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African Journal of Biochemistry Research

April-June 2022
ISSN 1996-0778
DOI: 10.5897/AJBR
www.academicjournals.org

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Vol. 16(2), pp. 32-38, April-June 2022
DOI: 10.5897/AJBR2022.1145
Article Number: A0BD44F69197
ISSN 1996-0778
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Properties of antioxidants produced by *Rhodobacter sphaeroides*

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Received 11 March, 2022; Accepted 20 May, 2022

This study investigated the properties of antioxidants produced by *Rhodobacter* (*R.*) *sphaeroides*, a purple, non-sulfur photosynthetic bacterium. *R. sphaeroides* was fractionated into water-soluble and ethanol-soluble fractions, and the ethanol-soluble fraction showed stronger activity. Light irradiation significantly decreased the antioxidant activity of the ethanol fraction. The absorption spectra of the ethanol fraction showed that the peaks of bacteriochlorophyll (BChl) and spheroidene decreased after light irradiation. BChls and carotenoid compounds, which are photosynthetic pigments, were believed to be among the antioxidants in the ethanol fraction. Therefore, BChl *a* was obtained from *R. sphaeroides* and evaluated its antioxidant activity. BChl *a* scavenged DPPH radicals in a concentration-dependent manner. The absorption spectra of the reaction solution showed a decrease in the peak of the DPPH radical at 500 nm, and a decrease in the peak of BChl *a* at 760 nm and a shift in wavelength, suggesting that BChls exhibit antioxidant activity through their own oxidation and destruction. Therefore, this study suggests that *R. sphaeroides* produces antioxidants, which include photosynthetic pigments, and thus highlights the potential of photosynthetic bacteria as a source of antioxidants.

Key words: *Rhodobacter sphaeroides*, Photosynthetic bacteria, Bacteriochlorophyll, Antioxidant, Physiological Functions

INTRODUCTION

Lifestyle-related diseases are increasing due to changes in dietary habits, leading to increasing health concerns. Reactive oxygen species (ROS) generated in the body are one of the causes of lifestyle-related diseases. In addition to the defense mechanisms in the body, ROS are eliminated by antioxidants ingested from the diet. Therefore, antioxidants are expected to help prevent lifestyle-related diseases (Yamakado, 2014). There are a variety of naturally occurring antioxidants. Naturally occurring antioxidants in plants include vitamins C and E, polyphenolic compounds such as anthocyanins and catechins, and carotenoid compounds (Nishibori, 1998; Terao and Nagao, 1999). In addition, microbe-derived antioxidants, such as those produced by microorganisms themselves and from fermented foods using microorganisms, have also been reported (Nishihara et al., 2009; Han et al., 2020; Li and Wang, 2021). The production of substances using microorganisms' benefits

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from the high microbial diversity, and mass production is possible at a low cost. Therefore, the use of microorganisms as a source of antioxidants is considered highly promising. Similar to plants, photosynthetic bacteria are autotrophs that use light energy to produce ATP and carbon dioxide to produce carbohydrates. Photosynthetic bacteria have been studied for a number of applications, including the production of useful substances such as hydrogen, environmental purification, and animal feed (Kobayashi, 1984; Asada et al., 2008; Kim et al., 2013; Lu et al., 2016). Photosynthetic bacteria produce various useful compounds, including photosynthetic pigments such as bacteriochlorophylls (BChls) and carotenoid compounds. In the first step of the light reaction in photosynthetic bacteria, light energy is received by BChls and carotenoids in a pigment-protein complex. The BChls are oxidized by receiving light energy and reduced by transferring light energy to the other pigments. The pigments are held by the polypeptides and protect them from light energy and photo-redox reactions (Fiedor et al., 2012). Therefore, photosynthetic pigments or polypeptides are expected to have antioxidant potential. Although the antioxidant activity of chlorophyll, a plant-derived photosynthetic pigment, has been previously reported (Nishibori and Namiki, 1988; Lanfer-Marquez et al., 2005), few studies have investigated the antioxidant activity of photo-synthetic pigments produced by photosynthetic bacteria (Li et al., 2017; An et al., 2019; Kars et al., 2020). This study investigated the antioxidant activities of carotenoid pigment-producing and-non-producing strains of *Rhodobacter* (*R.*) *sphaeroides*, a purple non-sulfur photosynthetic bacterium. In addition, other characteristics of the antioxidants produced by *R. sphaeroides* were also studied.

MATERIALS AND METHODS

Reagents

2-Morpholinoethanesulfonic acid, monohydrate (MES) was purchased from Dojindo Laboratories (Kumamoto, Japan). 1.1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). All other reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Photosynthetic bacteria

The purple non-sulfur photosynthetic bacteria *R. sphaeroides* strain 2.4.1 and R-26 were used in this study. *R. sphaeroides* 2.4.1 produces the carotenoid spheroidene, whereas *R. sphaeroides* R-26 does not produce carotenoids. *R. sphaeroides* was cultured in liquid medium, as previously reported (Kobayashi et al., 2004). After inoculation, the culture was incubated in a water bath at 25°C under light irradiation using 60 W incandescent lamp. The culture solution was then centrifuged (10,000×g, 10 min, 4°C), and the cells were collected. These recovered cells were then freeze-dried. The freeze-dry cells were grinding by mortar at room temperature for 10 min.

Extraction of antioxidants

To 0.1 g of the cell powder obtained by freeze drying as mentioned above, 10 ml of ethanol solutions of various concentrations (0, 50, 70, 80 and 100%) were added, and the mixture was stirred in the dark at 4°C for 24 h using a magnetic stirrer. The mixture was then centrifuged (3,000×g, 10 min), and the supernatant was used as the ethanol extraction. Meanwhile, 10 ml of distilled water was added to 0.1 g of the cell powder, and the mixture was stirred with a magnetic stirrer for a period of 24 h in the dark at 4°C. The mixture was then centrifuged, and the supernatant was used as the water fraction. The precipitate was lyophilized and the dried product was similarly extracted with ethanol. The obtained supernatant was used as the ethanol fraction. The absorption spectra of each sample were measured using a spectrophotometer (V-630, JASCO Corporation).

Measurement of DPPH radical scavenging activity

The samples were prepared to have a final ethanol concentration of 50%. The sample (0.6 ml) was placed in a test tube, 200 μM DPPH solution (0.3 ml) and 140 mM MES buffer solution (0.3 ml, pH7.0) were added, and the reaction solution was transferred to a micro tube after stirring for 10 s using a vortex mixer. The reaction solution was centrifuged (10,000 × g, 5 min), and 10 min after the DPPH solution was added, the absorbance at 517 nm was measured using a spectrophotometer (V-630, JASCO). The antioxidant activity was calculated by subtracting the absorbance of the sample itself, from the absorbance of 50% ethanol without the sample.

Effect of temperature on each fraction

To investigate the effect of temperature on the antioxidants, the water and ethanol fractions were incubated in the dark overnight at various temperatures (5, 25, and 40°C). The absorption spectra and DPPH radical scavenging activities of each fraction were measured before and after incubation.

Effect of light on each fraction

To investigate the effect of light on the antioxidants, the water and ethanol fractions were incubated overnight at 25°C under the light of an incandescent lamp. The absorption spectra and DPPH radical scavenging activities of each fraction were measured before and after incubation.

DPPH radical scavenging activity of BChl *a*

The cell powder (1 g) was added to 20 ml of distilled water, followed by addition of 15 ml of methanol. After 10 min, centrifugation (10,000×g, 5 min) was performed and the supernatant obtained was added to 2 ml of dioxane, followed by addition of 8 ml of distilled water. The mixture was incubated at -20°C. The mixture was centrifuged (10,000×g, 5 min), and BChl *a* was collected as a precipitate and lyophilized. BChl *a* was then dissolved in 50% ethanol, and DPPH radical scavenging activity and absorption spectra were measured.

Statistical analysis

The DPPH radical scavenging activity of the water and ethanol fractions obtained from the cell powder were tested for significant

difference using t-test for homemade program on Microsoft Excel for Microsoft 365. The difference was considered significant if the p-value, the probability of significance, was less than 0.05.

RESULTS

DPPH radical scavenging activity of *R. sphaeroides*

The culture medium of *R. sphaeroides* was separated by centrifugation into cells and culture supernatant, and the cells were lyophilized and extracted with water or ethanol solution to determine the DPPH radical scavenging activity. The DPPH radical scavenging activity was not observed in the lyophilized culture supernatant of either *R. sphaeroides* R-26 or 2.4.1 (data not shown). Therefore, neither *R. sphaeroides* was considered to produce extracellular antioxidants. However, the DPPH radical scavenging activity of extracts of both *R. sphaeroides* R-26 and 2.4.1 cells increased depending on the sample concentration in the extract obtained from the lyophilized cells powder with water or ethanol solution (Figure 1). For the extracts of *R. sphaeroides* 2.4.1 cell, the activity of the extract with distilled water was the lowest, and the higher the ethanol concentration, the stronger was the activity. The *R. sphaeroides* R-26 and 2.4.1 cells were extracted with water to obtain the water-soluble component, and the residue was then extracted with ethanol to separate the water-insoluble and ethanol-soluble components. The antioxidant activities of each component were evaluated. Both the *R. sphaeroides* R-26 and 2.4.1 cells showed higher activity in the ethanol fraction than in the water fraction (Figure 2). In the water fraction, the activity of the *R. sphaeroides* R-26 cell was approximately 40%, whereas that of *R. sphaeroides* 2.4.1 cell was only 4%. This suggests that the *R. sphaeroides* R-26 produces more water-soluble antioxidants than the *R. sphaeroides* 2.4.1. In the ethanol fraction, since the *R. sphaeroides* 2.4.1 produces spheroidene, it is considered to show higher antioxidant activity than the *R. sphaeroides* R-26 extraction. However, there was no significant difference in the antioxidant activities between the two *R. sphaeroides*. Therefore, it is possible that carotenoids have a weak effect on DPPH radical scavenging activity, or that *R. sphaeroides* R-26 produces other non-water-soluble antioxidants that make up for the lack of carotenoids in it. In both *R. sphaeroides*, the ethanol fraction was green in color, suggesting that BChls were present in this fraction and affected the antioxidant activity. Since there was a difference in antioxidant activity between the water-soluble and water-insoluble fractions, the absorption spectra of both fractions were measured. In the spectra of the water-soluble fraction, peaks were observed around 280 nm and 360-400 nm, in case of both *R. sphaeroides* 2.4.1 and R-26 cells (Figure 3a). In the ethanol fraction, characteristic peaks at 360 nm, 550-600 nm, and 780 nm, were observed in both *R. sphaeroides* (Figure 3b).

Effect of temperature and light on bacterial fractions

To evaluate the effect of temperature on the antioxidants produced by *R. sphaeroides*, the water and ethanol fractions were incubated for 24 h at 5, 25 and 40°C, after which the DPPH radical scavenging activity was measured. The DPPH radical scavenging activity of the *R. sphaeroides* R-26 slightly decreased at 40°C in the water fraction, and at 5 and 25°C in the ethanol fraction, after incubation (Figure 4). The DPPH radical scavenging activity of the *R. sphaeroides* 2.4.1 was not significantly different between the two fractions before and after incubation. The absorption spectrum of each fraction showed a slight decrease after incubation (data not shown). To evaluate the stability of the antioxidants under light irradiation, both fractions were incubated at 25°C for 24 h under light or dark conditions, after which the DPPH radical scavenging activity was measured. The DPPH radical-scavenging activity of the water fractions of both *R. sphaeroides* cells after incubation was not significantly different ($p<0.05$) from that before incubation, regardless of light exposure (Figure 5). In the ethanol fraction, there was a slight decrease in activity after incubation in the dark in case of both strains, but the difference was not significant. However, the activity after light irradiation was significantly ($p<0.05$) decreased, approximately 50% in the *R. sphaeroides* 2.4.1 cells and by approximately 60% in the *R. sphaeroides* R-26 cells. These results suggest that the activity of the water-soluble antioxidants produced by both *R. sphaeroides* cells was not affected by light, whereas that of non-water-soluble antioxidants was strongly affected by light. The absorption spectra of the ethanol fractions of both *R. sphaeroides* cells, before and after light irradiation, are shown in Figure 6. The absorption spectra did not change significantly when both the fraction of *R. sphaeroides* cell were incubated in the dark. However, after light irradiation, the peaks near 360 nm, 550-600 nm, and 780 nm, corresponding to BChl *a*, decreased significantly. For the *R. sphaeroides* 2.4.1 cells, the peak at 400-500 nm, corresponding to spheroidene, was also significantly reduced. Therefore, BChls and carotenoids, which are photosynthetic pigments, were considered to be involved in the antioxidant activity in the ethanol fraction. Among these compounds, BChl *a* is present in both strains; therefore, BChl *a* or its derivative were considered to be largely involved in the antioxidant activity of *R. sphaeroides*.

DPPH radical scavenging activity of BChl *a*

BChl *a* was isolated from the *R. sphaeroides* R-26 cells, and its DPPH radical scavenging activity was determined. BChl *a* scavenged DPPH radicals in a concentration-dependent manner (Figure 7a), indicating that it is an antioxidant. The absorption spectrum showed that the peak of the DPPH radical at 500 nm decreased with the addition of BChl *a*, the peak of BChl *a* at 760 nm

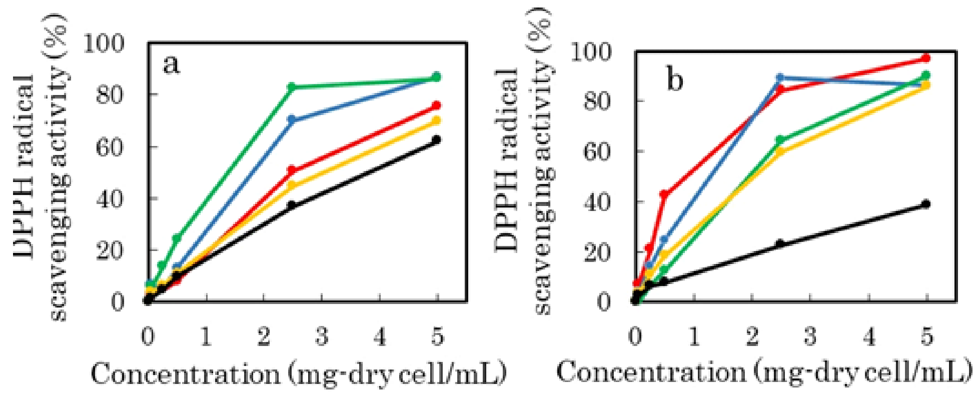

Figure 1. DPPH radical scavenging activity of *R. sphaeroides* R-26 (a) AND 2.4.1 (b) extracts. (●,100% Ethanol; ●,80% Ethanol; ●,70% Ethanol; ●, 50% Ethanol; ●, Water)
Source: Authors

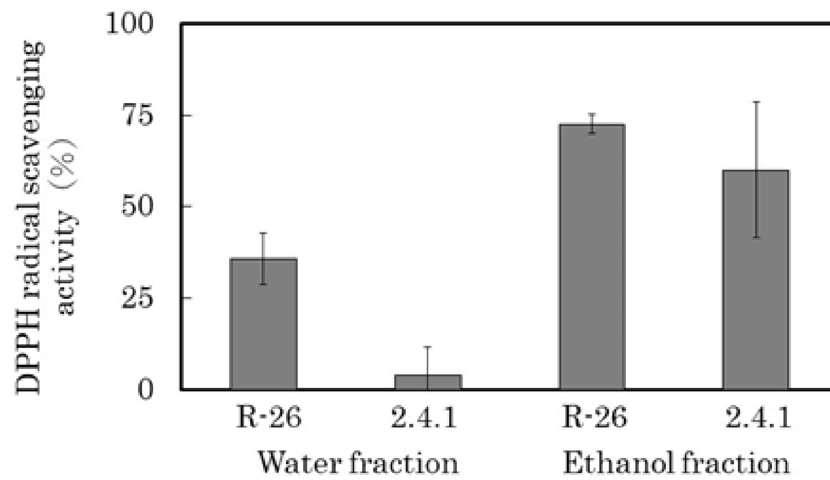

Figure 2. DPPH radical scavenging activity of each fraction.
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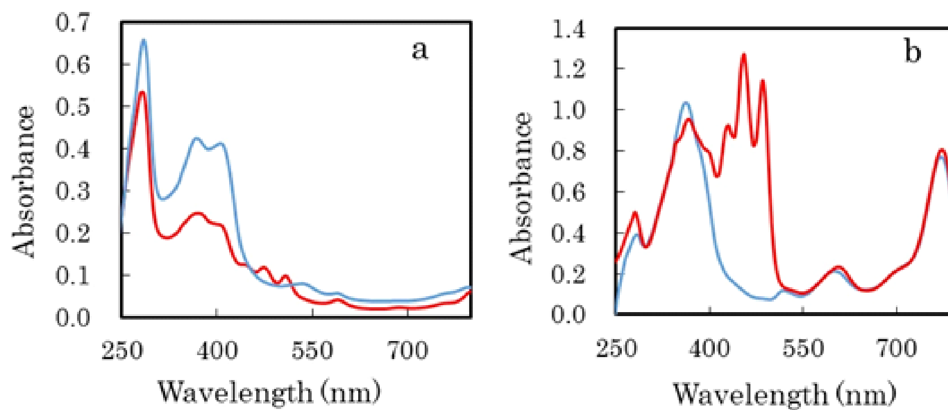

Figure 3. Absorption spectra of water fraction (a) and ethanol fraction (b). (▬, *R.sphaeroides* R26; ▬,*R. sphaeroides* 2.4.1).
Source: Authors

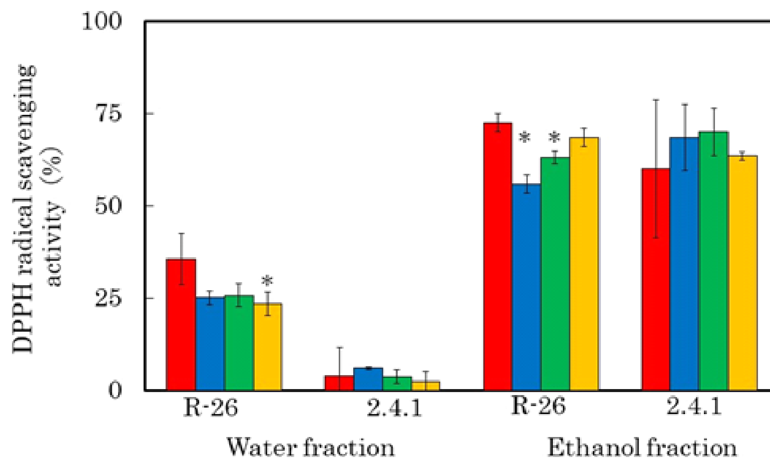

Figure 4. Effect of heat treatment on DPPH radical scavenging activity of water or ethnol fraction from *R. sphaeroides* R-26 and 2.4.1 cell. (■, Before; ■, 5°C; ■, 25°C; ■, 40°C).*$p<0.05$
Source: Authors

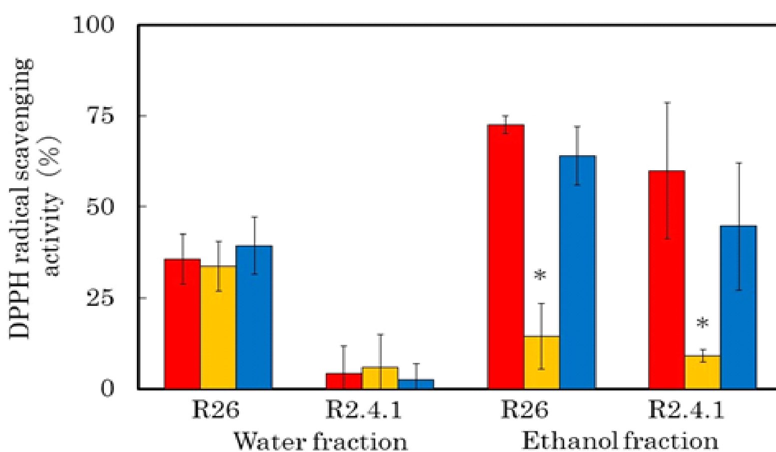

Figure 5. Effect of light iradiation on DPPH radical scavenging activity of water or ethnol fraction from *R. sphaeroides* R-26 and 2.4.1 cell. (■, Before; ■, Light; ■ Dark).*$p<0.05$.
Source: Authors

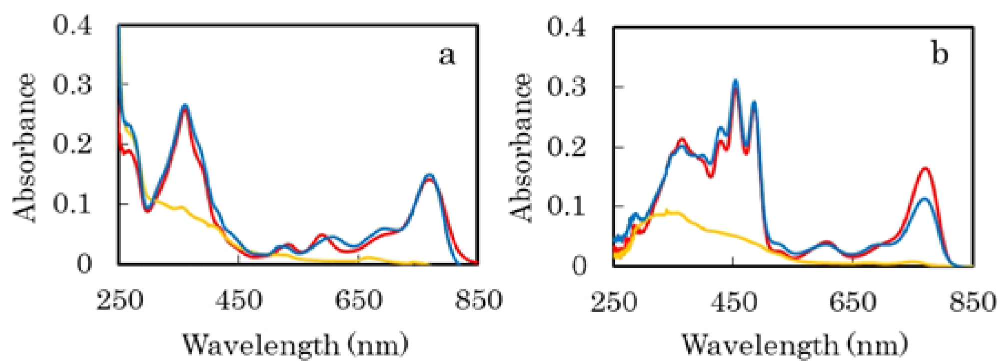

Figure 6. Absorption spectra of light and dark conditions for *R.sphaeroides* R-26 ethanol fraction (a), and *R. sphaeroides* 2.4.1 ethanol fraction (b). (▬, Before; ▬ , Light; ▬, Dark).
Source: Authors

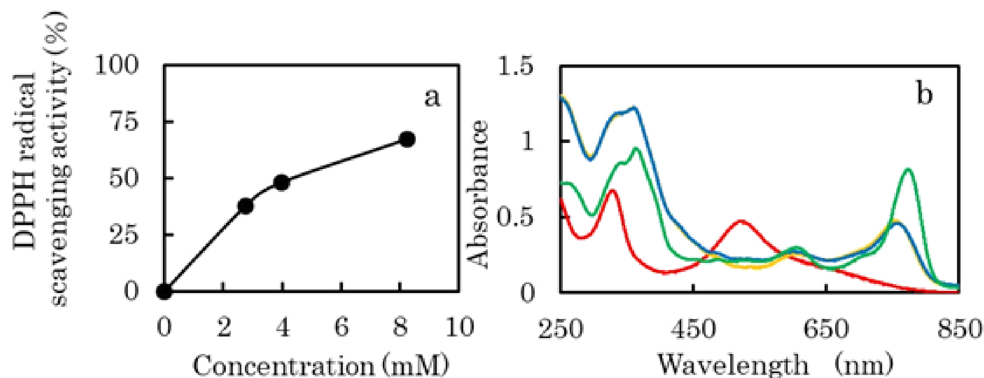

Figure 7. Concentration dependence of BChl *a* for DPPH radical scavenging activity (a), and absorbtion spectra of BChl *a* after elapsed time of DPPH radical scavenging activity measurement (b) (red, DPPH; green, 0 min; blue, 1.0 min; yellow, 8.5 min).
Source: Authors

decreased, BChl *a* was destructed (Figure 7b). In addition, the peak at 760 nm was shifted to the short wavelength side.

DISCUSSION

There is no DPPH radical scavenging activity of culture medium of *R. sphaeroides* R-26 and 2.4.1. However high DPPH scavenging activity of cell-free supernatant of *R.sphaeroides* 8513 has been reported (An et. al., 2019). There exist differences on DPPH radical scavenging activity of culture medium between *R. sphaeroides* 2.4.1 and R-26 and *R. sphaeroides* 8513. Although differences of detail of DPPH radical scavenging activity method was found, this suggest that presence of antioxidant on cell surface depends on bacterial strain and/or cultivation methods. We found differences of DPPH radical scavenging activity of cell extract by inorganic and organic solvents. The antioxidant activities of extract by acetone have been reported, which show the activities of extract by acetone increased with the increase in the concentration of lyophilized cells of *R. sphaeroides* 3757 (Li et al., 2017). The concentration dependence of lyophilized *R. sphaeroides* 3757 cells is very similar to that of lyophilized *R. sphaeroides* 2.4.1 cells. The study estimates the value of IC_{50} that is about 50 for ratio of acetone to *R. sphaeroides* 3757 cells. The value of IC_{50} is estimated about 1-mg *R.sphaeroides* 2.4.1 cell / ml-ethanol. These results suggest that ethanol is useful solvent for extract of the antioxidant compounds. Furthermore, the DPPH radical scavenging activities are changed by the treatments of lyophilized cell, that is, superfine grinding treatment and ultrasonic treatment (Li et al., 2017). Also estimated was the value of IC_{50} of extract by water for *R. sphaeroides* 2.4.1 cells is about 8 mg dry-cell / ml-water and that for *R. sphaeroides* O.U. 001 cells is about 0.3 mg dry-cell / ml-water (Kars et . al., 2020). The value of extract of water for *R. sphaeroides* 2.4.1 cells is much smaller than that for hydrolyzed *R. sphaeroides* O.U. 001 cells. These results were showed that the DPPH radical scavenging activity were depend on not only bacterial strain and cultivating and powdering method of cells but also extraction solvent.

The extract of *R. sphaeroides* R-26 cells showed stronger activity in the extract with 70% ethanol than in the extract with 100% ethanol. The extract of *R. sphaeroides* R-26 cells in distilled water showed the lowest activity, as in the case of the *R. sphaeroides* 2.4.1 cells, but showed higher activity than the water extract of *R. sphaeroides* 2.4.1 cells. Therefore, it seems that the optimal solvent composition for extracting the antioxidants differs between the *R. sphaeroides* 2.4.1 and R-26 cells, and the components exhibiting antioxidant activity differ as well. *R. sphaeroides* 2.4.1 produces a carotenoid called spheroidene, and carotenoid compounds are known to be sparingly soluble in water and exhibit antioxidant activity (Maoka, 2007). Therefore, the extract with 100% ethanol was considered to show the highest activity in the extract of *R. sphaeroides* 2.4.1 cells. On the other hand, the *R. sphaeroides* R-26 is a carotenoid less-mutant that does not produce spheroidene, but showed the same level of DPPH radical scavenging activity as the extract of *R. sphaeroides* 2.4.1 cells in case of the extract with 70% ethanol. And the extract of the *R. sphaeroides* R-26 cells with distilled water was approximately twice as active as that of the *R. sphaeroides* 2.4.1 cells. Therefore, the *R. sphaeroides* R-26 is thought to produce a water-insoluble antioxidant that takes the place of spheroidene, and a water-soluble antioxidant that the *R. sphaeroides* 2.4.1 does not produce. The profile and peak positions of absorption spectra in this region of water-soluble fraction are very similar to that of the suspended solution of intact cells. Because the cells were lyophilized, the water-soluble fractions might contain intra cytoplasmic membrane. The

280 nm and 360-400-nm peaks were higher in the *R. sphaeroides* R-26. The substance corresponding to these peaks was considered to be a water-soluble antioxidant, and the *R. sphaeroides* R-26 cell is considered to exhibit increased DPPH radical scavenging activity in the water fraction because it produced more water-soluble antioxidants. From the peak positions of absorption spectra of ethanol-soluble fraction of *R. sphaeroides* R-26, main component of ethanol-soluble fraction was considered to BChl *a* (Tamiaki and Mizoguchi, 2009). The peak at 400-500 nm was observed only in case of *R. sphaeroides* 2.4.1, and corresponds to spheroidene (Takaichi 2009). These results suggested that *R. sphaeroides* produces water-soluble and water-insoluble antioxidants. BChl *a* and spheroidene are water-insoluble. As antioxidant activity has been reported for plant chlorophyll *a* and pheophytin *a*, which is a derivative of chlorophyll *a* (Nishibori and Namiki,1988; Lanfer-Marquez et al., 2005), BChl *a* and its derivatives are expected to possess antioxidant activity as well.

BChl *a* shows DPPH radical scavenging activity, and the change in absorption spectrum in this reaction seems to be similar to the change in BChl *a* due to photooxidation. Therefore, it is considered that the antioxidant action of BChl *a* is not due to the reversible redox reaction, but due to the oxidation and destruction of BChl *a* itself.

Conclusion

Since photosynthetic bacteria can utilize carbon dioxide, effective utilization is important from the viewpoint of carbon dioxide reduction. In this study, *R. sphaeroides* was found to produce antioxidants including BChl. To date, there have been few reports on the antioxidant activity of BChl, a photosynthetic pigment found in bacteria. In the future, by investigating the properties and mechanisms of these antioxidants, it can be determined whether photosynthetic bacteria can be an effective source of physiologically functional substances, as well as those produced by other photosynthetic bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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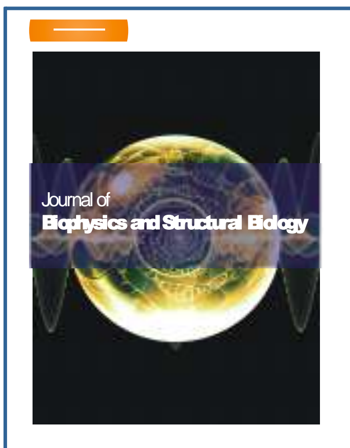

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