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ORIGINAL PAPER

Optimized *Rhodobacter sphaeroides* for the Production of Antioxidants and the Pigments with Antioxidant Activity

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Abstract

The photosynthetic bacterium, *Rhodobacter sphaeroides*, is a bacterium that can grow in a variety of environments and produces substances with antioxidant effects. In this study, we investigated the culture conditions to increase the production of antioxidants in *R. sphaeroides*, which can grow under both aerobic and anaerobic conditions. After incubation in the exponential phase and stationary phase under both conditions, a 2,2-diphenyl-1-picrylhydrazyl assay was used to confirm the antioxidant effect. Although the highest antioxidant effect was shown in the stationary phase under aerobic conditions, the antioxidant effect of each cell was found to be greater when cultured under anaerobic conditions. The antioxidant activity of *R. sphaeroides* was increased by sonication. In addition, the contents of carotenoids and bacteriochlorophyll, which are pigments with antioxidant effects, produced by *R. sphaeroides* were measured. We confirmed that the content of carotenoids was higher in anaerobic conditions than in aerobic conditions. However, when measuring the content of the bacterium, we found that there was more content in aerobic conditions. Therefore, we confirm that when grown in anaerobic conditions, the antioxidant effect of *R. sphaeroides* is higher, which suggests that this antioxidant effect comes from the effect of carotenoid.

Keywords *Rhodobacter sphaeroides* · Antioxidant activity · Cell culture condition · Pigments

Introduction

Rhodobacter sphaeroides is a purple non-sulfur bacterium (PNSB) that can grow aerobically, anaerobically, heterotrophically, and photoautotrophically [1]. PNS bacterium such as *R. sphaeroides* can produce high-value metabolites, such as coenzyme Q10, 5-aminolevulinic acid, poly-β-hydroxybutyrate, vitamin B12, biohydrogen, and carotenoid [2]. *R. sphaeroides* has a photosynthetic gene cluster of seven native crt genes involved in carotenoid synthesis [3]. Antioxidant activity is an important function of this strain, which is related to functional properties such as immunity promotion, including anti-inflammatory properties [4]. Carotenoids and bacteriochlorophyll are important functional and structural components included in *R. sphaeroides* and are involved in the capture of light energy generated by events that occur mainly during photosynthesis and the subsequent transfer of this energy to reaction center [5]. Carotenoids are plant pigments found in algae photosynthetic pigments and protein complexes [6]. Carotenoids are soluble pigments that exhibit colors such as red and orange and are important antioxidants and active ingredients in photosynthetic organisms, such as bacteria [7]. The carotenoids

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produced by *R. sphaeroides* exhibit significant antioxidant activity without cytotoxicity. In addition, the production of carotenoids may increase resistance to ROS, enhancing defense mechanisms against environmental stress [8].

In this study, compared with other strains, it was confirmed that *R. sphaeroides* was suitable as an antioxidant-producing substance. In addition, we found both an optimized culture condition in which the most antioxidants were generated and a treatment method to increase antioxidants in these culture conditions. To confirm and compare the antioxidant effect under each culture condition, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used. And the amount of the pigment with antioxidant activity produced for each condition was measured. Through these experiments, pigments derived from the antioxidant effect generated by each condition in *R. sphaeroides* were confirmed.

Materials and Methods

Cell Culture Condition

R. sphaeroides KCTC 1434 (Korean Collection for Type Cultures) and *Escherichia coli* BL21 (DE3) (Novagen Chemicals Inc., Germany) cultures were based on previously published protocols [9]. The growth of the cells was monitored by the optical density (OD) at 600 nm using a UV/VIS spectrophotometer (Mecasys Co., LTE., Daejeon, Korea), with a sufficient dilution for the culture broth. *R. sphaeroides* and *E. coli* were compared in the exponential phase, an aerobic condition.

Sonication Treatment for Antioxidant Extraction

Sonication (Sonics & Materials, Inc. USA) was used as an experimental method to increase the amount of antioxidants produced by *R. sphaeroides* [10]. Cells cultured in each condition were centrifuged at 3500 rpm for 10 min to obtain a pellet and then washed twice with sterile distilled water. Into the washed pellet, 10 mL of sterile distilled water and 100 µL of phenylmethylsulfonyl fluoride were added and dissolved, followed by sonication. Sonication was carried out for a total of 25 min at 20% pulses for 30 s and stopping for 59 s.

DPPH Radical Scavenging Activity

Among the reagents used in this study, DPPH purchased from SIGMA were used. The DPPH radical scavenging activity experiment followed the method of Dojindo's DPPH assay (Dojindo, Kumamoto, Japan). The DPPH reagent was used by dissolving it in ethanol at a concentration of 0.1 mM. To confirm the antioxidant activity, *R. sphaeroides* and *E. coli* cultured in exponential and stationary phases were used as samples. In addition, the antioxidant activity of *R. sphaeroides* cultured aerobically and anaerobically was compared, and *R. sphaeroides* treated with sonication as a method to increase the effect was also compared. The sample and DPPH solution were mixed 1: 1, reacted for 30 min in the dark at room temperature, and the absorbance was when measured at 517-nm wavelength. Ascorbic acid, a representative antioxidant, was used as a standard material at concentrations of (1, 5, 25, 50, and 100) µg/mL.

Measurement of Pigment Content by Culture Conditions

The antioxidant activity of *R. sphaeroides* cultured in the stationary phase under aerobic and anaerobic conditions was compared. The antioxidant activity of the cells for each condition was compared by dividing the measured antioxidant effect power for each condition by the OD_{600} value of the cell. Carotenoids and bacteriochlorophyll contained in R. sphaeroides are included as representative antioxidants. Experiments were conducted to measure the content of these pigments in cells cultured in the stationary phase of aerobic and anaerobic conditions. To measure the bacteriochlorophyll content [11], cells were obtained from 10 mL of the main culture medium, washed twice with distilled water, and then freeze-dried before use. Acetone, ethanol, and SIST medium and then 1 mL of the mixed medium were added to the dried cells, and the mixture was reacted in the dark for 1 h to measure the absorbance of the sample at 772-nm wavelength. Also, by measuring the carotenoid content [12], the cells obtained from 10 mL of culture medium were washed twice with distilled water and then lyophilized to measure the dry weight of the cells by measuring the weight of cells. To the dried cells, 1 mL of 3 M HCl was added, and the cells were incubated at 28 °C and 180 rpm for 30 min and centrifuged at 1,000 rpm for 20 min to separate the supernatant. After adding 1 mL of acetone to the pellet, reacting under the same conditions, and centrifuging again at 10,000 rpm for 20 min, the supernatant was recovered, and the absorbance was measured at 480-nm wavelength. After measurement, the content of carotenoids is measured by the method below.

$$\text{Carotenoid content (\%)} = \frac{1000 \times \text{Absorbance value} \times \text{Dilution rate} \times \text{Acetone amount}}{0.16 \times \text{Dry weight}}.$$

Data Analysis

The results of all experiments were obtained from three independent samples measured simultaneously for error analysis. The results are presented together with the mean and standard deviations the different experimental conditions. These data were analyzed using Sigma Plot (Systat Software, Inc., USA).

Results

Antioxidant Activity of *R. sphaeroides* and *E. coli* Using DPPH Assay

DPPH radical scavenging activity for each culture condition was tested to determine antioxidant productivity according to various culture conditions, such as aerobic and anaerobic conditions, and exponential or stationary phases. As a result of measuring the antioxidant activity by measuring the ability to remove DPPH radicals, the lowest cellular OD_{600} value was shown in the anaerobic exponential phase and the least number of cells and antioxidant activity. Conversely, the highest antioxidant activity was shown in the stationary phase of aerobic conditions and had the highest OD_{600} value, indicating that the greatest number of cells grew among the four culture conditions. Figure 1 suggests that the antioxidant activity is most effective in the stationary phase of aerobic conditions, and the ability of *R. sphaeroides* can be seen, in that the antioxidant activity is proportional to the amount of cell growth. To confirm the suitability of *R. sphaeroides* as a strain for producing antioxidants, the results were compared after culturing in the exponential phase and stationary phase under the same aerobic conditions as *E. coli*, a gram-negative bacterium, and then performing an antioxidant experiment (Fig. 2). The antioxidant activity of *R. sphaeroides* in both exponential and stationary phases was significantly higher than that of *E. coli*. As a result, the *R. sphaeroides* was shown to be suitable as a strain to produce antioxidants among Gram-negative bacteria. These results suggest that *R. sphaeroides* is suitable as an antioxidant.

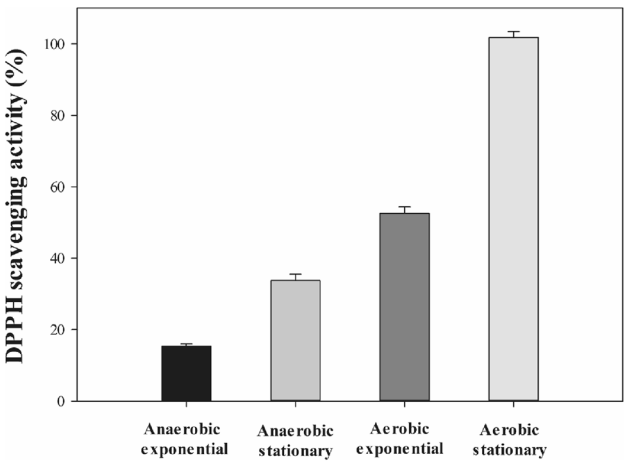

Fig. 1 Comparison of the antioxidant activity of *Rhodobacter sphaeroides* cultured until exponential or stationary phases under anaerobic and aerobic culture conditions

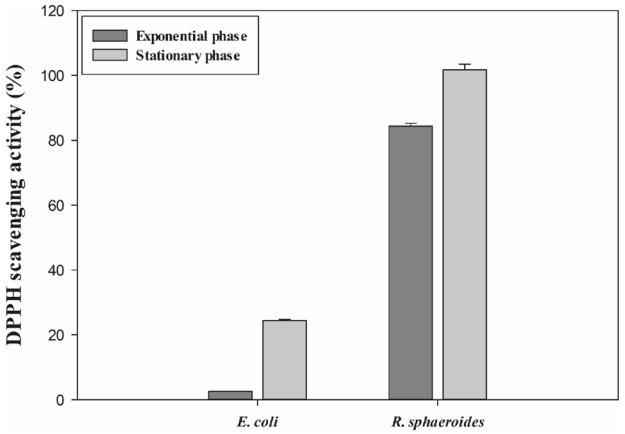

Fig. 2 Antioxidant activity of *Escherichia coli* and *R. sphaeroides* cultured until the exponential or stationary phase under aerobic conditions

Optimized Culture Conditions and Increase Antioxidant Effect

Sonication was performed as a method of extracting antioxidants to increase the antioxidant activity of cells. As a result, Fig. 3a shows that the antioxidant activity was about 2 times higher in the cells sonicated for each condition under anaerobic conditions. In addition, under aerobic conditions, the results of sonication in the exponential phase suggest the most effective results, and higher antioxidant activity was measured in the cells subjected to sonication in the stationary phase as well. As a result of this experiment, the antioxidant activity increased after sonication of *R. sphaeroides* under any culture conditions, suggesting that the antioxidant substances of these cells are more present in the cells. From the previous results, it was found that the antioxidant activity of *R. sphaeroides* was proportional to the OD_{600} value of the culture medium in which the cells were grown. In Fig. 3b the OD_{600} value of the cells is divided by the measured antioxidant activity value to determine the antioxidant effect by each cell under aerobic and anaerobic conditions. As a result, it was shown that the antioxidant activity of each cell was higher under anaerobic conditions than under aerobic conditions. That is, although the cells grew more in aerobic conditions than in anaerobic conditions, it suggests that the antioxidant activity of cells cultured in anaerobic conditions was higher.

Comparison of Antioxidant Activity and Pigment Content by Culture Condition

As the antioxidant activity of cells cultured under anaerobic conditions was measured to be higher, the contents of carotenoids and bacteriochlorophyll, which are representative pigments contained in *R. sphaeroides*, were measured to investigate the origin of the antioxidants produced in

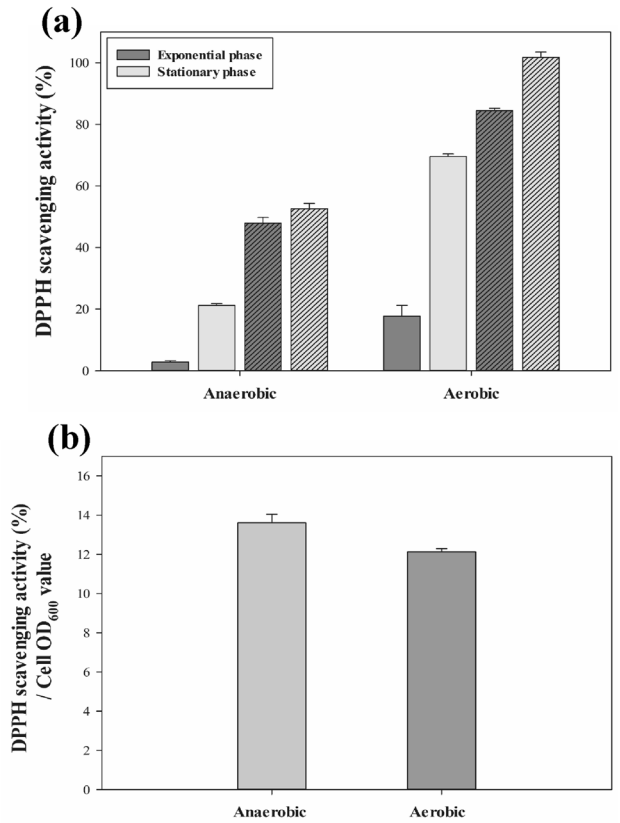

Fig. 3 Comparison of antioxidant activity using sonication of *R. sphaeroides* cultured until exponential and stationary phases under anaerobic and aerobic culture conditions and the antioxidant activity of each cell divided by OD_{600} value. **a** Antioxidant activity after sonication (The shaded area is the result after sonication). **b** Antioxidant activity of cells divided by OD_{600} value

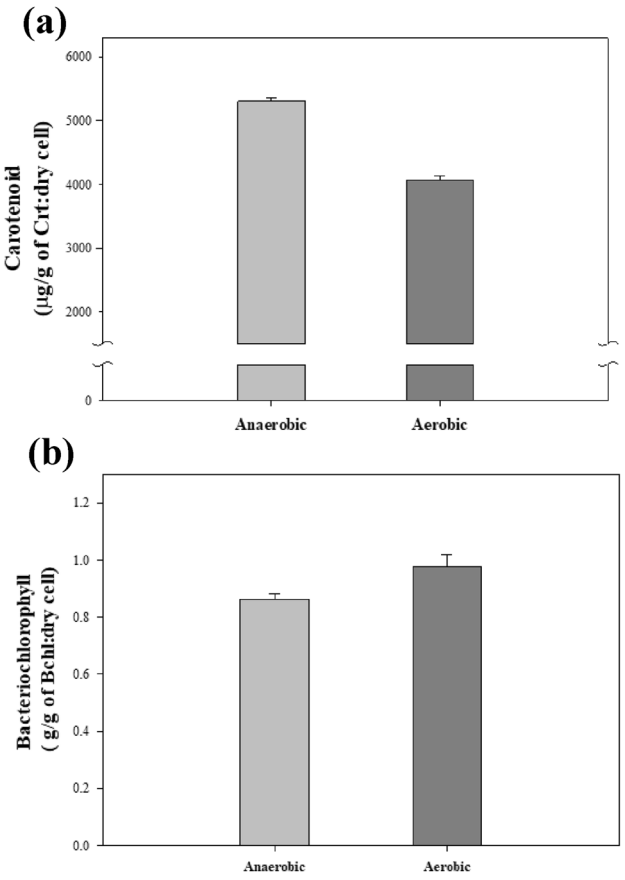

Fig. 4 Measurement of pigments contained in *R. sphaeroides* cultured until the stationary phase of aerobic and anaerobic conditions. **a** Content of carotenoids and **b** content of bacteriochlorophyll

these cells. (Fig. 4). Figure 4a shows that the carotenoid content was higher in anaerobic and showed the same tendency to Fig. 3b, suggesting that it is related to the antioxidant activity of each cell. Conversely, Fig. 4b shows that the content of bacteriochlorophyll was higher in anaerobic than in aerobic conditions, but there was no significant difference. Based on these results, the antioxidant substances and pigments that affect the antioxidant activity of each cell are derived from carotenoids.

Discussion

In this paper, optimal culture conditions for the *R. sphaeroides* production of antioxidants were studied. First, to prove that this *R. sphaeroides* is suitable as a strain for the production of antioxidants, the capacity was compared with *E. coli*, a gram-negative bacteria. The results showed that *R. sphaeroides* was suitable as an antioxidant-producing strain because it showed significantly higher antioxidant capacity than *E. coli*. To determine the antioxidant productivity according to various culture conditions, such as aerobic or anaerobic conditions and exponential or stationary phases, the DPPH radical scavenging activity was tested for each culture condition. As a result, the optimal culture conditions were identified by showing the highest DPPH radical scavenging activity in the aerobic stationary phase. In addition, to increase the productivity of antioxidants in *R. sphaeroides* cultured under each culture condition, we measured the antioxidant activity of cells with or without sonication treatment. As the antioxidant activity after sonication treatment was high, it was suggested that the antioxidant substances of *R. sphaeroides* exist inside the cell and can increase the antioxidant productivity due to sonication treatment. When comparing the anaerobic and aerobic conditions among various culture conditions, a higher antioxidant capacity was detected under the aerobic condition. Furthermore, to determine the antioxidant capacity of each cell under these conditions, the results were derived by dividing the cell culture amount, that is, the OD value, by the antioxidant activity for each condition. In addition, the contents of carotenoid and

bacteriochlorophyll were measured to confirm the antioxidant-derived substances of *R. sphaeroides*. The carotenoid content was higher in the anaerobic condition, and this trend was the same as that of the antioxidant activity of each cell [7, 8]. Conversely, the bacteriochlorophyll content may be higher under aerobic conditions, but not significantly different from anaerobic conditions. Based on this, it is suggested that the content of antioxidants produced by each of the anaerobic and aerobic culture conditions is different.

Conclusion

In conclusion, the optimal culture conditions for antioxidant production of *R. sphaeroides* show the highest antioxidant activity in the aerobically stationary phase. However, the antioxidant activity of cells is higher under the anaerobic condition, suggesting that it is related to the carotenoid content of *R. sphaeroides*.

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Author Contributions SL performed all of the experiments. SL and JY wrote the main manuscript text. Y-HK and JM designed the overall experimental concept and revised the manuscript; approved the final version: all authors.

Declarations

Conflict of interest Subin Lee, Jaeyoung Yu, Yang-Hoon Kim, and Jiho Min declare that they have no conflict of interest.

Informed Consent Informed consent was obtained from all individual participants included in the study

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

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