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Construction of Astaxanthin Overproducing Strain of *Phaffia rhodozyma* by Protoplast Fusion

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The availability of *Phaffia rhodozyma* as an astaxanthin sources in the aquaculture industry is limited because of the low carotenoid content of natural isolate. In this study, we have used the protoplast fusion technique to construct cell hybrids with an increased content of astaxanthin from *P. rhodozyma*. Cell hybrids (F307 and F406) obtained were very stable and produced considerably more astaxanthin (>1 mg/g yeast) than the wild parent. Karyogamy was confirmed by the isolation of recombinants after mitotic segregation of parental auxotrophic genetic markers, the increased amount of chromosomal DNA/cell and the presence of single nucleus/cell.

Astaxanthin is not only an important source for pigmentation and flavor in the aquaculture industry (1, 2) but also a scavanger of active oxygen radicals (10). The yeast *Phaffia rhodozyma* produces carotenoids containing a high proportion of astaxanthin (1-3), and could potentially provide a biological source of astaxanthin. *P. rhodozyma* is a basidiomycetous yeast, but its sexual cycle has not been demonstrated (11). Protoplast fusion has been used to bypass natural barriers to hybridization of intra- or inter-species, or to genetically improve the industrial yeasts (4, 6, 9, 12, 18). In this study, we have attempted the protoplast fusion between auxotrophic mutants from *Phaffia rhodozyma* to obtain hybrids with increased astaxanthin production.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

Yeast strains used were *Phaffia rhodozyma* CBS 6938 and CBS 5905. Microorganisms were maintained on slants of YPD (3% yeast extract, 3% peptone and 2% glucose) at 4℃. The isolation and induction of auxotrophic or color mutants from *Phaffia rhodozyma* was carried out by the method of Bai *et al.* (4). For analysis of auxotrophs, *P. rhodozyma* was grown in YPD or minimal medium (MM; yeast nitrogen base [YNB, Difco] without amino acids and ammonium sulfate) on shaker (120 rpm). The MM was supplemented as appropriate with amino acids, adenine and uracil. Plate culture was solidified with 2% agar. For pigment production, yeast cells were grown in YM broth (Difco) for 3 days.

Protoplast Formation and Fusion

Protoplast formation and fusion were performed by the methods previously described (4). The yeast cells were grown in 25 m*l* of YPD to an early growth phase (0.2 or 0.3 unit at 610 nm). Two m*l* of cells ($1-2\times10^7$ CFU) were harvested by centrifugation and washed twice with water. The washed cells were treated with 2 m*l* of protoplasting buffer (PB) consisting of 0.1 M Na-succinate buffer (pH 4.5) containing 0.7 M $(NH_4)_2SO_4$ and 0.6 M KCl as osmostabilizer, and 0.1 M 2-mercaptoethanol for 60 min at 22℃. Then, 2~5 mg of Novozym234 (Novo Industri, Denmark) was added and left at 22℃ for 2~3 hrs. Under the above conditions, yield of protoplast ranged from 19 to 81% depending on the mutants types. The protoplasts were washed twice with PB (pH 6.0). Each partner in a 1:1 ratio was mixed in 2 m*l*

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of PB containing 35%-polyethylene glycol(PEG: M.W., 4,000; Sigma) and 0.1 M $CaCl_2$ and left for 45 min. The mixed protoplast cells were centrifuged. Then, the cell pellet was resuspended in 2 *ml* of PB containing 0.1 M $CaCl_2$ to remove excess PEG and followed by plating 0.2 *ml* of samples on stabilized MM agar plates using soft agar overlay technique (17). The plates were incubated for 5 to 7 days. Fusion frequencies ranged from 2.3×10^{-5} to 6.0×10^{-5}. Colonies showing prototrophy on MM agar plate were tooth-picked onto selective medium. After incubation for 5 to upto 7 days, colonies showing prototrophy on MM agar plate were examined.

Analysis of Fusion Hybrid

DNA content per cell was determined for stationary populations grown in MM supplemented with appropriate amino acids. DNA was extracted by a procedure outlined by Stewart (16), and its content was estimated by diphenylamine assay using herring sperm DNA as a standard. The nuclei of yeast cells were stained for log phase populations with Giemsa according to the method of Fournier *et al.* (7). The cell volume was determined using an equation described by Sipiczki and Ferenczy (14). Segregation analysis was performed by plating fusion hybrids grown in YPD broth for 48 hrs onto YPD agar. After 7 days of incubation, colonies were replica-plated onto MM plates to obtain spontaneous segregants. Mitotic segregation was induced by growing hybrids in YPD containing 100 to 600 μg of p-fluorphenylalanine for 48 hrs before plating on YPD agar.

Carotenoid Extraction and Analysis

P. rhodozyma was grown in flasks containing 250 *ml* of YM broth for 3 days. The extraction and quantitation of carotenoids from harvested cells was carried out substantially by the methods of An *et al.* (1). The analysis of individual carotenoids was carried out by thin-layer chromatography (TLC) and electronic absorption spectra (1, 5). Carotenoid extracts were chromatographed by TLC on silica gel plates (Silica Gel 60, 5×20 cm, 0.25 mm thickness: E. Merck AG, Darmstadt, Federal Republic of Germany) with 20% acetone-80% petroleum ether. After development, each band was scraped and eluted in acetone through a pipette plugged with glass wool. Absorbancee maxima (1, 5), Rf values (1, 5), and cochromatography with standards were used for identification of pigments. Carotenoid standards for cochromatography, trans- and cis-astaxanthin, cis-echinenone, hydroxy-echinenone and phoenoxanthin, were donated from Dr E. Widmer (F. Hoffma La Roche Co., Swiss). β-Carotene and r-carotene were obtained from Sigma (U.S.A). Using the method of Davies (5), a kind of carotenoid, phytoene, was isolated and purified from *Phycomyces blakesleeanus* C5 CarB10(−) provided by Prof. Cerda-Olmedo, Sevilla University, Spain, and used as a standard.

RESULTS AND DISCUSSION

Single auxotrophs were isolated from NTG mutagenesis of *Phaffia rhodozyma*. Double auxotrophs from the single mutant isolate were induced by N-methyl-N'-nitro-N-nitrosoguanidine (NTG, Sigma) or ethylmethane sulfonate (EMS, Sigma). One mutant, *trp leu*, contained an increased amount of carotenoid compared with that of the parents. A *arg carA* mutant was chosen for comparison in carotenogenesis. This colorless auxotroph did accumulate about 96% of phytoene, suggesting that it may be blocked in the conversion of phytoene to subsequent biosynthesis intermediates.

A total of 10 cell hybrids were obtained, giving fusion frequencies of 2.3×10^{-5} to 6.0×10^{-5}. These values was very low in *P. rhodozyma* which is in sharp contrast to the frequency of 1.7×10^{-3} obtained from *Schizosaccharomyces pombe* (15). The difference could be in part ascribed to low regeneration of protoplast from *P. rhodozyma* auxotrophs (about 1~2%). This low regeneration rate may have been due to the relatively poor ability of auxotrophic mutants to reconstitute wall, as has been observed in *Candida albicans* mutants (13). Since the concern of this study was the formation of stable hybrids with increased carotenoid, two hybrids (F307 and F406) which displayed deep coloration were chosen. One fusion hybrid (F10) between white and color auxotrophic mutants were also included for comparison. Cell hybrids

Table 1. Wild types, mutants and cell hybrids and their carotenoids

Strains	Genotype[1]	DNA/cell (fg/cell)		Ploidy[2] (N)	Carotenoid[3] (μg/g yeast)
CBS 5905	wild	74.0	0.3	1	370
	lys met[4]	74.0	0.4	1	380
	arg carA[4]	75.0	0.2	1	2630
CBS 6938	wild	77.2	0.2	1	810
	trp leu	79.8	0.4	1	1640
Hybrids					
F10	*lys met* x *arg carA*	151.0	0.4	2.04	470
F307	*lys met* x *trp leu*	148.0	0.5	1.925	1503
F406	*lys met* x *trp leu*	152.0	0.4	1.977	1478

1: Single auxotrophs were induced by NTG mutagenesis; double auxotrophs from single auxotrophs was induced by NTG or EMS; the reversion frequencies were below 2.4×10^{-9}.
2: Ploid of hybrids is based on average of each parents.
3: For composition see Table 3.
4: For these mutants see Reference 10.

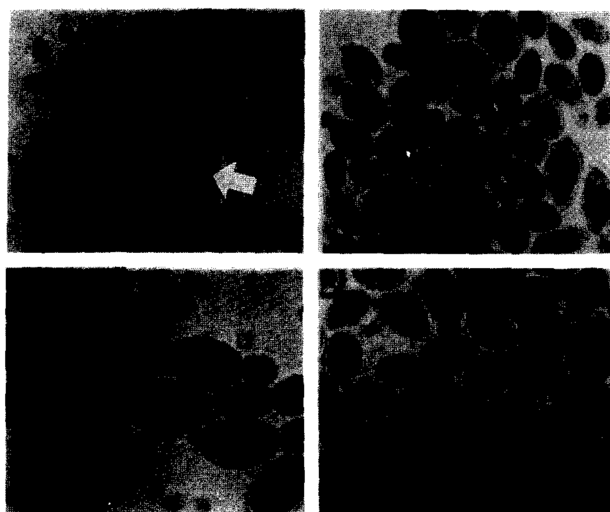

Fig. 1. Photomicroscopy of nuclei of cell hybrids and their parents. Arrows indicate a nucleus (×1,000).
F307 and F406 (*lys met* × *trp leu*)

Table 2. Mitotic segregation analysis of hybrids

Hybrids[5]	Segregants		Frequencies of segregation	
	SMS[1]	IMS[2]	SMS	IMS
F10	*lys*(2) *met*(1) *arg*(2) *lys carA*(1) *carA*(3)	*lys*(21)[3] *carA*(2) *met caeA*(2) *met arg caeA*(1) *lys carA*(10)	1.0×10^{-3} (8623)[4]	4.0×10^{-3} (9255)
F307	*trp leu*(10) *trp lys*(1) *trp met*(1)	*trp leu*(4) trp(1)	2.4×10^{-4} (8325)	7.0×10^{-4} (8560)
F406	*trp leu*(1) *trp met*(1) *trp lys*(1)	*trp met*(1) *trp leu*(3)	2.5×10^{-4} (7893)	1.1×10^{-3} (8923)

1: Spontaneous mitotic segregation.
2: Induced mitotic segregation.
3: Numbers in parentheses indicate auxotrophic segregants appeared.
4: Numbers in parentheses indicate total colonies screened.
5: For parents of hybrids see Table 1.

were characterized with respect to cell size and volume (data not shown). The cell volume of all hybrids was significantly larger than their parents. Fusion hybrids (F 10, F307, F406) doubled the DNA content of their parents (Table 1) and had a nucleus/cell (Fig. 1). Nuclear fusion appears to occur in fusion hybrids as was strongly suggested by the isolation of several auxotrophic recombinants (Table 2). When F10, F307 and F407 were analyzed for spontaneous mitotic segregation after bimonthly transfer for over a year, their segregation frequencies were 1.0×10^{-3}, 2.4×10^{-4} and 2.5×10^{-4}, respectively (Table 2), indicating a marked stability of cell hydrids.

The mutant, *trp leu*, showed a reduction of cell growth on three carbon sources (2% glucose, 2% succinate and

Table 3. Carotenoid composition of *Phaffia rhodozyma* strains

Carotenoids	μg of carotenoids/g yeast							
	CBS 6938		CBS 5905			Hybrids[6]		
	P	*trp leu*	P	*lys met*	*arg carA*	F10	F307	F406
DCD[2]	20	100	17	10	ND[1]	5	110	80
Astaxathin	475	1029	214	168	45	120	1010	1050
Phoenoxanthin	21	23	9	15	ND	10	50	20
HDCO[3]	95	249	38	44	ND	30	210	100
HDC[4]	28	36	ND	ND	ND	35	10	5
Hydroxyechinenone	58	60	35	52	ND	61	60	30
Echinenone	4	8	13	20	ND	45	5	3
γ-carotene	26	8	ND	ND	ND	ND	8	5
β-zeacarotene	19	5	ND	ND	ND	ND	4	3
β-carotene	14	12	24	60	20	160	5	2
Phytoene	ND	ND	ND	ND	2565	ND	ND	ND
Other carotenoid[5]	47	84	15	12	ND	10	20	10
Unknown	3	26	5	ND	ND	4	11	170

1: Not detected. 2: 3,3′-Dihydroxy-3′, 4-didehydro-β, ψ-carotene-4,4′-dione. 3: 3-hydroxy-3′,4-didehydro-β,ψ-carotene-4-one. 4: 4-Hydroxy-3′,4-didehydro-β,ψ-carotene. 5: the most polar and yellow pigment with an absorption peak at 460 nm in acetone. 6: For parents of hybrids see Table 1.

2% ethanol) compared with the parent, and hybrids thereof exhibited similar result (data not shown). Unlike the above growth, this mutant, *trp leu*, was more highly pigmented on each energy source, and two hybrids thereof consistantly produced >1 mg of astaxanthin/g yeast. The increased production of carotenoid in mutants may be associated with the reduction of growth on succinate or ethanol, and the greater susceptibility to antimycin A (data not shown) which may be caused by the alteration in respiratory chain (1, 2). This alteration could produce a more reduced respiratory component for the formation of oxygen radicals capable of promoting carotenoid synthesis (2).

Cell hybrids behaved similarly to their parents with regard to carotenoid composition and were also complementary to carotenogenesis (Table 3), indicating another evidence of cell fusion. F5 obtained between white (*arg carA*) and color (*lys met*) auxotrophs produced less astaxanthin and higher β-carotene compared with its parent (*lys met*) and wild type while F307 and F406 as well as their parents showed an opposite trend. This result suggests that β-carotene might be an important intermediate related to regulation of astaxanthin production.

If other carotenoid hyper-producing strains were available, cell fusion of *Phaffia rhodozyma* would increase efficiency of strain improvement and be preferable to the mutation selection by which carotenoid hyper-producing strains obtained were unstable (8).

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