{-1} d^{-1} with content of astaxanthin ranging 1.5-6.8 mg g^{-1} (Orosa et al., 2001; Del Campo et al., 2004; Liu et al., 2012; 2016; Chen et al., 2017). Another study showed accumulation of 13.1 mg g^{-1} astaxanthin where phytohormones were added to a high algal suspension (inoculum) in microplates (Chen et al., 2020). In above-described studies, the astaxanthin productivity was lower, so there were attempts to improve the productivity using a two-stage method, by increasing the cell density in dark via fermentation process and then inducing the stress (high light) to improve the astaxanthin productivity (5.24 and 9.9 mg L^{-1} d^{-1}) as described (Table V-2). However, the astaxanthin content was still lower than *H. pluvialis* (Zhang et al., 2017a; Sun et al., 2019). While this PhD study reached highest biomass productivity using mixotrophic fed-batch

cultivation (Table IV-1, 2) and achieved a comparable amount of astaxanthin productivity (2.73 mg L^{-1} d^{-1}) with the previous studies, however, the astaxanthin content was seemingly low. Thus, it can be inferred that it is important to not only increase the astaxanthin productivity via two- stage process but also the astaxanthin content on cell basis. This PhD study was a combination of three phase which specifically focused on preculture, biomass production and carotenoid induction phase. The effect of preculture on the following phases depicted the morphological changes, accumulation of nuclei, lipids and starch, which greatly affected the cell proliferation in the light mode, increasing the biomass production of *C. zofingiensis*. Even though the astaxanthin productivity was low as compared to the previous studies, it still showed the probability of increase in the astaxanthin production using a three-phase cultivation strategy method. There have been reports on heterotrophic fed-batch fermentation of *C. zofingiensis* for production of astaxanthin consisting of acclimation stage (Sun et al., 2019), but none of the studies focused on mixotrophic fed-batch for production of astaxanthin. The low light acclimation method of the high dense mixotrophic fed batch cells did not show many differences in the astaxanthin productivity of heterotrophic precultured cells. However, further studies are still needed to understand the changes during the acclimation period as well as the high light intensity period. The exhaustion of nitrate is also important to understand the trigger of astaxanthin biosynthesis. The combination of the proposed three phase transitional modes strategy with some previous knowledge such as addition of some phytohormones or chemical inducers, using column tubular reactors for equal distribution of light intensity to each cell (Chen et al., 2020; 2021) shall also be evaluated in the future. Furthermore, expression of enzymes and the important precursors for the improvement of the astaxanthin biosynthesis in the metabolic pathways should be clearly understood.

5.3 A suggested cell cycle approach for improving biomass and commercial astaxanthin production

The coordination of the cell cycle (multinucleation in the dark and cellular division in the light) in *C. zofingiensis* can be exploited as a unique approach for a higher biomass and astaxanthin productivity. In this study, the response of *C. zofingiensis* to nutritional modes transition was characterized by the switch between multinucleation and cellular division (Chowdhary et al., 2023). The heterotrophically precultured cells cultured in the dark mode (heterotrophic production mode) confirmed the triggering of multinucleation. However, the transition of heterotrophically precultured cells into the irradiated period (autotrophic production mode) showed nuclear division (multinucleation) was not as active as cellular division and the growth relied on multiple fission. The multiple fission in this study was supported by the utilization of the stored intracellular compounds. The transition of *C. zofingiensis* from the dark (for multinucleation) to continuous light (for multiple fission) phase can lead to successful mass cultivation. The mechanisms of multinucleation and rapid cellular division in *C. zofingiensis* can be a practical and economical approach for higher biomass productivities.

The triggers for nuclei accumulation and effect of intracellular compounds are not well understood. Future studies on optimization of culture conditions based on multinucleation and intracellular compound accumulation may further enhance the productivity of *C. zofingiensis*.

5.4 Further studies

Although all of the previous research studies on the cultivation of *C. zofingiensis* cells in heterotrophic conditions, for example; 7.03 g L^{-1} d^{-1} in (Zhang et al., 2017a) and 5.8 g L^{-1} d^{-1} in (Sun et al., 2019) showed high biomass productivities than this current study, but it can be confirmed that a higher or similar biomass productivity can be achieved by the suggested nutritional modes with mixotrophic cultivation (2.52 g L^{-1} d^{-1}, this PhD study). Thus, even higher biomass productivity can be aimed by controlling the wavelength and intensity of the light source, so that there is uniform light distribution throughout the reactor volume with higher initial cell density in a high-volume flat panel reactors and higher glucose concentrations might be considered with the mixotrophic fed-batch cultivations in the future.

In *Chromochloris zofingiensis*, the series of cell cycle events (multiple nuclei accumulation in the darkness and division of cells in the light) could be used as a distinctive biological strategy to increase biomass productivity. Whilst the impact of exposure time in dark heterotrophic cultivations was observed in our study by comparing autotrophically precultured cells in dark for 6 days (one time) and heterotrophically precultured cells in the dark for 18 days (three times). The triggers in the 2nd dark culture period for nuclei accumulation specifically, have not been explored. The role of intracellular components for the enhancement of the growth is also not fully implied either. Thus, enhancing the productivity of *C. zofingiensis* by the improvements of the cultivation conditions based on accumulation of multiple nuclei and intracellular components and the underlying mechanism of cell cycle division is needed in the future.

Even though this PhD study did not reach a higher astaxanthin productivity in comparison to the aforementioned research studies (Mulders et al., 2014; Sun et al., 2019), but

we propose the column reactors (Fig. V-2) with combination of the nutritional modes for the enhancement of astaxanthin accumulation and productivity. The enhancement or the triggers of the precursors and the genes involved in the biosynthesis of the astaxanthin should be highly considered. Also, high light is known as the best inducer for the astaxanthin accumulation (Liu et al., 2014; Zhang et al., 2017a; Sun et al., 2019), however, considering, the light path, thin column reactors, longer cultivation period, other light sources such as LEDs, for higher astaxanthin accumulation might be an ideal choice.

Future experimental design

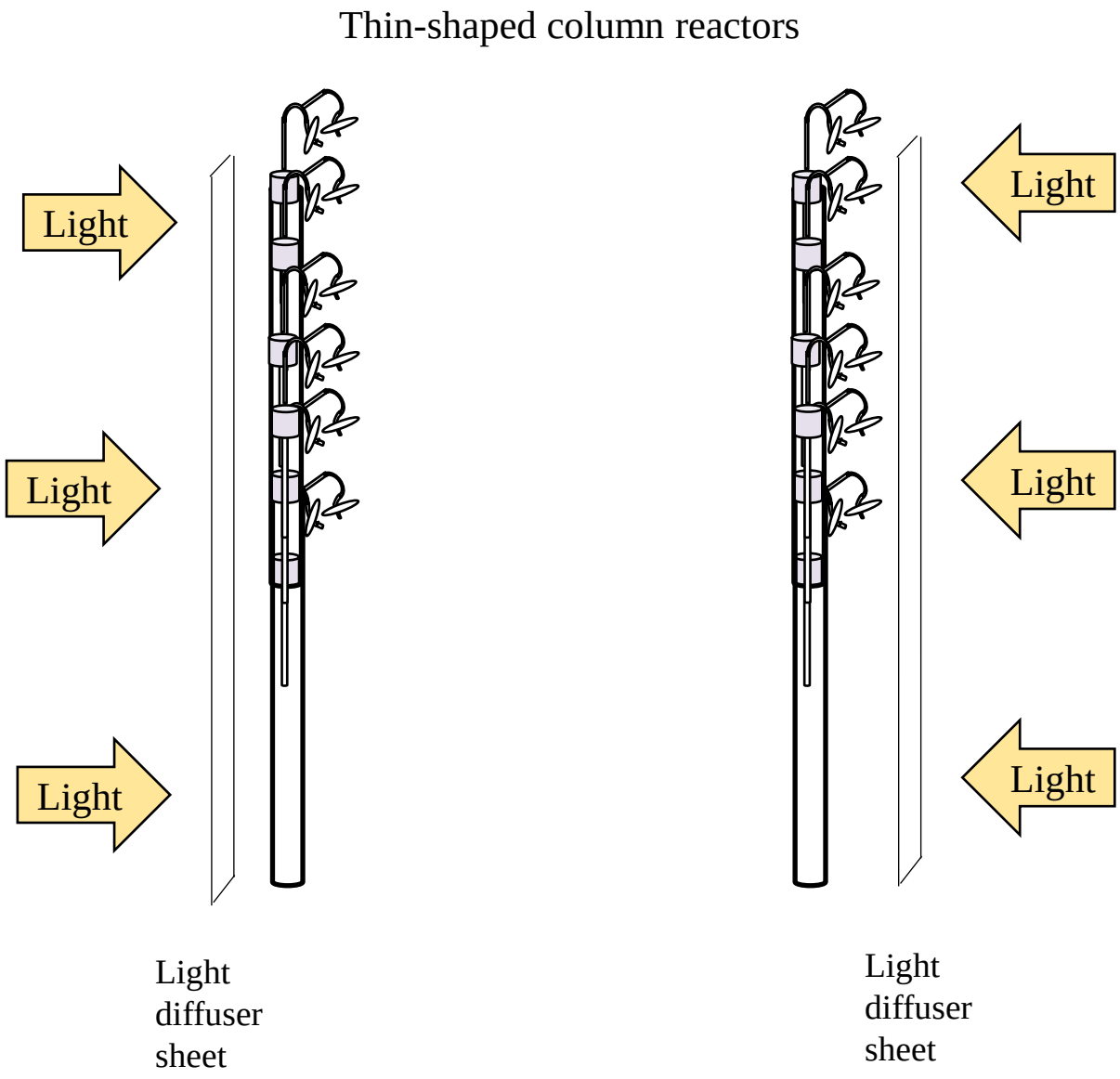


Fig. V-2. Long-thin column shaped reactor design for future experimental analysis

Tables

Table V-1. Comparison of the mixotrophic biomass productivities of *Chromochloris zofingiensis*.

Culture conditions			Preculture			Initial cell density	Biomass productivity	References
	Light	Glucose	Mode	Light	Glucose			
	(μmol m^{-2} s^{-1})	(g L^{-1})		(μmol m^{-2} s^{-1})	(g L^{-1})	(g L^{-1})	(g L^{-1} d^{-1})	
Batch	>80	30	M	10	10	< 0.5	0.59	Chen et al., 2017
Batch	30	5	A	30, continuous	–	N.D.[a]	0.72	Wang et al, 2013
Batch	N.D.	36	A	50	–	N.D.[a]	0.59	Oncel et al., 2010
Batch	100	5	H	Dark	5	0.5	2.63	Zhang et al., 2017b
Batch	N.D.	N.D.	H	Dark	5	0.6	2.50	Zhang et al., 2021
Batch	260	10	H	Dark	10	<0.1	1.28	This study
Fed-batch	260	10	H	Dark	10	0.5	2.51	

Notes: A: autotrophic; M: Mixotrophic; H: Heterotrophic; N.D.: No Data
[a] The cultures were inoculated with the seed at 10% volume to volume ratio.

Table V-2. Comparison of biomass and astaxanthin production in *Chromochloris zofingiensis* in previous studies

Seed culture/ Inoculum	**Preculture**	**Biomass phase**	**Astaxanthin induction phase**	**Initial cell density ($g\ L^{-1}$)**	**Growth rate (d^{-1})**	**Biomass yield ($g\ L^{-1}$)**	**Biomass productivity ($g\ L^{-1}\ d^{-1}$)**	**Astaxanthin content ($mg\ g^{-1}$)**	**Astaxanthin productivity ($mg\ L^{-1}\ d^{-1}$)**	**References**
Autotrophic	-	-	Autotrophic, -N, glass columns (0.25L)	0.5	-	2	0.53	4.9	1.79	Liu et al., 2016
Autotrophic	-	-	Autotrophic, NaCl	-	0.96	6.7	0.7	1.5	2.8	Del campo et al., 2004
Autotrophic	-	-	Autotrophic, NaCl (0.5L)	-	-	-	-	6.8	0.8	Orosa et al., 2001
Autotrophic	-	-	Heterotrophic, Molasses	0.5	0.8	12.9	1.55	1.18	1.7	Liu et a., 2012
Mixotrophic	-	-	Mixotrophic, Blue LEDs, microplates (0.25L)	< 0.5	-	5.05	0.42	5.53	2.33	Chen et al., 2017
Heterotrophic	-	Heterotrophic, Fed-batch (4L)	Mixotrophic (-N, dilutions, rotating photobioreactor ; >1L)	0.5	2.38	98.4	7.03	1.12	5.24	Zhang et al., 2017a

Continued Table V-2. Comparison of biomass and astaxanthin production in *Chromochloris zofingiensis* in previous studies

Seed culture/ Inoculum	**Preculture**	**Biomass phase**	**Astaxanthin induction phase**	**Initial cell density ($g\ L^{-1}$)**	**Growth rate (d^{-1})**	**Biomass yield ($g\ L^{-1}$)**	**Biomass productivity ($g\ L^{-1}\ d^{-1}$)**	**Astaxanthin content ($mg\ g^{-1}$)**	**Astaxanthin productivity ($mg\ L^{-1}\ d^{-1}$)**	**References**
Heterotrophic	-	Heterotrophic, Fed-batch (3L)	High light, column reactors	1.5	-	60	5.8	2.69	9.9	Sun et al., 2019
Autotrophic	Heterotrophic	Mixotrophic, Fed-batch (1L)	High light, test tubes (0.40L)	0.5	**1.07**	**9.06**	**2.52**	**0.35**	**1.00**	**This study**
			1D LL acclimation, High light					**0.13**	**0.37**	
			2D LL acclimation, High light					**0.28**	**0.95**	
			4D LL acclimation, High light					**0.32**	**0.87**	
Autotrophic	Autotrophic	Mixotrophic, Fed-batch (1L)	High light, test tubes (0.40L)	0.5	**0.74**	**8.15**	**1.72**	**0.18**	**0.68**	**This study**
			1D LL acclimation, High light					**0.23**	**1.03**	
			2D LL acclimation, High light					**0.31**	**0.89**	
			4D LL acclimation, High light					**0.30**	**2.73**	

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