

Cellular characteristics of *Chromochloris zofingiensis* in different nutritional modes

Anupreet Kaur Chowdhary^{1*}, Masatoshi Kishi^{2,3}, Tatsuki Toda⁴

- 1) Faculty of Science and Engineering, Soka University, 1-236 Tangi-cho, Hachioji, Tokyo 192-8577, Japan
- 2) Institute of Sustainable Processes, University of Valladolid, School of Industrial Engineering, Pso. Prado de la Magdalena 3-5, 47011, Valladolid, Spain
- 3) Research Center for Earth Surface System, Research Institute of Global Change, Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka-City, Kanagawa, 237-0061, Japan
- 4) Laboratory of Restoration Ecology, Graduate School of Science and Engineering, Soka University, 1-236 Tangi-cho, Hachioji, Tokyo 192-8577, Japan

* Corresponding author: Tel.: +81 42 691 9455

E-mail address: chowdhary@soka.ac.jp (A.K. Chowdhary).

Received May 15 2025, Accepted June 5 2025

Abstract *Chromochloris zofingiensis* has ability to produce high value pharmaceutical and nutraceutical pigments in autotrophic, mixotrophic or heterotrophic nutritional mode which makes it a promising microalgal species. In this study, we observed the effect of dilution on growth and biomass concentration in three different nutritional modes. When the cells in auto-, mixo- and hetero-trophic nutritional modes, in this study, reached the highest optical density (OD), they were diluted to ensure they remained in linear range of optical density detection and to understand the morphological differences between each nutritional mode. As a result, the cell behavior changed in each nutritional mode due to difference in conditions. The heterotrophic cells reached the highest biomass concentration (1.11 g L^{-1}) due to largest cell size and possible accumulation of intracellular compounds in the dark phase. On the other hand, cells in mixotrophic and autotrophic nutritional mode reached biomass concentration, 0.88 and 0.40 g L^{-1} , respectively. Our study showed a correlation between growth and dry weight, where cells in each nutritional mode showed almost similar growth with different biomass concentration. We observed an accurate comparison between biomass between conditions where cell shapes might have influenced OD independently of biomass. Thus, it can be said that the differences in nutritional modes affected the cell physiological characteristics of *C. zofingiensis*.

Keywords: biomass, *Chromochloris zofingiensis*, dilution, growth, nutritional modes

Chromochloris zofingiensis, a eukaryotic green microalga, has attracted attention due to its high growth rate, biomass and natural production of high-value compounds such as astaxanthin and canthaxanthin (Liu et al. 2014, Zhang et al. 2017, Chowdhary et al. 2022, 2025). *C. zofingiensis* can be easily cultured under several nutritional modes such as autotrophic, mixotrophic and heterotrophic based on various carbon sources (Sun

et al. 2008, Li et al. 2009, Chen et al. 2015, Sun et al. 2019). However, cells tend to change their cell growth, carotenoids, physiological characteristics such as cell size, and intracellular compounds such as starch, lipids when cultured into different nutritional modes. In our previous study, we showed that cultivation of *C. zofingiensis* through transition of nutritional modes affects not only the growth but also the physiological characteristics (Chowdhary et al. 2023). We also reported the mechanism of multinucleation followed by multiple fission and changes in intracellular compounds by transition of nutritional modes in *C. zofingiensis*. The high optical density can be affected by both cell numbers and cell density based on the nutritional modes in *C. zofingiensis*. However, the dilution of high biomass concentration to understand the relationship between growth and cell dry weight has not been explored. It is possible that diluting high optical density (OD) cells might help reduce the influence of cell shape and size on light scattering, by improving the reliability of OD as a proxy for biomass across varying nutritional modes. The difference in nutritional modes leads to change in cell behavior and

physiological characteristics which might be explained by possible dilutions. Thus, in this study we elucidated a more reliable relationship of whether cell physiology affects the biomass and growth of *C. zofingiensis* in different nutritional modes with dilution strategy.

Chromochloris zofingiensis (ATCC 30412) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The modified Bristol's medium (MBM) (Ip & Chen 2005) was used for maintaining the microalgal stock in 250 mL Erlenmeyer® flasks at 25 °C under a light intensity of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at a 12 h-L/12 h-D photoperiod. This study analyzed the relationship between optical density ($\lambda = 750 \text{ nm}$) with cell dry weight (g L^{-1}). Cells from the stock phase were cultured into three nutritional modes each, namely, autotrophic (with 260 $\mu\text{mol m}^{-2} \text{s}^{-1}$ continuous light intensity), mixotrophic (10 g L^{-1} glucose with 260 $\mu\text{mol m}^{-2} \text{s}^{-1}$ continuous light intensity), and heterotrophic (dark with 10 g L^{-1} glucose) mode with continuous aeration (0.2 vvm) in MBM at 25 °C until the stationary phase. When the cells reached optical density >1.2 (original sample, Ori.) in each nutritional modes, cells were diluted (1x,

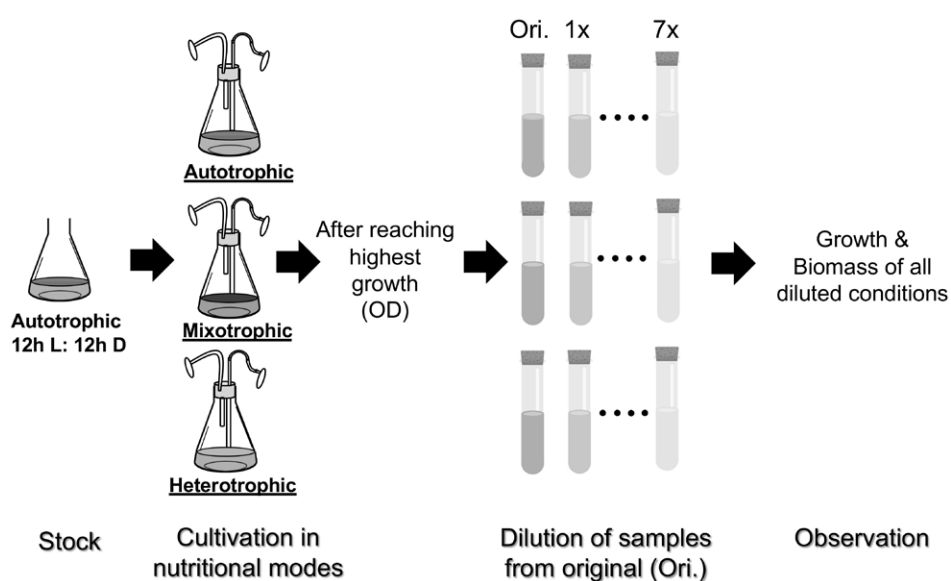


Fig. 1. Cultivation of *Chromochloris zofingiensis* in three nutritional modes and observation of growth using a dilution strategy from the original (Ori.) sample, with high optical density in each nutritional mode.

2x, 3x, 4x, 5x, 6x, and 7x) with distilled water. After preparation of different dilutions, the optical density and the cell dry weight of the diluted culture in each nutritional mode was observed (Fig. 1). The cultures were conducted in monoplicate.

The cell dry weight analysis was performed by using pre-weighed glass fiber filters with a pore size of 0.7 μm (GF/F, Whatman, USA). The filtered cells were washed three times with distilled water and dried at 80 $^{\circ}\text{C}$ for 8 h for the dry weight measurements. The growth was measured by using a UV-visible spectrophotometer (UV-2450, Shimadzu, Japan) at optical density (OD), $\lambda = 750 \text{ nm}$. Two different types of hemacytometer, a bacteria counter (SLGC, Koshigaya, Japan) and Thoma (Hirschmann, Germany), were used depending on cell length and density.

The growth of *Chromochloris zofingiensis* in all three nutritional modes, autotrophic, mixotrophic and heterotrophic, reached about 1.29, 1.40, and 1.21 (OD) respectively. The specific growth rate was the highest in mixotrophic (0.89 d^{-1}) as compared to autotrophic and heterotrophic nutritional modes (0.72 and 0.50 d^{-1} , respectively). The highest growth rate in mixotrophic cultivation mode was possibly because *C. zofingiensis* can utilize both light and carbon sources for growth (Chen et al. 2017, Sun et al. 2019, Chowdhary et al. 2022). However, the physiological characteristics of *C. zofingiensis* changed with respect to different nutritional modes. The cells were larger in size in heterotrophic ($14.6 \mu\text{m}$), as compared to mixotrophic and autotrophic nutritional modes (9.17 and $5.44 \mu\text{m}$, respectively) (Fig. 2). The largest cell size of *C. zofingiensis* in heterotrophic cultivation led to highest cell dry weight, 1.11 g L^{-1} , possibly due to accumulation of intracellular compounds and multiple nuclei (Chowdhary et al. 2023). The cell dry weight in mixotrophic and autotrophic nutritional mode reached 0.88 and 0.40 g L^{-1} , respectively. The lowest dry

weight in autotrophic nutritional mode can perhaps be due to smaller cell size and low accumulation of intracellular compounds (Chowdhary et al. 2022, 2023). Even though cells were smaller in size in autotrophic nutritional mode, the growth (OD) reached almost similar to cells cultured in two other nutritional modes; mixotrophic and heterotrophic. Different nutritional modes are known to stimulate production of nuclei, intracellular compounds such as starch, lipids, and biosynthesis of carotenoids (Chowdhary et al. 2023, 2025).

In this study, the relationship between *C. zofingiensis* cell growth (OD) and biomass concentration was also

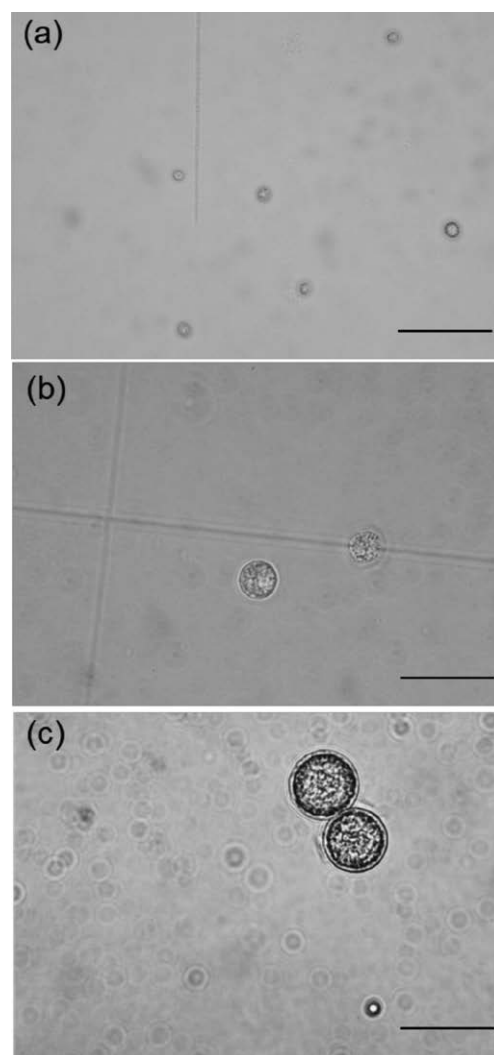


Fig. 2. Pictures of *Chromochloris zofingiensis* cells cultured in (a) auto-, (b) mixo-, and (c) hetero-trophic nutritional modes.

Scale bar 20 μm

observed. After reaching highest cell growth, cells were diluted (1x to 7x) and later the growth of cells in each nutritional mode was observed (Fig. 3). At high cell densities, OD sometimes can become non-linear due to light scattering or change in shapes. The dilution strategy in this study generated a more reliable relationship between OD and actual biomass, which enabled correction across the nutritional modes (Fig. 4). It also helped limit the influence of cell shape and size on OD measurements. It is known that *C. zofingiensis* has unique cellular characteristics which change with nutritional modes (Azaman et al. 2017, Chowdhary et al. 2022). In this study, it was noticed that *C. zofingiensis* cells cultured in autotrophic nutritional mode showed similar growth (OD, $\lambda = 750$ nm) to cells cultured in mixotrophic and heterotrophic modes, but due to smallest cell size the dry weight did not reach as high as other two nutritional modes (Fig. 4). It is possible that the cellular content or cell density of *C. zofingiensis* possibly led to higher growth in autotrophic nutritional mode (Chowdhary et al. 2022). However, the large size of heterotrophic *C. zofingiensis* cells probably led to accumulation of

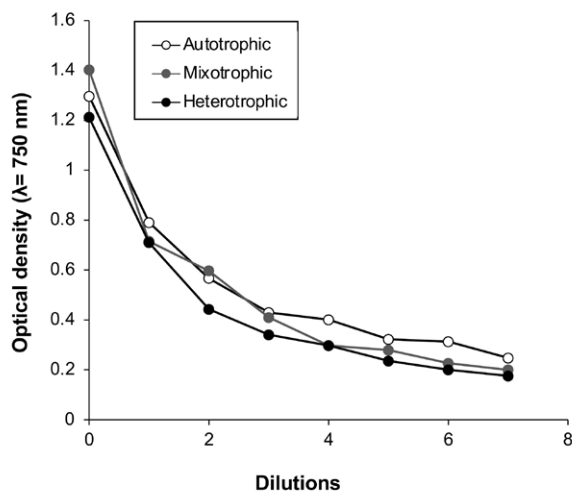


Fig. 3. Growth ($\lambda=750$ nm) of *Chromochloris zofingiensis* after dilutions, starting from 0 to 7, where 0 is the original sample and 1, 2, 3, to 7 are the dilution factors 1:1, 1:2, 1:3 until 1:7, respectively.

higher cellular content (lipids, starch, nuclei) producing highest biomass as compared to the cells in autotrophic nutritional mode (Chowdhary et al. 2023). The cells in mixotrophic nutritional mode, on the other hand, showed high growth and biomass possibly due to larger cell size (9 μ m) and high intracellular content. In our previous study, the highest growth rate and biomass productivity was observed in mixotrophic nutritional mode (Chowdhary et al. 2022). The highest dilution (7x) also showed that with the same growth (about 0.2, OD), biomass concentration in each nutritional mode was different. The differences can be owed to the change in cell size, or cell density in *C. zofingiensis* in autotrophic, mixotrophic and heterotrophic nutritional mode.

The differences in physiological characteristics were noticeable due to the dilution of the cells in different nutritional modes when they reached high biomass. In a previous study, it was found that the light efficiency increased when the cells were diluted and transferred for efficient production of astaxanthin (Wan et al. 2015, Zhang et al. 2017). It is possible that improved light efficiency in

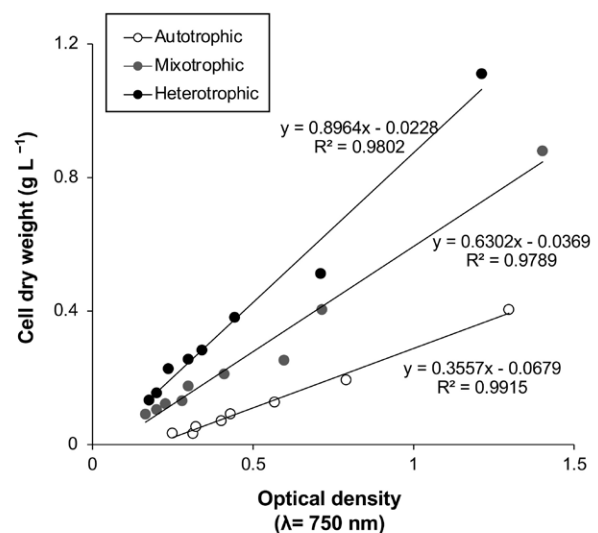


Fig. 4. Relationship between growth ($\lambda=750$ nm) and cell dry weight (g L^{-1}) in *Chromochloris zofingiensis* in auto-, mixo-, and hetero-trophic nutritional modes with dilution strategy.

C. zofingiensis during the biomass production phase with dilution might also improve the biomass and affect the cellular characteristics. Thus, future studies are required to examine the effect of dilution on cell biomass and cellular characteristics in different nutritional modes in *C. zofingiensis* for improved biomass production.

References

- Azaman SNA, Nagao N, Yusoff FM, Tan SW, Yeap SK (2017) A comparison of the morphological and biochemical characteristics of *Chlorella sorokiniana* and *Chlorella zofingiensis* cultured under photoautotrophic and mixotrophic conditions. *PeerJ* 5: e3473.
- Chen JH, Liu L, Wei D (2017) Enhanced production of astaxanthin by *Chromochloris zofingiensis* in a microplate-based culture system under high light irradiation. *Bioresour Technol* 245: 518–529.
- Chen T, Liu, J, Guo B, Ma X, Sun P, Liu B, Chen F (2015) Light attenuates lipid accumulation while enhancing cell proliferation and starch synthesis in the glucose-fed oleaginous microalga *Chlorella zofingiensis*. *Sci Rep* 5: 1–10.
- Chowdhary AK, Kishi M, Toda T (2022) Enhanced growth of *Chromochloris zofingiensis* through the transition of nutritional modes. *Algal Res* 65: 102723.
- Chowdhary AK, Kishi M, Toda T (2023) A novel process for the production of *Chromochloris zofingiensis* through dark-induced multi-nuclei formation. *Algal Res* 71: 103053.
- Chowdhary AK, Kishi M, Toda T (2025) Astaxanthin induction in *Chromochloris zofingiensis* by transition of nutritional modes. *J Appl Phycol*:1–8
- Ip PF, & Chen F (2005) Production of astaxanthin by the green microalga *Chlorella zofingiensis* in the dark. *Process Biochem* 40: 733–738.
- Li Y, Huang J, Sandmann G, Chen F (2009). High-light and sodium chloride stress differentially regulate the biosynthesis of astaxanthin in *Chlorella zofingiensis* (Chlorophyceae). *J Phycol* 45:635–641.
- Liu J, Sun Z, Gerken H, Liu Z, Jiang, Y, Chen F (2014). *Chlorella zofingiensis* as an alternative microalgal producer of astaxanthin: Biology and industrial potential. *Mar Drugs* 12: 3487–3515.
- Sun N, Wang Y, Li YT, Huang JC, Chen F (2008). Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic *Chlorella zofingiensis* (Chlorophyta). *Process Biochem* 43: 1288–1292.
- Sun Z, Zhang Y, Sun LP, Liu J (2019). Light elicits astaxanthin biosynthesis and accumulation in the fermented ultrahigh-density *Chlorella zofingiensis*. *J Agric Food Chem* 67: 5579–5586.
- Wan M, Zhang Z, Wang J, Huang J, Fan J, Yu A, Wang W, Li Y (2015). Sequential Heterotrophy–Dilution–Photoinduction Cultivation of *Haematococcus pluvialis* for efficient production of astaxanthin. *Bioresour Technol* 198: 557–563.
- Zhang Z, Huang JJ, Sun D, Lee Y, Chen F (2017). Two-step cultivation for production of astaxanthin in *Chlorella zofingiensis* using a patented energy-free rotating floating photobioreactor (RFP). *Bioresour Technol* 224: 515–522.