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Carotenoids Production by *Xanthophyllomyces dendrorhous* Mutant Grown on Molasses

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ABSTRACT

Astaxanthin, the main carotenoid pigment in dietary red yeast of *Xanthophyllomyces dendrorhous*, has a huge market potential as fleshy colourant and supplementary food to animal production and health. Cost of carotenoid production would be reduced by using a carotenoid-hyperproducing mutant and a cheap medium of molasses. Therefore, either mutant yeast [mutant GM807 (derived from gamma irradiation) or mutant n485 (from neutron irradiation)] was selected based on growth and carotenoid production in 5% (v/v) molasses containing 30 g/l urea and 4.5 g/l sodium phosphate under shaking condition for 10 days. The mutant GM807 was superior to mutant n485 in 1.5 fold higher of carotenoid content. Medium formula development for mutant GM807 revealed that beef extract and potassium nitrate enhanced higher carotenoid content than urea did. Concentration of molasses had an effect only on yeast growth but not on carotenoid formation. Total carotenoid content of 450 μg/g of yeast was obtained at the optimum pH of 4. Therefore, molasses (2% v/v) containing 4 g/l potassium nitrate, at pH 4, could possibly be an alternative culture medium for the mutant GM 807 to produce carotenoids.

Key words: astaxanthin, carotenoids, *Xanthophyllomyces dendrorhous*, mutant, molasses

INTRODUCTION

Astaxanthin is an orange-pink carotenoid pigment occurring naturally in a wide variety of living organisms, such as salmonids, lobsters, and egg yolks of chicken and quail (Gu *et al.*, 1977; Johnson *et al.*, 1980). Production of these farm animals usually uses astaxanthin as a supplementary food to enhance their flesh color and to improve growth and survival (Torrissen and Christiansen, 1995). Astaxanthin provides also beneficial properties of strong antioxidant activity and antitumor activity. Therefore, it has a huge market potential not only for food additives, but also for supplemetary additives in cosmetic and pharmaceutical products (Fang and Wang, 2002). Production of astaxanthin is primarily supplied by chemical means. However, the chemical synthesis is relatively difficult and expensive. Microorganisms synthesizing high level of astaxanthin, and being potential sources for commercial production of astaxanthin are the green microalga H*aematococcus pluvialis* and yeast *Xanthophyllomyces dendrorhous*.

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At present, *Xanthophyllomyces dendrorhous*, formerly known as *Phaffia rhodozyma*, is the only yeast that produces astaxanthin. Wild type yeast in yeast malt (YM) broth produces astaxanthin at low concentrations of 100-200 μg/g dry yeasts. Enhancing astaxanthin production has been reported either by controlling operational conditions such as oxygen supply (Liu *et al*., 2006) and pH (An *et al*., 1996; Hu *et al*., 2006) or by using appropriate types and concentrations of carbon source, nitrogen source and other components (Fang and Cheng, 1993; An *et al*., 1996; Sun *et al*., 2004). Mutagenesis to achieve carotenoid-hyperproducing mutants is also an alternative way to improve astaxanthin production (Fang and Cheng, 1993; An *et al*., 1996, 2001). Therefore, microbial production of this pigment may be improved through mutagensis or combined strategies, such as carotenoid-hyperproducing mutants and low-cost-based fermentation media. In this study, *X. dendrorhous* mutant GM807 and mutant n485 were selected based on growth ability and higher carotenoid production in molasses. Then nitrogen sources, molasses concentration, and pH were tested to obtain an optimal basic medium for astaxanthin production of the selected mutant yeast.

MATERIALS AND METHODS

Microorganisms and chemicals

Highly pigmented strains of *X. dendrorhous* mutant GM807 (derived from gamma irradiation) and *X. dendrorhous* mutant n485 (from neutron irradiation) were provided from The Office of Atoms for Peace, Bangkok, Thailand. These yeasts were maintained on slant of YM agar containing 3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l glucose and 20 g/l agar. They were stored at 4°C as working cell bank and the cultures with less than 10 passages were used for these experiments. The cultures were also kept in freezing medium containing 40% glycerol and 60% YM broth at -70°C as master cell bank.

Molasses were diluted and sterilized according to the method of An *et al*. (2001). They were used as medium. The reducing sugar in molasses measured by the dinitrosalicylate (DNS) method was 46 % (g/l).

Strain selection and conditions for carotenoid production

X. dendrorhous mutant GM807 and *X. dendrorhous* mutant n485 were cultured in 50 ml of 5 % (v/v) molasses containing 30 g/l urea and 4.5 g/l sodium phosphate, in 22°C rotary shaking incubator at 200 rpm for 10 days. The strains that well grew or highly produced carotenoid content were selected for further study on medium development for carotenoid production. These included nitrogen sources, molasses concentration, and pH.

To elucidate the effect of nitrogen sources on carotenoid production of the selected mutant in 5 % (v/v) molasses, individual nitrogen source (5 g/l peptone, 3 g/l beef extract and 3 g/l yeast extract) was replaced the original additives of urea and sodium phosphate. All cultures were grown under the same condition for 10 days and carotenoid contents were determined. Nitrogen source that gave the highest carotenoid concentration was used for the study of molasses concentrations. The molasses concentrations at 2, 5 or 8 % (v/v) were studied and the optimal molasses concentration was chosen from the highest carotenoid content. Furthermore, the formulated media containing appropriate nitrogen source and molasses concentration at pH ranged 3-6 were studied on carotenoid production. Potassium hydrogen phthalate buffer was used to prepare molasses and its initial pH at 3-6 in molasses was adjusted using HCl or NaOH.

Determination of growth and carotenoid content

Yeasts were grown at 22°C in 250 ml

flasks containing 50 ml of the medium (either YM broth or diluted molasses), on a rotary shaker at 200 rpm for 10 days. Cell growth was determined by dry cell weight after 1 ml of culture broth was washed and centrifuged at 12,000 g for 5 min, the cell pellet was removed with 0.5 ml distilled water and dried at 105°C for 4 days. All samples were carried out in triplicates, and their average weights were determined.

The total carotenoid content of yeast was determined according to An *et al*. (1996). One millilitre of yeast culture was harvested and resuspended in distilled water, washed twice and centrifuged to collect cell pellet. One millilitre of DMSO was added together with glass beads to break cells using vortex mixer. Then carotenoid extraction was performed by serially adding of each one millilitre of acetone, petroleum ether and 20% (w/v) NaCl solution, following with vortexing. After the solvent layers settled, the carotenoid in petroleum ether layers was monitored absorbance at wavelength 474 nm (A_{474}) by using visible spectrophotometer. All the samples were carried out in triplicate, and their average total carotenoid were determined**.** The total carotenoid composition was calculated by using the 1% extinction coefficient = 2,100 by the formula:

Total carotenoid (μg/g of yeast) = (ml of petrol)(A_{474})(100)/(21)(yeast dry weight)

RESULTS

Strain selection for carotenoid production in molasses

Both mutants of *X. dendrorhous* (mutant GM807 and mutant n485) were cultured in 5 % (v/v) molasses containing 30g/l urea and 4.5 g/l sodium phosphate, for 10 days. Time courses of cell biomass and total carotenoid content are shown in Figure 1. Molasses supported growth of both mutants to cell mass at 3.2-3.5 g/l. The carotenoid formation was observed simultaneously at the beginning of cell growth and continued till the cessation of cell growth. Carotenoid content of the mutant GM807 was the highest at 105 μg/g of yeast in day 9 of cultivation while that of the mutant n485 was the highest at 70 μg/g of yeast in day 8. Therefore, mutant GM807 which produced 1.5 fold higher carotenoid content than that of the

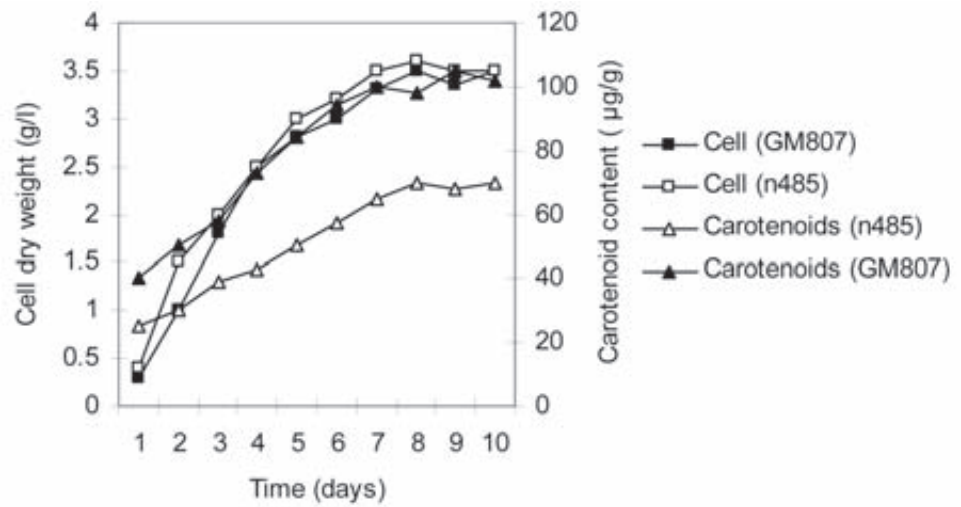

Figure 1 Growth and carotenoid content of *X. dendrorhous* mutant GM807 and *X. dendrorhous* mutant n485 in 5 % (v/v) molasses containing 30 g/l urea and 4.5 g/l sodium phosphate, in a 200 rpm rotary shaking incubator at 22°C for 10 days.

mutant n485 was chosen for subsequent study on carotenoid production in molasses.

Medium modification for carotenoid production

The molasses medium was modified to obtain an optimal medium for carotenoid production of the selected mutant GM807. Nitrogen sources of peptone, beef extract and yeast extracts were compared with the original nitrogen source of 30g/l urea and 4.5 g/l sodium phosphate. Result in Figure 2 showed that beef extract was the best organic nitrogen source for enhancing carotenoid production in molasses. Effects of inorganic nitrogen sources of ammonium sulphate and potassium nitrate compared to beef extracts at the concentrations of 1-4 g/l were examined. Result showed that both inorganic nitrogen sources did not affect carotenoid synthesis. However, the carotenoid content seemed to increase with the increasing nitrogen concentration and the highest carotenoid content was achieved from the nitrogen concentration at 4 g/l (Figure 3). The influence of molasses concentrations was also investigated in 4 g/l potassium nitrate. Result in Figure 4 showed that growth of *X. dendrorhous* mutant GM807 was increased in accordance with the increasing molasses concentration. In contrast, carotenoid content was decreased with higher molasses concentration. In this study, 2 % (v/v) molasses gave the highest carotenoid production at 325 μg/g of yeast. Finally, the effect of pH on carotenoid production showed that the optimal pH for carotenoid production of the mutant yeast was at pH 4. The carotenoid content at this pH was 450 μg/g of yeast, while pH 3 and pH 6 caused cell death and no carotenoid was detected (Figure 5).

DISCUSSION

The wild type *X. dendrorhous* and two mutant yeasts of *X. dendrorhous* (mutant GM807 and mutant n485) were originally cultivated in an expensive medium of YM broth. Carotenoid content of cultures in YM broth observed during study revealed that carotenoid content of *X. dendrorhous* mutant GM807 (600- 800 µg/g of yeast) was higher than the mutant n485 (400 - 600 µg/g of yeast) and the wild type strain (100-200 µg/g of yeast), respectively. Level of carotenoid production by mutant GM807 was comparable to the mutant ant-1 (670 µg/g of yeast) as reported by An *et al*. (1989). Cost of production could be reduced by carotenoid-hyperproducing mutants if they are able to grow and produce carotenoid in an inexpensive medium. Therefore, carotenoid production of both mutants was re-examined using molasses as a production medium.

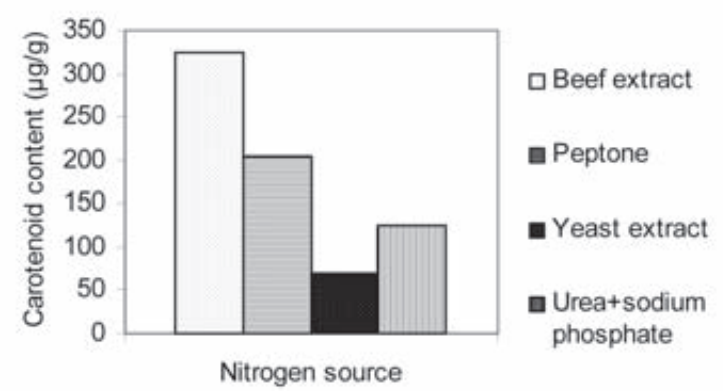

Figure 2 The effect of organic nitrogen sources on carotenoid production by *X. dendrorhous* mutant GM807 in 5 % (v/v) molasses under shaking at 200 rpm in 22°C incubator for 10 days.

Molasses, a by product of sugar production, contain sugars, organic and inorganic substances. Total sugars (mainly sucrose) constitute approximately 47-48% (w/w) of molasses, and total nitrogen containing compounds (mainly betaine and glutamic acid) is about 8-10% (w/w) (Ryan and Johnson 2001). Therefore, molasses containing both carbon and nitrogen source would be a suitable substrate for carotenoid production at the industrial scale. According to An *et al*. (2001), molasses (5%, v/v) containing 30g/l urea and 4.5 g/l sodium phosphate

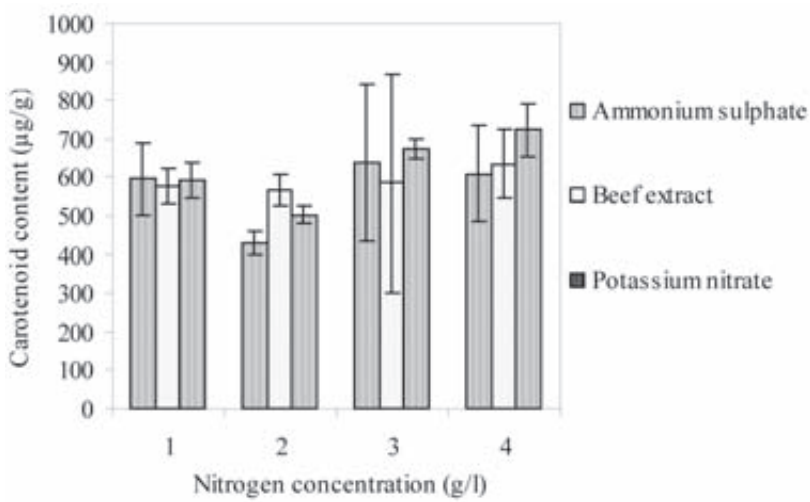

Figure 3 The effect of inorganic nitrogen of ammonium sulphate, potassium nitrate compared with beef extracts at concentration of 1-4 g/l medium on carotenoid content of *X. dendrorhous* mutant GM807 culturing in 5 % (v/v) molasses under shaking at 200 rpm in 22°C incubator for 10 days.

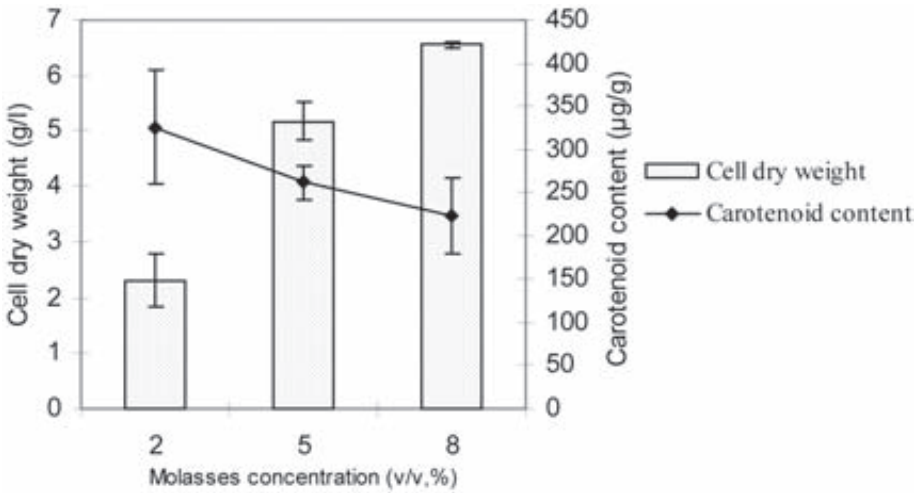

Figure 4 Effect of molasses concentration on cell dry weight and carotenoid content of *X. dendrorhous* mutant GM807. Cartotenoid contents were obtained from cultures incubated in a 200 rpm rotary shaking incubator at 22°C after 10 days cultivation.

were good for biomass production of *X. dendrorhous*. In our study, the diluted molasses medium containing 2.3 g/l sugar was sufficiently supported the growth of both mutants to similar cell dry weight at about 3.2-3.5 g/l. Considering the amount of carotenoid content during growth, Figure 1 shows growth-associated pigment formation pattern of the mutants. However, carotenoid content of both mutants in molasses was lower than when they were grown in YM broth; carotenoid content in molasses of *X. dendrorhous* mutant GM807 was105 μg/g of yeast, while of the *X. dendrorhous* mutant n485 was 70 μg/g of yeast. Thus, *X. dendrorhous* mutant GM807 was a better carotenoid-hyperproducing mutant in molasses.

Nitrogen sources in molasses were mainly betaine and glutamic acid. However, peptone, yeast extracts and beef extract were reported to be suitable nitrogen sources for carotenoid production (Sun *et al*., 2004). Furthermore, Yamane *et al*. (1997) reported that astaxanthin production was enhanced by an initial high carbon/nitrogen ratio (C/N ratio). Therefore, each of these organic nitrogen sources, at the similar molarity of nitrogen, were replaced the combination of urea and sodium phosphate in the original medium of 5 % (v/v) molasses. Beef extract enhanced the maximum carotenoid production of *X. dendrorhous* mutant GM807 (Figure 2). This was different from the study by Fang and Cheng (1993), that peptone was the best individual nitrogen source for astaxanthin production. This could probably result from the characteristic difference of the mutant to be able to use different nitrogen source.

Beef extracts would increase high cost in carotenoid production using molasses. In our study, ammonium sulphate and potassium nitrate could replace beef extract for carotenoid production by *X. dendrorhous* mutant GM807, as the carotenoid contents were all in similar ranges. This was similar to the study by Parajó *et al*. (1998) that *Phaffia* strain utilized inorganic nitrogen for pigmentation. However, we found that the initial concentrations of ammonium sulphate and potassium nitrate in the medium affected carotenoid content significantly (TWO-WAY ANOVA, $p<0.05$), and potassium nitrate at 4g/l molasses would be a good nitrogen source for carotenoid production in molasses.

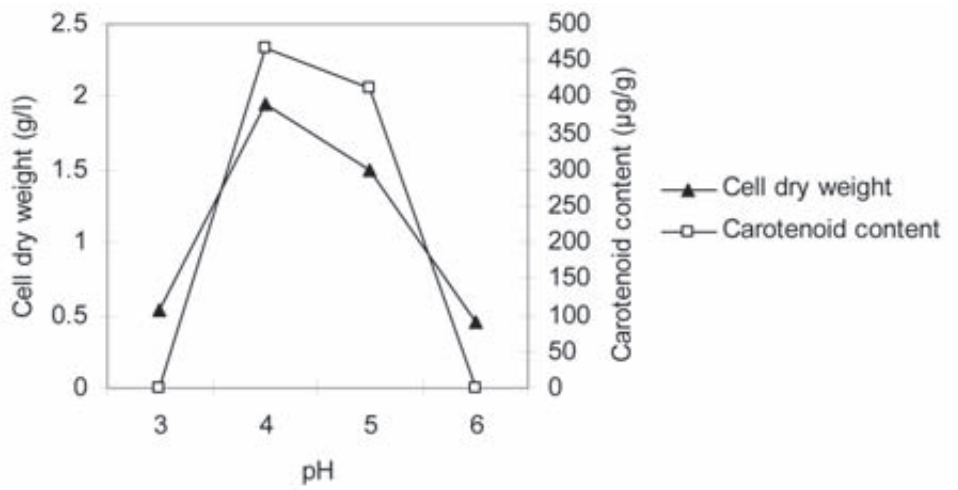

Figure 5 Effect of pH on cell dry weight and carotenoid content of *X. dendrorhous* mutant GM807 grown in 2 % (v/v) molasses medium supplemeted with 4 g/l potassium nitrate under shaking at 200 rpm in 22°C incubator for 10 days.

Carbon/nitrogen ratio in different molasses concentrations at a fixed nitrogen concentration affected mutant growth as the mutant grew better at highly molasses concentration. However, carotenoid production was decreased as the molasses concentration increased. Johnson and Lewis (1979) reported that carotenoid formation was decreased by high concentrations of glucose in *X. dendrorhous*. Moreover, Ryan and Johnson (2001) also reported that high potassium concentration in molasses decreased ethanol production of *S. cerevisiae* and *Z. mobilis*. Therefore, carotenoid formation of *X. dendrorhous* mutant GM807 was probably decreased by either high concentration of glucose or potassium in molasses, or both. However, molasses concentration revealed no significant effect on carotenoid production ($p>0.05$). Thus, the molasses concentration played role only on growth of the yeast, but not carotenoid formation.

Optimal pH for growth and carotenoid production of *X. dendrorhous* were varied. Hu *et al*. (2006) reported an optimal pH for growth at 6.0, and optimal pH for astaxanthin formation at 5.0. RamÌrez *et al*. (2001) reported optimal pH for astaxanthin production at pH 6. However, our optimal pH for carotenoid production was at pH 4, which was similar to that reported by An *et al*. (2001).

CONCLUSION

Mutagenesis for a carotenoid hyperproducing mutant combined with using an inexpensive substrate for fermentation would lead to cost reduction of carotenoid production. *X. dendrorhous* mutant GM807 in molasses produced higher carotenoid content than the mutant n485. The optimal medium for the carotenoid production of the mutant GM 807 was 2% v/v molasses as carbon source, 4 g/l potassium nitrate as nitrogen source and the initial pH of the medium at 4. This medium supported sufficiently high carotenoid production at 450 μg/g of yeast.

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