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Effect of Phenol on β-Carotene Content in Total Carotenoids Production in Cultivation of *Rhodotorula glutinis*

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Abstract−The composition of carotenoids produced by *R. glutinis* was observed to be dependent upon the addition of phenol into medium. A stimulatory effect of phenol on β-carotene of *Rhodotorula glutins* K-501 grown on glucose was investigated. Carotenoids produced by *Rhodotorula glutinis* K-501 were identified to torularhodin, torulene and β-carotene, whose composition was 79.5%, 6.4% and 14.1%, respectively. The β-carotene content increased up to 35% when phenol was added to culture media at 500 ppm. The ratio of torularhodin decreased with increasing phenol concentration, while torulene content was almost constant.

Key words: Phenol, β-Carotene, Carotenogenic Ratio, *Rhodotorula glutinis*

INTRODUCTION

Carotenoids are liposoluble tetraterpenes, usually red or yellow, and are one of the most important families of natural pigments. These pigments have several conjugated double bonds that act as chromophores and thus absorb light in the visible region, which gives them their strong coloration properties. They are precursors of vitamin A and are thus used as food supplements for modifying the color of fats, oils, cheese and drinks. They are also used in animal feeds. Use of these pigments is growing year after year because of user safety. Recently, it was reported that carotenoids had an anti-carcinogenic and antioxidant effect [Edge et al., 1997; Nesaretnam et al., 2000; Young et al., 2003], due to quenching singlet oxygen in humans and animals [Sies et al., 1987; Gerster, 1992].

Carotenoids are roughly classified into two groups. One is the hydrocarbon carotenes such as β-carotene, torulene, and the other is the oxygenated xanthophylls such as torularhodin and astaxanthin [Young et al., 1993]. Simpson et al. [1964] studied biosynthetic pathway of carotenoids in *Rhodotorula glutinis* by investigating intermediate substances with pathway blocking inhibitors such as methylheptenone vapor and ionone vapor. They proposed that some γ-carotene turned into metabolite, β-carotene, by cyclization. Other γ-carotene turned into torulene by dehydrogenation and then torulene was oxidized to final metabolite, torularhodin.

Carotenoids are mostly produced by extraction of plants. A variety of microorganisms have been examined for the industrial scale fermentative production of carotenoids, including fungi and green algae [Nelis and DeLeenheer, 1991; An et al., 2001; Parajo et al., 1998]. However, carotenoid biosynthesis is unfortunately seldom found in yeasts, which have some advantages such as well developed cultivation methods and easy extraction of carotenoids. Various yeasts, in particular *Rhodotorula*, *Cryptococcus*, *Phaffia* and *Sporobolomyces*, produce a variety of carotenoids that have a broad region of light absorption of 450-550 nm so that the culture broth has a colored appearance [Girad et al., 1994; Walker et al., 1973]. The fermentation conditions, such as cultivation temperature [Nelis and Deleenheer, 1991], lightening [Meyer et al., 1994], induced substances [Schroeder et al., 1993, 1995], and inhibitors [Girad et al., 1994; An et al., 1989; Feist et al., 1969] play important roles in the carotenoid-forming activity of yeasts as well as composition ratio of carotenoids (carotenogenic ratio).

Phenol is a precursor of dyes, pesticides and salicylic acid and is also raw material for phenolic resins such as epoxy resin and carbonate resin. However, phenol is a toxic compound that is hardly degradable [Park et al., 2003; Xiaoli et al., 2003]. Many microorganisms including bacteria in the genera of *Acetobactor* and *Pseudomonas* [Feist et al., 1969], and yeasts in the genera of *Candida*, *Rhodotorular* and *Trichosporon* can biodegrade phenol. Yeast strains of the genus *Rhodotorula* were already known to be capable of degrading phenol [Walker et al., 1973]. Katayama-Hirayama et al. [1991a, b] studied the metabolism of phenol in *Rhodotorula* genera. They reported that phenol was hydroxylated to catechol before cleavage and then oxidized to cis,cis-muconic acid in *Rhodotorula rubra*. In 1994, they also reported that the pathway of phenol degradation in *R. glutinis* was the same manner (ortho ring fission of catechol) as that in *R. rubra*. Up to now, considerable attention has been directed towards the biodegradation pathway of phenol by *R. glutinis*.

In this study, we found a stimulatory effect of phenol on β-carotene ratio in cultivation of *Rhodotorula glutinis* K-501 which had been isolated from soil. Similarly, an increase in β-carotene content using sea water medium in *R. glutinis* mutant has been reported [Bhosale and Gadre, 2001]. It was observed that the composition of carotenoids produced by *R. glutinis* K-501 was dependent upon the addition of phenol into medium. The relationship between the phenol addition and the carotenogenic ratio was examined. This study is the first report that phenol has a stimulatory effect on β-carotene

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production in cultivation of *Rhodotorula glutinis.*

MATERIALS AND METHODS

1. Microorganism

Rhodotorula glutinis K-501 was isolated from soil and was identified according to carbon and nitrogen assimilation tests [Kim et al., 1997]. The cells were maintained on malt agar slants at 4 ºC and transferred every month.

2. Culture Medium and Condition

The culture medium contained different levels of phenol in a basal medium. The basal medium consisted of 15 g/L glucose, 2 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2$, and 0.1 g/L NaCl. The initial pH of the medium was adjusted to 5.5 before sterilization. Phenol was autoclaved separately and added to the culture flask aseptically.

Erlenmeyer baffle flasks of 500 mL containing 200 mL of the basal medium were used for cultivation of the strain. The cells were pre-cultured in yeast malt media (YM media, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose). After inoculation with the pre-cultured cell suspension of *R. glutinis* (inoculation size, 2% (v/v)), flask cultures were performed at 22 ºC on a rotary shaker at 150 rpm. The culture broth was harvested by centrifugation at 10,000 g at 4 ºC for 15 minutes. After subsequent washing with 0.9% saline water (NaCl solution), the resting cell suspension containing 140 mg of cell dry weight per liter, was suspended in 0.67% YNB (yeast nitrogen base without amino acids and ammonium sulfate, Difco Laboratories, MI, USA) medium which was supplemented with phenol as a sole carbon source, and 2 g/L of ammonium sulfate as a nitrogen source. The incubation temperature was 22 ºC and the flasks containing YNB media were placed on the rotary shaker at 150 rpm.

3. Analytical Methods

The cell growth was monitored by determining the absorbance of the cultures at 660 nm with a UV-spectrophotometer (HP 8452A, Hewlett Packard, USA). The glucose concentration was measured by DNS (dinitrosalicylic acid) method [Miller, 1959]. The concentration of phenol was measured by an HPLC system (Young-In Scientific Co., Korea) using a UV detector with μ-Bondapak C_{18} column. For elution, a mixture of water and methanol (7 : 3, v/v) was used. The concentrations of total carotenoids were measured as follows. The cells were harvested and then washed twice with distilled water. The liquid was removed by centrifugation. Dimethyl sulfoxide (DMSO) of 1 mL preheated to 55 ºC, was added and then the mixture was vortexed for 10 sec. Acetone (1 mL), petroleum ether (1 mL) and saturated NaCl (1 mL) were added successively to extract the carotenoids. After centrifugation, the petroleum ether phase was withdrawn, and absorbance detection was conducted by using the spectrophotometer at 501 nm. The concentration of total carotenoids was calculated with an extinction coefficient of 2040.

4. Identification of Carotenoids

Carotenoids were separated by thin layer chromatography (TLC) on silica gel plates (5×20 cm, Kieselgel 60 F_{254}, Merck). The extracted carotenoids were loaded on the TLC with mixed elution solvent of acetone and hexane (3 : 7, v/v). The bands on the TLC plate were identified by using standard compounds such as β-carotene, torulene and torularhodin. After development, the bands were scraped in petroleum ether and separated by centrifugation. The composition of carotenoids was determined by HPLC using μ-Bondapak C_{18} column with a UV detector. The elution solvent was acetonitrile-tetrahydrofuran-water (5 : 3 : 1, v/v/v).

RESULTS AND DISCUSSION

1. Carotenogenesis and Identification of Carotenoids

Identification of carotenoids produced by *R. glutinis* K-501 was conducted. The extracted carotenoids were chromatographed by TLC on silica gel plates. The carotenoids were developed and separated by the difference of polarity [Boyer, 1993]. On the TLC plate, there were three spots indicating that *R. glutinis* K-501 had mostly three carotenoids (data not shown). Simpson et al. [1964] reported that the major carotenoids of *R. glutinis* were β-carotene, torulene and torularhodin. It was observed that β-carotene, torulene and tor- ularhodin were successively developed. Their R_f values were 0.97, 0.95 and 0.35, respectively.

Each carotenoid on silica gel plates was scraped in petroleum ether. Absorbance spectra of each carotenoid were measured by a spectrophotometer. The determined absorbance maxima of β-carotene, torulene and torularhodin were 450, 479 and 500 nm, respectively. Since carotenoids had specific absorbance spectra, the change of the carotenoids composition can be predictable by absorbance spectrum [Young and Britton, 1993; Simpson et al., 1964; An et al., 1989; Polulyakh et al., 1991]. The extracted carotenoids were eluted with a solvent composed of acetonitrile, tetrahydrofuran and H_2O as described in Materials and Methods. Since a reversed phase column (μ-Bondapak C_{18}) was used, the carotenoids were eluted in the order of decreasing polarity [Nam et al., 1988]: torularhodin, torulene and β-carotene, successively.

From the analysis of carotenoids by HPLC, in contrast to the study of Nam et al. [1988], the ratio of torularhodin was larger than that of torulene. The ratio of torularhodin, which was primary carotenoid, was 79.5% in *R. glutinis* K-501 and those of torulene and β-carotene were 6.4% and 14.1%, respectively, when the cells were grown on glucose for 100 h.

Fig. 1 shows a typical time course of batch cultivation of *R. glutinis* K-501. Glucose was utilized at the initial stage until it leveled off at 27 h. The cell growth occurred during the period of glucose consumption. The biosynthesis of carotenoids started in the growth phase and continued even after stationary or death phase. From this result,

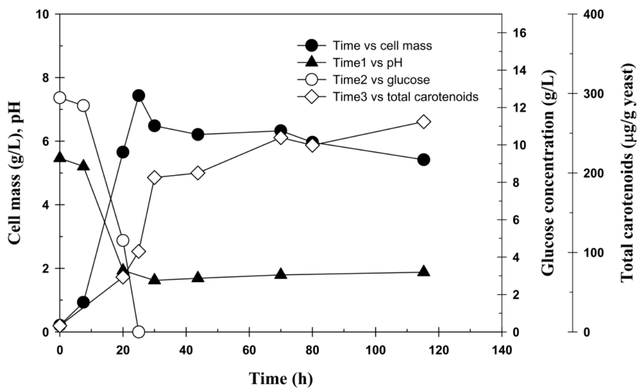

Fig. 1. Time course for the cultivation of *R. glutinis* K-501.

Table 1. Degradation of phenol by *R. glutinis* K-501

Initial phenol concentration (ppm)		100	200	300	400	500	700
Residual phenol concentration (ppm)	Using 35 h-old cells	9	16	4	26	181	597
	Using 85 h-old cells	0	0	0	0	6	536

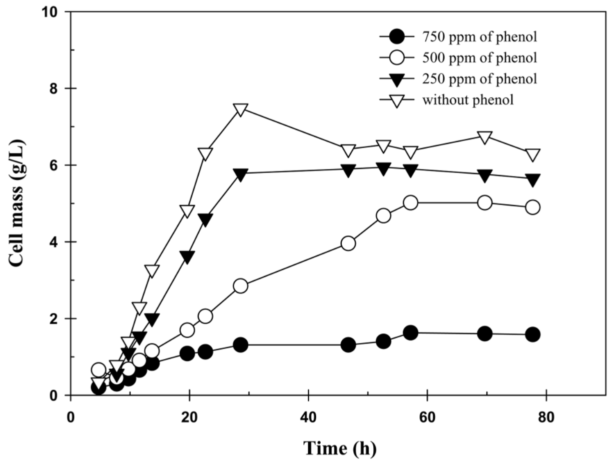

Fig. 2. The effect of the initial concentration of phenol on the cell growth.

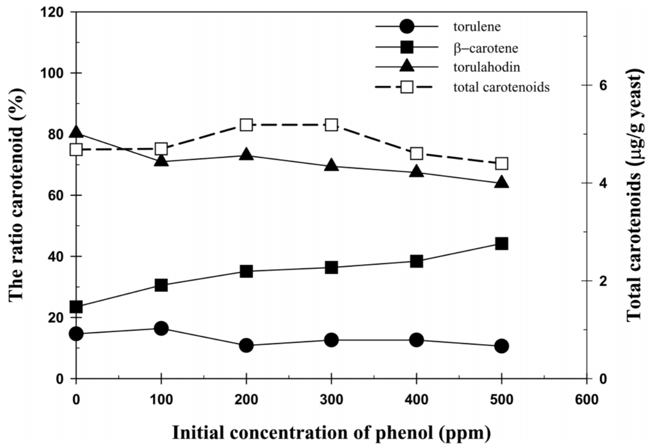

Fig. 3. The effect of phenol on total carotenoid content and carotenogenic ratio. Data were obtained after 120 h cultivation.

carotenogenesis seemed to have a mixed-growth-associated pattern.

2. Phenol Degradation

It has been already reported that *R. glutinis* consumed phenol as a carbon source and energy source [Katayama-Hirayama et al., 1991a, 1994]. The cells were incubated in YM medium, harvested and washed twice by distilled water. The washed cells (0.07 g) were suspended and incubated in YNB medium containing phenol as a sole carbon source of different concentrations. The concentration of residual phenol was measured by HPLC. It was observed that *R. glutinis* K-501 utilized phenol as a sole carbon and energy source up to 500 ppm of phenol as shown in Table 1. 85 h-old cells biodegraded phenol better than 35 h-old cells did, which is similar to the result of Walker [1973] with bacteria.

The effect of phenol on the cell growth was investigated in the basal medium. The cell growth was inhibited by phenol and the cells did not grow at 750 ppm of phenol as shown in Fig. 2. In this case, it appeared that glucose was used as a growth substrate and phenol was a toxic inhibitor for cell growth.

3. Effect of Phenol on Carotenogenic Ratio

Polulyakh et al. [1991] reported that astaxanthin was produced up to 80% of total biosynthetic carotenoids in *Phaffia rhodozyma* at 20 °C, but torulene and torularhodin were 90% of those at 30 °C. Nakayama et al. [1954] reported that β-carotene and γ-carotene were major carotenoids in *Rhodotorula glutinis* at 5 °C, but torulene and torularhodin were major carotenoids at 25 °C. Frengova et al. [1995] reported that light of a specific wavelength induced carotenogenesis, but did not change the composition ratio of carotenoids in incubation of *Phaffia rhodozyma* [Meyer and Du Preez, 1994]. Costa et al. [1987] investigated effects of nutrients on carotenogenesis.

The cells were harvested in the exponential phase of growth and then incubated in distilled water for 1 day. To investigate the effect of phenol on carotenoid production, phenol was added to a concentration of 500 ppm in the basal medium. The pH of culture broth was 5.5 at the beginning of the fermentation as shown in Fig. 1. The culture pH decreased through fermentation. The pH decrease is probably due to ammonium sulfate ion consumption in the medium [Krebs et al., 1983]. To compare the phenol effects in the basal medium and in a modified medium for a higher buffering capacity, the cells were incubated in both media containing phenol at a level of 500 ppm. Potassium hydrogen phthalate solution (0.05 M) was used as a buffering agent with which the pH of the cell broth was kept constant over a long-term fermentation. The absorbance spectra were observed for both cases after 120 hr cultivation (Fig. 3). Phenol had some effect on carotenogenic ratio when the cells were incubated in the basal medium. Absorbance intensity at 450 nm, which was the maximum peak of β-carotene, increased relatively by addition of phenol (data not shown). This means that the ratio of β-carotene to the other carotenoids increased. It was observed that in the buffered medium, phenol had little effect on carotenogenesis. Phenol probably inhibits the dehydrogenation of torulene to torularhodin by virtue of decreasing the intracellular pH. Then, the cyclization of γ-carotene to β-carotene was relatively activated. So the ratio of β-carotene increased and that of torularhodin decreased. This was confirmed by HPLC analysis.

The total carotenoids by incubation with different initial concentrations of phenol are shown in Fig. 3. Phenol inhibited the cell growth to some extent. However, the total concentration of carotenoids was almost constant. However, it cannot be explained by a strict correlation between the growth inhibition and total carotenoid biosynthesis to examine the effect of phenol in the ratio of carotenoids; a relationship between the ratio of carotenoids and the initial concentrations of phenol was observed. As shown in Fig. 3, the ratio of β-carotene increased and that of torularhodin decreased with increasing phenol concentration. The β-carotene content increased up to 35% when phenol was added to culture media at 500 ppm. However, the ratio of torulene was almost constant.

To reduce the inhibitory effect of phenol for the cell growth, phenol was extra-fed with 250 ppm just after consumption of the initially added phenol (250 ppm). The color of the culture broth containing more phenol became more yellowish (data not shown); its color was probably due to β-carotene content. It was clearly observed that phenol had a stimulatory effect on the β-carotene biosynthesis.

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