

Article

Modulation of Antioxidant Activity Enhances Photoautotrophic Cell Growth of *Rhodobacter sphaeroides* in Microbial Electrosynthesis [†]

Yu Rim Lee ^{1,2,‡} , Soo Youn Lee ^{1,‡}, Jiye Lee ¹, Hui Su Kim ^{1,3}, Jin-Suk Lee ¹, Won-Heong Lee ^{4,*} and Sangmin Lee ^{1,*} 

¹ Gwangju Bio/Energy R&D Center, Korea Institute of Energy Research, Gwangju 61003, Korea; rimin9@kier.re.kr (Y.R.L.); syl@kier.re.kr (S.Y.L.); jiye.lee@kier.re.kr (J.L.); gmltnwrl@kier.re.kr (H.S.K.); bmjslee@kier.re.kr (J.-S.L.)

² Interdisciplinary Program of Agriculture and Life Sciences, Chonnam National University, Gwangju 61186, Korea

³ Department of Advanced Chemicals and Engineering, Chonnam National University, Gwangju 61186, Korea

⁴ Department of Integrative Food, Bioscience and Biotechnology, Chonnam National University, Gwangju 61186, Korea

* Correspondence: wonhlee@jnu.ac.kr (W.-H.L.); silverism@kier.re.kr (S.L.)

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[‡] These authors contributed equally to this work.

Abstract: Global warming is currently accelerating due to an increase in greenhouse gas emissions by industrialization. Microbial electrosynthesis (MES) using electroactive autotrophic microorganisms has recently been reported as a method to reduce carbon dioxide, the main culprit of greenhouse gas. However, there are still few cases of application of MES, and the molecular mechanisms are largely unknown. To investigate the growth characteristics in MES, we carried out growth tests according to reducing power sources in *Rhodobacter sphaeroides*. The growth rate was significantly lower when electrons were directly supplied to cells, compared to when hydrogen was supplied. Through a transcriptome analysis, we found that the expression of reactive oxygen species (ROS)-related genes was meaningfully higher in MES than in normal photoautotrophic conditions. Similarly, endogenous contents of H₂O₂ were higher and peroxidase activities were lower in MES. The exogenous application of ascorbic acid, a representative biological antioxidant, promotes cell growth by decreasing ROS levels, confirming the inhibitory effects of ROS on MES. Taken together, our observations suggest that reduction of ROS by increasing antioxidant activities is important for enhancing the cell growth and production of CO₂-converting substances such as carotenoids in MES in *R. sphaeroides*.

Keywords: antioxidant; microbial electrosynthesis; *Rhodobacter sphaeroides*



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1. Introduction

A climate crisis has arisen as carbon dioxide emissions have increased due to industrialization [1]. Various technologies have been proposed to reduce carbon dioxide emissions, and biological CO₂ fixation is attracting attention as a desirable alternative. Recently, sustainable bioelectrochemical systems have been proposed to convert CO₂ into valuable chemicals by microbial electrosynthesis (MES) using electroactive autotrophs. In this system, electroactive microorganisms can utilize CO₂ using electrons as a reducing power [2]. Production of high value-added materials such as polyhydroxybutyrate (PHB), lycopene and α -humulene using *Cupriavidus necator* (*C. necator*) in an MES reactor has been reported [3–5]. Acetate was also continuously produced from CO₂ by a mixed microbial consortium via an MES system [6].

One of the purple non-sulfur bacteria, *Rhodobacter sphaeroides*, has emerged as a promising electroactive autotroph. *Rhodobacter sphaeroides* (*R. sphaeroides*) can interact directly with the cathode to utilize electrons for cell growth. In addition, exposure to antibiotic selective pressures enhanced biofilm formation and increased PHB accumulation under nitrogen-limited MES conditions [7]. *R. sphaeroides* is a suitable strain for CO₂ biorefinery because of its intracellular metabolic pathways and tractable genetic engineering. In particular, terpenoid and carotenoid biosynthesis naturally develops well, which is advantageous for the study of their production. Increases in the yield and productivity of amorpha-4,14-diene production were reported through the enhancement of terpene synthase activities, and lycopene was also produced through the modification of a carotenoid biosynthetic gene [8,9].

The generation of reactive oxygen species (ROS) is inevitable in cellular metabolism. Maintaining a balance between the formation and degradation of ROS is very important to increase cell viability. Various intracellular antioxidants, such as ascorbic acid, are known to be effective in reducing and maintaining endogenous levels of ROS [10]. Carotenoids synthesized in *R. sphaeroides* exhibit considerable antioxidant activities against cancer cells without any cellular toxicity [11]. The accumulation of carotenoids in *Deinococcus radiodurans* increases resistance to ROS, enhancing defense mechanisms against environmental stresses [12]. Furthermore, the addition of ascorbic acid helped to alleviate the side effects of saline stress and enhanced biosynthesis of secondary metabolites, including carotenoids, phenolics, and flavonoids [13].

In this study, we investigated the differences in the growth characteristics of *R. sphaeroides* according to reducing sources such as electricity and hydrogen. Many changes in the expression of ROS-related genes were observed through a transcriptome analysis, and it was confirmed that the endogenous levels of ROS and oxidative damage were greater in MES. Treatment of exogenous ascorbic acid, as an antioxidant, reduced the ROS contents and promoted cell growth. This suggests that modulation of antioxidant activity is important to enhance photoautotrophic growth and production of valuable metabolites, such as carotenoids, on MES systems.

2. Materials and Methods

2.1. Bacterial Strain and Preparation of Microbial Electrosynthesis Reactor

The *Rhodobacter sphaeroides* KCTC1434 strain was obtained from Korean Collection for Type Cultures (KCTC) and grown on Sistrom's medium without succinic acid [14]. The precultured cells for the microbial electrosynthesis (MES) reaction were cultured in modified Sistrom's medium under autotrophic conditions purged with 5% CO₂, 60% H₂, and 35% argon. Cell growth was estimated by measuring optical density (OD) using a spectrophotometer (BioSpectrometer, Eppendorf, Hamburg, Germany) at 660 nm.

The double-chamber H-type microbial electrosynthesis reactor had the same configuration as described previously [15]. The anode and cathode chambers were joined with a glass arm and separated using a proton exchange membrane (PEM, Nafion 117, DuPont Ltd., Wilmington, DE, USA). The anode and cathode electrodes were, respectively, a 4 cm × 10 cm and 4 cm × 5 cm piece of graphite felt. The thickness of the electrodes was 0.3 cm (GF030, FuelCellStore, College Station, TX, USA) and they were connected to titanium wire. The reference electrode, Ag/AgCl (in 3 M NaCl), was placed in the cathode chamber. The cathode electrode was poised with −0.6 V (vs. Ag/AgCl) using a potentiostat (WMPG1000, WonAtech, Seoul, Korea). The microbial electrosynthesis reaction was conducted under light-anaerobic conditions in batch mode with a gas composition of CO₂ 5% and argon 95% at a rate of 17.5 mL/min (0.05 vvm). Ascorbic acid (A92902, Sigma-Aldrich, St. Louis, MO, USA), which was used after adjusting the pH to 7 with KOH, was treated to final concentrations of 5 mM and 10 mM in the MES systems. All MES reactors were placed at 30 °C under white LED lamps and operated in triplicate.

2.2. Transcriptome Analysis

Preparation of total RNA for the transcriptome analysis was conducted by using a Quick-RNA Fungal/Bacterial Miniprep kit (Zymo Research, CA, USA). RNase-free DNaseI was treated to total RNA to remove any contaminating genomic DNA. Complementary DNA library construction and raw data processing were finished by Macrogen (Seoul, Korea). The cDNA libraries were sequenced with an Illumina HiSeq 2500 (Illumina, CA, USA) in pair-end mode. A differentially expressed genes (DEG) analysis was performed with edgeR. The genes were selected by p -value < 0.05 and fold-change (FC) > 2 .

2.3. Determination of ROS Levels

The levels of H_2O_2 and peroxidase activities were quantified using an Amplex[®] Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, OR, USA), as described previously [16]. The sonicated samples were prepared in potassium phosphate buffer (pH 7.5). First, 50 μ L of sample was mixed with the reaction reagent containing Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and horseradish peroxidase (HRP), and it was then incubated for 30 min at room temperature. For quantification of peroxidase activities, H_2O_2 was added instead of horseradish peroxidase. Fluorescence was measured using a SYNERGY H1 microplate reader (BioTek, VT, USA) with excitation/emission of 530/590 nm.

Endogenous reactive oxygen species levels were measured using the fluorescent dye CM- H_2 DCFDA (Invitrogen, MA, USA). The cells were washed and prepared in PBS buffer (pH 7.4). The CM- H_2 DCFDA solution was treated to a final working concentration of 1 μ M, and mixtures were incubated for 30 min at 30 °C in dark conditions. Fluorescence signals were read at excitation/emission of 495/527 nm.

2.4. Measurement of Total Carotenoid Contents

The measurement of total carotenoid was conducted as described previously [16]. Briefly, the freeze-dried cells were suspended in 1 mL of 3 M HCl and then incubated for 30 min at 30 °C, 100 rpm. The supernatants were discarded after centrifugation and the pellets were resuspended in 1 mL of acetone. The supernatants were harvested after incubation and the absorbance for quantification of carotenoids was read at 480 nm.

2.5. Statistical Analysis

The statistical analysis was carried out using a Student's t -test. The data were indicated as mean $\pm$ standard deviation. The differences were considered as statistically significant when the p -value was less than 0.05.

3. Results and Discussion

3.1. Comparison of Growth Behavior According to Reducing Source in *R. sphaeroides*

For CO_2 biological fixation using electroactive microorganisms in MES, an appropriate reducing power, such as organic compounds, hydrogen or electricity, is required. Although several studies on MES have been reported, there has been no direct comparison of growth behavior according to reducing source, such as hydrogen or electricity [17–19]. Thus, we first examined the cell growth of *R. sphaeroides* according to the supply of different electron donors in the presence of constant light and CO_2 (Figure 1). As a result, it was observed that cell growth was significantly slower when electricity was used as an electron donor than when hydrogen was used. These results indicate that bacterial cell growth is strongly influenced by the type of electron source. Hydrogen is generally one of the most preferred electron donors for autotrophic microorganisms. It readily generates intracellular proton motive force (PMF) to induce sufficient ATP synthesis and promote CO_2 transport. This proton gradient caused by hydrogen ion also promotes the generation of reducing cofactor, such as $NADPH_2$ [7,20,21]. $NADPH_2$ is an important biochemical redox cofactor responsible for the transfer of electrons and protons. It is used for reductive biosynthesis, such as biosynthesis of fatty acids and some amino acids. Moreover, it functions as

an oxidative stress defense because NADPH₂ is essential for the functions of several antioxidant enzymes, including glutathione peroxidase, glutathione S-transferase, and catalase [22–24]. When the hydrogen is used as a reducing source, the supply of NADPH₂ and ATP are sufficient for CO₂ fixation. However, when electricity is used as a reducing source, the supply of NADPH₂ and ATP is insufficient due to only electrons are provided and appropriate proton gradient is not formed. Thus, we suspected that the deficiency of ATP and NADPH₂ is one of the important factors limiting the bacterial cell growth in MES. For this reason, the direct supply of electrons or the indirect supply of permeated hydrogen ions after water decomposition at the anode is estimated to have lower utilization efficiency in *R. sphaeroides* compared to the direct supply of hydrogen.

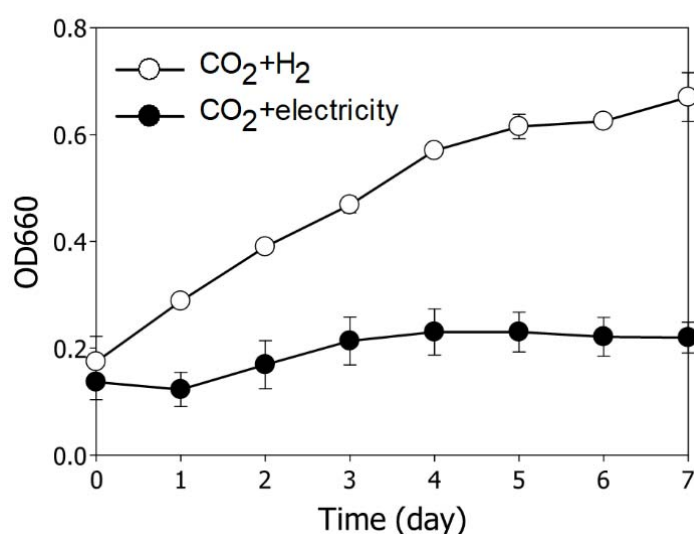


Figure 1. Comparison of growth characteristics according to different reducing power sources in *R. sphaeroides*. The cells were cultured using CO₂ and light, and only the reducing power source was varied. The white circle line denotes CO₂ + H₂, which represents normal photoautotrophic cultivation. The black circle line denotes CO₂ + electricity, which represents MES cultivation. The experiments performed in triplicate and error bars indicate the standard deviation of the mean.

We next performed a transcriptomic analysis to understand the variation of the molecular mechanism in MES systems compared to normal photoautotrophic conditions (Table 1). Consistent with slower cell growth in MES, the expression of hydrogenase (*hupSL*) gene for uptake of H₂ and the Calvin-Benson-Bassham (CBB) cycle (*cbbSL*, *cfxA*, *fbp1*, and *cbbR*) genes for the uptake of CO₂ were downregulated in MES [25,26]. PHB is a representative carbon storage material that accumulates in cells and the expression of PHB biosynthesis (*phaA*, *phaB*, *phaC1*, and *phaC2*) genes reported to be proportional to cell growth also decreased overall in MES [16]. It is speculated that this is due to the upregulation of the PHB synthesis repressor (PhaR), encoded by RSP_0380 [27]. The transcript levels of genes involved in flagellar biosynthesis (*flhAB*, *flgALK111HE*, *fliEF1KPRD*, and *motAB*) were significantly increased when electricity was used as an electron donor [28]. In MES systems, electron transfer occurs either directly or indirectly through an extracellular electron uptake mechanism. The flagellar mediate direct electron transfer by adhesion to the electrode, and this mechanism has been identified in *Desulfovibrio* [29,30]. Based on this, it is hypothesized that the upregulation of flagellar biosynthetic gene expression in MES is closely associated with direct electron uptake in *R. sphaeroides*.

Table 1. Comparison of transcript levels in MES conditions versus normal photoautotrophic conditions.

Gene Number	Gene Name	Function	Description	Log ₂ (FC)
RSP_0495	<i>hupS</i>	Hydrogenase protein small subunit	Energy production and conversion	−7.6
RSP_0496	<i>hupL</i>	Hydrogenase protein large subunit	Energy production and conversion	−9.7
RSP_1281	<i>cbbS</i>	Ribulose 1,5-bisphosphate carboxylase small subunit	Carbohydrate transport and metabolism	−6.0
RSP_1282	<i>cbbL</i>	Ribulose 1,5-bisphosphate carboxylase large subunit	Energy production and conversion	−6.3
RSP_1283	<i>cfxA</i>	Fructose-1,6-bisphosphate aldolase	Carbohydrate transport and metabolism	−6.5
RSP_1284	<i>prkA</i>	Phosphoribulokinase	Energy production and conversion	−6.7
RSP_1285	<i>fbp1</i>	Fructose-1,6-bisphosphatase	Carbohydrate transport and metabolism	−6.4
RSP_1286	<i>cbbR</i>	RuBisCO operon transcriptional regulator, CbbR	Transcription	−2.2
RSP_0382	<i>phaC1</i>	Poly-beta-hydroxybutyrate polymerase	Lipid transport and metabolism	−2.6
RSP_0745	<i>phaA</i>	Acetyl-CoA acetyltransferase	Lipid transport and metabolism	−1.8
RSP_0747	<i>phaB</i>	3-oxoacyl-(Acyl-carrier-protein) reductase	Function unknown	−3.7
RSP_1257	<i>phaC2</i>	Putative polyhydroxyalkanoic synthase	Lipid transport and metabolism	−3.6
RSP_0380		Polyhydroxyalkanoate synthesis repressor, PhaR	Function unknown	2.9
RSP_0034	<i>flhA</i>	Flagellar biosynthesis protein	Cell motility	8.4
RSP_0036	<i>flgA</i>	Flagella basal body P-ring formation protein	Cell motility	5.9
RSP_0052	<i>fliE</i>	Flagellar hook-basal body complex protein	Cell motility	7.0
RSP_0053	<i>fliF1</i>	Flagellar M-ring protein	Cell motility	8.2
RSP_0058	<i>fliK</i>	FliK, flagellar hook-length control protein	Cell motility	6.4
RSP_0063	<i>fliP</i>	Flagellar biosynthetic protein	Cell motility	9.2
RSP_0065	<i>fliR</i>	Flagellar biosynthetic protein	Cell motility	5.8
RSP_0066	<i>flhB</i>	Flagellar biosynthetic protein	Cell motility	7.3
RSP_0070	<i>fliD</i>	Flagellar hook-associated protein 2	Cell motility	4.0
RSP_0073	<i>flgL</i>	Flagellar hook-associated protein 3	Cell motility	5.9
RSP_0074	<i>flgK1</i>	Flagellar hook-associated protein 1	Cell motility	6.7
RSP_0076	<i>flgI1</i>	Flagellar P-ring protein	Cell motility	8.5
RSP_0077	<i>flgH</i>	Flagellar L-ring protein	Cell motility	6.0
RSP_0080	<i>flgE</i>	Flagellar hook protein	Cell motility	6.6
RSP_0231	<i>motB</i>	Flagellar MotB protein	Cell motility	5.9
RSP_0233	<i>motA</i>	Flagellar MotA protein	Cell motility	7.7
RSP_2380	<i>katC</i>	Catalase	Inorganic ion transport and metabolism	−1.3
RSP_2780	<i>oxyR2</i>	Transcriptional regulator, LysR family	Transcription	2.4
RSP_1796	<i>sodC</i>	Superoxide dismutase [Cu-Zn]	Inorganic ion transport and metabolism	1.1
RSP_1092	<i>rpoE</i>	ECF RNA polymerase sigma factor RpoE	Transcription	2.8
RSP_1093	<i>chrR</i>	Anti-sigma-E factor ChrR	Transcription	2.9
RSP_2143	<i>phrA</i>	DNA photolyase, Cryptochrome 1 apoprotein (Blue light photoreceptor)	Replication, recombination and repair	4.0
RSP_1194	<i>grxC</i>	Glutaredoxin	Posttranslational modification, protein turnover	1.1
RSP_2953		Glutaredoxin	Posttranslational modification, protein turnover	1.6
RSP_1529	<i>trxA</i>	Thioredoxin	Posttranslational modification, protein turnover	2.0
RSP_0725		Thioredoxin	Posttranslational modification, protein turnover	2.6
RSP_0264	<i>crtF</i>	Demethylspheroidene O-methyltransferase	Function unknown	−4.7
RSP_0265	<i>crtE</i>	Geranylgeranyl diphosphate synthase	Coenzyme transport and metabolism	−6.2
RSP_0266	<i>crtD</i>	Hydroxyneurosporene desaturase	Secondary metabolites biosynthesis	−3.9
RSP_0267	<i>crtC</i>	Acyclic carotenoid 1,2-hydratase	Function unknown	−3.2
RSP_0270	<i>crtB</i>	Phytoene synthase	Lipid transport and metabolism	−3.2
RSP_0271	<i>crtI</i>	Phytoene desaturase	Secondary metabolites biosynthesis	−3.8

In particular, the genes related to ROS signaling (*oxyR*, *rpoE*, *chrR*, and *phrA*), redoxins (*grxC*, RSP_2953, *trxA*, RSP_0725), and superoxide dismutase (*sodC*) were highly expressed in MES conditions [31]. In contrast, the expression of catalase gene (*katC*) involved in

hydrogen peroxide scavenging and the carotenoid biosynthetic genes (*crtBCDEFI*), which were reported to be involved in antioxidant activity, was notably decreased. Overall, the genes related to ROS signaling were upregulated, while the genes related to ROS-scavenging mechanisms were downregulated except for the *sodC* gene. It is confirmed that expression of the genes related to ROS and antioxidant activity were critically influenced by the direct supply of electricity. These transcriptome analysis results suggest that when CO₂ is used as a carbon source, cellular metabolisms are greatly changed depending on the type of reducing power. In particular, it causes a large difference in antioxidant activity, and the mechanism regulating it is very complex, and thus, further studies are needed to elucidate the change in MES.

Many studies have reported that ROS metabolism is very sensitive to changes of environmental growth conditions. In our transcriptome analysis data, it was confirmed that the ROS signaling was strictly associated with the reducing power under photoautotrophic growth conditions. To identify whether changes in transcript levels affected the generation of ROS, we next evaluated the endogenous levels of ROS in MES compared to those under normal photoautotrophic conditions (Figure 2a,b). The levels of endogenous hydrogen peroxide were relatively higher under MES systems than under photoautotrophic conditions. Consistent with this, higher fluorescence values were also observed in MES when the levels of ROS were measured by the detection of fluorescence caused by oxidation of H₂DCFDA, an indicator of general oxidative stress. We subsequently measured the activities of peroxidase, which is related to scavenging of hydrogen peroxide (Figure 2c). The peroxidase activities were 4.9-fold lower when electricity was utilized as the electron source than when H₂ was utilized. Generally, when the level of endogenous ROS exceeds the detoxification capabilities of microorganisms, cell damage and bacterial cell death occur. It has been reported that the production of endogenous ROS predicted by an ensemble approach of genome-scale increased the susceptibility of *E. coli* to oxidative attack, quickly leading to cell death [32]. Furthermore, cytotoxic ROS are generated in electrochemical systems through water splitting side reactions. It was also reported that the growth of *C. necator* in the hybrid microbial-water-splitting catalyst system is inhibited by ROS generated from the electrode in MES when the voltage is less than 2.3 V or higher than 4.0 V. In this system, carbon dioxide converts into biomass and isopropanol along with hydrogen and oxygen produced from water splitting. Since the minimum thermodynamic potential for water splitting is 1.23 V, the relatively high potential for microorganisms was applied in this system [33]. Afterward, to avoid the toxic effect of ROS against microbes and to decrease the potential for water splitting, the biocompatible catalyst system which used an ROS-resistant cobalt-phosphorus alloy cathodic electrode was reported [34]. It was also reported that ROS generated in the inorganic electrocatalytic system, such as O₂^{•−} and NO, has a toxicity to *C. necator*, resulting in the inhibition of cell growth and yield of the products [35]. To evade the toxicity of ROS to bacterial cell growth, it has been reported that the two-chamber MES reactor is efficient. This configuration, which separates the cathodic and anodic chamber by a proton exchange membrane (PEM) and has an applied potential at −0.6 V, is similar to our MES reactor [36]. Both the present and the previous observations support that ROS generated in MES systems unfavorably influence cell growth, indicating that the regulation of endogenous ROS is a key element for the improvement of growth in MES.

3.2. Enhancement of Bacterial Cell Growth and Carotenoid Production by Modulating ROS in MES

Based on our experimental results so far, when electricity was used as an electron source, we confirmed that the cell growth is slower and the endogenous ROS content is higher compared to when hydrogen is used as an electron source (Figures 1 and 2). Through this, we hypothesized that cell growth is poor in MES due to increased levels of ROS. Furthermore, the growth of microbial cells, including attached cells and planktonic cells, is an important factor in increasing the production of high-value added materials in MES. To improve the cell growth by modulation of ROS while confirming our hypothesis,

we introduced ascorbic acid, which is known as a powerful biological antioxidant. The cell growth was observed in MES with the addition of 5 mM and 10 mM ascorbic acid, respectively (Figure 3a). During 10 days of cultivation, the bacterial cell growth was significantly improved by the addition of ascorbic acid. When 5 mM or 10 mM of ascorbic acid was added, the cell density increased by 161% and 131%, respectively. Dosing of more than 10 mM ascorbic acid appears undesirable in MES because excess ascorbic acid can act as an oxidizing agent and inhibit cell growth. To confirm the effect of ascorbic acid on endogenous ROS, we subsequently analyzed the contents of H_2O_2 and the levels of endogenous ROS (Figure 3b,c). Compared to the control, the levels of H_2O_2 were reduced proportionally to the concentration of treated ascorbic acid. When ascorbic acid was supplied at 5 mM and 10 mM, the fluorescence value indicating the endogenous contents of ROS was also decreased by 60% and 75%, respectively. In addition, activities of peroxidase were improved 2.9-fold and 2.1-fold in the presence of 5 mM or 10 mM ascorbic acid, respectively (Figure 3d). These observations suggest that supplementation of exogenous antioxidants successfully reduced endogenous levels of ROS, which have a significant impact on the bacterial cell growth.

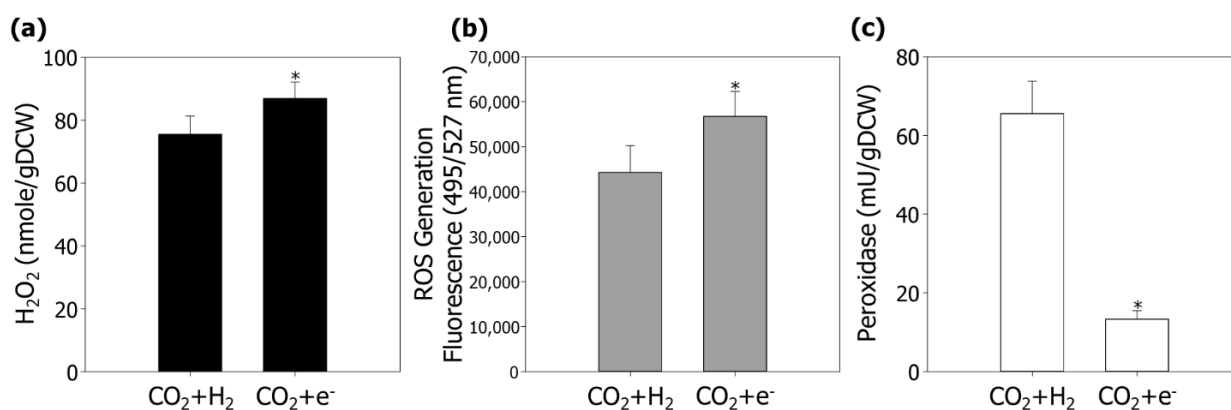


Figure 2. Endogenous levels of ROS in *R. sphaeroides* under photoautotrophic ($CO_2 + H_2$) and MES ($CO_2 + e^-$) conditions. (a) Endogenous contents of H_2O_2 . (b) Generation of endogenous ROS. The levels of ROS were measured using CM- H_2 DCFDA. The fluorescence is represented in arbitrary units and normalized by the optical density of the samples. (c) The measurement of peroxidase activity. Experiments were conducted in triplicate and error bars indicate standard deviation of mean. Asterisk represent statistically significant difference, as determined by a Student's *t*-test (* $p < 0.05$).

Ascorbic acid is one of the most important antioxidants present in cells. It prevents oxidative damage caused by ROS and mediates diverse cell metabolism. In plants, ascorbate serves as a cofactor of multiple enzymatic reactions and supports photosynthesis and flowering [37]. Ascorbic acid is able to scavenge hydrogen peroxide, which occurs through the photosynthetic electron transport chain, and thereby contributes to the control of photosynthetic efficiency in plants [38]. Furthermore, the addition of ascorbic acid also has various effects on microbial metabolism. In *R. sphaeroides*, the activity of light-dependent ATPase involved in ATP synthesis is usually observed to be lower under aerobic conditions than under anaerobic conditions. However, additional supply of ascorbic acid reduced ROS toxicity and recovered ATPase activity to anaerobic levels [39]. The photosynthesis of *R. sphaeroides* is mediated by the reaction centers (RCs). As the concentration of ascorbate increases, the generation of singlet oxygen (1O_2) decreases in the reaction centers [40]. These findings indicate that the supplement of ascorbic acid modulates the production of ROS during photosynthesis, affecting the microbial CO_2 fixation efficiency. According to both the present and previous results, ROS regulation by the supply of antioxidants meaningfully improves bacterial cell growth in MES. In order to better understand the effect of regulating antioxidant activity on microorganisms, it will be of great help if we further analyze changes of gene expression patterns and related mechanisms in the future.

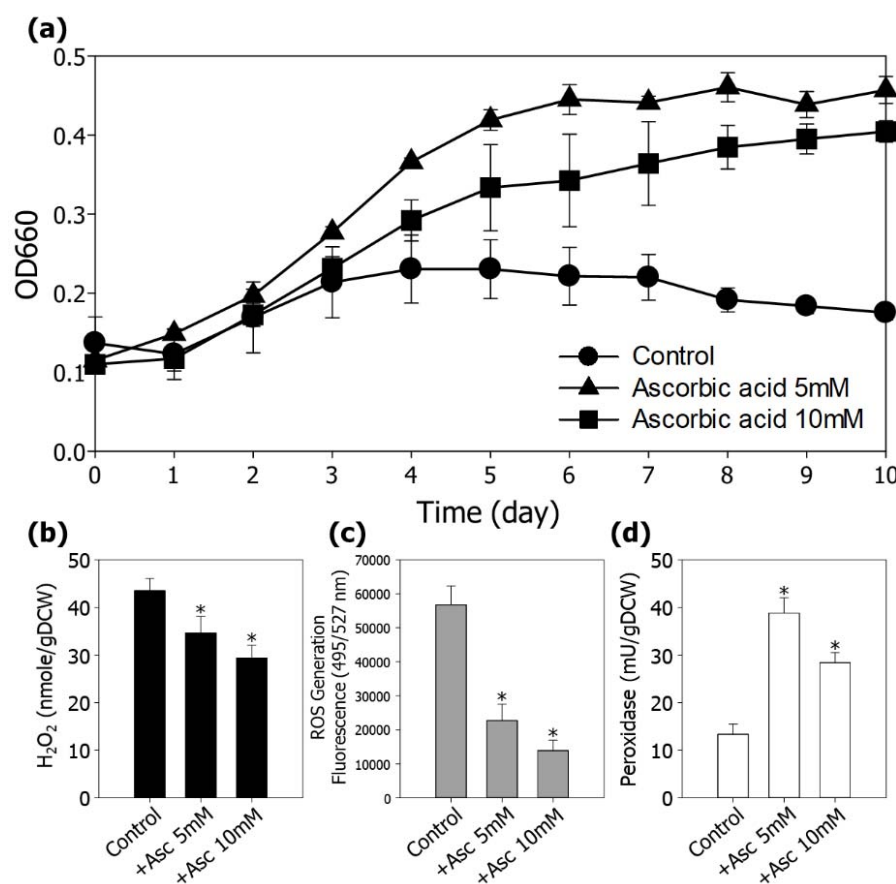


Figure 3. Effects of ascorbic acid on MES in *R. sphaeroides*. **(a)** Cell growth in the presence of 5 mM or 10 mM ascorbic acid. The cultivations were conducted in MES reactors using CO₂ as a carbon source and electricity as a reducing source under −0.6 V (vs. Ag/AgCl). Circle lines indicate control (untreated ascorbic acid). Upward triangle lines indicate the addition of 5 mM ascorbic acid. The square line represent the addition of 10 mM ascorbic acid. **(b)** Measurement of endogenous H₂O₂. **(c)** Generation of endogenous ROS. The levels of ROS were measured using CM-H₂DCFDA. The fluorescence is represented in arbitrary units and normalized by the optical density of the samples. **(d)** The analysis of peroxidase activity. Asc, ascorbic acid. Every experiment was conducted in triplicate and error bars indicate standard deviation of mean. Asterisk represents statistically significant difference, as determined by a Student's *t*-test (* *p* < 0.05).

The valorization of CO₂ into high value-added products is an important aspect of expanding the application of MES. In the context, we next investigated the production of carotenoids, which are naturally accumulated in *R. sphaeroides* and possess antioxidant activity, under MES systems (Figure 4). When 5 mM and 10 mM of ascorbic acid was added, the contents of total carotenoids were 2.7-fold and 3.3-fold elevated in cells, respectively. These results indicate that the production of total carotenoids in *R. sphaeroides* is increased in proportion to the concentration of ascorbic acid. Consistent with this, it was reported that a moderate positive correlation was observed between the levels of ascorbate and the contents of total carotenoid in spinach [41]. However, since plants and microorganisms have very different metabolic processes, it is necessary to conduct further research to elucidate the molecular mechanism between ascorbic acid and carotenoid production in *R. sphaeroides*. Moreover, the antioxidant activity of carotenoids is expected to be beneficial in utilization of CO₂ under MES conditions. Previous studies reported that the carotenoid pigments assist the modulation of photosynthetic electron transport and protect against the damage induced by light during photosynthesis [38]. If a synergistic effect between carotenoid biosynthesis and its antioxidant activity could be achieved, then a vigorous

electricity-driven cell factory could be established for sustainable carotenoid production in MES [4]. Taken together, the present results show that modulation of ROS by an exogenous antioxidant promoted the production of carotenoids as well as cell growth. These observations suggest that the enhancement of antioxidant activity can be used as a novel strategy to increase the bacterial cell growth and the yield and productivity of valuable products under MES systems.

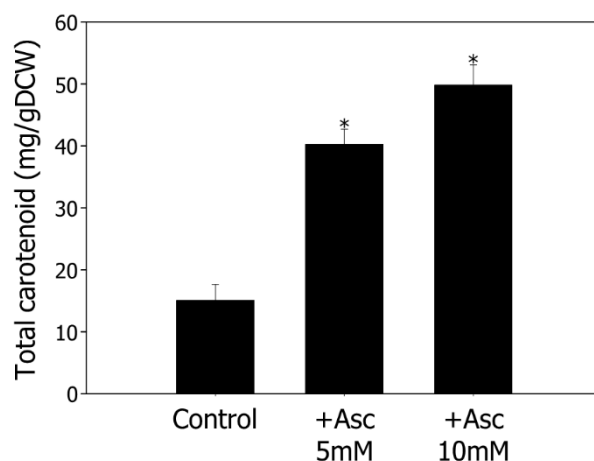


Figure 4. Measurement of total carotenoid contents in the presence of ascorbic acid under MES conditions. Asc, ascorbic acid. Experiments were performed in triplicate and error bars indicate standard deviation of mean. Asterisk represent statistically significant difference, as determined by a Student's *t*-test (* $p < 0.05$).

4. Conclusions

Improving bacterial cell growth is a crucial factor in converting CO₂ into valuable materials under MES systems. However, the cell growth in MES has been much slower than that under normal photoautotrophic conditions due to the generation of large amounts of endogenous ROS. To overcome this, we applied on exogenous antioxidant to MES. When ascorbic acid was treated, the cell growth and carotenoid accumulation were significantly increased. Furthermore, while the levels of endogenous ROS were decreased, the activity of ROS scavenging enzyme was increased. Altogether, our results suggest that modulation of ROS is very important to promote bacterial cell growth and carotenoid production under MES conditions.

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