

Importance of cell mass and total pigment level-dependant changes in the relative β -carotene proportion for carotenoid production by the yeast variants of the Sporidiobolales

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Abstract

The extent of variation in the composition and amount of carotenoids among a large number of phenotypic variants, induced from a single wild type parent by N-methyl-N'-nitrosoguanidin treatment, has been compared between two strains of the carotenoid-rich, pink yeasts belonging to the order Sporidiobolales (*Rhodotorula dairenensis* Sag 17 and *Rhodospiridium diobovatum* Sea 2-11). We report, for the first time, the weight ratios of monocyclic to dicyclic carotenes in the variants changed differently between those originated from Sag 17 and Sea 2-11, with the decrease of total pigment levels compared to their wild type parents. In contrast, a lack of changes in the relative proportion of β -carotene was apparent for the variants whose total pigment levels are higher than the wild types. Our data clearly showed that the choice of the parent with low level of relative β -carotene content is critical to improve these carotenogenic yeasts by mutagenesis treatment with a view to preferential synthesis of monocyclic pigments having potent antioxidant activities. We further examined the effect of substrate concentration on pigment production by the Sag 17 variants. Use of the variants exhibiting high level of cell mass in the stationary phase was found to be essential for carotenoid production under high substrate concentrations, although they need to be selected based primarily on the pigment contents.

Key words

antioxidant activity, β -carotene proportion, carotenoid species, mutagenesis, Sporidiobolales.

1. Introduction

Dietary carotenoids are recognized as playing an important role in the prevention of human diseases. These terpenoids are a source of vitamin A and excellent oxygen radical scavenger in humans and other animals (Nakano et al., 1999; Rao and Rao, 2007). Apart from astaxanthin-producing *Xanthophylomyces dendrorhous*, several species of the yeasts belonging to the genera *Rhodotorula* and *Rhodospiridium* (of the order Sporidiobolales) synthesize four monocyclic carotenoids (β -zeaxanthin, γ -carotene, torulene, and torularhodin) as well as dicyclic β -carotene as the major pigments (Perrier et al., 1995; Ueno et al., 2011). Of these, torularhodin scavenges peroxy radicals and quenches singlet oxygen more efficiently than β -carotene (Sakaki et al., 2001, 2002).

The members of *Rhodotorula* and *Rhodospiridium* occur in marine environments (Kutty and Philip, 2008). Therefore, the halo-tolerant species of both genera are expected to grow in a low-cost medium consisting of highly concentrated, organic waste and seawater which increase salinity and osmotic pressure in the culture. Since freshwater resources are finite, the use of these yeasts for carotenoid production would contribute not only to reducing the cost of cultivation but also to avoiding the depletion of freshwater resources by culturing them in media prepared with seawater.

Only a few studies have examined the levels of carotenoid production by the phenotypic variants induced from the Sporidiobolales strains (*Rhodotorula* spp.) by mutagenesis treatments, i.e., those derived from the wild type *R. glutinus* and *R. mucilaginosus* by UV irradiation and N-methyl-N'-nitrosoguanidin treatment, respectively (Bhosale and Gadre, 2001; Frenogova et al., 2004). However, the extent of variation in the composition and amount of carotenoids among a large number of variants, induced from a single wild type strain, has not been compared between organisms of different yeast species. There is a need for selection of the parent strains suitable for induction of variants which synthesize large amounts of particular carotenoid species with strong antioxidative activities. Therefore, it would be intriguing if composition or amount of the pigments produced by the UV or chemically-induced variants differs markedly between those derived from different parents. Likewise, several reports have described the effects of temperature (Simpson et al. 1964), carbohydrate source (Buzzini and Martini, 1999), white light (Sakaki et al., 2001), active oxygen species (Sakaki et al., 2002), aeration (Simova et al., 2003), co-cultivation with yogurt bacteria (Frenogova et al., 2004; Simova et al., 2003), and salinity (Ueno et al., 2011) on carotenoid production by the yeasts of the order Sporidiobolales. But we are not aware of any reports on the pigmentation of those yeasts (and variants) under high substrate concentrations.

Considering these situations, the primary objective of this work is to compare two groups of phenotypic variants, one originated from *Rhodotorula dairenensis* and the other from

Rhodospiridium diobovatum, induced by chemical mutagenesis based on the yields of biomass, total carotenoids, and β -carotene. We further examined the effect of substrate concentration on pigment production by the selected variants of *R. dairenensis*.

2. Materials and Methods

2.1 Microorganisms

Rhodotorula dairenensis Sag 17 and *Rhodospiridium diobovatum* Sea 2-11 were selected based on abundance of total carotenoids from the yeasts isolated from aquatic environments; both strains had the abilities to produce β -carotene, torularhodin, γ -carotene, torulene, and β -zeacarotene (Ueno et al., 2011). The cultures were maintained on YPD agar plates (2 % glucose, 2 % peptone, 1 % yeast extract, and 2 % agar, pH 6.2) at 25 °C.

2.2 Mutagenesis treatment

To induce phenotypic variants, we modified the method for chemical mutagenesis of *Xanthophyllomyces dendrorhous* (An et al., 1989). Cells harvested from the early stationary phase cultures of the wild type strains (Sag 17 and Sea 2-11) grown in YPD liquid medium (2 % glucose, 2 % peptone, 1 % yeast extract) were washed twice with sterilized de-ionized water and suspended in 9.8 ml of 50 mM Tris-maleate buffer (pH 6.5) to yield a mass fraction of 0.1 % (w/v). A 200- μ l aliquot of N-methyl-N'-nitrosoguanidin (NTG) solution (5 mg/ml) was added to the cell suspension to give a final concentration of 100 μ g/ml. The treated cells were incubated for 20 min at 25 °C with reciprocal shaking at 120 rpm, which resulted in a survival rate of less than 5 %. The cells were pelleted by centrifugation and washed twice with sterile water. After serial dilution with sterile water, the cell suspension (treated with NTG) was spread onto YPD agar plates. The plates were incubated at 25 °C for 5 d. The isolated colonies were examined for their colors and abilities to produce carotenoids by the following method.

2.3 Analyses of carotenoids

To initiate the cultures for the analyses of microbial carotenoids, a loopful of cells taken from a 5-d-old plate culture was inoculated into a test tube containing 10 ml YPD liquid medium. The tube was incubated at 25 °C with reciprocal shaking at 120 rpm for 36 h until the early stationary phase was reached. A 200- μ l aliquot of this culture was inoculated into a 500-ml flask containing 100 ml of YPD; the culture was incubated at 25 °C with reciprocal shaking at 120 rpm for 144 h until the stationary phase was reached. The cells were washed twice and resuspended in sterilized de-ionized water and lyophilized. Carotenoids were extracted by the method of Sedmak et al. (1990), with modifications. A centrifuge tube containing the freeze-dried cells (0.1 g) was supplied with 2 ml dimethyl sul-

foxide (DMSO), vortex agitated, incubated at 55 °C for 15 min, and subjected to sonication for 10 min. Subsequently, 3 ml of acetone : petroleum ether: 20 % NaCl (1:1:1, v/v) was added to the homogenates to partition the carotenoids into a layer of organic solvent. The tube was vortex agitated for 1 min and centrifuged at 1,200 \times g for 20 min to separate the aqueous and organic phases. The organic phase containing total carotenoid was transferred to a clean tube, and then the solvent was evaporated under nitrogen and stored in the dark at -20 °C. Total carotenoid content was estimated by spectrophotometry in petroleum ether (Davies, 1976).

Dried pigments were prepared for each of the variants as well as their parents, and were dissolved in 1 ml of methyl t-butyl ether, (MTBE). A 100- μ l aliquot of this solution was diluted 10-fold by adding 900 μ l of fresh MTBE, and filtered with a 0.2- μ m Teflon membrane prior to HPLC analyses. The HPLC system consisted of a pump (Shodex DS-3, Showa Denko K. K., Tokyo, Japan), a three-line degasser (DG-880-50, Jasco International, Tokyo, Japan), a ternary gradient unit (LG-2080-02, Jasco International), a HPLC mixer (MX-2080-32 dynamic mixer, Jasco International), a column oven (Shodex AO-50, Showa Denko K. K.), and a UV-VIS detector (L-7420, HITACHI, Tokyo, Japan). Chromatographic analysis was performed using an analytical scale (250 mm \times 4.6 mm i.d.) C30 reverse phase column with a particle size of 5 μ m (YMC carotenoid C30 S-5 column, YMC, Wilmington, North Carolina). The HPLC conditions were as follows: eluent A consisted of methanol, MTBE, and water (81:15:4, v/v); eluent B was prepared by mixing the same components at a ratio of 6:90:4. Individual carotenoids were separated by a linear gradient from 100 % A to 100 % B for 90 min at a flow rate of 1 ml/min. The compounds separated by HPLC were recovered in separate test tubes and dried with nitrogen. The isolated pigments were dissolved in four different solvents (ethanol, chloroform, hexane, and acetone). Their electronic absorption spectra were recorded using a spectrophotometer (UV-2400 PC, Shimadzu, Kyoto, Japan). Apart from HPLC, TLC on silica gel plates (Silica G 60, 10 \times 20 cm, 0.25 mm thickness; Wako, Tokyo) was performed with solvent A or B to separate the major pigments. Solvent A consisted of petroleum ether and toluene (1:9 v/v), and solvent B was prepared by mixing methanol, acetone, and toluene at a ratio of 5:20:75. By the method of Perrier et al. (1995), carotenoid species of the pigments isolated by HPLC were tentatively determined based on the absorption maxima (and shoulders) obtained from their spectral data in the aforementioned four solvents and the relative R_f (as compared with β -carotene) of the spots separated by TLC. Commercial β -carotene (Sigma Chemicals, St. Louis, Missouri) was used as a reference in TLC analysis and to calibrate the HPLC quantification of this compound.

2.4 Classification of yeasts based on abundance of total pigments and β -carotene

We classified the color variants based on abundance of total pigments and β -carotene using a following scheme. All of the permanently culturable variants induced from a single parent strain were classified by the total carotenoid levels. Next the variants, belonging to each of the groups defined by the total pigment levels, were further divided into four sub-groups based on relative abundance of β -carotene (to total pigments) in individual variants. Subsequently, the averaged values of the relative β -carotene proportion in individual variants were compared between the groups defined by the total carotenoid contents.

2.5 Effects of substrate concentration on production of carotenoids

The effect of substrate concentration on growth and carotenoid production was investigated using the variants induced from *Rhodotorula dairenensis* Sag 17. In this study, the amount of pigments contained in one gram of dry yeast cells is described as 'specific carotenoid production' ($\mu\text{g/g}$ dry cell), while that contained in one liter of culture broth is referred to as 'absolute carotenoid production' (mg/l). The variants used were selected based on the specific pigment levels. Cells were cultured in a 500-ml conical flask containing 100 ml of YPD supplied with different concentrations of glucose (1, 3, 5, and 8 % w/v) at 25 °C with reciprocal shaking at 120 rpm for up to 170 h. Cultures were grown in triplicate for each of the individual substrate concentrations. YPD contained 2% glucose unless specified in the text. An additional growth test was performed using Sag 17 and its pigment-rich variants in 100 ml of YPD at 25 °C with reciprocal shaking at 120 rpm for up to 192 h to classify the variants by their growth rate. Growth was monitored by measuring optical densities at 660 nm. Analytical methods followed those for microbial pigments described above.

3. Results and discussion

3.1 Classification of yeasts based on abundance of total pigments and β -carotene

Considerable color variation was observed among the colonies formed on YPD agar plates after mutagenesis treatment of the cells with NTG, i.e., red, deep pink, orange, yellow, tan, and white. Following several cycles of regular transfer on YPD plates, we chose phenotypic variants based on the colors of colonies by visual inspection which could not be observed for the wild type. Consequently, 33 and 26 color variants were collected for *Rhodotorula dairenensis* Sag 17 and *Rhodospiridium diobovatum* Sea 2-11, respectively. The HPLC and TLC analyses revealed differences in the composition and abundance of carotenoids between individual variants (e.g., Figure 1); some of them completely lost the ability to synthesize the pigments.

As for the wild type parents, the specific and absolute pro-

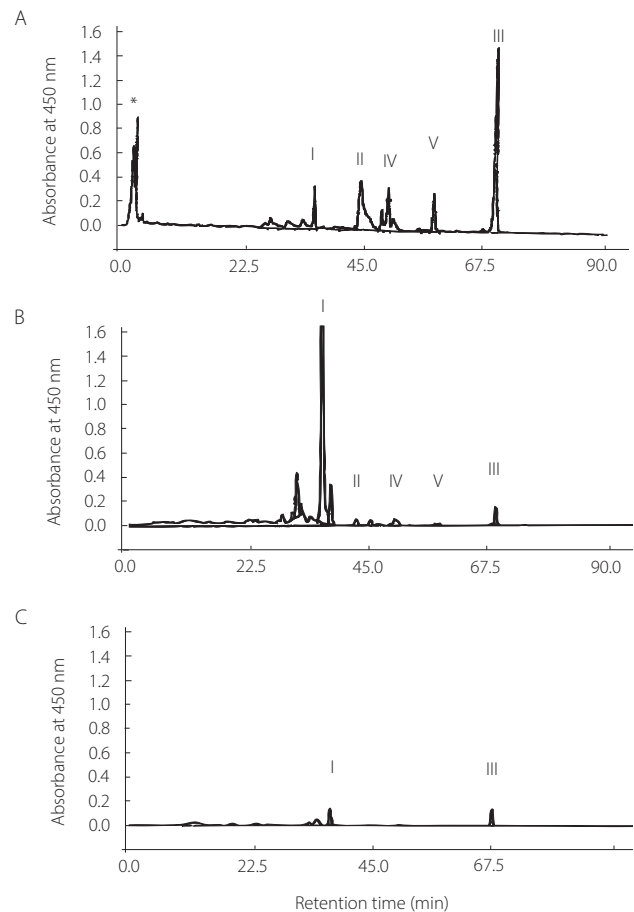


Figure 1: Typical HPLC chromatograms of a pigment extract from the variants forming a red colony (A), a yellow colony (B), and a pale orange colony (C) induced from Sea 2-11. (I), β -carotene. (II), torularhodin. (III), γ -carotene. (IV), torulene. (V), β -zeacarotene. (*), solvent.

duction levels of total pigments produced by Sag 17 (Sea 2-11) were respectively 730 (620) $\mu\text{g/g}$ dry cells and 9.49 (7.07) mg/l , with the relative β -carotene proportion of 82.2 (63.8) %. We compared the relative proportion of β -carotene in total carotenoids (%) between the groups of variants that were classified by the levels of total carotenoids (Figures 2 and 3). When the variants of *Rhodotorula dairenensis* Sag 17 were classified by the specific production levels of total carotenoids, the relative proportion of β -carotene increased with an increase of total carotenoid levels in individual variants (Figure 2, panels A and B). The same result was obtained when these variants were reclassified based on the absolute production levels of total carotenoids (Figure 2, panels C and D). In contrast to the situation with Sag 17, there was a negative correlation between the relative proportion of β -carotene and the level of total carotenoids when the variants induced from another parent *Rhodospiridium diobovatum* Sea 2-11 were examined (Figure 3).

The genes responsible for formation of terminal β -ionone ring to form β -carotene from γ -carotene may have been mu-

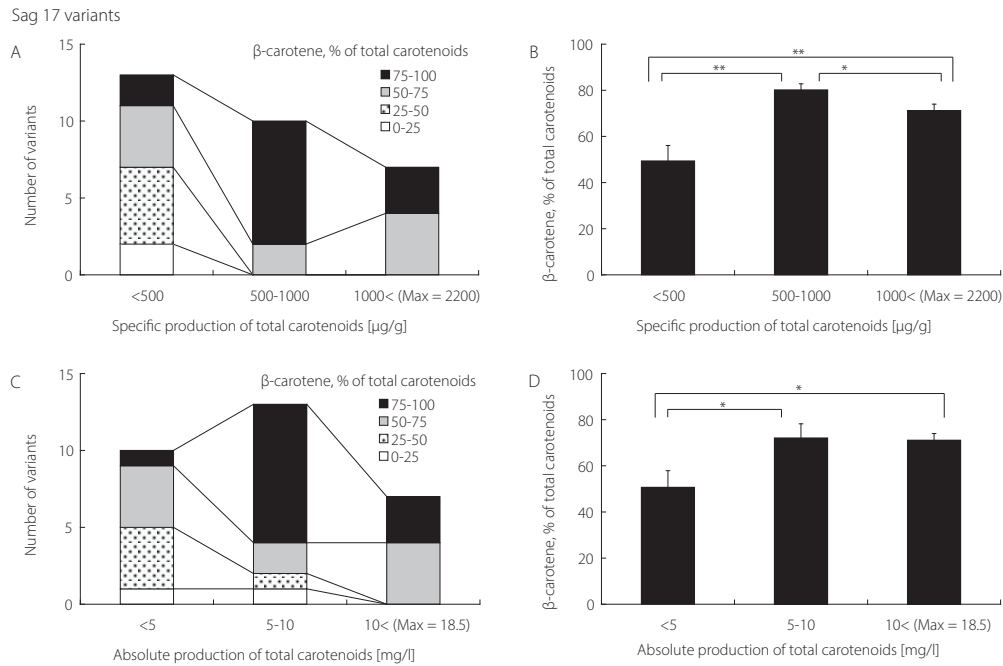


Figure 2: The relative proportion of β -carotene in total carotenoids compared between the groups of *Rhodotorula dairenensis* Sag 17 variants classified by the specific (A and B) and absolute (C and D) production levels of total carotenoids. (B and D) Values are expressed as mean of variants belonging to each of the groups classified by the amount of total pigments \pm SE (see panels A and C for numbers of the variants used in the statistical analyses). Colorless variants as well as parent Sag 17 were excluded from the analyses. The statistical analyses were performed using Student's t-test. Asterisks indicate significant difference between the groups of variants classified based on abundance of total carotenoids. * $P < 0.02$; ** $P < 0.01$.

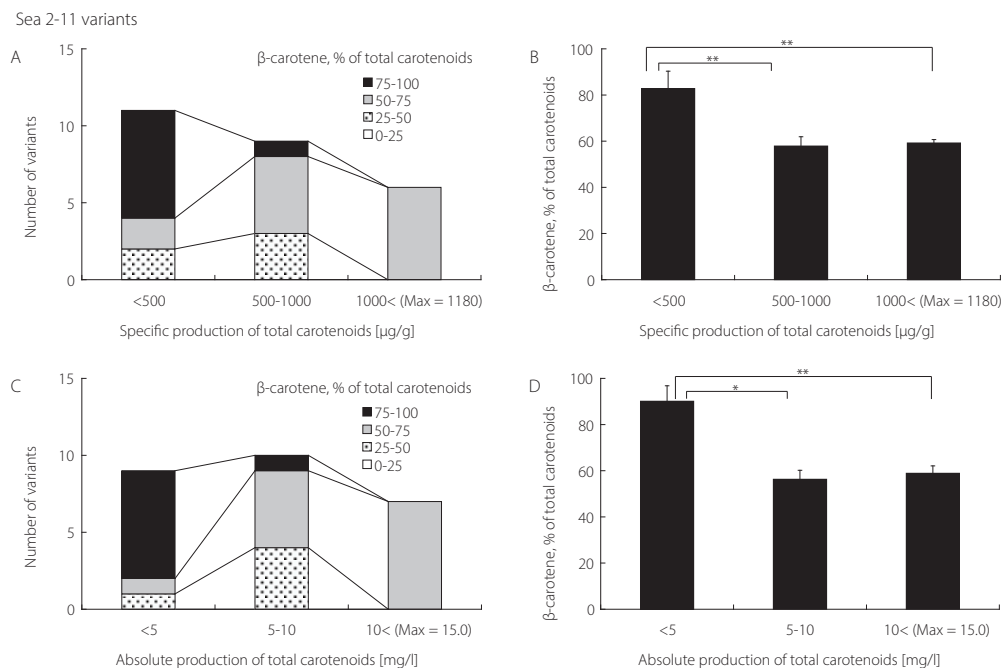


Figure 3: The relative proportion of β -carotene in total carotenoids compared between the groups of *Rhodosporidium diobovatum* Sea 2-11 variants classified by the specific (A and B) and absolute (C and D) production levels of total carotenoids. (B and D) Values are expressed as mean of variants belonging to each of the groups classified by the amount of total pigments \pm SE (see panels A and C for numbers of the variants used in the statistical analyses). Colorless variants as well as parent Sea 2-11 were excluded from the analyses. The statistical analyses were performed using Student's t-test. Asterisks indicate significant difference between the groups of variants classified based on abundance of total carotenoids. ** $P < 0.01$.

tated or eliminated in most of the Sag 17 variants whose specific production levels of total pigments were less than 500 $\mu\text{g/g}$ dry cells. In contrast, it was suggested that the genetic materials involved in the oxidation of monocyclic carotenes have been damaged in the case of Sea 2-11 variants with the reduced content of total pigments compared with the wild type. Nevertheless, these speculations does not account for a lack of changes in the relative proportion of β -carotene in the case of the variants whose total carotenoid levels are higher than the wild types (Figures 2 and 3). The relative β -carotene proportions of these variants (with elevated levels of total pigment contents) appeared to reflect those of the parents, suggesting the enhanced activities of both dicyclic and monocyclic carotenoid-biosynthetic pathways.

These phenomena are deemed very important in application of the carotenoid-rich variants for preferential production of particular pigments having excellent radical-scavenging activities. Of the aforementioned five major carotenoid species synthesized by both Sag 17 and Sea 2-11, polyene chains of four monocyclic carotenoids are longer than that of dicyclic β -carotene. The carotenoids with longer polyene chains, that are specific to marine organisms, exhibited potent abilities to quench singlet oxygen (Shimidzu et al., 1996). The same was true for the pigments in *Rhodotorula glutinis*; more potent quenching ability of torularhodin (as compared with β -carotene) was thought to be attributable to its longer polyene chain (Sakaki et al., 2001). Therefore, the variants which preferentially synthesize monocyclic pigments are useful in biotechnology from the perspective of anti-oxidation, provided that they have the ability to produce and accumulate high levels of total carotenoids. Since the relative proportion of β -carotene in individual variants of Sea 2-11 decreased (by increasing the relative amount of monocyclic carotenoids) with an increase of total pigment levels (Figure 3), this wild type strain is likely to be more useful as a parent to induce an efficient producer of monocyclic pigments via chemical mutagenesis as compared with Sag 17. Therefore, we suggest that choice of the parent species/strain is critical to improve the ability of the yeasts (that are belonging to the order Sporidiobolales) to synthesize monocyclic pigments using mutagenic agents. No past studies have focused on the relationship between abundance of total pigments and the relative β -carotene proportion using a large number of variants derived from a single parent.

3.2 Effects of substrate concentration on production of carotenoids by the variants

Three variants of *Rhodotorula dairenensis* Sag 17 (No. 3, 9 and 29) were used to investigate the effect of substrate concentration on carotenoid production. They were selected based on abundance of total pigments after growth in YPD for 144 h. The specific pigment production (2,200 $\mu\text{g/g}$ dry cells)

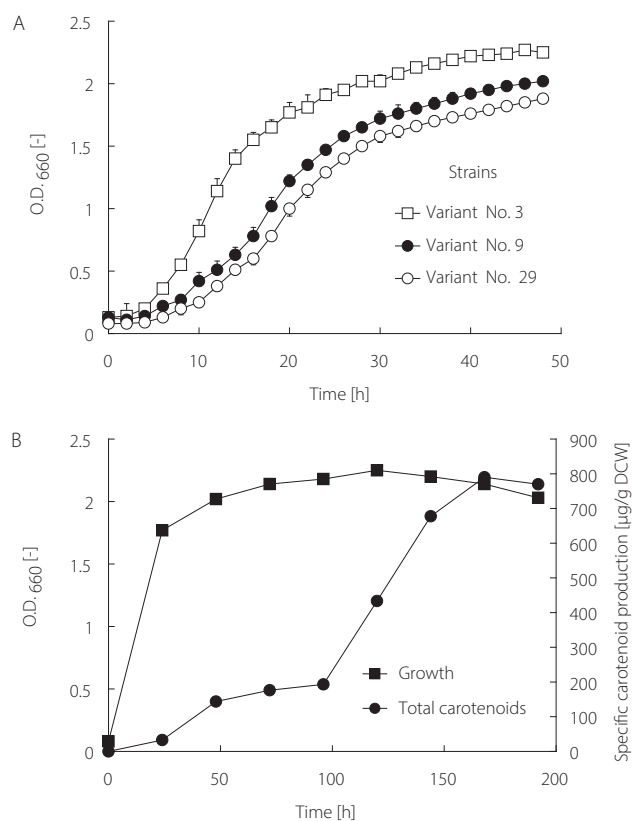


Figure 4: Growth of pigment-rich variants and their parent *Rhodotorula dairenensis* Sag 17 in YPD. (A) Growth of variants. Cultures were grown in triplicate. Data are expressed as mean \pm SE ($n = 3$); (B) Growth and carotenoid production by the parent culture.

for No. 3 was highest among all variants examined, which was 1.8, 1.6, and 3.0-fold higher respectively than those for No. 9, No. 29, and the wild type Sag 17. Figure 4 (A) shows growth of three variants in YPD at 25 $^{\circ}\text{C}$ with reciprocal shaking at 120 rpm. The specific growth rates (μ_{max}) of No. 3, 9, and 29 were 0.16, 0.13, and 0.12 (h^{-1}), respectively. Based on this result, No. 3 is referred to as the 'fast growing strain', while remaining variants are named as the 'slow growing strains'. An additional cultivation of Sag 17 under the same condition showed that the specific pigment production by this wild type reached a maximum after cell growth had terminated (Figure 4 (B)), and the same observation has been made for *Rhodotorula mucilaginoso* (Simova et al., 2003). Elevation of substrate concentration generally results in an increase of time required for growth termination, this was predicted to be most obvious for the slow growing strains. Considering these situations, both fast and slow growing strains were used to investigate the effect of sugar concentration on total pigment production.

Figure 5 (A) shows changes in the specific carotenoid production and biomass by the fast growing strain No. 3 as a function of initial glucose concentration. When glucose was raised from 1 to 3 %, the specific yields of total carotenoids in-

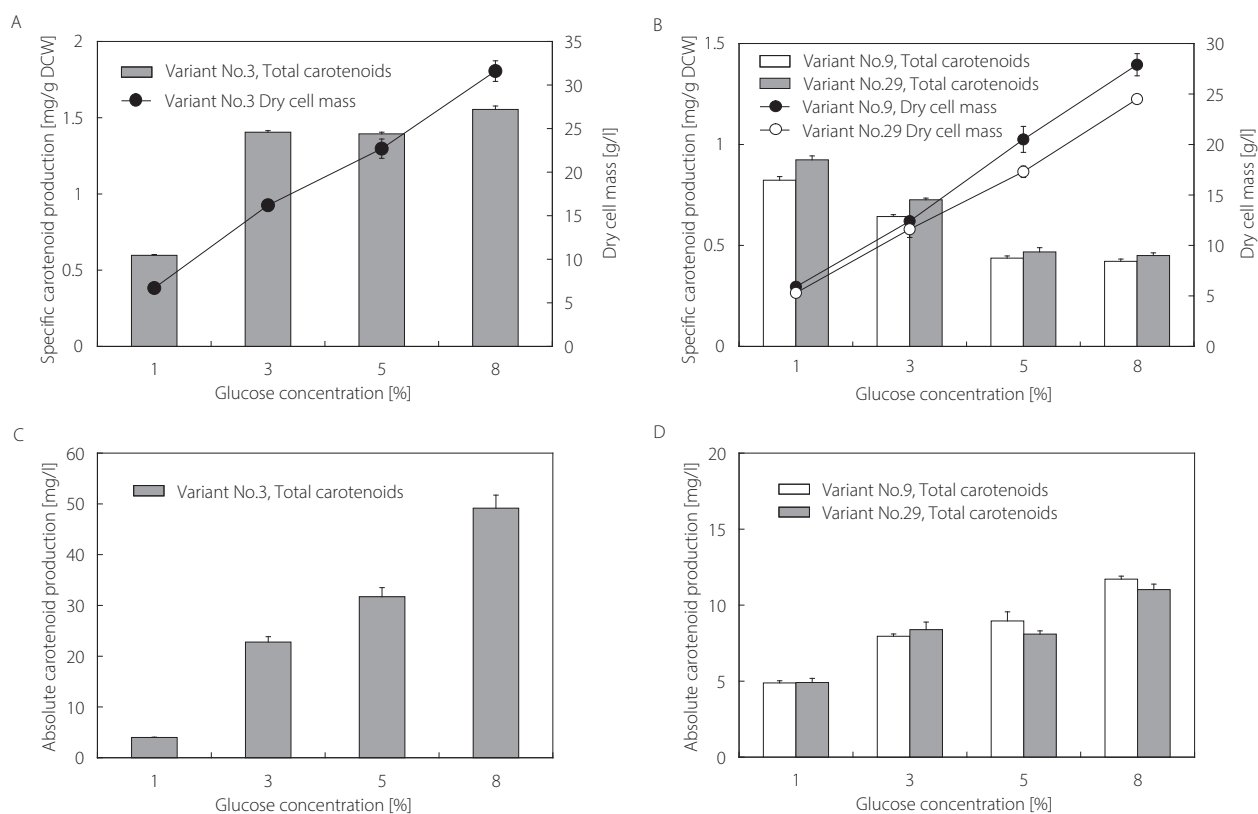


Figure 5: Production of carotenoids and biomass by the carotenoid-rich variants induced from *Rhodotorula dairenensis* Sag 17 as a function of glucose concentration in YPD. Cultures were grown at 25 °C for 170 h. Panels A and B show specific carotenoid production and biomass. The absolute carotenoid production (mg/l) in panels (C) and (D) were calculated from the values in (A) and (B), respectively. (A and C) Fast growing variant No. 3; (B and D) slow growing variants No. 9 and No. 29. (A-D) Data are expressed as mean \pm SE ($n = 3$).

creased by 2.4-fold. However, further supplementation of glucose did not result in a linear increase of the specific pigment production level, which slightly increased by 0.15 mg/g dry cells with elevation of the sugar concentration from 3 to 8%. In contrast, the levels of absolute pigment production and the dry cell mass in the No. 3 cultures increased linearly by 1.9 and 2.0-fold respectively with the elevation of glucose concentration from 3 to 8% (Figure 5, panels A and C). Therefore, the increase of cell mass is mostly responsible for the increase of the absolute pigment yields in the No. 3 cultures under high glucose concentrations (3-8%). Taking into account the fact that the specific carotenoid level in the parent Sag 17 culture (YPD) significantly increased by 4.1-fold 48-120 h after the culture entered the stationary phase (Figure 4 (B)), most possible explanation for this phenomenon is that the No. 3 cultures containing 5-8% glucose may not have reached the stage for pigments accumulation after arrest of cell growth.

This interpretation is further supported by the results from the cultivation of the slow growing strains. Figure 5 (B) shows the specific carotenoid production by the slow growing strains, i.e., No. 9 and No. 29, under glucose concentrations of 1-8%. The specific carotenoid levels of both strains decreased

by half with elevation of substrate concentration from 1 to 8%. However, the absolute production of total pigments increased (by 2.4-fold for No. 9 and 2.2-fold for No. 29) with the elevation of glucose amount from 1 to 8% (Figure 5(D)). Again, the level of biomass in the cultures of both strains increased linearly in proportion to the initial glucose content (Figure 5 (B)), and this contributed to an increase of the absolute pigment yield with an increase of sugar concentration. Therefore, the use of the fast-growing variants which also yield high level of cell mass in the stationary phase is expected to compensate for the delay in microbial pigmentation under high substrate concentrations, although they need to be pigment-rich in the first place.

4. Conclusion

To increase the efficiency of carotenoid production, selection of the Sporidiobolales strains has been reported in combination with the medium optimization, responses to various environmental factors (except for substrate concentration), co-cultivation with bacteria, and physiological comparison of the wild type isolate and a few pigment-rich variants induced there-from. Apart from those approaches, we first described

importance of relationship between the relative β -carotene proportion and total pigment level, as well as that of the ability to proliferate under high sugar concentrations, through investigating a large number of phenotypic variants derived from a single parent of the same order.

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For any correspondence, please contact Dr. Naoto Urano.

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