Algal Research 19 (2016) 178–183

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

The crosstalk between astaxanthin, fatty acids and reactive oxygen species in heterotrophic *Chlorella zofingiensis*

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ARTICLE INFO

Article history:
Received 7 June 2016
Received in revised form 6 August 2016
Accepted 22 August 2016
Available online xxxx

Keywords:
Chlorella zofingiensis
Heterotrophic
Astaxanthin
Fatty acids
NADPH
ROS

ABSTRACT

Chlorella zofingiensis can achieve ultrahigh cell density under heterotrophic growth conditions and has been considered as a promising producer of astaxanthin. Astaxanthin is predominantly esterified with fatty acids, in the form of either mono-ester or di-ester. The interrelationship between astaxanthin and fatty acids remains poorly understood. In the present study, we found that astaxanthin induction was accompanied by fatty acid accumulation in the glucose-fed heterotrophic *C. zofingiensis* cells. The presence of cerulenin, a specific inhibitor against fatty acid biosynthesis, led to a significant increase in the content of astaxanthin, di-ester in particular. When treated with sesamol, which is known to inhibit intracellular NADPH supply, a considerable decrease in astaxanthin was observed, together with a slight drop in fatty acids. Reactive oxygen species (ROS) level was found to correlate well with astaxanthin content in *C. zofingiensis* regardless of culture conditions. Collectively, these findings suggest that astaxanthin biosynthesis may compete with fatty acids for the carbon precursors and reducing power, and NADPH supply is important for astaxanthin accumulation, which will help us better understand astaxanthin biosynthesis in heterotrophic *C. zofingiensis* and benefit the future engineering for astaxanthin production.

1. Introduction

Astaxanthin is a high-value ketocarotenoid found in many marine animals and microorganisms [9]. Because of strong pigmentation function, powerful antioxidative activity and broad beneficial effects on human health, astaxanthin possesses a wide range of applications in feed, food, nutraceutical, and pharmaceutical industries [8,15]. Humans cannot synthesize astaxanthin and have to obtain it through the food chain. The green microalga *Haematococcus pluvialis* is believed to synthesize the highest level of astaxanthin in the world [8] and dominates the current market of natural astaxanthin. Nevertheless, *H. pluvialis* has inherent limitations such as slow grate rate, low biomass yield, ease of contamination by other fast-growing organisms, and a requirement of high light illumination for astaxanthin induction and accumulation [22,40]. *C. zofingiensis* has recently been proposed as a promising alternative microalgal producer of astaxanthin due to its fast growth, high cell density, low sensitivity to unfavorable environments, and astaxanthin accumulation under heterotrophic conditions with glucose as the sole carbon and energy source [27]. The astaxanthin yield of *C. zofingiensis* can reach up to 56 mg/L in fed-batch fermentation [25,28], comparable to that of *H. pluvialis* [17]. However, the relatively low content of astaxanthin in *C. zofingiensis* (up to 0.5% of dry weight) confers limitation on its industrial application and needs to be addressed [27].

Understanding the regulatory mechanisms for carotenoid biosynthesis and deposit is fundamentally important for metabolic engineering of astaxanthin production in microalgae. Hydroxylation and ketolation of β-carotene lead to the formation of astaxanthin, which is predominantly esterified with fatty acids, in the form of either mono-ester or di-ester in microalgae. Astaxanthin is believed to be deposited in the triacylglycerol (TAG)-rich lipid bodies (LBs) [3,10,46]. There is a strong correlation between fatty acids (particularly oleic acid) and astaxanthin content in *H. pluvialis* [3,37,47]. In the presence of fatty acid biosynthesis inhibitors, *H. pluvialis* showed a considerable decrease in fatty acids and TAG, with a concomitant and almost complete abolishment of astaxanthin [48]. Two roles of fatty acids in astaxanthin accumulation have been proposed: esterification with astaxanthin to form astaxanthin esters, and more importantly, forming LBs as the storage for astaxanthin [3]. Additionally, Reactive oxygen species (ROS), the well-known signal molecule involved in many cellar metabolic processes [1,19,31,45], is believed to play a crucial role in regulating carotenoid biosynthesis and astaxanthin accumulation in both *H. pluvialis* and *C. zofingiensis* [12,16,18].

Nevertheless, the above mentioned studies are conducted predominantly under photoautotrophic conditions. The heterotrophic production potential of *C. zofingiensis* drives us to explore the possible mechanisms for astaxanthin biosynthesis and accumulation under heterotrophic conditions, which remains largely unknown and has been so far rarely touched. Therefore, the main objective of this study is to investigate the relationship between fatty acids and astaxanthin in *C.*

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http://dx.doi.org/10.1016/j.algal.2016.08.015
2211-9264/

zofingiensis under heterotrophic growth conditions and the role of ROS in astaxanthin accumulation. To this end, we employed two inhibitors, the fatty acid biosynthesis inhibitor cerulenin [7] and the NAPDH formation inhibitor sesamol [43], and analyzed their effect on fatty acid and astaxanthin profiles in heterotrophic *C. zofingiensis* cultures. Cerulenin was shown to attenuate fatty acid biosynthesis while enhancing astaxanthin accumulation, particularly astaxanthin di-ester. By contrast, sesamol led to a decrease in both fatty acids and astaxanthin levels, with astaxanthin reduction being more significant. Meanwhile, the intracellular ROS level correlated well with astaxanthin accumulation. Collectively, our work represents the first effort to study the crosstalk between astaxanthin, fatty acid and ROS in heterotrophic *C. zofingiensis* cultures, which may provide valuable insights into future engineering of this alga for improved production of astaxanthin under heterotrophic conditions.

2. Materials and methods

2.1. Strains and culture conditions

C. zofingiensis (ATCC30412) was purchased from American Type Culture Collection (ATCC, Rockville, USA). The alga was maintained in dark at 25 °C in 500-mL Erlenmeyer flasks containing 100 mL Kuhl growth medium [30]. Glucose was used as the carbon source at a concentration of 5 g/L. The algal cells were allowed to grow 3 days to reach the late exponential phase and served as the seed cultures for nitrogen (N) starvation, from which the cells were collected, washed, and re-suspended at a cell density of 0.5 g/L in the N-free Kuhl medium containing 5 g/L glucose. When needed, the inhibitors cerulenin and sesamol were applied to the cultures upon N starvation at a concentration of 5–40 μM and 0.25–2 mM, respectively.

2.2. Fatty acid analysis

Cell samples were collected by centrifuging at 4000*g* for 3 min at 4 °C and lyophilized in a DW3 freeze-drier (Heto Dry Winner, Denmark). The lyophilized cell samples were directly methylated to fatty acid methyl esters (FAMEs) by incubating with 1% sulphuric acid in methanol according to the procedures described by Liu et al. [26]. The FAMEs were analyzed by using a GC-MS-QP 2010 SE (Electron Ionization type) gas chromatograph-mass spectrometer (SHIMADZU, Japan) and an Rtx-2330 capillary column (30 m × 0.25 mm) (Restek, Guangzhou, China). Helium was used as the carrier gas. The injection temperature, ion temperature and interface temperature were set at 250 °C, 200 °C and 260 °C, respectively. The initial column temperature was set at 150 °C. The column temperature subsequently rose to 200 °C at 10 °C/min and then to 220 °C at 6 °C/min, followed by a hold at 220 °C for 10 min. The quantification of FAMEs was performed according to Liu et al. [24]. All measurements were done in triplicate.

2.3. Pigment extraction and analysis

The lyophilized microalgal cell samples were grinded with mortar and pestle under liquid nitrogen and then extracted three times with acetone. The acetone layers were collected by centrifugation and dried under nitrogen gas. The dried pigments were dissolved in 1 mL acetone and filtered with a 0.22 μm Millipore membrane before HPLC analysis. Free astaxanthin and astaxanthin esters were quantified by HPLC (Waters, Milford, MA, USA) equipped with a waters 2695 HPLC and a 2998 detector. The method was used as described by Ip et al. [13]. All measurements were done in triplicate.

2.4. ROS measurement

ROS levels were determined with the ROS assay kit (Beyotime Institute of Biotechnology, China) by measuring the oxidative conversion of 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) to fluorescent compound dichlorofluorescin (DCF) [14,23]. Briefly, one milliliter culture samples were harvested by centrifugation at 4000*g* for 3 min and the cell pellets were resuspended in 200 μL Kuhl medium with 10 μM DCFH-DA, the cell pellets with Rosup reagent (in the kit) added was regarded as a positive control. After incubation for 20 min, the cells were washed three times with fresh Kuhl medium and then were resuspended in the collected original culture medium. Then the DCFH fluorescence was tested at 485 nm excitation and 520 nm emission by a fluorescence microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). All measurements were done in triplicate.

2.5. RNA isolation, cDNA synthesis, and real-time RT–PCR

RNA was extracted from about 10^8 cells using the RNA plant plus Reagent (Tiangen, Beijing, China) according to the manufacturer's instructions. The concentration of total RNA was determined spectrophotometrically at 260 nm. Total RNA (1 μg) extracted from different samples was reverse transcribed to cDNA by QuantScript RT Kit reagent (Tiangen, Beijing, China) according to the manufacturer's instructions. RT-PCR amplification was carried out using One Step SYBR PrimeScript PLUS RT-PCR Kit reagent (TaKaRa, Tokyo, Japan). Primers used for real-time PCR were listed in Table 1. The mRNA expression level was normalized using the *C. zofingiensis* actin (ACT) gene as the internal control, and the relative gene expression level was calculated by using $2^{-\Delta\Delta CT}$ method [29].

2.6. Statistical analysis

All the experiments were conducted in three biological replicates. The results in Figures are expressed as mean value ± SD. The significance of the results was calculated by one way ANOVA (analysis of variance) and Duncan's multiple range tests ($P < 0.05$) by using SPSS version 19.0.

3. Results

3.1. Astaxanthin induction is accompanied by fatty acid accumulation in heterotrophic C. zofingiensis under nitrogen deficiency condition

The DCW of heterotrophic *C. zofingiensis* increased fast under nitrogen deficiency condition with the presence of 5 g/L glucose, from a starting dry cell weight of 0.5 g/L to 1.6 g/L after 72 h of cultivation (data not shown). In order to investigate the relationship between astaxanthin and fatty acids, the contents of astaxanthin and total fatty acids (TFAs) in *C. zofingiensis* cells during the growth period were determined. Clearly, astaxanthin exhibited a drastic increase within the first 24 h, followed by a slight but steady increase within the next 48 h (Fig. 1). Similarly, TFA content showed a great increase from 9.6% to 31.2% of dry weight (DW) within 72 h (Fig. 1). Both TFA and astaxanthin followed similar accumulation patterns, suggesting that astaxanthin accumulation correlates with fatty acid synthesis in *C. zofingiensis*. Our results were consistent with photoautotrophic *H. pluvialis* in which astaxanthin accumulation was in accordance with TFAs [47].

Table 1
PCR primers used for RT-PCR to quantify the genes' expression level.

Gene	Forward (5′–3′)	Reverse (5′–3′)
PSY	CACCAGGTTGTCAGAGTCCA	ACTAGTGTGTTGCTGACTCT
PDS	GATGAATGTATTTGCTGAACT	GGCCAGTGCCTTAGCCATAG
LCYb	CGCAGGCGAAAAATTCCTGT	TAAGGAATGTCACACCGCTGG
CHYb	GCCAGCCATGAAACGTGTG	GTTCCTTCCAGTTATGTACACA
BKT	GGTGCTCAAAGTGGGGTGGT	CCATTTCCCACATATTGCACCT
ACT	TGCCGAGCGTGAAATTGTGA	CGTGAATGCCAGCAGCCTCCA