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 glutinis 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 anticarcinogenic 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 carotenoids 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 Sporabolomyces, 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 Acetobacter and Pseudomonas [Feist et al., 1969], and yeasts in the genera of Candida, Rhodotorula 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.
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 °C 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 (NH₄)₂SO₄, 1 g/L KH₂PO₄, 0.5 g/L MgSO₄·7H₂O, 0.1 g/L CaCl₂, 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 °C on a rotary shaker at 150 rpm. The culture broth was harvested by centrifugation at 10,000 g at 4 °C 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 °C 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 C₁₈ 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 °C, 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 (5x20 cm, Kieselgel 60 F₂₅₄, 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 C₁₈ 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 torularhodin were successively developed. Their Rₛ 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 H₂O as described in Materials and Methods. Since a reversed phase column (µ-Bondapak C₁₈) 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,