



# Enhanced Production of Astaxanthin by Co-culture of *Paracoccus haeundaensis* and Lactic Acid Bacteria

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This study aimed to determine the increase in astaxanthin production from *Paracoccus haeundaensis* by optimizing the medium conditions and co-culturing with lactic acid bacteria. In order to select a suitable strain to co-culture with *P. haeundaensis*, several strains were co-cultured with *P. haeundaensis*. *Lactobacillus fermentum* was selected as the optimal strain. Further to optimize the astaxanthin production in the co-culture, various medium conditions were tested and the PMF medium developed in this study was selected as the optimum medium. For the co-culture under optimal conditions, the dried cell mass and astaxanthin concentration were  $1.84 \pm 0.09$  g/L and  $821.09 \pm 30.98$   $\mu$ g/g-dried cell weight, respectively, which was 2.5 times higher than that of the *P. haeundaensis* strain in the Luria-Bertani broth medium. In order to confirm the transcription level of the carotenoid biosynthesis genes such as *crtE*, *crtB*, *crtl*, *crtY*, *crtZ*, and *crtW* of *P. haeundaensis* in the co-culture, real-time qPCR was performed. Among the six carotenoid biosynthesis genes, *crtl*, *crtY*, and *crtZ* showed a significant difference in relative RNA levels. The mass culture was performed using a 5 L jar fermenter and the astaxanthin concentration was the same as that at the laboratory scale. These results may be used as reference for co-culture and astaxanthin production in carotenoid biosynthesis microorganisms.

**Keywords:** astaxanthin, co-culture, ferric citrate, *Lactobacillus fermentum*, *Paracoccus haeundaensis*

## INTRODUCTION

Carotenoids are fat-soluble pigments that are widely present in nature and have red, orange, and yellow colors. These pigments are biosynthesized from bacteria, algae, yeast, fungi, and plants, and play essential roles in photosynthesis, such as electron transport and photoprotection (Domonkos et al., 2013). Also, carotenoids have essential physiological activities, such as antioxidant, anti-inflammatory, and anticancer activities in mammalian cells (An et al., 1991; Kobayashi et al., 1993; Castelblanco-Matiz et al., 2015). Carotenoids mainly have 40 carbon atoms backbones and are classified as either carotenes, which consist of only carbon and hydrogen atoms, and xanthophylls with cyclic or acyclic functional groups containing oxygen (Emmerstorfer-Augustin et al., 2016). Currently, more than 1,000 different carotenoids are known (Yabuzaki, 2017), and these

carotenoids are listed on the website <http://carotenoiddb.jp>. Among the carotenoids, astaxanthin has excellent physiological activity in humans including antioxidant and anti-inflammatory effects (Fassett and Coombes, 2009, 2011), and is used in cosmetic additives, dietary supplements, and aquaculture feed. In order to produce astaxanthin industrially, *Haematococcus pluvialis* and *Phaffia rhodozyma* have been used for decades (Miller et al., 1976; Bublick, 1991), and research has been conducted to produce astaxanthin from various microorganisms (Ide et al., 2012; Watanabe et al., 2018; Jhang et al., 2020).

Many studies have reported an increase in the production of carotenoids from carotene-producing microorganisms. The methods used to increase carotenoid production include mutations of microorganisms by chemical and physical mutagens (Seo et al., 2017), stress during microbial culture (Li et al., 2019), and the co-culture of microorganisms with other microorganisms. Co-culture methods have been used to produce a variety of valuable chemicals from microorganisms (Jones and Wang, 2018). Some studies have revealed that carotenoid production is increased when different microorganisms are co-cultured with carotenoid-producing microorganisms. Studies on carotenoid production using co-culture include *Rhodotorula glutinis* and *Debaryomyces castellii* in corn syrup (Buzzini, 2001), *R. glutinis* and *Chlorella vulgaris* in starch waste water (Zhang et al., 2019), *R. rubra*, and *Lactobacillus casei* in whey ultrafiltrate (Simova et al., 2003), *R. rubra* and yogurt starter in whey ultrafiltrate (Simova et al., 2004) and *H. pluvialis* and *B. subtilis* in starch-containing waste water (Bohutskyia et al., 2018). According to these studies, the cell mass and carotenoid production increased in the raw materials and byproducts of agro-industrial origin, such as corn syrup, whey, and starch wastewaters. These studies show that the production of carotenoids in carotenoid-producing microorganisms is increased by interactions with other bacteria, which aid in the use of nutrients in the media (such as starch and whey) that are not available to carotenoid-producing microorganisms.

*Paracoccus haeundaensis* is known as a strain that produces astaxanthin. However, it is difficult to use commercially due to low astaxanthin productivity. For this reason, in order to increase the production of astaxanthin from *Paracoccus* sp. strains, mainly physicochemical mutation methods were studied (Ide et al., 2012; Seo et al., 2017). However, no studies were reported to increase the production of astaxanthin using co-culture of *Paracoccus* sp. strains. The purpose of this study was to determine the production of astaxanthin using a co-culture method to establish the optimal production conditions for *P. haeundaensis* and to increase astaxanthin production. In order to increase the production of carotenoids in the *P. haeundaensis* strain, lactic acid bacteria and *Bacillus subtilis* were co-cultured with *P. haeundaensis* by changing the composition of the medium. This medium referred to the composition of LB medium (Luria-Bertani medium) known as nutrient medium and PPES-II medium for culturing marine bacteria. After optimizing the medium composition, lactic acid bacteria strains were selected for co-culture with *P. haeundaensis*. *P. haeundaensis* has been reported to possess the *crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, and *crtW* genes required for the biosynthesis of astaxanthin (Figure 1), and

astaxanthin production is directly related to the expression of these genes. To analyze the cause of the increase in astaxanthin production, the cell mass and astaxanthin produced were analyzed after the medium composition and strain were selected, and expression patterns of the carotenoid biosynthesis genes were analyzed using real-time quantitative polymerase chain reaction (qPCR). *Paracoccus haeundaensis* and lactic acid bacteria were co-cultured in a 5 L jar fermenter, and the production of the cell mass and astaxanthin were confirmed.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

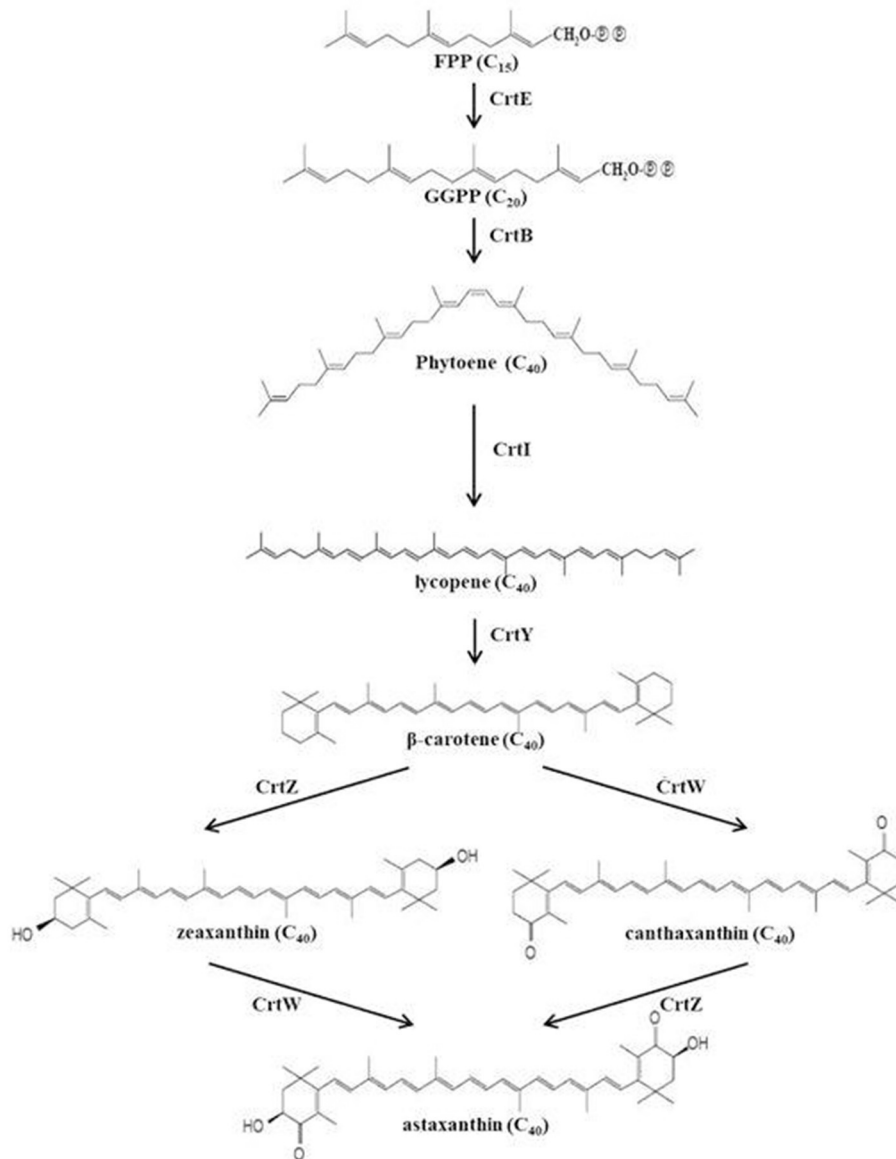
The *P. haeundaensis* (KCCM 10460) strain was cultured at 25°C for 24 h using LB medium. *Lactobacillus sakei* (KCCM 40264), *L. casei* (KCCM 35465), *L. brevis* (KCCM 11509), *L. fermentum* (KCCM 35461), *L. helveticus* (KCCM 11223), *Enterococcus faecium* (KCCM 12117), *E. durans* (KCCM 40711), *E. lactis* (KCTC 21015), and *Bacillus subtilis* (KCCM 11316) were cultured at 37°C for 24 h in MRS medium (10 g peptone, 10 g beef extract, 4 g yeast extract, 20 g glucose, 5 g sodium acetate trihydrate, 1 g polysorbate 80, 2 g dipotassium hydrogen phosphate, 2 g triammonium citrate, 0.2 g magnesium sulfate heptahydrate, 0.05 g manganese sulfate tetrahydrate, and distilled water 1 L). In a previous study, *P. haeundaensis* was found to have optimal growth and astaxanthin production at 25°C; thus, the co-culture of *P. haeundaensis* and lactic acid bacteria was performed at 25°C (Lee et al., 2004).

### Co-culture of *Paracoccus haeundaensis* With Lactic Acid Bacteria

The co-culture of *P. haeundaensis* with lactic acid bacteria was performed to increase the production of astaxanthin. *Paracoccus haeundaensis* were pre-cultured for 24 h at 25°C in LB medium, and the lactic acid bacteria strains for the co-culture were pre-cultured for 24 h at 37°C in MRS medium. After pre-culture, 1 L of the LB medium was inoculated with 10% volume of the *P. haeundaensis* cultured medium and each of the lactic acid bacteria culture media, then incubated at 25°C for 72 h. After incubation, the cells cultured in each medium were centrifuged at 4,000 rpm for 30 min, and the supernatant was removed and washed three times with physiological saline. After the collected cells were dried using a freeze dryer (FDU-2200, Eyela, Japan), the weight of the dried cell mass was measured. The freeze-dried cell mass samples were stored at -20°C.

### Cell Mass and Astaxanthin Production by Medium Composition

The media MLB (8 g tryptone, 5 g bacto yeast extract, 10 g NaCl, and 1 L distilled water), PPES-II (0.2 g polypeptone, 1.0 g bacto soytone, 1.0 g proteose peptone, 1.0 g bacto yeast extract, 30 g NaCl, 0.1 g ferric citrate, and 1 L distilled Water), MLB50 (5 g tryptone, 2.5 g bacto yeast extract, 10 g NaCl, and 1 L distilled water), PM (4 g tryptone, 0.2 g polypeptone, 1.0 g bacto soytone, 1.0 g proteose peptone, 3.5 g bacto yeast extract, 3% NaCl, 0.1% ferric citrate, and 1 L distilled water) were prepared. A co-culture of *P. haeundaensis* and *L. fermentum* was incubated for 72 h at



**FIGURE 1** | Schematic of the carotenoid biosynthesis pathways of *P. haeundaensis*.

25°C in each of the prepared media. After freeze-drying, the weight of the dried cell mass was measured.

In some studies, there were reported that  $\text{Fe}^{2+}$  ions enhance the production of astaxanthin (Fraser et al., 1997; Zhou et al., 2015). Based on these studies, in order to confirm the production of the cell mass and astaxanthin depending to the concentration of ferric citrate, ferric citrate was added into the PM medium at final concentrations of 0, 0.1, 0.05, 0.01, 0.005, and 0.001%, respectively. *Paracoccus haeundaensis* and *L. fermentum* were inoculated in each medium followed by incubation at 25°C for 72 h. The cells cultured in each medium were centrifuged and washed three times with physiological saline. After the collected

cells were dried, the weight of the dried cell mass was measured. The freeze-dried samples were stored at  $-20^{\circ}\text{C}$ .

### Cell Mass and Astaxanthin Production by the Inoculation Ratio

To confirm the ratio optimization of the inoculation for the co-culture, the amount of the culture solution of each strain was inoculated differently. Both cultures used for the inoculation were cultured for 24 h. To confirm the optimal inoculation conditions of the *L. fermentum* culture, the inoculation of the *P. haeundaensis* culture was kept at 10% of the volume of medium to be co-cultured, while *L. fermentum* cultures were inoculated

with 0, 1, 5, 10, 15, and 20% of the volume of medium to be co-cultured. The optimum conditions for the *P. haeundaensis* culture were also confirmed by the same method. After washing with physiological saline and freeze-drying, the weight of the dried cell mass was measured.

## Carotenoid Extraction From Bacteria and Astaxanthin Analysis by High Performance Liquid Chromatography

In order to analyze the production of astaxanthin produced from the collected cell samples, 0.1 g of the freeze-dried sample was treated with chloroform: methanol (v/v, 6:4), followed by vortexing at 3 h intervals for 24 h at room temperature in a dark room. After centrifugation at 4,000 rpm for 30 min, the upper solvent was collected and filtered through a 0.22  $\mu$ m filter. The pretreated extract and standard sample (Sigma, USA) were subjected to high performance liquid chromatography (HPLC). The analysis was performed using an Alliance e2695 Separations Module (Waters, USA), and the column was Poroshell 120 EC-C18 (4.6  $\times$  250 mm) (Agilent, USA). Methanol:acetonitrile (v/v, 80:20) mixed solvent was used as the mobile phase solvent, and the absorbance was measured at 470 nm.

## Real-time Quantitative Polymerase Chain Reaction for the RNA Level of the Carotenoid Biosynthesis Genes

Real-time qPCR was performed with cDNA synthesized from *P. haeundaensis* total RNA. The primers used for the cDNA synthesis and real-time qPCR of carotenoid biosynthesis genes were designed using the astaxanthin biosynthesis gene cluster (AY957386.1) of *P. haeundaensis*, and the primers of *rpoB* gene for housekeeping gene was designed using genome sequence of *Paracoccus marcusii* (NZ\_VDDD01000029.1). The primers are shown in **Table 1**. The equipment used for the real-time qPCR was QuantStudio 6 Flex (Thermo Fisher, US), and the reagents were AccuPower<sup>®</sup> 2X GreenStar<sup>™</sup> qPCR Master Mix (Bioneer, Daejeon, Korea). The real-time qPCR was performed under conditions of 15 s at 95°C denaturation, 20 s at 58°C annealing, and 20 s at 72°C extension. The RNA levels of each gene were analyzed with Ct values and normalized to the *rpoB* gene transcript level (Dahllöf et al., 2000; Rodrigues and Tiedje, 2007).

## Scale-Up With Optimum Production Conditions

To mass-produce astaxanthin from the *P. haeundaensis* strain, *P. haeundaensis*, and *L. fermentum* strains were co-cultured in a 5 L jar fermenter. For use as a seed culture, the *P. haeundaensis* strain was cultured for 24 h at 25°C in LB medium and the *L. fermentum* strain was cultured for 24 h at 37°C in MRS medium. The medium used for the mass culture of the strains was the PMF medium. The mass culture was inoculated with a seed culture of 10% of the volume to be cultured. The culture was incubated at 25°C with shaking at 160 rpm and cultured for 72 h at 1 vvm of aeration.

## Statistical Analysis

The experimental results of all groups were expressed as the mean  $\pm$  standard deviation (mean  $\pm$  SD), and the significance test of the control group and the experimental group was confirmed by a Student's *t*-test. When the significance probability was  $p < 0.05$ , it was determined that there was a significant difference between the control and experimental groups.

## RESULTS

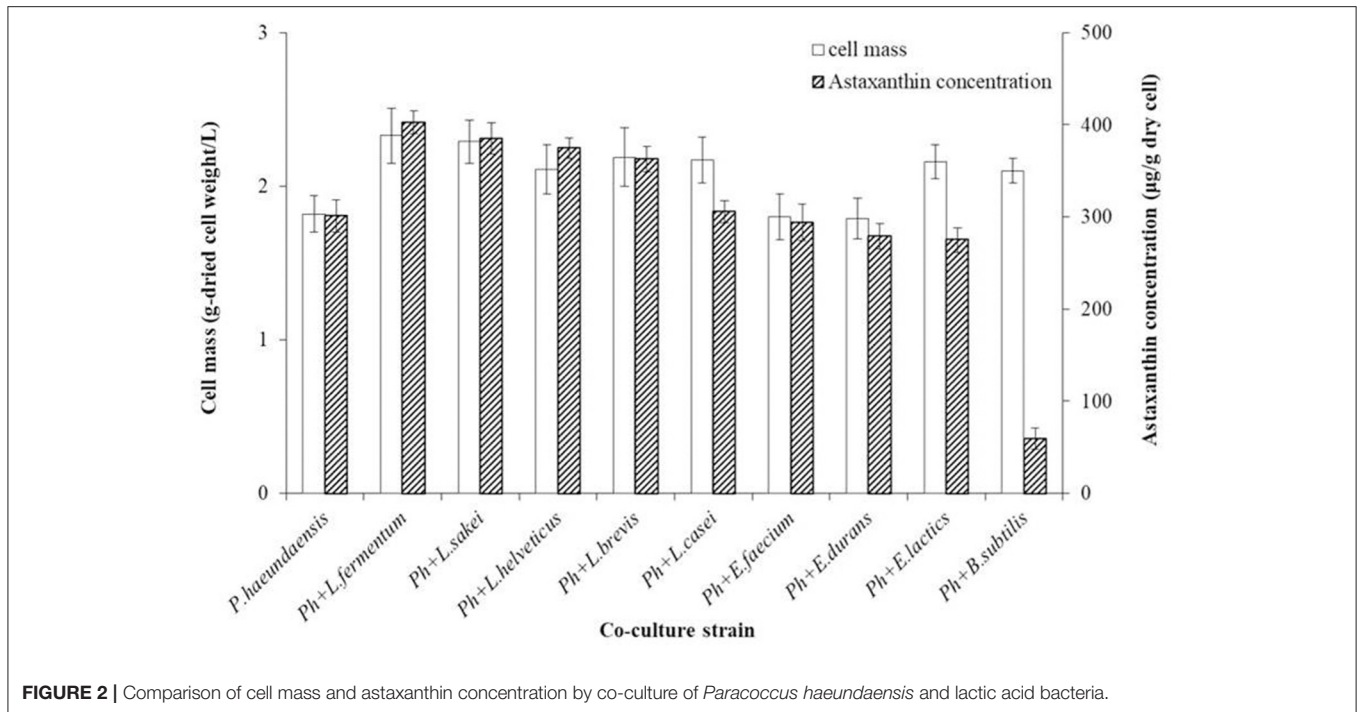
### Selection of Lactic Acid Bacteria for Co-Culture With *Paracoccus haeundaensis*

To increase the production of astaxanthin from *P. haeundaensis*, lactic acid bacteria were co-cultured with *P. haeundaensis*. The cell mass and astaxanthin concentrations of the co-cultures are shown in **Figure 2**. The co-culture of *P. haeundaensis* with *L. fermentum*, *L. sakei*, *L. brevis*, and *L. casei* significantly increased the cell mass and astaxanthin concentration compared to the monoculture of *P. haeundaensis* ( $p < 0.05$ ). In particular, the cell mass of the co-culture of *P. haeundaensis* and *L. fermentum* was the highest at  $2.33 \pm 0.18$  g/L, which was 1.28 times higher than that of  $1.82 \pm 0.12$  g/L of *P. haeundaensis* alone.

The cell mass and astaxanthin concentrations from each co-culture using different lactic acid bacteria were compared. Astaxanthin concentrations were highest in the co-culture of *P. haeundaensis* and *L. fermentum* at  $402.84 \pm 12.60$   $\mu$ g/g-dried cell weight, which was 1.34 times higher than that of *P. haeundaensis* alone ( $301.14 \pm 17.43$   $\mu$ g/g-dried cell weight). In addition, all co-cultures of *P. haeundaensis* and *Lactobacillus* spp. strains showed higher astaxanthin concentrations than *P. haeundaensis* monoculture, while the co-culture of *P. haeundaensis* with other

**TABLE 1** | Primer sequences for the real-time quantitative polymerase chain reaction.

Genes	Forward primer	Reverse primer	Length (bp)
<i>crtE</i>	5'-CGGGGGTTCGAACAGGAACAG-3'	5'-ATCGCGACCGGTATCCTTGC-3'	209
<i>crtB</i>	5'-TGTTGGATGAGGCGGAACCC-3'	5'-TCTTGGCAGCCTTGGACGTG-3'	180
<i>crtI</i>	5'-GCACAACTATCGCGACCTGC-3'	5'-CGGGCCCTTGAAGATCTCGT-3'	196
<i>crtY</i>	5'-CCAGAAATTCGTGGGCGTCG-3'	5'-ATCGGAATAGCGCGTGTCT-3'	163
<i>crtZ</i>	5'-TGGGCATGACCGTCTATGGG-3'	5'-GGTCTGCTTCAGCTTGTGCG-3'	207
<i>crtW</i>	5'-GACGACCCCGATTTCGACCA-3'	5'-CAGGTGCCGAACACGGAACAG-3'	203
<i>rpoB</i>	5'-CACCGCGGAATACGACAAGC-3'	5'-GGCGCATGACGCGATAGATG-3'	194



**FIGURE 2** | Comparison of cell mass and astaxanthin concentration by co-culture of *Paracoccus haeundaensis* and lactic acid bacteria.

genera, such as *Enterococcus* spp. and *Bacillus subtilis*, were even lower than those of *P. haeundaensis*.

When the amount of astaxanthin produced in 1 L of medium was calculated, the co-culture of *P. haeundaensis* and *L. fermentum* was highest at about 938.61 µg. This value is approximately 1.71 times higher than the 548.07 µg produced by *P. haeundaensis*, and 1.06 times higher than the 881.99 µg produced in the co-culture of *P. haeundaensis* and *L. sakei*. Therefore, *L. fermentum* was selected as the optimal strain for co-culture with *P. haeundaensis*.

## Analysis of Astaxanthin Production According to Medium Composition

The LB medium is a basic nutrient medium widely used for bacterial cultures, and the PPES-II medium is a marine bacterial culture medium used for culturing *P. haeundaensis* in a previous study (Lee et al., 2004). Based on these two media, the optimum composition was prepared by modifying the media composition. *Paracoccus haeundaensis* and *L. fermentum* were co-cultured in LB, MLB, MLB50, PM, and PPES-II media in order to optimize the medium to increase the cell mass and astaxanthin concentration.

The highest yield of cell mass was  $2.34 \pm 0.08$  g/L in the LB medium, while the cell mass in MLB and MLB50 was  $2.29 \pm 0.09$  g/L and  $1.66 \pm 0.08$  g/L, respectively. The lower the nutrient content of the medium, the less cell mass was produced. In the PPES-II medium, which contained lower nutrient contents than the modified LB medium, the cell mass was  $0.36 \pm 0.02$  g/L. In contrast, the astaxanthin concentrations in the LB, MLB, and MLB50 were  $401.46 \pm 28.31$  µg/g-dried cell weight,  $420.63$

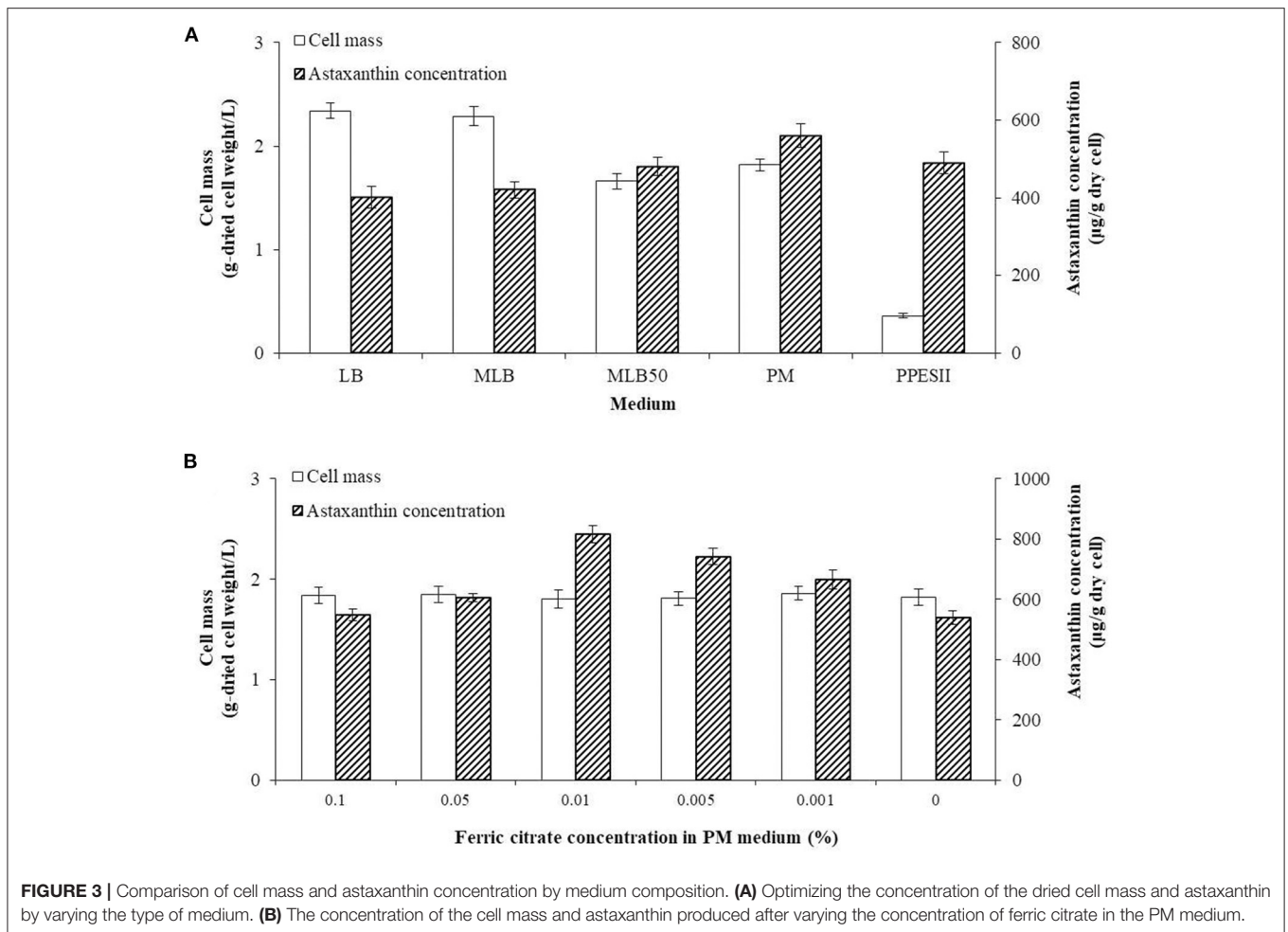
$\pm 28.31$  µg/g-dried cell weight, and  $480.66 \pm 23.65$  µg/g-dried cell weight, respectively, and the astaxanthin concentration increased with decreasing nutrients in the LB medium. The astaxanthin concentration in the PPES-II medium was  $490.12 \pm 27.36$  µg/g-dried cell weight. In order to supplement the astaxanthin production in the MLB50 and PPES-II media, the PM medium was prepared, which is a combination of the MLB50 and PPES-II media. The cell mass and astaxanthin concentration in PM were  $1.82 \pm 0.06$  g/L and  $560.31 \pm 29.97$  µg/g-dried cell weight, respectively, which was higher than that in the MLB50 and PPES-II media (Figure 3A).

To optimize the production of the co-culture cells and astaxanthin, the co-culture was performed in PM with different ferric citrate concentrations. The production of the cells did not significantly differ among the different concentrations of ferric citrate. As the concentration of ferric citrate in the PM medium changed, the astaxanthin concentration gradually increased from  $548.16 \pm 18.97$  µg/g -dried cell weight at 0.1% ferric citrate to  $817.49 \pm 28.73$  µg/g-dried cell weight at 0.01%. However, when the concentration of ferric citrate was lower than 0.01%, the astaxanthin concentration decreased (Figure 3B).

As a result of this experiment, we determined that the optimal medium for producing astaxanthin in the co-culture was a PM medium containing 0.01% ferric citrate, which was named as the PMF medium.

## Analysis of Astaxanthin Production According to Inoculation Ratio

In order to confirm the optimal inoculation amount in the co-culture, the inoculation amount of *P. haeundaensis* was fixed at 10%, and *L. fermentum* was inoculated at different



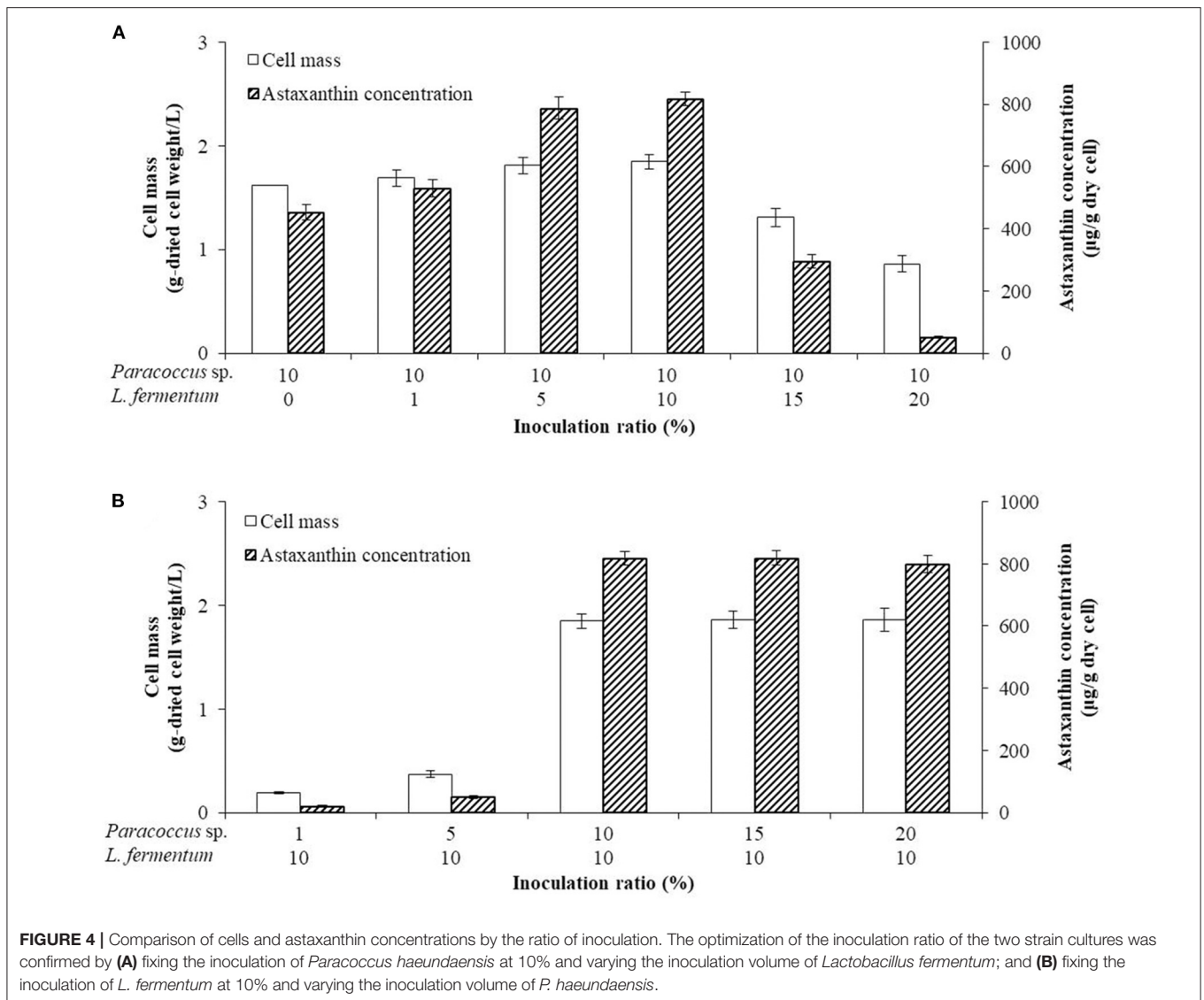
concentrations in the PMF medium. The cell mass and astaxanthin concentrations increased gradually up to 10% of the inoculation amount of *P. haeundaensis*, and the cell mass and astaxanthin concentrations were highest at  $1.85 \pm 0.07$  g/L and  $817.49 \pm 21.84$  µg/g-dried cell weight, respectively, when the inoculation amount of *L. fermentum* was 10%. However, when the inoculum of *L. fermentum* was 15%, the cell mass and astaxanthin concentration decreased to  $1.31 \pm 0.09$  g/L and  $294.36 \pm 22.07$  µg/g-dried cell weight, respectively (**Figure 4A**).

The inoculation amount of *L. fermentum* was fixed at 10%, and *P. haeundaensis* was inoculated at different concentrations. The cell mass and astaxanthin concentration at 1% of *P. haeundaensis* were  $0.19 \pm 0.01$  g/L and  $21.23 \pm 1.99$  µg/g-dried cell weight, respectively, and at 5% were  $0.37 \pm 0.03$  g/L and  $49.93 \pm 3.39$  µg/g-dried cell weight, respectively. In addition, the cell mass and concentration of astaxanthin at 15% of *P. haeundaensis* were  $1.86 \pm 0.08$  g/L and  $818.63 \pm 22.69$  µg/g-dried cell weight, respectively. Compared with the results of the 10% inoculation of *P. haeundaensis*, the cell mass and astaxanthin concentration in the 1 and 5% *P. haeundaensis* inoculations showed very low productivity, and the production at more than 15% *P. haeundaensis* was not significantly different compared to the production at 10% (**Figure 4B**). From these experiments, it

was confirmed that the productivity was the highest when the inoculation amounts of *P. haeundaensis* and *L. fermentum* were both at 10%. In order to confirm the production of cell mass over time, the growth curve was confirmed while incubating for 72 h. Cell mass was measured at 3 h intervals. At the end of the experiment, the concentration of dried cells in co-culture was  $1.82 \pm 0.05$  g/L, which was slightly increased compared to  $1.64 \pm 0.03$  g/L in a single culture of *P. haeundaensis*. In contrast, the dried cell mass of *L. fermentum* single culture was  $0.23 \pm 0.02$  g/L, and it was confirmed that the growth rate was slow at 25°C incubation temperature (**Figure 5**).

### Total Carotene in the Co-Culture of *Paracoccus haeundaensis* and *Lactobacillus fermentum*

To confirm the effect of media optimization on carotenoid synthesis, the carotenoids were analyzed by culturing *P. haeundaensis* in the LB and PMF media, respectively. In addition, to confirm the carotenoid change in the co-culture, carotenoids were analyzed from the co-culture of *P. haeundaensis* and *L. fermentum* in the PMF medium and compared with the results of *P. haeundaensis* single culture. The quantitative analysis of



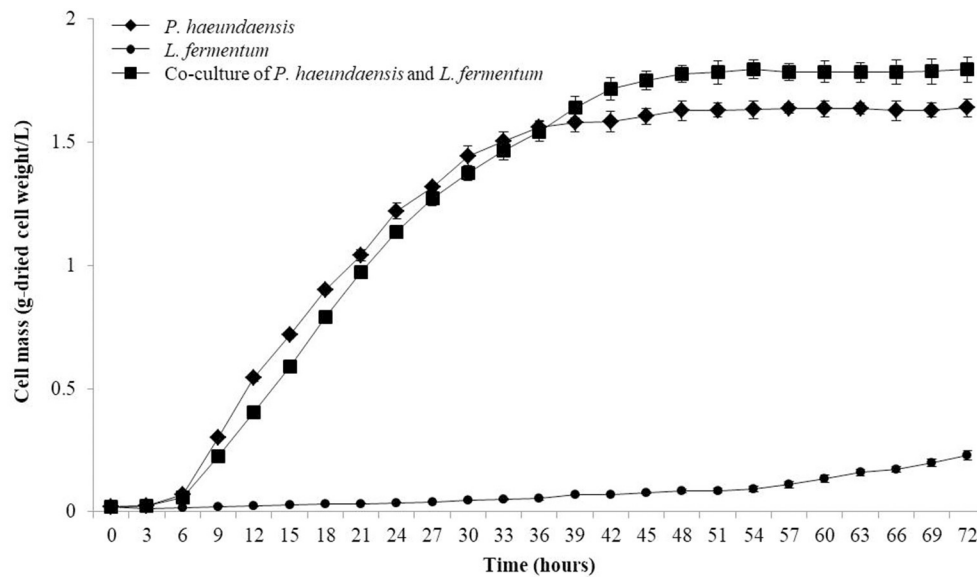
the carotenoids produced from *P. haendaensis* in the LB and PMF medium and the co-culture in the PMF medium used zeaxanthin, canthaxanthin,  $\beta$ -carotene, and lycopene standards in HPLC analysis and are shown in **Figure 6**.

The amount of carotenoids under each condition are shown in **Table 2**. When the concentrations of the carotenoids in the LB and PMF media were compared, the astaxanthin concentration increased significantly in PMF, while  $\beta$ -carotene and lycopene decreased significantly. The zeaxanthin and canthaxanthin tended to increase and decrease, respectively, but there was no significant difference in the concentrations. These results were related to the optimization of the composition of the medium, and in particular, it is assumed that the activity of CrtZ increased due to the  $\text{Fe}^{2+}$  ions contained in the PMF medium. As the activity of CrtZ increased, the biosynthesis of astaxanthin and zeaxanthin increased, and  $\beta$ -carotene and lycopene were shown to decrease due to their consumption as substrates in the carotenoid synthesis pathway.

When *P. haendaensis* and the co-culture carotenoid concentrations were compared in the PMF medium, the astaxanthin production increased 1.77 times, and the other carotenoids, except canthaxanthin, also increased significantly. It is assumed that the increase in the concentration of most carotenoids in the co-culture is related to the activity of enzymes involved in the carotenoid synthesis pathway.

## RNA Levels of Carotenoid Biosynthesis Genes

In order to confirm the transcription level of the carotenoid biosynthesis genes of *P. haendaensis* in the co-culture, cDNA was synthesized using reverse primers for each gene, and real-time qPCR was performed. The RNA levels of each gene were analyzed with Ct values and normalized to the *rpoB* gene transcript level (**Figure 7**). Among the six carotenoid biosynthesis genes, *crtE*, *crtB*, and *crtW* did not show a significant difference in relative RNA levels. The relative RNA



**FIGURE 5** | Growth curve of *P. haeundaensis* single culture, *L. fermentum* single culture and co-culture of *P. haeundaensis*, and *L. fermentum*.

level of the *crtI* gene was significantly increased by 3.2 times when co-cultured ( $0.879 \pm 0.023$ ) compared to that of *P. haeundaensis* alone ( $0.272 \pm 0.010$ ). In the co-culture, the relative RNA level of the *crtY* gene was  $0.596 \pm 0.029$ , which was 1.9 times higher than  $0.312 \pm 0.012$  of *P. haeundaensis*. In addition, the *crtZ* gene was  $8.748 \pm 0.265$  in the co-culture and increased by 1.9 times compared to  $4.683 \pm 0.172$  in *P. haeundaensis*.

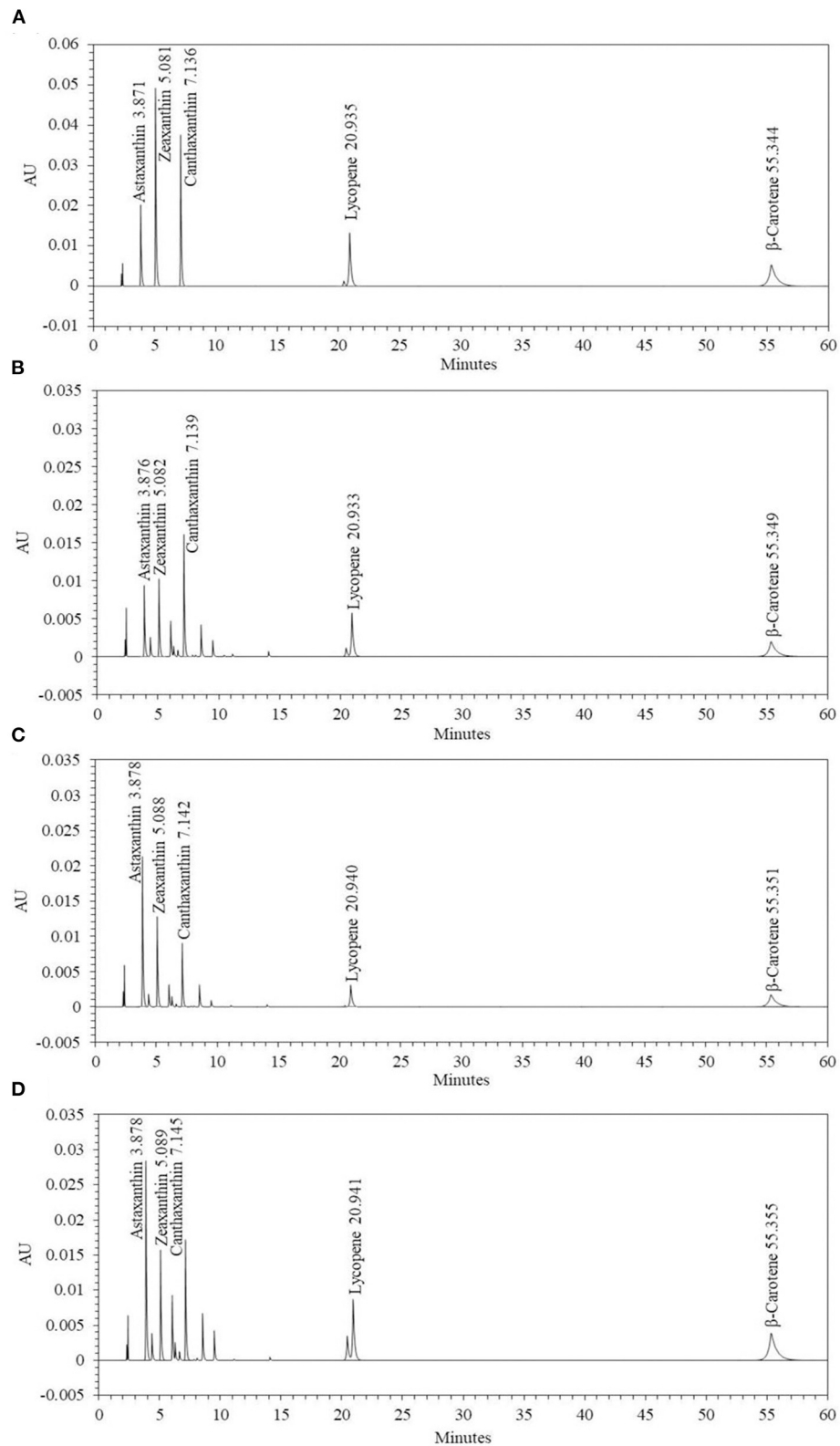
### Cell Growth and Astaxanthin Production in the 5 L Co-Culture System

The co-culture of *P. haeundaensis* and *L. fermentum* was performed in a 5 L jar fermenter. As a medium for the 5 L fermentation, the PMF medium was used, and *P. haeundaensis* and *L. fermentum* strains were simultaneously inoculated and incubated for 72 h. The concentration of cells and astaxanthin were measured at 6-h intervals. The growth rate of the cells and the production rate of astaxanthin remained constant and there was no difference between the laboratory scale production and the 5 L jar fermenter. Regarding the cell concentration rate in the co-culture, the stationary phase was reached in 42 h in both the laboratory scale and the 5 L jar fermenter. At the end of the experiment, the dried cell concentration of the 5 L jar fermenter was  $1.81 \pm 0.04$  g/L, which was about 99.6% compared to  $1.81 \pm 0.07$  g/L on the laboratory scale cultured in flask. In addition, the maximum astaxanthin production in the co-culture culture was reached in 54 h in both samples. The astaxanthin concentration of the 5 L jar fermenter sample at 72 h was  $808.72 \pm 15.58$   $\mu\text{g/g}$ -dried cell weight, which was about 99.3% compared to  $814.20 \pm 35.03$   $\mu\text{g/g}$ -dried cell weight on the laboratory scale (Figure 8).

### DISCUSSION

In this study, we confirmed the astaxanthin production according to the co-culture of *P. haeundaensis* and *L. fermentum*. First, in order to identify the optimal medium, various mediums were prepared to confirm astaxanthin production in co-culture. When the composition of the marine bacterial medium PPES-II medium was added with modified LB medium, the production of astaxanthin and cell mass were increased. The medium to be used for co-culture was optimized by adjusting the concentration of ferric citrate in the medium composition. In some studies, there were reported that  $\text{Fe}^{2+}$  ions enhance the production of astaxanthin (Zhou et al., 2017). In the composition of the PM medium, 0.1% ferric citrate is present, and it has been shown that ferric citrate increases the astaxanthin production of the PPES-II medium and PM medium. To optimize the production of the co-culture cells and astaxanthin, the co-culture was performed in PM with different ferric citrate concentrations. The production of the cells did not significantly differ among the different concentrations of ferric citrate. As the concentration of ferric citrate in the PM medium changed, the astaxanthin concentration highly increased at 0.01%. As a result of this experiment, we determined that the optimal medium for producing astaxanthin in the co-culture was a PMF medium which is PM medium containing 0.01% ferric citrate, and  $\text{Fe}^{2+}$  ions contained in the medium were shown to increase the production of astaxanthin. Second, as a result of confirming the astaxanthin production by the inoculation amount, it was confirmed that the initial inoculation amount affects astaxanthin production. When the inoculation amount of *L. fermentum* is higher than the optimal inoculation amount, it seems to inhibit the growth of *P. haeundaensis*. In addition, *L. fermentum* grows

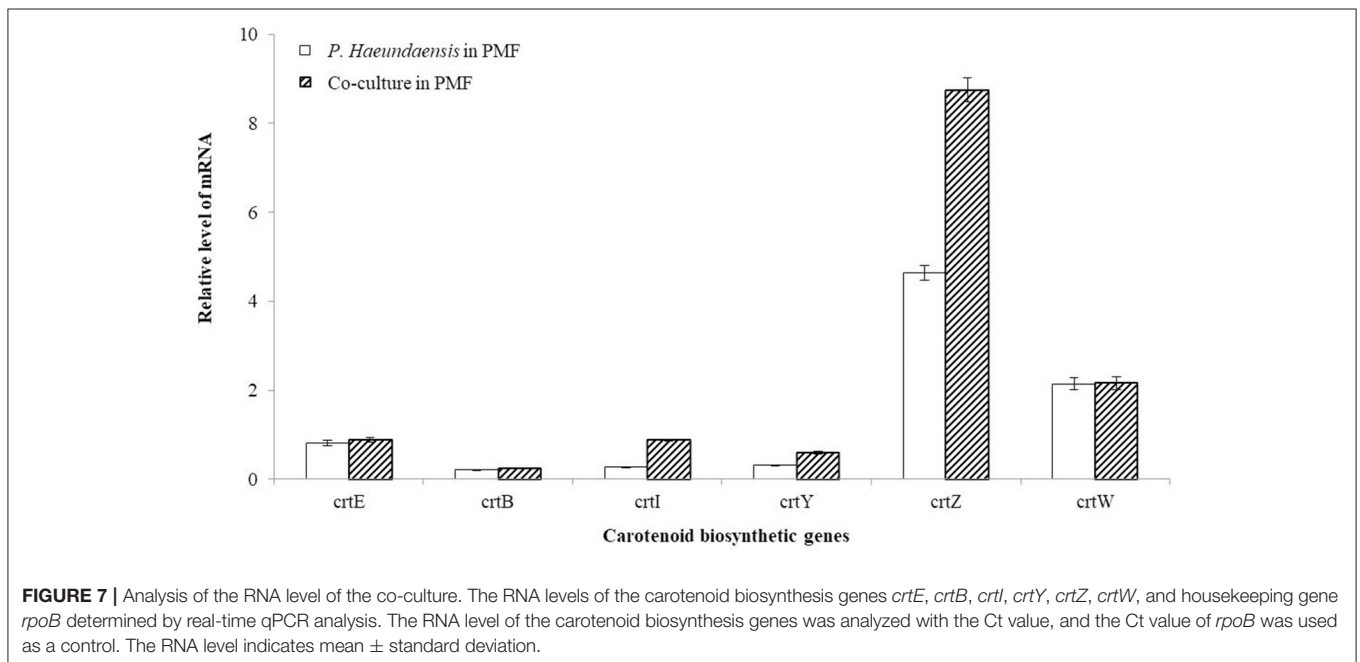




**FIGURE 6** | High performance liquid chromatography chromatograms of the carotenoids extracted from *Paracoccus haeundaensis* and the co-culture with *Lactobacillus fermentum*. **(A)** Standard carotenoids, **(B)** *P. haeundaensis* cultured in the LB medium, **(C)** *P. haeundaensis* cultured in the PMF medium, **(D)** co-culture of *P. haeundaensis* and *L. fermentum* in the PMF medium.

**TABLE 2** | Carotenoid concentrations according to culture medium and the co-culture of *Paracoccus haeundaensis* and *Lactobacillus fermentum*.

Carotenoid	Carotenoid concentration ( $\mu\text{g/g}$ -dried cell weight)		
	<i>P. haeundaensis</i> in LB	<i>P. haeundaensis</i> in PMF	Co-culture in PMF
Astaxanthin	303.34 $\pm$ 20.22	462.86 $\pm$ 29.39	821.09 $\pm$ 30.98
Zeaxanthin	151.19 $\pm$ 13.37	176.31 $\pm$ 15.26	220.76 $\pm$ 19.91
Canthaxanthin	548.51 $\pm$ 33.33	501.69 $\pm$ 18.71	547.71 $\pm$ 14.92
$\beta$ -carotene	223.21 $\pm$ 17.49	183.38 $\pm$ 16.96	416.33 $\pm$ 10.99
Lycopene	265.53 $\pm$ 8.81	202.26 $\pm$ 11.12	381.28 $\pm$ 26.65



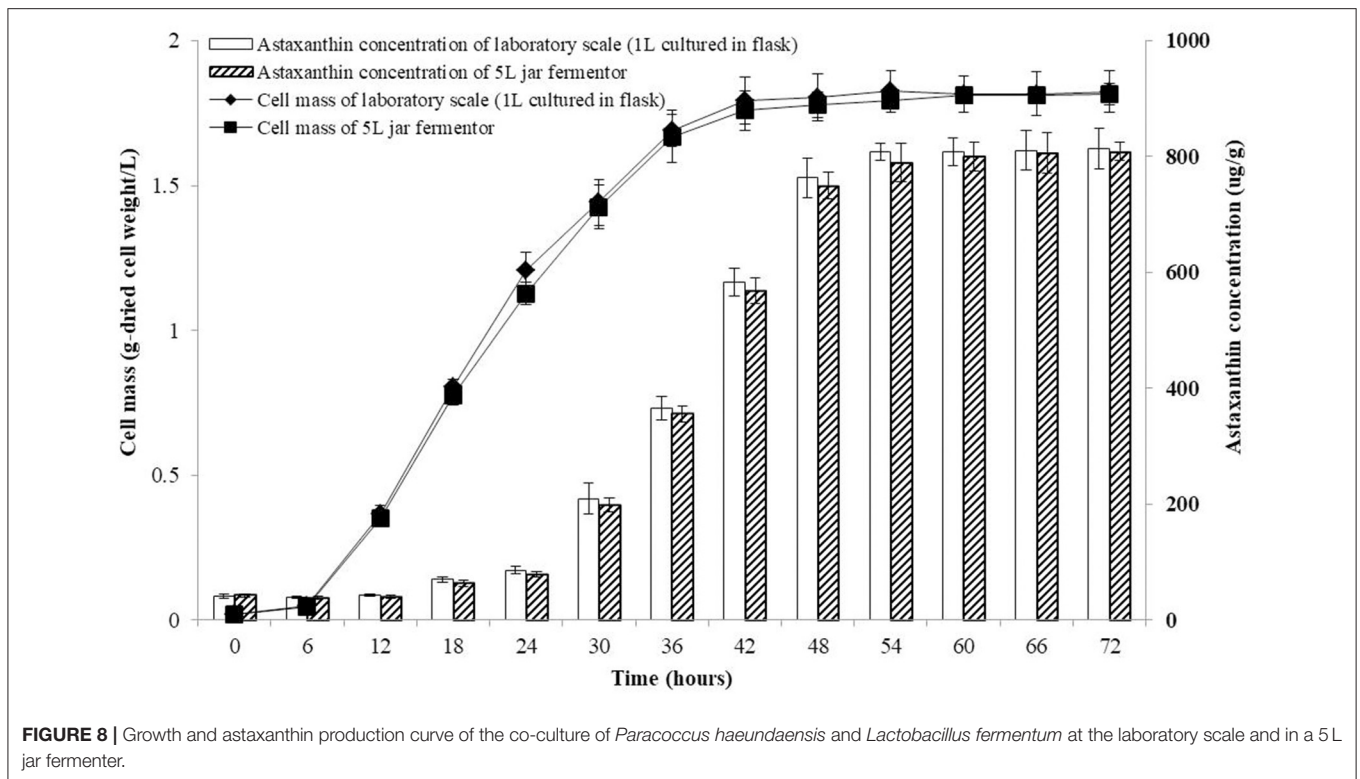
slowly at 25°C. For this reason, both strains cannot grow during co-culture, and as a result, the production of cell mass seems to decrease. These results indicate that the optimization of the medium and the inoculation amount are important for the production of and astaxanthin.

Compared with the single culture of *P. haeundaensis*, the cell mass was increased in co-culture. It was thought that the growth of *L. fermentum* strain was inhibited and the cell mass of *P. haeundaensis* increased at a culture temperature of 25°C. However, as a result of single culture of *L. fermentum* at 25°C, an increase in cell mass was confirmed, although the growth was slow. Therefore, it is assumed that the increase in cell mass in co-culture was due to the growth of the *L. fermentum* strain, not the growth of the *P. haeundaensis* strain.

An increase in the transcription level of the *crtI* gene plays an important role in carotenoid biosynthesis, as CrtI converts phytoene to lycopene by removing eight hydrogen atoms and inducing four double bonds. In plants, this step is involved in several enzymes, such as phytoene desaturase,  $\zeta$ -carotene desaturase,  $\zeta$ -carotene cis-trans isomerase, and carotene cis-trans isomerase, but only one enzyme is involved in CrtI in

bacteria. This step is the main regulatory step of the carotenoid biosynthesis pathway, and thus, CrtI plays a key enzyme role in the pathway (Schaub et al., 2012; Choi et al., 2018). Therefore, the increased transcription of the *crtI* gene from *P. haeundaensis* in the co-culture increases not only lycopene synthesis but also most of the carotenoids synthesized after this step.

The *crtZ* is a gene that converts  $\beta$ -carotene into zeaxanthin and synthesizes astaxanthin together with the *crtW* gene, and thus plays an important role in the synthesis of astaxanthin. When *P. haeundaensis* and *L. fermentum* were co-cultured, the RNA level of *crtW* did not significantly change, but *crtZ* increased approximately 1.9 times compared to that in the *P. haeundaensis* single culture. This result is closely related to the slight decrease in canthaxanthin and the increased content of zeaxanthin and astaxanthin in the co-culture. Increasing the RNA levels of the *crtI* and *crtY* genes enhances the induction of  $\beta$ -carotene biosynthesis, which increases the concentration of  $\beta$ -carotene, and the high concentration of the  $\beta$ -carotene, a substrate for CrtZ and CrtW enzymes, would enhance the synthesis of zeaxanthin and canthaxanthin with the CrtZ and CrtW enzymes, respectively. However, the increase in the expression of CrtZ



**FIGURE 8 |** Growth and astaxanthin production curve of the co-culture of *Paracoccus haeundaensis* and *Lactobacillus fermentum* at the laboratory scale and in a 5 L jar fermenter.

induces more conversion of  $\beta$ -carotene to zeaxanthin, and it is presumed that the production of canthaxanthin from  $\beta$ -carotene by CrtW is relatively small. In addition, it is presumed that a slight decrease in the concentration of canthaxanthin in the co-culture is competitively limited to the use of CrtW  $\beta$ -carotene by the increase of the CrtZ enzyme, and is caused by the competitive inhibitor of increased zeaxanthin, another substrate of CrtW.

In several studies, *L. fermentum* has been reported to produce useful substances such as organic acids and vitamins (LeBlanc et al., 2011; Khalil et al., 2018), which are thought to increase the RNA level of astaxanthin biosynthetic enzyme from *P. haeundaensis*. However, it was not known exactly how the substance or interaction stimulates *P. haeundaensis* to promote the production of astaxanthin. The co-culture conditions of the 5 L jar fermenter were established to conditions for astaxanthin concentration on a larger scale. However, compared to the laboratory-scale flask culture, astaxanthin production has not improved at the fermenter scale, despite better control of process parameters such as temperature, pH, DO, etc. in 5 L jar fermenter. It appears that further research is needed to search for substances produced by *L. fermentum* that stimulate astaxanthin production from *P. haeundaensis*, and to find the optimal conditions for increasing astaxanthin production in a 5 L jar fermenter.

In this study, the co-culture of *P. haeundaensis* and *L. fermentum* increased astaxanthin concentration. This indicates

that *L. fermentum* stimulates the astaxanthin production in the co-culture. These studies may be useful as reference for co-culture and astaxanthin production from carotenoid biosynthesis microorganisms.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

SC conducted an overall experiment and wrote this paper. YS provided ideas for the initial experiment, and gave interpretation and advice on the experimental results. S-WN gave advice on conducting the experiment. G-DK was the corresponding author. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** YS was employed by the company CBS Bioscience Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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