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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.

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 [13], β-carotene hydroxylase (Genbank accession EU016205) in the dark-grown alga.

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2. Materials and methods

2.1. Microalga 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 adjusted to pH 6.5 prior to autoclaving at 121 °C for 20 min.

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 (Bioengineering 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 (μ, 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 extracted with acetone until the cells became colorless. After centrifugation at 14,000 rpm at 4 °C for 5 min, the supernatant was collected and evaporated under nitrogen gas and the residue was redissolved in 1 ml acetone which was filtered through a 0.22-μm Millipore organic membrane prior to HPLC analysis. The HPLC system was equipped with a Waters 2695 separations module and a Waters 2996 photodiode array detector. Twenty microliters of each extract was separated by HPLC on a Waters Spherisorb® 5 μm ODS2 4.6 × 250 mm analytical column (Waters, Milford, MA, USA) according to the method described by Ref. [14]. Pigments were eluted at a flow rate of 1.2 ml min^{-1} with a linear gradient from 100% solvent A (acetonitrile:methanol:0.1 M Tris–HCl, pH 8.0 [84:2:14]) to 100% solvent B (methanol:ethyl acetate [68:32]) for 15 min, followed by 20 min of solvent B. The absorption spectra of the pigments were shown between 300 and 700 nm. Peaks were measured at a wavelength of 450 nm. The concentration of individual carotenoids was determined using standard curves of standard pigments at known concentrations.

Sugar concentration (g l^{-1}) was determined according to the method of Miller [15].

2.5. RNA isolation

RNA techniques were followed according to the standard method described in Ref. [16]. RNA was isolated from aliquots of about 10^8 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.

2.6. 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′-GATGAATGTATTTGCTGAACTGGGC-3′ and reverse, 5′-GGCCAGTGCCTTAGCCATAGCG-3′), *BKT* (forward, 5′-GTGCTCAAAGTGGGGTGGTATG-3′ and reverse, 5′-CCATTTCCCACATATTGCACCT-3′) and *CHYb* (forward, 5′-GCCAGCCATGAAACGTGTG-3′ and reverse, 5′-GTTCCTTCCAGTTATGTAC ACA-3′). *C. zofingiensis* actin (act) primers (5′-TGCCGAGCGTGAAATTGTGA G-3′ and reverse, 5′-CGTGAATGCCAGCAGCCTCCA-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 EU016205, 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 alga 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 D-glucose and several other hexoses across the plasmalemma [20,21]. Our study

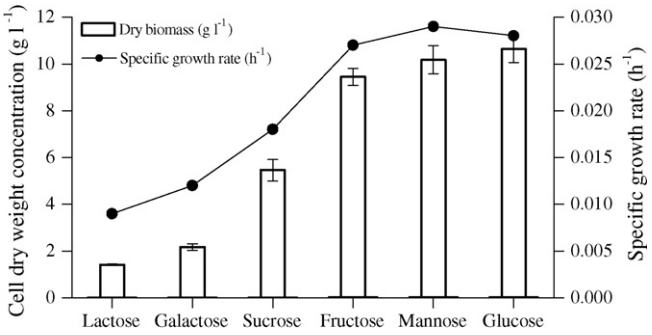

Fig. 1. Heterotrophic growth of *C. zofingiensis* at 50 g l^{-1} of various sugars. Cell dry weight concentration (□) and specific growth rate (●). Data are mean values ± S.D. of three independent measurements.