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Carotenoid Production of *Rhodosporodimum Toruloides* under Light Irradiation

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ABSTRACT

Carotenoids are widely used as an antioxidant, a precursor to vitamin A, and food colorants in the medical, cosmetic, chemical, food, and feed industries. They were distributed in diverse microorganisms including bacteria, algae, and fungi. Among them, the yeast *Rhodosporidium toruloides* is convenient for large-scale fermentation due to its unicellular nature and high growth rate. However, many factors influence the biotechnological synthesis of carotenoids. Light is a crucial consideration while making microbial carotenoids. Carotenogenic is a photo-protective technique used by microorganisms to defend themselves from the light that causes oxidative damage. *R. toruloides* raised their carotenoid productivity under light conditions in this study. Various lights were used to compare the effect of color light in carotenoid productivity. Results showed that *R. toruloides* produced more carotenoid content when cultured in blue or white light as compared to a red light or in the dark. Besides, results from microarray showed that this light influence was in the transcription gene level, the light irradiation encouraged the formation of antioxidants such carotenoids, causes a protective mechanism against DNA damage and oxidative stress.

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1. Introduction

With approximately 600 different structural component types in nature, carotenoids are regarded as one of the most significant pigment families. Formulas for them consist of 40 carbons, have molecules that are either oxygenated or not, conjugated double bonds, and have rings at the end [1], [2]. Two classes – xanthophylls, which have oxygen structure, and carotenes, which do not contain oxygen structure – have been included [3]. Additionally, carotenoids are lipophilic compounds that produce pigments ranging from yellow to red color [4]. As a result, they are employed as natural colorants in the food sector. Additionally, some carotenoids function as antioxidants or vitamin A precursors. They are all utilized as dietary supplements and vitamins [5]. The bioactive phytochemicals known as carotenoids are also useful in lowering the chance of contracting degenerative diseases including cancer, cataracts, and cardiovascular disease and etc [6], [7].

Rhodosporidium toruloides is a non-pathogenic basidiomycetous fungus that has a red color cell (Figure 1). *R. toruloides* can produce over 70 % lipid in their body. They can also create carotenoids, as well as vital enzymes for biotechnology [8], [9]. Many scientists have investigated the carotenoid production of *R. toruloides*. According to the researchers, *R. toruloides* produced a variety of amounts of γ -carotene, β -carotene, torulene, and torularhodin [10]–[13]. A well-known pigment, β -carotene, is used in numerous industries, including healthcare products, food additives, feed additives, and many more goods. The compound known as β -carotene has two retinyl groups and eleven conjugated double bonds, giving it a yellowish-orange color. The polyene chain and ring include a lot of double bonds, which renders β -carotene easily oxidized by free radicals. β -carotene has antioxidant effects and has been added to meals as a result [14]. Cancer, and tumor growth are all thwarted by β -carotene's antioxidant qualities in the healthcare and medical areas. The isomer of β -carotene is called γ -carotene. In addition to having eleven conjugated double bonds, γ -carotene also has a retinyl group, one non-conjugated double bond. Despite having one retinyl group less than β -carotene, γ -carotene performs

similarly to β -carotene. Consequently, both β -carotene and γ -carotene are a precursor of vitamin A [15], [16]. Acid pigments include torulene and torularhodin. γ -carotene is transformed into torulene via an additional double-bond in the 13th Carbon position. One carboxyl group is substituted for one methyl group to produce torulerhodin. Given that both of these carotenoids have the β-ionone ring, which serves as the foundation for vitamin A, in their structures, they are both likely precursors to vitamin A. Because β -carotene and γ -carotene lack extra-conjugated C = C double bonds at the C-13 position of their structures, torulene and torularhodin have demonstrated greater antioxidant properties. Mutants with high levels of torularhodin production have been shown to reduce oxidative damage brought on by active oxygen species [17], [18].

Figure 1. *Rhodosporidium toruloides NBRC 10032's morphology on a YPD plate*

The carotenoid yields of NBRC 10032 are higher than those of other microorganisms. The dry mass of the cells in the *Rhodotorula glutinis* DBVPG 6081 contained 112.2 $\mu g \times g^{-1}$ of carotenoids containing 30.57% torulene, 46.8% torularhodin, 18% β -carotene, and 55% γ -carotene. Additionally, it was recorded that the *R. toruloides* DBVDG 6739 had a carotene content of 122.6 $\mu g \times g^{-1}$ cell dry mass [10]. There were 5.4 $mg \times g^{-1}$ cell dry of carotenoids in additional *Rhodotorula* strains, such as the *R. glutinis* mutant 32, with β -carotenoid, torularhodin, and torulene, being present at 80%, 2.3%, and 17%, respectively [19]. Research on the practical manufacture of carotenoids from microbes has been stimulated by the benefits of carotenoids as ingredient in food and specific anticancer medicines. Numerous techniques of media manipulation and cultural conditions have been proposed for the development of carotenoid composition since cultural conditions like temperature, light, carbon supply, and nitrogen source are dependent on carotenoid production. This study investigated how light affected *R. toruloides* NBRC10032 's ability to produce carotenoids. Although *R. toruloides* NBRC10032 was found to be a strong lipid generator, it also has the capacity to generate carotenoids. In this work, it was shown that the transcription level regulates carotenoid biosynthesis production in carotenoid synthesis in reaction to illumination.

2. Materials and Methods

2.1. Strains and culture conditions

The *R. toruloides* NBRC 10032 was made available by the National Institute of Technology and Evaluation (Japan). 2% glucose, 1% yeast extract, 1% peptone, and 2% agar were placed on YPD 2 agar plates (Yeast Peptone D-Glucose), *R. toruloides* was cultivated for four days at 30°C before being stored at 4°C. For pre-cultivation, the YPD 2 medium was utilized, and for carotenoid accumulation, the YPD 10 medium (10% D-Glucose, 1% Yeast extract, 1% Peptone) was used. After being pre-cultivated for 24 hours in YPD 2 medium with an initial optical density of 660 nm = 0.1, they were cultured in YPD 10 medium for carotenoid production. 24 m thick in total Aluminum foil was used to cover the flasks to create the dark circumstances, and a fluorescent lamp with a power output of 27 watts and a photon density of 98 mol/m2/s was used to create the light conditions.

2.2. Different color light source experiment

NBRC 10032 was developed in four different environments: blue color light , red color light, white color light, and darkness when it was plated on the YPD 2 agar plate. The LED Lighting Unit 3LH Series utilized sources of blue color light and red color light with an irradiation wavelength of 445 nm and 660 nm respectively. We employed a combination of 445, 520, and 660 nm for the white color light. As a control, the incubator was set up in the darkness.

2.3. Biochemical analysis

Using the UV mini-1240 (Japan) for ultraviolet-visible spectroscopy, the optical density (OD) of each cultivation was determined at 660 nm wavelength. To obtain dry cells, 1mL of broth was removed, cleaned with distilled water, and frozen.

The Multi Beads Shocker was used to extract carotenoids from dry cells in 100% acetone, in accordance with the K.D. Pham technique [20]. 500 mg of 0.5 mm glass beads and 200 µL of acetone were combined with dry cells. Utilizing 30 cycles of ON time 30 seconds and OFF time 30 seconds at 4°C, condition for the Multi Beads Shocker was performed at 2500 rpm. 800 µL of acetone were added after cell disruption. The mixture was centrifuged for 10 minutes at 4 °C / 14000 rpm. Then, 100 µL was measured using Infinite M200 PRO (Japan).

2.4. HPLC analysis

Following acetone extraction, a DISMIC Cellulose Acetate Syringe Filter Unit filter with a 0.45 µm pore size was used to filter the carotenoid solution. By using High Performance Liquid Chromatography (HPLC) system including a UV/VIS detector, carotenoids were examined. Shim-Pack XR-ODS HPLC, 75 × 3 mm, a 2.2 µm packing particle size separation C18 column was used with P. Davoli and Roland W.S. Weber both made changes to the approach [21], [22]. The gradient ranged from 70% to 100% acetone and the flow rate at 1 mL/minute. All data were recorded at 450 nm.

2.4. Microarray analysis

For total RNA extraction, yeast cells were first cultivated in the dark for 24 hours, then moved to either light or dark conditions, and sampled at the appropriate intervals after the transfer. After that, total RNA was extracted by a modified hot-phenol method using TRIzol®LS (Invitrogen, USA) after the cells were frozen. Then, an RNA spin follows the GE healthcare RNA extraction protocol for additional purification.

For microarray analysis, Agilent Array Genomics software, which was supplied by Agilent, was used. Customized microarray to hold all the potential genes for the *R. toruloides* NBRC 10032 was used. To achieve a probe length of 60 mers, a total of 6 probes were created, with 2–3 probes corresponding to each gene. They were then positioned on the substrate of the microarray chip. Cell Innovator was hired to do the analysis. Data analysis was done using the Gene Spring software from Agilent.

2.5. Statistical analysis

All experiments were replicated at least three times.All standard deviations (not shown) were less than 5% of the mean. The student's *t*-test was used to determine the statistically significant differences ($p \leq 0.05$).

3. Results and Discussion

3.1. Effects of different visible light wavelengths

Figure 2. *The color change of cultures after one day cultivation of R. toruloides NBRC 10032.*

From Figure 2, resulting in the culture's color changed to redder color after only one day under light irradiated cultivation when grown in the YPD 10 broth medium in dark condition and light conditions. It was hypothesized that carotenoid was formed in the medium when exposed to light.

Compared to red color light condition and darkness condition grown cultivation, *R. toruloides* colonies became reddish color when exposed to blue color light and white color light, as seen in Figure 3. According to this discovery, *R. toruloides* is responsive to blue color light. It was therefore hypothesized that *R. toruloides* NBRC 10032 has a light-responsive mechanism for producing carotenoids.

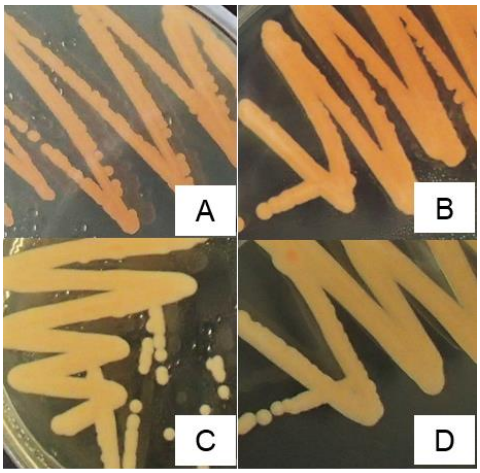

Figure 3. *R. toruloides NBRC 10032's color changes when exposed to different visible light wavelengths. (**A**: blue color light; **B**: white color light; **C**: red color light; **D**: darkness condition).*

In the studies of *Dunaliella salina*, the red (not blue or white light) increased the carotenoid production [23]. However, the mechanism of carotenoid biosynthesis in the plant or algae that photosynthetic organisms might be different to non-photosynthetic organisms. In the fungal light response, most fungi have two different types of photoreceptors. While some blue light photoreceptors are located in the nucleus and have the ability to directly regulate gene expression, red light photoreceptor phytochrome has both nuclear and cytoplasmic functions and is connected to the transcriptional machinery through other signaling modules [24].

3.2. Comprehensive gene expression study for light response gene identification

The results below show that after one day of exposure to light, a darker red color was obtained, which indicated that this strain's genes controlled the route for producing carotenoids. To further comprehend this fact, detailed expression study on the NBRC 10032 genes was carried out during one hour of light exposure and compared to one hour of darkness and zero hour of darkness.

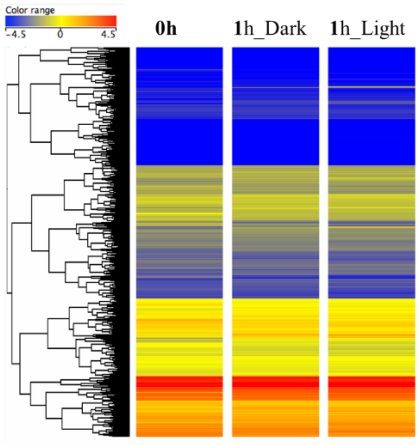

Figure 4. *The thoroughness of the gene expression was shown by the heat map. Red color represents a high expression intensity, whereas blue color represents a low expression intensity.*

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From cells that had been exposed to light for zero- and one-hour, total RNA was extracted. Genes were grouped according to the degree of expression after microarray data analysis (Figure 4). Red color one represented the high intensity expression, whereas blue color one represented the low intensity expression. Most genes' expression levels appeared unaltered in all the darkness condition and the light illumination compared at zero hour after continuous. However, compared to zero-hour expression, forty-eight genes and twenty-three genes, respectively, demonstrated a five-fold or greater increase in expression within one hour of light illumination condition and at one hour of darkness condition. Among of them, sixteen genes were presented under both circumstances, and only **thirty-two** genes differ by five times in their degree of expression as they respond to light irradiation (Figure 5). As a result, these genes were proposed as the genes that react to light.

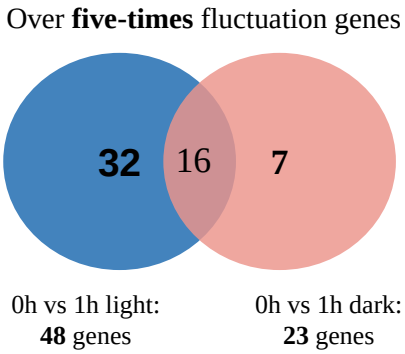

Figure 5. *Using a Venn diagram, identify gene groups that only exhibit over five-fold fluctuation expression in light-induced cultivation.*

Some interesting data were obtained about how the *R. toruloides* strain responded to light with regard to of carotenoid synthesis. When NBRC 10032 was cultivated in the light irradiation, redder colorations were noticed, indicating increased carotenoid synthesis. The quick color change also demonstrated how strongly the light affected the carotenoids in NBRC 10032.

In comparison to cells grown in the dark, samples of carotenoid from light-irradiated cells showed a significantly increased absorbance. Previous studies, after six days of cultivation, the cell-free extract revealed that cells grown in the light produced five times as much carotenoids as cells grown in the dark [20]. Another study had also shown that growing the fungus *Mucor hiemalis* under light increased the number of carotenoids it produced. Additionally, the amount of carotenoid produced under blue and white light was 2.5 and 3 times higher than it was under dark-grown conditions, respectively [25]. *R. glutinis* has also shown similar results that torularhodin production was activated by white light irradiation or high irradiation increased the yields of carotenoids [18], [26]. We therefore hypothesized that the carotenoid synthesis processes in *Rhodosporidium* species are light-responsive.

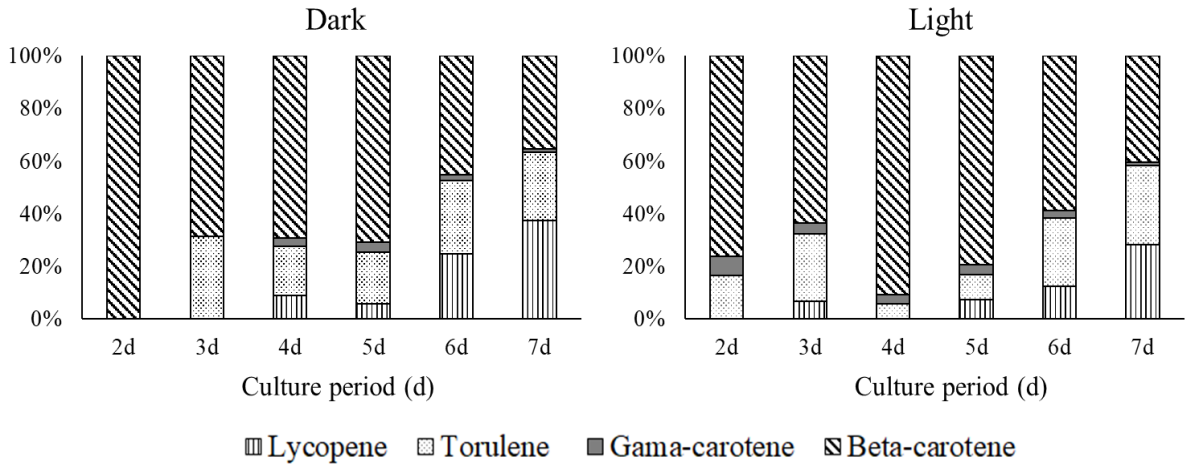

Figure 6. *Carotenoid composition varies with light and darkness according to cultural time.*

Table 1. *List of the thirty-two genes with a five-fold fluctuation expression in response to light.*

Annotation	(+: increased gene, -Dropened gene)
hydroxymethylglutaryl-CoA synthase	-
proteinof caleosin family	+
### Not characterized ###	+
proteinof choline transporter-like family	+
FAD dependent oxidoreductase	+
### Not characterized ###	+
deoxyribodipyrimidine photo-lyase	+
phytoene dehydrogenase (*CAR1*)	+
DUF427-domain-containing protein	+
geranylgeranyl diphosphate synthase, type III(*GGPSI*)	+
### Not characterized ###	+
Allergen	+
cell division control protein Cdc54	+
short-chain dehydrogenase/reductase SDR family protein	+
D-arabinono-1,4-lactone oxidase	+
beta-Ig-H3/Fasciclin	+
Ribonuclease	+
minichromosome maintenance protein 6	+
proteinof bacterial rhodopsin family	+
### Not characterized ###	+
GATA transcription factor	+
UV DNA damage endonuclease	+
mnng and nitrosoguanidine resistance protein	+
Hypothetical Protein RTG_01011	+
Proteophosphoglycan ppg4	+
phenolic acid decarboxylase	+
Proteophosphoglycan 5	+
phytoene synthase *(CAR2)*	+
zinc-type alcohol dehydrogenase	+
SH3 domain protein	+
arabinose-5-phosphate isomerase	+
START domain containing protein	+

In the previous studies, the carotengenesis genes were suggested the regulation by a two steps photoresponse mechanism [20]. The production of carotenoids increased as a result of the activation of light receptor and carotenoid genes. What is the function of step two, which modifies the carotenoid composition by altering the carotenoid synthesis steps. Results of the daily analysis of the carotenoid

content are shown in Figure 6. The raising torulene content on days two and six in the light, when compared to days two and six in the dark, revealed that in the absence of the light impact, α-carotene is the priority product, followed by the production of torulene and torulahordin. In the condition of the light effect, light increased the production of torulene and γ-carotene as well as carotenoid precursors. Since torulene was not seen on day two in the dark and the majority of the compound was α -carotene, torulene gradually grew over the course of the next two days of cultivation. The formation of torulene was stimulated by light on day two; however, as there was no rise in gene expression after that day, the concentration of α -carotene increased until day five. The step two was initiated on day 6 and manufacture of torulene was encouraged. As a result, the amount of torulene was increased while the amount of α -carotene was decreased.

A list of these thirty-two genes was discovered in the complete gene expression findings and is provided in Table 1 with expression variations of at least five-fold only in response to light irradiation. Light irradiation increased the expression levels of the genes *CAR1*, *CAR2*, and *GGPSI* that are involved in the production of carotenoids. The transcription levels were also boosted by the oxidoreductase enzyme in response to light exposition. The examples of these enzymes are D-arabinono-1,4-lactone oxidase, flavin adenine dinucleotide dependent oxidoreductase, and zinc-type alcohol dehydrogenase. The FAD-dependent oxidoreductase enzyme belongs to the superfamily of FAD binding domains of FAD flavoenzymes. The oxidoreductase D-arabinono-1,4-lactone oxidase (like Cryptochrome DASH gene) has one flavin adenine dinucleotide cofactor. This cofactor demonstrates further *R. toruloides* sensitivity to light induced oxidative damage. In addition, the transcription of the deoxyribose-pyrimidine photolyase and UV DNA damage endonuclease was elevated in response to light exposure. It has been hypothesized that *R. toruloides* still has a broad DNA damage repair mechanism that is activated by exposure to light. Thus, it is theorized that light irradiation, which encourages the formation of antioxidants such carotenoids, causes a protective mechanism against DNA damage and oxidative stress. In addition, the fact that these enzymes' binding sites contained FAD showed that a flavin-based photoreceptor was likely involved in photo-carotenogenesis.

4. Conclusions

In conclusion, the light had an impact on the carotenoid biosynthesis producing of *R. toruloides* NBRC10032, and under light conditions, *R. toruloides* NBRC 10032 generated more carotenoid content than it did under dark conditions, as shown by comparing the color of the cells or the medium. In addition, the kind of light source also influences the creation of carotenoid pigments, with blue and white light producing higher carotenoid than red light or dark. The transcriptional regulation of carotenoid synthesis was demonstrated in response to light. This studies also finding the type of genes were enhanced expression level. Most of these genes involved the reactive oxygen species related genes with the protective mechanism.

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Conflict of Interest

The authors declare no conflict of interest.

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