Improvement of Carotenoid-Synthesizing Yeast *Rhodotorula rubra* by Chemical Mutagenesis

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A mutant *Rhodotorula rubra* with enhanced carotenoid-synthesizing activity for synthesizing total carotenoids and β-carotene was obtained by N-methyl-N′-nitro-N-nitrosoguanidine mutagenesis. When co-cultivated with yogurt starter bacteria (*Lactobacillus bulgaricus* + *Streptococcus thermophilus*) in whey ultrafiltrate it produced 15.7 mg total carotenoids l^{-1} culture fluid or 946 μg total carotenoids g^{-1} dry cells of which 71% was β-carotene. Grown as a monoculture in glucose substrate, the mutant shown 1.4 times lower carotenoid-synthesizing activity, and the relative share of β-carotene in the total carotenoids was lower (63%). The individual pigments torulene and torularhodin were identified, whose mass fractions were (29% and 7%) and (24% and 4%), respectively, for the mutant grown as a monoculture and as a mixed culture with the yogurt bacteria.

Key words: Carotenogenesis Improvement, N-methyl-N′-nitro-N-nitrosoguanidine, *Rhodotorula rubra*

Introduction

The best known function of carotenoids, such as α-carotene, β-carotene, β-cryptoxanthin, torulene and torularhodin, is acting as provitamin A. β-Carotene possesses the highest provitamin A activity (Simpson, 1983; Ershov *et al.*, 1992). The application of carotenoids as natural pigments in food and forages is a well-known practice (Nelis and De Leenheer, 1991). Carotenoids, and especially β-carotene, also act as antioxidants by reacting with active oxygen species (Edge *et al.*, 1997) and as anti-carcinogenic agents (Hennekens, 1997).

Several bacteria, fungi and yeasts are good carotenoid producers (Nelis and De Leenheer, 1991) of which the yeast species of *Rhodotorula* and *Phaffia* are well known (Martin *et al.*, 1993a,b; Meyer and Du Preez, 1994; Buzzini and Martini, 1999; Vijayalakshmi *et al.*, 2001). The major carotenoid pigments produced by *Rhodotorula* are β-carotene, torulene and torularhodin in various proportions (Perrier *et al.*, 1995; Buzzini and Martini, 1999). Astaxanthin is produced by *Phaffia rhodozyma* (Martin *et al.*, 1993b; Meyer and Du Preez, 1994). For effective carotenogenesis, of vital importance is the use of: inexpensive alternative carbohydrate sources found in natural substrates, which typically are by-products from various industries and tend to contaminate the environment; and strain-producers of high carotenoid-synthesizing activity.

The present work reports an improvement of *Rhodotorula rubra* to synthesize carotenoids using mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine.

Materials and Methods

Microorganisms and cultivation conditions

Rhodotorula rubra GED8, selected from 10 strains and screened for carotenoid production, was grown in glucose medium. The parent strain *R. rubra* GED8 and its mutants were maintained on YM agar containing (g l^{-1}): glucose (40.0), malt extract (3.0), yeast extract (3.0), pepton (5.0), agar (20.0); pH 5.5. The mutant strains were tested for carotenoid production in YM broth with the aforementioned composition.

Two fermentation media were used from the parent strain *R. rubra* GED8 and the mutant *R. rubra* 56–13 for comparative evaluation of carotenogenesis: 1. Synthetic medium with glucose as a carbon source (45 g l^{-1}) and the following components (g l^{-1}): $(NH_4)_2SO_4$ (6.0), KH_2PO_4 (5.5), Na_2HPO_4 (3.0), $MgSO_4 \cdot H_2O$ (0.5), yeast extract (5.0), pH 5.5; 2. Natural substrate with lactose as a carbon source (45 g l^{-1}) – cheese whey ultrafiltrate (WU) (Frengova *et al.*, 1994) supplemented

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with the components of nutrient medium 1. In the synthetic medium the parent and mutant strains were cultivated as single-strain cultures. In the natural substrate the yeast cultures were grown in association with yogurt starter (*Lactobacillus bulgaricus* 2–11 + *Streptococcus thermophilus* HA15). The monocultures *R. rubra* GED8, *R. rubra* 56–13 and the microbial associations *R. rubra* GED8 + (*L. bulgaricus* 2–11 + *S. thermophilus* HA15), *R. rubra* 56–13 + (*L. bulgaricus* 2–11 + *S. thermophilus* HA15) were grown on a rotary shaker with 220 rpm for 8 d, at 30 °C, in 1000-ml flasks each containing 100 ml of the respective fermentation medium.

The pure yogurt cultures were maintained and transferred on a weekly basis in skim cow's milk and stored at 4 °C. They were associated with a yogurt starter according to a method described earlier (Beshkova *et al.*, 1998). The amount of yogurt starter inoculum for the fermentation medium was 1% (v/v) and its preparation was described in detail in a previous publication (Beshkova *et al.*, 1998).

The inocula of the yeast cultures were grown in 1000-ml Erlenmeyer flasks containing 100 ml culture medium with 2% malt extract, at 30 °C, in the course of 48 h, on a rotary shaker with 220 rpm. The inoculum size for all fermentation was 5% (v/v). The inoculums from each yeast culture (parent and mutant) and from the yogurt starter were introduced in the WU simultaneously.

Mutagenesis

Freshly grown wild type cells were washed twice with sodium citrate (0.1 M, pH 5.5) and suspended to yield a mass fraction of 0.1% (w/v). N-methyl-N′-nitro-N-nitrosoguanidine (NTG) (1 mg ml^{-1} in sodium citrate) was added to the cell suspension (final concentration of NTG, 60 μg ml^{-1}). The treated cells were incubated for 20 min at 30 °C (~ 95% kill). The cells were then harvested by centrifugation and were washed three times with sterile water. The suspension of mutagenically treated cells was appropriately diluted and sprayed onto YM agar. The dishes were incubated for 7 d at 30 °C. The isolated colonies were tested in YM broth for carotenoid production.

Analytical methods

Cell dry weight was determined after heating them at 105 °C to a constant weight. Lactose, glucose, galactose and lactic acid were determined by enzymatic methods as described by Boehringer Mannheim. Extraction of carotenoids from the cells, determination of total carotenoids (spectrophotometrically) and individual carotenoid pigments (by HPLC) were described earlier (Frengova *et al.*, 1994). Data represent the mean values of three independent experiments and standard deviation.

Results and Discussion

The mutagen (NTG) was observed to generate considerable variation in pigmentation among the colonies screened. A large number of colour variants (white, cream, intense pink, brown, pink orange, red orange and yellow orange colonies on plates) were isolated after NTG mutagenesis of *R. rubra* GED8. Such colour variants were never generated spontaneously by the original strain. During the first stage of mutation, 226 large colonies coloured in intensive pink and in orange tinges were selected by visual examination of the isolated colonies. Those isolates were screened spectroscopically for carotenoid production in YM broth. Individual pigments constituting total carotenoids were identified in five mutants with the highest carotenoid-synthesizing activity (Table I). The mutants showed colour instability during continuous cultivation, which required a second stage of NTG mutagenesis of mutant *R. rubra* 56. A second-generation mutant *R. rubra* 56–13 was obtained by appropriate selection as described above. The result of the two-stage NTG mutagenesis and subsequent selection from the parent culture *R. rubra* GED8 was mutant *R. rubra* 56–13, which was able to synthesize 3.4 times more total carotenoids and 8.3 times more β-carotene. The cell biomass synthesizing activity was not influenced significantly (Table I).

The cultivation of the parent culture and mutant as monocultures in glucose synthetic medium and as mixed cultures with yogurt starter (*L. bulgaricus* 2–11 + *S. thermophilus* HA15) in natural lactose substrate (WU) revealed that the maxima of accumulated cell mass and carotenoid formation did not coincide (Fig. 1). The amount of carotenoids in wild type cells and mutant type cells reached a maximum after growth had ended, *i.e.* in the stationary phase of growth of the yeast. The mutagenically treated yeast strain *R. rubra* GED8 did not assimilate lactose but actively synthesized

Table I. Carotenoid production by the parent culture and its mutants.

Organism/mutant	Dry cell mas [g l^{-1}]	Total carotenoids [mg l^{-1} culture fluid]	[μg g^{-1} dry cells]	Proportion (β-carotene:torulene: torularhodin,%)
Rhodotorula rubra GED8	14.3 ± 0.98	2.67 ± 0.28	187 ± 7.00	26:30:42
Mutant 12	12.5 ± 0.62	3.40 ± 0.19	272 ± 7.21	38:49:10
Mutant 38	11.0 ± 0.40	3.71 ± 0.21	338 ± 9.64	33:50:15
Mutant 56	12.0 ± 0.79	5.46 ± 0.14	455 ± 5.00	54:37:8
Mutant 159	11.6 ± 0.56	4.39 ± 0.10	379 ± 8.89	50:40:9
Mutant 204	9.8 ± 0.52	2.99 ± 0.25	306 ± 10.15	46:39:12
Mutant 56–13	12.7 ± 0.30	8.12 ± 0.11	640 ± 8.66	63:29:7

carotenoids when cultivated in synthetic media containing carbon carriers like glucose, galactose and sucrose. Carotenoid synthesis by lactose-negative yeast in lactose substrates can be achieved by providing proper conditions for transforming lactose into carbon carriers (glucose, galactose, lactic acid) that are easily assimilated by yeast (Frengova *et al.*, 1994). The selected mutant 56–13 also was lactose-negative.

In previous studies the microbial association *R. rubra* GED8 + yogurt starter (*L. bulgaricus* 2–11 + *S. thermophilus* HA15) was selected for active synthesis of carotenoids in WU. *R. rubra* GED8 demonstrated higher activity of cell mass production (1.2 times) and carotenoids (1.9 times) in associated cultivation with yogurt starter in the natural substrate than the yeast culture in glucose substrate. That characteristic was also shown by the mutant isolate 56–13. No significant difference in assimilating the carbon substrate by the parent culture or mutant was found during cultivation in either fermentation medium (data not shown). WU lactose was entirely assimilated by the mixed cultures by the 6th day. Yogurt bacteria actively transformed lactose into glucose, galactose and lactic acid. The analyses in the course of the process for glucose and galactose availability showed absence of glucose and galactose, while lactic acid concentrations were 1.4 and 1.1 g l^{-1} for the association with *R. rubra* 56–13 and the association

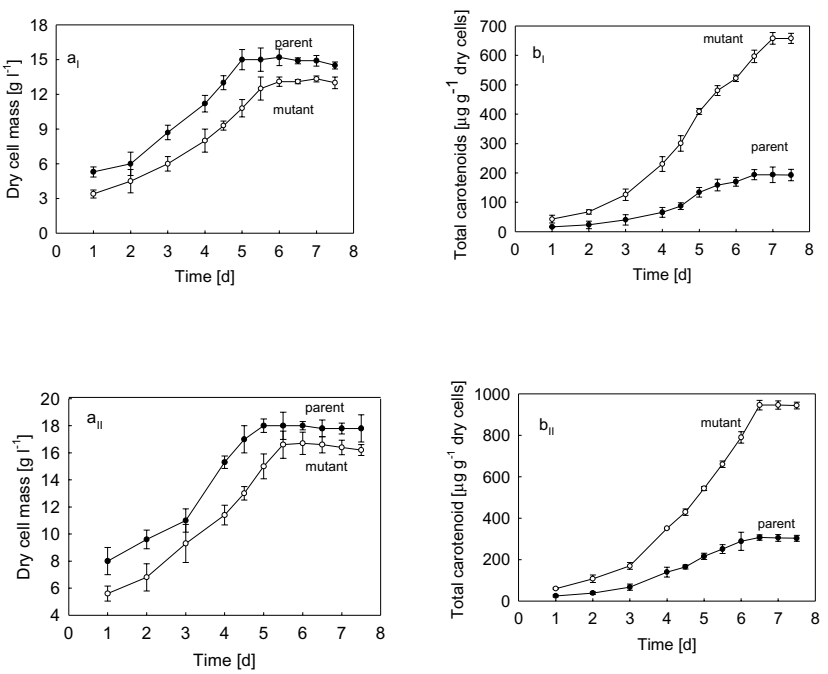

Fig. 1. Growth and carotenoid production by the parent culture *R. rubra* GED8 (●) and mutant *R. rubra* 56–13 (○) in glucose medium ($_{I}$) and in whey ultrafiltrate* ($_{II}$): (a) dry cell mass; (b) total carotenoid. In whey ultrafiltrate, the parent culture and mutant 56–13 were grown in association with a yogurt culture (*L. bulgaricus* 2–11 + *S. thermophilus* HA15).

with *R. rubra* GED8, respectively. Both monosaccharides and lactic acid are easily assimilated substrates for yeast growth and they quickly passed into a phase of exponential growth in a medium, in which the carbon substrate is directly inassimilable. Nearly all glucose from the synthetic medium was assimilated by the monocultures: on the 6th day 2.5 and 2.0 g glucose l^{-1} was recorded for *R. rubra* 56–13 and *R. rubra* GED8, respectively. The lactose-negative mutant *R. rubra* 56–13 manifested high carotenoid-synthesizing activity in associated cultivation with yogurt starter (946 μg carotenoids g^{-1} dry cells): 1.4 times higher than that of the monoculture *R. rubra* 56–13 grown in glucose synthetic medium; 4.7 times higher than that of the mixed culture *R. glutinis* 22P + *L. helveticus* 12A grown in WU (Frengova *et al.*, 1994); 11.3 times higher than the lactose-positive strain *R. lactosa* BKM-1264 cultivated in whey reported in literature (Zalashko, 1990).

The identified individual pigments β-carotene, torulene and torularhodin forming total carotenoids (Table II) are typical of the species of the *Rhodotorula* genus as supported reported by other authors (Perrier *et al.*, 1995; Buzzini and Martini, 1999). The resulting mutant *R. rubra* 56–13 manifested enhanced activity in synthesizing β-carotene during associated cultivation with yogurt starter. That activity significantly exceeded the activity received by us with the microbial association *R. glutinis* 22P + *L. helveticus* 12A (Frengova *et al.*, 1994) and in comparison with the activities given in literature for mutants *R. gracilis* and *Phaffia rhodozyma* (Vijayalakshmi *et al.*, 2001; Girard *et al.*, 1994). Depending on the species specificity of the strain-producer and the cultivation conditions, the authors report diverse data for the amounts and correlations between pigments. According to the biosynthetic pathway for carotenoid synthesis in the yeast *Rhodotorula* proposed by Simpson *et al.* (1964), γ-carotene is the major branch-point and acts as precursor for β-carotene and torulene. Depending on the activity of enzymes β-carotene synthase and torulene synthase, γ-carotene can be transformed into either of the carotenoid pigments. Hydroxylation and oxidation of torulene by mixed function oxydase leads to the formation of torularhodin (Goodwin, 1980). The mutant *R. rubra* 56–13 produced insignificant amount of torularhodin, which can be related to affected oxydase activity. However, no direct correlation was established between the decrease in torularhodin concentration and the increase in β-carotene concentration as the increase in β-carotene was several times higher.

Table II. Concentrations of individual pigments in carotenoids synthesized by the wild strain *Rhodotorula rubra* GED8 and the mutant strain *Rhodotorula rubra* 56–13 grown in glucose and lactose media.

Yeast	Total carotenoids [mg l^{-1} culture fluid]	[μg g^{-1} dry cells]	Proportion (β-carotene:torulene: torularhodin, %)
Rhodotorula rubra GED8*	2.9 ± 0.19	194 ± 5.57	26:30:42
Rhodotorula rubra 56–13*	8.7 ± 0.25	658 ± 7.00	63:29:7
Rhodotorula rubra GED8**	5.5 ± 0.16	305 ± 8.66	33:28:38
Rhodotorula rubra 56–13**	15.7 ± 0.31	946 ± 5.00	71:24:4

* *R. rubra* GED8 and *R. rubra* 56–13 were grown as monocultures in glucose medium.
** *R. rubra* GED8 and *R. rubra* 56–13 were grown as mixed cultures with yogurt bacteria (*L. bulgaricus* 2–11 + *S. thermophilus* HA15).
The concentrations of total carotenoids are the maximum reached on the respective day.

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