Citrus Residues Isolates Improve Astaxanthin Production by *Xanthophyllomyces dendrorhous*

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The wild strain and two astaxanthin-overproducing mutant strains, W618 and GNG274, of *Xanthophyllomyces dendrorhous* were analyzed in order to assess their ability to grow and synthesize astaxanthin in a minimal medium containing (per liter): 2 g KH_2PO_4, 0.5 g $MgSO_4$, 2 g KNO_3, and 1 g yeast extract, and supplemented with citrus residues isolates as a carbon source (citrus medium). The selected strain W618 was evaluated under various contents of citrus juice. At the content of 20% (v/v), the highest astaxanthin production reached 22.63 mg L^{-1}, which was two-fold more than that observed in yeast malt medium. Addition of 8% (v/v) *n*-hexadecane to the citrus medium was found to be optimal, increasing the astaxanthin yield by 21.7%.

Key words: Astaxanthin, *Xanthophyllomyces dendrorhous*, Citrus Residue

Introduction

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) is a valuable keto-carotenoid pigment which confers a characteristic colouration to some birds, crustaceans, and salmons (Meyer and Du Preez, 1994). Recent studies showed that it has a nearly ten-fold higher antioxidant activity than other carotenoids and has a 100- to 500-fold higher activity than α-tocopherol (Miki, 1991; Naguib, 2000). It has been increasingly used as feed and food pigment in the aqua-culture industry, and also regarded as a potential functional food and pharmaceutical supplement because of its excellent antioxidant activity (Guerin *et al.*, 2003; Johnson and Schroeder, 1995).

The red yeast *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) is one of the most promising microorganisms for biotechnological production of dietary astaxanthin (Cruz and Parajó, 1998). However, the wild strains of *X. dendrorhous* produce very low levels of astaxanthin, so its production on a large scale is not yet economically feasible. Recently, the research focus was to diminish production costs either through obtaining astaxanthin-overproducing strains or the development of low-cost culture media that would increase the pigment synthesis. Previous studies using low-cost by-products and residues of agroindustrial origin have shown the possibility of astaxanthin production from several materials such as molasses (An *et al.*, 2001), wood hydrolysates (Cruz and Parajó, 1998), hemicellulose hydrolysates of eucalyptus (Parajó *et al.*, 1998a), peat hydrolysates (Vázquez and Martin, 1998), and pineapple juice (Jirasripongpun *et al.*, 2008). Nevertheless, the use of citrus juice as a culture medium for astaxanthin production has not been reported.

The propensity of *X. dendrorhous* for growing 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.*, 1998b), is a remarkable advantage. So in China, citrus juice, elicited from waste, is a very potential source as the medium of astaxanthin production because it contains utilizable carbon and is of acidic pH. Moreover, China is one of the world's largest production countries of mikan, satsuma and mandarin orange, and occupies more than 40% of total yield in the world. In this study, we report the development of a low-cost culture medium based on juice elicited from residues of citrus, which significantly increases the growth of *X. dendrorhous* and astaxanthin synthesis compared to yeast malt (YM) medium. In

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addition, *n*-hexadecane as an oxygen vector was also investigated.

Material and Methods

Microorganisms

P. rhodozyma (now *X. dendrorhous*) AS 2.1557 was obtained from China General Microbiological Culture Collection Center (CGMCCC, Beijing, China), maintained on slants of YM agar at 4 °C, and transferred monthly. The astaxanthin-overproducing mutant *P. rhodozyma* W618 was obtained in our laboratory by mutagenesis with ^{60}Co gamma irradiation of *P. rhodozyma* AS 2.1557. The mutant GNG274 of *X. dendrorhous* was performed using *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG).

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 the citrus medium were (per liter): 2 g KH_2PO_4, 0.5 g $MgSO_4$, 2 g KNO_3, and 1 g yeast extract; citrus juice was added to this as carbon source as required. Astaxanthin standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of citrus waste isolates

The residues of citrus were collected, blended, and then incubated at 55 °C for 4 h. The juice was separated from the residues by filtrations, and the resulting solution was centrifuged at 8,000 × *g* to remove suspended solids. The juice was sterilized by autoclaving, and it was added to the minimal medium as required. The dinitrosalicylate (DNS) method was used for quantitative analysis of reducing sugars (Miller, 1959). The pH value of the citrus juice was 5.0.

The lipophilic compounds of citrus juice were extracted with *n*-hexane, and deionized water was added to the organic phase. The mixture was evaporated on a rotary evaporator to remove *n*-hexane, and the aqueous phase was autoclaved at 121 °C for 15 min, ready for tests.

Flask cultures

X. dendrorhous AS 2.1557, and mutants W618 and GNG274 were grown in 250-mL Erlenmeyer flasks containing 30 mL of 20% (v/v) citrus juice medium for 8 d at 22 °C in shaking incubators on a shaker operating at 200 rpm, in order to assess their ability to grow and synthesize astaxanthin.

The selected strain was evaluated under different citrus juice contents, which varied from 5%, 10%, 20% to 30% (v/v), in order to determine the appropriate citrus juice content for astaxanthin production, compared with YM medium. Cultivation was done for 6 d in triplicate.

The liquid hydrocarbon *n*-hexadecane, selected based on previous studies (Liu and Wu, 2006a), was tested as oxygen vector in the shake-flask cultures of *X. dendrorhous*. *n*-Hexadecane, which was sterilized by filtration through 0.22-μm syringe filters, was added to the citrus medium at the time of inoculation.

Analytical procedures

Biomass was determined based on dry cell weight (DCW). The reducing sugar concentration in the culture medium was determined by the DNS method (Miller, 1959). Astaxanthin was determined by high-performance liquid chromatography (HPLC) with a Waters 2695 instrument equipped with a 5-μm Dikma Diamonsil TM-C18 reversed-phase column (250 × 4.6 mm; Dikma Technologies Inc., Beijing, China), using UV detection at 480 nm. The eluting solvent was methanol/methyl cyanide (9:1, v/v) and the flow rate was 1 mL min^{-1}. All samplings and assays were carried out in triplicate, and the results were determined as the mean values ± standard deviation.

Results

Strain selection for astaxanthin production in citrus residues isolates medium

The juice extracted from citrus waste was analyzed. The pH value was 5.0. Total sugar and reducing sugar concentrations were 200 and 150 g L^{-1}, respectively.

The time courses of culture (Fig. 1) showed that *X. dendrorhous* strain AS 2.1557, *X. dendrorhous* mutant W618 and mutant GNG274 grew rapidly during the first 2 days after inoculation, and then cell weight slightly increased and remained in a stationary phase in the later days with little change. The mutant W618 grew most fast which resulted in the highest amount of cell weight. The time courses of total astaxanthin formation

(Fig. 2) showed that they proceeded in parallel with the cell growth and continued through the stationary phase. The astaxanthin content of *X. dendrorhous* mutant W618 was higher than that of the mutant GNG274 and *X. dendrorhous* AS 2.1557 throughout the whole cultivation period. After a 6-day cultivation, the highest contents of astaxanthin in the cells of *X. dendrorhous* strain AS 2.1557 and mutants W618 and GNG274 were (0.53 ± 0.03), (1.23 ± 0.06), and (0.83 ± 0.03) mg g^{-1} DCW (mean ± S.D.; $n = 3$), respectively. It was calculated that mutant W618 produced 48% and 132% higher amounts of astaxanthin than the mutant GNG274 and strain AS 2.1557, respectively. Therefore, the mutant W618 was chosen for a subsequent study on astaxanthin production in citrus juice medium.

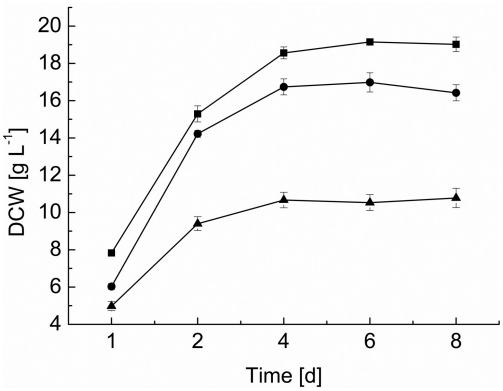

Fig. 1. Growth of *Xanthophyllomyces dendrorhous* mutant W618 (■), *X. dendrorhous* mutant GNG274 (●), and *X. dendrorhous* AS 2.1557 (▲) in 20% (v/v) citrus juice medium incubated at 22 °C in a rotary shaking incubator at 200 rpm for 8 days.

Culture conditions for astaxanthin production

The influence of citrus residues isolates contents on the biomass and astaxanthin production in the culture of *X. dendrorhous* mutant W618 was examined (Table I). The biomass production increased with an increase in the content of citrus juice. The citrus juice content of 30% resulted in the maximal biomass, which was 86% higher than that of YM medium. The astaxanthin content was enhanced with increasing citrus juice content to the maximum at 20% (v/v), after which the astaxanthin content decreased. Citrus juice at 20% (v/v) content supported the highest astaxanthin yield of (22.63 ± 0.27) mg L^{-1} and the highest astaxanthin content of (1.19 ± 0.030) mg g^{-1} DCW, which were 2-fold and 1.3-fold more than that of YM medium, respectively. Therefore, this showed that the 20% citrus residues isolates medium was a more efficient low-cost production medium for production of astaxanthin by *X. dendrorhous*.

As shown in Table II, the astaxanthin yield and content of *X. dendrorhous* mutant W618 in-

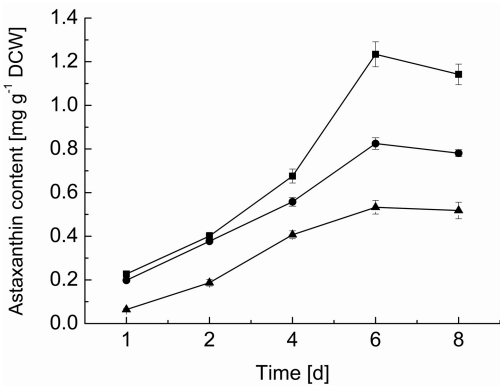

Fig. 2. Astaxanthin content of *Xanthophyllomyces dendrorhous* mutant W618 (■) *X. dendrorhous* mutant GNG274 (●), and *X. dendrorhous* AS 2.1557 (▲) in 20% (v/v) citrus juice medium incubated at 22 °C in a rotary shaking incubator at 200 rpm for 8 days.

Table I. Effect of citrus residues isolates contents on growth, astaxanthin yield, and astaxanthin content of *Xanthophyllomyces dendrorhous* mutant W618 compared with yeast malt (YM) medium in a 6-day culture ($n = 3$).

Citrus residues isolates content (% v/v)	Biomass [g L^{-1}]	Astaxanthin yield [mg L^{-1}]	Astaxanthin content [mg g^{-1} DCW]
5	5.980 ± 0.29	8.17 ± 0.04	0.86 ± 0.013
10	13.55 ± 0.38	14.52 ± 0.28	1.07 ± 0.029
20	19.03 ± 0.12	22.63 ± 0.27	1.19 ± 0.030
30	22.51 ± 0.22	16.13 ± 0.14	0.72 ± 0.017
YM medium	12.10 ± 0.20	11.01 ± 0.05	0.91 ± 0.022

Table II. Effect of lipid-soluble extract of citrus residues isolates added to YM medium (C) on growth, astaxanthin yield, and astaxanthin content of *Xanthophyllomyces dendrorhous* mutant W618 compared with 20% citrus residues isolates medium (B) and YM medium (A) in a 6-day culture (n = 3).

Medium	Biomass [g L^{-1}]	Astaxanthin yield [mg L^{-1}]	Astaxanthin content [mg g^{-1} DCW]
A	13.05 ± 0.41	12.43 ± 0.51	0.952 ± 0.046
B	18.43 ± 0.21	21.03 ± 0.32	1.141 ± 0.049
C	16.28 ± 0.54	17.87 ± 0.66	1.098 ± 0.036

creased when lipid-soluble extract of citrus residues isolates was added to YM medium. It caused a 43.8% increase in astaxanthin yield and a 15.3% increase in astaxanthin content, compared to YM medium. However, its maximal astaxanthin yield and content were lower than those of 20% citrus residues isolates medium.

Effects of n-hexadecane on cell growth and astaxanthin production

The effect of *n*-hexadecane on *X. dendrorhous* mutant W618 culture was examined at different doses, 4%, 6%, 8%, and 10% (v/v) (Fig. 3). The addition of 8% *n*-hexadecane to 20% citrus residues isolates resulted in the highest biomass yield of 19.71 g L^{-1} and the total astaxanthin yield of 25.03 mg L^{-1}, which were 11.3% and 21.7% higher than those of the control, respectively. Correspondingly, the astaxanthin content at the optimum was increased by 11.4%, from 1.14 to 1.27 mg g^{-1} DCW. The results showed that the astaxanthin yield was enhanced with increasing *n*-hexadecane volume fraction until the maximum at 8% (v/v), after which the astaxanthin yield decreased.

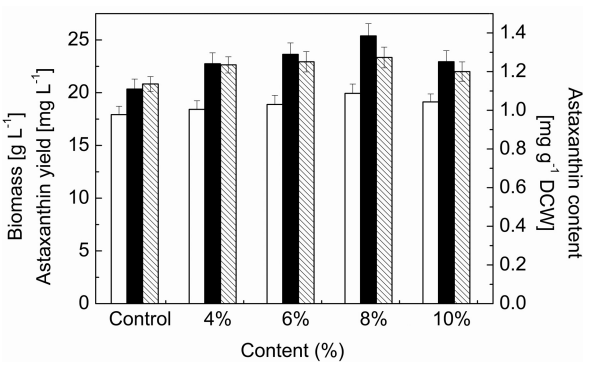

Fig. 3. Effect of *n*-hexadecane volume fraction on biomass (□), astaxanthin yield (■), and astaxanthin content (▨) of *Xanthophyllomyces dendrorhous* mutant W618 in a 6-day culture (n = 3).

Discussion

Astaxanthin is an industrially important carotenoid. The development of low-cost culture media that diminish production costs is proved to be one of the most important strategies for mass production of astaxanthin at industrial scale. The present work examined the use of juice from citrus waste processing as a carbon source for astaxanthin production from *X. dendrorhous*. 20% juice medium supported the highest astaxanthin yield of (22.6 ± 0.3) mg L^{-1} and the highest astaxanthin content of (1.19 ± 0.030) mg g^{-1} DCW, which are much higher than the result by Jirasripongpun *et al.* (2008), reporting a highest carotenoid content of *X. dendrorhous* in a 6-day culture with (0.49 ± 0.01) mg g^{-1} using pineapple juice medium, and the result by Ramírez *et al.* (2000), reporting that the yucca medium supported the highest astaxanthin yield of 6.2 mg L^{-1} by a *P. rhodozyma* mutant. Therefore, it showed that the citrus medium was a more efficient low-cost medium for production of astaxanthin by *X. dendrorhous*.

Previous studies have shown that the astaxanthin production rate in liquid cultures of *X. dendrorhous* increases with increasing oxygen uptake (Prevatt *et al.*, 1991; Yamane *et al.*, 1997). Moreover, *n*-hexadecane can be easily separated from the culture medium for reuse. Therefore, the addition of *n*-hexadecane as an oxygen vector is an efficient and economical measure for scale-up production of astaxanthin by *X. dendrorhous*. Liu and Wu (2006a) reported that the addition of 9% (v/v) *n*-hexadecane to the liquid medium at the time of inoculation increased the carotenoid yield by 58%. In the present study, the astaxanthin yield was only increased by 21.7%. The different strains used in two tests may cause this big difference in increasing astaxanthin production. The astaxanthin yield of control in our experiments was 20.35 mg L^{-1}, *i.e.* much higher than 9.2 mg L^{-1} which was reported by Liu and Wu (2006a).

Citrus juice used in this study was elicited from waste, and the optimal content used as a carbon source was only a small amount of 20% (v/v). It was reported that citrus juice contains small amounts of several carotenoids, such as lycopene, α-carotene, β-carotene, β-zeacarotene, and β-cryptoxanthine, which could be used as intermediates or precursors for astaxanthin production by the yeast as reported by Liu and Wu (2006b). As showed in Table II, the astaxanthin yield and content increased obviously after addition of lipid-soluble extract of citrus juice to YM medium. This was probably the contribution of some carotenoids in the citrus juice. The above results showed that the citrus medium is a cheap and satisfactory medium for astaxanthin production by *X. dendrorhous* mutant W618. An intensive study of effective ingredients in the citrus juice, may be a scope for further enhancement of astaxanthin production.

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