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Molecular Profiling and Optimization Studies for Growth and PHB Production Conditions in *Rhodobacter sphaeroides*

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Abstract: In the recent climate change regime, industrial demand for renewable materials to replace petroleum-derived polymers continues to rise. Of particular interest is polyhydroxybutyrate (PHB) as a substitute for polypropylene. Accumulating evidence indicates that PHB is highly produced as a carbon storage material in various microorganisms. The effects of growth conditions on PHB production have been widely studied in chemolithotrophs, particularly in *Rhodobacter*. However, the results on PHB production in *Rhodobacter* have been somewhat inconsistent due to different strains and experimental conditions, and it is currently unclear how diverse environmental factors are linked with PHB production. Here, we report optimized growth conditions for PHB production and show that the growth conditions are closely related to reactive oxygen species (ROS) regulation. PHB accumulates in cells up to approximately 50% at the highest level under dark-aerobic conditions as opposed to light aerobic/anaerobic conditions. According to the time-course, PHB contents increased at 48 h and then gradually decreased. When observing the effect of temperature and medium composition on PHB production, 30 °C and a carbon/nitrogen ratio of 9:1 or more were found to be most effective. Among PHB biosynthetic genes, *PhaA* and *PhaB* are highly correlated with PHB production, whereas *PhaC* and *PhaZ* showed little change in overall expression levels. We found that, while the amount of hydrogen peroxide in cells under dark conditions was relatively low compared to the light conditions, peroxidase activities and expression levels of antioxidant-related genes were high. These observations suggest optimal culture conditions for growth and PHB production and the importance of ROS-scavenging signaling with regard to PHB production.

Keywords: *Rhodobacter*; polyhydroxybutyrate; growth conditions; reactive oxygen species

1. Introduction

Biopolymers are very important materials in responding to environmental pollution and global warming, and their production is increasing. Among them, polyhydroxyalkanoate (PHA), which is used

as a representative biopolymer, is a highly interesting and useful biochemical, because it can replace petrochemicals such as polypropylene [1]. In particular, poly-β-hydroxybutyrate (PHB), one the PHAs, has been the most widely studied product and has already reached a level of commercialization [2]. However, in order to compete with petrochemicals, there is a need to dramatically improve the economic feasibility, especially in the feed supply.

PHB is known as a bacterial energy storage material that accumulates in cells under a carbon-rich environment [3,4]. It is biosynthesized in three steps from acetyl-CoA in most microorganisms. Two molecules of acetyl-CoA are condensed to an acetoacetyl-CoA by acetyl-CoA acetyltransferase (*PhaA*) and then reduced to β-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (*PhaB*). Lastly, PHB synthase (*PhaC*) catalyzes polymerization to PHB [5,6]. PHB depolymerase, encoded by *PhaZ*, hydrolyzes PHB to utilize it as a carbon and energy source under energy starving conditions [7].

PHA production has been studied in diverse microorganisms such as *Escherichia coli* and *Pseudomonas* species, under various conditions [8–11]. Recombinant *E. coli* harboring PHB synthesizing genes from *Streptomyces aureofaciens* showed different PHB accumulation according to the medium composition including amino acid sments, carbon source, and nitrogen source [8,9]. Smentation of amino acids except glycine and valine enhance PHB accumulation, and the maximum PHB content (60% of dry cell weight) was accomplished in a medium with glycerol as the sole carbon source, and yeast extract and peptone as the nitrogen source [9]. In *Pseudomonas* species, medium-chain length PHAs were produced from sludge palm oil or vegetable oil under a phosphate and nitrogen limited medium, suggesting that waste carbon resources can be used to reduce feed costs of PHB production [10,11].

The chemotrophs, such as *Ralstonia*, *Rhodospirillum*, and *Rhodobacter*, have many advantages in producing biochemicals. These microorganisms contribute to reducing global warming and environmental pollution, because they utilize carbon dioxide as well as organic wastes [12–17]. Optimization of the nutrient composition in the medium for PHB production in *Ralstonia eutropha* has been reported [12]. When *Ralstonia eutropha* was grown in a medium that contains urea and corn steep liquor instead of ammonium sulphate and yeast extract, PHB contents of up to 45.1% was observed in the cells, indicating that medium conditions are critical to increase the productivity and price competitiveness of PHB.

Notably, *Rhodobacter sphaeroides* is a highly favorable microorganism for PHB production. Under nitrogen-limiting conditions, PHB accumulated up to 70% of cellular dry weight in *R. sphaeroides* [16]. Among various carbon sources, acetate yielded the highest PHB production [17]. Moreover, PHB productivity can be greatly improved within *R. sphaeroides*, since it is possible to enhance the performance of strains using well-established genetic engineering tools and by integrating advances in previous studies [5,18–20].

The versatile metabolism of *R. sphaeroides* is very useful for research on intracellular processes including photosynthesis, respiration, and various signaling networks in bacteria [21,22]. There have also been many reports on oxidative stress signaling in *R. sphaeroides*. In the microbial cells, oxidative stress is mainly caused by reactive oxygen species (ROS), which are generated from diverse cellular processes and environmental factors such as light, oxygen, and temperature [23–26]. ROS scavenging mechanisms have been evolutionally developed to combat oxidative stress, which leads to severe cellular damage in *R. sphaeroides*. Glutathione peroxidase, catalase, and superoxide dismutase, which remove ROS such as superoxide and hydrogen peroxide, have been identified in *Rhodobacter*, and the effects of antioxidants such as carotenoids and ascorbate have also been verified [27–30]. A hydrogen peroxide-inducible transcriptional activator, OxyR, has been studied as the main regulator of ROS signaling, and a homolog of *E. coli* OxyR exists in *R. sphaeroides* [31]. The OxyR-defective *oxyR* mutants were more sensitive to hydrogen peroxide compared with the hydrogen peroxide sensitivity of the wild-type strain of *R. sphaeroides*, indicating that *R. sphaeroides* OxyR also has a similar function with *E. coli* OxyR in the oxidative stress response [31].

In this work, we investigated cell growth and PHB production under varied conditions in *R. sphaeroides*, such as light, aeration, incubation time, and temperature. We also found that PHB-biosynthetic genes,

PhaA and *PhaB*, are critical for PHB production according to environmental change, and accumulation of ROS and carotenoids changed depending on PHB production. Our observations provide optimized growth conditions for PHB production and demonstrate the close relationship between ROS metabolism and PHB production.

2. Materials and Methods

2.1. Bacterial Strain and Growth Conditions

Rhodobacter sphaeroides KCTC1434 strain was purchased from Korean Collection for Type Cultures (KCTC) and grown on Sistrom's minimal medium [32]. The cells were cultured at 30 °C and 150 rpm under aerobic-dark conditions. The growth of cells was monitored by measuring optical density (OD) using a spectrophotometer (Mega-800, Scinco, Seoul, Korea) at 660 nm, with sufficient dilution of the culture broth. The values of optical density were calculated to dry cell weight using our standard curve of cell mass for OD_{660} (OD_{660} 1 = 0.5678 g/L).

To examine the effects of light, oxygen, temperature, and C/N ratio on cell growth and PHB production, the precultured cells were added to 100 mL of Sistrom's medium in 250 mL Erlenmeyer flasks after being diluted to an OD_{660} of 0.1 and cultured under various growth conditions for 48 h. For anaerobic experiments, all serum bottles were purged with argon gas.

2.2. PHB Extraction and Analysis

For the PHB analysis, approximately 10 mg of lyophilized whole cells was reacted in a small screw-cap test tube with a 2 mL solution containing 85% (*v/v*) methanol, 15% (*v/v*) concentrated sulfuric acid, and 250 mg/L benzoic acid as an internal standard. After addition of 2 mL chloroform, the mixture was incubated for 3.5 h in a heating block at 100 °C. The tubes were cooled to room temperature. After addition of 1 mL of 1 M NaCl to each tube, the mixtures were shaken for 1 min and centrifuged at 4200 rpm for 10 min. The bottom organic layer containing PHB was dried over sodium sulfate before analysis. The organic phase was analyzed by a gas chromatograph (7890, Agilent, Santa Clara, CA, USA) equipped with a flame ionization detector (FID). The capillary column was a HP-5 from Agilent J & W Scientific, 30 m in length with 0.25 mm internal diameter. The injection split ratio was 60:1. The injection port and the detector temperatures were 180 and 200 °C, respectively. The initial oven temperature was maintained for one minute at 90 °C with an increase of 8 °C/min to a final temperature of 150 °C maintained for five minutes. The flow rate of the helium carrier gas was 1 mL/min. The PHB polymer (363502, Sigma-Aldrich, St. Louis, MO, USA) was used as an external standard.

2.3. HPLC Analysis for Organic Acids

The organic acids from the cell culture were analyzed by an HPLC (1260, Agilent, Santa Clara, CA, USA) equipped with a UV detector. The samples were centrifuged, and the supernatants were collected and filtered through 0.2 μm pore size membrane syringe filter. The capillary column was an Aminex® HPX-87H, 300 mm in length with 7.8 mm internal diameter. The separation was achieved isocratically, using a mobile phase of 5 mM sulfuric acid pumped at a flow rate 0.6 mL/min at 50 °C. The injection volume was 20 μL, and the detection was made at 210 nm. Succinic acid and fumaric acid were used as organic acid standards.

2.4. RNA Isolation and Transcript Analysis by qRT-PCR

To analyze the transcript levels of PHB biosynthetic genes and ROS-related genes, total RNA samples were isolated from the harvested cells using a Quick-RNA Fungal/Bacterial Miniprep kit (Zymo Research, Irvine, CA, USA). RNase-free DNaseI was treated to total RNA samples to remove any contaminating genomic DNA. The cDNA was synthesized by using a GoScript Reverse Transcription System (Promega, Madison, WI, USA). qRT-PCR was performed in 96-well blocks with QuantStudio 6 Flex (Applied Biosystems, Foster City, CA, USA) using the SYBR Green I master mix. The parameter

is one cycle of 95 °C for 20 s followed by 40 cycles of 95 °C for 1 s, 60 °C for 20 s. *RpoZ* gene, encoding DNA-directed RNA polymerase ω-subunit, was designated as the endogenous reference gene for normalizing RNA levels. Relative expression of genes was analyzed using the comparative Ct method, as described previously [33].

2.5. Determination of H_2O_2 Concentration and Peroxidase Activity

The endogenous levels of hydrogen peroxide (H_2O_2) concentration and peroxidase activities were measured using an Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR, USA), as described previously [34]. Briefly, cell lysis was carried out by sonication, and samples were prepared in potassium phosphate buffer (pH 7.5). Fifty microliters of sample was mixed with the reaction reagent, including the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and horseradish peroxidase (HRP) and incubated for 30 min according to the manufacturer's protocol. For measurement of peroxidase activities, H_2O_2 should be added instead of HRP. Fluorescence was read at excitation/emission of 530 nm/590 nm. Intracellular H_2O_2 levels were quantified using the H_2O_2 standard curve, and peroxidase activities were assessed using the horseradish peroxidase (HRP) standard curve. Fluorescence measurements were carried out using a SYNERGY H1 microplate reader (BioTek, Winooski, VT, USA).

2.6. Total Carotenoids Extraction

To extract total carotenoids from *R. sphaeroides*, 33.3 mg of freeze-dried cells were soaked in 1 mL of 3 M HCl and incubated for 30 min in a shaking incubator at 30 °C, 100 rpm. The suspensions were centrifuged at 10,000 rpm for 20 min, and the supernatants were discarded. One milliliter of acetone was added to the pellet, which was put in the shaking incubator for 30 min. Afterwards, the suspension was centrifuged for 20 min, and the supernatants were transferred to new tubes. The supernatant should be sufficiently diluted depending on the sample. One hundred microliters of sample was moved into 96-well microplate, and the absorbance was read at 480 nm.

2.7. Statistical Analysis

All statistical significance of the measurements was determined using a Student *t*-test. The data were expressed as mean ± standard deviation. A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of Light and Aeration on PHB Production

The purple non-sulfur bacterium *Rhodobacter sphaeroides* grows under both phototrophic and chemolithotrophic conditions and utilizes a wide range of substrates, including carbon dioxide, succinic acid, and acetic acid [16,35,36]. Although many studies have been conducted on the production of PHB under various growth conditions in *R. sphaeroides*, varying results on how each environmental factor affects cell growth and PHB production have been obtained because of differences in genotypes and experimental conditions [16,17,37–39].

We set up four different growth conditions, light-aerobic, dark-aerobic, light-anaerobic, and dark-anaerobic, to elucidate the effects of light and aeration on cell growth and PHB production. Whereas cell growth and PHB contents were much higher in the dark than in the light under aerobic conditions, they were only measured in the light because cells did not grow in the dark under anaerobic conditions (Figure 1A). In anaerobic fermentation, the light is essential to photosynthesis and anaerobic respiration for the cells [40]. Consistent with the results of cell growth and PHB accumulation, succinic acid was most highly consumed under dark-aerobic conditions (Figure 1B). The concentration of fumaric acid, an intermediate of succinic acid metabolism in the TCA cycle, was higher in the light than in the dark under aerobic conditions, indicating that succinic acid metabolism slightly differs between the light and dark conditions.
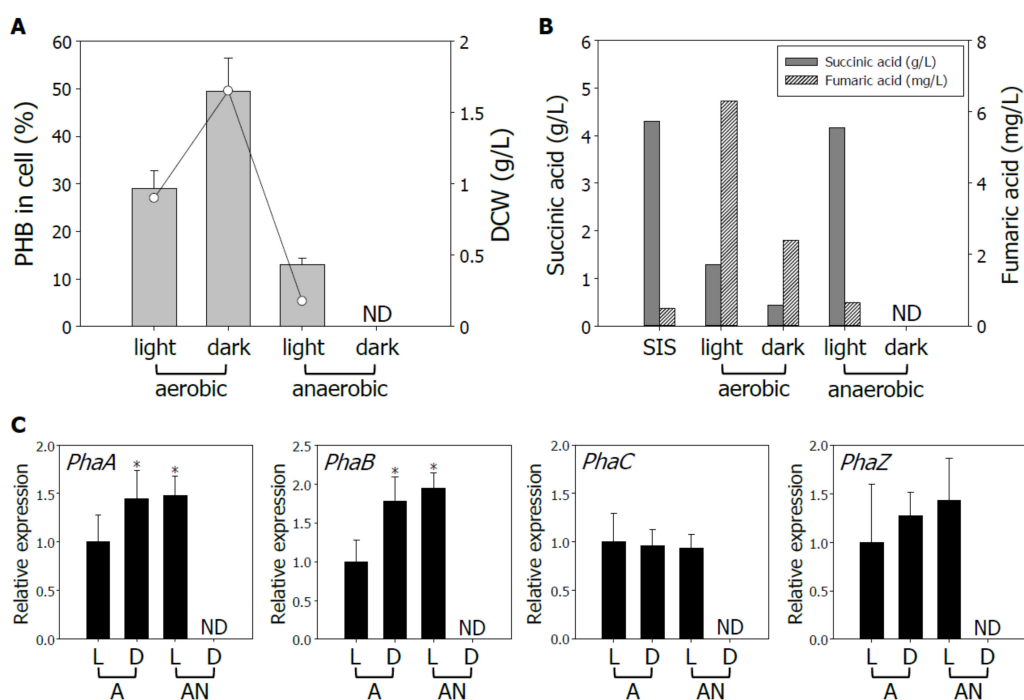
Figure 1. Effects of light and aeration on polyhydroxybutyrate (PHB) production in *R. sphaeroides*. (**A**) PHB accumulation (% of dry cell weight) and cell growth. The samples were harvested at 48 h after inoculation. Gray bars for PHB contents and open circle lines for dry cell weight (DCW). (**B**) Measurement of organic acids concentrations. SIS, Sistrom's medium. (**C**) Expression of genes encoding PHB biosynthetic enzymes. *PhaA*, acetyl-CoA acetyltransferase (RSP_0745); *PhaB*, acetoacetyl-CoA reductase (RSP_0747); *PhaC*, PHB polymerase (RSP_0382); *PhaZ*, PHB depolymerase (RSP_0383). L, light; D, dark; A, aerobic; AN, anaerobic. Experiments were performed in triplicate and bars indicate standard error of the mean. Asterisks represent a statistically significant difference, as determined by a Student *t*-test (* $p < 0.05$). ND is not determined.

Based on these observations, we next examined expression of PHB biosynthetic genes under light/dark and aerobic/anaerobic conditions. The *PhaA*, encoding acetyl-CoA acetyltransferase, and *PhaB*, encoding acetoacetyl-CoA reductase, genes were slightly induced more than 1.5-fold under dark-aerobic and light-anaerobic conditions. However, the *PhaC*, encoding PHB polymerase, and *PhaZ*, encoding PHB depolymerase, genes were expressed almost consistently under all conditions except for dark-anaerobic (Figure 1C). Together, these results suggest that dark-aerobic is optimal conditions for cell growth and PHB production.

3.2. PHB Production According to Time-Course

Depending on the growth stage, intracellular metabolism is dramatically different [41]. To confirm the change in PHB production according to time-course, we analyzed cell growth and PHB contents under dark-aerobic conditions where PHB accumulation was the highest. The cells were cultured at 30 °C and harvested at 24, 48, 72, and 96 h for analysis.

The dry cell weight reached its maximum in 48 h and then decreased. Likewise, the PHB content was the highest at 49.45% of dry cell weight in 48 h and continued to decrease (Figure 2A). After 48 h, it is considered that the cell vitality have decreased, and their PHB content dropped by almost 50% compared to 48 h. Succinic acid, the main carbon source in the medium, was almost totally consumed in *R. sphaeroides* after 48 h. The initial succinic acid concentration of approximately 4 g/L reduced to 0.6 g/L at 48 h and remained constant thereafter. In contrast, the concentration of fumaric acid continued to increase reaching 8.4 mg/L at 96 h (Figure 2B). These observations suggest that PHB

accumulates significantly in the early stage of cell growth in *R. sphaeroides*, and when the carbon source is depleted in the medium, PHB begins to decline.
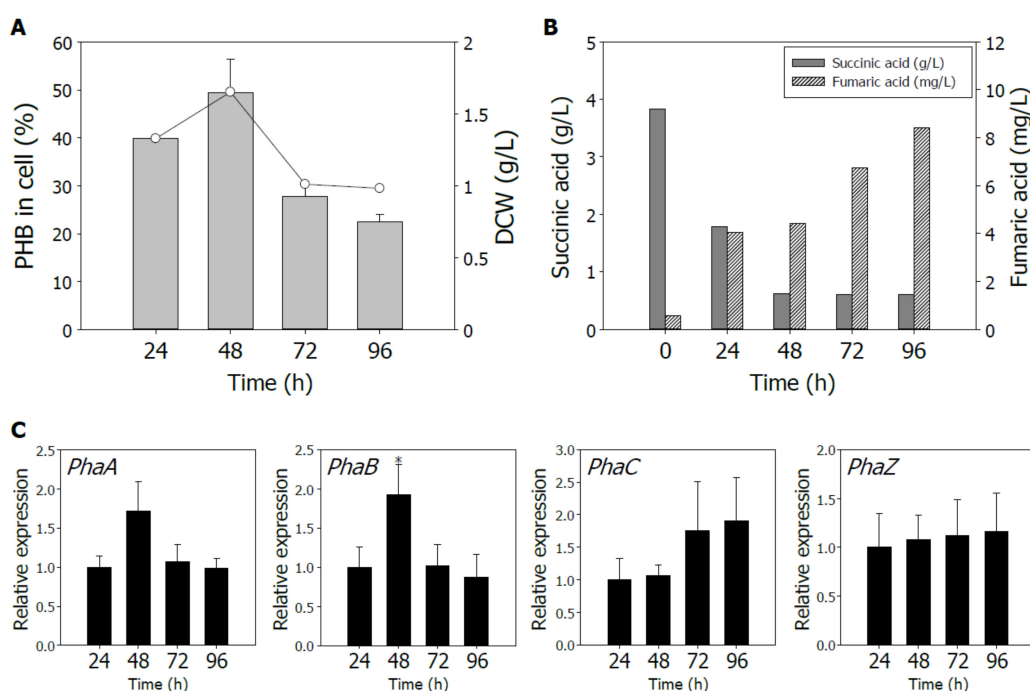

Figure 2. Growth stage-dependent PHB production in *R. sphaeroides*. (**A**) PHB accumulation (% of dry cell weight) and cell growth. The cells were grown under dark-aerobic conditions and harvested at the indicated time points. Gray bars for PHB contents and open circle lines for DCW. (**B**) Measurement of organic acids concentrations. (**C**) Expression of genes encoding PHB biosynthetic enzymes. Experiments were performed in triplicate and bars indicate standard error of the mean. Asterisks represent statistically significant difference, as determined by a Student *t*-test (* $p < 0.05$).

To further investigate the link between PHB biosynthesis and time-course, we analyzed transcript levels of PHB biosynthetic genes according to the culturing time. The expression of *PhaA* and *PhaB* genes was, respectively, found to be higher at 48 h than at 24 h, after which they decreased. In the case of the *PhaC* gene, expression levels were slightly higher at 72 and 96 h than at 24 and 48 h. The expression of the *PhaZ* gene expression revealed insignificant differences in all samples (Figure 2C). It is therefore evident that the *PhaA* and *PhaB* genes among PHB biosynthetic genes are strongly correlated to the PHB accumulation according to time-course in *R. sphaeroides*.

3.3. Effects of Temperature on PHB Production

PHB production is highly dependent on the temperature conditions in *R. sphaeroides*, as previously reported [39]. We next examined the effects of various temperature conditions on cell growth and PHB accumulation. In the previous results, cell growth and PHB production were highest at 48 h, so even though the growth rate was different for each temperature, the *R. sphaeroides* cells were cultivated for 48 h at 20, 25, 30, 35, and 40 °C under aerobic conditions in darkness.

The results showed that PHB contents at each temperature among 20 to 40 °C were 18.25, 25.02, 49.45, 23.96, and 21.55%, respectively (Figure 3A). The change in cell growth also showed similar patterns to the PHB production. These findings support that 30 °C, known as the general *R. sphaeroides* growth temperature, is the optimal temperature condition for cell growth and PHB production, and they are critically inhibited at higher or lower temperatures than at 30 °C.
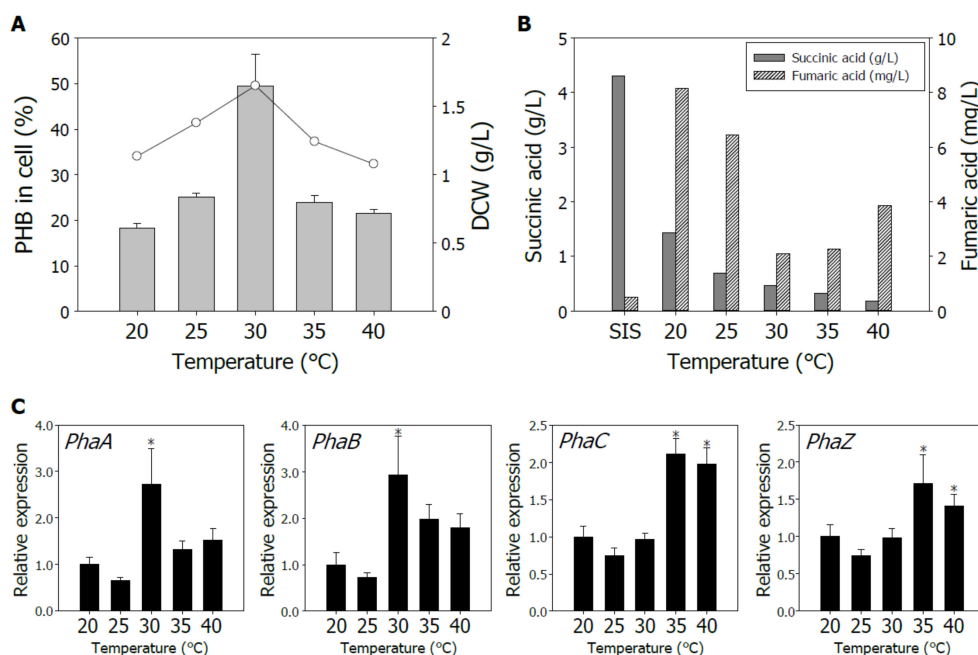
Figure 3. Effects of temperature on PHB production in *R. sphaeroides*. (**A**) PHB accumulation (% of dry cell weight) and cell growth at different temperatures. The cells were grown under dark-aerobic conditions and harvested at 48 h after inoculation. Gray bars for PHB contents and open circle lines for DCW. (**B**) Measurement of organic acids concentrations. (**C**) Expression of genes encoding PHB biosynthetic enzymes. Experiments were performed in triplicate and bars indicate standard error of the mean. Asterisks represent statistically significant difference, as determined by a Student *t*-test (* $p < 0.05$).

An organic acid analysis using high-performance liquid chromatography (HPLC) revealed that all samples except those grown at 20 °C consumed almost all succinic acid during cultivation under various temperature conditions (Figure 3B). At 20 °C, 1.43 g/L of succinic acid remained and 8.14 mg/L of fumaric acid was produced in *R. sphaeroides*. The lowest concentration of fumaric acid was measured to be 2.09 mg/L at 30 °C. Our results indicated that succinic acid metabolism is very different depending on the temperature.

Gene expression assay by quantitative real-time RT-PCR (qRT-PCR) showed that the *PhaA* and *PhaB* genes were strongly induced approximately three-fold at 30 °C (Figure 3C). Meanwhile, the transcript levels of *PhaC* and *PhaZ* genes were higher at high temperature including 35 °C and 40 °C compared with low temperature, indicating that the *PhaC* and *PhaZ* genes are involved in different signaling from PHB production at a high temperature.

3.4. Effects of C/N Ratio in Growth Medium on PHB Production

It is known that PHB is highly accumulated in an environment where the nitrogen source is insufficient and the carbon source is rich under aerobic conditions [3,4]. We therefore decided to examine the effects of the carbon to nitrogen ratio in the medium on PHB production in *R. sphaeroides*.

To determine the optimal C/N ratio in the medium for PHB production, we prepared media with carbon to nitrogen ratios of 3:1, 6:1, 9:1, 12:1, and 15:1 using succinic acid and ammonium chloride as carbon and nitrogen sources, respectively. The C/N ratio was altered by maintaining the carbon content at a constant level and adjusting the nitrogen content. The cells were harvested at 48 h under dark-aerobic conditions, as before. PHB content was highest at 51.57% at the C/N ratio of 15:1 and, overall, was high when the C/N ratio was greater than 9:1 (Figure 4A). On the other hand, the cell growth was the highest at the C/N ratio of 3:1 as opposed to PHB content, and biomass tended to increase as the C/N ratio decreased. The organic acids in the medium, including succinic acid and fumaric

acid, were not detected at C/N ratios of 3:1 and 6:1. Whereas the rate of succinic acid consumption was slower with a higher C/N ratio, fumaric acid content was higher (Figure 4B). These observations suggest that nitrogen-rich conditions enhance cell growth and carbon-rich conditions cause PHB accumulation under aerobic conditions in *R. sphaeroides*.
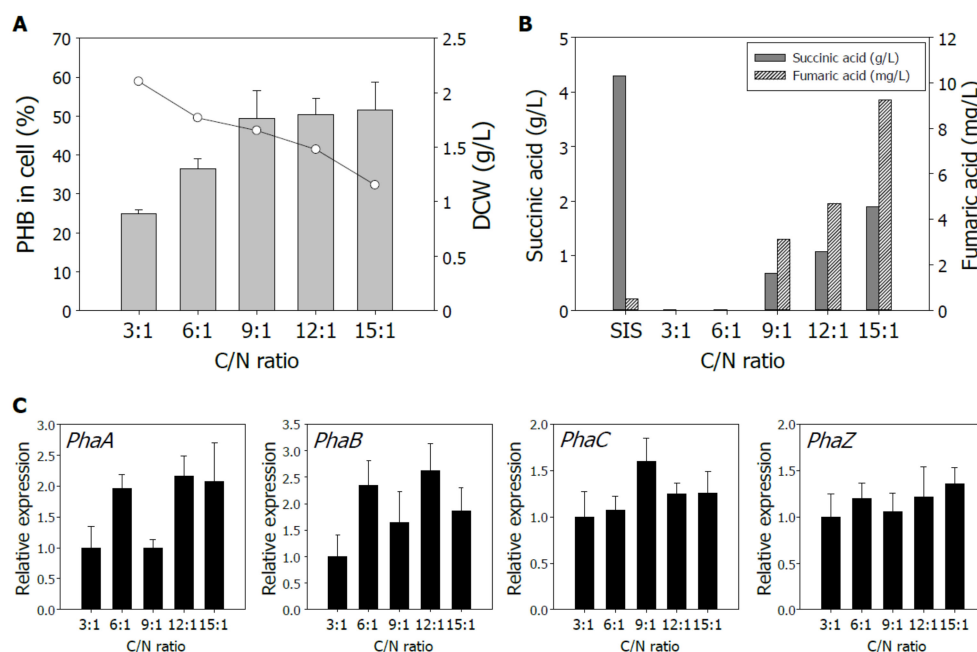

Figure 4. Effects of carbon to nitrogen ratio on PHB production in *R. sphaeroides*. (**A**) PHB accumulation (% of dry cell weight) and cell growth under indicated C/N ratio. The cells were grown under dark-aerobic conditions and harvested at 48 h after inoculation. Gray bars for PHB contents and open circle lines for DCW. (**B**) Measurement of organic acids concentrations. (**C**) Expression of genes encoding PHB biosynthetic enzymes. Experiments were performed in triplicate and bars indicate standard error of the mean. Asterisks represent statistically significant difference, as determined by a Student *t*-test.

Unlike the previous high correlation between PHB production and the expression of PHB biosynthetic genes, there were no distinct expression patterns depending on the C/N ratio in the medium. Whereas the transcript levels of *PhaA* and *PhaB* genes were slightly higher at the C/N ratio of 6:1, 12:1, and 15:1, those of *PhaC* gene were a little higher in the C/N ratio of 9:1 (Figure 4C). The *PhaZ* gene was constantly expressed regardless of the C/N ratio. The previous and the present results indicated that *PhaA* and *PhaB* genes have similar expression patterns, because they are located within the same operon [19]. In addition, the expression patterns of these genes showed a high correlation with PHB production except for changes in the C/N ratio in the medium, suggesting that PHB biosynthesis is mainly regulated via *PhaA* and *PhaB* genes under various growth conditions in *R. sphaeroides*.

3.5. ROS Regulation in R. sphaeroides

ROS are rapidly generated in the cells depending on the levels of light and oxygen, growth stage, and temperature change [26,42–44]. Although ROS mediates adaptive responses to environmental change, high levels of ROS cause oxidative stress such as degradation of proteins and lipid peroxidation [24,25]. Considering the high correlation between light and ROS generation, we hypothesized that the differences between light and dark conditions led to changes in levels of ROS, which affected cell growth and PHB production.

To examine this hypothesis, we measured endogenous H_2O_2 contents and peroxidase activity under light and dark conditions in *R. sphaeroides*. As expected, the levels of H_2O_2 were higher

under light conditions than under dark conditions (Figure 5A). In contrast, the activity of peroxidase, which scavenges H_2O_2, was higher under dark conditions (Figure 5B), confirming that the light causes a change in the ROS levels in *R. sphaeroides*.
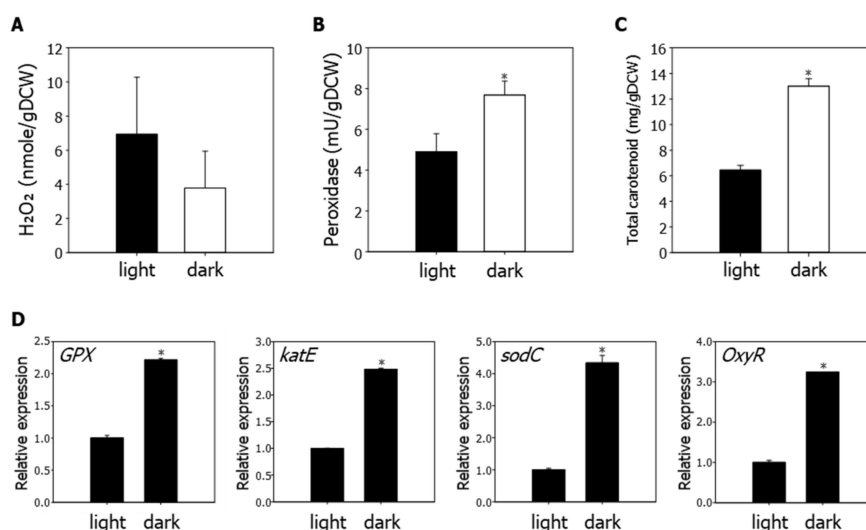

Figure 5. Regulation of endogenous reactive oxygen species in *R. sphaeroides*. Samples were harvested at 48 h after inoculation. (**A**) The endogenous levels of hydrogen peroxide (H_2O_2) under light or dark aerobic conditions. (**B**) Measurement of peroxidase activity. (**C**) Measurement of total carotenoid contents. (**D**) Expression of reactive oxygen species (ROS)-related genes. *GPX*, glutathione peroxidase (RPS_2389); *katE*, catalase (RSP_2779); *sodC*, superoxide dismutase (RSP_1796); *OxyR*, hydrogen peroxide-inducible genes activator (RSP_2780). Experiments were performed in triplicate, and bars indicate standard error of the mean. Asterisks represent a statistically significant difference, as determined by a Student *t*-test (* $p < 0.05$).

We next measured the contents of carotenoids under light and dark conditions in *R. sphaeroides*. Carotenoids, which are highly accumulated in *R. sphaeroides*, are known to function as an antioxidant as well as pigments [27]. Measurement of carotenoid levels showed that the contents of carotenoids were approximately two times higher under dark conditions compared to the levels under light conditions (Figure 5C). Interestingly, transcript levels of genes encoding antioxidant metabolizing enzymes, such as glutathione peroxidase (*GPX*), catalase (*katE*), and superoxide dismutase (*sodC*), were elevated by more than two-fold under dark conditions (Figure 5D). Higher transcript levels of *OxyR* gene, which functions as an H_2O_2-inducible transcriptional activator, were also induced under dark conditions. These results indicate that ROS levels were elevated under light conditions due to decreased antioxidant activity.

Altogether, we have derived optimal growth conditions, including light, culturing time, temperature, and medium composition, for cell growth and PHB production in *R. sphaeroides*. It is envisaged that alterations of growth conditions cause changes in ROS levels. Consequently, ROS is expected to have a huge influence on cell growth and PHB production, such as the effects of light.

4. Discussion

In this study, we carried out optimization of culture conditions, such as light presence, oxygen existence, harvesting time, temperature, and composition of medium, for cell growth and PHB production in *Rhodobacter sphaeroides*. We found that PHB content is the highest under dark-aerobic conditions. In dark-aerobic conditions, the cell growth is promoted with the application of oxygen as a terminal electron acceptor and succinic acid as a carbon and energy source. The photo-sensitive cyclic electron transfer pathway is shut down under the dark conditions due to the termination of the photo-oxidation in the reaction center (RC) [45]. Consequently, the reducing equivalents are

accumulated within the cytoplasmic membrane, causing greater production of PHB as an electron and carbon sink. We observed maximum cell growth as well as PHB accumulation at 48 h. After 48 h, the cell vitality reduced, and the PHB contents decreased by almost 50% compared to the level at 48 h. Initiation of PHB degradation when the cells reached the stationary phase was reported previously, confirming the importance of harvesting time on PHB production [3]. Similar to our results on the effects of temperature, PHB synthesis decreased at higher and lower temperatures than 30 °C in *Bacilllus subtilis*, which is likely caused by the decline of PHB synthetic enzyme activity [46]. PHB production is significantly affected by nitrogen availability when the amount of carbon remains the same and is not required under nutrient-rich conditions [5,47], supporting our data on the effects of the carbon/nitrogen ratio in the medium. Carbon and nitrogen sources are also very closely related to PHB production. In *Rhodobacter*, it has been reported that organic acids and inorganic nitrogen sources produce better PHB than sugar and organic nitrogen sources, respectively [17,48]. Adding medium optimization to our optimal PHB production conditions will allow us to achieve higher PHB production.

We examined the expression patterns of four genes, *PhaA*, *PhaB*, *PhaC*, and *PhaZ*, involved in the biosynthesis and metabolism of PHB in *R. sphaeroides*. The overall transcript levels of *PhaA* and *PhaB* genes were similar under diverse experimental conditions, because *PhaA* and *PhaB* genes are located in the same operon, while the *PhaC* and *PhaZ* genes are located in a different operon [19]. The *PhaA* gene is important to initiate PHB production by acetyl-CoA condensation, and the *PhaB* gene is the most critical genetic component for PHB productivity [49]. Therefore, expression levels of *PhaA* and *PhaB* genes indicated a strong correlation with PHB production, whereas those of *PhaC* and *PhaZ* genes were independent relatively. The *PhaZ* gene, which mediates PHB depolymerization, is downregulated during the active growth phase by PhaR, a regulator protein of phaZCPR operon [5]. Recent studies revealed that disruption of the *PhaZ* gene and overexpression of the *PhaA*, *PhaB*, and *PhaC* genes elevated PHB production in *R. sphaeroides* [18]. Moreover, PHB biosynthetic enzymes, including PhaC, which is the limiting step for PHB production in *R. sphaeroides*, altered their activities very easily in response to changes in the external environment [18,50]. It is expected that PHB production will be further enhanced through combination with optimization for the expression of PHB biosynthesis-related genes and growth conditions.

Since *R. sphaeroides* has a versatile metabolism, modification of the substrate instead of succinic acid in the medium can be a solution to improve the economics of PHB production. In terms of bioremediation, municipal and industrial organic waste, including food waste and sludge, are good alternatives. CO_2 will be utilized as the main carbon source for PHB production in the future, because *R. sphaeroides* also has the ability to utilize CO_2. Furthermore, to compensate for the physical properties of PHB, including brittleness and poor processability, it is possible to produce copolymers, such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), by utilizing long-chain carbohydrates such as propionic acid as a carbon source [51]. In some cases, interactions between *R. sphaeroides* and other microorganisms lead to better production results. When the three different types of *R. sphaeroides* were mixed, they showed higher hydrogen yield and specific production rate than each pure culture [52]. Through co-culture with *Clostridium butyricum*, acetic acid produced by *C. butyricum* was used as a substrate to increase the rate of hydrogen production [53]. In this way, mixed microorganisms can create synergy that balances energy and substances by constructing various metabolic pathways through interdependent relationships.

Air and light cause oxidative stress, such as cell death, growth retardation, and alteration of metabolism, in bacteria. Our results also indicate that light leads to changes in intracellular ROS levels and antioxidant activities such as peroxidase activities and carotenoid accumulation. In the presence of carotenoids, the cells can be partially protected from oxidative damage. The carotenoid-deficient mutants were more sensitive to ROS compared to wild-type *R. sphaeroides* [27], indicating that carotenoids act as an antioxidant. These observations indicate that PHB production is strongly influenced by ROS. Whereas the concentration of H_2O_2 was slightly higher under light conditions than under dark conditions, PHB contents were higher under dark conditions compared to light

conditions in *R. sphaeroides*. In *Caulobacter crescentus*, H_2O_2-induced gene expression involved in PHB depolymerization [54]. Methyl-esterified 3-hydroxybutyrate (ME-3HB) oligomers, synthesized by PHB depolymerization, protect the bacteria cells from ROS due to their ROS scavenging activity [55]. *R. sphaeroides* is also known to produce PHB well, and thus, it will be very interesting to study the mechanism of ROS regulation by these oligomers in the future.

Furthermore, *R. sphaeroides* has diverse biosynthetic pathway and is capable of growth under various environments, such as light/dark, aerobic/anaerobic, and autotrophic/heterotrophic conditions. This implies that ROS generated by light, air, and high/low temperatures has a critical impact on intracellular metabolism. These features suggest that *R. sphaeroides* is a good model system to study ROS signaling adaptation to environmental changes and mediation of biochemical production.

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