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Ethanol increases carotenoid production in *Phaffia rhodozyma*

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Addition of ethanol (0.2%) to cultures of the yeast *Phaffia rhodozyma* increased the specific rate of carotenoid production [(carotenoid)(cell mass)$^{-1}$(time)$^{-1}$]. The incremental increase in carotenoid synthesis with ethanol was highest in carotenoid-hyperproducing strains. Ethanol increased carotenoid production when it was added at various points during the lag and active growth phases. Ethanol increased alcohol dehydrogenase and hydroxy-methylglutaryl-CoA (HMG-CoA) reductase activities. Our results indicate that increased carotenoid production by ethanol is associated with induction of HMG-CoA reductase and possibly activation of oxidative metabolism.

Keywords: ethanol; carotenoids; astaxanthin; *Phaffia rhodozyma*

Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is one of the most abundant carotenoids in nature and occurs in certain microorganisms [16] and animals [13] where it imparts vivid pigmentation. Since animals cannot synthesize carotenoids, these must be provided in their diets for deposition into the flesh or carapace. Astaxanthin is valuable commercially mostly for pigmentation of aquacultured salmon. Salmon aquaculture harvest was expected to exceed 400 000 metric tons in 1995 and the corresponding market for astaxanthin as a feed component would be >$80 million [1]. Astaxanthin is important not only as a pigment source in aquaculture [14,16], but also as a potent antioxidant [16] that may delay aging and degenerative diseases in humans and animals. Carotenoids have been proposed to prevent some cancers [6] and to stimulate the immune system [9]. The high antioxidant capacity of astaxanthin could lead to expanded commercial applications.

Astaxanthin is the principal carotenoid in the heterobasidiomycetous yeast *Phaffia rhodozyma*, but commercial production of astaxanthin is limited because of the low content in wild strains [14,15]. Several mutants have been isolated by industrial and academic laboratories that have increased levels of astaxanthin, but the majority of these mutants grow more slowly than the wild strains and accumulate high levels of carotenoid intermediates or byproducts [4]. Various nutritional supplements including valine [20], yeast extract [11], acetic acid [19], and mevalonic acid [8] have been shown to increase carotenoid production in *P. rhodozyma*. Mevalonate significantly increased carotenoid production [8], but is too expensive for use in large-scale fermentations. In this study, we show that addition of low quantities of ethanol increases carotenoid production in *P. rhodozyma* which could provide an economical method for increasing astaxanthin levels in the yeast without lowering growth.

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Materials and methods

Yeast strains and culture conditions

P. rhodozyma strains used in this study were the natural isolate UCD-FST-67-385 [4] and mutants derived from this strain. Mutant 2A2N was obtained by serial nitrosoguanidine mutagenesis of strain 67-385 and antimycin selection [2]. Strain P-5-6 was isolated by manganese-induced selection: strain 2A2N was inoculated into 5 ml of YEP (yeast extract, 0.3%; bactopeptone, 0.5%; glucose, 1%) containing manganous chloride (79 mg ml^{-1}), and incubated for 7 days at 20°C. The yeast culture was diluted with 0.85% NaCl solution, plated on YEP-glucose agar plates, and grown for 7 days at 20°C. Colonies were replicated onto YEP-succinate agar, and the small colonies (impaired in respiratory activity) were screened for carotenoid production. One mutant, P-5, was selected and grown in Schopfer's medium (glucose, 3 g L^{-1}; asparagine, 1 g L^{-1}; KH_2PO_4, 1.5 g L^{-1}; and $MgSO_4 \cdot 7H_2O$, 0.5 g L^{-1}) [27] for 7 days at 20°C. Strain P-5-6 was isolated as a colony that stained weakly after tetrazolium treatment [21] for 3 h indicating respiratory deficiency. Strain Dp-41 was isolated from strain P-5-6 by manganese-induced selection as described above. Yeasts were maintained on YM broth (yeast extract/malt extract/peptone/dextrose medium) (Difco, Detroit, MI, USA) agar slants at 4°C [4] or in 40% glycerol/60% YM broth at −70°C.

P. rhodozyma was grown in 300-ml baffled flasks containing 30 ml YM broth at 20°C with 150 rpm orbital shaking for 5 days. Ethanol (200 proof, USP grade) (Sigma Chemical Co, St Louis, MO, USA) was supplemented to designated incubations. Cell mass was determined by the optical density (660 nm) of a washed cell suspension; 1 mg dry cell weight per ml corresponds to an OD of 1.35 [4].

Carotenoid and enzyme analyses

For quantitative analysis of total yeast carotenoids, yeast cells were harvested by centrifugation, washed with water, and carotenoids extracted by the dimethylsulfoxide

(DMSO) method [26]. One milliliter each of DMSO, acetone, petroleum ether and 20% NaCl solution were serially added to the yeast pellet with vortexing [5]. The upper petroleum ether layer containing the carotenoids was collected and analyzed by thin layer chromatography and electronic absorbance spectroscopy [4].

For measurement of enzyme activities, yeasts from 15 ml of culture were centrifuged for 5 min at $4000 \times g$ and washed with distilled water. Yeasts were suspended in 3 ml of phosphate buffer (0.1 M, pH 7.0) and disrupted by sonication for 6 min (High Intensity Ultrasonic Processor, 600 watt model, Vibra Cell, Sonic & Materials, Danbury, CT, USA) with continued cooling on ice. The yeast homogenate was centrifuged for 4 min at $20\,000 \times g$. The activity of alcohol dehydrogenase and HMG-CoA reductase were measured by the methods of Racker [23] and Qureshi *et al* [22], respectively. All experiments and analyses were conducted in duplicate and replicated at least once, and the means and standard deviations for the data are presented.

Results

Our laboratory previously demonstrated that carotenoid-hyperproducing mutants obtained by antimycin selection were impaired in ethanol utilization [4]. In this study mutants were isolated after manganese treatment and screening for impaired tetrazolium reduction, conditions that select for alterations in respiratory metabolism. The effects of a low concentration (0.2%) of ethanol on growth and carotenoid synthesis were investigated in various strains. Cell mass accumulation was increased by ethanol in the wild-type 67-385 and the parental strain 2A2N, but total carotenoid formation was not appreciably increased (Table 1). In contrast, in the mutants P-5-6 and Dp-41, cell mass was increased compared to the parental strains in the absence of ethanol, and carotenoid formation was increased by the addition of 0.2% ethanol to the medium (Table 1). These results suggest that ethanol increased the flow of carbon precursors to carotenoids in the mutants.

Incremental increases of ethanol concentrations of 0, 0.1, and 0.2% were accompanied by increased carotenoid formation (1420 ± 90, 2010 ± 120, and 2170 ± 130, respectively), and decreased final pH (5.3 ± 0.2, 5.0 ± 0.1, and 4.8 ± 0.1, respectively) but did not appreciably affect growth (4.6 ± 0.2, 4.7 ± 0.1, and 4.8 ± 0.2, respectively). The final pH probably decreased due to the production of organic acids. The effect of addition of ethanol to cultures at various times was examined. Addition of 0.2% ethanol at 0, 24, 48, or 72 h of cultivation did not affect cell mass or carotenoid accumulation (Table 2). Incremental additions of ethanol at 0, 24, and 48 h also did not affect cell mass and carotenoids (Table 2).

Since carotenoid formation was not affected by the time of ethanol addition, it was assumed that ethanol increased carotenoid production as the cells enter into stationary phase (during days 3–5). Therefore, growth and carotenoid formation were measured over time in strain P-5-6. Growth of the mutant was slightly inhibited by ethanol at the beginning of cultivation resulting in a slightly longer lag phase than the control (Figure 1a). Carotenoid formation was continuously enhanced by ethanol but the effect of ethanol was greatest at 3 and 5 days (Figure 1b).

The activities of certain enzymes related to carotenogenesis and alcohol metabolism were assayed to determine if they were affected by ethanol. Ethanol slightly increased the activity of NAD^+-alcohol dehydrogenase (Table 3). The activity of HMG-CoA reductase, a key enzyme for isoprenoid and carotenoid production was increased about three-fold by ethanol (Table 3). The activities of enzymes related to TCA and glyoxalate cycle activity including malate synthase, malate dehydrogenase, isocitrate dehydrogenase, and isocitrate lyase were not significantly affected by ethanol (data not shown).

Discussion

Ethanol has been shown to affect morphology, sexual activity, and metabolic activities of various yeasts and fungi [17]. In humans and other mammals, ethanol oxidation results in alteration in the size of mitochondria, disruption of the cytoplasmic reticulum, and increased formation of reactive oxygen species [17]. Ethanol is oxidized in cells to acetaldehyde by alcohol dehydrogenase or by a cytochrome P-450 enzyme system [17]. Acetaldehyde is known to be catabolized by at least three routes: (a) acetaldehyde + H_2O + O_2 $\rightarrow$acetate + superoxide by aldehyde oxidase; (b) acetaldehyde + $CO_2$$\rightarrow$pyruvate by pyruvate decarboxylase; and (c) acetaldehyde + H_2O + NAD(P)$\rightarrow$NAD(P)H + acetate + H^+ by aldehyde dehydrogenase [17]. The reaction catalyzed by pyruvate decarboxy-

Table 1 Effect of ethanol on growth and carotenogenesis of *P. rhodozyma*

Strain	No ethanol		Ethanol (0.2 %, v/v)	
	Cell mass (g L^{-1})	Carotenoid (μg g^{-1} yeast)	Cell mass (g L^{-1})	Carotenoid (μg g^{-1} yeast)
67-385	3.2	520	5.2	570
2A2N	2.9	1730	4.1	2040
P-5-6	4.3	1790	4.6	2310
Dp-41	4.3	2240	4.3	2800

Yeasts were grown in 300-ml baffled flasks containing 30 ml YM broth at 20°C with 150 rpm orbital shaking for 5 days. The standard deviations for cell mass and carotenoid were 0.3–0.5 g L^{-1} and 60–120 μg g^{-1} yeast, respectively.

Table 2 Effect of ethanol on carotenogenesis of *P. rhodozyma* strain P-5-6 when added at various time points

Time (h)	Ethanol (%, v/v)	Cell mass (g L^{-1})	Carotenoid (μg g^{-1} yeast)
Control	0	4.8	1680
0	0.2	4.8	2190
24	0.2	4.6	2050
48	0.2	4.6	2230
72	0.2	4.7	2140
0, 24, and 48	0.07 each	4.7	2240

The standard deviations for cell mass and carotenoid were 0.1–0.2 g L^{-1} and 60–120 μg g^{-1}, respectively.

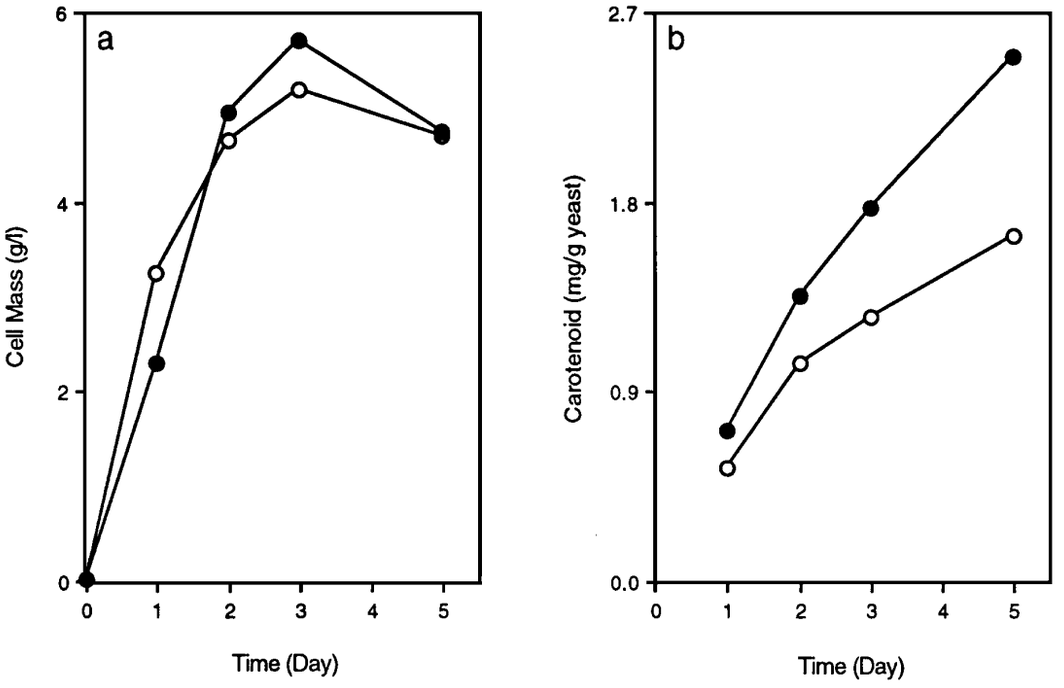

Figure 1 Effect of ethanol on cell mass and carotenoid production of *P. rhodozyma* P-5-6. (a) Cell mass production; and (b) carotenoid production during growth. Symbols: (—●—), growth in the presence of ethanol (0.2%, v/v); and (—○—), growth in the absence of ethanol.

Table 3 Effect of ethanol on induction of alcohol dehydrogenase and HMG-CoA reductase in *P. rhodozyma* strain P-5-6

	No ethanol	Ethanol (0.2%, v/v)
Cell mass (g L^{-1})	4.7 ± 0.1	4.8 ± 0.1
Carotenoid (μg g^{-1} yeast)	1650 ± 160	2490 ± 150
Alcohol dehydrogenase (unit mg^{-1} protein)	42 ± 5	54 ± 8
HMG-CoA reductase (unit mg^{-1} protein)	0.33 ± 0.06	0.91 ± 0.24

lase almost always proceeds in the reverse direction because of thermodynamic constraints. The reaction catalyzed by aldehyde dehydrogenase produces NAD(P)H, which when present in excess may present a redox imbalance, which slows activity of the TCA cycle resulting in respiratory inhibition. Previously, our laboratory demonstrated that respiratory inhibition with accompanying formation of reactive oxygen species increased carotenoid production [3,24,25]. Growth of superoxide dismutatse mutants (Mn-SOD or Cu/Zn-SOD) of *Saccharomyces cerevisiae* was significantly decreased with ethanol compared to glucose as the carbon source [18]. Lowered aeration, however, alleviated the inhibitory activity of ethanol [18]. Therefore, increased carotenoid production by ethanol is probably related to alteration in respiratory metabolism and formation of reactive oxygen species. However, it is also possible that ethanol induces specific enzymes such as P-450 systems, oxidases, or glyoxalate cycle enzymes involved in oxidative metabolism and formation of acetyl-CoA.

The reactions catalyzed by aldehyde oxidase and aldehyde dehydrogenase form acetate as a product. The observed decrease in pH by growth of *P. rhodozyma* with ethanol may have been caused by acetate formation. Addition of acetate to the medium increased carotenoid formation by *P. rhodozyma* [19]. Acetate has been postulated to inhibit enzymes of the glyoxalate bypass resulting in increased levels of acetyl-CoA [17]. However, of various nutrient supplements evaluated, mevalonate has been most effective in enhancing carotenoid accumulation in *P. rhodozyma* [8], but addition to production fermentations is not economical.

It is intriguing in the present study that ethanol increased the activity of HMG-CoA reductase, a key enzyme in isoprenoid biosynthesis. The biosynthesis of ergosterol, an important membrane sterol, was increased by ethanol in *S. cerevisiae* and *S. carlsbergensis* [12]. HMG-CoA reductase in *S. cerevisiae* is subject to feed-back regulation by mevalonate [10], and the enzyme is known to be intricately regulated in mammalian cells [7]. Our results support the conclusion that increased flux through the isoprenoid pathway induced by ethanol probably results in part from deregulation of HMG-CoA reductase.

In conclusion, this study provides evidence that biosynthesis of isoprenoids including astaxanthin and other carotenoids in *P. rhodozyma* is enhanced by ethanol. This method should provide a simple and inexpensive means to increase the yields of astaxanthin in industrial fermentations.

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References

1 An G-H. 1995. Case studies on the potential demand estimation of biotechnological products. MBA thesis, Yunsei University, Seoul, Korea.
2 An G-H, J Bielich, R Auerbach and EA Johnson. 1991. Isolation and characterization of carotenoid hyperproducing mutants of yeast by flow cytometry and cell sorting. Bio/technology 9: 70–73.
3 An G-H and EA Johnson. 1990. Influence of light on growth and pigmentation of the yeast *Phaffia rhodozyma*. Antonie van Leeuvenhoek 57: 191–203.
4 An G-H, DB Schuman and EA Johnson. 1989. Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. Appl Environ Microbiol 55: 116–124.
5 An G-H, C-H Kim, E-S Choi and S-K Rhee. 1996. Medium optimization for cultivation of carotenoid hyperproducing *Phaffia rhodozyma* mutant HT-5FO1C. J Ferment Bioeng 82: 515–518.
6 Bertram JS. 1993. Cancer prevention of carotenoids: mechanistic studies in cultured cells. Ann NY Acad Sci 691: 177–191.
7 Brown MS and KL Goldstein. 1980. Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J Lipid Res 21: 505–517.
8 Calo P, T de Miguel, JB Velazquez and TG Villa. 1995. Mevalonic acid increases trans-astaxanthin and carotenoid biosynthesis in *Phaffia rhodozyma*. Biotechnol Lett 17: 575–578.
9 Chew BP. 1993. Role of carotenoids in the immune response. J Dairy Sci 76: 2804–2811.
10 Dimster-Denk D, MK Thorsness and J Rine. 1994. Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Saccharomyces cerevisiae*. Mol Biol Cell 5: 655–665.
11 Fang TJ and Y-S Cheng. 1993. Improvement of astaxanthin production by *Phaffia rhodozyma* through mutation and optimization of culture conditions. J Ferment Bioeng 75: 466–469.
12 Gal'tsova RD and TS Lyappunova. 1968. Effect of ethanol on biosynthesis of sterols by yeasts. Mikrobiol 37: 672–675.
13 Goodwin TW. 1984. Biochemistry of Carotenoids. vol 2, Animals. Chapman and Hall, London, UK.
14 Johnson EA and G-H An. 1991. Astaxanthin from microbial sources. Crit Rev Biotechnol 11: 297–326.
15 Johnson EA and MJ Lewis. 1979. Astaxanthin formation by the yeast *Phaffia rhodozyma*. J Gen Microbiol 115: 173–183.
16 Johnson EA and WA Schroeder. 1995. Microbial carotenoids. Adv Biochem Eng 53: 119–178.
17 Jones RP. 1989. Biological principles for the effects of ethanol. Enzyme Microb Technol 11: 130–153.
18 Longo VD, EB Gralla and JS Valentine. 1996. Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. J Biol Chem 271: 12275–12280.
19 Meyer PS and JC du Preez. 1993. Effect of acetic acid on astaxanthin production by *Phaffia rhodozyma*, Biotechnol Lett 15: 919–924.
20 Meyer PS, JC du Preez and SG Kilian. 1993. Selection and evaluation of astaxanthin-overproducing mutants of *Phaffia rhodozyma*. World J Microbiol Biotechnol 9: 514–520.
21 Ogur M, RC St John and S Nagai. 1957. Tetrazolium overlay technique for population studies of respiration deficiency in yeast. Nature 125: 928–929.
22 Qureshi N, S Nimmannit and JW Porter. 1981. 3-Hydroxy-3-methylglutaryl-CoA reductase from yeast. Meth Enzymol 71: 455–461.
23 Racker E. 1955. Alcohol dehydrogenase from baker's yeast. Meth Enzymol 1: 500–503.
24 Schroeder WA and EA Johnson. 1995. Carotenoids protect *Phaffia rhodozyma* against singlet oxygen damage. J Ind Microbiol 14: 502–507.
25 Schroeder WA and EA Johnson. 1995. Singlet oxygen and peroxyl radicals regulate carotenoid biosynthesis in *Phaffia rhodozyma*. J Biol Chem 270: 18374–18379.
26 Sedmak JJ, DK Weerasinghe and SO Jolly. 1990. Extraction and quantitation of astaxanthin from *Phaffia rhodozyma*. Biotechnol Tech 4: 107–112.
27 Spencer JFT and DM Spencer. 1988. Yeast genetics. In: Yeast; a Practical Approach (Campbell I and JH Duffus, eds), pp 65–105, IRL Press, Washington DC.