0273-2289/97/63-65—0305$10.50

# Astaxanthinogenesis in the Yeast *Phaffia rhodozyma*

## Optimization of Low-Cost Culture Media and Yeast Cell-Wall Lysis

JOSÉ D. FONTANA,*[,1] MIRIAM B. CHOCIAL,[2] MADALENA BARON,[1] MANOEL F. GUIMARAES,[1] MARCELO MARASCHIN,[1] CIRANO ULHOA,[3] JOSE A. FLORÊNCIO,[1] AND TANIA M. B. BONFIM[2]

[1]*LQBB-Biomass Chemo Biotechnology Laboratory/Biochemistry/UFPR, P.O. Box 19046 (81531-990), Curitiba-PR-Brazil;* [2]*Pharmacy Course/UFPR; and* [3]*Celular Biology Department, UnB-Brasilia-DF-Brazil*

## ABSTRACT

Astaxanthin is a diketo-dihydroxy-carotenoid produced by *Phaffia rhodozyma*, a basidiomicetous yeast. A low-cost fermentation medium consisting of raw sugarcane juice and urea was developed to exploit the active sucrolytic/urelolytic enzyme apparatus inherent to the yeast. As compared to the beneficial effect of 0.1 g% urea, a ready nitrogen source, mild phosphoric pre inversion of juice sucrose to glucose and fructose, promptly fermentable carbon sources, resulted in smaller benefits. Corn steep liquor (CSL) was found to be a valuable supplement for both yeast biomass yield (9.2 g dry cells/L) and astaxanthin production (1.3 mg/g cells). Distillery effluent (vinace), despite only a slightly positive effect on yeast growth, allowed for the highest pigment productivity (1.9 mg/g cells). Trace amounts of $Ni^2$ (1 mg/L, as a cofactor for urease) resulted in controversial effects, namely, biomass decrease and astaxanthin increase, with no effect on the release (and uptake) of ammonium ion from urea. Since the synthesized astaxanthin is associated with the yeast cell and the pigment requires facilitated release for aquaculture uses (farmed fish meat staining), an investigation of the yeast cell wall was undertaken using detergent-treated cells. The composition of the rigid yeast envelope

*Author to whom all correspondence and reprint requests should be addressed.

was found to be heterogeneous. Its partial acid or enzymatic depolymerization revealed glucose and xylose as common monomeric units of the cell-wall glycopolymers. Yeast cell-wall partial depolymerization with appropriate hydrolases may improve the pigment bioavailability for captive aquatic species and poultry.

**Index Entries:** *Phaffia*; astaxanthin; corn steep liquor; distillery effluent; cell wall.

## INTRODUCTION

The basidiomicetous yeast *Phaffia rhodozyma* has gained an outstanding place in the industrial microbiology market (>100 million US$/year) solely by its peculiar biochemical ability to synthesize the highly oxygenated carotenoid astaxanthin *(1)*.

There are two main applications for this biotechnological product, aquaculture (farmed salmonid meat staining) *(2)* and orthomolecular medicine (as a quencher/scavenger of active oxygen species) *(3,4)*. Our first report on the subject dealt with the convenient and productive utilization of 1:10 diluted raw sugarcane juice and reduced supplementation with urea (0.1 g%) for *P. rhodozyma* growth and astaxanthin production *(5)*. The preceding report focused on a low-cost media improvement using soya meal and tannery shavings as alternative nitrogen sources to urea and enzymolyzed starches or ligno(hemi)cellulosics as disaccharide-enriched carbon sources. These substrates take advantage of the polyvalent fermentative capability of this microrganism *(6)*.

Several recent contributions on the basic and technological aspects of carotenoid production deserve mention. The astaxanthin deposition inside the yeast cell was clarified through cytofluorometry *(7)*. By comparison, a small protein (39 kD) was isolated as the wrapper element for the β-carotene globules observed in the halotolerant alga *Dunalliela bardawil (8)*. Genetic characterization and engineering of natural and cloned carotenoid producers are being examined. The electrophoretic karyotype patterns of three *P. rhodozyma* strains recalled its description as a single genus/species *(9)* and revealed 9–17 chromosomal bands. *Escherichia coli* recombinants were obtained after separated and sequential steps with the insertion of particular genes from *Erwinia* and then from the marine bacteria *Agrobacterium* or *Alcaligenes*. *E. coli*(r) incorporated the biochemical ability for the production of 303–494 μg of canthaxanthin (a diketo-carotenoid)/g cells *(10)*.

Two aspects of *P. rhodozyma* growth and astaxanthin production stand as valid prospects on carotenoid (bio)technology: culture media optimization owing to its alternative respiratory and fermentative metabolism *(1)* and yeast cell (envelope) lysis for the facilitated pigment release. New contributions for both aspects are presented in this article.

## MATERIALS AND METHODS

### Yeast Source, Maintenance, Growth in Shaken Cultures, and Routine Analytical Procedures

All small-scale procedures followed in this article use the guidelines already described in our previous publications *(5,6)*. For inversion, samples of sugarcane juice (1:10 dilution; total sugar content 2.0–2.5 g%) were adjusted to several pH values (4.5–1.5) with aqueous phosphoric acid, boiled for 30 min., and then titrated with aqueous ammonia to adjust the pH to 6.0 prior to yeast inoculation. The catalyst remained thus incorporated in the fermentation broth as ammonium phosphate. The control also received ammonia adjustment until pH 6.0. Corn steep liquor (CSL; 40 g% solids content) was provided by Refinacoes de Milho Brasil (Balsa Nova-PR, Brazil). Novo Nordisk hydrolases (Novozym 234 and SP-299 Mutanase) were a gift from E. Bordin. A *Trichoderma* sp. (ex-Brazilian "Cerrados") enzyme prepared by C. Ulhoa, UnB-Brasilia-DF, Brazil was also used. Gastric juice enzymes from the snail *Megalobulimus paranaguensis* were prepared at LQBB/UFPR.

### Other Analytical Procedures

Ammonium ion, protein, and total carbohydrate were quantified by the Nessler, Coomassie (Bradford), and phenol-sulfuric reagents *(11)*, respectively. Optical and electronic micrography was carried out either in a Nikon/Labophot AFX-II (Tokyo, Japan) or a Philips SEM-505 apparatus (Amsterdam, The Netherlands). For the optical imaging, yeast cells or cell walls were prestained with 0.25% Coomassie blue, followed by destaining with methanol:acetic acid:glycerol:water (46:46:2:6) *(12)*. For scanning micrography, the biomaterial was fixed, buffered, and metalized (glutaraldehyde, $Na^+$ cacodilate, $OsO_4$, gold dust) according the classical technique. Acid hydrolysis of yeast cell walls or fractions was carried out with 2 or 4*M* trifluoroacetic acid (TFA) at 100°C from 1.5–5.0 h. The released sugars were analyzed by thin-layer chromatography (TLC) on silica-gel 60 plates (Merck, Darmstadt, Germany) using isopropanol:ethyl acetate:nitroethane:acetic acid:water (45:25:10:1:19) as mobile phase and staining with sulfuric orcinol or *p*-anisaldehyde *(11)*. Monosaccharide sylil derivatives were resolved by gas-liquid chromatography (GLC) on a HP-5 (Hewlett-Packard, Wilmington, DE) column programmed from 160–260°C (at 10°C/min) with an FID detector and using silylated *p*-nitro-Ø-D-galactopyranoside as an internal standard.

## RESULTS AND DISCUSSION

*P. rhodozyma* growth in 1:10 diluted crude sugarcane juice alone (2.0–2.5 g% sugars) resulted in low biomass yield (<0.2 g cells dry wt/L) and intermediate astaxanthin content (0.5 mg/g cells) (Fig. 1; first bars of

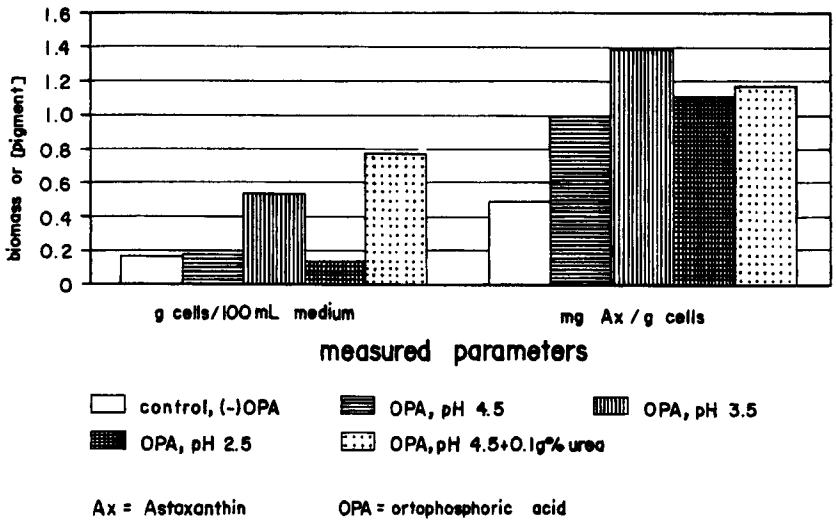


Fig. 1. Effect of OPA-mediated pre-inversion of raw diluted sugarcane on *P. rhodozyma* growth and astaxanthin production, OPA = orthophosphoric acid. All culture media, prior to yeast inoculation for growth at 25°C and 100 rpm for 96 h, were partially neutralized to pH 6.0 with aqueous ammonia. Yeast growth measured as dry wt biomass. Astaxanthin spectrophotometrically quantified at 478 nm.

both parameters). These control values were significantly increased as a result of a previous juice pretreatment, i.e., its mild inversion (sucrose → glucose + fructose) with drops of aqueous phosphoric acid (OPA), followed by a 30-min boiling. Of the pH values tested, best results for promoting growth were obtained at pH 3.5. This corresponded to biomass and astaxanthin yields of 0.54 g cells/L and 1.4 mg of pigment/g cells, respectively (Fig. 1; third bars). Deviation to either milder (pH 4.5) or stronger (pH 2.5) hydrolytic conditions resulted in diminished yields either to biomass or pigment, although a greater amount of reducing sugars was initially released at pH 2.5. Thus, a positive role for the catalyst itself (OPA → ammonium phosphate under neutralization with ammonia) cannot be ruled out. In addition, the supplementation of the mildest hydrolysate (pH 4.5) with 0.1 g% urea (last bars for both measured parameters) as compared to the urea-free control (second bars) implied an increase in both biomass and astaxanthin yields. This may be explained by the lesser amount of ammonia (a nitrogen source) employed for the neutralization of inversion done at pH 4.5. OPA inversion at pH 1.5 led to juice browning and yeast inhibition.

CSL (Fig. 2) was evaluated as an alternative to urea or ammonium phosphate, since the beneficial effect of complex nitrogen sources, like soya bean and tannery shavings proteolyzates (*5*), was already established. With respect to the control (first bars of the two blocks), and considering that all media were adjusted to pH with ammonia, CSL (0.2 mL% addition;

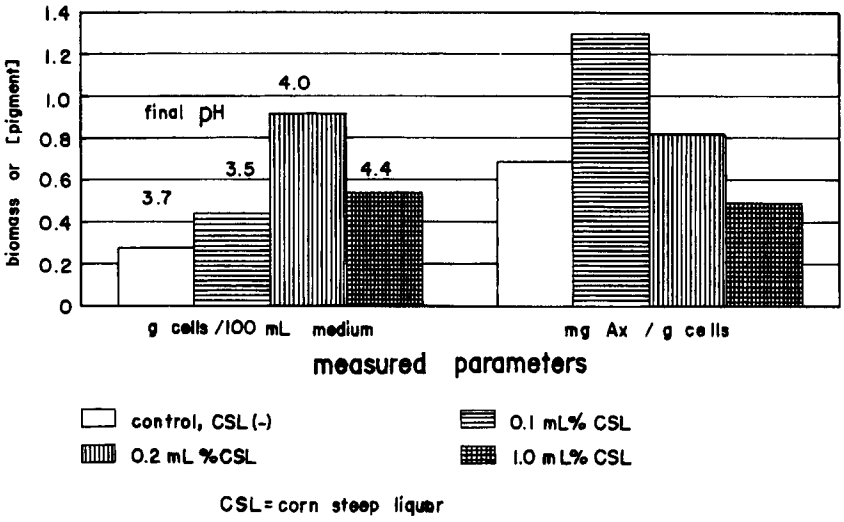


Fig. 2. Effect of CSL supplementation of sugarcane juice on *P. rhozoyma* growth and astaxanthin production (CSL = 40 g% solids; other details as in Fig. 1).

third bars) caused a maximum enhancement of 3.2-fold in biomass (almost 10 g cells/L) and 1.9-fold in astaxanthin (1.3 mg/g cells). Five to 10-fold increase CSL supplementation proved inhibitory for both yields. It may be also pointed out that moderate CSL addition (0.1 → 0.2 mL%) resulted in a progressively better consumption of the juice sugars by the yeast and better values for the biomass dry weights.

Distillery effluent (vinace), which is a major polluting industrial liquid, has found only limited application as a soil cofertilizer ($K^+$ supply for coffee and cane crops). It contains some mevalonic acid, a precursor for isoprenoid biosynthesis *(13)*. Addition of vinace (0.5%, v/v) to raw sugarcane juice resulted in improved astaxanthin productivity (1.9 mg/g cells; third bar, second block, Fig. 3), despite a smaller effect on biomass enhancement (1.6-fold; fourth bar, first block; Fig. 3) as compared to that obtained with CSL.

Nickel may be a bound metallic cofactor for urease. Since this enzyme plays an important role for nitrogen uptake by *Phaffia*, the effect of this cation was investigated in the 1–10 mg/L range. Experiments following the inclusion of dimethyl glyoxime (DMG), (stoichmetric amounts of 4–40 mg/L), an $Ni^{2+}$ chelator *(11)* in the yeast culture media (irrespective the presence or absence of the cation), resulted in growth inhibition for the yeast. However, when $Ni^{2+}$ was administered alone (1 mg/L), the biomass was reduced, but astaxanthin doubled (second bars, both blocks; Fig. 4). Addition of urea (0.1 g%) ensured an apparent better yeast performance for both parameters (third bars, both blocks; Fig. 4). This may be explainable by the effect of urea alone as indicated by the respective control (no $Ni^{2+}$

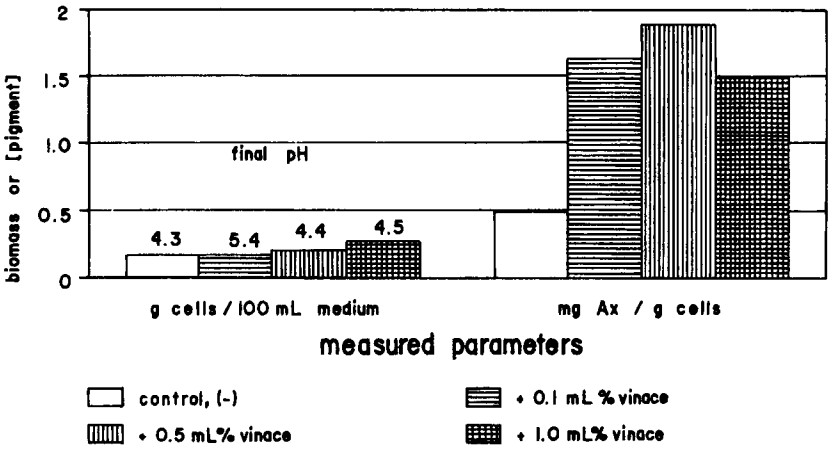


Fig. 3. Effect of distillery effluent (vinace) supplementation of sugarcane juice on *P. rhodozyma* growth and astaxanthin production. (Details as in Fig. 1.)

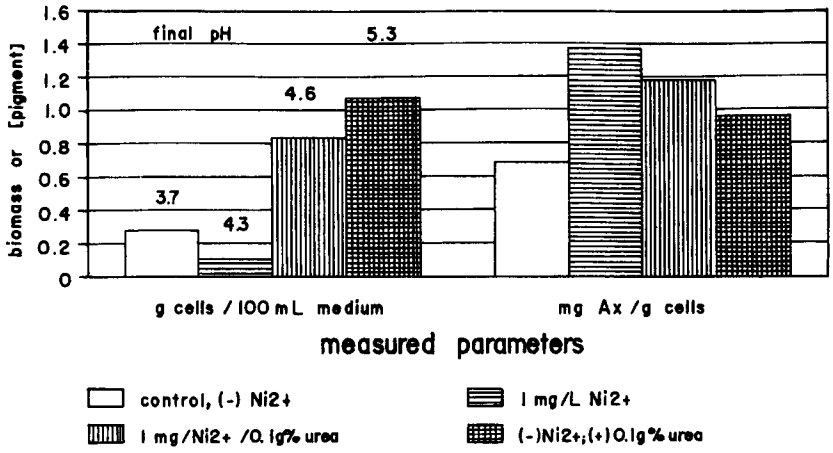


Fig. 4. Effect of $Ni^{2+}$ supplementation of sugarcane juice containing or not containing urea on *P. rhodozyma* growth and astaxanthin production. (Details as in Fig. 1.)

addition; compare the first and last bars in each block; Fig. 4). Urea-derived ammonium ion was monitored (Nessler reagent) in the time-course of the fermentation from 2 until 96 h. There was only a small difference between the experiments with or without 1 mg/L $Ni^{2+}$. Urea hydrolysis and ammonium ion onset started in both cases at 6 h and reached a maximum at 48 h (30.9 mg% $NH4^+$ for the experiment with 0.1% urea alone) and at 72 h (29.3 mg% $NH4^+$ for the experiment with urea + $Ni^{2+}$). Both experiments finished (96 h) with an equivalent free $NH^{4+}$ content in the culture media (17.6 and 17.9 mg%).

*P. rhodozyma* cells had previously proven very resistant to mechanical rupture (French Press or ultrasound procedures *(5)* for the purpose of cell

lysis and invertase/urease release). One gram of yeast cells was rendered free of membrane and cytoplasmatic components (low-mol-wt metabolites, nucleic acids, proteins) through exhaustive washings with the detergent SDS (100 mL 2 g% sodium dodecylsulfate; threefold overnight agitated extractions). The extract was spectrophotometrically monitored at 260 (nucleic acids) and 280 nm (proteins). A reduction from 12–13 OD units (first cycle) to about 1/10 to 1/20 (third cycle) occurred. The aspect of fresh and SDS-washed (and also alkali-treated) cell walls can be seen in Fig. 5A and B. SDS cell walls were then fractionated by sequential 6-h extractions with half-saturated lithium isothiocyanate (a chaotropic agent) (I), and at room temperature (II) or boiling 5*M* KOH (III). All fractions produced polymeric material, which was then precipitated with 3 vol of ethanol. Yields were 125 mg (I), 155 mg (II), 115 mg (III), and final residue (405 mg), representing an 80% recovery. All of them also gave positive reactions for protein (Bradford reagent) and total carbohydrate (sulfuric phenol reagent). The chaotropic extractant yielded the richest protein fraction, and hot alkali produced the richest carbohydrate-containing preparation. The partial strong acid hydrolysis (TFA) of these materials indicated in every instance the dominant presence of xylose and glucose in the hydrolyzates, except for fraction III (hot alkali), which was richer in another hexose(s). GLC analyses (Fig. 6) confirmed the TLC initial data on sugar composition. The fraction arising from room temperature KOH extraction was preliminarily inspected by $^{13}C$-nuclear magnetic resonance (NMR). Low-field signals at 105.1 and 104.5 ppm corresponded to to C-1 of β-D-glucopyranosyl and D-xylopyranosyl rings as nonreducing units (results not shown). Since in the time-course of TFA hydrolysis of whole SDS cell walls from 1.5 to 5.0 h, xylose + glucose were always detected together and in progressive amounts, a xyloglucan is indicated as a component of *P. rhodozyma* cell wall. SDS cell walls were also submitted to enzymatic attack with *Trichoderma* spp. (poly)hydrolases (*see* Materials and Methods; overnight incubation at 35°C). "Cerrados" *Trichoderma* sp. enzyme was more effective for sugar release on the final residue than on the original SDS cell walls. Glucose was found as the major released monosaccharide in all enzymatic incubations. Snail enzyme also released minor amounts of other sugars (mannose and less probably rhamnose). Neither xylose or *N*-acetyl-glucosamine was found by TLC in the enzymic digests. In *Phaffia* SDS cell walls chitin (resistant to TFA hydrolysis) remains to be characterized.

Since residual SDS cell walls remained pink-colored (despite the chaotropic and strong alkali pretreatments), a preliminary supercritical fluid extraction (SFE) was attempted on fresh yeast cells (about 1 mg astaxanthin/g cells) using a homemade steel reactor vessel and thermopressurized $CO_2$ just above the critical conditions for this gas (45°C and 80 atm). The extraction, under this condition, was not efficient. The procedure is being repeated under higher thermopressurization conditions

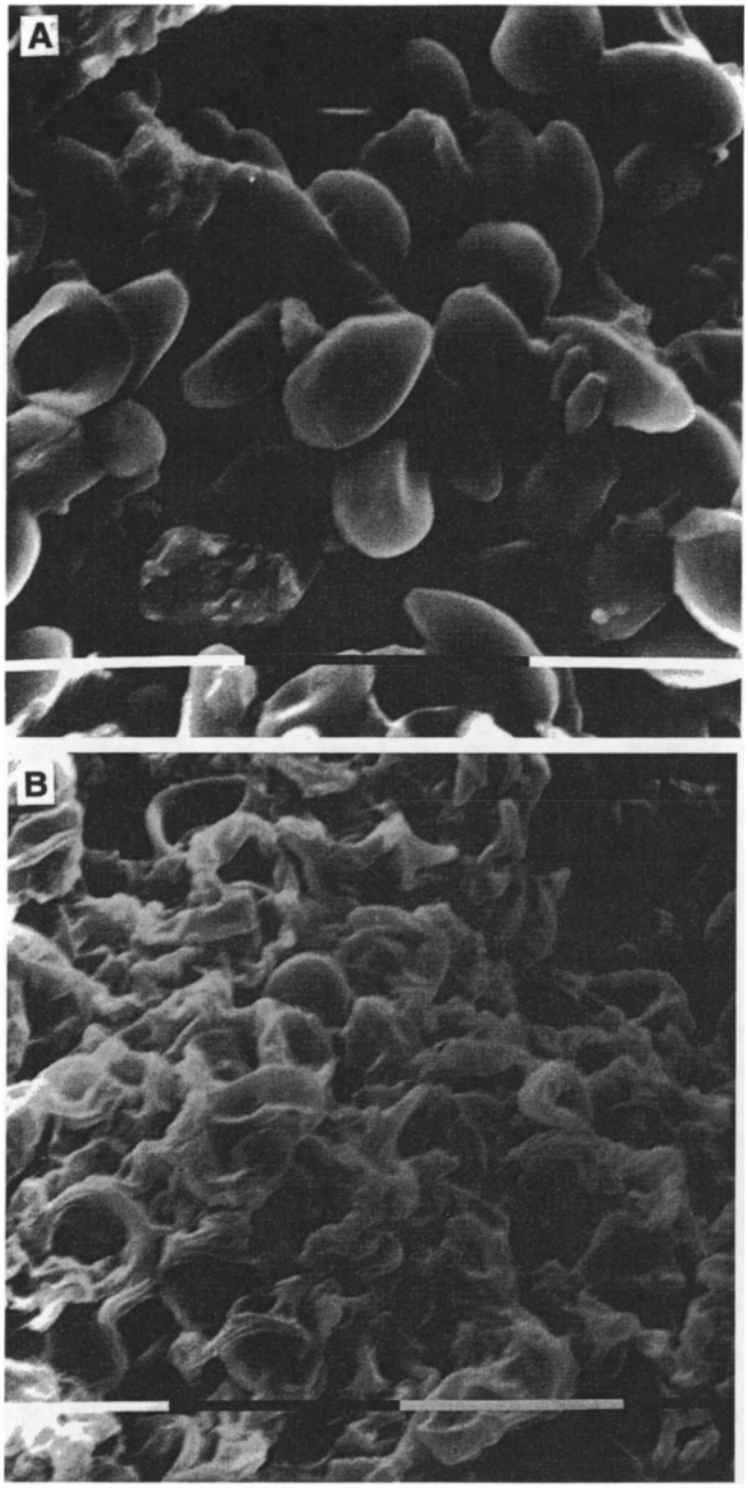


Fig. 5. Scanning micrography for *P. rhodozyma* fresh cells **(A)** and SDS/cold alkali-treated cell walls **(B)** (Nominal magnification: ×11,300).

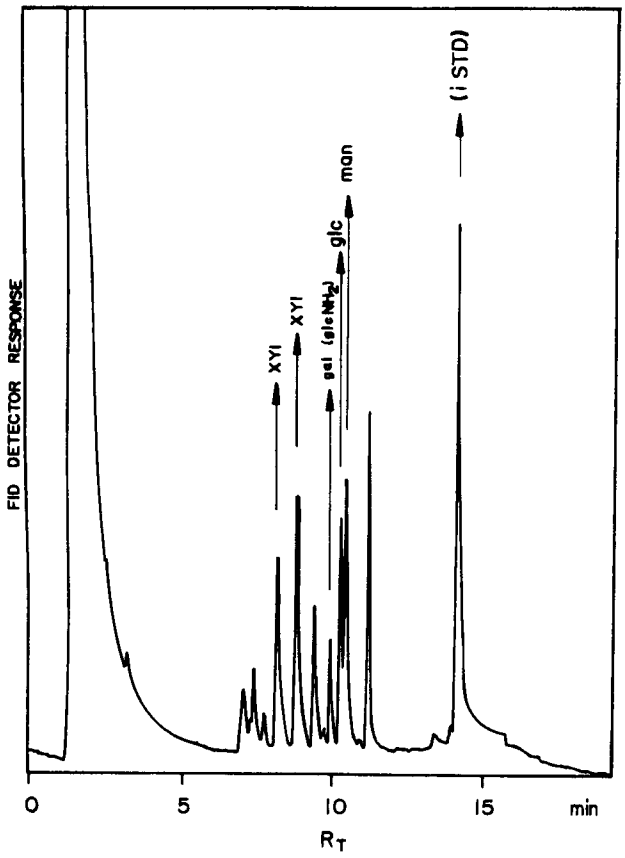


Fig. 6. GLC analysis of the TFA hydrolyzate of *P. rhodozyma* SDS cell walls (3.5 h of 2*M* TFA hydrolysis at 100°C; direct presilylated sugar derivatives; *see* Materials and Methods).

and including organosolvent moderators (e.g., methanol). The results will be published elsewhere.

## CONCLUSIONS

The optimization of low-cost culture media for *P. rhodozyma* growth and astaxanthin production was attained through the addition of two new conutrients, CSL and distillery effluent. The superior sucrolytic/ureolytic activity possessed by this yeast, both genetically and physiologically well expressed, indicated that preinversion of sugarcane juice (sucrose) may not be essential for fermentation improvement. Purified yeast cell walls and their isolated subfractions revealed a heterogeneous nature concerning the polymeric carbohydrate architecture with an apparent dominance of xylosyl and glucosyl units. Cell-wall lysis and facilitated pigment release will require improvements concerning degradative enzyme induction/use or supercritical extraction procedures.

## ACKNOWLEDGMENTS

The authors thank CNPq/PADCT-SBIO and CAPES (Brazilian funding agencies), J. A. Florencio (technical assistance), and M. Lima (drawings).

## REFERENCES

1. Johnson, E. A. (1995), *Stud. Mycology* **38,** 81–90.
2. Starebakken, T. and No, N. K. (1992), *Aquaculture* **100,** 209–229.
3. Yokoyama, A., Izunida, H., and Miki, W. (1994), *Biosci. Biotechnol. Biochem.* **58(10),** 1842–1844.
4. Lambert, C. R. (1995), *Proc. SPIE—Int. Soc. Opt. Eng.* **2391** (Laser-Tissue Interaction VI), 218–224.
5. Fontana, J. D., Chocial, M. B., Baron, M., Guimaraes, M. F., Marines, N. T., Fontana, C. A., Maraschin, M., Ulhoa, C., Florêncio, J. A. and Bonfim, T. M. B. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 413–422.
6. Fontana, J. D., Czeczuga, B., Bonfim, T. M. B., Chocial, M. B., Oliveira, B. H., Guimaraes, and M. F., Baron (1996), *Biores. Technol.* **58(2),** 121–125.
7. Johnson, E. A. (1995), *Proc. 1995 Intl. Chem. Congress of Pacific Basin Soc.* Honolulu, Hawaii, ref. 339 (Agrochemistry).
8. Katz, A., Jimenez, C., and Pick, U. (1995), *Plant Physiol.* **108(4),** 1657–1664.
9. Miller, M. W., Yokoyama, M., and Soneda, M. (1976), *Int. J. Syst. Bacteriol.* **26,** 73.
10. Misawa, N., Kajiwaya, S., Kondo, K., Yokoyama, A., Satomi, Y., Saito, T., Miki, W., and Ohtani, T. (1995), *Biophys. Biochem. Res. Commun.* **209(3),** 867–876.
11. Dawson, R. M. C., Elliot, D. C., and Jones, K. M. (1986), *Data for Biochemical Research,* Clarendon, Oxford.
12. Fontana, J. D., Souza, A. M., Fontana, C. K., Torriani, I., Moreschi, J. C., Gallotti, B. J., Souza, S. J., Narciso, G. P., Bichara, J. A., and Farah, L. F. X. (1990), *Appl. Biochem. Biotechnol.* **24/25,** 253–264.
13. Armstrong, G. A. and Hearst, J. E. (1996), *FASEB J.* **10,** 228–237.