Expression of Carotenogenic Genes and Astaxanthin Production in *Xanthophyllomyces dendrorhous* as a Function of Oxygen Tension

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This report gives an insight into the specific changes in the transcription of four key carotenogenic genes [encoding geranylgeranyl diphosphate synthase (crtE), phytoene desaturase (crtI), phytoene synthase lycopene cyclase (crtYB), and astaxanthin synthase (ast), respectively] in *Xanthophyllomyces dendrorhous* cultures, with regard to dissolved oxygen (DO) contents of 10%, 25%, and 40% air saturation, respectively. 25% DO proved to be the most beneficial for yeast growth, transcription of carotenogenic genes, and astaxanthin content.

Key words: Carotenogenic Genes, Astaxanthin, *Xanthophyllomyces dendrorhous*, Dissolved Oxygen

Introduction

The red yeast *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) serves as the major microorganism for the biotechnological production of dietary astaxanthin (Cruz and Parajó, 1998; Rodríguez-Sáiz *et al*., 2010; Schmidt *et al*., 2011). The ketocarotenoid astaxanthin has attracted much attention due to its strong ability to quench singlet oxygen, involvement in cancer prevention, and enhancement of the immune response (Park *et al*., 2010). Recent studies showed that it has a nearly 10-fold higher antioxidant activity than other carotenoids and 100- to 500-fold higher activity than α-tocopherol (Miki, 1991; Naguib, 2000). It has been increasingly used as a feed and food pigment in the aquaculture industry, and is also regarded as a potential functional food and pharmaceutical supplement because of its excellent antioxidant activity (Guerin *et al*., 2003; Johnson and Schroeder, 1995).

X. dendrorhous is an aerobic organism whose growth (primary metabolism) is dependent on the oxygen supply in the culture system. Previous studies have shown that the astaxanthin production rate in liquid cultures of *X. dendrorhous* increases with increasing oxygen uptake (Yamane *et al.*, 1997). As oxygen supply to the culture medium is a key factor for *X. dendrorhous* growth and astaxanthin production, in the present study, we investigated the effect of dissolved oxygen (DO) content on the biosynthesis of astaxanthin and the transcription of four key carotenogenic genes, *i.e.* geranylgeranyl diphosphate (GGPP) synthase (crtE, Genbank accession no. DQ016502), phytoene desaturase [crtI (Verdoes *et al.,* 1999a), Genbank accession no. AY177424], phytoene synthase lycopene cyclase [crtYB (Verdoes *et al.*, 1999b), Genbank accession no. AY177204], and astaxanthin synthase [ast (Ojima *et al.*, 2006), Genbank accession no. DQ002007], in the cells cultured in fermentors.

Material and Methods

Microorganisms

X. dendrorhous AS 2.1557 was obtained from the China General Microbiological Culture Collection Center (CGMCCC, Beijing, China), maintained on slants of YM agar at 4 °C, and transferred monthly.

Media and chemicals

YM agar medium, which contained the following components (per liter): 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, and 20 g agar, was used to maintain the yeast strains. The

components of 1 L production medium were 30 g glucose, 3 g KH_2PO_4, 3 g $MgSO_4$, 1 g Na_2HPO_4, and 5 g yeast extract. Astaxanthin standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Batch culture experiments

The culture experiments were carried out in three 5-L stirred fermentors (Biostat, B. Braun, Melsungen, Germany) with a working volume of 3–3.2 L (initial filling volume 3.0 L). The following probes were installed on the top plate: InPro 6000 series O_2 probe (Mettler-Toledo, Greifensee, Switzerland), pH electrode, pt-100 temperature sensor. The fermentation parameters were controlled by a digital measurement and control system. The air flow rate into the fermentors was fixed at 3,000 mL min^{-1} (equivalent to 1.0 vvm), and the DO content was controlled at various air saturations (10%, 25%, and 40%) by regulating the agitation rate. The temperature of the fermentor was kept at 20 °C.

Analytical procedures

Dry cell weight (DCW) was determined after drying at 60 °C in a hot-air oven till dry constant weight was obtained. Astaxanthin content was determined by high-performance liquid chromatography (HPLC) according to Lu *et al.* (2010). All samplings and assays were carried out in triplicate, and the results were determined as the average mean values ± standard deviation (SD).

RNA isolation

RNA techniques followed the standard methods described by Sambrook *et al.* (1989). RNA was isolated from 40-mL aliquots of cultures grown with various DO contents for 2 d. Cells were collected by centrifugation and powdered under liquid nitrogen using a mortar and pestle. The total RNA content in the extract was determined at 260 nm, and the purity was determined by the ratios of optical densities both at 260:230 nm and 260:280 nm (greater than 1.8).

RT-PCR assay

Reverse transcription (RT) and polymerase chain reaction (PCR) were done according to Lodato *et al.* (2007). The primers used for amplification of crtI, crtYB, crtE, ast, and actin were as described by Lodato *et al.* (2007). Following the separation of the PCR products on ethidium bromide-stained 1.8% agarose gels, the bands were quantified using the Herolab E.A.S.Y Win 32 software (Wiesloch, Germany). Each band was normalized against the intensity obtained with the same cDNA using actin-specific primers.

Results and Discussion

Effects of various DO contents on cell growth and carotenoid production of X. dendrorhous

The influence of the DO content on growth and astaxanthin synthesis in the batch culture of *X. dendrorhous* AS 2.1557 in a 5-L stirred fermentor was examined (Table I). Both cell growth and carotenoid production increased with increasing DO content from 10% to 25%, however, it seemed to result in a rapid decrease when the DO content was increased to 40%. DO at 25% (v/v) supported both the highest astaxanthin yield of (12.78 ± 0.63) mg L^{-1} and the highest astaxanthin content of (0.97 ± 0.05) mg g^{-1} DCW.

It was also apparent that the astaxanthin production in cultures grown with different DO contents (Table I) was correlated with cell growth. Cultures with 25% DO not only grew fastest leading to the highest dry biomass, but also produced the highest quantities of astaxanthin. In contrast, 10% and 40% DO poorly supported growth and astaxanthin production of *X. dendrorhous*. Previous reports have stated that the DO tension affects productivity, cell autolysis, and rigidity of the cell wall in fungal fermentations. It was shown that lipase production of *Rhizopus delemar* decreased when the DO tension was lower than about 18% of air saturation (Giuseppin, 1984); and that in cultures of *Aureobasidium pullulans*, the yield of pullulan at 50% DO tension of air saturation was higher than that at 100% DO tension (Wecker and Onken, 1991). From

Table I. Effect of various DO contents on growth, astaxanthin yield, and astaxanthin content of *X. dendrorhous* AS 2.1557 in 5-L stirred fermentors (n = 3).

DO (%)	Biomass [g L^{-1}]	Astaxanthin yield [mg L^{-1}]	Astaxanthin content [mg g^{-1} DCW]
10	9.88 ± 0.39	5.97 ± 0.28	0.60 ± 0.03
25	13.12 ± 0.55	12.78 ± 0.63	0.97 ± 0.05
40	11.12 ± 0.48	9.52 ± 0.37	0.86 ± 0.04

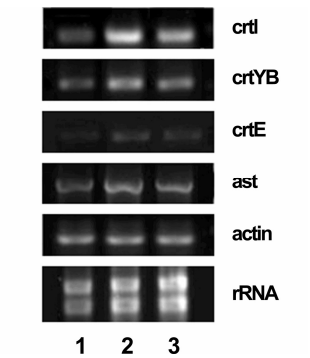

Fig. 1. RT-PCR analysis of the influence of DO content on the expression of carotenogenic genes. RT-PCR was performed using RNAs from cells grown at 10% DO (lane 1), 25% DO (lane 2), and 40% DO (lane 3). For comparison, total RNA was stained with ethidium bromide (lowest panel).

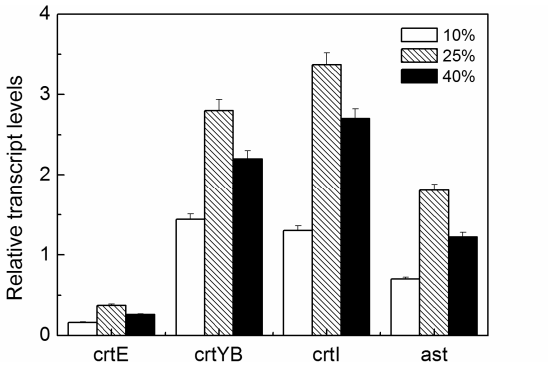

Fig. 2. Influence of 10%, 25%, and 40% DO, respectively, on the expression of carotenogenic genes determined by RT-PCR. The band intensity of each transcript was adjusted to that of actin. Data shown are means ± SD of three independent experiments.

these results, the DO content optimal for growth and astaxanthin production was determined to be 25% of air saturation.

Differential transcription of the crtI, crtYB, crtE, and ast genes in X. dendrorhous cells grown with various DO contents

Since astaxanthin yields were a function of the DO content, we investigated whether the DO content likewise differentially regulates the related carotenogenic genes. An RT-PCR approach was used to detect the transcript levels of the genes encoding GGPP synthase (crtE), the bifunctional enzyme phytoene synthase lycopene cyclase (crtYB), phytoene desaturase (crtI), and the bifunctional enzyme β-carotene ketolase β-carotene hydroxylase (astaxanthin synthase, ast) in cells cultured with various DO contents. The results are shown in Fig. 1 and Fig. 2. The various DO contents differentially affected the transcription of the genes. 25% DO, which supported the best growth and astaxanthin production of the yeast cells, also resulted in the highest transcript levels of all the genes. 40% DO only moderately enhanced the transcription of the genes, while 10% DO did not up-regulate the transcription of the carotenogenic genes. As shown in Fig. 2, the transcript level of crtI at 10% DO was only 39% of that at 25% DO, and 48% of that at 40% DO. Our results suggest a correlation between biomass, astaxanthin production, and the transcript levels of carotenogenic genes in cells cultured under different oxygen tensions.

In conclusion, our experimental results have shown that *X. dendrorhous* cell growth and carotenoid production in fermentors depend strongly on the oxygen supply, and different DO contents greatly affected the transcription of four key carotenogenic genes involved in the synthesis of astaxanthin. Since *Vitreoscilla* hemoglobin (VHb) is an oxygen-binding protein that allows the bacterium to grow aerobically even under microaerophilic conditions (Zhu *et al.*, 2011), in further studies, we will exam the effect of VHb on astaxanthin production in *X. dendrorhous*.

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