Effect of magnesium chloride on microalgal biomass and canthaxanthin accumulation in *Chromochloris zofingiensis*

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Abstract

Chromochloris zofingiensis has been regarded as a potential producer of natural high-value bioproducts and is capable of accumulating secondary carotenoids under environmental stress conditions. Canthaxanthin is a secondary carotenoid that possesses powerful antioxidant activity and provides beneficial effects for human and animal health. Various means to improve carotenoid production have been employed including treatment of microalgal cultures with exogenous chemical treatments to instigate environmental stress conditions. However, information of exogenous chemical treatment on biomass and canthaxanthin production in *C. zofingiensis* is still limited. This study aimed to determine the effects of different concentrations of magnesium chloride ($MgCl_2$) on biomass and canthaxanthin accumulation in *C. zofingiensis*. The algal cells were cultured under the mixotrophic condition in the proteose medium supplemented with glucose 20 g L^{-1} for 8 days. $MgCl_2$ was supplied into mixotrophic *C. zofingiensis* cultures at various doses of 2.5, 5, and 10 mM. Specific growth rate, cell density, and biomass concentration of each $MgCl_2$-treated culture were determined and the accumulation of canthaxanthin in *C. zofingiensis* was evaluated by HPLC analysis. The results revealed that $MgCl_2$ did not significantly inhibit *C. zofingiensis* growth rate. Under the mixotrophic condition, the cell density of *C. zofingiensis* was greatly increased on day 8 of cultivation compared to day 0. $MgCl_2$ treatment at 5 and 10 mM could considerably induce canthaxanthin accumulation in *C. zofingiensis* by about 28.87-42.40% compared to control, in which the highest canthaxanthin content of 0.937 mg g^{-1} was found upon treatment with 5 mM $MgCl_2$, whereas low concentration at 2.5 mM gave slightly lower canthaxanthin content than the control. The results of this study thus have an implication for the development of the strategy to improve canthaxanthin accumulation in *C. zofingiensis*.

Keywords*: *Chromochloris zofingiensis, magnesium chloride, biomass, canthaxanthin

1. Introduction

Carotenoids are a group of fat-soluble compounds appearing in yellow to reddish-brown colors and contain a central C40 polyene backbone of eight isoprene units (Ambati et al., 2019; D'Alessandro & Antoniosi, 2016; Varela et al., 2015). Biosynthesis of carotenoids can be found in various organisms such as in higher plants, yeasts, bacteria, and algae, but humans and animals cannot synthesize carotenoids and must obtain them from their diet (Breithaupt, 2007; Jackson, Braun, & Ernst, 2008). Carotenoid pigments exhibit the important biological action as potent antioxidant molecules in which they offer beneficial effects to human health, especially their ability in scavenging reactive oxygen species (ROS) and promote protective actions against several human diseases caused by oxidative damage (Breithaupt, 2007; J. Zhang et al., 2014). Moreover, the use of natural compounds has proven to be safe and more potent than those of synthetic derivatives, leading to increasing demand for natural carotenoids in the global markets (D'Alessandro & Antoniosi, 2016; Spolaore et al., 2006; Varela et al., 2015).

Microalgae are an important biological source of nutritional contents and high-value bioproducts, including carbohydrates, proteins, lipids, essential fatty acids, and carotenoids (Spolaore et al., 2006). Among diverse microalgal species, *Chromochloris zofingiensis* is a unicellular green microalga that has been suggested as a potential producer of secondary carotenoids such as canthaxanthin (Cezare-Gomes et al., 2019). This species is capable of being cultured not only under photoautotrophic conditions but also

under heterotrophic and mixotrophic conditions (Liu et al., 2014). Besides, the fast growth property of *C. zofingiensis* can produce high cell density. Among carotenoids produced by microalgae, canthaxanthin is considered a strong antioxidant compound, in which its activity is much stronger than other carotenoids such as β-carotene. Especially, the Food and Drug Administration (FDA) has approved canthaxanthin for safe use as feed additives to improve skin colors in poultry, salmon, and trout (Breithaupt, 2007). Moreover, this pigment has beneficial biological activities such as immunomodulatory and anti-cancer activities, making it an attractive high-value carotenoid for healthcare applications (Cezare-Gomes et al., 2019; Gorgich et al., 2021).

It is observed that secondary carotenoids can be produced in *C. zofingiensis* when exposed to environmental stress (Liu et al., 2014). Some chemicals can be used to induce stress conditions and stimulate microalgal growth and astaxanthin pigment production in *Haematococcus pluvialis* (Yu, Chen, & Zhang, 2015). Metal ion such as magnesium plays an important role in cellular metabolisms and serves as an essential component in chlorophyll (Dong et al., 2019). A previous study in *Monoraphidium* sp. FXY-10 indicated that the addition of Mg^{2+} can induce algal growth and lipid production (Huang et al., 2014). However, to our knowledge for the *C. zofingiensis* microalga, information on the effects of magnesium chloride ($MgCl_2$) on cell growth and pigment production, especially canthaxanthin, remains limited. The present study mainly focused on the investigation of the effects of various concentrations of $MgCl_2$ treatment on biomass and canthaxanthin production in *C. zofingiensis* mixotrophic cultures. Results obtained from this study would be the first report on the use of $MgCl_2$ to enhance canthaxanthin production in *C. zofingiensis*. This cultivation strategy would be beneficial and feasible for developing the commercial production of high-value carotenoids in microalgae.

2. Objectives

This study aimed to evaluate the effects of different concentrations of magnesium chloride ($MgCl_2$) on microalgal biomass and canthaxanthin production in *Chromochloris zofingiensis* under mixotrophic culture conditions. $MgCl_2$ is classified as a metal ion group, in which Mg^{2+} is a trace element involved in important cellular components and mechanisms. Moreover, the application of exogenous $MgCl_2$ could be a feasible and inexpensive manner to enhance high-value pigment production in *C. zofingiensis*.

3. Materials and Methods

3.1 Algal cultivation and $MgCl_2$ treatment

Chromochloris zofingiensis UTEX 32 was kindly provided by Miss Pokchut Kusolkumbot at the Biodiversity Research Centre, Thailand Institute of Scientific and Technological Research (TISTR). The microalgal cultures were maintained in the proteose medium, as described by the University of Texas Culture Collection of Algae (UTEX, Austin, USA). The seed cultures were inoculated into a 40 mL proteose medium (without $MgCl_2$ in the medium) containing 20 g L^{-1} glucose in a 250 mL flask at an inoculum size of 10% inoculum (Ip, Wong, & Chen, 2004). When algal cells reached the early stationary growth phase, magnesium chloride ($MgCl_2$) was added to the algal cultures with the final concentration ranging 2.510 mM (Mg 2.5-10 mM) for inducing stress conditions. The shake flask batch cultures were incubated at 25°C with shaking at 150 rpm and illuminated under fluorescence light (18/6 h light/dark) for 8 days. A culture without the addition of $MgCl_2$ (Mg 0 mM) was used as a control. All chemicals used for medium preparation and $MgCl_2$ treatment were analytical grades obtained from Sigma Aldrich (St. Louis, MO, USA) and Ajax Finechem (Seven Hills, NSW, Australia).

3.2 Determination of specific growth rate, cell density, and biomass concentration

The algal cell growth of the control and $MgCl_2$ treatments was monitored at OD680 every two-day interval using a UV-1800 spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan). Cell density was measured using a Neubauer hemocytometer under a light microscope. The specific growth rate (μ) at the exponential growth phase was calculated according to the following equation:

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Specific growth rate (h^{-1}) = $\frac{\ln X_2 - \ln X_1}{t_2 - t_1}$; where X_1 and X_2 are OD680 at time t_1 and t_2, respectively

For determination of biomass concentrations, 10 mL of algal culture on day 8 was collected by centrifugation at 4,476 × *g* for 5 min, washed with distilled water 3 times, transferred to a pre-weighed tube (W_0, g), and dried in the ScanVac Coolsafe 110-4 Pro freeze-dryer (LaboGene ApS, Vassingerød, Denmark) to constant weight (W_1, g) (Ding et al., 2019). The biomass concentration (g L^{-1}) was calculated according to the following equation:

$$\text{Biomass concentration (g L}^{-1}) = \frac{W_1 - W_0}{10} \times 1000$$

3.3 Pigment analysis

Approximately 10 mg of the lyophilized cells were extracted in the dark using the extraction solvent containing methanol/dichloromethane (3:1). The extracts were subsequently filtrated through 0.22 uM Millipore membrane before subjection to HPLC analysis. All solvents used for pigment analysis were HPLC-grade (Merck, Darmstadt, Germany) and the canthaxanthin standard (Sigma-Aldrich, St. Louis, MO, USA) was kindly provided by Miss Pokchut Kusolkumbot at the TISTR.

The extracted pigments were identified and quantified by HPLC analysis using Shimadzu LC20A (Shimadzu Scientific Instruments, Kyoto, Japan), equipped with the C18 column (5 uM; 250 x 4.6 mm) (GL Science Inc., Tokyo, Japan). The mobile phase and HPLC gradient were set according to the previously described protocol (Ip et al., 2004). In brief, the HPLC gradient was run at a flow rate of 1 ml min^{-1} using mobile phase A (dichloromethane/methanol/acetonitrile/water, 5:85:5.5:4.5, v/v) and mobile phase B (dichloromethane/methanol/acetonitrile/water, 25:28:42.5:4.5, v/v) as follows: phase B at 0% for 8 min, phase B gradient from 0 to 100% for 6 min, and phase B at 100% for 50 min. The oven temperature was operated at 30°C, and the injection volume was 20 µL. The diode array detector (DAD) was employed and peaks were monitored at 480 nm. The canthaxanthin contents of each of the *C. zofingiensis* cultures upon $MgCl_2$ treatment, together with the control, were quantified and compared.

3.4 Statistical analysis

All treatments were performed in three biological replicates. The obtained results were expressed as mean ± SEM and analyzed using a statistical *t*-test, in which $p < 0.05$ is considered to bestatistical significance (X. H. Yu et al., 2015).

4. Results and Discussion

4.1 Growth of mixotrophic *C. zofingiensis* cultures

C. zofingiensis is capable of growing under a mixotrophic condition, in which glucose is the best carbon source for promoting cell growth (Ip et al., 2004). In this study, glucose at the final concentration of 20 g L^{-1} was chosen for supplementation into the proteose medium for stimulating algal cell growth. As shown in Figure 1, the specific growth rates at the exponential growth phase of all cultures did not considerably change (ranging from 0.0143-0.0152 h^{-1}) and a slight reduction in specific growth rate was not statistically significant. For future studies, data on the algal growth profile of each cultivation day could be collected and analyzed to define a more detailed specific growth rate of *C. zofingiensis* upon treatment with $MgCl_2$. Mixotrophic cultivation is a merging mode between phototrophic and heterotrophic cultivations, in which the mixotrophy cells use both photosynthesis and carbon metabolisms as their energy and carbon sources (Pang et al., 2019). This cultivation mode is favorably applied for culturing diverse microalgal

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species, such as *Chlorella sorokiniana*, *H. pluvialis*, and *Botryococcus braunii* (Kobayashi et al., 1992; Li et al., 2014; H. Zhang et al., 2011), offering an effective strategy to enhance high microalgal growth rates.

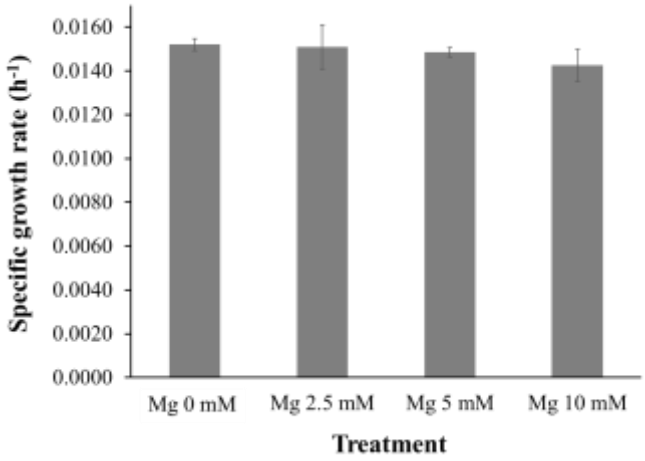

Figure 1 Specific growth rate of mixotrophic *C. zofingiensis* cultures

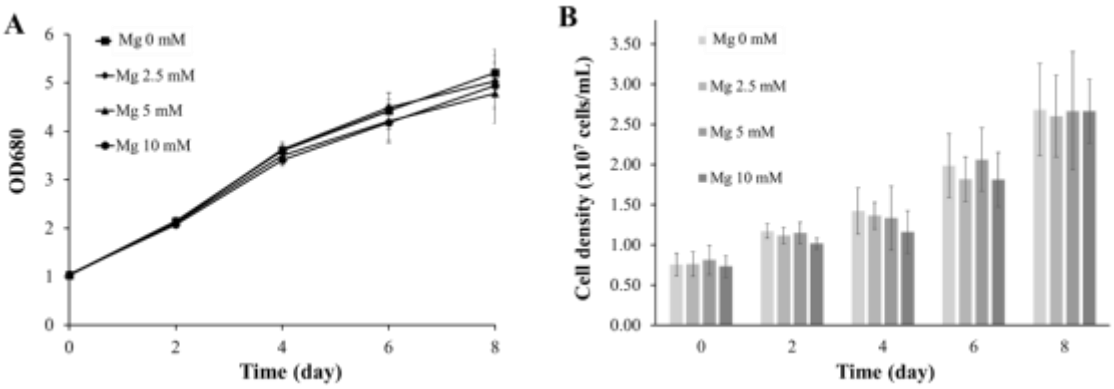

Figure 2 Growth curve and cell density of mixotrophic *C. zofingiensis* cultured under $MgCl_2$ treatment: (A) OD680 for growth curve measurement and (B) cell density measured by hemocytometer of all cultures treated with different $MgCl_2$ concentrations

Table 1 Biomass concentrations and canthaxanthin contents obtained from mixotrophic *C. zofingiensis* cultured under $MgCl_2$ treatment

Treatment	Biomass concentration (g L^{-1})	Canthaxanthin content (mg g^{-1})
Mg 0 mM	2.233 ± 0.291	0.658 ± 0.019
Mg 2.5 mM	2.356 ± 0.332	0.628 ± 0.056
Mg 5 mM	2.039 ± 0.099	0.937 ± 0.120*
Mg 10 mM	2.071 ± 0.211	0.848 ± 0.014*

*Significant difference at $p < 0.05$

4.2 Biomass production in *C. zofingiensis* under $MgCl_2$ treatment

To determine the effect of $MgCl_2$ on *C. zofingiensis* biomass production, different concentrations of $MgCl_2$ ranging2.5-10 mM were applied to algal cultures after cells reaching the early stationary phase. All tested $MgCl_2$ concentrations did not inhibit *C. zofingiensis* growth, as the OD680 on day 8 of control and $MgCl_2$-treated cultures was in a similar range at 4.781 ± 0.605 to 5.208 ± 0.482 (Figure 2A). Likewise, the cell density of all algal cultures treated with $MgCl_2$ was not significantly different from the control on day 8 (Figure 2B). Notably, a drastic increase by about 3.29-3.64% in cell density was obtained on day 8 of mixotrophic cultivation, as compared to that on day 0. For measurement of biomass concentration, the highest value of 2.356 ± 0.332 g L^{-1} was found in Mg 2.5 mM treatment (Table 1). There was a slight decrease in biomass concentrations observed in Mg 5 and 10 mM cultures, as compared to the control culture (Mg 0 mM). These results suggested that the growth of *C. zofingiensis* was not significantly affected by the $MgCl_2$ treatment. Similarly, this growth effect was also observed in a previous study that added Mg^{2+} ion into *Monoraphidium* sp. FXY-10 cultures (Dong et al., 2019).

4.3 Canthaxanthin accumulation in *C. zofingiensis* under $MgCl_2$ treatment

C. zofingiensis can accumulate high-value carotenoid compounds under abiotic stresses caused by various nutrient and environmental factors (Liu et al., 2014). To determine the canthaxanthin accumulation in mixotrophic *C. zofingiensis* cultures, different $MgCl_2$ concentrations of 2.5-10 mM were added to the algal cultures for inducing canthaxanthin production. The corresponding $MgCl_2$ concentrations were chosen according to the appropriate concentrations of metal ion treatment that induce astaxanthin production in the *H. pluvialis* microalgal strain (X. H. Yu et al., 2015). The extracted pigments of *C. zofingiensis* were subsequently identified and quantified using HPLC analysis. Identification of canthaxanthin was analyzed by comparing its identical retention time and absorption spectrum with the authentic canthaxanthin standard (Sigma-Aldrich, St. Louis, MO, USA), and accordingly, quantification of the extracted canthaxanthin was calculated from a comparison of peak areas. As shown in Figure 3, according to the retention time and absorption spectrum, the peak of canthaxanthin in the experimental cultures could be clearly identified in the HPLC chromatogram, compared to the standard peak shown in the inserted inlet that reveals the corresponding absorption spectrum at 477 nm. Under the tested condition, the $MgCl_2$-treated cultures with 5 and 10 mM resulted in a significant increase of canthaxanthin contents by about 28.87-42.40% (Table 1). Especially, the highest canthaxanthin content of 0.937 ± 0.120 mg g^{-1} was found at the concentration of 5 mM. However, a slight decrease of canthaxanthin content was observed at 2.5 mM $MgCl_2$ (0.628 ± 0.056 mg g^{-1}). Similarly, a decreased astaxanthin production in *H. pluvialis* was found at the low concentration of 2 mM $MgCl_2$ (X. H. Yu et al., 2015). The effect of different types of magnesium ions (such as $MgSO_4$ or $Mg(NO_3)_2$) has not been reported in *C. zofingiensis*. Investigation of microalgal growth and canthaxanthin production in response to various types of magnesium could be further carried out to provide useful information on cultivation strategies for inducing microalgal biomass and pigment production. In the present study, high levels of canthaxanthin accumulation could be achieved under our cultivation conditions. Magnesium ion is an important component of chlorophyll and involves several microalgal cellular metabolisms, such as photosynthesis and cell division. (Dong et al., 2019). Additionally, magnesium serves as a cofactor of enzymes involved in carotenogenesis and fatty acid biosynthesis in microalgae (Raman & Ravi, 2011). Therefore, the exogenous application of $MgCl_2$ could be used to promote other important bioproducts such as astaxanthin, lutein, and fatty acids in *C. zofingiensis*. This cultivation strategy would be a feasible approach for further developing the commercial production of high-value bioproducts in microalgae.

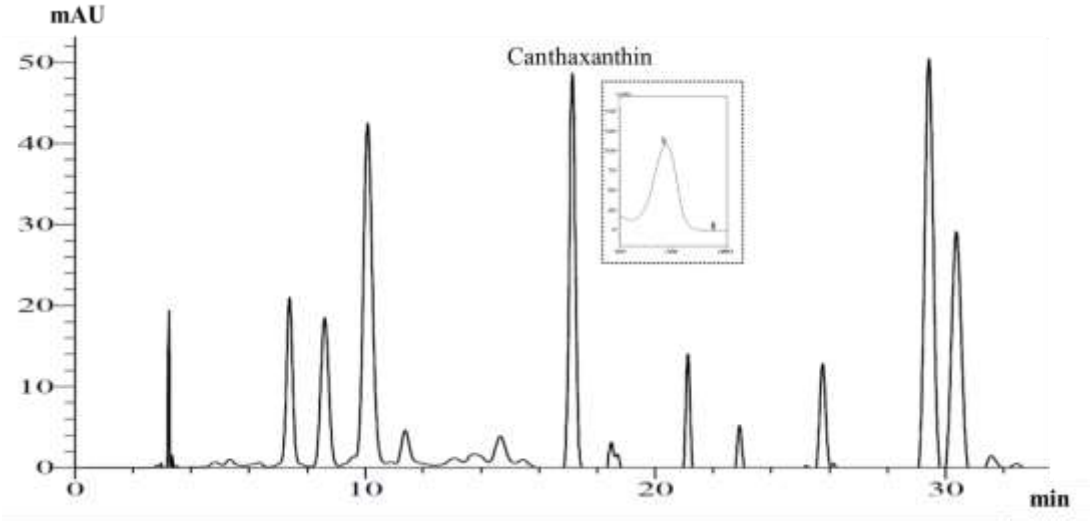

Figure 3 Representative pigment profile analyzed by HPLC in mixotrophic *C. zofingiensis* treated with 5 mM $MgCl_2$

5. Conclusion

Mixotrophic cultivation is an effective strategy that could improve microalgal growth rate to achieve high cell density. In addition, the application of exogenous chemicals can be used to induce high amounts of high-value bioproducts in microalgae. In this study, the growth of *C. zofingiensis* was not affected by exogenous $MgCl_2$ treatment and a drastic increase by about 3.29-3.64% in cell density was especially obtained on day 8 of cultivation under mixotrophic culture condition. The exogenous $MgCl_2$ treatment could induce a significant increase in canthaxanthin accumulation by about 42.40% in *C. zofingiensis*. These results suggested that mixotrophic cultivation combined with the addition of $MgCl_2$ may be a useful cultivation strategy for stimulating the production of high-value secondary carotenoids in microalgae. Further investigation of additional carotenoid compounds produced in *C. zofingiensis* is required in order to ultimately gain more information on carotenoid profiles in response to $MgCl_2$ treatment.

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