Bioresource Technology 214 (2016) 319–327

Contents lists available at ScienceDirect

Bioresource Technology

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

Simultaneous production of triacylglycerol and high-value carotenoids by the astaxanthin-producing oleaginous green microalga *Chlorella zofingiensis*

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HIGHLIGHTS

- Stress conditions induced TAG and astaxanthin production in *C. zofingiensis*.
- A strong correlation between TAG and astaxanthin accumulation was observed.
- Simultaneous TAG and astaxanthin production in semicontinuous modes was optimized.
- High TAG and astaxanthin productivities (297 and 3.3 mg L^{-1} day^{-1}) were obtained.
- Inhibiting *de novo* fatty acid biosynthesis enhanced astaxanthin accumulation.

ARTICLE INFO

Article history:
Received 24 March 2016
Received in revised form 21 April 2016
Accepted 22 April 2016
Available online 26 April 2016

Keywords:
Astaxanthin
Biofuels
Chlorella zofingiensis
Integrated production
Stress conditions
Triacylglycerol

ABSTRACT

The production of lipids and astaxanthin, a high-value carotenoid, by *Chlorella zofingiensis* was investigated under different culture conditions. Comparative analysis revealed a good correlation between triacylglycerol (TAG) and astaxanthin accumulation in *C. zofingiensis*. Stress conditions promoted cell size and weight and induced the accumulation of neutral lipids, especially TAG and astaxanthin, with a concomitant decrease in membrane lipids. The highest contents of TAG and astaxanthin achieved were 387 and 4.89 mg g^{-1} dry weight, respectively. A semi-continuous culture strategy was developed to optimize the TAG and astaxanthin productivities, which reached 297 and 3.3 mg L^{-1} day^{-1}, respectively. Additionally, astaxanthin accumulation was enhanced by inhibiting *de novo* fatty acid biosynthesis. In summary, our study represents a pioneering work of utilizing *Chlorella* for the integrated production of lipids and high-value products and *C. zofingiensis* has great potential to be a promising production strain and serve as an emerging oleaginous model alga.

1. Introduction

Owing to the ever-increasing energy consumption and drastic depletion of fossil fuels, alternative energy forms that are green, renewable and sustainable are highly sought after. Biodiesel, or fatty acid methyl esters (FAMEs) derived from oil transesterification, is renewable, carbon neutral and portable, and has received great interest of the public and research (Knothe, 2009). Currently, biodiesel is produced mainly from plant oils. Nevertheless, plant oil-based biodiesel has inherent limitations and cannot realistically substitute the petroleum fuels (Chisti, 2007). In contrast, microalgae possess significant advantages over plants for biodiesel production and are considered to be the next-generation biodiesel feedstock capable of meeting the existing demand for transportation fuels (Chisti, 2007; Wijffels and Barbosa, 2010).

Although the utilization of microalgae as biodiesel feedstock has achieved substantial progress during the recent decades, significant challenges remain yet to be addressed before the cost-effective production of algal biodiesel can be realized (Richardson et al., 2012). Efforts have been made with an aim to improve the production economics of algal biodiesel, including alga selection, strain trait improvement, exploration of novel cultivation technologies and downstream processes, etc (Radakovits et al., 2010; Breuer et al., 2012; Pribyl et al., 2012; Kim et al., 2013; Liu and Chen, 2014; Wang et al., 2014; Sun et al., 2015). Alga selection,

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http://dx.doi.org/10.1016/j.biortech.2016.04.112
0960-8524/

the starting point of algal biodiesel production pipeline, is considered fundamentally important. Because of the rapid growth, ease of cultivation, high lipid content and excellent lipid productivity, *Chlorella* has been regarded to be among the most promising producers of biodiesel precursors (Liu and Chen, 2014; Sun et al., 2015). While many studies focus on the optimization of algal culture conditions, the integrated production of lipids with co-products emerges as a new research direction (Liu and Chen, 2014), which is believed to be a promising approach toward offsetting the algal biodiesel production cost. Several oleaginous *Chlorella* species have been proposed to produce other useful components than lipids, including but not restricted to protein, lutein, and astaxanthin (Liu and Chen, 2014; Liu et al., 2014a). Nevertheless, protein and the primary carotenoid lutein inversely correlate with the accumulation of storage lipids (Guccione et al., 2014; Sun et al., 2015), thus impeding the integrated production to a great extent. In contrast, astaxanthin is a secondary carotenoid and tends to accumulate in *Chlorella zofingiensis* under such stress conditions as high light irradiance and nitrogen deprivation (Liu et al., 2010a, 2014b), which are also the typical triggers of storage lipids especially triacylglycerol (TAG), an ideal precursor for making biodiesel (Liu et al., 2012a; Sun et al., 2015). The characteristic that TAG and astaxanthin are induced by the same conditions offers *C. zofingiensis* substantial advantages for the simultaneous production of these two products. While there have been many studies exploiting the production of lipids or astaxanthin by *C. zofingienesis* (Del Campo et al., 2004; Liu et al., 2010a,b, 2011, 2012a, 2012b, 2013, 2014a, 2014b; Sun et al., 2015), to date have been few attempts to concurrently produce both products (Liu et al., 2012b; Mulders et al., 2014). The correlation between TAG and astaxanthin accumulation, and how they respond to the different stress conditions, remains to be elucidated in *C. zofingiensis*.

The main objective of the present study was to investigate the growth and accumulation of TAG and astaxanthin in response to four culture conditions, namely the regular low light (LL) growth condition and three stress conditions of nitrogen deprivation (ND), high light (HL), and the combination of ND and HL (ND + HL). Comparative results revealed that TAG and astaxanthin were induced by stress conditions and accumulated in a well-correlated manner. The production of both TAG and astaxanthin was greatly promoted by the adoption of a semi-continuous cultivation strategy coupled with nitrogen limitation. Our work represents a pioneering effort for comprehensive study of *C. zofingiensis* lipids and carotenoids and provided valuable insights into the utilization of this alga for the integrated production of TAG and astaxanthin.

2. Materials and methods

2.1. Algal strain and culture conditions

C. zofingiensis (UTEX B32) was purchased from the University of Texas Culture Collection of Algae (UTEX, Austin, USA) and maintained at 14 °C on agar plates of the modified BG-11 medium (150 mg L^{-1} of nitrate-N). Briefly, 10 mL of liquid BG-11 was inoculated with cells from agar plates and the alga was grown aerobically in flasks at 25 °C for 6 days with orbital shaking at 150 rpm and illuminated with continuous light of 30 μE m^{-2} s^{-1}. The cells were then inoculated at 10% (v/v) into 100-mL columns provided with constant illumination of 70 μE m^{-2} s^{-1} and aeration of 1.5% CO_2 enriched air, grown to late exponential phase and used as seed cultures for subsequent experiments.

2.2. Batch culture for the induction of oil and astaxanthin

The seed cultures were harvested, washed with N-deficient medium and suspended in N-replete (150 mg L^{-1} of nitrate-N) or N-deficient medium, yielding a final cell density of 0.5 g L^{-1}. Both cultures were grown in 250-mL glass columns aerated with 1.5% CO_2 enriched air, illuminated with 70 μmol photons m^{-2} s^{-1} (LL and ND) or 350 μmol photons m^{-2} s^{-1} (HL and ND + HL).

For the inhibition of fatty acid biosynthesis, cerulenin (Sigma–Aldrich, St. Louis, MO, USA), which specifically inhibits β-ketoacyl-ACP synthase I (KAS I), was added upon N-deprivation at a concentration of 10 μM in the cultures under ND conditions.

2.3. Semi-continuous culture for the production of oil and astaxanthin

The seed cultures were harvested, washed with N-deficient medium and suspended in N-limited (5, 10, or 20 mg L^{-1}) medium, giving a cell density of 0.5 g L^{-1}. The cultures were grown under HL (350 μmol photons m^{-2} s^{-1}) conditions for three days and then subject to semi-continuous cultivation at a daily dilution rate of 0.5 using the corresponding N-limited medium, namely, half of the cultures were taken and replaced by fresh medium manually. Additional semi-continuous cultures (10 mg L^{-1} N) were conducted, with half cultures replaced every 2, 1, and 0.5 days, corresponding to a dilution rate of 0.25, 0.5 and 0.75, respectively.

2.4. Determination of cell number, dry weight, and diameter

Cell numbers were counted using a hemocytometer under a light microscope. For dry weight determination, the algal cells were filtered through a pre-dried Whatman GF/C filter paper (1.2 μm pore size), washed three times with deionized water, dried at 100 °C in a vacuum oven until constant weight was achieved, and were subsequently cooled down to room temperature in a desiccator before weighting. Cell diameter was determined by using a microscope equipped with an internal reticle scale.

2.5. Lipid and pigment extraction

Cell samples were harvested, washed and freeze-dried on a DW3 freeze-drier (Heto Dry Winner, Denmark). The lyophilized cells were disrupted by homogenization with liquid nitrogen and extracted by a solvent mixture of chloroform/methanol/0.75% KCl solution (2:1:0.75, by volume). The lower chloroform phase containing lipids and pigments were collected and dried under nitrogen gas and then dissolved in chloroform or acetone for immediate use or stored at −20 °C.

2.6. Lipid analysis

Neutral lipids were separated on a Silica gel 60 TLC plate (Merck, Darmstadt, Germany) using a mixture of hexane/tert-butylmethyl ether (TBME)/acetic acid (80/20/2, by volume) as the mobile phase, while polar lipids were separated on a TLC plate using a mixture of chloroform/methanol/acetic acid/water (25/4/0.7/0.3, by volume) as the mobile phase. Lipids were detected by spraying the TLC plate with 10% $CuSO_4$ in 8% phosphoric acid, followed by charring at 180 °C for 3 min. For quantification, individual lipids on TLC plate were visualized with iodine vapor, recovered, transesterified and analyzed by Gas Chromatography Mass Spectrometry (GC–MS). The contents of these lipids were expressed as their fatty acids relative to dry weight (%).

Fatty acid methyl esters (FAMEs) were prepared by direct transesterification of lipids with 1% sulfuric acid in methanol. The FAMEs were separated and identified by GC–MS using a PerkinElmer CLarus 680 capillary gas chromatograph equipped with a SQ8 Mass Spectrometry detector and apolar TR-WAX column (Thermo Fisher Scientific; length 30 m, diameter 0.25 μm, film thickness 0.25 μm). Helium was used as the carrier gas. Samples were injected in split mode (5:1 split ratio) at an oven temperature