

Effect of Different Carbon Source on Expression of Carotenogenic Genes and Astaxanthin Production in *Xanthophyllomyces dendrorhous*

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Abstract: The present research gives an insight into astaxanthin production, as well as transcription differences of four key carotenogenic genes, in *Xanthophyllomyces dendrorhous* when cultured with various carbon sources and soybean oil as co-substrates. Glucose was found to be the carbon source with best culture growth and astaxanthin production and the addition of 2% (v/v) soybean oil resulted in even higher astaxanthin producing. In addition, four carotenogenic genes encoding geranylgeranyl diphosphate synthase (*crtE*), phytoene desaturase (*crtI*), phytoene synthase lycopene cyclase (*crtYB*) and astaxanthin synthetase (*ast*), respectively, were demonstrated to be associated with different transcription levels under various substrates. The present study suggests the effectiveness of manipulating the metabolic regulation by using different carbon sources, in order to improve the production of astaxanthin.

Keywords: Astaxanthin, carbon source, carotenogenic genes, *Xanthophyllomyces dendrorhous*

INTRODUCTION

It is known that *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) is one of the promising microorganisms for biotechnological production of dietary astaxanthin (Cruz and Parajó, 1998). Astaxanthin (3, 3'-dihydroxy- β , β '-carotene-4, 4'-dione), which can also be produced by *Haematococcus pluvialis* (Kobayashi, 2003; Kim and Lee, 2005), is used as a red pigmentation product in aqua-culture industry. Furthermore, it is also used as a potential functional food and pharmaceutical supplement because of its excellent antioxidant activity (Guerin *et al.*, 2003; Johnson and Schroeder, 1995).

Previous studies have shown that *X. dendrorhous* can grow on a variety of carbon sources, such as glucose, cellobiose, maltose, sucrose, lactose, xylose and arabinose (Johnson and An, 1991; Fang and Cheng, 1993; Parajó *et al.*, 1998; Palágyi *et al.*, 2001), but rapid catabolism of these sugars could cause a decrease in the biosynthesis of many bioactive metabolites (Rokem *et al.*, 2007). Nowadays slowly metabolized carbon, such as vegetable seed oils, is used as a part of the carbon source, which can reduce the glucose suppressive effect. As the organic carbon source is the most important factor for *X. dendrorhous* growth and astaxanthin production, in the present study, we investigated the effect of various carbon sources and soybean oil on the biosynthesis of astaxanthin and the transcription of four key carotenogenic genes, which are Geranylgeranyl Diphosphate (GGPP) synthase (*crtE*, Genbank accession no. DQ016502), phytoene desaturase (*crtI* (Verdoes *et al.*, 1999b), Genbank accession no. AY177424), phytoene synthase lycopene

cyclase (*crtYB* (Verdoes *et al.*, 1999a), Genbank accession no. AY 177204) and astaxanthin synthase (*ast* (Ojima *et al.*, 2006), Genbank accession no. DQ002007) in the cells. This report is focused on the effect of a series of media containing various carbon sources and supplementation of soybean oil as co-substrates on accumulation astaxanthin. This body of work highlights for the first times the contribution of these substrates to transcription differences of four key carotenogenic genes.

MATERIALS AND METHODS

Microorganism and culture conditions: The *X. dendrorhous* AS 2.1557 (obtained from the China General Microbiological Culture Collection Center, Beijing, China) was used in this study, which was stored at -80°C. The medium for maintenance of the yeast was made of 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 20 g agar (per L). The fermentation mediums were numbered from one to five in our test and the composition of each medium is given in Table 1. The medium was adjusted to pH 5.0 and sterilized by autoclaving at 121°C for 20 min. Liquid culture was maintained in shake-flasks with 250-mL Erlenmeyer flasks on an orbital shaker at 200 rev/min and 20°C. Each flask was filled with 30 mL of fermentation medium. The overall culture period was 4 days, unless otherwise specified. Soybean oil was added to medium 3 (not including soybean oil) according to the detailed arrangement of each experiment, in order to determine the appropriate oil content for astaxanthin production. The soybean oil used in this study was purchased from Tianjin Jiali Oil

Table 1: Medium composition

Medium	Composition (g/L) ^a			Composition (% v/v) Soybean oil
	Glucose	Sucrose	Xylose	
1	30	-	-	2
2	-	30	-	2
3	30	-	-	-
4	-	30	-	-
5	-	-	30	-

^a: All media also contain (g/L): 3 g KH₂PO₄, 3 g MgSO₄, 1 g Na₂HPO₄ and 5 g yeast extract

Plant (China) and other chemicals were from Sigma (St. Louis, MO, USA). All experiments were performed triplicate.

Analytical procedures: Yeast biomass was separated from the liquid medium by centrifuging and rinsed twice with double distilled water and then dried at 60°C to constant weight, yielding the Dry Cell Weight (DCW). Astaxanthin content was determined by High Performance Liquid Chromatography (HPLC) according to Lu *et al.* (2010). Briefly, the dried yeast was disrupted with Dimethyl Sulphoxide (DMSO) and then extracted with acetone. The crude extract was taken up in acetone to 4.0 mL and freezing was done at -20°C to precipitate the colourless lipids. The defatted crude extracts were filtered through a 0.45-µm syringe filter and 10-µL aliquots were subjected to reversed-phase HPLC analysis. Astaxanthin standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All samplings and assays were carried out in triplicate and the results were determined as the average mean values±Standard Deviation (S.D.).

RNA isolation: Yeast cells from 30 mL aliquots of culture for 2 days were collected by centrifugation and powdered under liquid nitrogen using a mortar and pestle. RNA extraction was performed with TRIzol Reagent (Invitrogen) according to the manufacturer's procedure and then treated with RNase-free DNase I (Invitrogen) for 30 min at 37°C to remove residual DNA. The total RNA content in the extract was determined at 260 nm and the purity was determined by the ratio of optical densities both at 260:230 and 260:280 nm (greater than 1.8).

RT-PCR assay: Reverse transcription and PCR were done according to Lodato *et al.* (2007). The primers used for amplification of *crtI*, *crtYB*, *crtE*, *ast* and *actin* were 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 the actin-specific primers.

RESULTS AND DISCUSSION

Effect of soybean oil concentrations on cell growth and carotenoid production: Soybean oil has been reported to be beneficial to the biomass and to increase the production of bioactive metabolites in several fungi (Silva *et al.*, 2007; Yang and He, 2008). In this research, the effect of soybean oil on astaxanthin production of *X. dendrorhous* was studied, where oil was added at the concentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (v/v) (Fig. 1). Three percent (v/v) soybean oil concentration resulted in the maximum biomass (15.03±0.35) g/L, which was 22.8% higher than the control. The stimulation of biomass by soybean oil might be caused by partial incorporation of lipids in the cell membrane, thereby facilitating the uptake of nutrients from the medium (Yang *et al.*, 2000). The maximal astaxanthin production (11.93±0.32) g/L and astaxanthin content (0.86±0.01) mg/g DCW were obtained in the medium containing 2% (v/v) soybean oil, which was 1.31-fold and 1.16-fold higher than the control, respectively. As we all know, soybean oil is rich of various fatty acids, which may have a influence on astaxanthin production of *X. dendrorhous*, but the results also showed that redundant fatty acids were suitable for biomass but not for astaxanthin production.

Effects of different carbon sources on cell growth and carotenoid production: Previous studies showed that *X. dendrorhous* can grow well heterotrophic ally with glucose as sole carbon and energy source, so we investigated if this yeast is capable of metabolizing other organic carbon sources to grow and synthesize astaxanthin. The composition of the different media investigated here for carotenoid production is given in Table 1 and the results are presented in Table 2. *X. dendrorhous* could use all the sugars and soybean oil for growth but their dry biomass concentrations and carotenoid production were rather different. Medium 1 and medium 2, containing sugars and soybean oil, were the best choices for growth and astaxanthin production of *X. dendrorhous* which supported high dry biomass concentration and high astaxanthin yield. Glucose (medium 3) and sucrose (medium 4) were also good carbon sources, with which the yeast cells exhibited lower dry biomass and astaxanthin yield. Xylose (medium 5) was the poorest carbon source, resulting in a very low biomass and low astaxanthin yield, which were 68 and 86% lower than that of medium 1, respectively.

Our study showed that *X. dendrorhous* cells could transport and assimilate various carbon sources with different efficiency. Astaxanthin production from cultures with different carbon sources correlated with cell growth and a similar conclusion was drawn by Yamane *et al.* (1997). Among the three sugars surveyed, glucose and sucrose were good carbon source for yeast growth and astaxanthin synthesis. The addition of soybean oil to glucose-supplemented culture

Table 2: Effect of various medium composition on growth, astaxanthin yield and astaxanthin content of *X. dendrorhous* AS 2.1557 (n = 3)

Medium	Biomass (g/L)	Astaxanthin yield (mg/L)	Astaxanthin content (mg/g DCW)
1	13.44±0.57	11.31±0.46	0.84±0.04
2	12.37±0.51	10.10±0.39	0.82±0.03
3	12.28±0.39	8.90±0.41	0.72±0.03
4	11.95±0.62	8.24±0.32	0.69±0.02
5	4.36±0.11	1.62±0.05	0.37±0.02

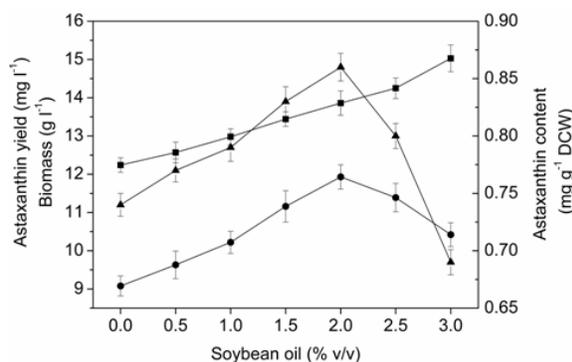


Fig. 1: Effects of soybean oil concentration on the biomass and astaxanthin production in shake flask. Symbols are for the mycelia biomass (■), astaxanthin yield (●) and astaxanthin content (▲)

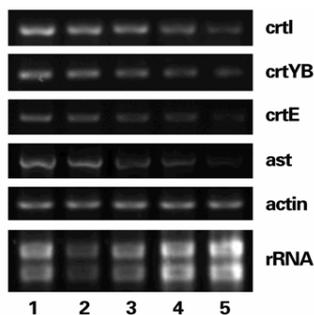


Fig. 2: RT-PCR analysis of the influence of various carbon sources on carotenogenic genes expression. RT-PCR was performed using RNAs from cells cultured at medium 1 (lanes 1), medium 2 (lanes 2), medium 3 (lanes 3), medium 4 (lanes 4) and medium 5 (lanes 5). The composition of medium 1-5 was given in Table 1. For comparison, ribosomal RNA was stained with ethidium bromide (lower panel)

not only supported higher biomass, but also resulted in higher astaxanthin content of *X. dendrorhous*. Just as reported by Bhosale (2004) that the carotenoid cellular accumulation is generally associated with increased activity of enzymes involved in their biosynthesis, thus addition of soybean oil is likely to stimulate carotenoid biosynthesis. Breitenbach *et al.* (2011) also found that over-expression of *crtE* is a useful target to divert and enhance metabolite flow into carotenogenesis.

Effect of various carbon sources on carotenogenic genes expression: To further study differential

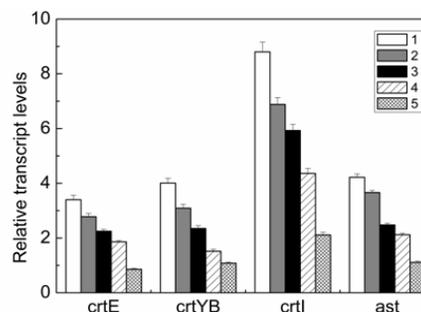


Fig. 3: Influence of various carbon sources (medium 1-5) on expression of carotenogenic genes determined by RT-PCR. The composition of medium 1-5 was given in Table 1. The band intensity of each transcript was adjusted to that of *actin*. Data shown were mean±S.D. of three independent experiments

transcription of carotenogenic genes in *X. dendrorhous* cells grown with various carbon sources. RNA was extracted from cells cultured with different media and transcript levels were compared by RT-PCR (Fig. 2 and 3). Four carotenogenic genes are selected for RT-PCR in this study, which are geranylgeranyl diphosphate synthase (*crtE*), phytoene desaturase (*crtI*), phytoene synthase lycopene cyclase (*crtYB*) and astaxanthin synthetase (*ast*). It is shown in Fig. 2 and 3 that various carbon sources differentially regulated the transcript levels of the four key genes. Medium 1 and 2, which supported the best growth and astaxanthin production of the yeast cells, also resulted in the highest transcript levels of all the genes. Glucose (media 3) and sucrose (media 4) only moderately enhanced the transcription of the genes; while xylose (medium 5) which was poorly metabolized by the cells did not up-regulate the transcription of the carotenogenic genes. The expression levels of *crtI* was even 4.2-fold higher in cells grown on medium 1 than those of cells grown on medium 5 and 1.5-fold higher of than those of cells grown on medium 3 (Fig. 3).

Our results suggested that there exists a correlation between astaxanthin production and the transcripts of carotenogenic genes, which was evident in the cells cultured with various carbon sources and addition of soybean oil as co-substrates could enhance the expression of carotenogenic genes. We found that the various carbon sources differentially regulated the expression levels of carotenogenic genes in *X. dendrorhous* and this phenomenon was discovered in green microalgae *Chlorella zofingiensis* (Sun *et al.*, 2008). Besides, Wu *et al.* (2011) reported that dissolved oxygen may affect the transcription level of carotenogenic genes of *X. dendrorhous*. But our data is not sufficient to explain that the oil act to at post-translational or enzyme level in addition to their action at transcriptional level in the carotenoid biosynthesis pathway. Just as reported by Wozniak *et al.* (2011) that besides transcriptional regulation, there exist additional regulatory mechanisms which may be operating in the carotenogenic pathway of *X. dendrorhous*.

CONCLUSION

Comparing the various carbon sources in this research, we found that glucose and sucrose could be sufficiently used as carbon sources by *X. dendrorhous* and whereas xylose was a poor carbon source, resulting in low fungal biomass and astaxanthin production. The supplement of soybean oil as co-substrates was very effective in enhancing astaxanthin accumulation and it may be an area for further increasing astaxanthin production by supplying effective ingredients isolated from soybean oil.

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