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Screening and effect evaluation of chemical inducers for enhancing astaxanthin and lipid production in mixotrophic *Chromochloris zofingiensis*

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Abstract

A two-stage screening approach of chemical inducers was carried out to evaluate the positive effect on enhancing astaxanthin and lipid production in mixotrophic *Chromochloris zofingiensis*. The results demonstrated that oxidants were able to increase the astaxanthin content high up to 11.4 mg g^{-1} biomass, while pyruvic acid promoted the cell growth and increased the contents of astaxanthin and lipids up to 10.7 mg g^{-1} and 66.1% dry weight. The highest yield (87.0 mg L^{-1}) and productivity (7.3 mg L^{-1} day^{-1}) of astaxanthin were obtained in parallel with high lipid productivity (459.0 mg L^{-1} day^{-1}). The correlations between the dosage effect of chemical inducers and metabolites variation were further examined by statistical analyses. Additionally, *C. zofingiensis*-derived lipids were estimated to be more viable and feasible as edible oils based on fatty acid characteristics assessment. The present work highlights the effective screening strategy of chemical inducers for enhancing astaxanthin and lipid production and the potential application in mixotrophic *C. zofingiensis*.

Keywords *Chromochloris zofingiensis* · Astaxanthin · Lipid · Chemical inducer · Regulatory mechanism

Introduction

Microalgae have been considered one of the most promising and renewable bioresources of lipids and carotenoids for the production of biofuels, edible oils, and natural pigments with commercial applications in food, nutraceuticals, and pharmaceuticals. Currently, the green microalga *Chromochloris zofingiensis* (Chlorellaceae, Chlorellales, Trebouxiophyceae), has gained the wide interest due to its ability to synthesize astaxanthin and lipids with fast growth rate, achieving high density in scaled-up cultivation system under stress in either phototropic or heterotrophic conditions (Liu et al. 2014). Similar to phototrophic *Haematococcus pluvialis*-derived astaxanthin, astaxanthin from *C. zofingiensis* is also mainly composed of the (3S, 3S′) stereoisomer of astaxanthin with the superior strong anti-oxidative activity and possesses food safety standards (Ip et al. 2004; Liu et al. 2014). Yet, *C. zofingiensis* is still not economically use for industrial-scale production, which is mainly due to the high content of canthaxanthin and low contents of astaxanthin and lipids in biomass and also the lack of information on the ideal inducers to stimulate the coordinated accumulation of desired valuable compounds (Bar et al. 1995; Liu et al. 2014; Sun et al. 2019a; Zhang et al. 2021a).

To address these limitations of *C. zofingiensis*, more efforts are urgently required to effectively improve the content and yield of astaxanthin and lipids. So far, there have been many studies succeeding in utilizing the chemical induction strategy to significantly improve the accumulation of valuable metabolites in microalgae and identification of large numbers of bioactive potential chemicals such as exogenous ROS

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(reactive oxygen species)-inducers, synthetic antioxidants scavenging excess intracellular ROS, metabolic regulators of certain biological pathways, inorganic salts, and organic acids in the tricarboxylic (TCA) cycle (Franz et al. 2013; Yu et al. 2015a; Sun et al. 2019a). However, in these studies, the widely-used model species for phenotypic screening are limited to *H. pluvialis*, *Xanthophyllomyces dendrorhous*, and other lipid and carotenoid-producing organisms and rarely in mixotrophic *C. zofingiensis*. Considering the unique physiological characteristics of *C. zofingiensis* and its species-specific responses to exogenous stimuli, it is important to systematically estimate the feasibility of these chemical inducers to enhance the accumulation of astaxanthin and lipids, providing theoretical guidance for the scale-up cultivation of *C. zofingiensis* for commercial production of astaxanthin and lipids.

Pigments and fatty acids are two typical intracellular valuable metabolites in *C. zofingiensis* cells, and their composition and respective contents substantially varied in cultures treated with different exogenous stimuli (Liu et al. 2014; Mulders et al. 2015; Chen et al. 2020). Accordingly, the regulatory mechanisms of these chemical inducers might be inferred from physiological and biochemical responses of algal cells to chemical induction. Statistical techniques such as cluster analysis and multivariate statistical analysis, have been proven to be powerful approaches to uncover the potential underlying relationships between exogenous chemical induction and endogenous carotenoid and lipid biosynthesis (Yu et al. 2015b; Chen et al. 2020). Recent studies demonstrated that the full characterization of intracellular metabolites (i.e., carotenoids and fatty acids) and their comparative composition could be utilized to assess the characteristics of microalgae-derived products especially as edible oils (Huang et al. 2016; Minyuk et al. 2020). However, up to date, there is still a lack of solid scientific evidence to verify whether *C. zofingiensis*-derived biomass or lipids could be utilized to produce edible oils and frying oils besides astaxanthin while possessing superior advantages in comparison to other resources, which is worth of in-depth systematic investigation.

The objectives of this present study were to identify potential chemical inducers that may efficiently improve the accumulation of astaxanthin and lipids in mixotrophic *C. zofingiensis* and characterized its fatty acids for potential applications. A series of potential chemical inducers were assessed by preliminary screening using a fluorescence-based rapid estimation method and then were further verified in the secondary screening to confirm their abilities. Subsequently, the variations of pigments and fatty acids compositions were investigated by statistical analysis to provide a better understanding of the mechanisms of these chemical inducers involving astaxanthin and lipid biosynthesis. The present work highlights the capabilities of screened chemical inducers in stimulating the biosynthesis of astaxanthin and lipids in *C. zofingiensis* and its potential in promoting applications of this microalga in the food industry.

Materials and methods

Microalga and culture conditions

The green microalga *Chromochloris zofingiensis* (ATCC 30412) was purchased from the American Type Culture Collection (ATCC, Rockville, USA). The algal cells were cultured in a modified Bristol medium (Ip et al. 2004). Briefly, algal cells from agar plates were mixotrophically cultivated in 250-mL flasks containing 100 mL sterilized medium with 10 g L^{-1} glucose and maintained in an orbital shaker for 4 days at 150 rpm and 26 °C under continuous illumination of ca. 10 μmol photons m^{-2} s^{-1}. The low light intensity (10 μmol photons m^{-2} s^{-1}) was only utilized in the pre-cultivation for favorable growth under the mixotrophic mode. The seed culture in the exponential phase was used as the inoculum in the following experiments.

Chemicals and reagents

Analytical grade chemical reagents were obtained from Guangzhou Huaqisheng Biotechnology Co., Ltd. (Guangzhou, China), including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), epigallocatechin gallate (EGCG), 2,2′-azo-bis(2-amidinopropane)-dihydrochloride (AAPH), glycine betaine (GB), sodium orthovanadate (SOV), 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), glyphosate (Gly) and atrazine (At). Acetylcholine (ACE) was purchased from Topscience Biotech Co., Ltd. (Shanghai, China). Organic acids including pyruvic acid (PA), citric acid (CA), malic acid (MA), and α-ketoglutaric acid (KG) were obtained from Macklin Biochemical Reagent Co., Ltd. (Shanghai, China). Ethanol (EtOH), hydrogen peroxide (H_2O_2), potassium chloride (KCl), and magnesium chloride ($MgCl_2$) were purchased from local suppliers. Pigment standards were from Sigma-Aldrich Chemical Co. (USA). HPLC (high-performance liquid chromatography)-grade methanol and methyl tert-butyl ether (MTBE) were from Thermo Fisher Scientific Inc. (USA).

Microplate-based cultivation for the screening of various chemical inducers

To identify positive chemical inducers for the accumulation of astaxanthin and lipids in *C. zofingiensis*, a two-phase screening procedure was utilized in the present study for high screening efficiency and accuracy. In

the first phase, *C. zofingiensis* cells were inoculated into 24-well microplates each containing 1.5 mL modified Bristol medium with different potential chemical inducers at designated dosages (Table 1) (Chen et al. 2009; Tran et al. 2009; Chalifour and Juneau 2011; Doddaiah et al. 2013; Franz et al. 2013; Parsaeimehr et al. 2015; Yu et al. 2015b; Zhao et al. 2016). The initial biomass concentration was kept at 2–3 g L^{-1} and maintained at 26 °C under high light irradiation for 12 days at a shaking speed of 400 rpm, followed by flow cytometry (FCM) analysis of algal autofluorescence intensity as primarily screening (Chen et al. 2020). These chemical inducers with stimulatory effects on the mean autofluorescence intensity (MFI) of the bandpass filter FL2 (585 nm ± 20 nm) were regarded as potential positive triggers for astaxanthin enhancement (Chen et al. 2017a). The second screening phase involved quantification and comparison of astaxanthin and lipids in chemical-induced algal cells using traditional HPLC and gravimetric analysis to verify the capability of the primarily selected chemical inducers. Algal cells were cultivated in the same culture systems under the above-described conditions to obtain adequate algal biomass for all required analyses as mentioned below (Chen et al. 2020).

Analytical methods

To measure algal dry weight, approximately 2 mL of the culture was collected by centrifugation at 4000 rpm for 3 min and washed twice with pure water, followed by oven dried overnight at 60 °C until constant weight. Total pigments were extracted from dry biomass repeatedly using methanol/dichloromethane (3:1, v/v) as solvent until algal cells become colorless, and the combined extracts were analyzed on a reversed-phase HPLC system according to the referenced protocol (Chen et al. 2017a). The detailed procedure of flow cytometric analysis was described previously using a BD Accuri C6 flow cytometer (Accuri Cytometers, Inc., USA) equipped with two 50 mW air-cooled lasers (488 nm and 640 nm) and four interference filters (FL1: 533 nm ± 15 nm, FL2: 585 nm ± 20 nm, FL3 > 670 nm, and FL4: 675 nm ± 25 nm) (Chen et al. 2017a). Total lipids were extracted from dry biomass using methanol/dichloromethane (3:1, v/v), and the extract of

Table 1 Dose-dependent effects of potential chemical inducers on MFI of mixotrophic *C. zofingiensis*

Types	Potential chemical inducers	Abbreviation	Cosolvents	Designated dosages		MFI increase (%)[a]	
				Low dosage (L)	High dosage (H)	MFI in FL1[b]	MFI in FL2[b]
Oxidants	2,2′-Azo-bis(2-amidinopropane)-dihydrochloride	AAPH	Water	2.7 mg L^{-1}[c]	27.1 mg L^{-1}	10.1 ± 0.9	9.4 ± 1.0
	Hydrogen peroxide	H_2O_2	Water	32.3 mg L^{-1}	64.5 mg L^{-1}[c]	23.1 ± 1.8	24.1 ± 1.9
	Ethanol	EtOH	Water	1.0%	2.0%[c]	16.8 ± 8.4	12.1 ± 8.2
Antioxidants	Butylated hydroxyanisole	BHA	DMSO	540.0 μg L^{-1}	5410.0 μg L^{-1}[c]	5.4 ± 1.0	− 3.4 ± 0.8
	Butylated hydroxytoluene	BHT	DMSO	660.0 μg L^{-1}	6610.0 μg L^{-1}[c]	− 3.1 ± 0.6	− 9.9 ± 0.6
	Propyl gallate	PG	DMSO	85.0 μg L^{-1}	849.0 μg L^{-1}[c]	− 13.2 ± 3.5	− 16.5 ± 2.9
	Epigallocatechin gallate	EGCG	Water	0.2 mg L^{-1}	1.8 mg L^{-1}[c]	3.0 ± 2.3	0.1 ± 2.0
Metabolic inhibitors	Glycine betaine	GB	Water	590.0 mg L^{-1}	1180.0 mg L^{-1}[c]	− 16.4 ± 3.6	− 12.3 ± 4.1
	Sodium orthovanadate	SOV	Water	92.0 mg L^{-1}[c]	185.0 mg L^{-1}	− 9.0 ± 3.6	− 4.2 ± 3.5
	Acetylcholine	ACE	Water	0.6 μg L^{-1}	5.6 μg L^{-1}[c]	− 28.4 ± 8.7	− 30.3 ± 8.4
	3-(3,4-Dichlorophenyl)-1,1-dimethyl urea	DCMU	Ethanol	120.0 μg L^{-1}[c]	1160.0 μg L^{-1}	28.2 ± 4.0	32.9 ± 4.3
	Glyphosate	Gly	Water	0.2 mg L^{-1}[c]	1.7 mg L^{-1}	8.4 ± 4.4	5.2 ± 4.3
	Atrazine	At	DMSO	21.6 μg L^{-1}	215.7 μg L^{-1}[c]	13.1 ± 13.6	20.0 ± 18.9
Metabolic precursors	Pyruvic acid	PA	Water	0.9 g L^{-1}	4.3 g L^{-1}[c]	18.8 ± 1.0	15.8 ± 0.9
	Citrate acid	CA	Water	1.9 g L^{-1}	9.6 g L^{-1}[c]	14.5 ± 0.8	15.3 ± 0.8
	Malic acid	MA	Water	1.3 g L^{-1}[c]	6.7 g L^{-1}	15.0 ± 0.3	11.9 ± 0.4
	α-Ketoglutaric acid	KG	Water	1.5 g L^{-1}[c]	7.3 g L^{-1}	− 40.5 ± 0.1	− 43.7 ± 0.1
Metal ions	Potassium chloride	KCl	Water	1280.0 mg L^{-1}	12.7 g L^{-1}[c]	− 1.8 ± 0.9	− 3.6 ± 0.8
	Magnesium chloride	$MgCl_2$	Water	1620.0 mg L^{-1}[c]	16.2 g L^{-1}	22.2 ± 1.1	20.4 ± 1.0

Notes: *DMSO*, dimethyl sulfoxide; *MFI*, mean autofluorescence intensity; *FL1*, 533 nm ± 15 nm; *FL2*, 585 nm ± 20 nm

[a]Comparison of MFI of *C. zofingiensis* cells in chemical-induced cultures vs. the control cultures (with/without cosolvents); [b]calculated by comparing MFI of algal cells in the cultures with optimal dosages of chemical inducers with the control cultures; [c]optimal dosages of specific chemical inducers were selected based on their effects on MFI of algal cells in FL1 and FL2

lipids was collected by centrifugation and blow to be dry under nitrogen flow gas to constant weight for lipid content measurement (Chen et al. 2015, 2020; Yu et al. 2015b). Fatty acid composition in biomass was analyzed by gas chromatography-mass spectrometry (GC–MS) according to our previous study (Chen et al. 2020). Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), the ratio of omega-6 to omega-3 polyunsaturated fatty acids (ω-6/ω-3 ratio), calculated oxidation stability value (COX), and unsaturation index were calculated based on the fatty acid composition and compared with previous studies (Dubois et al. 2007; Ismail and Ali 2015; Bialek et al. 2017; Giakoumis 2018).

Data analysis

Experimental data in the figures are presented as the mean ± standard deviation (SD). The statistical significance of the data was analyzed by the one-way analysis of variance (ANOVA) followed by a Tukey's HSD test or the Student's *t*-test at a significant level of $p < 0.05$. The Shapiro–Wilk test for normality check and Levene's test of homogeneity of variances were used to verify these assumptions of the one-way ANOVA. Agglomerative hierarchical cluster analysis was performed by the between-groups linkage method using squared Euclidean distance as the measure of similarity to cluster groups. These statistical evaluations were performed using SPSS software (Version 19.0, SPSS Inc., USA) and Origin software (Version 9.1, OriginLab Corp., USA).

Results

Preliminary screening of positive chemical inducers

To screen positive chemical inducers for efficiently improving the accumulation of astaxanthin and lipids in mixotrophic *C. zofingiensis*, a two-phase screening process was utilized in the present study for high screening efficiency and accuracy. As shown in Table 1, nineteen potential chemical inducers belonging to five classifications at two suitable dosages based on previous literature were first chosen by using flow-cytometry-based fluorescence measurement to estimate their abilities to enhance the production of astaxanthin and/or lipids. Figure 1 demonstrates the effects of all tested chemical inducers on the MFI of algal cells. The autofluorescence of *C. zofingiensis* cells at specific wavelengths (530 nm and 580 nm) could be applied for rapid evaluation and prediction

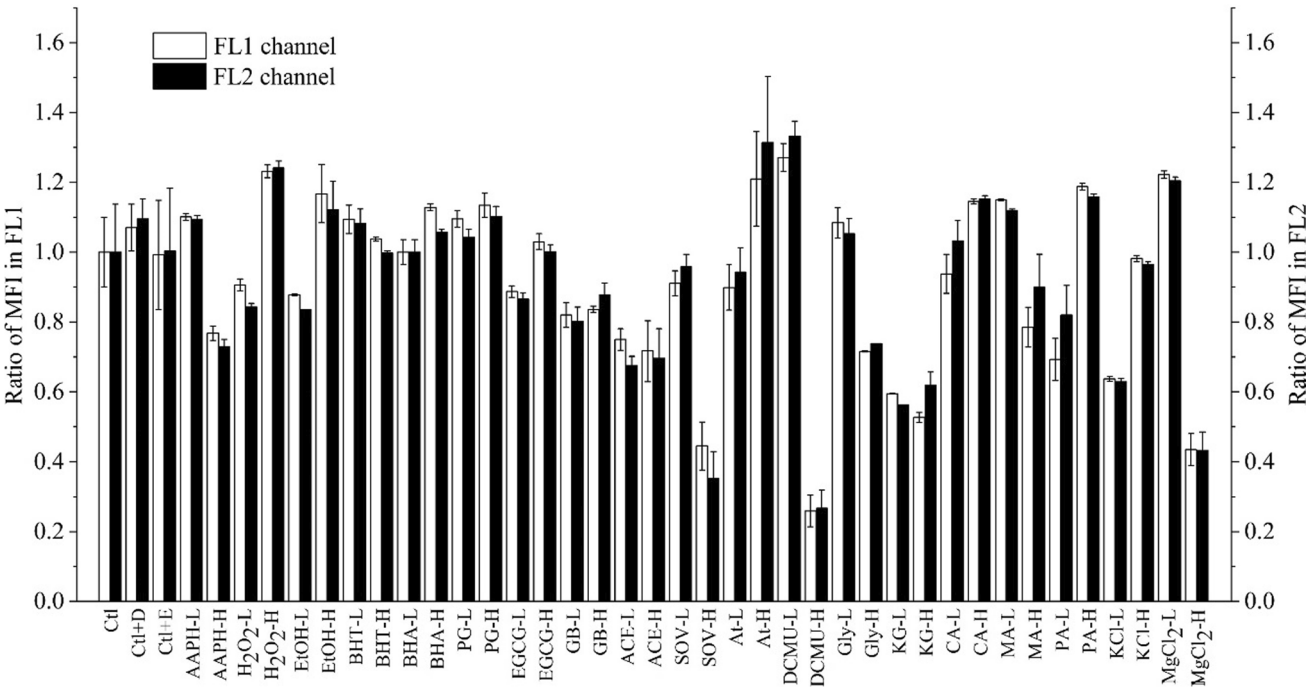

Fig. 1 Mean autofluorescence intensity of mixotrophic *C. zofingiensis* cells at FL1 and FL2 channel in a flow cytometer using various potential chemical inducers. The results are presented as ratios by comparison to the control (referred to as 1.0). Bar values represent averages of three replicates ($n = 3$), and error bars indicate standard deviations (SD) across all samples. All the abbreviations used for potential chemical inducers are defined in Table 1 for easy reference. Capital letters L and H present the low and high dosages of each potential chemical inducer, respectively

of fluorescent pigments especially astaxanthin since the autofluorescence intensity of algal cells correlated closely with the content of cellular pigments (Chen et al. 2017a). Results clearly showed that each chemical inducer had significantly different effects on MFI in FL1 (one-way ANOVA: $F_{40,\ 82} = 65.8$, $p < 0.001$) and FL2 (one-way ANOVA: $F_{40,82} = 47.1$, $p < 0.001$). The chemicals classified as oxidants were the most efficient activators enhancing MFI at suitable dosages, especially H_2O_2 and ethanol with MFI increases of 24.1 and 12.1%, respectively. Comparatively, antioxidants seemed to show negative or slight increases of MFI in FL2 even at their optimal dosages. As for metabolic regulators, DCMU, glyphosate, and atrazine could potentially be more suitable for astaxanthin biosynthesis since MFI in FL2 of algal cells in these treatments increased 32.9%, 5.2%, and 20.2%, respectively. However, GB, SOV, and ACE decreased MFI by 19.9%, 4.2%, and 30.3%. Metabolic precursors, i.e., pyruvic acid, citrate acid, and malic acid exerted almost similar positive influences on MFI in FL2 with the increases between 11.9 and 15.8%, while α-ketoglutaric acid as a key molecule in the TCA cycle significantly inhibited MFI in FL2 by 43.7%. Similarly, not all metal ions activated the increase of MFI in FL2. Unlike the 20.4% increase of MFI in the $MgCl_2$ treatment, the addition of KCl was unfavorable for MFI increase at both dosages compared to the control. Thus, *C. zofingiensis* cells were cultured at the desirable dosage (Table 1) of each chemical inducer under the same culture conditions for accurate quantification and comparison of astaxanthin and lipids in chemical-induced algal cells using traditional HPLC and gravimetric analysis to further confirm the abilities of the preliminary selected chemical inducers to trigger astaxanthin and lipid biosynthesis.

Secondary screening of the selected chemical inducers

The preliminary screening identified the desired dosage of each potential chemical inducer by using flow cytometry-based fluorescence measurement. In this section, *C. zofingiensis* cells were cultured at the desirable dosage of each chemical inducer to further confirm their abilities in triggering astaxanthin and lipid biosynthesis. The results demonstrated the positive or inhibitory effects of different chemical inducers on biomass production (Fig. 2). Oxidants and metabolic regulators except acetylcholine exhibited significant inhibitory effects on biomass concentration with a more than 27.7% decrease. Among these antioxidants, PG and EGCG also exerted detrimental influences on biomass production; however, BHA and BHT improved biomass production with increases of 17.4 and 11.4%, respectively. Additionally, α-ketoglutaric acid was the most efficient stimulator for biomass production, which considerably improved biomass to the maximum concentration of 9.9 g L^{-1} with a marked significant increase exceeding 49.7% in comparison to the control (Student's *t*-test: $t(5) = 27.2$, $p = 0.0013$). The other three organic acids, i.e., pyruvic acid, malic acid, and citrate acid, also exerted positive effects on biomass production with increases of 26.8, 25.6, and 23.0%, respectively. The addition of metal ions showed no negative or slightly positive effects on algal cell growth in which biomass production was promoted by 7.6 and 8.3%, respectively.

Different chemical inducers at desirable dosages had significant effects on the production of pigments and lipids in *C. zofingiensis* (Figs. 3 and 4). As illustrated in Fig. 3a, all oxidants greatly improved the astaxanthin content, especially ethanol-induced up to 12.7 mg g^{-1} astaxanthin accumulation with a 48.6% increase compared to the control, followed by glyphosate, H_2O_2, and AAPH with increases of 36.9, 33.8,

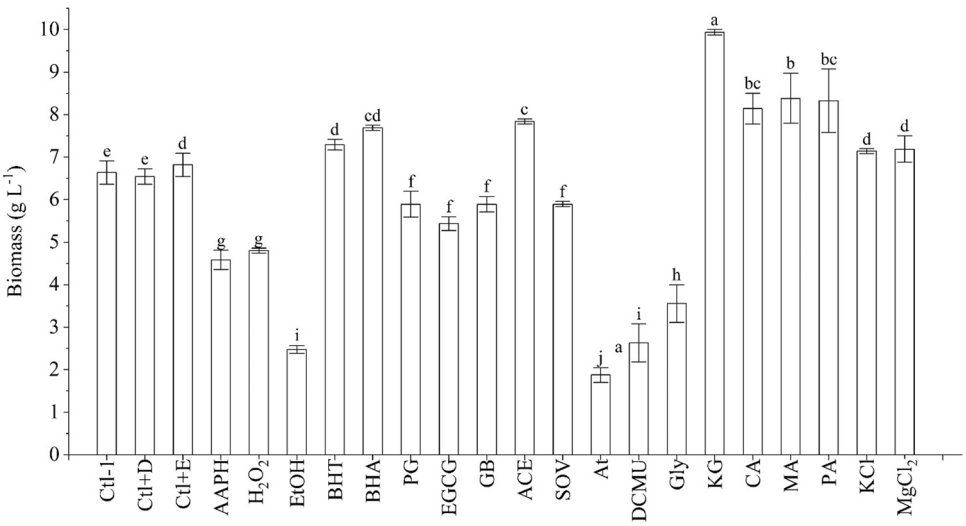

Fig. 2 Biomass concentration of mixotrophic *C. zofingiensis* induced by the desirable dosages of each potential chemical inducer. The bars represent the averages of three biological replicates and the corresponding standard errors. The letter display was used to present multiple-comparison results, in which values followed by identical letters were not significantly different, whereas values followed by different letters present significant differences among groups ($p < 0.05$, one-way ANOVA)

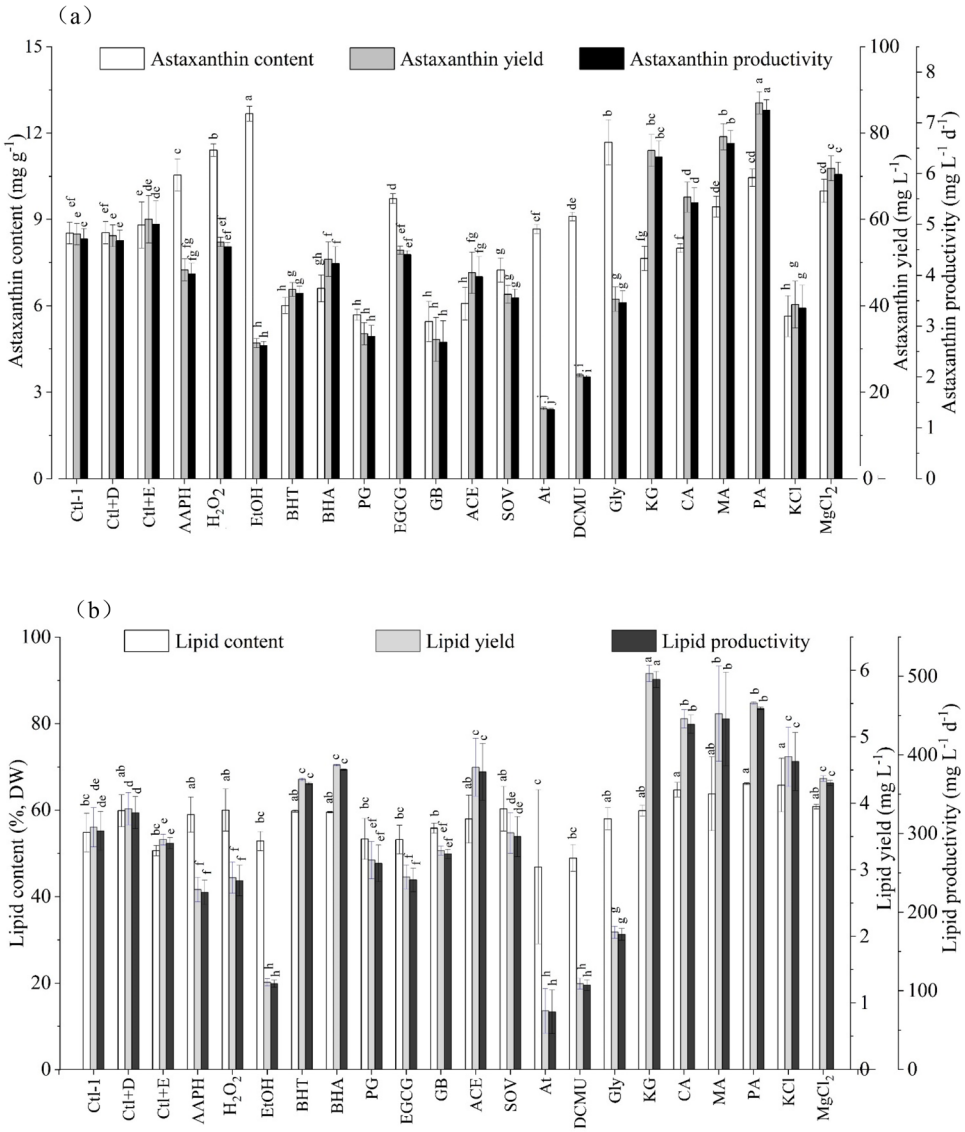

Fig. 3 Production of astaxanthin (**a**) and lipids (**b**) by *C. zofingiensis* in a microplate-based cultivation system with the addition of chemical inducers at desirable dosages. The data shown represent averages of three replicates with standard deviations and different letters indicate significant differences. Values followed by different letters represent significant difference levels ($p < 0.05$, one-way ANOVA)

and 23.7%, respectively. The addition of antioxidants such as BHA, BHT, and PG resulted in low astaxanthin content, while EGCG was an exception that promoted astaxanthin accumulation. In contrast, organic acids (i.e., pyruvic acid and malic acid), as well as $MgCl_2$, were able to simultaneously promote biomass production (Fig. 2) and astaxanthin accumulation (Fig. 3). Pyruvic acid addition, in particular, resulted in a 26.8% increase of biomass in parallel to higher astaxanthin content at 10.4 mg g^{-1} compared to the control, and thus, the highest astaxanthin yield (87.0 mg L^{-1}) and productivity (7.3 mg L^{-1} day^{-1}) were obtained in the PA-added culture. Figure 4a shows the composition of pigments in *C. zofingiensis* cells affected by exogenous chemical inducers. Astaxanthin, canthaxanthin, lutein, and ketolutein comprised more than 92% of total pigments, in which astaxanthin accounted for about 62.3 to 75.1% of total pigments followed by 3.5–13.0% of canthaxanthin. Noticeably, ethanol exhibited different effects on pigments composition, especially chlorophylls and lutein significantly increased to 7.3 and 8.0% of total pigments by ethanol, respectively.

Additionally, as shown in Fig. 3b, these selected chemical inducers stimulating astaxanthin accumulation were not able to trigger lipid biosynthesis, e.g., oxidants, organic acids, glyphosate, and $MgCl_2$; while these chemical inducers with detrimental effects on astaxanthin biosynthesis, such as BHT, BHA, ACE, α-Ketoglutaric acid, and KCl, were identified to improve lipid yield. Among these chemical inducers, organic acids were the most efficient stimulators for lipid accumulation, resulting in a more than 16.3% increase in lipid content. The highest lipid content (66.1%, DW) was obtained in the pyruvic acid-added culture, while in KG-added cultures, the maximum lipid yield (496.0 mg L^{-1} day^{-1}) was obtained with a slightly lower lipid content (59.9%, DW). Figure 4b illustrates the variations of fatty

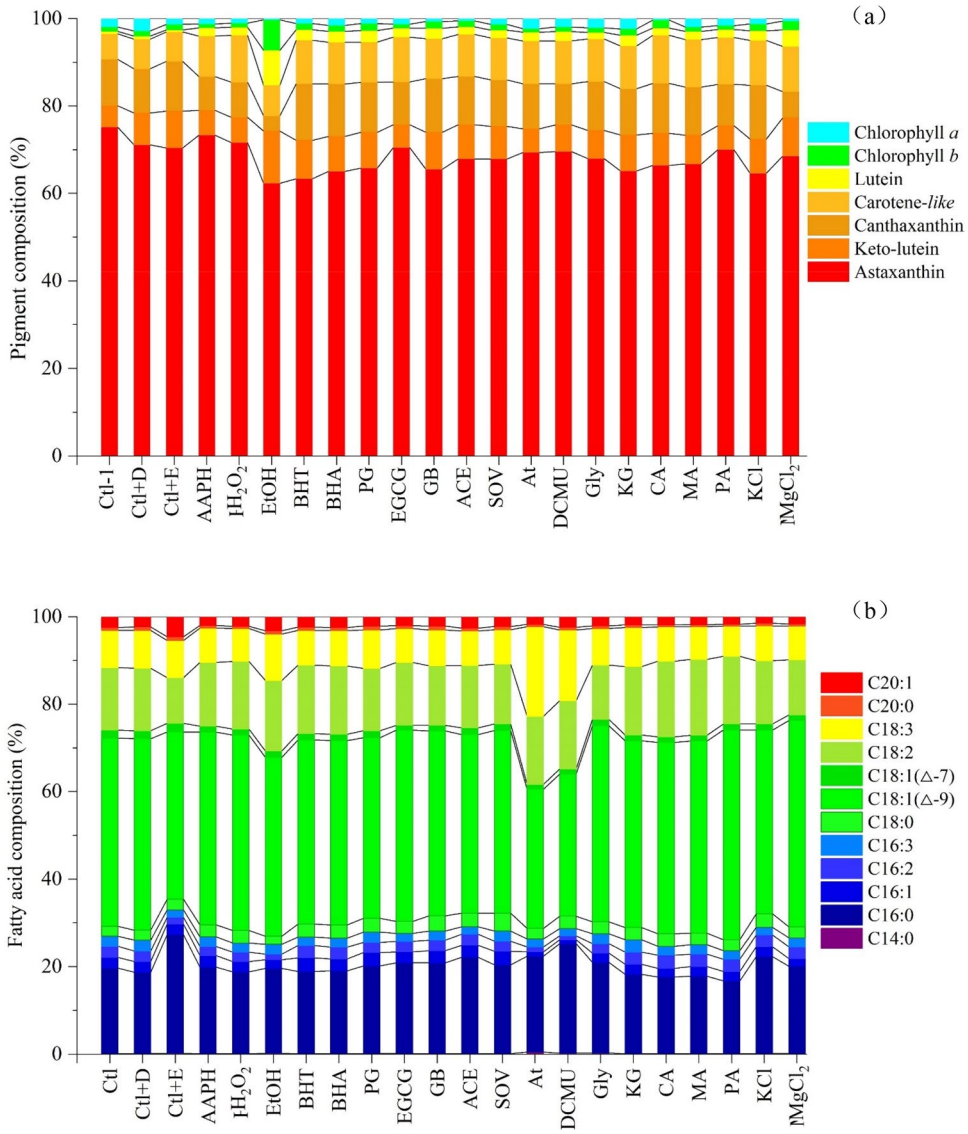

Fig. 4 Compositions of carotenoids (**a**) and fatty acids (**b**) in *C. zofingiensis* treated with different chemical inducers at desirable dosages

acid composition in *C. zofingiensis* cells in the cultures treated with different chemical inducers. Obviously, most of the chemical inducers had slight degrees of effects on the fatty acid composition, which were predominantly consisted of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). However, atrazine and DCMU had different degrees of effects on fatty acid profile, i.e., oleic acid in At-added cultures decreased significantly from above 43.1 to 31.8% of total fatty acids followed by DCMU treatment with about 32.5% of oleic acid.

Statistical analysis of abundance changes in cellular pigments and fatty acids

The changes of physiological parameters of *C. zofingiensis* cells as well as pigment and fatty acid abundance in total lipids were evaluated by hierarchical cluster analysis (HCA) and heatmap analysis (Fig. 5). As shown in the dendrogram based on HCA analysis of cell growth and targeted metabolite biosynthesis in *C. zofingiensis*, all of these chemical inducers were mainly clustered into two groups when the rescaled distance was 15. Compared to the majority of chemical inducers in one group, ethanol, glyphosate, atrazine, and DCMU belonged to the other group since they all had serious detrimental impacts on cell growth. While when the distance was 10, ethanol and glyphosate were separated from atrazine and DCMU. Organic acids and $MgCl_2$ were also clustered into the same group, while the other chemical inducers were in the same cluster as the control.

To further interpret the valuable information from these results, heatmap analysis of pigments and fatty acids in *C. zofingiensis* cells as percentages of total lipids was conducted and illustrated in Fig. 5b. It was shown that specific pigments and fatty acids in *C. zofingiensis* cells were divided

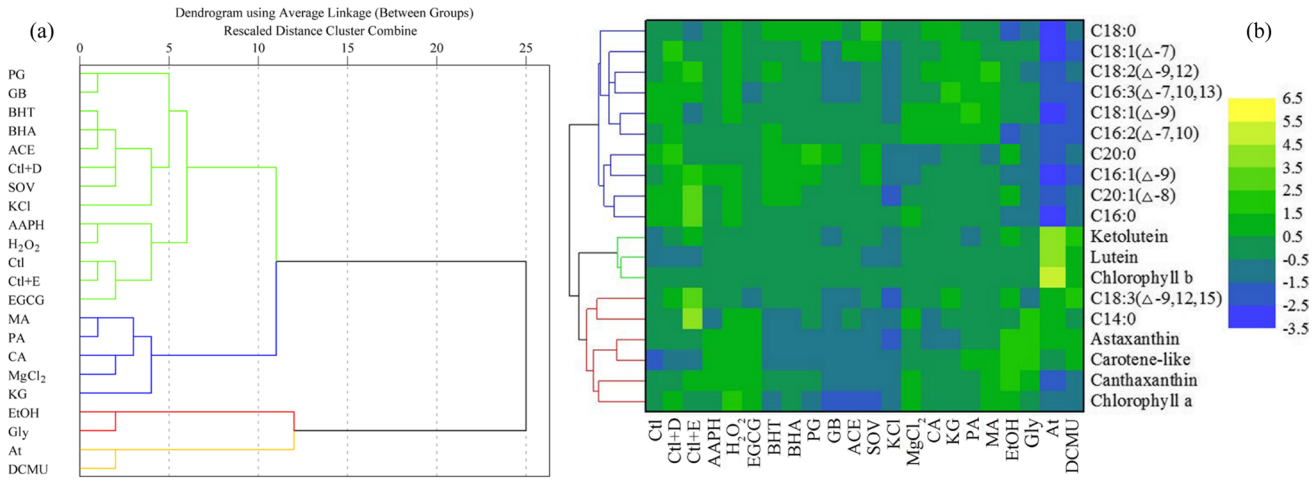

Fig. 5 Schematic dendrograms of agglomerative hierarchical cluster analysis (**a**) and heatmap analysis (**b**) based on astaxanthin and lipid abundance in mixotrophic *C. zofingiensis* cells after chemical induction

into three clusters based on their abundance changes in these chemical induction treatments. The first cluster mainly consisted of fatty acids that were simultaneously up-regulated at a different extent in many treatments of exogenous chemical inducers but down-regulated significantly in At and DCMU treatments. Furthermore, lutein, ketolutein, and chlorophyll *b* in the second cluster all represented upregulation patterns in the At and DCMU treatments, whereas their levels decreased slightly in other treatments. The third cluster majorly included pigments and parts of fatty acids, e.g., astaxanthin, carotene, canthaxanthin, and C18:3, which showed similar regulation patterns among these chemical treatments, although the diagram showed that their levels changed slightly. The results indicated that the exogenous addition of chemical inducers could link intracellular metabolites to certain biosynthesis pathways to yield higher accumulation of astaxanthin and lipids (Table 2).

Characteristics of C. zofingiensis-derived lipids

In the following context, we carried out the evaluation of the feasibility of *C. zofingiensis* lipids for the potential production of microalgae-derived edible oils. The fatty acid compositions of *C. zofingiensis* biomass from the control and PA-added cultures, as well as the ratios of saturated and unsaturated fatty acids, were analyzed and compared as presented in Table 3. It was shown that *C. zofingiensis* was mainly comprised of C16 and C18 fatty acids which accounted for more than 96.7% of total fatty acids in both cultures. Although the percentage of PUFA showed no significant difference in these two cultures, more saturated fatty acids were converted into monounsaturated fatty acids by the addition of pyruvic acid, in which the percentage of MUFA increased from 19.7 to 53.2% along with the decrease of SFA percentage from 22.5 to 19.7%. Additionally, the addition of metabolic precursors pyruvic acid further promoted the elongation of 16 carbon fatty acids and resulted in a higher percentage of C18 fatty acids at 74.2%. The calculated unsaturation index and degree of unsaturation of algal oils were above 3.4 and 1.2, respectively, which means that they have similar characteristics to edible vegetable oils.

Discussion

A two-phase screening process was utilized in the present study to identify the positive chemical inducers for improving the accumulation of astaxanthin and lipids in mixotrophic *C. zofingiensis*. In brief, the preliminary screening of the desired dosage of each chemical inducer was conducted by using flow cytometry-based fluorescence measurement, and subsequently, the stimulatory effects of these selected chemical inducers on astaxanthin/lipids production were verified and compared for identification of potential positive chemical inducers. Generally, among these chemical inducers, oxidants, metabolic regulators, organic acid precursors, and metal ions at their optimal dosages exerted stimulatory effects on MFI of agal cells, which was consistent with previous studies utilizing the chemical induction strategy to promote the accumulation of astaxanthin and/or lipids in microalgae (Franz et al. 2013; Yu et al. 2015a; Sun et al. 2019a). However, certain chemical inducers such as GB, SOV, ACE, and α-ketoglutaric acid decreased MFI even though their positive effects on carotenoid and/or lipid biosynthesis in other microalgae were reported (Tran et al. 2009; Parsaeimehr et al. 2015; Zhao et al. 2016). These differences might be due to the species-specific physiological characteristics of multi-trophic *C. zofingiensis* with unique

Table 2 Levels of astaxanthin and lipid production by *C. zofingiensis* under various trophic modes in the latest 5-year reports

Strains	Culture mode	Biomass production		Astaxanthin			Lipids			References
		Biomass (g L^{-1})	Productivity (g L^{-1} day^{-1})	Content (mg g^{-1})	Yield (mg L^{-1})	Productivity (mg L^{-1} day^{-1})	Content (% DW)	Yield (g L^{-1})	Productivity (mg L^{-1} day^{-1})	
ATCC30412	M, batch, MCS[a]	8.3	0.7	10.4	87.0	7.3	66.1	5.5	459.0	This study
	M, batch, MCS[b]	2.5	0.2	12.7	31.4	2.6	52.9	1.3	109.0	
ATCC30412	P, batch, TR	0.6–3.8	0.3–0.9	0.5–3.1	2.0–6.5	0.5–1.6	5.1–18.0[c]	0.2–0.5[c]	40.0–120.0[c]	(Mao et al. 2020)
ATCC30412	P, batch, 200 mL column	3.6–9.9	0.6–1.65	0.8–6.0	4.9–41.8	0.8–7.0	1.9–21.6[c]	0.1–1.4[c]	20.0–240.0[c]	(Kou et al. 2020)
ATCC30412	M, batch, MCS	3.1–8.3	0.3–0.7	5.4–13.1	29.1–89.9	2.4–7.5	43.7–64.5	1.4–5.3	116.7–445.7	(Chen et al. 2020)
ATCC30412	P, two stage, F.–C	71.1–73.7	4.8–5.8	0.7–2.7	47.3–194.5	4.0–9.9	-	-	-	(Sun et al. 2019b)
ATCC30412	M, batch, 250-mL flask	~9.5	0.7	~1.9	~18.0	1.3	~22.5[d]	~0.2[d]	15.3[d]	(Zhang et al. 2019)
ATCC30412	P, batch, TR	1.2–3.3	0.1–0.4	0.7–3.9	2.2–4.5	0.3–0.6	5.8–27.2[c]	0.2–0.3[c]	19.6–52.4[c]	(Mao et al. 2018)
ATCC30412	P, batch, TR	~1.8	0.5	4.1–4.8	7.3–8.6	2.0–2.2	32.5–38.0[d]	0.6–0.7[d]	150.0–175.0[d]	(Zhang et al. 2018)
ATCC30412	M, batch, MCS	4.0–6.0	0.3–0.5	4.3–7.1	17.2–38.9	1.4–3.2	-	-	-	(Chen et al. 2017b)
ATCC30412	H, two-step, RFP	98.4	7.0	0.7	73.3	5.2	-	-	-	(Zhang et al. 2017)
UTEX B32	P, semi-continuous, GC	-	0.7–1.2	2.5–3.2	-	2.3–3.3	22.4–31.4[c]	-	213.0–297.0[c]	(Liu et al. 2016)
UTEX B32	P, batch, 250 mL flask	4.0–4.3	0.3	0.7–1.6	3.1–6.5	0.2–0.5	~12.0[d]	~0.5[d]	35.7[d]	(Mulders et al. 2015)

Notes: The values in this table were calculated based on the values presented in tables and figures in these reports

P, photoautotrophic culture; *M*, mixotrophic culture; *H*, heterotrophic culture; *MCS*, microplate-based culture system; *GC*, glass column; *TR*, tubular reactor; *F*, fermenter; *AP*, airlift-loop photobioreactor; *RFP*, an energy-free rotating floating photobioreactor; *F.-C.*, 3 L fermenter–thin column illuminated with high light (400 μmol photons m^{-2} s^{-1}); *DW*, dry weight; ~, estimated value; -, data items not measured

[a]Pyruvic acid-added culture; [b]ethanol-added culture; [c]TAG (triacylglyceride) instead of lipids; [d]total fatty acids instead of lipids

Table 3 Fatty acid composition and characteristics from mixotrophic *C. zofingiensis* in comparison with edible vegetable oils

Fatty acid composition and characteristics	Abbreviation	Control (without additions)	Pyruvic acid-added culture	Edible vegetable oil [a]		
				Olive	Peanut	Soybean
16-carbon fatty acids (%)	∑C16	26.8 ± 0.3	23.5 ± 0.1	12.1–12.1	10.0–10.6	11.0–12.5
18-carbon fatty acids (%)	∑C18	70.0 ± 0.4	74.2 ± 0.1	83.7–87.6	81.6–84.6	82.0–87.7
16- and 18-carbon fatty acids (%)	∑(C16 + C18)	96.7 ± 0.1	97.7 ± 0.1	95.8–99.8	91.8–94.9	94.5–99.4
Saturated fatty acids (%)	SFA	22.6 ± 0.2	19.7 ± 0.0	14.2–15.3	18.4–18.6	15.6–17.9
Monounsaturated fatty acids (%)	MUFA	49.7 ± 0.4	53.2 ± 0.1	73.6–75.7	49.5–57.8	24.0–25.5
Polyunsaturated fatty acids (%)	PUFA	27.8 ± 0.6	27.1 ± 0.0	6.5–10.3	22.4–32.2	52.0–60.1
Unsaturation index (USFA/SFA)	UI	3.4 ± 0.0	4.1 ± 0.0	5.5–5.8	4.3–4.5	4.3–5.4
Degree of unsaturation (▽/mole)	DU	1.2 ± 0.0	1.2 ± 0.0	0.9–1.0	1.0–1.5	1.4–1.5
Calculated oxidation stability value (*h*)	COX	3.8 ± 0.0	3.6 ± 0.0	1.5–1.9	2.9–3.9	6.3–7.3
Ratio of *ω*-6 to *ω*-3 polyunsaturated fatty acids	(*ω*-6)/(*ω*-3)	1.7 ± 0.0	2.2 ± 0.0	9.8–19.3	49.3–111.0	6.7–7.9

Notes: *USFA*, unsaturated fatty acids

[a]Data of fatty acid composition (% of total fatty acids) were collected from the references (Dubois et al. 2007; Bialek et al. 2017; Giakoumis 2018)

metabolic pathways and associated regulatory mechanisms. The preliminary screening based on fluorescence intensity-based astaxanthin measurement succeeded in effectively identifying potentially positive chemical inducers and their optimal dosages.

Considering the possible false negatives of fluorescence intensity-based astaxanthin measurement in the screening approach (Chen et al. 2017a), the desirable dosages of selected chemical inducers were required to be verified in the secondary screening phase. The results demonstrated that different chemical inducers had significant effects on algal cell growth and the accumulation of astaxanthin and lipids in *C. zofingiensis* (Figs. 2 and 3). The inhibitory effects of oxidants and metabolic regulators on biomass production could be probably due to the excessive and toxic ROS induced to oxidize membrane lipids and destruct cellular membrane structure instead of inhibiting cell proliferation (Bai et al. 2015; Zhang et al. 2020a). This could be further supported by the results that antioxidants especially BHA and BHT improved biomass production significantly. Detrimental influences of PG and EGCG on biomass production might be possibly due to the specific resistance of algal cells to these chemicals. Inorganic salts have also been reported previously to induce algal cells to accumulate carotenoids and/or lipids at the expense of biomass (Janchot et al. 2019); however, the present study demonstrated that, for *C. zofingiensis*, the addition of KCl and $MgCl_2$ conversely promoted the biomass production. The results were consistent with a previous report that the addition of $MgCl_2$ resulted in the enhanced production of biomass as well as carotenoids, which was mainly due to magnesium ions supplementation improved organic carbon accumulation even under nitrogen-deprived conditions (Saeki et al. 2017). Additionally, all these organic acids considerably improved algal biomass in comparison to the control, although to different degrees. Through a rough mass balance analysis constructed on the dry-weight basis, the general biomass yield on the glucose of mixotrophic *C. zofingiensis* was estimated at about 0.4 g g^{-1} within the maximum yield of above 0.7 g g^{-1} of glucose consumed (Zhang et al. 2021b). The above results clearly demonstrated the existence of probably different mechanisms in regulating the cell growth of *C. zofingiensis*.

These potential astaxanthin-inducing chemicals not only had different effects on biomass production but also exerted significant effects on the accumulation of astaxanthin and lipids in mixotrophic *C. zofingiensis*. Oxidants, especially AAPH and H_2O_2, triggered the significant increase of astaxanthin content, which was mainly due to their direct induction of high oxidative stress that disturbed the balance between ROS generation and scavenging probably via the inhibition of vital antioxidant enzymes (Liu et al. 2014; Yu et al. 2015b). Unfortunately, these increases of astaxanthin content were at the expense of biomass production and thus the obtained astaxanthin yields and productivities in these cultures were lower than the control. These were consistent with previous studies that ROS-inducing chemicals were able to shift the metabolic pathways to lipid biosynthesis with the inhibition of central carbon metabolism for cell growth and division (Zhang et al. 2020a). The addition of antioxidants such as BHA, BHT, and PG resulted in the low astaxanthin content, which was probably due to cellular ROS levels being scavenged, while EGCG was an exception that promoted the astaxanthin accumulation by regulating antioxidant enzyme activity. The reasonable explanation might be that these antioxidants may have different degrees of capabilities of ROS scavenging and antioxidant systems development that are highly dependent on chemical structures and physiological characteristics of microorganism species

(Zhang et al. 2020b). Organic acids such as pyruvic acid and malic acid were able to promote cell growth of mixotrophic algae in parallel with high contents of astaxanthin and lipids probably by supplying more substrates as carbon sources. A similar phenomenon was also observed in astaxanthin-producing *H. pluvialis*, where the exogenous addition of sucrose did not induce cellular ROS level, but rather increased the levels of substrates from sucrose catabolism and induced relative nitrogen deficiency stress responses accompanied with up-regulation of carotenogenesis genes and direct interactions with signaling pathways (Du et al. 2020). Furthermore, secondary carotenoids include astaxanthin, canthaxanthin, lutein, and keto-lutein were major pigments in chemical induced-algal cells, which was consistent with previous studies investigating pigment accumulation in *C. zofingiensis* under nitrogen limitation stress (Mulders et al. 2015; Borowitzka 2018). The increased content of chlorophylls in ethanol-induced cells could be easily explained that ethanol suppressed gene expressions and activities of chlorophyll catabolic enzymes, i.e., chlase, Mg-dechelatase, and peroxidase, by which algal cell degraded chlorophylls to valuable nitrogen for the biosynthesis of new enzymes and stress-resisting molecules (Opio et al. 2017). While considering the coordination of the biosynthesis of chlorophylls and primary carotenoids including lutein, it might be reasonable to deduce that lutein catabolism was also inhibited by ethanol which resulting in the high lutein percentage. Taken into account of the astaxanthin content of 12.7 mg g^{-1} in ethanol-added cultures, it was inferred that chlorophyll degradation, as well as primary carotenoid catabolism, might have close correlations with astaxanthin biosynthesis which is worthy of further in-depth studies for the utilization of the chemical induction strategy for astaxanthin enhancement. Furthermore, it was deduced from the results that carotenoids and lipids are induced to biosynthesize synergistically and/or antagonistically in algal cells in response to chemical induction (Yu et al. 2015a; Zhao et al. 2019; Chen et al. 2020). For instance, the addition of organic acids was efficient for lipid production associated with high astaxanthin content in the present study, which was comparable to a previous study that achieved the highest lipid content and yield at 45.5% and 473.0 mg L^{-1} day^{-1}, respectively (Liu et al. 2016). The underlying explanation might be that lipid synthesis is essential for secondary carotenoid inclusion since the secondary carotenoids are deposited in lipid droplets (Liu et al. 2014; Mulders et al. 2015). However, the excess ROS caused by herbicides enhanced the production of astaxanthin in algal cells but exerted adverse effects on lipid accumulation. Additionally, the slight variation of fatty acid profiles in algal cells treated with different chemical inducers was obtained in the present study except for atrazine and DCMU that significantly decreased the content of oleic acids. It might be explained that algal chromoplasts where oleic acids were biosynthesized, were disrupted by these herbicides-induced ROS generated by inhibiting the photosynthetic electron transport chain (Doddaiah et al. 2013; Majewska et al. 2018). Besides, the induction effects were also species-dependent, for example, the signaling molecule acetylcholine, also as a catalyst accelerating hydroperoxide decomposition to ROS, was stimulatory for algal biomass production and lipid yield of *Chlorella sorokiniana* (Parsaeimehr et al. 2015), but demonstrated no significant increase in astaxanthin accumulation in *C. zofingiensis*, indicating unique acting mechanisms of regulating the coordinated or non-coordinated accumulation of astaxanthin and lipids in algal cells. These results suggested that these chemical inducers had the capability of improving astaxanthin and lipid production significantly, although their stimulatory effects on astaxanthin and lipid accumulation were not coordinated.

The current production levels of astaxanthin and lipids by *C. zofingiensis* in the latest 5-year studies were investigated and compared with our present study (Table 2). Through comparing the production levels of astaxanthin and lipids by mixotrophic *C. zofingiensis* in comparison with previous studies, it was shown that the highest astaxanthin yield and productivity reported to date were achieved at 194.5 mg L^{-1} and 9.9 mg L^{-1} day^{-1}, respectively, by employing the heterotrophy-photoinduction cultivation strategy (Sun et al. 2019b). Similarly, the maximum values of biomass (98.4 g L^{-1}) and astaxanthin yield (73.3 mg L^{-1}) were reported in two-step cultivation of *C. zofingiensis* integrated with the fermenter and outdoor rotating floating photobioreactor (Zhang et al. 2017). However, the astaxanthin content in all of these cultures is limited to a range of 0.7 to 2.7 mg g^{-1} because of the lack of a high-efficient and economical induction strategy, which seriously affected the utilization of *C. zofingiensis* for future commercial applications. The strategy of chemical induction combined with high light irradiation and nitrogen deprivation was utilized in the present study and substantially improved the accumulation of astaxanthin in *C. zofingiensis*. For example, the addition of ethanol resulted in the highest astaxanthin content of as high as 12.7 mg g^{-1}. Furthermore, the integrated production of astaxanthin and lipids with high levels in *C. zofingiensis* was realized in the study, which was superior or comparable to previous studies especially on astaxanthin accumulation. In contrast to ethanol induction that merely triggered astaxanthin biosynthesis but did not stimulate lipid accumulation as well as cell growth, the addition of PA resulted in the maximum astaxanthin yield and productivity of 87.0 mg L^{-1} and 7.3 mg L^{-1} day^{-1} along with high accumulation levels of lipid content (66.1%) and productivity (459.0 mg L^{-1} day^{-1}). The results of the present study demonstrated that the addition of chemical induction (e.g., ethanol and pyruvic acid) was able to significantly promote the accumulation

of astaxanthin in *C. zofingiensis*. Especially, the extra supply of organic acids, integrated with high light irradiation and nitrogen limitation, would be an effective approach to improve the accumulation of carotenoids and lipids, and the improved production levels were superior or comparable to previous studies, thus highlighting the commercial potential of *C. zofingiensis* for future applications. To the best of our knowledge, this is the first study reported to date which systematically evaluated the feasibility of chemical induction for integrated production of astaxanthin and lipids in *C. zofingiensis*. This work, in accordance with our previous study using phytohormone induction strategy, provides novel insights into the use of exogenous metabolic modulators or stimuli for enhanced coproduction of astaxanthin and lipids in *C. zofingiensis* and further strengthens the potential of *C. zofingiensis* as a promising production strain for multiple value-added bioproducts in the future.

In-depth investigations of the variations of certain metabolite compositions correlated to chemical induction are necessarily required since they might provide valuable information for better elucidating regulatory mechanisms of *C. zofingiensis* and advancing its commercial applications. The results demonstrated that all these chemical inducers could be clustered into different groups by HCA analysis based on their effects on the cell growth and intracellular accumulation of astaxanthin and lipids. It was suggested that these chemical inducers in the same cluster with the control might act on similar metabolic sites or interact with identical signaling pathways with the control conditions, i.e., nutrient-limited stress and high light irradiation. Organic acids and $MgCl_2$ were also clustered into the same group mainly due to their positive effects on cell growth as well as on astaxanthin and lipid accumulation. Ethanol and glyphosate were separated from atrazine and DCMU, which might be predominantly attributable to their different degrees of stimulatory effects on astaxanthin accumulation. Most importantly, it was deduced from HCA analysis that these chemical inducers belonging to the same groups might involve in certain metabolic pathways related to astaxanthin and/or lipid biosynthesis, and accordingly, their acting mechanisms might be inferred from their effects on the physiological responses of algal cells.

The heatmap analysis clearly demonstrated the abundance changes of pigments and fatty acids in algal cells under different chemical induction treatments, indicating their close correlation in resistance to exogenous stresses. The results demonstrated that these metabolites changed their expression patterns in different chemical treatments, which were in accordance with the phenotype performance of *C. zofingiensis* for astaxanthin and lipid production. Fatty acids in the first cluster were significantly inhibited by atrazine and DCMU mainly due to that these two metabolic regulators both have toxicological effects on phytoplankton cells by inhibiting the electron transport chain and thereby resulting in the inhibition of photobiosynthesis and the reduced production of organic carbon and reducing power (Bai et al. 2015). Thus, it would be deduced from the results that the increased supply of substrates and reducing power might be necessary for enhanced fatty acid production. Additionally, lutein, ketolutein, and chlorophyll *b* belonging to the second cluster were up-regulated to a large extent in At and DCMU treatments. Generally, under stress conditions of high light and nitrogen starvation, cellular chlorophylls and primary carotenoids in algal cells would degrade rapidly to supply carbon-skeleton and nitrogen for essential cellular processes such as the biosynthesis of fatty acids and new resistance enzymes (Mulders et al. 2015). The high levels of chlorophyll *b* and lutein indicated their degradation could be suppressed by At and DCMU to a large extent, thereby resulting in the redirection of carbon flux from fatty acids towards other organic carbon metabolites, which could also partly explain the aforementioned inhibitory effects of atrazine and DCMU on fatty acid biosynthesis. In the third cluster, pigments and parts of fatty acids especially astaxanthin, canthaxanthin, and C18:3 showed a close correlation in resistance to exogenous stresses; however, these metabolites changed their expression patterns in different chemical treatments. For example, the levels of all these metabolites were simultaneously decreased in the treatments of antioxidants and metabolic regulators, whereas the levels of astaxanthin and canthaxanthin in AAPH, H_2O_2, EGCG, EtOH, and Gly treatments were all increased synergistically. It indicated that different types of oxidants, antioxidants, and metabolic regulators probably have certain mechanisms to regulate algal responses to exogenous stresses. Other than that, it was found that the synergistic and antagonistic biosynthesis of fatty acid and carotenoids (mainly astaxanthin) coexisted in algal cells through the comparisons among different treatments, suggesting unique mechanisms of regulating the synergistically coordinated accumulation of carotenoids and fatty acids in *C. zofingiensis*. However, detailed coordinated relationships between astaxanthin and fatty acids and underlying regulatory mechanisms are still lacking until now, hence further in-depth investigations for the reference of astaxanthin and lipid production by *C. zofingiensis* are recommended.

In nature, microalgae have evolved different regulatory mechanisms to shift intracellular metabolic pathways to deal with exogenous environmental disturbances (Sun et al. 2019a; Zhang et al. 2020a). To date, numerous studies utilizing chemical inducers to improve astaxanthin and/or lipid accumulation have been widely carried out in *H. pluvialis* and other lipid-producing microalgae, whereas systematical verification of their positive effects on *C. zofingiensis* is yet to be conducted and underlying regulatory mechanisms are still unclear. For a better

understanding of the stimulatory effects of positive chemical inducers on the biosynthesis of astaxanthin and/or lipids in *C. zofingiensis*, the hypothetical regulatory mechanisms of various potential chemical inducers were proposed based on the above results and previous studies as illustrated in Fig. 6. These hypothetical regulator mechanisms, to a certain extent, facilitate understanding and decoding the results and phenomena obtained in the present study that these chemical inducers exerted different effects on *C. zofingiensis*. Generally, stimulatory mechanisms of these chemical inducers could be classified into four groups: the induction or reduction of cellular ROS levels, the enhancement of carotenogenesis and fatty acid biosynthesis, the inhibition of crucial carbon/nitrogen metabolic pathways, and the adjustment of cellular morphological properties (e.g., membrane permeability, cell size, and shape) (Yu et al. 2015a; Sun et al. 2019a), which were discussed in detail as follows:

Oxidants and antioxidants Intracellular ROS is the most important nuclear signaling molecule that is mainly generated in three different organelles of algal cells due to the inevitable oxidative metabolisms in living cells such as photosynthesis and respiration (Zhang et al. 2020a). In terms of molecular mechanisms of ROS, it was systematically summarized previously that ROS induction may be executed through three aspects of microalgal metabolism, i.e., the manipulation of biosynthesis genes and transcription factors, the enhanced supply of precursors and reducing powers, and the activation of endoplasmic reticulum stress closely related to the

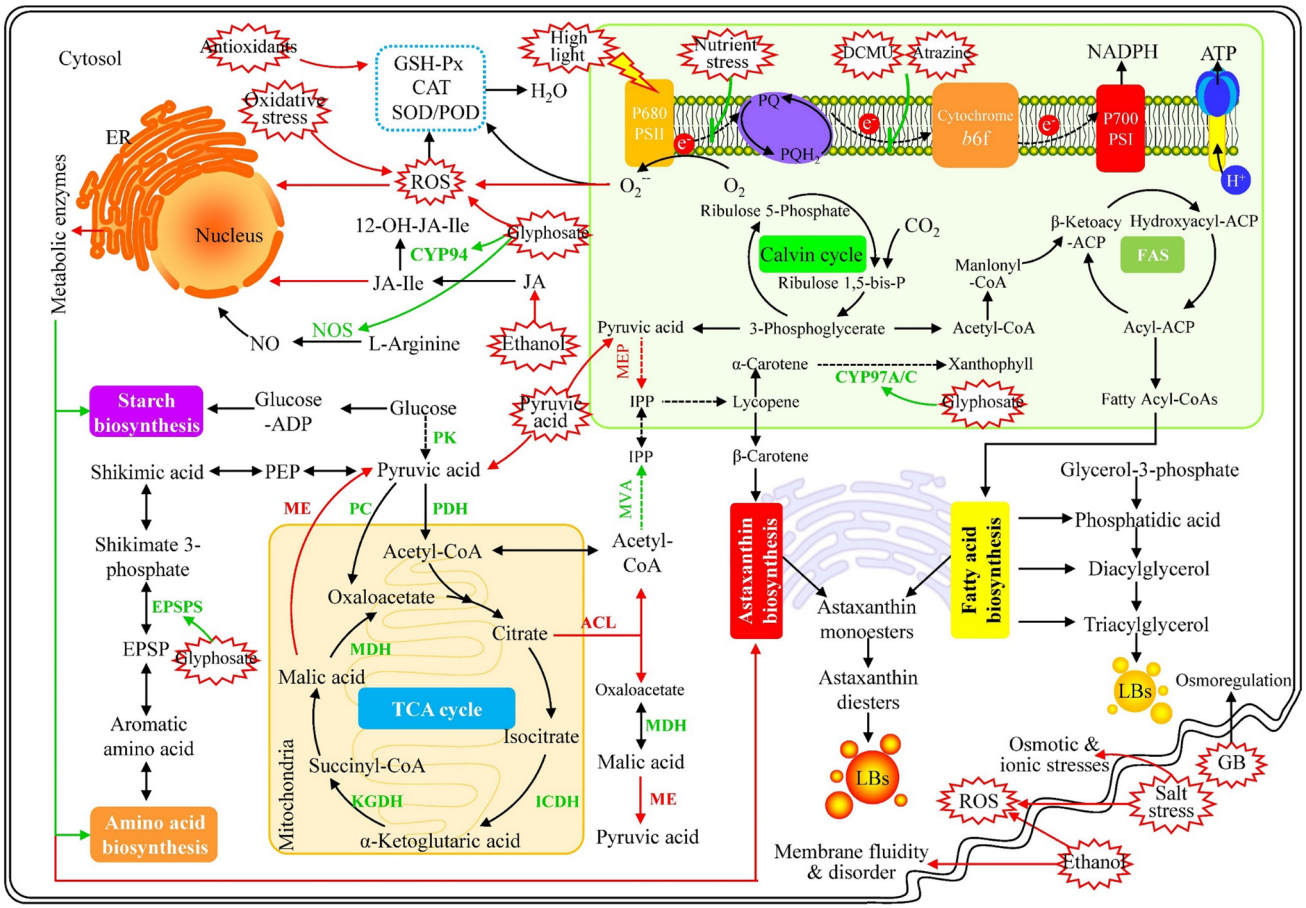

Fig. 6 Proposed mechanisms underlying the effects of positive chemical inducers on astaxanthin and lipid biosynthesis in mixotrophic *C. zofingiensis*. The red or green solid arrows represent stimulatory or inhibitory effects on the targeted enzymes and pathways, respectively. Dash line arrows indicate putative connections between the metabolites. ROS: reactive oxygen species; ER: endoplasmic reticulum; NO: nitric oxide; NOS: nitric oxide synthase; JA: jasmonic acid; JA-Ile: jasmonoyl-isoleucine conjugate; PEP: phosphoenolpyruvate; IPP: isopentenyl pyrophosphate; EPSP: 5-enolpyruvylshikimate-3-phosphate; LB: lipid droplets; PQ: plastoquinone; PQH_2: plastohydroquinone; TCA: tricarboxylic acid cycle; EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase; ACL: ATP citrate lyase; ME: malic enzyme; PK: pyruvate kinase; PC: pyruvate carboxylase; PDH: pyruvate dehydrogenase; MDH: malate dehydrogenase; KGDH: α-Ketoglutarate dehydrogenase; ICDH: isocitrate dehydrogenase; MVA: the cytoplasmic mevalonate pathway; MEP: the methylerythritol 4-phosphate pathway; CYP: cytochrome P450 enzymes; GSH-Px: glutathione peroxidase; CAT: catalase; SOD: superoxide dismutase; POD: peroxidase

formation of lipid droplets (Sun et al. 2019a; Zhang et al. 2020a). In the present work, the oxidants AAPH and H_2O_2 were found to trigger the significant increase of astaxanthin content but show slight influences on lipid accumulation in *C. zofingiensis* cells. It was suggested that the induced low level of ROS would stimulate the accumulation of secondary metabolites especially astaxanthin with the strongest capacity to quench ROS than the defensive enzyme system; however, when cellular ROS levels exceed a threshold limit, it would not continuously promote lipid biosynthesis but induce lipid droplet remodeling or storage lipid degradation as energy reserves for redox homeostasis maintenance. Although the antioxidants such as BHA and BHT were reported to be able to enhance astaxanthin accumulation by scavenging intracellular ROS, only EGCG was proved to promote the production of astaxanthin by *C. zofingiensis*. The simplest explanation could be that these antioxidants may have different degrees of capabilities of ROS scavenging and antioxidant systems development, which are highly dependent on chemical structures and physiological characteristics of microorganism species. Additionally, it was inferred that the EGCG stimulating astaxanthin accumulation not merely through efficiently reducing excess harmful oxygen-free radicals due to its unique chemical structure of the C ring gallate group associated with multiple phenolic hydroxyl groups, but also by activating the activities of defensive enzymes (e.g., catalase, superoxide dismutase, and glutathione peroxidase) and upregulating gene expression of protein kinases (e.g., mitogen-activated protein kinase, protein kinase C, and phosphoinositide 3-kinases) in signaling pathways (Zhang et al. 2020b).

Organic acid precursors Organic acids involved in the glycolytic pathway and the TCA cycle are capable of enhancing the pools of acetyl-CoA and essential reductants by the presence of related key enzymes (e.g., ATP citrate lyase and malic enzyme) and improving the channeling of carbon fluxes into carotenoids and lipids (Yu et al. 2015a; Sun et al. 2019a). According to the biosynthesis pathways of astaxanthin and lipids, as shown in Fig. 6, acetyl-CoA serves as the precursor for lipid biosynthesis in which the redox-sensitive pyruvate kinase (PK) is responsible for the biosynthesis of pyruvic acid in glycolysis and thiamine-dependent pyruvate dehydrogenase (PDH) and ATP-dependent ACL (ATP citrate lyase) further promote the conversion of pyruvic acid into acetyl-CoA. In contrast, astaxanthin is mainly derived from acetyl-CoA and pyruvic acid via the cytosolic mevalonate and the chloroplastidic MEP (methylerythritol 4-phosphate) pathway, respectively, thereby yielding the substrate for two necessary precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) for astaxanthin biosynthesis (Vranova et al. 2013). Based on our results, both pyruvic acid and citrate acid, capable of being converted into acetyl-CoA, induced different stimulatory effects on the biosynthesis of astaxanthin and lipids. It was deduced that the enhanced supply of acetyl-CoA may promote lipid biosynthesis but fail to simultaneously improve astaxanthin accumulation as their biosynthetic processes compete for the same substrate. Pyruvic acid seems to be a preferred precursor for astaxanthin biosynthesis through integrating with 3-phosphoglycerol aldehyde via the non-mevalonate pathway. The main advantage of the MEP pathway over the mevalonate (MVA) pathway is that MEP is energetically more balanced in terms of the generation of reducing equivalents and energy (Nguyen et al. 2020). Also, MEP has higher theoretical yields of IPP pools compared with MVA. For example, the cellular abundance of MEP precursors pyruvic acid and glyceraldehyde-3-phosphate (G3P) were reported to be nearly 150 times and 30 times higher than that of acetyl-CoA pools in cells, which serves as a pushing force to shift carbon flux to astaxanthin biosynthesis (Nguyen et al. 2020). This could also partly explain the phenomenon that pyruvic acid gave better astaxanthin production than other organic acids investigated. The stimulatory effects of malic acid might also be due to its conversion to pyruvic acid associated with NADPH production catalyzed by a malic enzyme (ME) instead of by malate dehydrogenase (MDH) to oxaloacetate. These results were consistent with a previous study that reported that pyruvate metabolism has a direct positive correlation with astaxanthin biosynthesis through altering carbon fixation to provide more precursors (Fang et al. 2020). Furthermore, it was reported that microalgae respond to nitrogen limitation by cascade reactions such as thiamine exhaustion, adenosine monophosphate (AMP) deamination, and the elevated cellular oxidative level, which further results in inhibited activities of related key enzymes, including redox/energy-sensitive catabolic enzymes (e.g., pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase) to generate pyruvic acid, AMP-dependent isocitrate dehydrogenase (ICDH) to degrade isocitrate, and thiamine-dependent pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH) to generate acetyl-CoA and succinyl-CoA (Semkiv and Sibirny 2019). The activity changes of these key enzymes in the glycolytic pathway and TCA cycle thus would change central carbon metabolism with different degrees, which could partly explain none or limited stimulatory effects of KG on astaxanthin and lipid accumulation. As to citrate acid, it is generally catalyzed by two typical enzymes, i.e., ACL and ICDH, to provide acetyl-CoA and ATP for cellular metabolism, respectively, and thus the enhanced activity of ACL accompanied with the lower activity of ICDH is required for lipid accumulation (Sun et al. 2019a). This is consistent with our results that citrate acid improved lipid accumulation level but failed to enhance astaxanthin accumulation, indicating that the increase of acetyl-CoA pools might promote

lipid biosynthesis but would not enhance the chances of their conversion into astaxanthin. In other words, increasing substrate availability associated with the regulation of enzyme activities would shift cellular carbon partitioning and drive more carbon fluxes towards the biosynthesis of astaxanthin and lipids, although to a different extent. Altogether, our work provides useful information into regulatory mechanisms of organic acids and concludes that improving the conversion of pyruvic acid into IPP by the MEP pathway instead of by the acetyl-CoA-mediated MEV pathway would be more favorable for astaxanthin accumulation.

Metabolic regulators Metabolic regulators are another wide range of active compounds capable of regulating diverse metabolic pathways for the targeted compound production even at the expense of necessary detrimental physiological changes. As shown in Fig. 6, GB (an osmoprotectant biosynthesized under abiotic stress), ACE (a stress-resisting signaling molecule), and SOV(an inhibitor of phosphoprotein tyrosine phosphatases capable of regulating mitogen-activated protein kinase signaling cascade) acted on different metabolic sites and pathways (Tran et al. 2009; Parsaeimehr et al. 2015; Shankar et al. 2015; Zhao et al. 2016), but induced antagonist effects on astaxanthin and lipid accumulation. Atrazine and DCMU also failed to further improve the accumulation of astaxanthin and lipids in *C. zofingiensis*, although photosynthetic inhibitors especially DCMU were reported to induce the cytoplasmic and mitochondrial ROS and subsequently enhance the pentose phosphate pathway to produce more NADPH (Schwarz et al. 2016; Sun et al. 2018). The results provided evidence that the significant increase of ROS through blocking the chloroplast electron transport chain would not certainly promote the biosynthesis of astaxanthin and lipids. Intriguingly, it was found that glyphosate was able to promote astaxanthin accumulation, although it was detrimental to cell growth. Through literature review, it is shown that glyphosate is a competitive inhibitor of various crucial enzymes, mainly 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and cytochrome P450 (CYP) family enzymes, implicated in important biological processes (Samsel and Seneff 2013; Gomes et al. 2014). The suppression of EPSPS by glyphosate on the biosynthesis of essential aromatic amino acids, including the auxin precursor tryptophan, could not explain its stimulatory effects since it was reported previously that the addition of exogenous auxins was favorable for the accumulation of astaxanthin and lipids (Chen et al. 2020). Thus, it is plausible that the potential inhibition by glyphosate of CYP enzymes and related pathways underline its stimulatory promotion. It was reported that carotene hydroxylases especially belonging to the CYP97 clan play physiologically crucial roles in the hydroxylation of carotene and subsequent biosynthesis of lutein, thus the inhibition of these enzymes would theoretically enable to inhibit the consumption of carotenoid precursors and thus promote astaxanthin accumulation. CYPs also participate in the signaling pathways related to the stimulation of astaxanthin/lipid biosynthesis. The essential signaling molecule jasmonic acid conjugated with isoleucine was reported to mediate in the regulation of astaxanthin accumulation, in which excess jasmonic acid and jasmonoyl-isoleucine (JA-Ile) conjugate were inactivated via hydroxylation by series of CYPs, suggesting closely potential relationships between CYPs and astaxanthin accumulation mediated by jasmonic acid (JA) signaling (Caarls et al. 2017). Our present study provides new insights into regulation mechanisms by which these metabolic regulators act on algal cells.

Salinity and osmotic stresses The modulation of the membrane permeability is another effective measure to improve the accumulation of astaxanthin and lipids in algal cells. The recently developed approach named membrane engineering with the aim to improve the permeability and storage capacity of membranes has proven successful in improving carotenoid production (Wu et al. 2017; Lu and Liu 2019), demonstrating the close correlation of membrane property modulation and enhanced carotenoid production. In the present study, besides being signaling molecules, ROS would react with components in cell membranes, change membrane permeability to small molecules, and eventually influence functions of cell membranes to stimulate astaxanthin and lipid accumulation to resist stress conditions (Horvath and Daum 2013). In contrast, ethanol adjusts cellular morphology and related biological processes (e.g., ATPase activity and transport systems in membranes) instead of reacting with membrane composition to indirectly induce astaxanthin accumulation (Da Silveira et al. 2003; Liu et al. 2019). Furthermore, the stimulatory effects of salinity stress on astaxanthin accumulation are also due to its adverse influences on cellular morphology and ion homeostasis. This is supported by a previous study that reported that excess KCl led to cell enlargement accompanied by the increases of carotenoid and lipid accumulation (Janchot et al. 2019). Noticeably, the superiority of $MgCl_2$ over KCl might contribute to its essential roles as an indispensable cofactor and structural constituent of many important metabolic enzymes, thereby directly activating the activities of diverse metabolic enzymes responsible for carbon partitioning and cellular energy transfer. Altogether, the present work systematically summarized the hypothetical mechanisms underlying the positive effects of chemical inducers on *C. zofingiensis*, although there is a lack of direct evidence for these hypotheses due to limited studies. Further studies are still required to clarify the regulatory mechanisms of chemical inducers, providing new insights and direction for the modulation of

metabolic pathways of *C. zofingiensis* to biosynthesize valuable targeted compounds.

Until now, techno-economic astaxanthin production by microalgae is still challenging. This chemical addition approach in the present study could be utilized in an algal biorefinery to maximize the outputs of astaxanthin and lipids from *C. zofingiensis* biomass and thus to a large extent exploit the efficiency of *C. zofingiensis* biorefinery. Besides, another feasible approach for economic microalgae cultivation is to make full utilization of high-value components especially lipids in algal cells which will bring in higher revenue and will be able to absorb the overall production costs. Currently, it is able to achieve the extraction and separation of lipids from carotenoids through the integration of supercritical extraction and fractionation process technologies and then maximize the total value of all outputs from the same material (Kadam et al. 2013; Liu et al. 2021). Previously, it was reported that *C. zofingiensis*-derived biodiesel was more suitable to be blended with other types of diesel instead of directly being utilized for engine applications based on its properties (Minyuk et al. 2020), which was consistent with our present study that *C. zofingiensis*-lipids with the high percentage of unsaturated fatty acids were not appropriate for biodiesel production directly. However, our present study proved the feasibility of *C. zofingiensis* lipids for the potential production of microalgae-derived edible oils with similar characteristics to edible vegetable oils. Moreover, through comparing the COX values of algal oils with other vegetable oils, it was found the potential of *C. zofingiensis*-derived lipids as edible oils with the resistance to the thermal and photo-induced during processing and storage. Most importantly, algal oils presented a relatively balanced (ω-6)/(ω-3) ratio (1.7–2.2) in comparison to that of vegetable oils which was characterized by the high percentage of linoleic acid (LA, C18:2, ω-6) and a low proportion of linolenic acid (ALA, C18:3, ω-3). As it is commonly known, ω-6 and ω-3 fatty acids are the two most essential FA families with specific precursors, namely ALA and LA, and they have a competition that exists with each other to act antagonistically in the regulation of diverse sets of body homeostasis (Saini and Keum 2018). It has been shown that a balanced dietary intake ratio of ω-6 and ω-3 fatty acids is recommended to be below 5:1 (Candela et al. 2011). However, the present dietary ratio of ω-6 and ω-3 fatty acids is much higher than that due to the excess of ω-6 fatty acids specifically LA in the human diet. The high (ω-6)/(ω-3) ratio can activate the persistent acute inflammation that is beneficial in protecting hosts against infections and wounds but considerably promotes the risks of cancer and acute cardiovascular diseases (Candela et al. 2011). One possible solution is to rebalance the (ω-6)/(ω-3) ratio by incorporating dietary supplements and functional foods rich in ω-3 fatty acids such as oil extracts from *C. zofingiensis* biomass. Thus, *C. zofingiensis*-derived algal oils abundant in astaxanthin and with a balanced (ω-6)/(ω-3) ratio have the promising potential for edible oil production.

Conclusions

This study developed a two-stage screening approach associated with chemical induction for enhancing the simultaneous production of astaxanthin and lipids in mixotrophic *C. zofingiensis*. The correlated relationships between the dosage effect of chemical inducers and abundance changes of metabolic parameters were investigated to help elucidate underlying regulatory mechanisms. The potentials of *C. zofingiensis* to be used as feedstocks for the production of edible oils were also evaluated. Our work provides a convincing basis of the chemical addition strategy in enhancing the accumulation of astaxanthin and lipids and highlights the viability of *C. zofingiensis* as feedstocks in future applications.

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Data availability The datasets generated during and/or analyzed during the current study are present in the paper. Additional data are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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