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Culture of the Astaxanthinogenic Yeast *Phaffia rhodozyma* in Low-Cost Media

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ABSTRACT

Growth of the yeast *Phaffia rhodozyma* was carried out in a simplified medium based on less expensive nutrient sources, such as diluted sugar cane juice, urea, and sodium phosphate. The usual content of the astaxanthin, an oxygenated pink carotenoid useful for fish flesh staining, was improved along with with good cell yields (respective values of >1300 μg/g cells and >5 g cells/L were observed). Yeast invertase and urease must therefore play an important role in the implementation of low-cost culture media.

Index Entries: *Phaffia rhodozyma;* astaxanthin; sugar cane juice; urea; carotenoids.

INTRODUCTION

Sales in the increasing world market for the oxygenated carotenoids astaxanthin and canthaxanthin were $130 million US dollars in 1992. The bioproduced and synthetic counterparts of these pigments are expected to be 67 and 33%, respectively, of the $455 million dollars market projected for 2000. Their current prices average from $1500–$4000/kg for a 100% product formulation *(1)*. The main use of astaxanthin is in farming of selected species, such as salmon or trout. Pink staining of salmonid flesh is a natural process owing to their feeding on colored marine live sources, such as krill and pigmented alga, since they are not able to perform the *de novo* biosynthesis of the pigments *(2)*. Similar or improved patterns of flesh staining may be achieved using feed supplemented with synthetic astaxanthin or whole *Phaffia rhodozyma* yeast cells. Markets for both synthetic and natural carotenoid pigments are now firmly established; in both cases, the question of isomeric forms of the highly oxygenated carotenoids should be considered *(3)*. *P. rhodozyma*, a single genus and single species yeast, was described only two decades ago *(4)*. This basidiomycetous yeast displays two striking features, namely, an intense dark-

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orange color of mature cultures, and, as opposed to other pigmented yeasts, the biochemical ability to ferment several mono- and disaccharides. The major pigment produced by *Phaffia* is 3,3'-dihydroxy β-carotene-4,4'-dione or astaxanthin *(5)*. An upper limit of 15 g/L was recorded for glucose as an efficient carbon source, if supplemented with yeast extract and salts, but the resulting astaxanthin concentration of 421 μg/g dry cell yeast could be further improved to a maximum of 652 μg/g when the monosaccharide was replaced by reagent-grade cellobiose. Other disaccharide sources, such as reagent-grade maltose and sucrose, resulted in intermediate values of astaxanthin productivity. A mildly acidic medium and temperatures below 25°C were reported as other optimal culture conditions *(6)*. *P. rhodozyma* whole yeast cells and their main carotenoid pigment should be biotechnologically more advanced than the fish farming basic processes, since recent reports indicate outstanding biological functions for astaxanthin, which serves as a provitamin A, active oxygen species scavenger and/or quencher, tumorogenesis supressor, and in in vitro antibody enhanced production *(7)*. Furthermore, singlet molecular oxygen quenching was proposed to be 500–1000 times more efficient with astaxanthin than with tocopherol *(8)*. The antioxidant role of carotenoids during the aging of *Phaffia* cells was recently documented *(9)*. Another example for the use of astaxanthin from whole or broken *Phaffia* yeast cells is as a poultry feed, since both egg production and yolk coloration are increased *(10)*. Aquaculture is an active and growing enterprise in developed and developing countries *(11,12)*, farming of channel catfish in Georgia, is one example. Exportation of salmon by Chile is one of its outstanding income sources. Paraná State (South of Brazil) has recently set up factories for processing of tilapia. There is an urgent need for novelties and technology improvement in Brazilian fish farming, since highly priced fish, such as salmonids, are being introduced. The flesh pigmentation pattern, being gastronomically attractive, remains an important requirement of fish and seafood consumers, and is directly related to their commercial value. *Phaffia* cells, in addition to astaxanthin, can provide carbohydrates, protein, and glyceride lipids as useful (co)nutrients for the purpose of fish farming. Considering that natural astaxanthin is getting an increased volume in the market of carotenoids *(1)*, and *Phaffia* is one of the best sources of this compound *(5)*, we started investigations on less expensive and readily available carbon and nitrogen sources for growth of *P. rhodozyma* and astaxanthin production.

MATERIALS AND METHODS

Yeast Source and Culture Maintenance

P. rhodozyma (reference strain 24202) was purchased from ATCC (Rockville, MD) and was maintained on agar slants containing 2 g/L glucose and 1 g/L yeast extract at 26°C.

Small-Scale Culture Growth and Routine Analytical Procedures

The preferential carbon source adopted was freshly collected sugar cane juice, previously clarified by centrifugation in order to remove cell debris and solid impurities arising from milling. The clarified juice was then diluted 10 times to ensure an appropriate sugar concentration. The routine supplements were 1 urea and 1.25 g/L sodium phosphate, pH = 6.5. Culture media components (sugars and

their complements) were separately autoclaved at 1 atm for 20 min. The comparative cultures of yeast (multifactorial approach) were grown in Erlenmeyer flasks containing 25 mL of medium at a 5:1 air:medium ratio and 100 rpm of agitation for 5 d at 25–26°C (26–27°C in the first trials). Flasks were loosely plugged with cotton wool in order to allow free gas exchange. Inoculation from 48 to 72 h solid medium precultures resulted in an initial value of $A_{650\,nm}$ = 0.05–0.075.

The same wavelength was used to measure growth by turbidimetry. Cell-free supernatant analyses were carried out for residual sugars by high-performance liquid chromatography (HPLC) in a SugarPak column (Waters do Brazil, Sao Paulo), using water at 85°C as eluant and a differential refractometer as detector. Reducing sugars were measured by the dinitrosalicylate method *(13)*. Samples of lyophilized cells (up to 200 mg) were swollen with dimethyl sulfuxide (DMSO) (1 h) and extracted with acetone. Residues were reswollen in DMSO and extracted with chloroform-methanol (1:1). Both extracts were combined and astaxanthin spectrophotometrically quantitated at 478 nm. A purified (silicic acid column) preparation of the pigment from the commercial product Caryophill Pink-8% (Hoffmann-La Roche, Basel, Switzerland) was used for calibration.

Scaled-Up Cultures

Phaffia cultures were scaled up to 2.5 L using a BioFlo IIc fermenter (New Brunswick, Edison, NJ) at 200 rpm, aeration of 0.5–0.75 working vol/min, and 25°C. Antifoam (Sigma, St. Louis, MO; 2 mL/run) was added, and sampling carried out at 12–24-h intervals.

Spectral and Chromatographic Analyses

The spectroscopic scan for pink pigments in acetone was obtained using either a Varian DMS-80 or a Hewlett Packard 8542 spectrophotometer. The composition of the whole and purified (silicic acid column) lipid extracts was assessed using chromatoplates of silica gel 60 (Merck, Darmstadt, Germany) developed with hexane:acetone (7:3). Plates were inspected with long-wave UV and developed at 110°C with 0.1% *p*-anisaldehyde in methanol containing 5% sulfuric acid or with saturated chloroform solution of antimony trichloride *(13)*. The tentative identification of carotenoids was carried out according a previously established protocol *(14)*. The β-carotene standard (all trans) was purchased from Sigma.

RESULTS AND DISCUSSION

Culture of *P. rhodozyma* on pure and expensive carbon and nitrogen sources, such as glucose or pure disaccharides supplemented with yeast/malt extracts and/or peptone, produced yeast cell and astaxanthin yields in the range of 3–4 g/L and 500–650 μg/g dry yeast cell, respectively *(6)*. In order to launch a strategy of double and simultaneous replacement of C and N sources, we first determined 1:10 diluted sugar cane juice to be the C source (mean content of 175 g/L sucrose in the fresh juice) *(15)*. Because of the huge production of sugar cane in Brazil (250 million tons in the 1994/1995 year crop; 13 billion liters of resulting ethanol) (Amorim, H. V., personal communication), sugar cane juice may be considered the most inexpensive C source. Paraná State, in addition to its extensive cane plantations also has one of the Brazilian petrochemical plants that produces urea as a traditional fertilizer. Urea was thus selected as the N source. Despite the physiological importance of

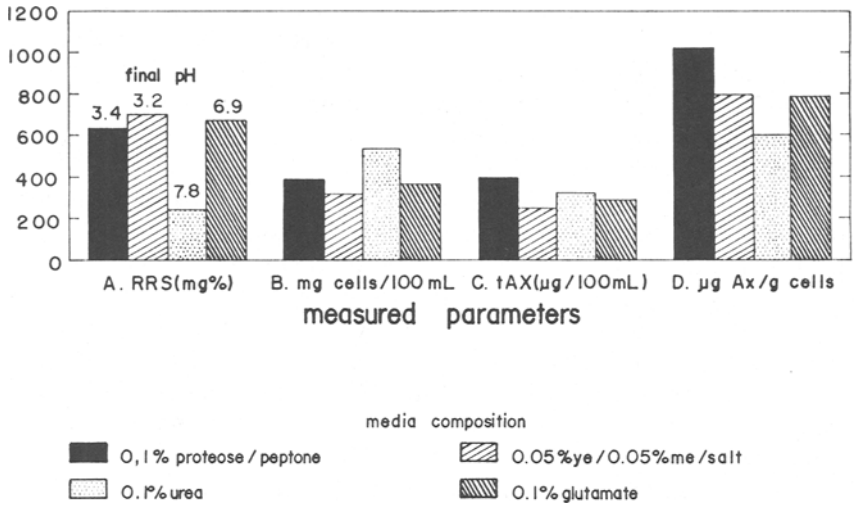

Fig. 1. Effect of nitrogen sources on *P. rhodozyma* growth and pigmention when using sugar cane juice as C source. The four column blocks refer to residual reducing sugars (RRS), cell growth, total astaxanthin (tAx), and astaxanthin productivity as measured in the indicated units.

phosphate, its addition as the third and last ingredient of the medium was to control the alkaline pH imbalance resulting from hydrolysis of urea. Significantly, a slightly acidic condition has been reported as favorable for *Phaffia* growth *(6)*.

Using yeast/malt extracts or proteose/peptone as complex reference sources of N, the results obtained with an initial multifactorial trial are summarized in Fig. 1. If the comparison is restricted to the more successful experiments, the main following parameters for the 25-mL cultures (measured parameters extrapolated to 1/10 L volume) were found. The highest efficiency of sugar consumption and yeast cell yield was obtained in an experiment using 1 g/L urea supplement: 2.4 g/L of residual reducing sugar and 536 mg cells/100 mL medium. Experiments with 1 g/L proteose/peptone and with 0.33 g/L of ammonium phosphate reinforcing the yeast/malt extracts yielded 392 and 249 µg of total astaxanthin/cells from 100 mL of culture, respectively, as compared to 323 µg in the urea-based experiment. The proteolyzed N supply resulted in the deepest colored cells. However, cell yields (g/100 mL media) in these complex nitrogen supplies were only 384 and 312 mg, respectively, and more residual reducing sugar was left over (6.3 and 7.0 g/L, respectively). The corresponding data for the urea-based run were thus superior. The only other result worthy of note was with 1 g/L sodium glutamate as N source, which yielded 364 mg cells and 286 µg of pigment/100 mL medium, but with a less efficient use of sugars (6.7 g/L residual). A remarkable acidification of the stationary-phase media (5 d) resulted from complex N sources (pH 3.4–3.2), whereas urea alone led to a mild alkalinization, which, as further confirmed, could be avoided by the inclusion of diluted phosphate buffer. Since the degree of medium acidity was previously shown to affect more the qualitative carotenoid profile than the content of astaxanthin *(6)*, phosphate salt was maintained as an appropriate cosupplement to urea, except for data in Fig. 2, where a specific focus was pursued as explained below. For this set of experiments, a fine control was set up for some operational

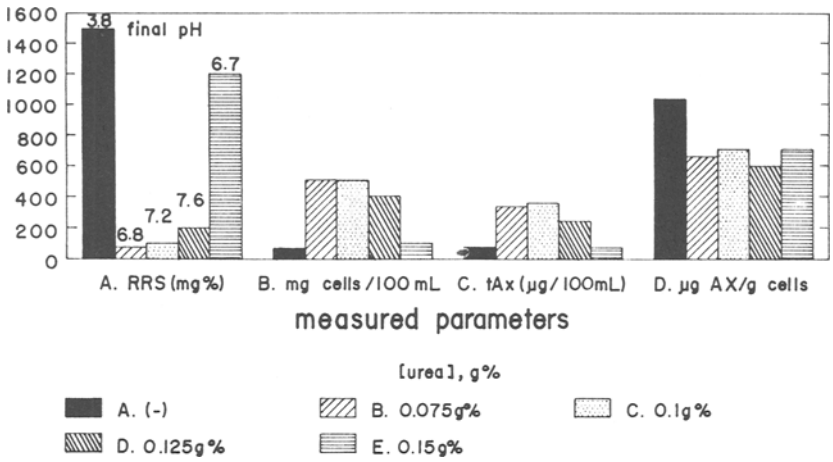

Fig. 2. Effect of urea concentration on *P. rhodozyma* growth and pigmentation when using sugar cane juice as C source (measured parameters as in Fig. 1).

parameters, namely the incubation temperature was lowered by 1° (that is, from 26–27°C to 25–26°C), owing to the high thermo-sensitiviy of *Phaffia;* and inoculum started from 48-h grown cells, which ensured a slightly higher initial optical density ($A_{650\,nm}$ = 0.06–0.07). With the goal of optimizing cane juice and urea pair, the effect of the C and N sources ratio was then determined (results in Fig. 2). The extremities of the dose–response curve, i.e., no urea addition and 1.5 g/L, led to poor cell yields as a result of drastically diminished sugar uptake. Urea supplementation in the 0.75–1.0 g/L range resulted in the highest production of cells (512 and 507 mg/100 mL medium, respectively) and similar astaxanthin production (339 and 360 µg), the pigment production being slightly superior in the second case (709 µg/g cells). The actual increase in astaxanthin could be related to the operational changes, but no net increase in cell biomass was observed. Addition of urea to sugar cane juice resulted in a pH shift from 3.8 (control; no urea addition) to the neutral range, as desired. Accordingly, the optimum urea concentration range also ensured a maximum of C source consumption (<1 g/L of residual reducing sugars). The apparent high productivity of astaxanthin in the urea-free control (first bar; last block of columns; Fig. 2) is not to be considered since the cell yield (only a threefold increase in the inoculation absorbance) is insignificant as compared to the most successful experiments. With the establishment of the beneficial effect of urea, we decided to introduce a small buffering effect through the phosphate input itself. The choice was the sodium salt (1.25 g/L, pH adjusted to 6.5). In the the first trial, maintaining 1 g/L urea as N source, the addition of phosphate resulted in a pH decrease of 1 U with a concomitant increase in cell yield from 404 to 536 mg/100 mL medium and total astaxanthin from 360 to 720 µg (as a reference proteose/peptonle resulted in 260 mg cells and 488 µg of pigment) (Fig. 3). Doubling the sugar cane concentration (i.e., using an 1:5 dilution) and maintaining the C/N ratio (i.e., also doubling urea concentration) did not result in a higher astaxanthin production, probably because of the inhibitory effect of higher urea inputs (as previously shown in Fig. 2). Proteose/peptone (0.5 g/L) allowed the best pigment production, but the corresponding cell yield was less than half that obtained from urea/phosphate. An enhancement

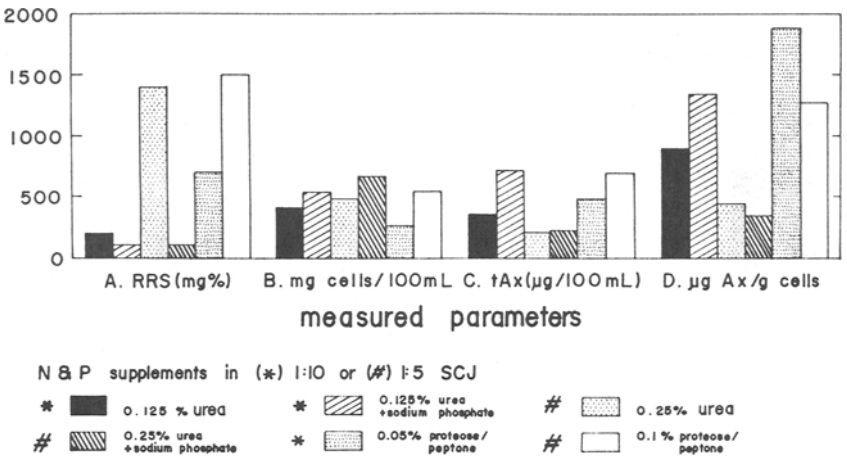

Fig. 3. Effect of phosphate addition on *P. rhodozyma* growth and pigmentation when using sugar cane juice and urea as C and N sources. Buffering pH 6.5 salt at a final concentration of 0.125 g%; (*) and (#), 1:10 and 1:5 dilutions of fresh sugar cane juice; the last two columns of each block.

of this yield (but not in astaxanthin production; last bar; last block of columns, Fig. 3) could be achieved at the expense of more concentrated C and N sources (1:5 diluted juice and 1 g/L of N supply as proteosepeptone), implying a higher cost. As a final attempt dealing with the nature of the N source, multifactorial assessment was carried out with a selected mix of free amino acids and native or proteolyzed leather and soya meal. Complete data will be reported elsewhere, but some of the main conclusions are presented below. The best cell yield arose from a urea supplement with phosphate (699 mg cells), the highest absolute pigment production being obtained with enzyme hydrolyzed soya meal (1089 µg/100 mL culture), and the optimum astaxanthin productivity was found with a mixture of valine, threonine, methionine, and leucine (>2000 µg pigment/g dry cells). The culture of *P. rhodozyma* and moderate productivity of astaxanthin was also feasible using corn, potato, and cassava starches, as well as sugar cane bagasse. The native polysaccharides were previously depolymerized with commercial enzymes (NOVO's disaccharidogenic Thermamyl and Celluclast), and the C input was maintained at 20 g/L along with urea/phosphate supplementation. Cell growth showed no variation, and astaxanthin reached a 550–750 µg/g cell range. The best pigment concentration (ca. 800 µg/g dry cells) resulted from a cassava starch hydrolyzate, a value that was surpassed, however, using, 20 g/L brown sugar utilized as a reference for the fresh sugar cane juice at the same concentration. Sugar cane molasses, the dark mother liquor after sucrose crystallization from concentrated sugar cane juice, also supported yeast growth and astaxanthin production.

P. rhodozyma, grown in sugar cane juice/urea/Na phosphate, was scaled up to 2.5 L in a New Brunswick IIc fermenter. Similar results were obtained to those of shake flasks (as an example, 12 g of dry yeast cells displaying a carroty orange hue). Thin-layer chromatography (TLC) indicated a coincident spot of $R_f = 0.24$ for both astaxanthin standard and the resulting reddish purified pigment. Sugar consumption was monitored by HPLC in the time course of the experiment. Following inoculation (even with washed cells), sucrose was quickly hydrolyzed into its

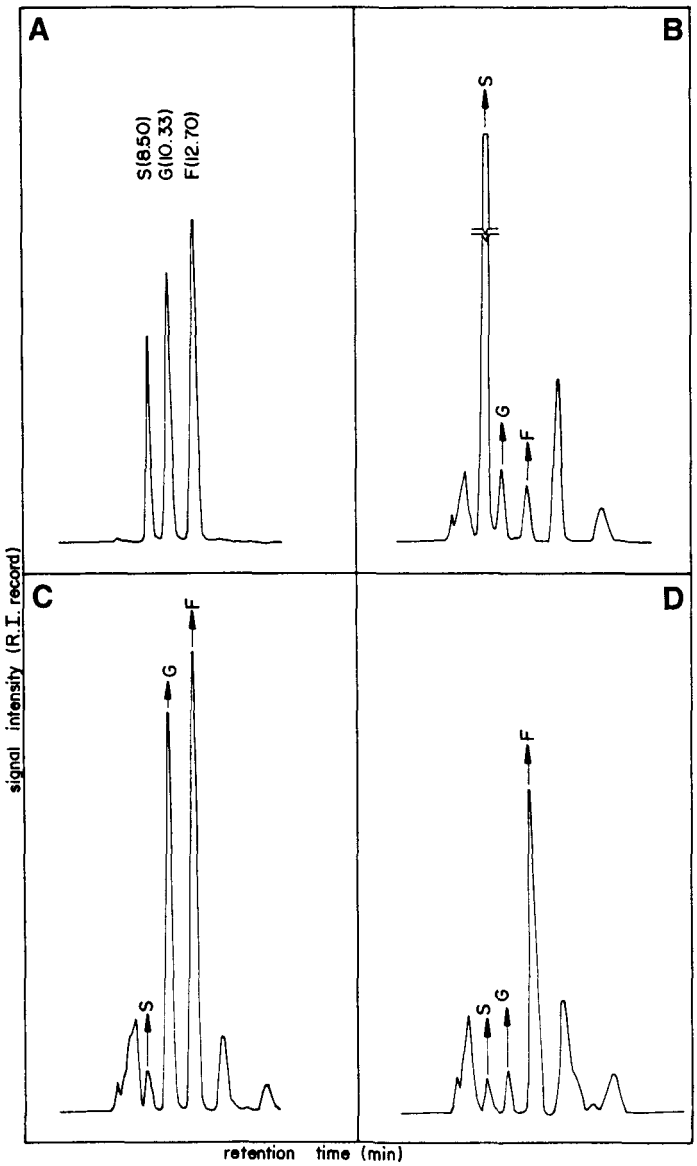

Fig. 4. HPLC analysis for sugar cane juice saccharide composition in this time course of a scaled-up culture of *P. rhodozyma*. The retention times are indicated only for the sugar standard **(A)**, fermenter sampling just after inoculation **(B)**, at 37 h **(C)**, and at 72 h **(D)**. S, sucrose; G, glucose; F, fructose.

halves (Fig. 4B), glucose being preferentially consumed (Fig. 4C), and some fructose remaining at the end of fermentation. Measurements of invertase and urease in the washed cells and in the cell-free supernatant indicated that most, if not all, of these enzymes are associated with the cells. Mild ultrasonication did not release the enzymes into the supernatant.

In order to confirm astaxanthin as the pink to orange carotenoid pigment being produced by *P. rhodozynma*, a spectroscopic analysis was carried out using acetone as solvent (Fig. 5). The pure astaxanthin standard prepared from

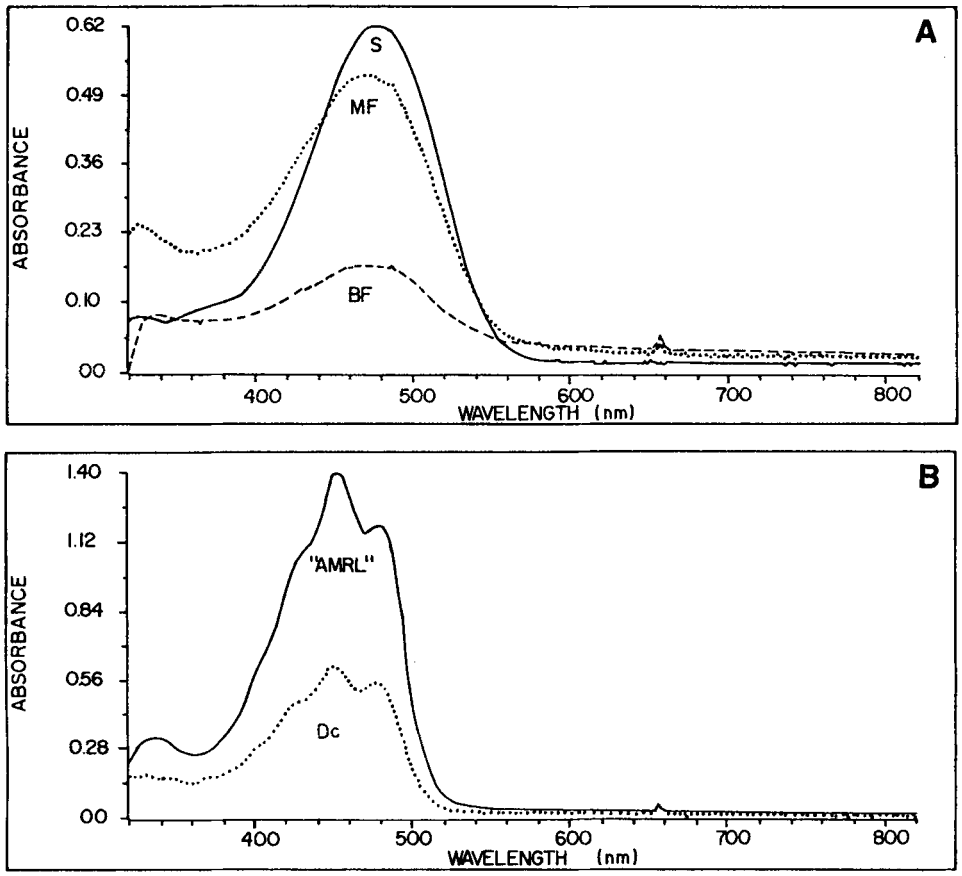

Fig. 5. Spectroscopic analysis of pigments. **(A)** Astaxanthin standard (S) and *P. rhodozyma* pigmented crude lipids from agitated flasks (MF; multifactorial) and scaled-up cultures (BF, BioFlo IIc fermenter). **(B)** Carrot *(Daucus carota)* chloroform extract (Dc); crude carotenoid extract from a carotenogenic bacterium ("AMRL" isolate).

Caryophill Pink, as expected, gave maximum absorbance at 482 nm (Fig. 5A). Absorbance peaks, for two different samples of *Phaffia* pigments obtained either in a multifactorial experiment (MF) or in the fermenter (BF), showed a small displacement toward a lower wavelength (ca. 472 nm) possibly owing to interference of the predominant contamination with triglycerides (as verified by TLC developed with the generic chromogenic reagent *p*-anisaldehyde; results not shown). The spectroscopic runs for carrot chloroform extract (Dc) and for a crude carotenoid extract from a yet uncharacterized carotenogenic bacterium (isolate "AMRL") are shown in Fig. 5A, for comparison purposes. Pigment quantitation *(14)* (Fig. 6A) on *Phaffia* cells from scaled-up cultures indicated 3R, 3'R-astaxanthin as the major component (63%) followed by the β-carotene (12%) (Fig. 6B) and the less oxygenated carotenoids (25%).

CONCLUSIONS

Inexpensive sugar cane juice, because of its high content of sucrose, proved to be a suitable carbon source for the growth of the yeast *P. rhodozyma* and for

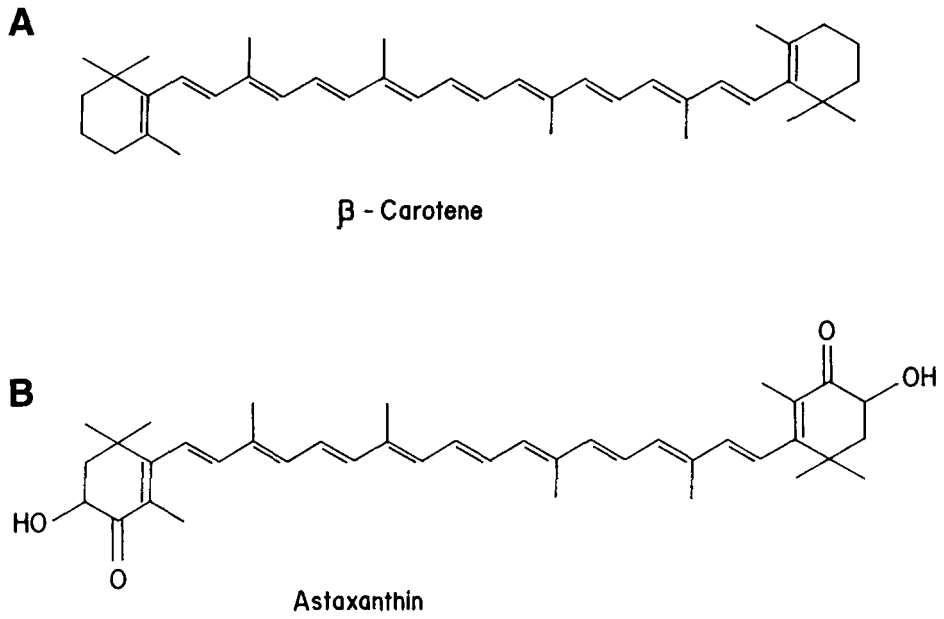

Fig. 6. Carotenoid structures: **(A)** β-carotene and **(B)** astaxanthin.

astaxanthin production. The contribution of the minor components of sugar cane juice to yields of yeast cells and to the carotenoid pigment remains to be established. Urea, a simple and also less expensive source of nitrogen, manifested itself as a productive supplement. The sugar cane juice and urea formulation was further improved by addition of sodium phosphate. Astaxanthin was also produced from disaccharide-rich C supplies, that is, crude maltose or cellobiose as the respective components from depolymerized starches and lignocellulose (bagasse).

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