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Production of carotenoids by *Phaffia rhodozyma* grown on media composed of corn wet-milling co-products

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SUMMARY

Natural isolates of the carotenoid-producing yeast *Phaffia rhodozyma* were analyzed for their ability to grow and to produce carotenoids in culture media composed exclusively of co-products of corn wet-milling for fuel ethanol production. Five *P. rhodozyma* strains were tested for biomass produced (dry weight) and carotenoid yield. Six co-products were examined, ranging in cost from approximately $0.02 per kg to $0.11 per kg, all less expensive than conventional or agricultural growth substrates previously tested. The three co-products allowing the greatest accumulation of biomass and carotenoids by *P. rhodozyma* were thin stillage (TS), corn condensed distiller's solubles (CCDS) and corn gluten feed (CGF). Of the medium compositions tested, 10–15% CGF, 70% TS and 6–8% CCDS generally allowed maximum carotenoid production. Cultures grown in these three media produced up to 65%, 148% and 104% of the carotenoid yield per ml of yeast extract/malt extract (YM) cultures, respectively. Under the conditions tested, this was at an approximate medium cost of $0.67 per g carotenoids for CCDS and $0.73 per g for CGF as compared to $385.00 per g for YM. These results indicate that certain co-products of corn wet-milling can serve, at the appropriate concentration, as efficient, economical substrates for growth and carotenoid production by *Phaffia rhodozyma*.

INTRODUCTION

The yeast *Phaffia rhodozyma* produces a number of carotenoids, predominantly astaxanthin [3,12]. Astaxanthin is of value as a supplement for poultry and aquaculture feeds, since the pigment is absorbed in the intestine and deposited in egg yolks and flesh [1,7], imparting the desirable coloration necessary for consumer acceptance. Astaxanthin obtained from natural sources may serve as an attractive alternative to the chemically synthesized pigment. A simple and inexpensive growth medium would reduce the cost of yeast-produced carotenoids and increase the commercial potential of *P. rhodozyma* as a natural source of astaxanthin.

The processing of corn by wet-milling for fuel ethanol production generates several co-products [10] (Fig. 1). These are of proven and potential interest as low cost growth medium components for production of value-added products by microorganisms [8]. Milling kernels yields primarily starch and separates the germ, which is sold for oil extraction and is the most valuable product of the process, and corn fiber (CF), which is largely composed of the seed pericarp, or corn bran. Bran contains approximately 70% xylan, 23% cellulose and 0.1% lignin. Gluten (G), the second most valuable product, is separated from the starch by centrifugation to form gluten wet cake (GWC), dried into gluten, and sold as a chicken feed additive. The starch is then saccharified to glucose, combined with the steepwater as a diluent and a nutritional additive, and fermented by yeast to ethanol. After fermentation, the remaining thin stillage (TS), a major product of the process, containing approximately 2.2% carbohydrate after clarification, is evaporated to form corn condensed distiller's solubles (CCDS). This co-product, which contains approximately 20% carbohydrate, 18% protein, is sprayed onto the CF and fermented to generate corn gluten feed (CGF), which is sold as cattle fodder. These six wet-milling co-products were tested as substrates for growth and carotenoid production by *Phaffia rhodozyma*.

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MATERIALS AND METHODS

Strains

P. rhodozyma strains and their sources are listed in Table 1.

Media and growth conditions

Liquid cultures (5 ml or 50 ml) were inoculated with 0.001 volume of a water-washed 48-h YEPD culture, and were grown in baffled 50-ml or 250-ml flasks for approximately 72 h at 300 r.p.m., 20 °C. Media consisted of YM (1.0% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% Bacto-Peptone; Difco, Detroit, MI, USA), or different corn co-products suspended at selected percentages (w/v) in distilled water, autoclaved, clarified by centrifugation at approximately

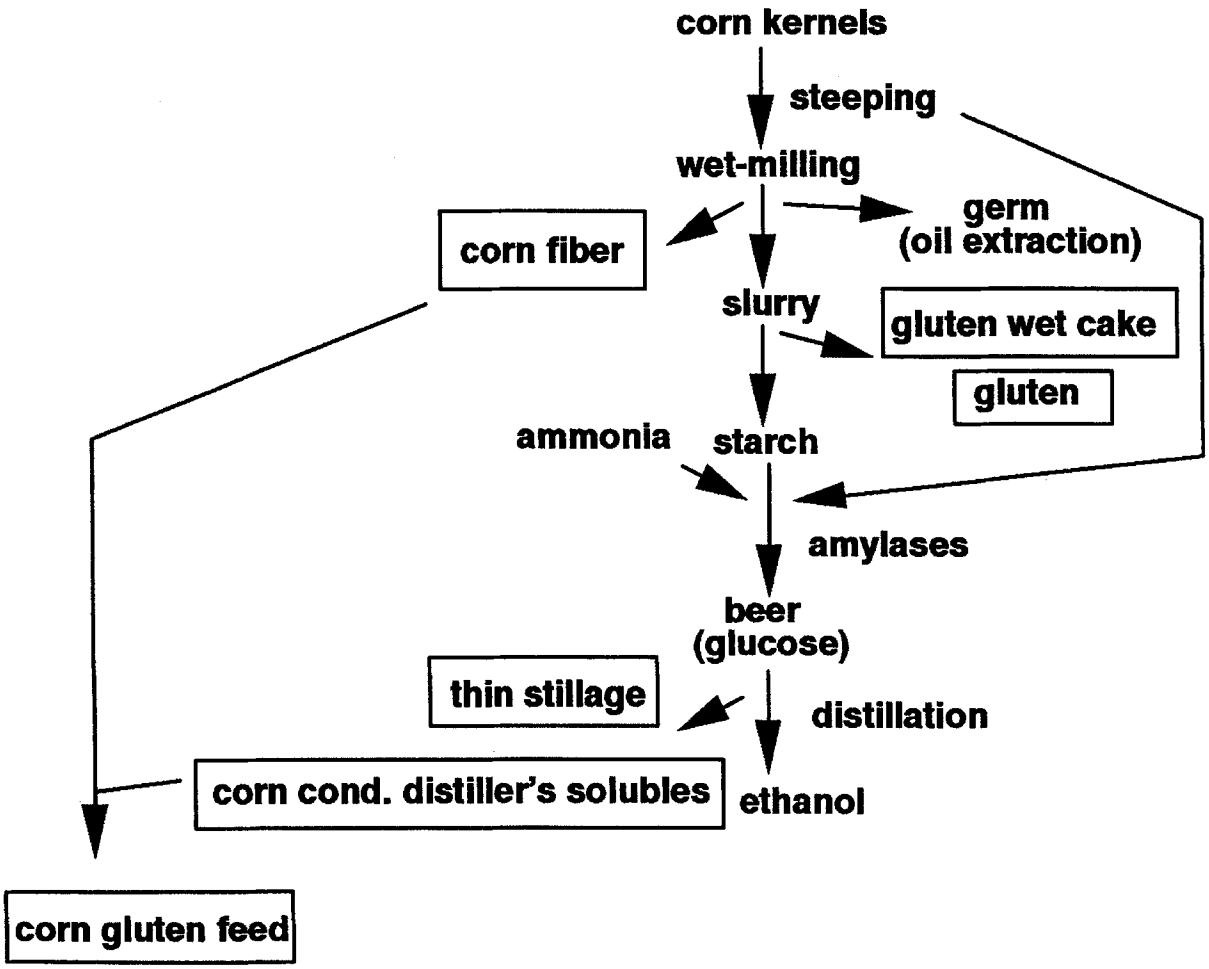

Fig. 1. Flow chart showing origin of co-products generated during corn wet-milling for fuel ethanol production. Co-products used in this study are shown in boxes.

TABLE 1

Phaffia rhodozyma strains used

Strains[a]	Alternate designation	Origin or source
NRRL Y-10921	UCD 67-210	Kyoto, Japan
NRRL Y-10922	UCD 76-18	W.I. Golubev[b]
NRRL Y-17268	BKM Y-2059	W.I. Golubev
NRRL Y-17269	BKM Y-2268	W.I. Golubev
NRRL Y-17270	BKM Y-2273	W.I. Golubev

[a] All strains were obtained for this study from the ARS Culture Collection, USDA, Peoria, IL, USA.
[b] Institute of Biochemistry and Physiology of Microorganisms, Moscow, Russia.

12 000 × *g* for 10 min, and diluted to appropriate concentrations. Corn co-product samples were kindly supplied by Pekin Energy Co., Pekin, IL, USA. Corn gluten feed (CGF) and gluten (G) were stored at room temperature. The remaining components were kept at 4 °C.

Cell yield determination

Cells were harvested by centrifugation and washed once with distilled water. Resuspended cell pellets were transferred to tared preheated aluminum pans and dried overnight in an oven at 110 °C prior to dry weight determination.

Carotenoid extraction and analysis

Carotenoids were extracted from aliquots of cultures essentially as described by Okagbue and Lewis [13]. Cells were extracted three times with 2 N HCl-acetone. After each HCl treatment, the samples were centrifuged and the acid supernatant fluid was removed prior to addition of acetone. After extraction, cell pellets no longer appeared pigmented. Carotenoids in extracted pigments dissolved in acetone were quantitated using a DU-65 spectrophotometer (Beckman, Fullerton, CA, USA), with synthetic astaxanthin (Hoffmann-La Roche, Nutley, NJ, USA), generously supplied by K. Eskins, as the standard. Biomass and carotenoid quantitations are presented as the average of determinations from two or more experiments.

High performance liquid chromatography (HPLC)

Astaxanthin was measured by HPLC essentially as described previously [5], using an Econosphere C_{18} 5-μm column (Alltech Associates, Deerfield, IL, USA). The mobile phase was an aqueous methanol (20:80)/ethyl acetate gradient from 10% to 75% ethyl acetate at a flow rate of 1 ml min^{-1}. Detection was at 476 nm using an ABI Analytical Spectraflow absorbance detector (Kratos Div., ABI, Ramsey, NJ, USA). Synthetic astaxanthin and β-carotene (Hoffmann-La Roche) were used as external standards.

RESULTS

Growth and carotenoid production in media containing different corn co-products

Different corn co-products were tested as medium components for their ability to support growth and carotenoid production of two *P. rhodozyma* isolates, type strain NRRL Y-10921 and a highly pigmented natural isolate, NRRL Y-17270. The co-products used included GWC, G, CF, TS, CCDS and CGF, each tested at 10% (w/v or v/v). Three co-products, TS, CCDS and CGF, were selected for further testing. These three co-products supported the most growth and carotenoid production, and had the lowest levels of insoluble medium components, allowing clearest determination of growth for the *P. rhodozyma* strains tested (data not shown).

Optimization of co-product concentration in growth media

P. rhodozyma strains NRRL Y-10921 and NRRL Y-17270 were grown in media containing different concentrations of the three co-products TS, CCDS and CGF. Accumulated biomass and carotenoid production are shown in Table 2. Maximum yields of biomass and carotenoids were obtained with 70% TS, 6–8% CCDS, and 10–15% CGF.

Carotenoid production by different strains grown in optimized co-product media

Using the optimal co-product concentrations of those examined, as determined in Table 2, wild-type strains of *P. rhodozyma* were tested for growth and pigment accumulation (Fig. 2(A and B)). All strains grew well in the three co-product media tested, with biomass yields usually greater than those obtained in YM medium. Carotenoids were formed in all cultures, with, on the whole, best pigment and biomass production obtained in TS and CCDS co-product cultures of strains NRRL Y-10922 and NRRL Y-17269 (approximately 0.3 μg g^{-1} dry weight in each case).

Accumulation of carotenoids and cell mass over time

Two *Phaffia* strains were analyzed over a 96-h period for biomass and carotenoid production when grown in 6% CCDS (strain NRRL Y-10921) or 8% CCDS (strain NRRL Y-17270). Daily aliquots were removed from cultures, and biomass and

TABLE 2

Effect of co-product concentration on growth and carotenoid production

Media	NRRL Y-10921			NRRL Y-17270		
	mg yeast ml^{-1}	μg astaxanthin ml^{-1}	μg astaxanthin mg^{-1} yeast	mg yeast ml^{-1}	μg astaxanthin ml^{-1}	μg astaxanthin mg^{-1} yeast
YM	4.1 ± 1.8	1.7 ± 0.3	0.4 ± 0.1	6.8 ± 1.9	2.6 ± 0.4	0.4 ± 0.2
% TS						
40	3.4 ± 1.1	1.1 ± 0.01	0.3 ± 0.1	7.7 ± 0.5	1.4 ± 0.1	0.2 ± 0.03
50	4.8 ± 0.3	1.2 ± 0.2	0.3 ± 0.03	9.7 ± 0.5	1.4 ± 0.8	0.2 ± 0.1
60	6.0 ± 0.8	1.8 ± 0.3	0.3 ± 0.1	10.9 ± 2.3	1.8 ± 0.1	0.2 ± 0.1
70	6.9 ± 0.3	1.9 ± 0.2	0.3 ± 0.04	11.5 ± 2.3	2.1 ± 0.3	0.2 ± 0.1
80	7.2 ± 0.04	1.2 ± 0.7	0.2 ± 0.1	10.0 ± 0.7	1.0 ± 0.03	0.1 ± 0.004
% CCDS						
2	1.1 ± 0.4	0.2 ± 0.2	0.2 ± 0.1	1.5 ± 0.1	0.6 ± 0.0	0.4 ± 0.04
4	2.5 ± 0.2	0.6 ± 0.3	0.2 ± 0.1	3.4 ± 0.5	1.2 ± 0.4	0.4 ± 0.2
6	4.5 ± 0.2	1.1 ± 0.5	0.2 ± 0.1	5.6 ± 1.1	1.6 ± 0.9	0.3 ± 0.2
8	3.2 ± 1.4	0.8 ± 0.7	0.3 ± 0.3	7.6 ± 1.6	2.4 ± 0.7	0.3 ± 0.2
10	0.3 ± 0.1	<0.1	—	3.3 ± 2.2	0.7 ± 0.5	0.1 ± 0.1
% CGF						
1	0.1 ± 0.1	<0.1	—	0.6 ± 0.01	<0.1	—
5	3.0 ± 1.0	0.4 ± 0.2	0.1 ± 0.03	3.6 ± 0.04	0.8 ± 0.1	0.2 ± 0.03
10	6.6 ± 1.0	1.1 ± 0.3	0.2 ± 0.02	7.6 ± 0.7	1.3 ± 0.4	0.2 ± 0.04
15	11.9 ± 3.5	0.9 ± 0.4	0.1 ± 0.01	14.5 ± 1.9	1.5 ± 0.5	0.1 ± 0.02

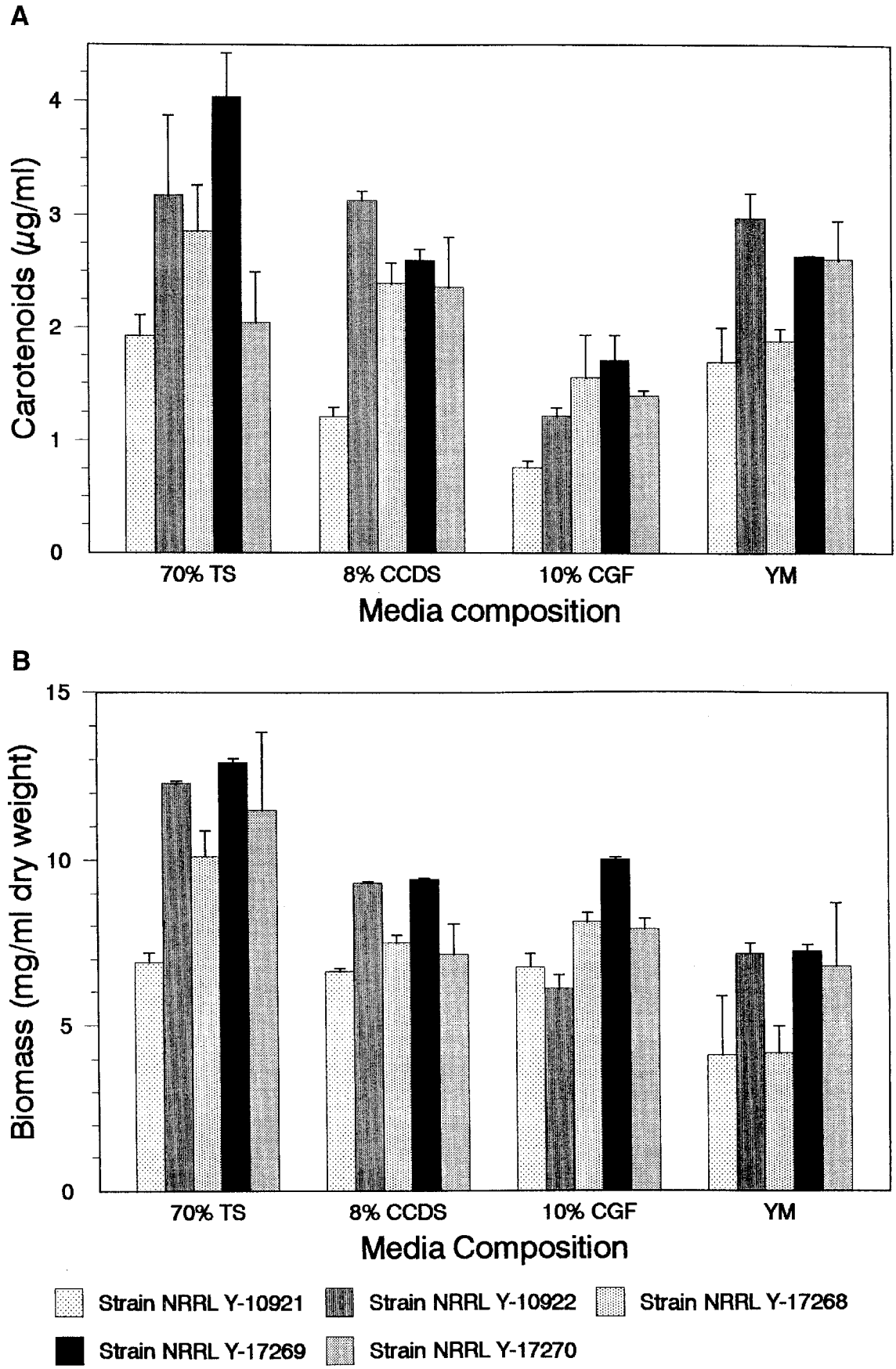

Fig. 2. Biomass and carotenoid yields of selected *P. rhodozyma* strains grown in 70% TS, 8% CCDS and 10% CGF media. Strains were grown and samples analyzed as described in Materials and Methods. Bars indicate standard error.

carotenoid level measurements are shown in Fig. 3. Highest observed carotenoid and cell yields were achieved by 72–96 h.

Effect of co-product batch on carotenoid yield

Variation in composition from batch to batch of a particular corn co-product may affect reproducibility of yields. Therefore the effect of different batches of one co-product, CCDS, on carotenoid production was analyzed. Four different batches of CCDS, produced on different days, were used to make growth media at 8% w/v, the optimal CCDS concentration for carotenoid production as determined using media made from one of the batches. These were inoculated with *P. rhodozyma* strains NRRL Y-10921 and NRRL Y-17270. Carotenoid levels produced in the four different lots of media are shown in Fig. 4. The mean cell and carotenoid yields across the four media lots were 5.1 ± 0.9 (standard error) mg ml^{-1} and 1.0 ± 0.2 μg ml^{-1}, respectively, for *P. rhodozyma* strain NRRL Y-10921, and 5.6 ± 1.0 mg ml^{-1} and 1.8 ± 0.3 μg ml^{-1}, respectively, for strain NRRL Y-17270.

Absorption spectra and HPLC profiles of carotenoids produced in TS, CCDS and CGF media

Acetone extracts of cultures from all five strains, each grown in three different co-product media (10% CGF, 8% CCDS, 70% TS) and YM were analyzed by HPLC. The primary peaks observed were identified as astaxanthin, and a secondary peak that coeluted with β-carotene was occasionally observed. HPLC analysis of uninoculated clarified corn co-product media showed no such peaks (data not shown).

DISCUSSION

Growth and carotenoid production at useful levels by *Phaffia rhodozyma* strains were obtained using simple culture media composed only of inexpensive co-products of corn wet-milling in water. The observation that acids used during extraction have adverse effects on carotenoids [4] may indicate that the carotenoid levels reported here, using a simple HCl-acetone extraction method, are underestimates of the actual levels produced. Previous reports have demonstrated growth of *P. rhodozyma* on other agricultural products, such as grape juice [11], alfalfa residual juice [13] and molasses [6]. Unfortunately, when alfalfa juice was used as a medium component, carotenoid synthesis was inhibited. High levels of biomass and carotenoids were demonstrated using molasses as a carbon and energy source [6], but molasses is a comparatively expensive medium component (approximately \$0.06 per kg) relative to corn co-products used in this study (approximately \$0.02 per kg for CCDS). Also, biomass and pigment levels produced using corn co-product media might be raised by supplementing media with carbohydrates, e.g. from enzymatic digests of other co-products (such as CF) and by adding buffer and ion combinations. Another approach would be to identify and eliminate inhibitory compounds in media containing higher co-product concentrations (Table 2, 80% TS, 10% CCDS, 15% CGF).

P. rhodozyma strain NRRL Y-17270 was originally selected for testing along with the type strain because it appeared highly pigmented. Interestingly, *P. rhodozyma* strains NRRL Y-10922 and NRRL Y-17269 produced higher carotenoid levels in most cases than strain NRRL Y-17270 when tested in optimized co-product media (Fig. 2).

Of the three co-products which supported the highest carotenoid production, TS and CCDS are perhaps of greatest interest, since they supported optimal pigment yields and are the predominant, low cost co-products of fuel ethanol production by corn wet-milling. Since they support good growth and carotenoid production by *P. rhodozyma*, they are most attractive as medium components.

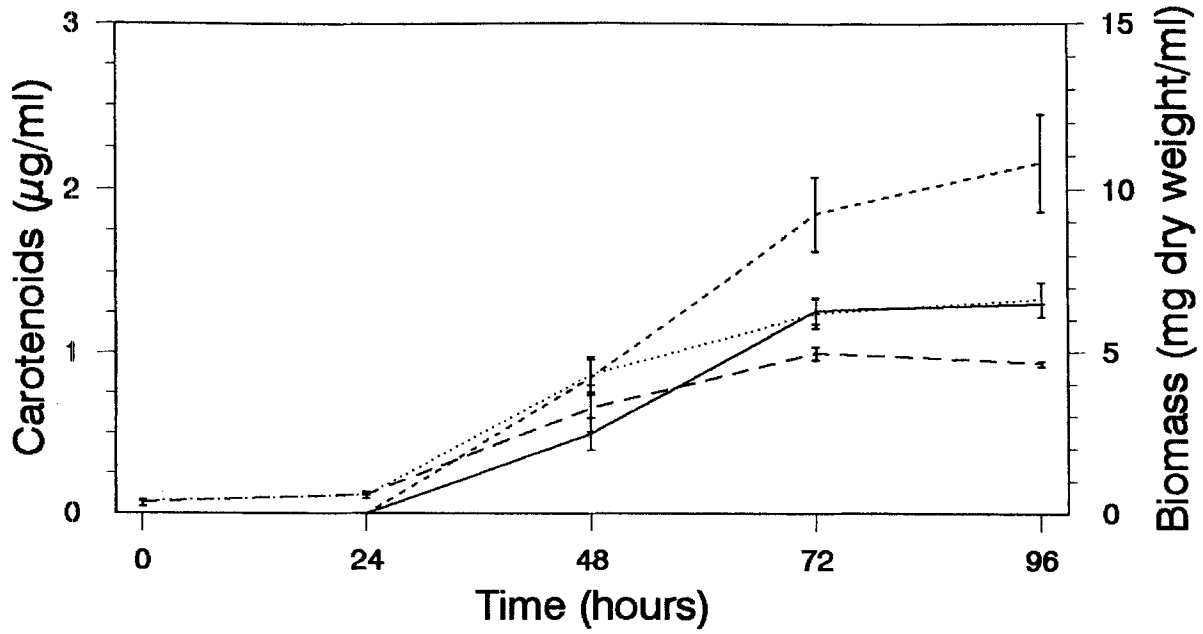

Fig. 3. Carotenoid and cell yields produced by *P. rhodozyma* strains NRRL Y-10921 (carotenoids, solid line; biomass, long-dashed line) and NRRL Y-17270 (carotenoids, short-dashed line; biomass, dotted line) over a 96-h period. Standard error bars are included.

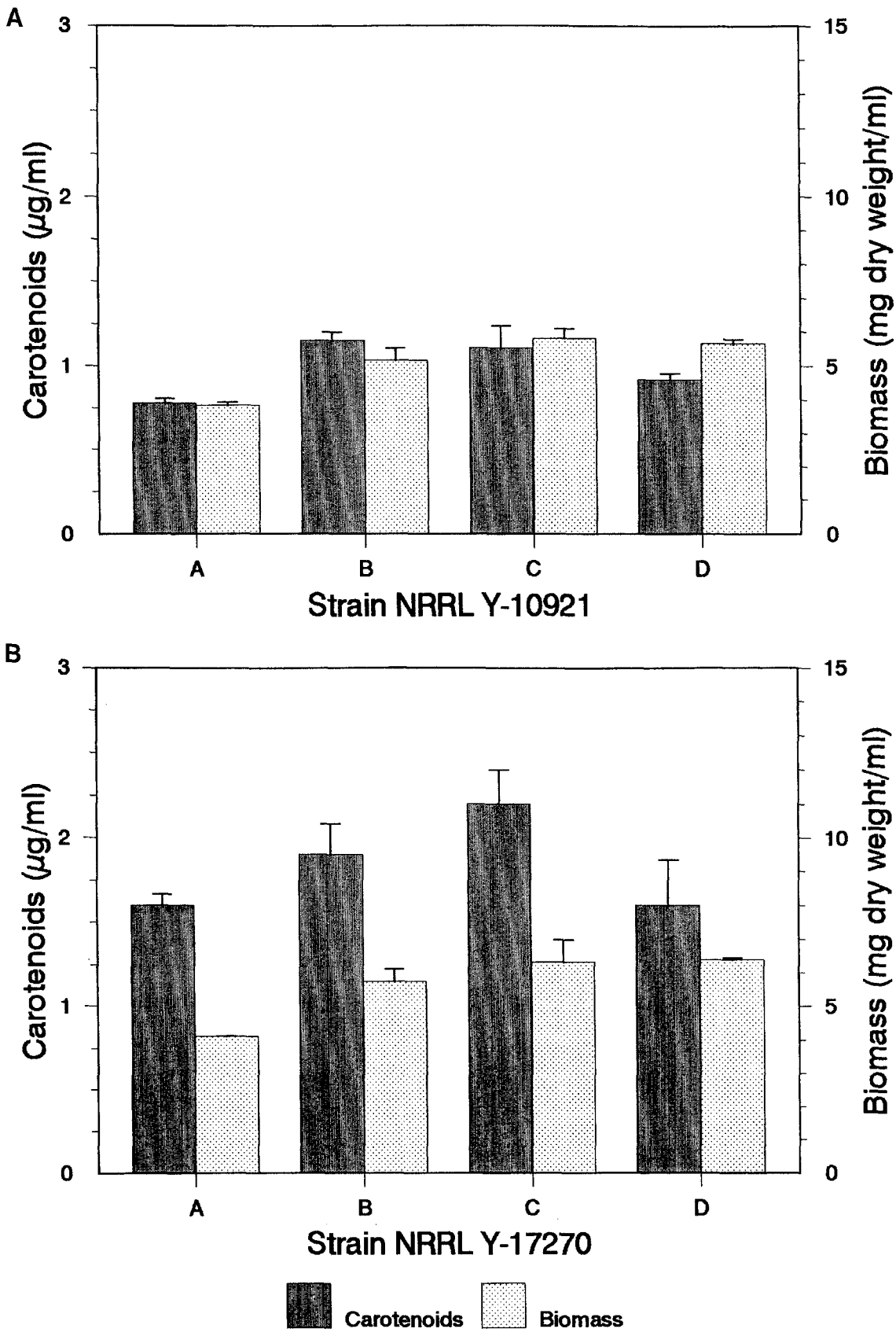

Fig. 4. Growth and carotenoid production of *P. rhodozyma* strains NRRL Y-10921 (A) and NRRL Y-17270 (B) in 8% CCDS media made from different batches of CCDS. Carotenoids, dark shading; biomass, light shading. Bars indicating standard error are shown.

Batch to batch variation of co-products is of concern, as it may affect consistency of carotenoid production. Our analyses of different batches of CCDS indicated that either there is no great batch variation, or it does not appear to impact significantly on growth and carotenoid synthesis.

Analyses by HPLC of cell extracts and growth media provided evidence that the predominant pigment present in the samples was astaxanthin, and that carotenoids were not present in significant amounts in the growth media prior to inoculation.

This study demonstrated production of significant levels of astaxanthin using natural isolates of *P. rhodozyma* grown in clarified corn co-product media. Elimination of the clarification step may further reduce the cost of using such media, although the solids may impede carotenoid extraction or further processing of whole cells. The use of these media to grow mutants that synthesize enhanced levels of carotenoids [2,9] should allow production of even higher pigment levels.

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