Sugar-based growth, astaxanthin accumulation and carotenogenic transcription of heterotrophic *Chlorella zofingiensis* (Chlorophyta)

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Abstract

The green microalga *Chlorella zofingiensis* can grow and produce the ketocarotenoid astaxanthin in the dark with glucose as sole carbon and energy source. In the present study, we reported that glucose, mannose, fructose, sucrose, galactose and lactose could differentially support the cell growth and astaxanthin biosynthesis. Of the sugars surveyed, glucose and mannose were the best carbon sources for the algal growth in the dark, as indicated by the relatively high specific growth rates (ca. 0.03 h⁻¹) and high cell dry densities (ca. 10 g l⁻¹). Furthermore, the algal cells cultured with glucose and mannose accumulated the highest amounts of astaxanthin (ca. 1 mg g⁻¹), indicating a correlation between cell growth and astaxanthin formation. In addition, various sugars differentially regulated the transcription of three carotenogenic genes encoding phytoene desaturase (PDS), β-carotene ketolase (BKT), and β-carotene hydroxylase (CHYb) respectively. By using glucose fed-batch fermentation, high cell dry weight concentration (ca. 53 g l⁻¹) and high astaxanthin production (ca. 32 mg l⁻¹) were obtained, which are much higher than those ever reported in the alga. The present study suggests that *C. zofingiensis* is suitable for the production of natural astaxanthin on a massive scale.

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1. Introduction

Green microalgae have already served as a major natural source of valuable carotenoids [1,2]. The ketocarotenoid astaxanthin has attracted much attention due to its strong quenching ability of singlet oxygen, involvement in cancer prevention, and enhancement of immune response [3,4]. Accumulating evidence has shown that astaxanthin is beneficial to human health by preventing degenerative diseases [4]. Potential production of astaxanthin from microorganisms has been a subject of intensive investigation in recent years [5–10].

Compared with other astaxanthin-producing microorganisms, some green algae, such as *Haematococcus pluvialis* and *Chlorella zofingiensis*, have the potential to accumulate high amounts of astaxanthin because they possess sequestering systems to storing up astaxanthin in lipid bodies [7,11]. *H. pluvialis* has been commercially used for producing natural astaxanthin, whereas *C. zofingiensis* has only recently attracted much attention due to its ease of growing under various conditions with high growth rate, and minimal negative environmental influence [8,9]. Furthermore, *C. zofingiensis* can grow and produce astaxanthin without light when exogenous glucose is supplemented [10]. Thus, dark-grown *C. zofingiensis* might be more economical for commercial production of astaxanthin than any light-dependent cultivation systems, because the well-developed and economical fermentation systems can be used to cultivate the algal cells on a large scale. However, heterotrophic *C. zofingiensis* accumulates much less astaxanthin (ca. 1 mg g⁻¹ or 10 mg l⁻¹) than phototrophic *H. pluvialis* (ca. 40 mg g⁻¹ or 35 mg l⁻¹) although the former one may reach much higher biomass (ca. 10 g l⁻¹) [7,10]. As the organic carbon source is the most important factor for dark-grown *C. zofingiensis*, in the present study, we investigated the effect of various sugars on the biosynthesis of astaxanthin and the transcription of three carotenogenic genes including the phytoene desaturase (*PDS*) [12], β-carotene ketolase (*BKT*), and β-carotene hydroxylase (Genbank accession EU016205) in the dark-grown alga.
2. Materials and methods

2.1. Microalgae and medium

Chlorella zofingiensis ATCC 30412 was obtained from the American Type Culture Collection (ATCC, Rockville, USA). This alga was maintained at 4 °C on an agar slant containing the modified Bristol's medium CZ-M1 [10]. Briefly, 10 ml of CZ-M1 broth was inoculated with cells from slants and the alga was grown aerobically in flasks at 27 ± 1 °C for 4 days with orbital shaking at 150 rpm and illuminated with continual florescence light at 60 µmol m⁻² s⁻¹ measured at the surface of the flask. The cells were then inoculated at 5% (v/v) into a 200-ml Erlenmeyer flask containing 36 ml of the medium, and were grown for 4 days and used as seed for batch culture. All media were prepared using distilled water and nutrient solutions (pH 6.6). The medium supplemented with various carbon sources (glucose, fructose, galactose, mannose, lactose, and sucrose) were inoculated with 10% (v/v) of exponentially growing inoculum and then incubated at 25 °C in an orbital shaker at 150 rpm in the dark.

2.2. Batch culture

In shake flask batch culture, Erlenmeyer flasks (250 ml), each containing 90 ml medium supplemented with various carbon sources (glucose, fructose, galactose, mannose, lactose, and sucrose) were inoculated with 10% (v/v) of exponentially growing inoculum and then incubated at 25 °C in an orbital shaker at 150 rpm in the dark.

2.3. Fed-batch fermentation

A two-stage fed-batch culture was carried out in a 3.7-l fermenter (Bioengineer AG, Wald, Switzerland). The working volume of the culture was 2.0 l. The cultivation conditions in the fermenter were controlled as follows: pH 6.6; temperature 25 °C; agitation 480 rpm; and dissolved oxygen concentration 50% saturation. During fed-batch cultivation, the sterilized stock nutrient solution was fed into the fermenter at intervals so that glucose concentration in the cultures was kept at 5–20 g l⁻¹.

2.4. Analytical methods

For cell dry biomass (DW, g l⁻¹) determinations, 5-ml aliquots of the cell culture were filtered through a pre-dried Whatman GF/C paper, washed three times, and the filters containing cells were dried until constant weight. Specific growth rate (h⁻¹) was calculated from the DW, during the logarithmic phase of growth, using the equation \( \mu = (\ln X_2 - \ln X_1)/(t_2 - t_1) \), where \( X_2 \) and \( X_1 \) represent DW values at times \( t_2 \) and \( t_1 \), respectively.

For pigment analysis, cell pellets were obtained by centrifuging the culture samples at 3000 rpm at 4 °C for 3 min and dried in a DW3 freeze-drier (Heto Dry Winner, Denmark). The freeze-dried cells (0.01 g) were ground with nitrogen and reverse, 5°C for 20 min.

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2.5. RT-PCR assay

RNA techniques were followed according to the standard method described in Ref. [16]. DNA was isolated from aliquots of about 10⁶ cells cultured with various sugars (50 g l⁻¹) for 2 days. Cells were collected by centrifugation and powdered under liquid nitrogen using a mortar and pestle. RNA was then isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s instructions. The concentration of total RNA was determined spectrophotometrically at 260 nm.

2.5. RT-PCR assay

Total RNA (1 µg) extracted from different samples was reverse transcribed to cDNA by using a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) for reverse transcription PCR (RT-PCR) primed with oligo(dT) according to the manufacturer’s instructions. PCR amplification was carried out using specific primers of PDS (forward, 5'-GCAATATGATTTTCTGCAATGCCGG-3' and reverse, 5'-GGCCGATCCCTAAGGATCATGCTG-3'), BKT (forward, 5'-GTCCTCAAAGCTGGGCTG-3') and reverse, 5'-CATATATGTTGACACTGCTG-3'). CHYb (forward, 5'-GGCCAGGATGAACTGCTG-3') and reverse, 5'-GTTCCTCCAGTATGCTG-3'). C. zofingiensis actin (act) primers (5'-TGCCTAGCTGAAATTTGGA-3' and reverse, 5'-CTCTGATCAGCAGGCTGCA-3') [13] were used to demonstrate equal amounts of templates and loading. The GenBank accession numbers for PDS, BKT, and CHYb were EF621405, AY772713, and EU16205, respectively. Amplification of the cDNA was done by conventional PCR [94°C for 2 min followed by 24 cycles (for PDS, BKT and actin genes) or 26 cycles (for CHYb gene) of 94°C for 15 s, 58°C for 20 s, 72°C for 30 s]. PCR products were separated on a 2% agarose gel and stained with ethidium bromide for photography (Biorad, USA). The relative transcript levels of the specific genes were determined based on gel visualization.

3. Results and discussion

3.1. Heterotrophic growth and astaxanthin synthesis of C. zofingiensis with various carbon sources

Since C. zofingiensis can grow well heterotrophically with glucose as sole carbon and energy source, we investigated if this alga is capable of metabolizing other organic carbon sources (e.g. oligosaccharides) for heterotrophic cultivation to achieve high cell concentrations. Four monosaccharides (glucose, fructose, galactose, and mannose) and two disaccharides (lactose and sucrose) were surveyed and the results are presented in Fig. 1. C. zofingiensis could use all the sugars for heterotrophic growth but their specific growth rates and dry biomass concentrations were rather different (Fig. 1). Glucose and mannose were the best carbon sources for heterotrophic growth of C. zofingiensis which exhibited high specific growth rates (0.028 h⁻¹/0.029 h⁻¹) and high dry biomass concentrations (10.63 g l⁻¹/10.17 g l⁻¹). Fructose was also a good carbon source for the heterotrophic growth of C. zofingiensis, with which the algal cells demonstrated a slightly lower specific growth rate (0.027 h⁻¹) and dry biomass (9.44 g l⁻¹) than that with glucose or mannose. Sucrose and galactose led to low specific growth rates (0.018 h⁻¹/0.012 h⁻¹) and low dry biomass (5.46 g l⁻¹/2.17 g l⁻¹). Lactose was the poorest carbon source assimilated by the algal cells, as indicated by the very low specific growth rate (0.009 h⁻¹) and high sugar residual in the medium (more than 40 g l⁻¹, data not shown). The dry biomass from lactose containing culture was only 1.42 g l⁻¹. 87% lower than that from glucose-supplemented culture.

Some green algae can grow heterotrophically, making use of sugars, amino acids and organic acids [17]. The uptake of sugars by the unicellular alga Chlorella was shown inducible [18,19]. When the algal shifts from carbon autotrophy to heterotrophy, hexose transport activity increases more than 200 fold [18]. In response to hexoses, Chlorella cells can induce monosaccharide-H⁺ symport catalyzing the energy-dependent transport of ω-glucose and several other hexoses across the plasmalemma [20,21]. Our study

![Fig. 1. Heterotrophic growth of C. zofingiensis at 50 g l⁻¹ of various sugars. Cell dry weight concentration (□) and specific growth rate (●). Data are mean values ± S.D. of three independent measurements.](image-url)
showed that *C. zofingiensis* cells could transport and assimilate various exogenous sugars with quite different efficiency.

Interestingly, the astaxanthin production from cultures with different carbon sources (Fig. 2) demonstrated a correlation to the cell growth. Cultures with glucose or mannose not only grew fastest leading to the highest dry biomass, but also produced the highest quantities of astaxanthin (1 mg g$^{-1}$ or 10 mg l$^{-1}$). In contrast, lactose is a poor carbon source for the heterotrophic growth of *C. zofingiensis*, with which the algal cells grew slowly and only produced a trace amount of astaxanthin (0.06 mg g$^{-1}$). Cultures with medium containing fructose, sucrose or galactose exhibited a decreasing trend in cell growth rates, dry biomass as well as astaxanthin production. These results revealed that the biosynthesis of astaxanthin in *C. zofingiensis* might be associated with the cell growth. In the red yeast *Phaffia rhodozyma*, the cell respiration rate was shown to correlate positively with the astaxanthin production rate [22]. Heterotrophic *C. zofingiensis* may share a similar regulation of astaxanthin synthesis as the yeast.

### 3.2. Differential transcription of BKT, CHYb, and PDS genes in *C. zofingiensis* cells grown with various sugars

We recently found that glucose induced the expression of the *BKT* that catalyzed the formation of both canthaxanthin from β-carotene as well as astaxanthin from zeaxanthin in *C. zofingiensis* [13]. Since various sugars resulted in different astaxanthin yields by the alga, we investigated whether the sugars differentially regulated the related carotenogenic genes. RT-PCR approach was used to detect the transcription of β-carotene ketolase (BKT), β-carotene hydroxylase (CHYb), and PDS in cells cultured with various sugars. The results are shown in Fig. 3. The various sugars differentially regulated the transcription of the genes. Glucose and mannose, which supported the best growth and astaxanthin production of the algal cells, also resulted in the highest transcript levels of all the genes. Fructose, galactose, and sucrose only moderately enhanced the transcription of the genes; while lactose which was poorly metabolized by the cells did not up-regulate the transcription of the carotenogenic genes. Glucose was reported to induce expression of carotenogenic genes in the cyanobacterium *Syneocystis* sp. PCC 6803 [23]. The glucose effects on the expression of genes are suggested to result from the glucose-sensing and signaling with the hexokinase as the key sensor in a variety of yeasts, mammals and higher plants [24–27], or from changes in the cytosolic redox state or electron flow of respiratory electron transport [24,28,29]. A correlation between biomass, astaxanthin production, and the transcripts of BKT and CHYb genes was evident in the cells treated with the sugars. Our results suggested that the uptake and/or the metabolism of sugars might mediate the transcription of the carotenogenic genes. By using glucose analogs and specific inhibitors of respiratory electron transport, it is possible to reveal how the cells regulate transcription of the carotenogenic genes in response of sugars.

In the present study, *C. zofingiensis* was shown to differentially utilize not only different monosaccharides but also different disaccharides (Fig. 1). Furthermore, different carbon sources not only supported differential cell growth, but also differential astaxanthin production in the cells (Fig. 2). As glucose and mannose benefit cell growth as well as astaxanthin production, it is possible to enhance the astaxanthin production of *C. zofingiensis* via optimizing the culture conditions (e.g. glucose concentration in medium) or/and culture approaches (e.g. fed-batch fermentation).

#### 3.3. Fed-batch fermentation enhanced the production of astaxanthin by *C. zofingiensis*

We previously showed that the highest specific growth rate of *C. zofingiensis* was obtained when 20 g l$^{-1}$ glucose was involved in the medium; whereas the highest cell dry weight and astaxanthin content were obtained when 50 g l$^{-1}$ glucose was used [10]. We therefore designed different culturing systems to investigate the role of carbon source on the cell growth and astaxanthin production. Batch culture with 50 g l$^{-1}$ glucose was used as control. In fed-batch cultures, 20 g l$^{-1}$ glucose was initially used. When the glucose concentration in medium dropped to around 5 g l$^{-1}$ (monitored by sugar detection each day), additional glucose or medium with glucose was fed to the cultures to reach the initial glucose concentration (20 g l$^{-1}$). Two times of feeding were carried out with a total of 50 g l$^{-1}$ glucose used by the fed-batch culture. The biomass and astaxanthin production of the culture are summarized in Table 1. The fed-batch culture (glucose/medium with glucose) obtained much higher biomass concentration (17.8 g l$^{-1}$/20 g l$^{-1}$) than the batch culture (10.3 g l$^{-1}$). However, the astaxanthin contents of the fed-batch culture (0.69 mg g$^{-1}$/0.1 mg g$^{-1}$) were much lower than that of the batch culture.
(1.02 mg g⁻¹). One reason for the low content of astaxanthin may be the low dissolved oxygen concentration in the flask cultures at high cell density, as revealed in the case of red yeast P. rhodozyma [22]. To answer this question, a 3.7-l fermenter, which could maintain high dissolved oxygen concentration, was used coupled with a fed-batch procedure that contained two stages of feeding: three times of medium and glucose feeding followed by four times of glucose feeding. The feeding time, time course of cell growth and astaxanthin production are shown in Fig. 4.

The fed-batch fermentation greatly increased the cell dry weight concentration from 10.3 g l⁻¹ to 51.8 g l⁻¹ and astaxanthin yield from 10.5 mg l⁻¹ to 32.4 mg l⁻¹ (Fig. 4 and Table 1). During the first stage of medium and glucose feeding, the cells grew fast but the astaxanthin content was less than 0.2 g g⁻¹ (data not shown). A high C/N ratio was found to facilitate astaxanthin formation by the alga [10]. The low content of astaxanthin in cells fed with medium with glucose may be due to the relatively low C/N ratio. This explanation was supported by the fact that astaxanthin accumulation was greatly enhanced when glucose was fed to the cultures (Fig. 4). Photoautotrophic C. zofingiensis accumulated astaxanthin up to 1.5 mg g⁻¹ or 15 mg l⁻¹ when induced with high light [30]. In contrast, dark-grown C. zofingiensis could accumulate astaxanthin up to 32.4 mg l⁻¹ (this study), which may be further enhanced by employment of reactive oxygen species as described in our previous study [31,32] or by optimization of fermentative medium [33]. Thus, dark-grown C. zofingiensis may be potentially employed for commercial production of astaxanthin on a large scale.

In conclusion, C. zofingiensis can uptake and metabolize a variety of sugars for heterotrophic growth. Glucose and mannose are the most suitable carbon source for heterotrophic growth of the alga. Carbon source and nutrient are two important factors influencing the growth and astaxanthin production by C. zofingiensis. The astaxanthin-producing capacity by C. zofingiensis could be greatly enhanced by using a fed-batch fermentation strategy.

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References
