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# Increased astaxanthin production by a *Phaffia rhodozyma* mutant grown on date juice from *Yucca fillifera*

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**The wild strain and the astaxanthin-overproducing mutant strain 25–2 of *Phaffia rhodozyma* were analyzed in order to assess their ability to grow and synthesize astaxanthin in a minimal medium composed of g $L^{-1}$: $KH_2PO_4$ 2.0; $MgSO_4$ 0.5; $CaCl_2$ 0.1; urea 1.0 and supplemented with date juice of *Yucca fillifera* as a carbon source (yuca medium). The highest astaxanthin production (6170 $\mu$g $L^{-1}$) was obtained at 22.5 g $L^{-1}$ of reducing sugars. The addition of yeast extract to the yuca medium at concentrations of 0.5–3.0 g $L^{-1}$ inhibited astaxanthin synthesis. The yuca medium supported a higher production of astaxanthin, 2.5-fold more than that observed in the YM medium.** *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 187–190.

**Keywords:** astaxanthin; *Phaffia rhodozyma*; *Yucca fillifera*; date juice

## Introduction

Astaxanthin (3,3′-dihydroxy-, $\beta$-carotene-4,4′ dione) is widely distributed in nature, especially in the marine environment, where it confers an orange coloration to several species [18]. Since astaxanthin cannot be synthesized by animals, it is necessary to include it in feed to obtain adequate pigmentation; however, to supply this pigment in feeds is an expensive practice, as synthetic astaxanthin is currently sold above $2000 per kg [17]. This has prompted the search for new sources of astaxanthin. *P. rhodozyma* is a carotenoid-producing yeast that synthesizes astaxanthin as its main carotenoid [1]. Recently, there has been considerable commercial interest in using *P. rhodozyma* as a dietary source of astaxanthin for poultry and pen-reared salmonids [7]. However, wild strains produce very low levels of carotenoids (200–300 $\mu$g $g^{-1}$ of yeast) so their production on a large scale is not yet economically feasible. The current trend is to diminish production costs either through obtaining astaxanthin-overproducing strains and/or the development of low-cost culture media that would increase pigment synthesis. Diverse natural substrates have been tested as supplements of media for the growth of *P. rhodozyma*, including molasses [5], peat hydrolysates [8], grape juice [9], alfalfa residual juice [13], corn wet-milling co-products [6] and hemicelullosic hydrolysates of eucalyptus [14]. In this study, we report the development of a low-cost culture medium based on date juice obtained from *Yucca fillifera*, which significantly increases growth and astaxanthin synthesis compared to YM medium. In contrast to other commercial varieties of date, the Chinese palm fruit possesses very little pulp and a high content of seeds which does not make it a very attractive product for commercialization. For this reason, thousands of tons of dates are wasted as no technological alternatives are currently available for their use.

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## Materials and methods

### *Microorganisms and culture conditions*

The wild-type strain of *Phaffia rhodozyma* ATCC 24202 was obtained from the Culture Collection of the Centro de Investigación y Estudios Avanzados del IPN, México. The astaxanthin-overproducing mutant 25–2 is derived from the wild-type 24202 and was obtained in our laboratory by mutagenesis with nitrosoguanidine and selected for its dark orange color. Both strains were grown in YM broth (1% glucose, 0.5% Bacto-peptone, 0.3% malt extract and 0.3% yeast extract, Difco) or minimal medium (2 g $L^{-1}$ $KH_2PO_4$; 0.5 g $L^{-1}$ $MgSO_4$; 0.1 g $L^{-1}$ $CaCl_2$ (Sigma) supplemented with nitrogen sources and date juice as carbon source as required).

### *Preparation of date juice*

Dates were collected in the state of Coahuila, México. The juice was extracted by dissolution for 1 h at 60°C in a stirred tank. The juice was separated from the pulp by successive filtrations and the resulting solution was centrifuged at 5000 × *g* to remove suspended solids. Finally, the juice was clarified using $H_3PO_4$ and CaO as described by Sigüenza [16] and adjusted to a final concentration of 160 g reducing sugars $L^{-1}$ (RS). The dinitrosalicylate (DNS) method was used for quantitative analysis of reducing sugars [10]. The juice was sterilized by autoclaving it and it was added to the minimal medium as required.

### *Inoculum*

Loopfuls of both strains of *P. rhodozyma* were used separately to inoculate 10 ml of YM broth, and were incubated at 20°C for 24 h on a rotary shaker. Five milliliters of this preinoculum were inoculated into a 250-ml Erlenmeyer flask containing 50 ml of minimal medium and were grown

with shaking at 300 rpm for 24 h; 5 ml of inoculum was used for each shake flask.

### *Flask cultures*

Experiments using shake-flasks (250-ml conical flasks with 50 ml medium) were carried out in YM and minimal medium at 21°C for 3 days on a rotary shaker at 300 rpm. Tryptone, yeast nitrogen base (YNB) (Difco), ammonium sulphate, ammonium phosphate, ammonium nitrate, ammonium chloride and urea (Sigma) at 1.06 g $L^{-1}$ nitrogen were used to study nitrogen supplementation. Media were buffered at pH 5.5 with 0.1 M potassium hydrogen phthalate buffer (Sigma).

### *Analytical procedures*

Cell growth was measured as turbidity at 600 nm. Biomass formation was measured as dry cell mass. Reducing sugar concentration in the culture medium was determined by the DNS method [10]. The carotenoid content was determined by the DMSO method [15]. Astaxanthin was measured by HPLC [15]. Chromatographic analyses were performed in a Beckman 166 chromatograph with an adapted Alltech carbamate column 5 $\mu$m (4.6 × 250 mm) and a 4.6 × 45-mm guard column. The eluting solvent was hexanes:ethyl acetate 50:50 (% v/v) and the flow rate was 1 ml $min^{-1}$. The eluant was monitored at 480 nm. Synthetic astaxanthin (Hoffman-La Roche) and $\beta$-carotene (Sigma) were used as external standards.

### *Statistical analysis*

The ANOVA and the Least Statistical Difference (LSD) tests, were performed according to Montgomery [12], using the Statgraphics software in order to determine significant differences between the treatments. Biomass and carotenoid quantities were obtained from two independent experiments.

## Results

### *Effect of nitrogen source on growth and astaxanthin production by P. rhodozyma*

The best carotenoid production yields in volumetric or mass basis for the mutant 25–2 strain were obtained in the medium with urea (4078 $\mu$g $L^{-1}$ and 1047 $\mu$g $g^{-1}$ yeast respectively); whereas no significant differences in carotenoid production on a mass basis were observed in the wild strain for all nitrogen sources ($P$ = 0.481) (Figure 1). An increase of 25% in biomass formation in comparison to the YM medium was observed in media supplemented with tryptone and ammonium phosphate; while in the media with YNB and urea, low yeast growth was observed (3.0 and 3.9 g $L^{-1}$, respectively).

### *Effect of sugar concentration on carotenoid production in yuca medium*

The wild and mutant strains were cultivated in yuca medium containing several concentrations of RS. The carotenoid production of the mutant strain increased up to 6430 $\mu$g $L^{-1}$ and 1203 $\mu$g $g^{-1}$ of yeast in medium containing 22.5 g $L^{-1}$ of RS (Figure 2). The lowest carotenoid production for both strains was observed at 5 g $L^{-1}$ of RS.

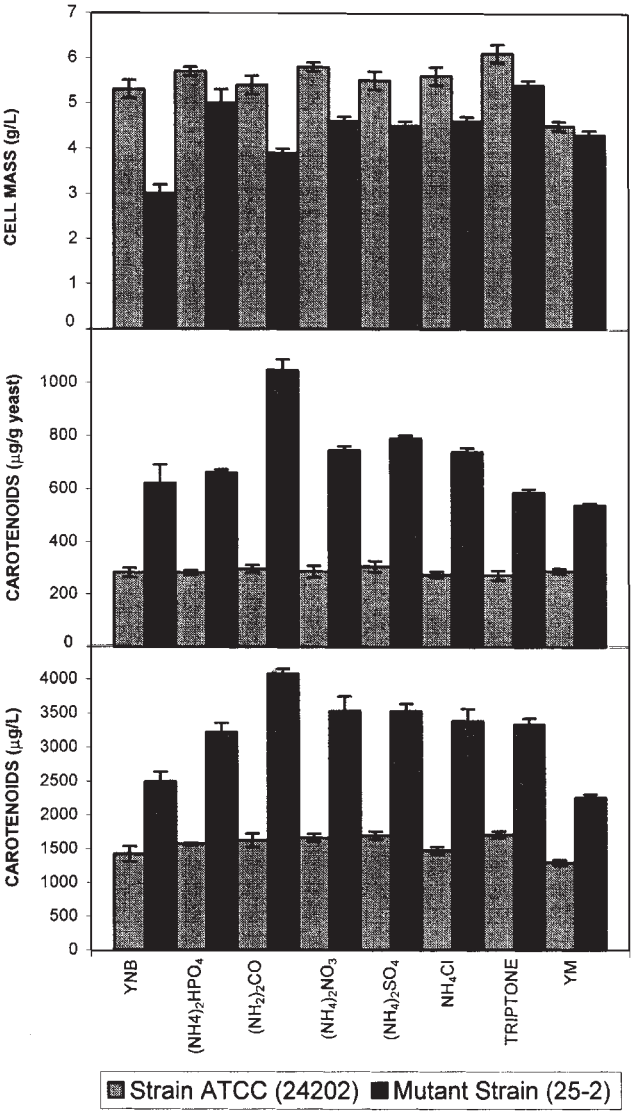


**Figure 1** Effect of nitrogen source on growth and carotenoid production by *P. rhodozyma*. Yeast strains were grown in minimal medium with date juice (15 g $L^{-1}$ as reducing sugars) and the indicated nitrogen sources at 1.0 g $L^{-1}$. Data are expressed as the mean of two independent experiments run in duplicate ± standard error.

Growth was dependent on the sugar concentration, and maximum biomass formation for both strains was observed at 30 g $L^{-1}$. Significant differences in growth and astaxanthin production in volumetric and mass bases were observed among the different concentrations of RS ($P$ = 0.000).

### *Effect of yeast extract on growth and astaxanthin production*

Since urea supported high pigment production, we investigated its effect combined with yeast extract to increase astaxanthin production. Contrary to what we expected, yeast extract (YE) inhibited astaxanthin synthesis on volumetric and mass bases in both strains; the best yields were obtained in the absence of YE: 6170 $\mu$g $L^{-1}$ and 1063 $\mu$g $g^{-1}$ of yeast for the mutant strain, whereas for the wild-type maximum astaxanthin production was attained at 0 and at

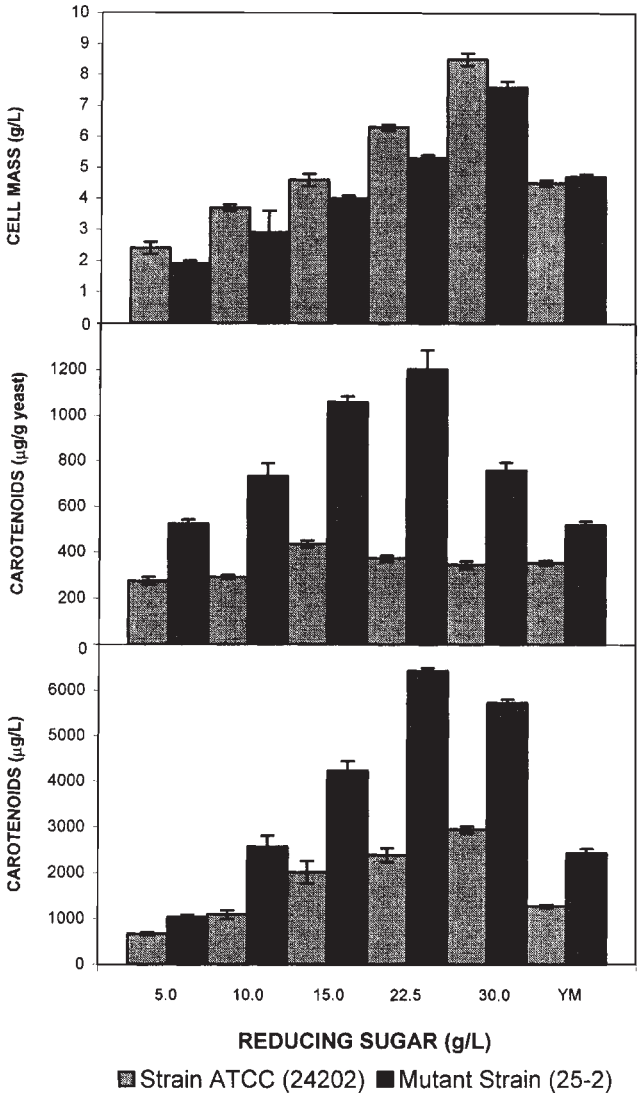


**Figure 2** Effect of sugar concentrations on carotenoid production by *P. rhodozyma*. Yeast strains were grown in yuca medium containing several reducing sugar concentrations and 1.0 g urea $L^{-1}$. Data are expressed as the mean of two independent experiments run in duplicate ± standard error.

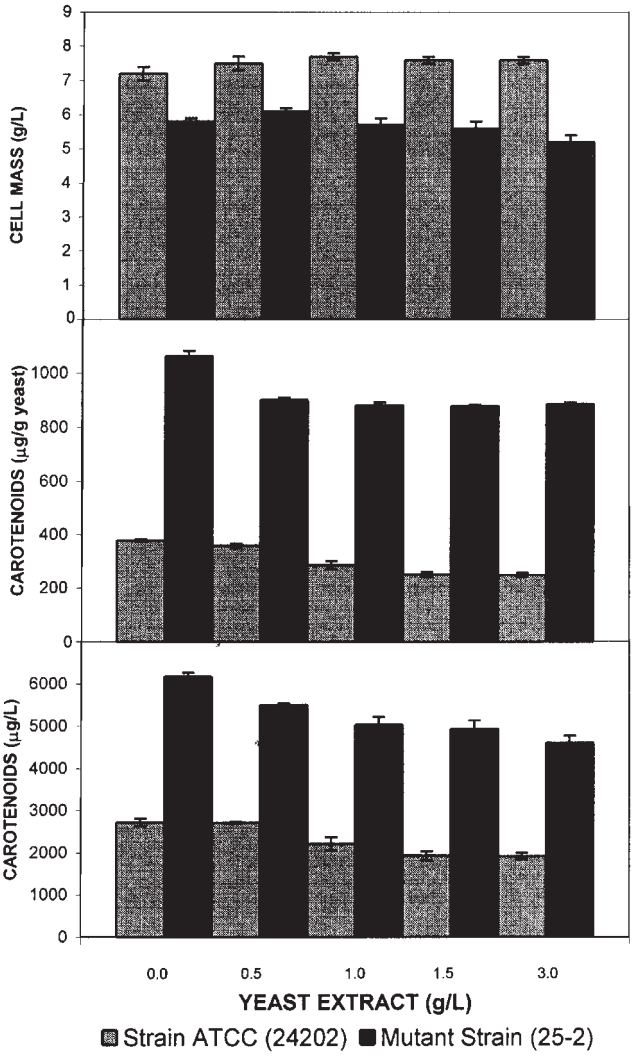


**Figure 3** Effect of yeast extract on growth and astaxanthin production by *P. rhodozyma*. Yeast strains were grown in a yuca medium containing 22.5 g $L^{-1}$ of reducing sugars and 1.0 g $L^{-1}$ urea. Data are expressed as the mean of two independent experiments run in duplicate ± standard error.

0.5 g $L^{-1}$ of YE (Figure 3). YE inhibited astaxanthin production in the mutant strain and in the wild strain up to 24% and 30% respectively. In regard to growth, significant differences in biomass production were not observed in the wild strain ($P = 0.2037$). On the contrary, in the mutant strain, YE inhibited growth up to 9% ($P = 0.0002$). Analysis of pigments by the HPLC method showed that astaxathin was the main pigment present (90–95%) in mutant strain 25–2 (data not shown).

## Discussion

Minimal medium supplemented with urea stimulated the highest pigment production in the 25–2 mutant strain; these results contrast to those obtained by Fang and Cheng [4] who observed neither growth nor pigment synthesis of *P. rhodozyma* NCHU-FS301 (mutant strain) grown in urea. In contrast in the wild strain, tryptone and ammonium sulphate supported the best astaxanthin production. These results suggest metabolic differences between the wild and the mutant strain 25–2. Carotenoid overproducing mutants show physiological characteristics quite different from those of the wild-type strains: high requirements of ammonia and low cellular biomass [2,3]. Urea could be a promising nitrogen source replacing YE and/or peptone in developing a less expensive culture medium for industrial production of astaxanthin. *P. rhodozyma* is the only known carotenogenic yeast-fermenting glucose [11]. A high glucose concentration in the growth medium causes significant reductions in the efficiency of pigment production [7,9]. In this study, pigment production ($\mu$g $L^{-1}$) was inhibited in the presence of high RS concentrations, whereas biomass formation increased linearly as a function of the concentration of date juice. YE has been successfully used for cultivation of *P. rhodozyma* [1]. However, YE is an expensive nutrient source for use at an industrial level and notably increases astaxanthin production costs. Surprisingly, addition of YE to yuca medium with urea as a nitrogen

**Table 1** Comparison of astaxanthin production obtained in flask cultures using different carbon sources

| Carbon source | Additional supplement | Strain | Astaxanthin production $\mu g\ g^{-1}$ | References |
|---|---|---|---|---|
| Molasses cane | Peptone | UCD 67–210 | 1182 | [5] |
| Glucose | Peptone and YE | NCHU-FS301[a] | 1297 | [4] |
| Hydrolysates peat | Peptone | ATCC 24202 | 1567 | [8] |
| Grape juice | Peptone and YE | N9[a] | 1240 | [9] |
| Corn wet-milling | None | NRRL Y-17270 | 400 | [6] |
| Hydrolysates Eucalyptus | Peptone | NRRL Y-17268 | 448[b] | [14] |
| Xylose | Peptone, YE and malt extract | NRRL Y-17268 | 571 | [14] |
| Date juice | None | 25–2[a] | 1203 | This work |

[a]Mutant strain.
[b]Fermentor batch culture.

source did not stimulate pigment synthesis, but caused a decrease in astaxanthin production at all concentrations tested; this effect was dose-related, and the greatest decrease (25% and 30%) was observed with the 3 g $L^{-1}$ concentration. The YE inhibition mechanism observed in the mutant strain 25–2 is unknown but it could be caused by the decrease in the C/N ratio, mainly at high concentrations of YE (3.0 g $L^{-1}$). The results obtained offer a new alternative to the development of an economically feasible production process, above all, if we consider that: (i) The experimental results were achieved in flasks without using statistical methodologies for optimization of culture conditions; this suggests that further improvements could be attained. (ii) Bioreactors provide higher oxygen transfer rates than flask cultures, therefore higher carotenoid yields could be obtained. (iii) In contrast to most media reported for industrial production of astaxanthin (Table 1), yuca medium doesn't require additional supplements such as peptone and yeast extract which are expensive and increase production costs. In conclusion, date juice combined with an appropriate source of nitrogen, that could well be urea, represents an excellent culture medium for the industrial production of astaxanthin by *P. rhodozyma*.

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