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ORIGINAL PAPER

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Utilization of mustard waste isolates for improved production of astaxanthin by *Xanthophyllomyces dendrorhous*

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Abstract Astaxanthin production in the wild strain *Xanthophyllomyces dendrorhous* TISTR 5730 was investigated using different mustard waste media, including mustard waste residue extract (MRE), mustard waste residue hydrolysate (MRH), mustard waste precipitated extract (MPE), and mustard waste precipitated hydrolysate (MPH). The growth of *X. dendrorhous* and the production of astaxanthin were dependent on the type and initial concentrations of mustard waste media. The MPH medium was the best substrate resulting in yields of biomass and astaxanthin of 19.6 g/L and 25.8 mg/L, respectively, under optimal conditions. MPH medium improved astaxanthin production 11-fold compared to the commonly used commercial yeast malt medium, and 1.3–2.1-fold compared to other mustard waste media.

Keywords Astaxanthin · *Xanthophyllomyces dendrorhous* · Mustard waste · Low-cost medium · HPLC analysis

Introduction

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is an important and valuable keto-carotenoid pigment. It has been widely used as a food colorant and in cosmetic and medical applications due to its high antioxidant activity [12]. Studies report that it has a nearly tenfold higher antioxidant activity than other carotenoids and has a 100–500-fold higher activity than α-tocopherol [16–17]. Astaxanthin has been produced commercially by chemical synthesis but the product contains only 8% astaxanthin and costs \$25,000–30,000 kg^{-1}. When substances are used as food additives, consumers prefer materials made using biological and/or biotechnological processes rather than chemical synthesis. Among the microorganisms that contain astaxanthin that might be utilized for commercial bio-production are *Brevibacterium*, *Mycobacterium lacticola* [18], *Agrobacterium auratium* [26], *Haematococcus pluvalis* [5], and the red yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodoxyma*) [1]. However, to date, no commercially viable processes have been developed to produce significant quantities.

Among these microorganisms, the yeast *X. dendrorhous* may be suitable for commercial production because astaxanthin accounts for 80–90% of the total carotenoid compounds. Drawbacks include the low astaxanthin level in wild-type strains (200–300 μg/g yeast) and the rigid cell wall that makes it difficult to extract intracellular products [11]. Strategies employed to increase product yields from microorganisms include (a) the use of overproducing strains, (b) addition of bacterial and fungal enzymes that disrupt the yeast cell wall, and (c) the development of low-cost culture media that diminish production costs [4, 6–9].

Previous studies using low-cost by-products and residues of agro-industrial origin have shown the possibility of astaxanthin production from several materials such as molasses [9], grape juice [15], hemicelullose hydrolysates of eucalyptus [19], peat hydrolysates [25], and hydroysates from *Yucca fillifera* [22]. Use of these low-cost culture media showed significantly increased growth and astaxanthin synthesis (1.1–3.8-fold) compared to yeast extract/malt extract (YM) medium. The current study focuses on the possibility of using mustard waste derivatives as low-cost alternative substrates for astaxanthin production by *X. dendrorhous*. The mustard waste is produced after the production of allyl isothiocyanate (AIT) from mustard seed (*Brassica juncea* var, Forge). Ten tons of mustard seed produces 70 tons of mustard waste suspension (in water), which is usually

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discarded. The process generates about 2,400 metric tons of waste per year so finding another value-added use for this material through further processing would provide economic benefits as well as reducing its environmental impact.

Materials and methods

Microorganism

Xanthophyllomyces dendrorhous TISTR 5730 (formerly *Phaffia rhodozyma*) was obtained from the Thailand Institute of Science and Technological Research (TISTR). The yeast strain was maintained on YM agar (Difco, USA) slants at 4°C and subcultured monthly.

Initial mustard residue and precipitated powder preparation

The mustard waste suspension resulting from the production of AIT was collected from Lanna Product Co., Ltd., Lamphun, Thailand. Mustard waste residue (MR) powder was prepared directly from the waste suspension by centrifugation and drying in a hot air oven at 50°C. The mustard waste precipitate (MP) powder was prepared from the mustard waste suspension by adding 0.2% citric acid to adjust to pH 5. The precipitate, which was produced was washed with hexane to remove fat and then was dried in the hot air oven. The MP powder contained protein (43.6%), carbohydrate (33.9%), fiber (8.5%), and ash (2.2%).

Mustard waste media preparation

The mustard waste extracts (E) media were prepared by adding a specific amount of either MR or MP powder to distilled water in an Erlenmeyer flask, followed by boiling and stirring for 30 min. The amount of MR or MP and distilled water used defined the percent composition [as % (w/v)] of the medium for later experiments (0–30%). The mixture was centrifuged and filtered [24]. The liquid supernatant was designated as either mustard waste residue extract (MRE) or mustard waste precipitate extract (MPE).

Acid hydrolysates (H) media from the MR or MP powders were obtained by mixing a specified amount of either MR or MP powder with 0.2 N H_2SO_4, followed by autoclaving at 121°C for 15 min. The amount of MR or MP and sulfuric acid used defined the percent composition [as % (w/v)] of the medium for later experiments (0–30%). Centrifugation and adjustment of the supernatant to pH 6.0 with 2 M NaOH yielded the mustard waste residue hydrolysate (MRH) or mustard waste precipitated hydrolysate (MPH).

Culture conditions

Inocula were prepared in YM broth. *X. dendrorhous* cells were incubated at 20°C for 28 h on a rotary shaker (200 rpm). The yeast cells were harvested by centrifugation and washed twice in sterile distilled water. One milliliter of the inoculum suspension (8.0×10^7 cells) was incubated in a 125 mL Erlenmeyer flask containing 30 mL of a specific mustard waste medium. All media were incubated at 20°C on a rotary shaker (200 rpm) for 120 h. Experiments were performed in quadruplicate. Yeast extract/malt extract (YM medium) was used as the reference medium for comparisons of the effectiveness of the different mustard waste media.

Analytical methods

Total nitrogen content was determined by the Kjeldahl method [2]. Total sugar was measured by the phenol-sulfuric method [3]. For cell dry weight determinations, 5 mL broth samples were centrifuged for 10 min at 3,500 *g* and washed twice with distilled water. The washed cells were dried in aluminium pans for 48 h at 95°C, allowed to cool in a desiccator and weighed. Carotenoids were extracted from cells by disruption with DMSO [23] and centrifugation. The total carotenoids were determined from the optical density at 474 nm, using an absorption coefficient value of 2,100 mg/L cm [23] with a Hitashi U-2000 UV/Vis spectrophotometer. To prepare pigment extracts, *X. dendrorhous* cells were disrupted by the freeze-thaw treatment and sonication at 3-minute intervals ten times. Multiple extractions (3–4 times) with sonication were carried out until the extract was nearly colorless [24]. The pooled acetone extracts were re-extracted with hexane and then concentrated to 5 mL by evaporation with nitrogen gas. The pigment extracts were kept at −20°C until analyzed.

Astaxanthin was determined by high performance liquid chromatography (HPLC) with a Hewlett-Packard series 1100 instrument equipped with a 150×4.6 mm, 5 μm Microsorb-MV C_{18} reversed-phase column, using UV detection at 450 nm. The isocratic eluent consisted of acetonitrile: methanol (9:1 v/v) with a flow rate of 0.5 mL/min. A 0.05 g/L standard solution of astaxanthin (Sigma, USA) was prepared in chloroform and used as the external standard. Samples and standard were stored at −20°C.

Results

The utilization of various mustard waste media as substrates for growth and astaxanthin production by *X. dendrorhous* was investigated. Comparisons of cell growth and astaxanthin production were made using four different mustard waste media as sole carbon and nitrogen sources: MRE, MRH, MPE, and MPH.

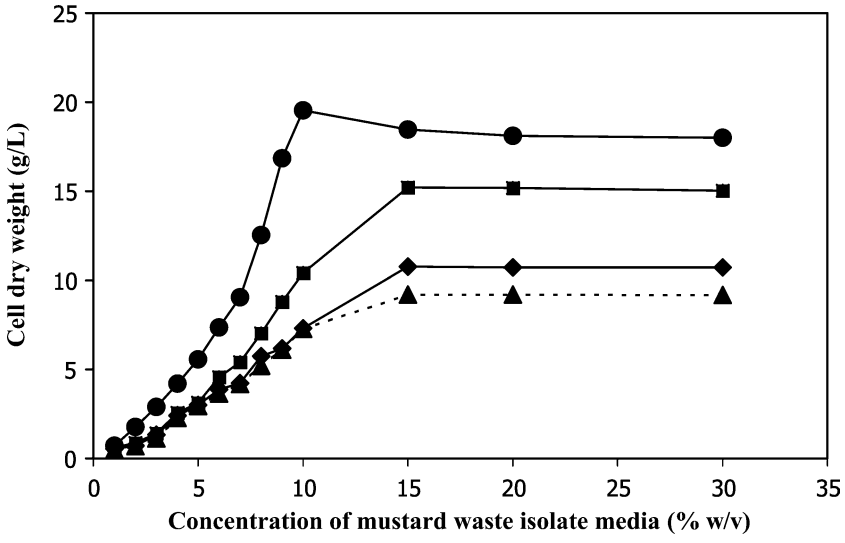

Fig. 1 Cell dry weight (g/L) of *X. dendrorhous* TISTR 5730 cultured in mustard waste media at different concentrations under optimal conditions: 20°C, pH 5.5, 200 rpm for 120 h. (*filled triangle* = MRE, *filled diamond* = MRH, *filled square* = MPE and *filled circle* = MPH)

Higher concentrations of the media yielded higher biomass (Fig. 1) and higher astaxanthin levels (Fig. 2). The highest yields of biomass and astaxanthin content were obtained from cultivation of *X. dendrorhous* in 15% w/v MRE, MRH, and MPE media and remained constant up to 30% w/v. For MPH the highest production of astaxanthin was at 10%, with a slight decrease over 10%, remaining constant until 30%. MPH was the most effective medium for both growth and astaxanthin production at all concentrations investigated.

The carbon and nitrogen contents at optimal concentration for all four media (Table 1) show that the mustard waste extract media, MRE and MPE, have lower sugar and nitrogen contents compared to the mustard waste hydrolysate media, MRH and MPH. Sugar and nitrogen in MRE (or MPE) are expected to be lower than those in the MRH (or MPH) since hydrolysis of the suspended materials would make more sugar and nitrogen available to the yeast. The mustard waste hydrolysate media showed higher average nitrogen and sugar utilization rates, and higher average astaxanthin production rate, than mustard waste extract media. The sugar (20.7 g/L) and nitrogen (38.3 g/L) content in MPH medium were suitable and sufficient for growth of *X. dendrorhous* and production of astaxanthin.

At optimal concentrations of media (15% for MRE, MRH, MPE, and 10% for MPH), the maximum yields of astaxanthin were 12.1 mg/L, 14.1, 19.0, and 25.8 mg/L, respectively (Table 1). MRE, MRH, MPE, and MPH were able to supply adequate levels of both nitrogen and carbon. The results showed that the growth of

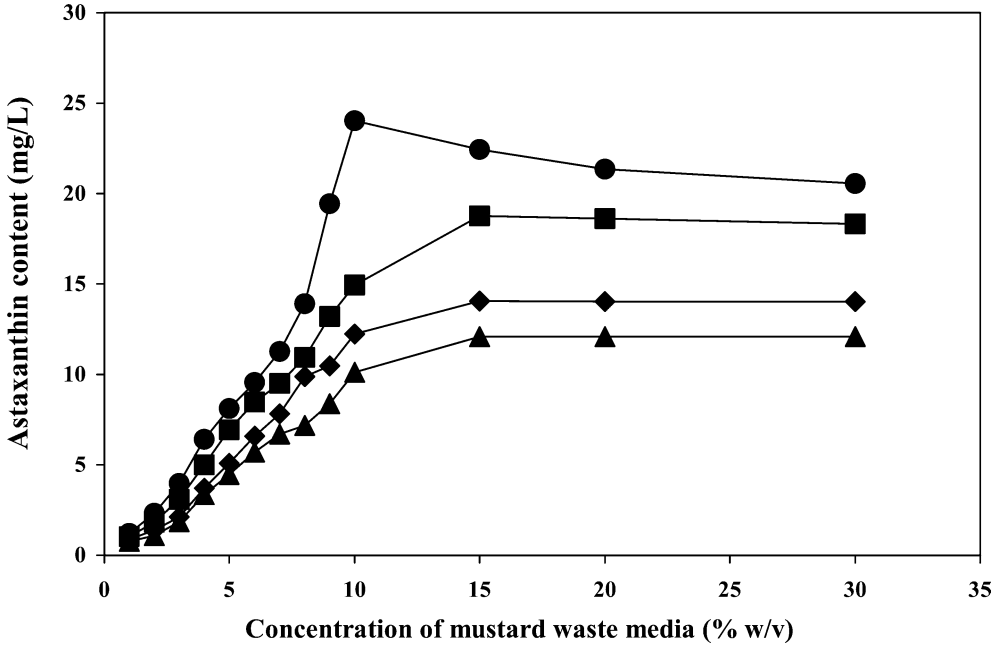

Fig. 2 Astaxanthin content (mg/L) by *X. dendrorhous* TISTR 5730 cultured in mustard waste media at different concentrations under optimal conditions: 20°C, pH 5.5, 200 rpm for 120 h. (*filled triangle* = MRE, *filled diamond* = MRH, *filled square* = MPE and *filled circle* = MPH)

Table 1 The growth of *X. dendrorhous* and its production of astaxanthin in mustard waste media under optimal conditions[a]

Parameters	Mustard waste media			
	MRE	MRH	MPE	MPH
Cell dry weight (g/L)	9.34 ± 0.10	10.9 ± 0.09	15.3 ± 0.40	19.6 ± 0.40
Total carotenoid production				
Cellular (mg/g)	1.29 ± 0.15	1.30 ± 0.19	1.37 ± 0.10	1.43 ± 0.30
Volumetric (mg/L)	12.0 ± 0.07	14.2 ± 0.11	21.1 ± 0.20	28.2 ± 0.30
Productivity (mg/L day)	2.40 ± 0.20	2.84 ± 0.10	4.22 ± 0.25	5.64 ± 0.20
Astaxanthin production				
Cellular (mg/g)	1.16 ± 0.20	1.16 ± 0.25	1.25 ± 0.50	1.31 ± 0.35
Volumetric (mg/L)	12.1 ± 0.20	14.0 ± 0.30	19.0 ± 0.15	25.8 ± 0.25
Productivity (mg/L day)	2.16 ± 0.02	2.52 ± 0.05	3.84 ± 0.50	5.18 ± 0.20
Total nitrogen				
Nitrogen content of medium (g/L)	11.8 ± 0.10	16.8 ± 0.15	32.6 ± 0.50	38.3 ± 0.20
Nitrogen utilization rate (g/L day)	1.32 ± 0.20	1.40 ± 0.20	1.58 ± 0.30	1.72 ± 0.05
FN/TN ($\times 10^{-3}$)	2.00 ± 0.20	5.32 ± 0.10	6.82 ± 0.50	7.85 ± 0.25
Total sugar				
Sugar content of medium (g/L)	13.2 ± 1.0	15.7 ± 0.20	18.7 ± 0.20	20.7 ± 0.20
Sugar utilization rate (g/L day)	2.64 ± 0.08	3.14 ± 0.12	3.74 ± 0.40	4.15 ± 0.30

[a]Values are means and standard deviations of quadruplicate determinations. The optimal conditions 15% of MRE, MRH and MPE and 10% of MPH cultured at 20°C, pH 5.5, cultivation time 120 h (96 h for MPH) and 200 rpm. *FN/TN* are formaldehyde nitrogen/total nitrogen ratio

X. dendrorhous and astaxanthin production using the mustard waste hydrolysate media (MRH and MPH) were higher than on mustard waste extract media (MRE and MPE). The results also showed the biomass and astaxanthin yields in MP media were higher than the MR media. The highest amount of astaxanthin was obtained when *X. dendrorhous* was cultured in 10% (w/v) MPH media. The astaxanthin content was 1.3–2.1-fold higher than from other mustard waste media. The results indicate that MPH media enhanced astaxanthin synthesis in the yeast *X. dendrorhous*. As a result, the MPH medium at concentration 10% (w/v) was the medium selected for further experiments.

Using the optimum conditions of 10% MPH, the time course of growth and astaxanthin production were monitored in parallel with the cell growth and the depletion of nutrients (nitrogen and carbon sources). Figure 3 shows that both biomass and astaxanthin production increased over time as total nitrogen and carbon in the mustard waste media decreased. The maximum levels of biomass (19.6 g/L) and astaxanthin (25.8 mg/L) were reached at 96 h, when the carbon source had been depleted, reaching a constant level from 96 to 144 h. Similar time course relationships were found for other media and other concentrations.

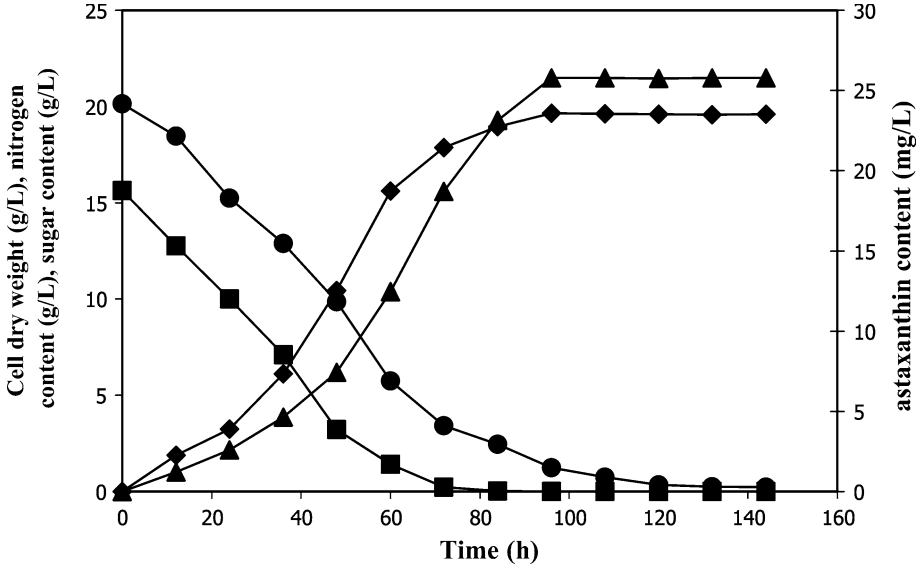

Fig. 3 Time-course of the growth and production of astaxanthin by *X. dendrorhous* in 10% (w/v) of MPH media. Experiments were carried out at 20°C and initial pH 5.5 (*filled diamond* = cell dry weight, *filled triangle* = astaxanthin content, *filled circle* = sugar content and *filled square* = nitrogen content)

Table 2 Comparison of astaxanthin production obtained in flask cultures using different media

Media	Additional supplement	Astaxanthin production (mg/L)	Astaxanthin production (μg/g)	Reference
Xylose	Peptone, YE, and malt extract	5.2	571	[20]
Molasses	Peptone	15.0	1,182	[9]
Peat hydrolysate	Peptone	5.52	1,567	[25]
Eucalyptus hydrolysate	Peptone	2.14	448	[19]
Corn wet-milling	None	0.1–2.6	400	[10]
20% Mustard meal extract	None	3.83	667	[24]
YM extract medium	–	2.34	497	This work
10% Hydrolyzed mustard waste isolate (MPH)	None	25.8	1,312	This work

Discussion

The present work examined the use of waste agro-industrial material from mustard processing as a potential medium for astaxanthin production from *X. dendrorhous*. Boiling water extraction and acid hydrolysis of mustard waste produce useful culture media, but the precipitated and hydrolyzed medium MPH was the most effective, yielding a biomass of 19.6 g/L and astaxanthin at 25.8 mg/L with a medium concentration of 10% w/v. Nutrient availability, as carbon and nitrogen sources, limit growth and astaxanthin production, and hydrolysis clearly increases these components. However, at high levels (above 15%) there is no additional enhancement, consistent with previous work [7] suggesting that high carbon and nitrogen levels can inhibit growth. The time course of the culture development shows the characteristic exponential and stationary phases [13, 14, 25], reaching a maximum level after about 100 h under the present conditions.

Another possible explanation for decreased growth and astaxanthin production in MRE and MRH compared to MPE and MPH is the presence of high levels of erucic acid, phenolic acid compounds and glucosinolate, considered to be antinutritional factors [21]. Acid precipitation during the preparation of the MP powder can reduce the amounts of these toxic substances.

Table 2 provides comparisons among a number of potential culture media for astaxanthin production and demonstrates that MPH, without supplementation, gives the highest yield on mg/L basis. Comparisons between the MPH medium and the reference YM medium demonstrate that the astaxanthin content produced by *X. dendrorhous* TISTR 5730 in MPH was 11 times higher than the YM medium (2.34 mg/L, Table 2). Astaxanthin production under optimal conditions in MPH was more than ten times that obtained from supplemented hydrolysates of eucalyptus and un-supplemented corn wet-milling [10], five times that from supplemented peat hydrolysates, and nearly double that of the supplemented molasses medium. Comparison to mustard meal extract [24], the astaxanthin yield was nearly seven times higher. Production of astaxanthin in MPH was five times higher than in xylose supplemented with peptone, glucose and yeast extract [20]. MPH could be an effective medium for commercial production of astaxanthin at low cost because supplementation is not necessary and the waste material is widely available during AIT production.

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