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

## *Chromochloris zofingiensis* のバイオマス生産とアスタキサンチン生産における多段階培養プロセスの栄養モードの最適化

17D5707 Chowdhary Anupreet Kaur 指導教員名 戸田 龍樹

## SYNOPSIS

*Chromochloris zofingiensis* is an emerging alternative for the commercial production of astaxanthin, to *Haematococcus pluvialis*, due to its high productivity and ability to grow in multiple nutritional modes namely, autotrophic, mixotrophic and heterotrophic. The cultivation of microalgae in multiple culture steps (stock, preculture, and biomass production phase) in different nutritional modes can contribute to the enhancement of cell biomass and has hardly been performed. Moreover, it's effect on cell growth and biomass productivity is still unknown. Therefore, in this study, we firstly observed the cell growth in different nutritional modes in preculture phase (Chapter II, Study 1). Later, the effect of preculture on the biomass production via combination of nutritional modes for determining the optimum conditions (stock, preculture and biomass production phase) was evaluated (Chapter III, Study 2). As a result, heterotrophically precultured cells showed higher biomass productivities than with auto- and mixotrophically precultured cells in autotrophic and mixotrophic in biomass production phase. The increase in productivity was due to the stored cellular materials such as lipids, starch and nuclei in heterotrophically precultured cells (Study 2). In the three-phase culture, a highest biomass productivity was obtained in mixotrophic condition (Chapter IV). This study suggested that the optimum combinations of nutritional modes for higher biomass and astaxanthin productivity are autotrophic for stock (to maintain the following heterotrophic growth), heterotrophic for preculture (to store growth-promoting factors), mixotrophic for biomass production phase (to achieve high productivity), and autotrophic (for astaxanthin induction), respectively.

Keywords: *Chromochloris zofingiensis*, biomass production, astaxanthin, multiple fission, nutritional modes.

## Introduction

The microalgal pigment production has attracted a lot of attention due to their high valuable commodities in several industries, including cosmetics, feed additives, and health food production[1,2,3,4]. *Haematococcus lacustris* is applied in the industrial astaxanthin production currently, however, its low cell density, easy contamination, long cultivation period limits the scale of its production[2]. However, the green microalga *Chromochloris zofingiensis* is an emerging alternative for astaxanthin production to due to its high productivity and its fast-growing ability in multiple nutritional modes[5,6,7]. The possible improvements in the production of *C. zofingiensis* in optimum conditions makes it a potential candidate in meeting many commercial demands.

The different nutritional modes such as autotrophic, mixotrophic and heterotrophic modes, is known to affect the biomass productivity, cell morphology, and chemical compositions of *C. zofingiensis*[8]. However, there are some advantages and disadvantages of each nutritional modes in terms of productivity, pigments, and growth rate. Therefore, combinations of different nutritional modes for biomass and astaxanthin production can be an effective strategy. The rapid cellular division due to the formation of multinucleated cells in a cell cycle can be beneficial for increasing the biomass production in *C. zofingiensis.* In addition, intracellular energy storage compounds may also contribute to enhance the growth upon the transition of nutritional modes. The clarification of the multinucleation trigger can also contribute to the improvement of the mass-cultivation of *C. zofingiensis.*

The biomass productivity of *C. zofingiensis* has been optimized for light intensity[9,10], glucose concentration[11,12], and nitrate concentration[11,13]. As for astaxanthin induction, light intensity[1,13,14,15], nutrient concentration[14,16], and salinity[17,18] have been optimized. Furthermore, a two-stage process can be conducted where firstly, optimization of the biomass production phase followed by the astaxanthin induction phase under different culture condition has been performed[2,15]. For example, increase in the biomass using a heterotrophic fed-batch cultivation, followed by high light induction for increasing the astaxanthin content has been conducted previously However, the effect of transition of the culture conditions in a culture phase (i.e., stock, preculture, or biomass production) to the following phase has hardly been studied. There is also minimal information regarding the cell growth, morphology, chemical compositions induced by the transition of different nutritional modes.

Thus, the overall objective of this Ph.D. thesis is to suggest the optimum combinations of nutritional modes for stock, preculture and biomass production phase. Then, the effect of these culture phase on astaxanthin induction phase was also conducted. The specific objectives are, Chapter 2- To enhance the growth of *C. zofingiensis* in different nutritional modes at the preculture stage. Chapter 3- To suggest and clarify the best nutritional conditions for stock, preculture and biomass production phase. Thereafter, the feasibility of the suggested nutritional modes for multiple phases was evaluated in terms of improvement of the mass-cultivation of *C. zofingiensis* as a part of general discussion.

## Materials and Methods

Our study conducted the optimization of nutritional modes (autotrophic, mixotrophic and heterotrophic) in preculture and biomass production phase. In this process we evaluated the effect of stock (as

described below) and preculture (in different nutritional modes) on biomass production phase in different nutritional modes.

**Stock culture**: The green microalga, *Chromochloris zofingiensis* (Dönz) Fucíková & L.A. Lewis ATCC 30412, was obtained from American Type Culture Collection (ATCC) Rockville, USA. The algal stock was maintained in a modified Bristol's medium (MBM)[11] in 250-mL Erlenmeyer® flasks and was illuminated with a light intensity of 60 µmol $m^{-2}$ $s^{-1}$ with 12-h light:12-h dark at 25 °C.

***Study 1. Growth of* C. zofingiensis *in different nutritional modes***

**Preculture**: Cells from the stock culture were inoculated into the 1st preculture, where algal cells were maintained in three different nutritional modes (autotrophic, mixotrophic and heterotrophic) with continuous aeration (0.2 vvm) in MBM at 25 °C for 6 days. A set of sequential precultures was performed, where cells from the 1st preculture were inoculated to the 2nd preculture for the next 6 days under the same nutritional modes.

**Biomass production phase**: Cells from the 2nd preculture were collected, washed, and resuspended at an optical density of 0.1. Then, they were subjected to batch cultures of the three) for the biomass production phase, in 1L medium bottles with 700 mL working culture volume, with continuous aeration with atmospheric air (0.2 vvm) and agitation (260 rpm) in MBM until they reached the stationary phase. The light was irradiated continuously (24-h) for autotrophic and mixotrophic condition, and the heterotrophic condition was kept under complete darkness. The light intensity and the glucose concentration were 260 µmol $m^{-2}$ $s^{-1}$ and 10 g $L^{-1}$, respectively. Sampling was aseptically performed every 24-h, and all the experiments were conducted in triplicates.

The following parameters were used for this experiment: cell dry weight, optical density, nutrient concentration ($NO_3$-N and $PO_4$-P) cell length (µm), pigment compositions, starch, and lipid.

***Study 2. Modified three-phase culture of* C. zofingiensis *for astaxanthin production***

The cells from the stock were cultured in aerated (0.2 L $m^{-1}$) MBM at 25 ℃ for 6 days as precultures (1st) in two modes: autotrophic (A) using 260 µmol $m^{-2}$ $s^{-1}$ continuous light and heterotrophic (H) using 24-h darkness and 10 g $L^{-1}$ glucose. Another set of precultures (2nd) was prepared using the same experimental conditions (A and H) as described above for an additional 6 days to allow the cells to adapt/acclimatize to the same conditions. The cells were then collected on the last day of each 2nd preculture, washed, and resuspended at an optical density of 0.1 and exposed to the following two experimental culture conditions: 1) irradiated (24-h light at 260 µmol $m^{-2}$ $s^{-1}$) and 2) dark (24-h dark and 10 g $L^{-1}$ glucose). Both A and H resuspended cells were subjected to irradiation and denoted as A−A and H−A, respectively. Similarly, the cells from each preculture transferred to the dark culture mode were denoted as A−H and H−H, respectively. The experiments were conducted with continuous aeration (0.2 L $m^{-1}$) and agitation (260 r $min^{-1}$) in MBM until cell growth reached the stationary phase.

The following parameters were used for this experiment: cell dry weight, cell count and length, and nuclei.

The total number of nuclei (N; nuclei $mL^{-1}$) was calculated using equation (1):

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

where C, n, and % $\mathrm{distribution}_n$ are the culture cell density (cells $mL^{-1}$), number of nuclei in each cell (nuclei $cell^{-1}$), and the fraction of cells that had the respective number of nuclei, respectively.

The specific cell number growth rate $\mu_c$ ($d^{-1}$) was calculated using equation (2):

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

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

The specific growth rate of nuclei per cell, µn ($d^{-1}$), was calculated using equation

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

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

Nuclear division is represented as the increase in the number of nuclei per cell, and cytokinesis is represented as the increase in cell number. This study defined the relative nuclear division rate, which was calculated using equation (4):

$$\text{Relative nuclear division rate} = \mu_n - \mu_c, \quad (4)$$

where $\mu_n$ and $\mu_c$ are specific growth rates of nuclei and cell number ($d^{-1}$), respectively. According to the equation, a positive relative nuclear division rate indicates a higher nuclear division, a negative rate indicates higher cytokinesis, and a zero-rate value indicates normal cell division wherein nuclear growth and cytokinesis occur at the same rate.

After the optimization of the stock, preculture and the biomass production phase, these particular nutritional modes were continued for the induction phase for astaxanthin production.

**Fed-Batch**: For fed-batch biomass production, the cells from the stock were cultured in aerated (0.2 L $m^{-1}$) MBM at 25 ℃ for 6 d as precultures (1st) in two modes: autotrophic using 260 µmol $m^{-2}$ $s^{-1}$ continuous light and heterotrophic using 24-h darkness and 10 g $L^{-1}$ glucose. Another set of precultures (2nd) was prepared using the same experimental conditions as described above for an additional 6 days to allow the cells to adapt/acclimatize to the same conditions. The cells collected at the end of the cultivation period of each 2nd preculture were washed, resuspended at 0.5 g $L^{-1}$, and were subjected to mixotrophic fed-batch conditions (24-h light at 260 µmol $m^{-2}$ $s^{-1}$ and 10 g $L^{-1}$ glucose).

**Astaxanthin Induction phase**: After reaching the highest biomass productivity (linear growth phase), cells from auto- to mixo- and hetero- to mixo- 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 in

tubular shaped reactors. After acclimatizing for days 0, 1, 2 and 4, cells were directly transferred for astaxanthin induction phase in high light intensity (380 µmol photon $m^{-2}$ $s^{-1}$).

The following parameters were used for this experiment: cell dry weight, pigment compositions.

## Results and Discussion

### *Study 1. Enhanced growth of* C. zofingiensis *through the transition of nutritional modes*

**Growth in different preculture modes:** The differential growth curves in the sequential precultures (i.e., autotrophic, mixotrophic, or heterotrophic) to the same mode were observed in this study (Fig. 1). The specific growth rates of the mixotrophic, autotrophic, and heterotrophic 1st precultures were 0.867 ± 0.067 $d^{-1}$, 0.586 ± 0.069 $d^{-1}$, and 0.387 ± 0.040 $d^{-1}$, respectively. The high specific growth rate of the mixotrophic culture was probably due to the synergistic relation between photosynthesis and carbon assimilation. The autotrophically and mixotrophically precultured cells exhibited similar growth curves for the 1st and 2nd precultures (Fig. 1a and b), indicating that the cells were possibly adapted to the same environmental conditions.

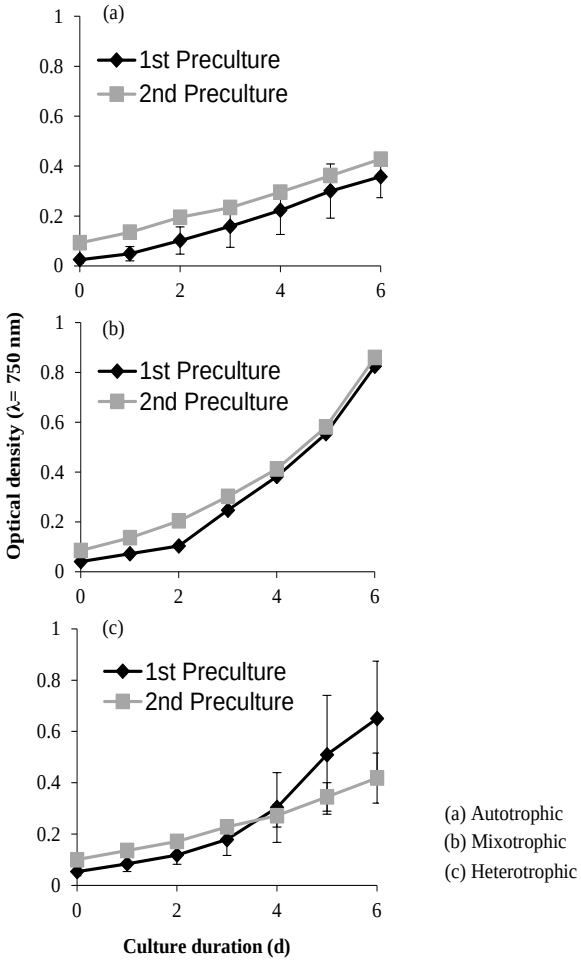


Fig. 1. Growth curve of *C. zofingiensis* in nutritional modes

However, the heterotrophically precultured cells exhibited lower specific growth rates in the 2nd preculture and later in the biomass production phase indicating that the cells continued to react with the environmental change.

**Biomass production phase**: The biomass productivity in biomass production phase were, found to be from high to low, in mixotrophic, autotrophic, and heterotrophic conditions, as with the precultures (Fig. 2). The average of all the preculture conditions in each biomass production phase was calculated. The mixotrophic culture showed the highest maximum specific growth rate (0.868 ± 0.281 $d^{-1}$) as compared with the autotrophic (0.578 ± 0.306 $d^{-1}$) and heterotrophic (0.447 ± 0.070 $d^{-1}$) nutritional modes. Similarly, the maximum biomass productivity was higher 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 $L^{-1}$ $d^{-1}$) biomass production phase. In the previous studies, it was shown that *C. zofingiensis* can simultaneously utilize both light and carbon for growth[1,5]. Therefore, the synergistic relationship between photosynthesis and carbon assimilation could have led to an increase in the biomass productivity in the mixotrophic cultivation of *C. zofingiensis*. The lower biomass productivities in heterotrophic biomass production phase in our study (0.448 ± 0.363 g $L^{-1}$ $d^{-1}$), as compared to

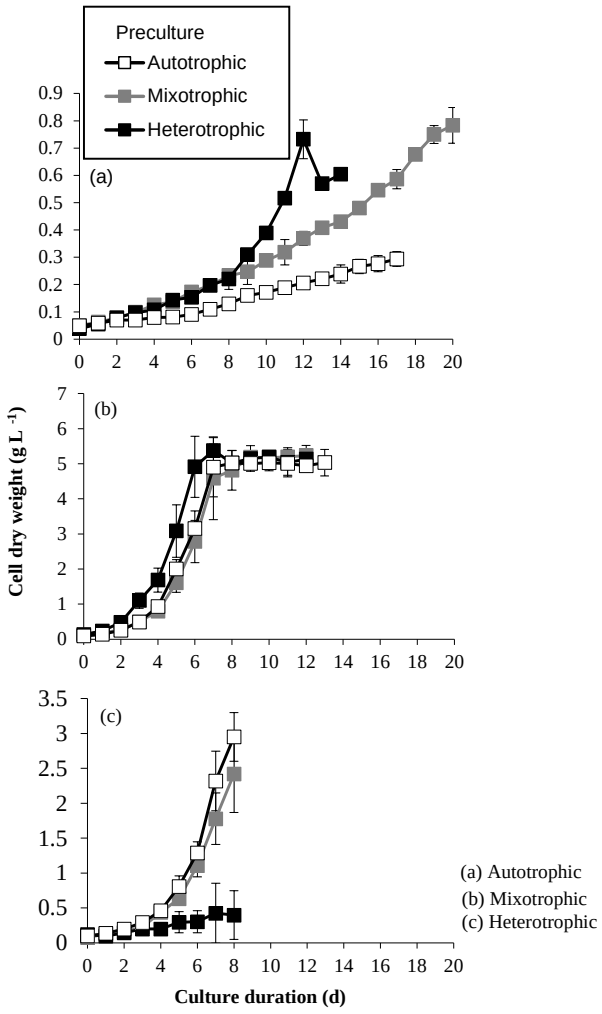


Fig. 2. Growth curve of *C. zofingiensis* in nutritional modes

previous study[2] 7.03 g $L^{-1}$ $d^{-1}$, can be due to the difference in the feeding condition, where this study implemented a one-time batch culture, while the other study implemented a fed-batch culture.

The effect of precultural nutritional mode on the biomass productivity differed between the dark (heterotrophic) and irradiated (auto- and mixotrophic) conditions. In the dark heterotrophic biomass production phase, the lowest growth rate was observed with the heterotrophically precultured cells. As opposed to the dark condition, in the irradiated conditions (autotrophic and mixotrophic biomass production phases), the heterotrophically precultured cells showed higher specific growth rates and biomass productivities than the autotrophically and mixotrophically precultured cells. In the autotrophic biomass production phase, the heterotrophically precultured cells had approximately a 2.2 to 2.4-times higher maximum specific growth rate compared with the autotrophically and mixotrophically precultured cells ($n$ = 3, $p$ < 0.05). Similarly, in the mixotrophic biomass production phase, the heterotrophically precultured cells had a 1.6 to 1.8-times higher maximum specific growth rate compared with the autotrophically and mixotrophically precultured cells ($n$ = 3, $p$ < 0.05). The higher growth in heterotrophically precultured cells were possibly due to accumulation of nuclei and intracellular compounds (Study 2).

**Suggested Conditions**: In this study, the highest biomass productivity was observed in the mixotrophic nutritional mode, especially when the culture was inoculated with heterotrophic cells (Fig. 2). Thus, the combination of a heterotrophic preculture with the mixotrophic biomass production phase is the most appropriate strategy for improving the biomass productivity of *C. zofingiensis*. Therefore, our results suggest that the optimum combinations of nutritional modes are as follows: autotrophic for stock (to induce growth factors for the following heterotrophic culture), heterotrophic for preculture (to accumulate intracellular growth-enhancing compounds, such as starch/lipids), and mixotrophic for the biomass production phase (to maximize biomass productivity).

***Study 2. Modified three-phase culture of* C. zofingiensis *for astaxanthin production***

**Cell proliferation in light**: Our findings showed that heterotrophically multinucleated precultured cells exhibited a high specific cell growth rate upon transition to autotrophic conditions, with the highest rate (1.85 $d^{-1}$) observed on day 1. The increased growth rate in heterotrophically precultured *C. zofingiensis* cells could be due to multiple fission. According to previous studies[19,20], high growth rates are related to larger cells having more than 6–8 nuclei that can divide into several daughter cells. In our study, a higher average number of nuclei (8.07) was observed on day 0 and a rapid reduction in number of nuclei was observed with transition of heterotrophically precultured inoculum to autotrophic conditions than that in the autotrophically precultured cells (Fig 3a).
Based on the fact that only the heterotrophic preculture exhibited multinucleated cells, we concluded that the dark conditions induced multinucleation (Fig. 3b).

**Three-phase culture**: The proposed suggested conditions for higher biomass productivity was then further evaluated to check the effect on the astaxanthin induction phase in *C. zofingiensis.* We observed the highest biomass productivity in fed-batch mixotrophic cultivation mode with heterotrophically than autotrophically precultured cells, 2.52 and 1.72 g $L^{-1}$ $d^{-1}$, respectively (Fig. 4). Astaxanthin content and yield obtained at the end of the cultivation period, 0.50 mg $g^{-1}$ and 9.0 mg $L^{-1}$ was higher in heterotrophically than autotrophically precultured cells, 0.30 mg $g^{-1}$ and 6.5 mg $L^{-1}$, respectively.

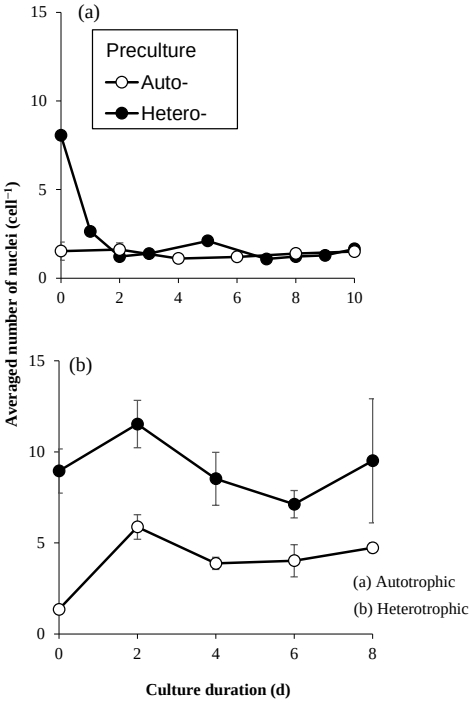

Fig. 3. Growth curve of *C. zofingiensis* in nutritional modes

## Conclusion

1) The present work demonstrated that heterotrophically grown precultured *C. zofingiensis* cells can accumulate intracellular compounds, which can improve the growth rate and productivity when exposed to light.
2) Our findings have shown that the combinations of suitable nutritional modes in the stock (autotrophic), preculture (heterotrophic), and biomass production (mixotrophic) phases can be a feasible way for increasing biomass productivity in *C. zofingiensis*.
3) Moreover, the transition of *C. zofingiensis* from the dark (for multinucleation) to continuous light (for multiple fission) phase can lead to successful mass cultivation.
4) Further, culturing of the cells from high cell density (mixotrophic mode) to high light intensity for astaxanthin accumulation could lead to a successful three-phase cultivation for *C. zofingiensis*.

## References

[1] Zhang et al., 2017, Algal Res. 109-116 [2] Zhang et al., 2017, Bioresour. Technol. 515-522 [3] Liu et al., 2014, Mar. Drugs 3487-3515 [4] Hui Chen et al., 2014, Bioresour. Tech 518-529 [5] Z. Zhang et al., 2021 Bioech. Biofuels 1-16 [6] Roth et al., 2019 Plant Cell 579-601 [7] Sun et al., 2009 Process Biochem. 1288-1292 [8] Azman et al., 2017, PeerJ [9] Imaizumi et al., 2014, Bioresour. Tech 53-59 [10] Imaizumi et al., 2016 Algal Res. 110-117 [11] Ip and Chen., 2005, Process Biochem. 733-738 [12] Wang et al., 2008, Worl J. Microbiol. Biotechnol. 1915-1922 [13] Chen et al. 2017, Bioresource. Technol. 518-529 [14] Bar et al., 1995, J. Plant Physiol. 527-534 [15] Sun et al., 2019, J. Agric. Food Chem. 5579-5586 [16] Minyuk et al., 2020, J. Appl. Phycol. 923-935 [17] Kou et al., 2020, Algal Res. 2211-9264 [18] Pelah et al., 2004, World J. Microbiol. Biotechnol, 483-486 [19] Vítová et al., 2014, Biotechnol. Adv. 1204–1218 [20] Koren et al., 2021, Biology (Basel), 1–17