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Astaxanthin hyperproduction by *Phaffia rhodozyma* (now *Xanthophyllomyces dendrorhous*) with raw coconut milk as sole source of energy

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Abstract Natural carbon sources, such as those present in cane sugar molasses and grape juice, promote the synthesis of astaxanthin in different *Phaffia rhodozyma* yeasts. One of these, coconut milk, has a very rich nutrient composition. The aim of this work was to investigate the utility of coconut milk as sole source of energy for astaxanthin pigment production by *P. rhodozyma* strains. Currently, coconut pulp is widely used in industrial processes in Mexico for the production of shampoos, candies, food, etc. However, coconut milk is a waste product. We show that coconut milk enhances astaxanthin production. The fermentation yielded 850 μg/g yeast with the NRRL-10921 wild-type strain and 1,850 μg/g yeast with the mutated R1 strain. Production was better than reported results employing other natural carbon sources.

Introduction

Among the appealing properties of coconut milk (CM), it is a chemically pure liquid, has good taste and is rich in mineral salts, sugars and vitamins (Table 1). In particular, the carbohydrates present in CM suggest that it could be an excellent culture medium for *Phaffia rhodozyma*. This yeast is able to ferment glucose and other sugars and produces carotenoids, such as astaxanthin, during its growth (Johnson and An 1991). The use of the natural astaxanthin pigment is of great interest in the chemical, pharmaceutical and alimentary industries due to its antioxidant properties (Yamane et al. 1997). In aquaculture, it is employed as a source of natural pigmentation and dietary supplement for trout and salmon (An et al. 1991). However, natural or synthetic processes for astaxanthin production are very expensive (U.S. $ 2,500–3,000 per kg; Olaizola 2000). As a consequence, researchers are studying the use of alternative carbon sources for the production of astaxanthin by *P. rhodozyma*. There have been reports on cane molasses (Haard 1988), sugar cane juice (Fontana et al. 1996), corn wet-milling co-products (Hayman et al. 1995), alfalfa residual juice (Okagbue and Lewis 1984), grape juice (Meyer and du Preez 1994) and hydrolyzed peat (Acheampong and Martin 1995; Martin et al. 1993). Frequently, additional carbon sources were used to enrich the substrate media.

In Mexico, coconut pulp is widely used in industrial processes for the production of shampoos, candies, food, etc. In contrast, despite its rich nutrient composition, CM is an unexploited industrial residual which is a waste product dumped into drainage water.

The main goal of this work was to explore the capacity of raw agro-industrial residual CM as sole source of energy for astaxanthin production by *P. rhodozyma*.

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Materials and methods

Microorganisms

The strain *P. rhodozyma* NRRL-10921 (sexual state *Xanthophyllomyces dendroohous*) was obtained from the ARS culture collection (US Department of Agriculture, Peoria, III). The mutated R1 strain was obtained after two treatments with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG; Jiménez 1999). The yeast was maintained on yeast/malt (YM) agar plates containing (per liter): 10 g glucose, 5 g Bacto-peptone, 3 g malt extract, 3 g yeast extract and 20 g agar.

Mutagenesis was performed on freshly grown *P. rhodozyma* using NTG (Sigma Chemical Co., St Louis, Mo.) according to the method described by An et al. (1989). After *P. rhodozyma* was treated with different concentrations of NTG several times, the cells were harvested by centrifugation and washed twice in 1 ml of 0.1 M sodium citrate buffer and samples were inoculated in YM broth for overnight growth before plating them on a selective medium (YM agar with 3×10^{-4} M β-ionone; Lewis et al. 1990).

Table 1 Chemical composition of yeast/malt (YM) medium and CM

Component	YM medium	CM
Glucose	10 g/l	–
Carbohydrates total	–	16 g/l
Yeast extract	3 g/l	–
Peptone	5 g/l	–
Malt extract	3 g/l	–
Sodium	–	0.25 g/ l
Magnesium	–	0.1 g/ l
Potassium	–	2,94 g/ l
Chlorite	–	1 g/ l
Distilled water	1 l	–

Inoculum preparation

The cell inoculum was grown aseptically in a 250-ml baffled Erlenmeyer flask containing 50 ml of YM medium (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract), at a constant temperature of 22°C and stirrer speed of 150 rpm, for 48 h. Fresh medium was then inoculated with the seed culture at 10% volume. The yeast was grown in the same culture conditions.

Growth kinetics

Cultures were grown aseptically in a 250-ml baffled Erlenmeyer flasks containing 50 ml of CM or YM medium and incubated using a gyrating shaker (Gyrating water batch shaker model G76; New Brunswick Scientific, Edison, N.J.) at 150 rpm and 22°C, for 5 days. Raw residual CM was obtained from a local candy industry located in Mexico City (Col. Vicentina 09340, Delegación Iztapala). It was sterilized by filtration and no nutrient was added. The cultures were inoculated with 5 ml YM medium in the exponential phase. Kinetic experiments were carried out in triplicate.

Analytical methods

Dry weight determination

Samples of 2 ml taken from the cultured medium were centrifuged for 5 min at 3,500 *g* at room temperature in a model 5415 centrifuge (Brikmann Instruments, N.Y.). The cell pellet was washed twice with 2 ml of distilled water and filtered through a 0.45-μm Millipore preweight filter. The filters containing the biomass were dried at 60°C for 24 h.

Pigment extraction

Triplicate 1-ml aliquots of the saturated culture from three different Erlenmeyer flasks were centrifuged at 3,500 *g* for 5 min. The cell pellets were washed twice with 1 ml of distilled water and 1 ml of acetone, until the solvent evaporated. One milliliter of glass pearls (425–600 μm diam.) was then added with 1 ml of dimethylsufoxide at 60°C. At this stage, in order to extract the colorant from its organic phase, cells were broken by vigorous shaking, using a vortex (model G-560; Scientific, N.Y.) for 5 min. Subsequently, 1 ml of acetone, 1 ml of petroleum ether and 1 ml of NaCl at 20% w/v were added. Finally, to separate the petroleum ether containing the colorant, the samples were centrifuged at 3,500 *g* for 5 min. Carotenoids were monitored by spectrophotometry at 474 nm using an extinction coefficient of 1%=2,100 (Sedmak et al. 1990). Astaxanthin was further quantified using a commercial standard (Hoffmann-La Roche, Switzerland) with the HPLC method of Jian-Ping et al. (1997). HPLC was conducted on the LDC 3200 analytical system (LDC, N.Y.) equipped with a UV-vis detector and a stainless steel Nova-Pack C18 (5 μm) reversed-phase column (4×250 mm). The eluting solvent was methanol/water/hexane (95:4:1, by vol.), with a flow rate set at 0.5 ml/min.

Sugar determination

The 3,5-dinitrosalicylic acid (DNS) method of Miller (1959) was used to determine residual reducing sugars in the culture media. A 1-ml sample was centrifuged at 3,500 *g* for 5 min, after which 1 ml of DNS reagent was added to the supernatant. The tubes were covered, heated to boiling point for 5 min and then immediately placed in an ice-bath for rapid cooling. Then, 8 ml of distilled water was added. The tubes were shaken using a vortex (model G-560; Scientific, N.Y.) for 5 min. A spectrophotometer, set at a wave-length of 575 nm, was employed for optical density measurement and the data were calibrated with a suitable standard reference curve to determine the glucose concentration of the samples.

Statistical analyses

All experiments were performed in triplicate. A tri-factorial analysis of variance was applied to cell density values of *P. rhodozyma*; and post hoc comparisons were carried out through the Newman–Keuls test (P=0.05; Sokal and Rohlf 1981). A similar statistical analysis was made for astaxanthin content; and differences among applied treatments were determined by means of the post hoc Newman–Keuls test (P=0.05).

Results

The main aim of the study was to explore the capacities of raw CM as sole substrate in the production of astaxanthin by a specific yeast. In line with this objective, CM and YM medium were compared with respect to yeast growth and astaxanthin production by wild yeast (NRRL-10921). The results showed that CM yielded hyperproduction of astaxanthin. To demonstrate consistency, similar experiments were developed with a mutated R1 strain, chosen for its greater astaxanthin production capacity.

CM versus YM medium In this study, we experimented with the wild-type strain *P. rhodozyma* NRRL-1092. With CM as substrate, the maximal growth was 9.2 g/l, while the maximal corresponding astaxanthin production was 850 μg/g yeast, at day 5 (Fig. 1). In contrast, the maximal registered yeast growth in the control medium YM was 5.6 g/l and the maximal astaxanthin production was 450 μg/g yeast, at day 5 (Fig. 1).

The mutated R1 strain In this study, experiments were carried out using the mutated R1 strain. When CM was employed as the culture medium, the maximal yeast growth was 6.2 g/l, while astaxanthin production was 1,850 μg/g yeast, at day 5 (Fig. 2). In comparison, using the control medium YM, the maximal yeast growth was 4.8 g/l, while astaxanthin production was 1,060 μg/g yeast, at day 5 (Fig. 2).

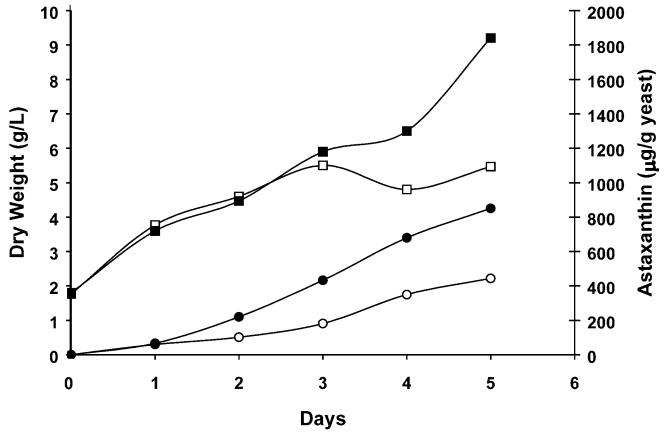

Fig. 1 Wild-strain growth and astaxanthin production in YM medium and CM at a constant temperature of 22°C and stirrer speed of 150 rpm. *Open squares* Growth in YM medium, *filled squares* growth in CM. *Open circles* Astaxanthin production in YM medium, *filled circles* astaxanthin production in CM. Values given are averages and confidence intervals (P=0.05). *L* Liters

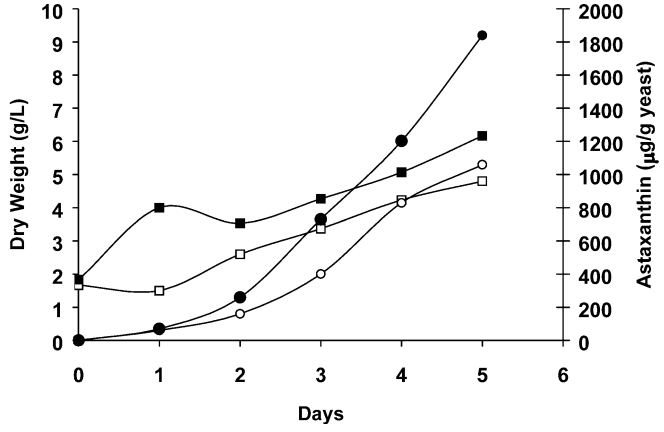

Fig. 2 Mutated R1 strain growth and astaxanthin production in YM medium and CM at a constant temperature of 22°C and stirrer speed of 150 rpm. *Open squares* Growth in YM medium, *filled squares* growth in CM. *Open circles* Astaxanthin production in YM medium, *filled circles* astaxanthin production in CM. Values given are averages and confidence intervals (P=0.05)

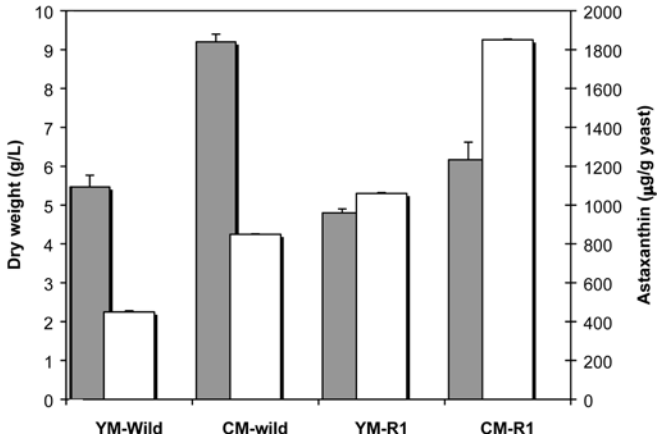

Fig. 3 Dry weight biomass (*filled squares*) and astaxanthin content in cells (*open squares*) of *P. rhodozyma* in 5-day cultures. Values given are averages and comparison intervals (P=0.05)

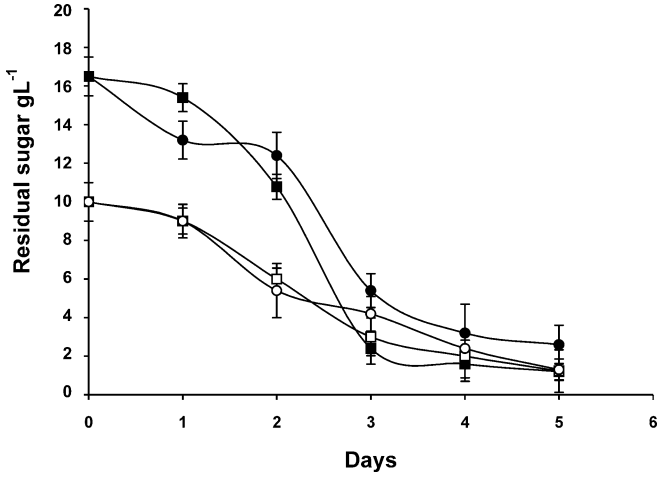

Fig. 4 Residual reducing sugars of *P. rhodozyma* NRRL-10921 and R1 in YM medium and CM at a constant temperature of 22°C and stirrer speed of 150 rpm. *Open squares* NRRL-10921 strain in YM medium, *filled squares* NRRL-10921 strain in CM, *open circles* R1 strain in YM medium, *filled circles* R1 strain in CM. Values are means of three determinations

It is worth noting that, in both culture media (YM, CM), the growth of the wild strain was greater than that of the mutated R1 strain. However, the growth of both species increased when cultivated in CM, from 5.6 g/l to 9.2 g/l for the wild yeast and from 4.8 g/l to 6.2 g/l for the R1 yeast (Fig. 3).

As expected, astaxanthin accumulation obtained with the mutated R1 was greater than that obtained with the wild strain. The important factor was that astaxanthin production by both yeast types was higher with CM, with values rising from 450 µg/g to 850 µg/g yeast for the wild yeast and from 1,060 µg/g to 1,850 µg/g yeast for the mutated R1 (Fig. 3).

In Fig. 4, residual sugar is shown for both *P. rhodozyma* strains in the two culture media (CM, YM). The growth kinetics at day 3 are such that sugar substrate consumption by NRRL-10921 was 85% in CM and 70% in YM medium. In the case of the mutated R1 strain, sugar consumption was 67% in CM and 58% in YM medium. At 120 h, NRRL-10921 sugar consumption was 92% in CM and 87% in YM medium, while mutated R1 sugar consumption was 84% in CM and 87% in YM medium.

Covariance analyses were carried out by means of the Newman–Kuels test. Results showed that the highest significant growth was recorded by the wild strain of *P. rhodozyma* grown in CM, whereas the lowest significant growth was recorded by the mutated R1 strain grown in YM medium ($P \ll 0.001$). However, the reported growth values of the wild strain in YM medium and the mutated R1 strain in CM did not show a significant difference ($P > 0.05$).

In contrast, the highest significant astaxanthin accumulation was recorded by the mutated R1 strain of *P. rhodozyma* grown in CM, while the lowest significant accumulation was recorded by the wild strain grown in YM medium ($P \ll 0.001$). Meanwhile, the registered values

of astaxanthin accumulation by the wild strain grown in CM and the mutated R1 strain grown in YM medium were not significantly different ($P>0.05$).

With respect to the interaction between the culture media and the strain of yeast, the registered values were not significantly different, neither for yeast growth ($P=0.61$), nor for astaxanthin production ($P=0.43$).

Discussion

It is clear that astaxanthin synthesis was only partially associated with biomass growth and still occurred even after growth stopped and sugars were exhausted (Fig. 4). Such behavior is certainly related to the fact that *P. rhodozyma* excretes and stocks some extracellular carbon intermediates, which stimulate late carotenogenesis (Johnson and An 1991).

Different carbon sources have been employed to enhance pigment synthesis. Among others, Meyer and du Preez (1994) employed a mutated CBS5905T strain to grow in grape juice and obtained a maximal production of 1,140 μg/g yeast at 120 h. In our case, we did not use any additional carbon source; and fermentation yielded 850 μg/g yeast with the NRRL-10921 wild-type strain and 1,850 μg/g yeast with the mutated R1 strain.

Some influential factors on pigment production are not specific to the medium or the strain, but are more related to cellular metabolism (Meyer and du Preez 1994; Meyer et al. 1993; Calo et al. 1995; Johnson and An 1991). In this sense, we claim that the presence of vitamins, amino acids and a major carbon source in the raw residual CM are responsible factors that enhance astaxanthin synthesis in *P. rhodozyma* cells (Bramley and Mackenzie 1988).

This work shows that CM medium may play a key role in astaxanthin production by suitable *P. rhodozyma* yeasts. The culture medium and strain are key factors that independently influence growth and enhance pigment production, as confirmed by covariance analysis.

CM appears to be an excellent culture medium, since a remarkable astaxanthin production increase of 95% was obtained with the wild-type yeast and a 75% increase with the mutated R1 strain. Therefore, the combination of a suitable *P. rhodozyma* yeast with CM medium provides significant potential for astaxanthin hyperproduction.

CM, as an industrial residual, is an inexpensive source of energy that enhances both biomass growth and astaxanthin production. In the context of industrial production, process engineers should thus assess the costs and possible instability of the mutated R1 strain with respect to the benefits and availability of residual CM. A further advantage is that maximal production comes from mutated yeast growth in CM substrate.

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