Journal of Microbiological Methods 160 (2019) 20–28

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Multiple promoters driving the expression of astaxanthin biosynthesis genes can enhance free-form astaxanthin production

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ARTICLE INFO

Keywords:
Astaxanthin
Escherichia coli
Multiple promoters

ABSTRACT

Astaxanthin possesses various biological properties and is used in the animal and fish feed, food, and beverage industries. In this study, we derived zeaxanthin biosynthesis genes (*crtE*, *crtB*, *crtI*, *crtY*, and *crtZ*) from *Erwinia uredovora* and *crtW* from *Agrobacterium aurantiacum*. We fused inducible and constitutive promoters to astaxanthin biosynthesis genes to construct a novel plasmid (dubbed PTP3-6) that can effectively enhance free-form astaxanthin (FFAX) production. The PTP3-6 plasmid contains one T7 promoter, driving IPTG inducible *crtW* expression, and three constitutive promoters (isolated from *E. uredovora*) driving expression of the other zeaxanthin biosynthesis genes. *Escherichia coli* BL21 (DE3) cells carrying the PTP3-6 plasmid produced 8.3 mg/g dry cell weight astaxanthin, which is 69.4-fold higher than has been previously reported. Using multiple promoter fusions of astaxanthin biosynthesis genes could be applied in other hosts to enhance astaxanthin production. FFAX was identified in recombinant *E. coli* cells through ultra-performance liquid chromatography-mass spectrometry.

1. Introduction

Astaxanthin (3, 3′-dihydroxy-β, β'-carotene-4, 4′-dione) is a natural carotenoid pigment found in various marine bacteria, yeast, algae, and aquatic animals (Higuera-Ciapara et al., 2006). Astaxanthin is approved as a food colorant and is used in the animal and fish feed, food, and beverage industries (Ambati et al., 2014; Asker, 2017). The antioxidant activity of astaxanthin is higher than that of β-carotene, lutein, and vitamin E (Ambati et al., 2014). Therefore, astaxanthin can protect cells, lipids, and membrane lipoproteins against oxidative damage (Kamath et al., 2008; Ambati et al., 2014). In addition, astaxanthin possesses various other biological properties, including anti-lipid peroxidative, anti-inflammatory, anti-diabetic, anticancer, and immune-modulating properties; it can also help prevent cardiovascular disease (Miki, 1991; Higuera-Ciapara et al., 2006; Rao et al., 2013; Ambati et al., 2014). Astaxanthin exists naturally in free and esterified forms. The natural compositions of astaxanthin esters are quite complex, therefore free-form astaxanthin (FFAX), which can be easily digested and absorbed by animals, is added to feed (Choubert and Luquet, 1983; Sommer et al., 1991; Ma and Chen, 2001; Chen et al., 2015). The addition of an appropriate amount of FFAX to high-cholesterol diets has been shown to effectively increase anti-oxidative activity in the liver and reduce lipid peroxidase concentrations (Chen et al., 2015). Furthermore, FFAX, which can be linked to functional fatty acids or water-soluble groups through its hydroxyl groups, could be used in development of specific astaxanthin esters or antioxidant therapeutics (Ma and Chen, 2001; Zhao et al., 2011). Therefore, compared with astaxanthin esters, FFAX is commercially more valuable, and has potential applications in developing healthy food, and feed additives, as well as nutraceutical and pharmaceutical products. FFAX can also enhance the value of fish farming (Ma and Chen, 2001; Higuera-Ciapara et al., 2006; Zhao et al., 2011; Ambati et al., 2014; Chen et al., 2015).

Astaxanthin can be produced by metabolic engineering of recombinant microorganisms, such as algae, yeast, and bacteria (Misawa and Shimada, 1998; Mao et al., 2017). The alga *Haematococcus pluvialis* was shown to produce a high content of astaxanthin, but the major products were esterified (70% w/w monoester and 25% w/w diester) with only a small amount of FFAX (5% w/w) (Miao et al., 2006; Zhao et al., 2011). Since FFAX is difficult to generate from hydrolysis of astaxanthin esters, the astaxanthin produced by this strain has limited value in drug development. Astaxanthin esters can be hydrolyzed to form FFAX by anaerobic saponification under alkaline conditions, but this method may produce by-products, such as astacene, that might have different biological functions than astaxanthin (Zhao et al., 2011).

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https://doi.org/10.1016/j.mimet.2019.03.012
Received 17 August 2018; Received in revised form 15 March 2019; Accepted 16 March 2019
Available online 16 March 2019
0167-7012/

Algal production of astaxanthin has a few disadvantages, such as the long culture period, at least 7–10 days in a two-stage batch process (Schmidt et al., 2011), and complicated extraction procedures, due to coexistence of chlorophylls, fatty acids, glycerides and other contaminants (Yokoyama et al., 1994). Compared with algae, the yeast *Xanthophyllomyces dendrorhous* is more widely used to produce FFAX. However, the high cost of industrial-scale production, resulting from the expensive fermentation procedure (two-stage phase process), and poor yield remain limiting factors for large scale production of FFAX using *X. dendrorhous* (Schmidt et al., 2011; Zhao et al., 2011; Ambati et al., 2014). Other recombinant hosts, such as *Candida utilis* (Misawa and Shimada, 1998), *Saccharomyces cerevisiae* (Zhou et al., 2015), and *Escherichia coli* (Misawa and Shimada, 1998; Mao et al., 2017), produce lower levels of FFAX than *H. pluvialis* (Steinbrenner and Sandmann, 2006) and *X. dendrorhous* (Gassel et al., 2013), but the higher growth rate of these recombinant hosts may compensate for lower astaxanthin production (Yuan et al., 2006).

E. coli is a non-carotenogenic bacterium that can naturally synthesize farnesyl pyrophosphate (FPP), a β-carotene precursor (Misawa and Shimada, 1998; Samoudi et al., 2015). *E. coli* is an appropriate host for FFAX production because it readily expresses many astaxanthin biosynthesis genes (Yuan et al., 2006). Additionally, *E. coli* is preferred for industrial-scale production because it has a shorter production time, smaller cultivation space, easier extraction processes, and more feasible incubation conditions. Several studies have reported novel, recombinant *E. coli* cell lines that synthesize FFAX (Misawa and Shimada, 1998; Mao et al., 2017), but the amount of FFAX accumulated in the recombinant *E. coli* cells (pACCAR25△crtX and pAK916) accounted for only 50% of the total carotenoid content (Misawa et al., 1995a, 1995b). It has been suggested that overexpression of the isopentenyl diphosphate isomerase (*idi*) gene from *E. coli* could increase the carbon flux of the isoprenoid pathway leading to the formation of FPP and a corresponding increase in the amount of FFAX accumulated (Mao et al., 2017). Additionally, plasmids constructed using two promoters (pTrc*CrtW*-pBAD*CrtZ*) (Scaife et al., 2009) or strong promoters (pMH1-pFZ81-pFZ153) (Ma et al., 2016) fused with astaxanthin biosynthesis genes could increase the amount of astaxanthin accumulated in recombinant *E. coli* cells. In this study, our goal was to increase astaxanthin production in a recombinant *E. coli* using multiple promoters fused to astaxanthin biosynthesis genes. To this end, a novel plasmid containing four promoters (one inducible and three constitutive) that drive the expression of astaxanthin biosynthesis genes at different times was constructed. It is likely that this strategy of incorporating multiple promoters fused to astaxanthin biosynthesis genes can be applied in other hosts to enhance astaxanthin production.

2. Materials and methods

2.1. Plasmid construction and growth conditions

The detailed procedure used to construct the 7 expression vectors is shown in Fig. 2. The zeaxanthin biosynthesis genes *crtE*, *crtY*, *crtI*, *crtB* and *crtZ* were isolated from *Erwinia uredovora* (GenBank: D90087.2), and *crtW* was isolated from *Agrobacterium aurantiacum* (PRF: 1096128) (Misawa et al., 1990; Misawa et al., 1995a, 1995b). An artificial operon containing a multigene construct under control of an *E. uredovora* constitutive promoter (native promoter) was made (Weber et al., 2011). Firstly, a single PCR product containing the *crtY-crtI-crtB* fragment was digested using *HindIII* and *XhoI* and ligated into the *HindIII/XhoI* sites of the pET-30a(+) expression vector (Novagen, Darmstadt, Germany) to generate pT-YIB (Fig. 2A). Physically, *crtY-crtI-crtB* are closely linked; for example, the ribosome-binding site of *crtI* overlaps with the stop codon of *crtY*. Another PCR product comprising the constitutive promoter sequence fused with the *crtY-crtI-crtB* fragment was digested using *HindIII* and *XhoI* and ligated into the *HindIII/XhoI* sites of the pET-30a(+) expression vector to generate pYIB. Next, a PCR product containing the constitutive promoter sequence fused with the *crtE* fragment was digested using *BamHI* and *SacI* and ligated into the *BamHI/SacI* sites of both pT-YIB and pYIB vectors to generate Pp-4 and Pp2-4, respectively. Third, a PCR product of the *crtW* fragment was digested using *KpnI* and *BamHI* and ligated into the *KpnI/BamHI* sites of Pp2-4 to generate Pp2-4a. Another PCR product of constitutive promoter sequence fused with the *crtZ* fragment was digested using *KpnI* and *BamHI* and ligated into the *KpnI/BamHI* sites of Pp2-4 to generate Pp2-4b. Finally, a PCR product of the constitutive promoter sequence fused with the *crtZ* fragment was digested using *XbaI* and *KpnI* and ligated into the *XbaI/KpnI* sites of Pp2-4a to generate PP3-6. Another PCR product of the *crtZ* fragment was digested using *NdeI* and *KpnI* and ligated into the *NdeI/KpnI* sites of Pp2-4a to generate PTP1-6, and another PCR product of the *crtW* fragment was digested using *NdeI* and *KpnI* and ligated into the *NdeI/KpnI* sites of Pp2-4b to generate PTP3-6. The six genes and promoters were inserted in the same orientation.

The recombinant *E. coli* BL-21 (DE3) cells (dubbed DE3 cells) were cultured in Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/mL). Cells were incubated for 18 h at 37 °C, followed by culturing at 16 °C for 20 h with or without isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration: 0.1 mM) addition.

2.2. RNA purification and cDNA synthesis

Total RNA from DE3 cells was prepared using TOOLS EasyPrep Cell &Bacteria RNAprep Purification Kit (Tools, Taiwan, R.O.C.). Total RNA was collected from samples in quintuplicate at each treatment time point. RNA concentration was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). The cDNA was synthesized from 500 ng of total RNA in a 50 μL reaction using ToolScript M-MLV Reverse Transcriptase (Tools, Taiwan, R.O.C.) and random octamers.

2.3. Quantitative real-time PCR

The cDNA levels were analyzed using a QuantStudio 12 K Flex Real-Time PCR System (Thermo Fisher Scientific Inc., MA, USA) with SYBR Green I detection. Each sample was measured in triplicate in a 96-well plate in a reaction mixture (10 μL final volume) containing TOOLS 2× SYBR qPCR Mix (Tools, Taiwan, R.O.C.) and 200 nM primer mix. Quantitative real-time PCR (qRT-PCR) was performed with an initial denaturation of 15 min at 95 °C, followed by 40 cycles of 10s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. Amplifications were run in triplicate together with controls that contained no template and no reverse transcription for each of the examined genes. Relative transcript levels were normalized to that of an internal control *cysG* (Zhou et al., 2011) using the Pfaffl method (Pfaffl, 2001). The gene-specific primers are listed in Table 1.

2.4. Extraction and purification of carotenoid pigments from recombinant E. coli cells

DE3 cells were harvested by centrifugation (4600*g* for 10 min at 4 °C). Carotenoid pigments were extracted from cell pellets with

Table 1
Primers used for qRT-PCR analysis.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
cysG	TTGTCGGCGGTGGTGATGTC	ATGCGGTGAACTGTGGAATAAACG
crtE	CGTGATTGCCTGCATCGTT	CAGCAGTTGAAATGCCTGACC
crtY	TCAGACACTGCTGCGAGAAGAA	GGCATTGCCCGACAGAGTAAT
crtI	TTGATTTTCGCGACCAGCTT	CCACAGAAAAGGCTGAGCCAT
crtB	ACAGCCAACTGGATGATACGCT	ACAACGCCTGCAACGTGATAG
crtZ	CGTTGGCCATTCCGCTATATT	CGCCATATACAACCGTTTGAGG
crtW	AGCTTGTCCTGTGGCTGTATGC	TGCTTGACGATCATCTTGCG

acetone, and then vigorously vortexed for 20 min. The acetone extracts were centrifuged at 10,000 *g* for 15 min at 4 °C. After centrifugation, the supernatant was loaded onto a rotary evaporation system (Rotavapor R-210 Series, Buchi Labortechnik, Flawil, Switzerland) at 40 °C and 80 MPa for 30–45 min until dry granules were formed. The dry granules were added to a mixture of n-hexane and ddH_2O (3:2, v/v) to separate the two phases. The collected upper layer and precipitate were freeze-dried *in vacuo* with a freeze-dryer (Christ Alpha 1–4, Osterode am Harz, Germany) to obtain a powder, which was stored at −20 °C for subsequent analyses. All processes were conducted in darkness.

2.5. Analytical procedures

FFAX and β-carotene standards were purchased from Sigma Co. (St. Louis, MO, USA). The content of β-carotene or FFAX was determined by high-performance liquid chromatography (HPLC, Agilent 1100, Agilent Technologies, Waldbronn, Germany); the HPLC instrument comprised an 1100 series device with a diode array detector (450 nm for β-carotene detection, and 470 nm for FFAX detection). The separations processes were performed on a 250 mm × 4.6 mm Syncronis C18 column (Thermo Fisher Scientific Inc., MA, USA) at 30 °C. The separation of the acetone extracts from the DE3 cells was achieved using a mobile phase composition of acetone-water (75:25, v/v, buffer A) and acetone-methanol (75:25, v/v, buffer B) mixtures. The elution process followed a linear gradient of 0% to 65% buffer B for 10 min. After 10 min, a linear gradient of 65% to 100% buffer B for 60 min was applied, followed by a final linear gradient of 100% to 0% buffer B for 10 min at a flow rate of 1 mL/min. The injection volume was 20 μL for all acetone extracts.

For liquid chromatography-mass spectrometry (LC-MS) measurements, an ultra-performance liquid chromatography (UPLC, Waters Corp., Milford, CT, USA) module was coupled to a Q-Tof (Waters Synapt G1 high definition mass spectrometer, Waters Corp., Milford, CT, USA) system equipped with an atmospheric pressure chemical ionization (APCI) interface and an ion trap. The separations processes were performed on a 100 mm × 2.1 mm Acquity UPLC BEH C18 column (Waters Corp., Milford, CT, USA) at 30 °C. The mobile phase composition of acetone-water (75:25, v/v, buffer A) and acetone-methanol (75:25, v/v, buffer B) mixtures was also used. The analyte elution process followed a linear gradient of 1% to 99% buffer B for 5 min. After 5 min, a linear gradient of 99% to 1% buffer B for 1 min at a flow rate of 0.4 mL/min was applied. The injection volume was 10 μL for all acetone extracts. The detection was performed using APCI in the positive ionization mode. The APCI of MS/MS parameters was performed as described by Miao et al. (2006). The spectrometer was calibrated in the positive mode, and $[M + H]^+$ ions were recorded.

2.6. Statistical analysis

All experiments were conducted in triplicate; the data were averaged and are presented as the mean ± standard deviation. One-way analysis of variance followed by Tukey's test was used to determine significant differences using the SPSS (version 22) package. Statistical significance was defined as $P < .05$.

3. Results and discussion

3.1. Gene selection and function

Many β-carotene biosynthesis genes (*crtE*, *crtB*, *crtI*, and *crtY*) have been cloned from various organisms and their functions have been determined (Armstrong, 1994). Although *E. coli* strains cannot synthesize β-carotene, they can synthesize a precursor of β-carotene, FPP (Misawa and Shimada, 1998; Samoudi et al., 2015). Therefore, we used genetic engineering technology to construct novel plasmids containing multiple promoters fused with β-carotene synthesis genes (*crtE*, *crtY*, *crtI*, and *crtB*), and transformed these into *E. coli* cells. The zeaxanthin biosynthesis genes (*crtE*, *crtY*, *crtI*, *crtB* and *crtZ*) used in this study were all isolated from *E. uredovora* and were transformed into and expressed in *E. coli* cells that produced more zeaxanthin than reported in previous studies has been proven (Li et al., 2015). Astaxanthin exists in geometric isomers, stereoisomers, as well as in free and esterified forms (Higuera-Ciapara et al., 2006). In animal studies, 3S, 3′ S-astaxanthin was found to exert no harmful effects on reproduction or fetal development and had higher antioxidative activity than other stereoisomers (Schneider et al., 2016; Liu et al., 2016). Therefore, we used *crtW* isolated from *A. aurantiacum*, which produces only the 3S, 3′ S-astaxanthin (Yokoyama et al., 1994) in construction of plasmids in the current study. Additionally, a constitutive promoter, which can effectively drive multigene expression in *E. coli* expression systems, was also isolated from *E. uredovora* (Misawa et al., 1990; Weber et al., 2011).

crtE encodes geranylgeranyl pyrophosphate (GGPP) synthase, which catalyzes the formation of GGPP from FPP; *crtB* encodes phytoene synthase, which catalyzes the formation of phytoene from GGPP; *crtI* encodes phytoene desaturase, which catalyzes the formation of lycopene from phytoene; *crtY* encodes lycopene cyclase, which catalyzes the formation of β-carotene from lycopene. *crtZ* encodes β-carotene hydroxylase, which catalyzes the hydroxylation of β-carotene to synthesize zeaxanthin; *crtW* encodes β-carotene ketolase, which can catalyze the conversion of methylene to keto groups of β-carotene to synthesize canthaxanthin. Zeaxanthin and canthaxanthin synthesize astaxanthin through the catalysis of β-carotene ketolase and β-carotene hydroxylase, respectively (Misawa et al., 1990; Misawa et al., 1995a, 1995b) (Fig. 1).

3.2. Expression of β-carotene genes

The DE3 cells containing the Pp-4 and Pp2-4 plasmids both produced β-carotene, but the yield of β-carotene was different. Expression β-carotene biosynthesis genes in Pp2-4 and Pp-4 plasmids were investigated by qRT-PCR. Using *cysG* as an internal control, analyses were made based on threshold cycle (Ct) values of the target genes. *cysG* was selected as a reference gene because it is known to be a suitable internal control for gene expression profiling in BL21 cells, and the its expression was found to be the most constant in the recombinant *E. coli* cells in different growth conditions, such as growth temperature and inducer concentrations (Zhou et al., 2011). The △Ct values were calculated by subtracting the Ct of the sample cDNA from that of the control cDNA at the same time measurement (Eom et al., 2006). The △Ct value represents the exponent in the calculation for amplification and reflects the relative expression of the target gene compared with the control. According to the Pfaffl method, normalization of the target gene with an endogenous standard was done *via* reference gene expression, which is calculated as $2^{-\triangle\triangle Ct}$ (Pfaffl, 2001).

The purpose of this study was to increase the production of astaxanthin in a recombinant *E. coli* cells. Previous research has indicated that *crtE* overexpression is necessary for increasing astaxanthin production (Wang et al., 1999), therefore, the *BamHI*-constitutive promoter-*crtE-SacI* fragments were ligated into the pT-YIB and pYIB vectors, respectively, to generate the Pp-4 and Pp2-4 plasmids (Fig. 2B). Because the Pp-4 and Pp2-4 plasmids both contained one T7 promoter and the *BamHI*-constitutive promoter-*crtE-SacI* fragment, DE3 cells carrying the Pp-4 and Pp2-4 plasmids had higher level of *crtE* expression when IPTG was added to medium than when it was omitted, but when IPTG addition to DE3 cells carrying the Pp-4 and Pp2-4 plasmids had no significantly different of *crtE* expression level to each other ($P > .05$) (Fig. 3A). DE3 cells carrying the Pp2-4 plasmid had higher levels of *crtY*, *crtI*, and *crtB* expression when induced with IPTG. When IPTG was added into DE3 cells carrying the Pp-4 and Pp2-4 plasmids, the former had lower levels of *crtY*, *crtI*, and *crtB* expression than DE3 cells carrying the Pp2-4 plasmid. DE3 cells carrying the Pp-4 plasmid with IPTG addition had higher levels of *crtY*, and *crtI* expression than

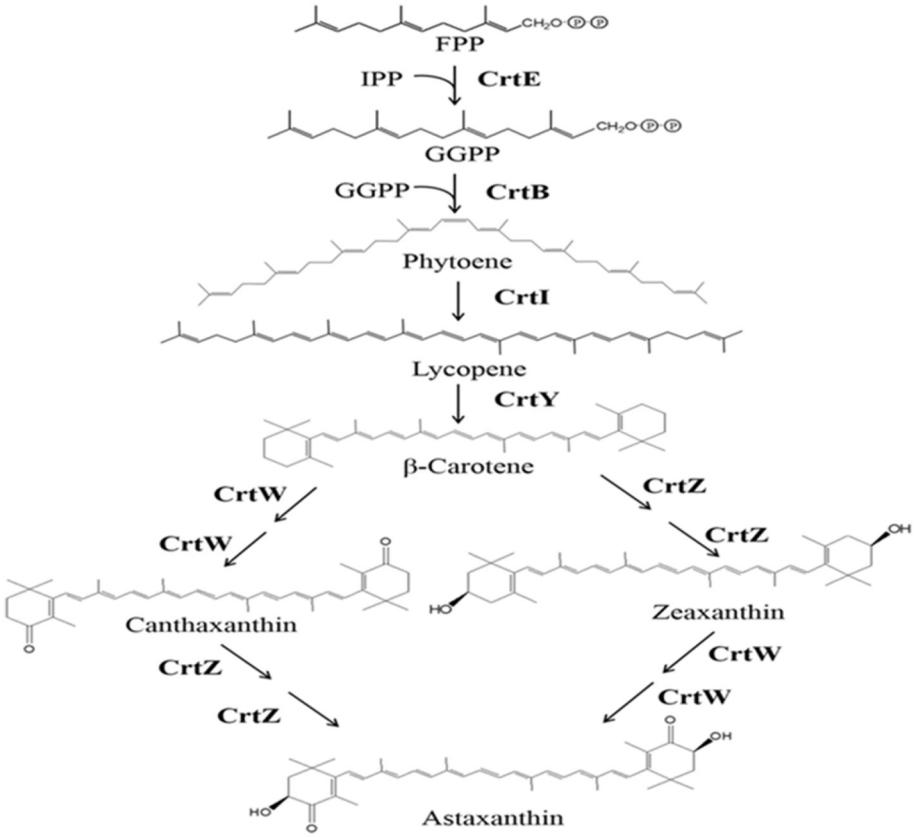

Fig. 1. Astaxanthin biosynthetic pathway model. β-carotene formation from FPP, and astaxanthin formation from β-carotene in recombinant *E. coli* cells. FPP: farnesyl pyrophosphate, GGPP: geranylgeranyl pyrophosphate. IPP: isopentenyl pyrophosphate (diphosphate).

DE3 cells carrying the Pp2-4 plasmid without IPTG addition. (Fig. 3B–D). We speculate that when IPTG was added, the expression of downstream genes might be driven by the T7 promoter through the constitutive promoter. Interestingly, contrary to the levels of *crtY* and *crtI* expression, the expression level of *crtB* in DE3 cells carrying the Pp-4 plasmid with IPTG addition were not significantly different ($P > .05$) to DE3 cells carrying the Pp2-4 plasmid without IPTG addition (Fig. 3D). We speculate that the distance of the *crtB* is too far from the T7 promoter to be affected by the regulation from the T7 promoter (Fig. 2B).

3.3. β-Carotene production

As shown in Fig. 4, DE3 cells carrying the Pp2-4 plasmid had the highest β-carotene production. Previous studies have reported that *idi* overexpression can increase the formation of isoprenoid compounds in recombinant *E. coli* cells (Kajiwara et al., 1997; Misawa and Shimada, 1998; Mao et al., 2017). The pRH1, pSI1, and pHP11 plasmids, carrying three exogenous *idi* genes, were separately isolated from *X. dendrorhous*, *S. cerevisiae*, and *H. pluvialis* (Kajiwara et al., 1997). The pACCAR16△crtX plasmid had one constitutive promoter driving expression of *crtE*, *crtB*, *crtI*, and *crtY* for catalyzing FPP to form β-carotene [488 μg/g dry cell weight (DCW)] in recombinant *E. coli* cells (Misawa et al., 1995a, 1995b; Misawa and Shimada, 1998). When the pRH1, pSI1, or pHP11 plasmid was transformed into recombinant *E. coli* cells (pACCAR16△crtX), the concentration of β-carotene was 709, 758, or 1310 μg/g DCW, respectively (Misawa and Shimada, 1998). In the current study, the β-carotene concentration (4540 μg/g DCW, Fig. 4) produced by DE3 cells carrying the Pp2-4 plasmid was higher than that reported by previous studies of recombinant *E. coli* cells carrying different plasmids. Based on the results transcriptional analyses (Fig. 3) and β-carotene production (Fig. 4), the Pp2-4 plasmid was used as the backbone of subsequent vectors constructed in the current study.

3.4. Astaxanthin biosynthesis genes expression

Three plasmids, PP3-6, PTP1-6, and PTP3-6, were constructed from the Pp2-4 vector (Fig. 2C). DE3 cells carrying the PP3-6, PTP1-6 or PTP3-6 plasmid synthesized astaxanthin from FPP (Fig. 1). Expression of *crtZ* and *crtW* in PP3-6, PTP1-6 and PTP3-6 plasmids were quantified, and although the expression level of *crtZ* in DE3 cells carrying the PTP3-6 plasmid had the highest value under IPTG induction, the expression level of *crtZ* in DE3 cells carrying the PTP3-6 plasmid was not significantly different with DE3 cells carrying the PP3-6 or PTP1-6 plasmid under IPTG induction (Fig. 5A). Similarly, when not provided IPTG, the level of *crtZ* expression in DE3 cells carrying the PTP3-6 plasmid was not significantly different to DE3 cells carrying the PP3-6 plasmid. This result indicated that *crtZ* expression in DE3 cells carrying the PTP3-6 or PP3-6 plasmid could be driven by the T7 promoter in the presence of IPTG or by the constitutive promoter in the absence of IPTG (Fig. 2C). The expression level of *crtW* in DE3 cells carrying the PTP3-6 plasmid had the highest value under IPTG induction. The expression level of *crtW* in DE3 cells carrying the PP3-6 plasmid under IPTG induction was not significantly different to DE3 cells carrying the PP3-6 plasmid under without IPTG induction, as well as to DE3 cells carrying the PTP1-6 plasmid under IPTG induction. When not provided IPTG, the expression level of *crtW* in DE3 cells carrying the PTP3-6 plasmid was not significantly different to DE3 cells carrying the PTP1-6 plasmid, and both had the lowest level of *crtW* expression (Fig. 5B). This result indicated that DE3 cells carrying the PTP3-6 plasmid had high *crtZ* and *crtW* expression in the presence of IPTG, but also had high *crtZ* expression in the absence of IPTG.

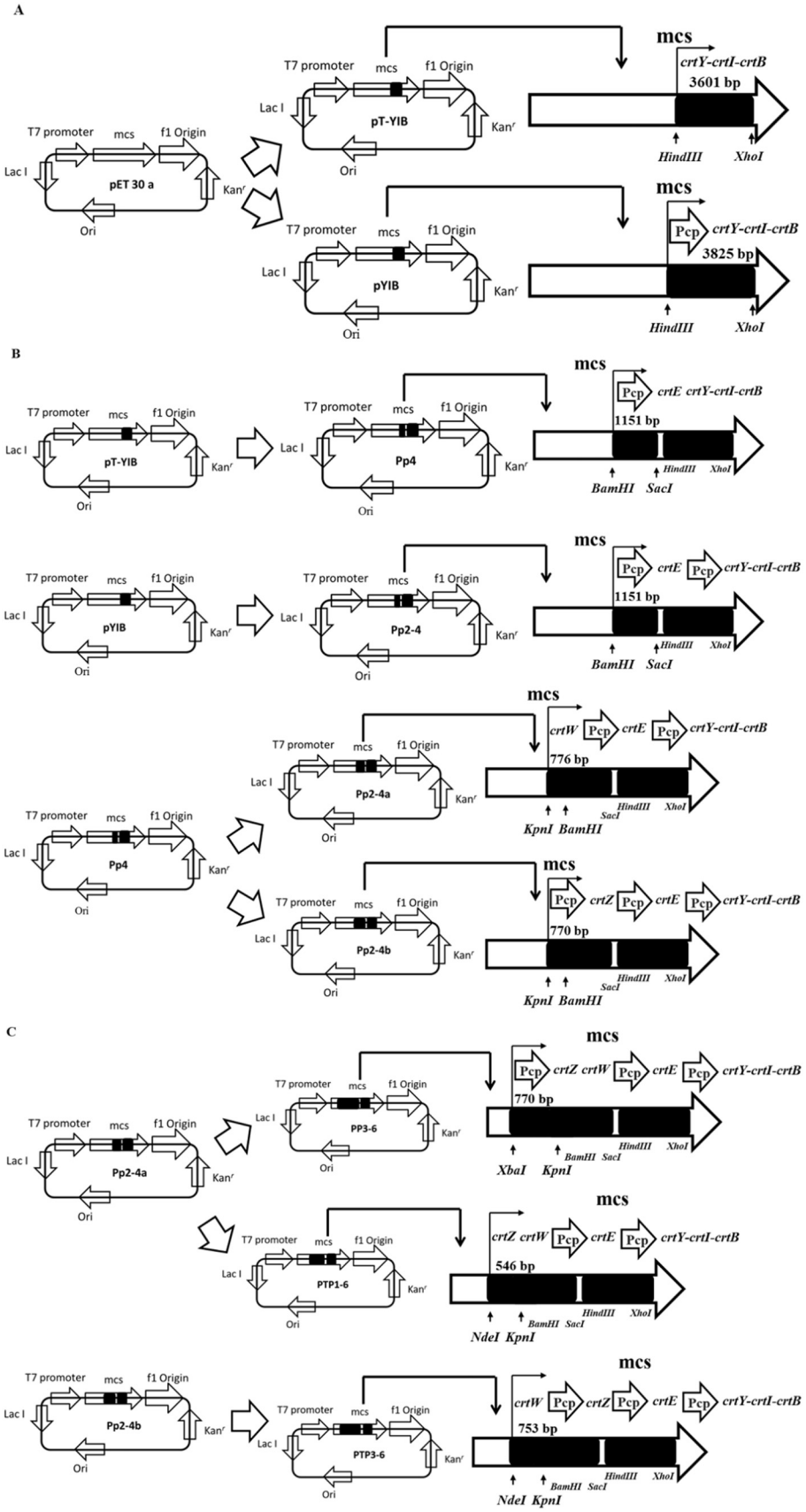

Fig. 2. Construction of astaxanthin biosynthesis genes in expression vector. The Pcp is a constitutive promoter (isolated from *E. uredovora*). All the T7 promoters are from a pET-30a (+) expression vector.

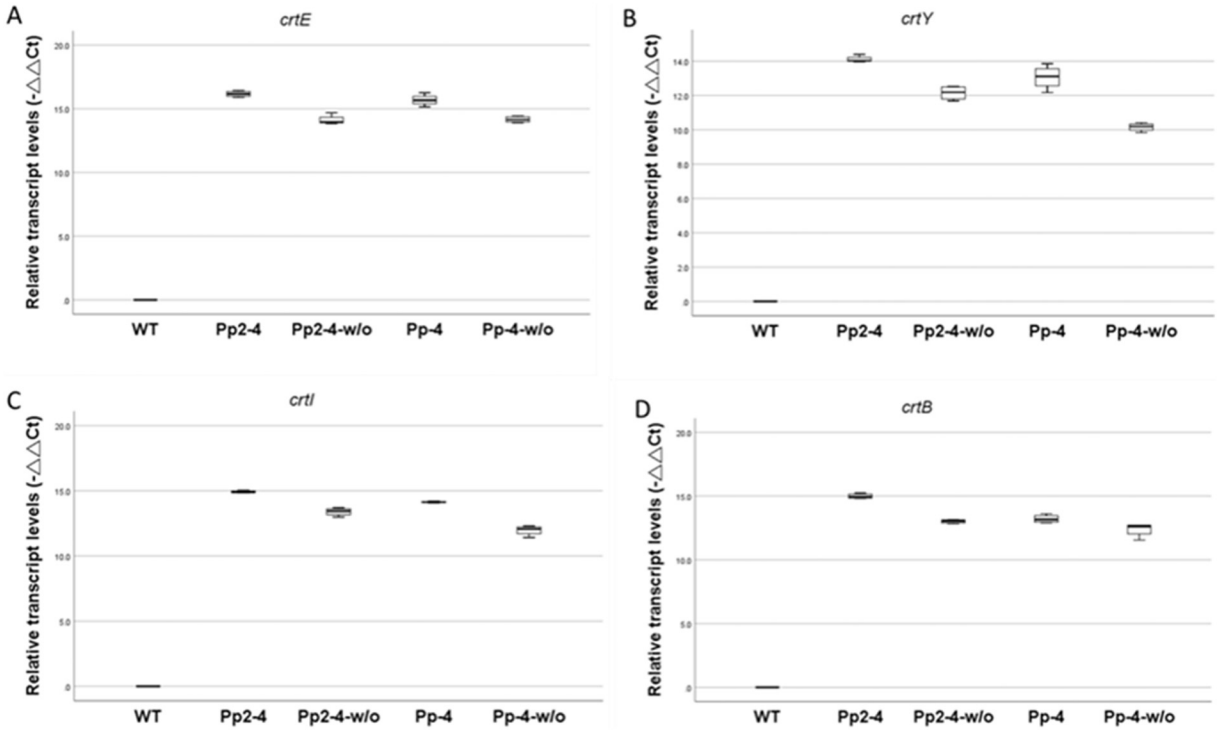

Fig. 3. Reference gene normalized transcript levels of β-carotene biosynthesis genes in DE3 cells carrying either the Pp2-4 or Pp-4 plasmid, with (Pp2-4 and Pp-4) or without (Pp2-4-w/o and Pp-4-w/o) IPTG. (A) *crtE*; (B) *crtY*; (C) *crtI*; (D) *crtB*. One-way analysis of variance followed by Tukey's test was to determine significance. Statistical significance was defined as $P < .05$.

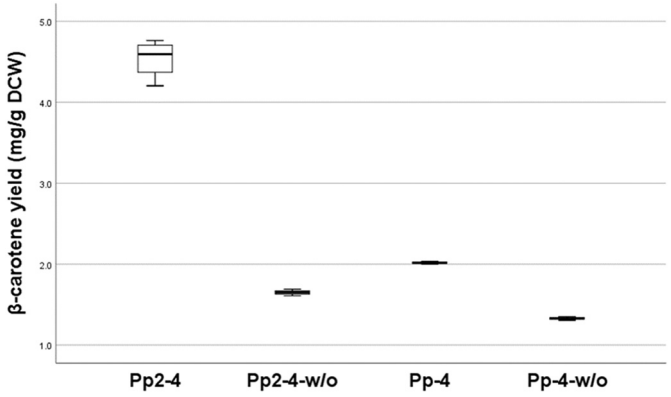

Fig. 4. β-carotene production in DE3 cells carrying either the Pp2-4 or Pp-4 plasmid, with (Pp2-4 and Pp-4) or without (Pp2-4-w/o and Pp-4-w/o) IPTG. One-way analysis of variance followed by Tukey's test was to determine significance. Statistical significance was defined as $P < .05$.

3.5. Astaxanthin production

The amount of astaxanthin produced in cells carrying the PTP3-6 plasmid was higher than in DE3 cells carrying either the PP3-6 or PTP1-6 plasmid, regardless of whether IPTG was added (Fig. 6). Because both CrtZ and CrtW enzymes require the same cofactors (O_2, Fe^{2+}, and 2-oxoglutarate) to activate enzyme catalytic functions (Yuan and Chen, 2000; Mao et al., 2017), simultaneous expression of CrtZ and CrtW results in competition for cofactors between the two enzymes and consequent instability in the catalytic activity of both. This leads to reductions in astaxanthin yield of (Fraser et al., 1997; Misawa and Shimada, 1998). Thus, balanced expression of CrtZ and CrtW is critical to achieving high levels and purity of astaxanthin (Cheng and Tao, 2012). Furthermore, the CrtZ and CrtW enzymes from different bacterial sources have different substrate affinities and specific activities for carotenoids, which can affect the efficiency of the conversion from β-carotene to astaxanthin (Fraser et al., 1997; Fraser et al., 1998; Misawa and Shimada, 1998). The specific activity for converting β-carotene to zeaxanthin by the CrtZ enzyme from *E. uredovora* was 37 ± 6.0 pmol/h/mg protein and the specific activity for converting β-carotene to canthaxanthin by the CrtW enzyme from *A. aurantiacum* was 5.0 ± 0.7 pmol/h/mg protein (Fraser et al., 1997). Additionally, the yield of astaxanthin may be reduced by accumulation of echinenone and canthaxanthin (Cheng and Tao, 2012). Hence, in the PTP3-6 plasmid, in which an IPTG-inducible T7 promoter is driving expression of *crtW* (Fig. 5B), accumulation of echinenone and canthaxanthin might be reduced, thereby enhancing astaxanthin formation in DE3 cells carrying the PTP3-6 plasmid. By contrast, when a T7 promoter or constitutive promoter is driving simultaneous expression of both *crtZ* and *crtW*, as in DE3 cells carrying the PP3-6 or PTP1-6 plasmid, low levels of astaxanthin were observed (Figs. 5 and 6). However, antibiotic selection must be applied to the recombinant cells generated in this study in order to maintain the stability of the strain. In the future, the astaxanthin biosynthesis gene fusion construct will be integrated into the genome of *E. coli* cells to facilitate large-scale production of astaxanthin.

Since DE3 cells carrying the PTP3-6 plasmid produced the highest yield of astaxanthin, they were used to produce astaxanthin for further analyses. These cells 8323 μg/g DCW astaxanthin, 69.4 times higher than the amount produced in recombinant *E. coli* cells carrying the pHP51 or pACCAR25△crtX plasmids (120 μg/g DCW) (Mao et al., 2017). The pHP51 plasmid, which contains *bkt* (β-carotene ketolase) from *H. pluvialis* was transformed into pACCAR25△crtX recombinant *E. coli* cells (Kajiwara et al., 1995). Multiple promoters driving the expression of astaxanthin biosynthesis genes, as in the pTrc*CrtW*-pBAD*CrtZ* dual expression system (Scaife et al., 2009), or co-transformation of three plasmids (pMH1, pFZ81, and pFZ153) into *E. coli* cells (Ma et al., 2016) have been shown to effectively increase production of astaxanthin. In addition, the transformation of *idi* into recombinant *E. coli* cells could increase the carbon flux to the isoprenoid pathway,

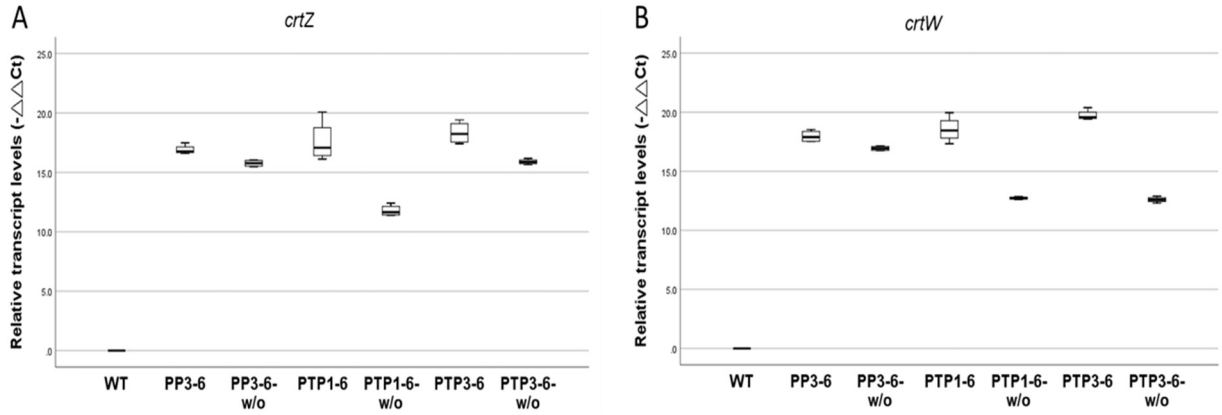

Fig. 5. Reference gene normalized transcript levels of β-carotene hydroxylase and ketolase genes in DE3 cells carrying the PP3-6, PTP1-6 or PTP3-6 plasmid, with (PP3-6, PTP1-6 and PTP3-6) or without (PP3-6-w/o, PTP1-6-w/o or PTP3-6-w/o) IPTG. (A) *crtZ*; (B) *crtW*. One-way analysis of variance followed by Tukey's test was to determine significance. Statistical significance was defined as $P < .05$.

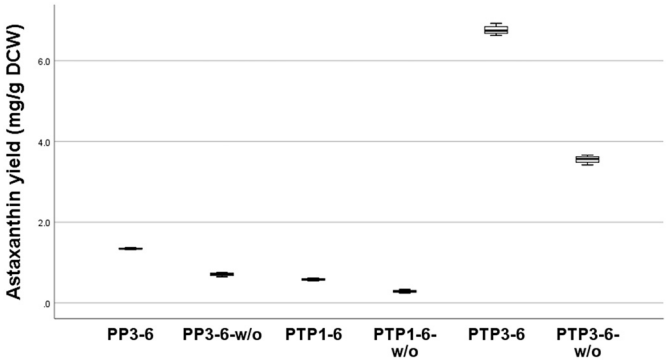

Fig. 6. Astaxanthin production in DE3 cells carrying the PP3-6, PTP1-6 or PTP3-6 plasmid, with (PP3-6, PTP1-6 and PTP3-6) or without (PP3-6-w/o, PTP1-6-w/o or PTP3-6-w/o) IPTG. One-way analysis of variance followed by Tukey's test was to determine significance. Statistical significance was defined as $P < .05$.

increasing astaxanthin production (Mao et al., 2017). For example, the pMH1, pFZ81, and pFZ153 plasmids, which were co-transformed into *E. coli* cells, also had two promoters driving the expression of downstream gene casettes (astaxanthin biosynthesis genes and *idi*), and the yield of astaxanthin was 6600 μg/g DCW (Ma et al., 2016). In future studies, we will investigate whether addition of *idi* into the PTP3-6 plasmid can enhance astaxanthin production in recombinant *E. coli*.

3.6. LC-MS/MS analysis of astaxanthin

The presence of astaxanthin in whole cell extracts of DE3 cells carrying the PTP3-6 plasmid was confirmed HPLC, and LC/MS/MS was used to verify that the produced astaxanthin was in the FFAX form. The FFAX standard (Fig. 7A) and the astaxanthin extract obtained from DE3 cells carrying the PTP3-6 plasmid had the same retention time, 12.5 min (Fig. 7). Moreover, no β-carotene peak formation was observed (Fig. 7B), indicating that the DE3 cells carrying the PTP3-6 plasmid effectively converted β-carotene to astaxanthin.

The protonated astaxanthin molecule was detected as the base peak in positive ion APCI mass spectra, and the base peaks of the FFAX standard corresponded to hydrogen ion gain or water ion loss, depicted as $[M + H]^+$ and $[MH\text{-}H_2O]^+$, respectively. The fragment ions were detected at *m/z* values of 597.4 (100) and 579.4 (6.5) in the FFAX standard, and those same peaks were also observed in extracts obtained from DE3 cells carrying the PTP3-6 plasmid. The presence of the two base peaks [m/z 597.4 (100) and 579.4 (6.7)] provided further confirmation that the extracts obtained from DE3 cells carrying the PTP3-6

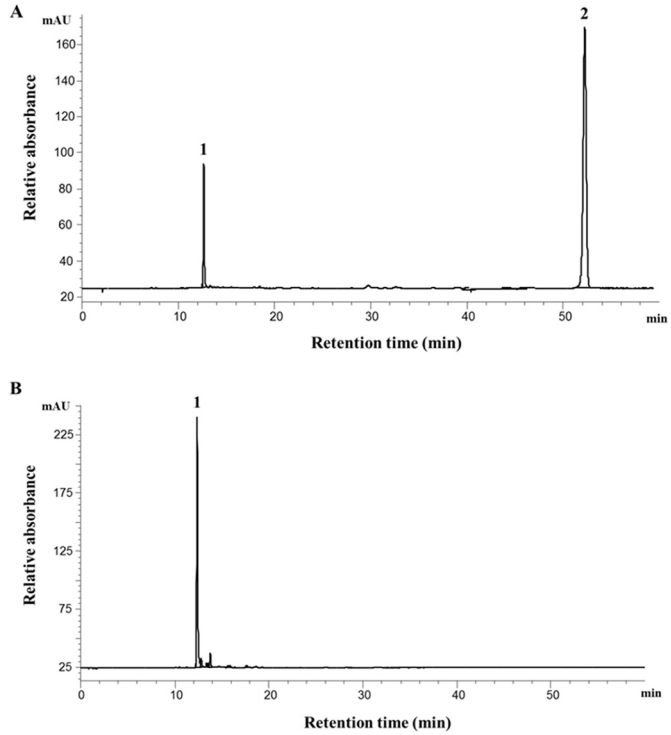

Fig. 7. HPLC analysis of astaxanthin. (A) FFAX and β-carotene standards. (B) FFAX extracted from DE3 cells carrying the PTP3-6 plasmid. (1) FFAX. (2) β-carotene. The retention times of the FFAX and β-carotene standard peaks were 12.5 and 52.3 min, respectively. All measurements were obtained at 470 nm.

plasmid contained astaxanthin. As measured by LC/MS/MS the base peaks of extracts (Fig. 8B) were nearly the same as those of the FFAX standard (Fig. 8A), indicating that FFAX was produced by the DE3 cells carrying the PTP3-6 plasmid. The base peak detected at the *m/z* value of 147.1 corresponds to a dehydrated terminal ring of FFAX with cleavage of the bond between the seventh and eighth carbons. Another fragment detected at the *m/z* value of 201.1 represents the cleavage of the bond between the tenth and eleventh carbons with loss of water from a dehydrated terminal ring. These two fragment ions were also observed in a previous study (van Breemen et al., 2012). These results demonstrated that DE3 cells carrying the PTP3-6 plasmid predominantly produced FFAX. In the future, FFAX can be linked to functional fatty acids or water-soluble groups to produce specific astaxanthin esters or astaxanthin derivatives for pharmaceutical

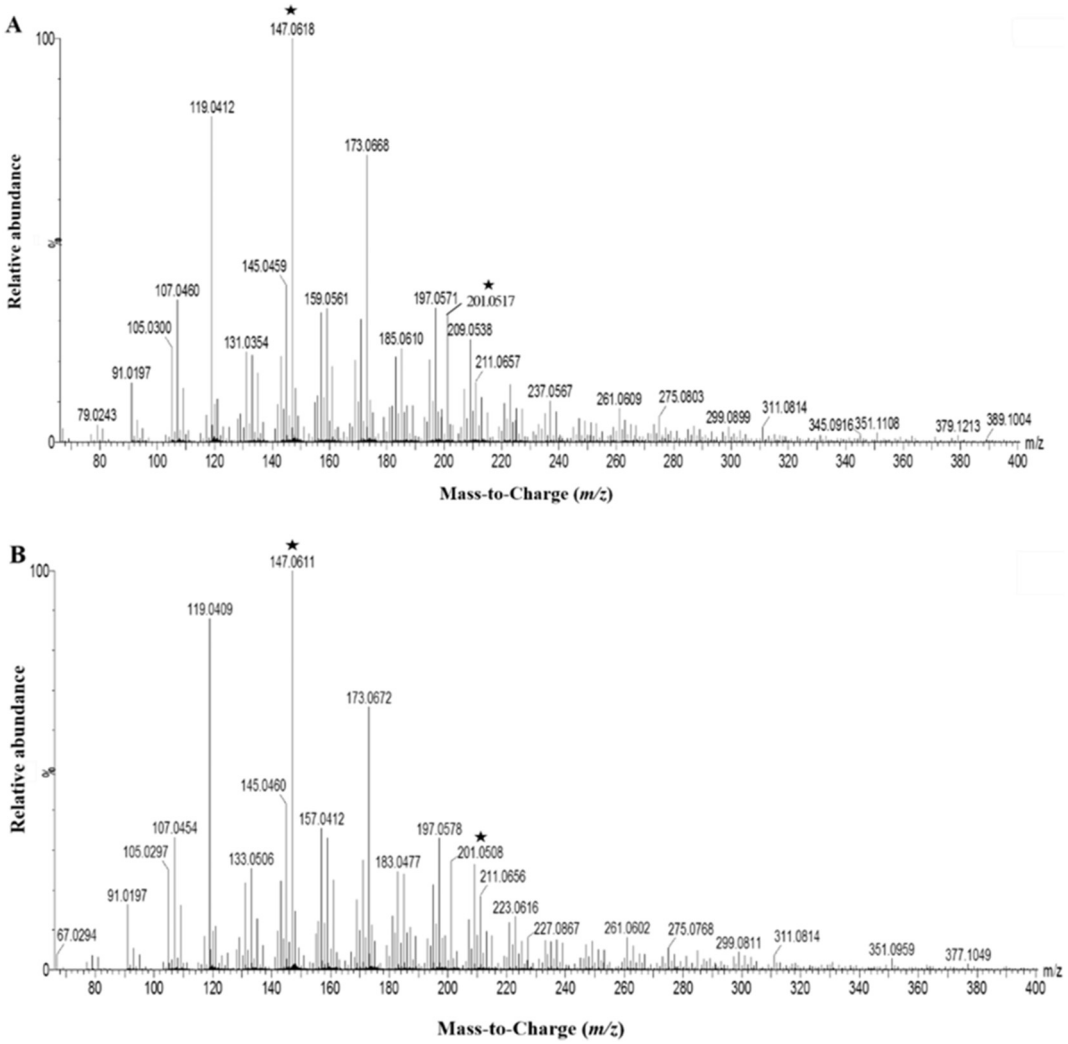

Fig. 8. LC-MS/MS (APCI, positive ion mode) analysis of FFAX. (A) FFAX standard. (B) FFAX was extracted and purified from DE3 cells carrying the PTP3-6 plasmid. Black stars represent fragment ions detected at *m/z* value of 147.1 (100), and 201.1 (30). Numbers in brackets represent relative abundance.

development (Ma and Chen, 2001; Zhao et al., 2011).

In this study, we used a T7 promoter and constitutive promoters fused with astaxanthin biosynthesis genes to construct a novel plasmid (dubbed PTP3-6). Transcriptional analyses and characterization of astaxanthin production demonstrated that DE3 cells carrying the PTP3-6 plasmid could produce high amounts of FFAX (8.3 mg/g DCW), due to *crtW* and *crtZ* expression at different times. Moreover, very low levels of residual endotoxin were observed in FFAX extracted from DE3 cells carrying the PTP3-6 plasmid (Table S1). These results suggest that fermentation technology can be used in the future for large-scale production of FFAX for applications in antioxidants, food colorants, food additives, animal feed, nutraceuticals, cosmetics, and pharmaceuticals.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported in part by the Ministry of Education, Taiwan, R.O.C. under the ATU plan; Ministry of Science and Technology (MOST), Taiwan, R.O.C. grants 106-2311-B-214-001- and 106-2632-B-214-001- were provided to Jei-Fu Shaw. We acknowledge the Metabolomics Core Facility, the Small Molecule Metabolomics core facility sponsored by the Institute of Plant and Microbial Biology, and Scientific Instrument Center, Academia Sinica, and its assistant, Miss Yu-Ching Wu, who provided technical assistance in the data acquisition and analysis processes during Synapt HDMS experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.03.012.

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