Research article

Expression of synthetic phytoene synthase gene to enhance β-carotene production in *Scenedesmus* sp. CPC2†

Chun-Yen Chen[1], Ai-Ling Kao[2], Zheng-Chia Tsai[2], Yu-Mei Shen[1], Pei-Hsun Kao[3], I-Son Ng[3,4*], and Jo-Shu Chang[3,4*]

[1] University Center of Bioscience and Biotechnology, National Cheng Kung University, Tainan 701, Taiwan

[2] Department of Biotechnology, Green Technology Research Institute, CPC Corporation, Taiwan

[3] Department of Chemical Engineering, National Cheng Kung University, Tainan 70101, Taiwan

[4] Research Center for Energy Technology and Strategy, National Cheng Kung University, Tainan 70101, Taiwan

[*]**Corresponding author:** Jo-Shu Chang (email: changjs@mail.ncku.edu.tw)

I-Son Ng *(*email: yswu@mail.ncku.edu.tw)

Tel: +886-62757575 ext. 62651; Fax: +886-62357146

[†]This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/biot.201700204].

Received: April 19, 2017 / Revised: August 30, 2017 / Accepted: August 31, 2017

Abstract

β-carotene is a valuable pigment abundant in some microalgal species but the low β-carotene productivity of microalgae has become the major obstacles against its commercialization. This work aims to improve the productivity of algae-based β-carotene via genetic engineering approaches. First, a synthetic *psy* gene construct (891 bp) encoding 297 amino acids was expressed in *Scenedesmus* sp. CPC2 host to enhance the β-carotene production. The synthetic *psy* gene was designed by considering the highest consensus of amino acids (i.e., 62% identity) from *Chlamydomonas reinhardtii*, *Dunaliella salina* and *Mariella zofingiensis*. The original β-carotene content in wild type *Scenedesmus* sp. CPC2 is 10.8 mg/g-cell when grown on BG11 medium under 2% CO_2 aeration, 150 μmol/m^2/s light intensity and 25°C. After transformation of the *psy* gene into the microalgal host, the β-carotene content of the best recombinant strain (i.e., transformant CPC2-4) significantly increased to over 30 mg/g-cell. The optimal production of β-carotene with the CPC2-4 recombinant strain was achieved when the strain was grown on BG11 medium amended with 0.075 g of $MgSO_4$, giving approximately 3-fold higher β-carotene content than that of the wild-type strain. The best cellular β-carotene content obtained (i.e., 31.8 mg/g) is superior to most algae-based β-carotene production performance reported in the literature.

Keywords

Phytoene synthase (psy), β-carotene, carotenoids, *Scenedesmus*, microalgae, genetic engineering

1. Introduction

In the recent years, microalgal biomass is considered a promising source for producing a variety of biofuels and biochemicals, such as biodiesel, bioethanol, carotenoids, lutein, beta-carotene and poly-unsaturated fatty acids [1-6]. Microalgal carotenoids as the sustainable and natural source of nutritional supplements in high-value functional foods and biomedical components are becoming much attractive. Therefore, it is of significant importance that the microalgal cultivation is scaled up to meet the growing demands and applications. The increasing demands of microalgae in global market of healthy food will breakthrough 8 billion USD in 2020.

Carotenoids are isoprenoids synthesized in photosynthetic system and are widespread in plants and algae [7]. Carotenoids are the important dietary source of provitamin A for animals, as they cannot synthesize Vitamin A. Therefore, carotenoids are a part of the massive commercial food or feed additives and it must be accomplished through an effective process. Phytoene synthase (PSY) is the key enzyme involved in carotenoid synthesis and has been originally reported in plants [8]. Microalgae share similar carotenoid biosynthetic pathway to that of plant. As shown in **Fig. S1**, phytoene synthase (*PSY*) catalyzes the first step of carotenoid synthetic pathway, leading the carbon flux towards carotenes and xanthophylls production [8]. The carbon flux control from geranylgeranyl diphosphate (C20), activation and regulation of carotenoid biosynthesis (phytoene, lycopene, carotene, lutein and zeaxanthin) has been elucidated [9]. It is worth mentioning that the down regulation of PSY caused by the binding of phytochrome-interacting factors (PIF) to the promoter of PSY to repress the PSY expression could result in a decrease in carotenoids production. This indicate that PIF

and PSY are both involved in the carotenoid biosynthesis [10].

The synthesis of carotenoids and its derivatives could be improved significantly by enhancing *psy* gene, as it is the rate determining step of carotenoid synthesis in many plants, such as tobacco [11], maize [12], and tomato [13]. Recently, the overexpression of carotenoids by microalgae has been reported, by applying *psy* gene from *Dunaliella salina* [14] or from *Chlorella zofingiensis* [15]. This provides an important insight into the use *psy* for enhancing carotenoid production as an alternative opportunity.

The highest β-carotene content has been recorded in the halotolerant algae, *Dunaliella bardawil* (Chlorophyceae), which produced about 80 mg/g cell originally but the biomass content was not improved [16]. Except for *D. bardawil*, the cellular content of β-carotene in recombinant *C. zofingiensis* and *Saccharomyces cerevisiae* cells was less than 6 mg/g cell [17] and higher quantities are needed to simplify the downstream processing and improve the economics of the production processes. The production of β-carotene with recombinant *S. cerevisiae* was not achieved by overexpressing PSY because carotene production in yeasts is through *crt* cluster genes (instead of psy gene) in carotenogenic pathway [17]. In recombinant *C. zofingiensis*, both PSY and PDS (phytoene desaturase) were cloned by its native genes [17]. It is thus presumed that *psy* is the key gene in the carotenoids production pathway of microalgae. Therefore, in this study, a new synthetic psy gene based on the combination of *psy* genes from *Chlamydomonas reinhardtii, Dunaliella salina and Mariella zofingiensis was designed* and cloned in a *Scenedesmus* sp. host for β-carotene production. Our previous study has successfully expressed type 2 diacylglycerol acyltransferase gene (DGTT1) in *Scenedesmus obliquus* to enhance lipid production [18]. It is also known that the growth rate and biomass production of *Scenedesmus* sp. are much higher than those of *Chlamydomonas* sp. [18-19]. In addition, the potential of using *Scenedesmus* sp. for

CO_2 fixation as well as biofuels and function compounds production (e.g., lutein) has been demonstrated [20]. Therefore, in this study, *Scenedesmus* sp. CPC2 was chosen as the host to express the synthetic *psy* gene to regulate the carotenoid synthesis.

2. Method and materials

2.1 Design of phytoene synthase gene

To design the *psy* cDNA, the amino acid sequences were aligned by VectorNTI 10.0 (Invitrogen, USA) from three different species of microalgae (*Chlamydomonas reinhardtii*: AAT38475, *Dunaliella salina:* ABY50091 and *Mariella zofingiensis*: CBW37867). Highly consensus regions were identified, and the coding region of gene was optimized based on *C. reinhardtii* which was synthesized by Genomics BioSci & Tech (Taipei, Taiwan). The full-length sequence of synthesized *psy* cDNA fragment and the genes harboring in pUC57 vector are shown in **Fig. S2** and **Fig. S3**.

2.2 Construction of the overexpression vector

To insert the *psy* gene in expression vector pHSN401, the plasmid was digested by restriction enzymes *Xba*I and *Eco*RI (Takara, Dalian, China), and then ligated with the *psy* gene-containing DNA fragment that was treated with the same sets of enzymes. Plasmids were propagated in *Escherichia coli* C43 (DE3) and purified using Plasmid DNA Extraction Mini Kit (Favorgen, Taiwan).

2.3 Genetic transformation by electroporation

Cells of wild-type strains were grown in 20 mL Tris-acetate phosphate (TAP) medium (detail composition is shown in **Table S1**) in a 100 mL flask with shaking (120 rpm) and maintained at room temperature under continuous white light (50 μmol

photons/m^2/s). The cells were harvested (OD_{680} = 0.3-0.5) by centrifugation at 2,500 x *g* for 10 min and re-suspended in 250 μL of fresh TAP medium with 40 mM sucrose. A 2 μg of linearized plasmid DNA was added into the resuspended cells and were then transferred into a 0.2-cm electroporation cuvette (Bio-Rad, USA). Nuclear transformation was performed by Gene Pulser Xcell™ electroporation systems (Bio-Rad, USA) with 300 V, 50 μF and infinity of resistance. After electroporation, cells were recovered in 10 mL TAP with 40 mM sucrose with the same culture conditions as mentioned above. After 24 hr recovery, cells were collected by centrifugation once again and were spread on TAP-Hygromycin agar plates containing 5 μg/mL for the screening of pHSN401-psy. The plate was incubated at a temperature of 25°C under continuous illumination at 50 μmol photons/ m^2/s.

2.4 PCR confirmation of the transformants

To isolate the genomic DNA, transformed cells were harvested by centrifugation and re-suspended in 200 μl of DNA extraction buffer (100 mM Tris-Cl at pH 8.0, 20 mM EDTA, 500 mM NaCl, 1.5% SDS) at 65°C for 20 min. DNA was extracted using 200 μL of phenol-chloroform solution (Sigma-Aldrich, USA) and vigorous vortex mixing. Following the centrifugation, the upper aqueous phase was transferred to a new 1.5 mL centrifuge tube. To precipitate DNA, an equal volume of isopropanol was added and mixed by inverting the tube several times. DNA was harvested by centrifugation at 15,000 x *g* for 10 min at room temperature. The DNA pellet was washed with 70% ethanol and air-dried in a 37°C oven for 10 min. DNA was re-suspended with EB buffer (10 mM Tris-Cl at pH 8.1). DNA concentration and purity were confirmed by agarose gel electrophoresis. To verify the *psy* gene, primer set psy-F2 and psy-R2 were designed to amplify the *psy* fragments. The detailed sequences were shown in **Table S2**. The

PCR program was as following: 94°C for 5 min, denaturation (94°C for 40 s), annealing (56°C for 20 s) and extension (72°C for 1 min) for 30 cycles, with a final extension at 72°C for 10 min. PCR products were examined on a 1.2% agarose gel.

2.5 Microalgal culture conditions

BG11 medium (detail composition is shown in **Table S3**) was used to cultivate CPC2 cells in a glass-made vessel with a working volume of 1L. The initial pre-cultured microalga was inoculated into the photobioreactor with inoculums size of approximately 40 mg/L. The culture conditions were 2% CO_2 aeration at an aeration rate of 0.1 vvm, 300 rpm agitation, light intensity of ca. 150 μmol/m^2/s (LI-COR Inc., Lincoln, USA) and a temperature of 25°C [19]. Two different organic carbon sources (i.e., 0.73 g/L glucose and 1.0 g/L sodium acetate; both of them contain 24.3 mM C equivalent each) and $MgSO_4$ at different concentrations (0.0375 g, 0.075 g, 0.15 g) were examined in the culture of transformants in BG11 medium to optimize β-carotene production and yield.

2.6 Determination of cell concentration

The cell density was monitored regularly by optical density at a wavelength of 680 nm (i.e. OD_{680}) using a spectrophotometer (model U-2001, Hitachi, Tokyo, Japan). The dry cell biomass was obtained by centrifugation at 5500 x *g* for 5 min and dried at 105°C until the weight was constant. The calibration between OD_{680} and dry cell biomass (g/L) were converted by the equation Y (OD_{680}) = 0.354 X (g/L).

2.7 β-carotene extraction and quantification with HPLC

A 10 mg of dry microalgae was mixed with 60% (*w/v*) KOH and cell wall was

disrupted in a bead beater for 25 min, and was followed by saponification at 40ºC for 1 h. The β-carotene was extracted with ether at room temperature until the organic extract was clear, with β-carotene content analyzed by high-performance liquid chromatography (YMC30 RP-18 column, (4.6 mm×250 mm×5 μm), Schermbeck, Germany) as previously described [21]. The mobile phase was 3 % ddH_2O in methanol containing 0.05 M ammonium acetate (solvent A); 100% TBME (solvent B) at 1 ml/min. Both solvents contained 0.1 % (w/v) butylated hydroxytoluene and 0.05 % (v/v) trimethylamine (TEA). Elution was carried out according to the following program: isocratic at 3 % B for 2 min followed by a linear gradient from 2 % to 38 % B in 1 min, isocratic at 15 % for 12 min, a linear increase to 68 % B. The β-carotene was detected by measuring the absorbance at 450 nm and a standard (Sigma-Aldrich, No. 22040) was used for quantification. All the experiments were conducted three times and the data presented are average values with error bars.

3. Results and discussion

3.1 Selection of recombinant Scenedesmus sp. CPC2 *strains expressing psy gene*

As shown in **Fig. S1**, *psy* (phytoene synthase) is the key gene of the carotenoid biosynthetic pathway in microalgae [9, 14-15]. PSY catalyzes the first step, which leads the carbon flux towards carotenes and xantophylls production. The synthetic *psy* gene was designed by considering the highest consensus amino acids (i.e., 62% identity) from *Chlamydemonas reinhardtii* (AAT38475) [17], *Dunaliella salina* (ABY50091) and *Mariella zofingiensis* (CBW37867) [15]. The final sequence of synthetic *psy* is 891 bp encoding 297 amino acids, and was cloned in algae adapted plasmids.

Figure 1A shows the *psy* expression plasmids obtained by inserting the *psy* gene into the vector pHSN401, where *psy* expression was driven by promoter CaMV35S.

The transformants were plated on TAP agar plate with 5 μg/L hygromycin for selection of pHSN401-psy. As shown in Fig. 2B, PSY transgenic strains under CaMV35S promoter had slower growth rate (i.e., 10 days). Through the second isolation, 6 transformants of pHSN401-psy for CPC2 were obtained (**Fig. 1B**). Of the 6 transformants obtained, transformant no. 4 (pHSN401-Psy-CPC2-4 or CPC2-4 in brief) with the highest β-carotene content was chosen for further studies. Verification of successful clones was done by PCR amplification of the psy gene from genomic DNA. All the isolated clones possessed *psy* gene (**Fig. 1C**). The results indicated the pHSN401 could express *psy* gene and thus can be used to enhance carotenoids production in microalgae.

3.2 Enhanced β-carotene production in the recombinant CPC2 strain

The carotenoid production and biomass content of the wild type CPC2 and the transformants expressing *psy* gene were evaluated and the results are shown in **Fig. 2**. The biomass of wild type and genetic transformants were similar, which was about 3 g/L at 10 days. The wild type CPC2 produced only 10.8 mg/g-cell (14.8 mg/L) β-carotene, while the β-carotene production of the transformant pHSN401-Psy-CPC2-4 (or in brief, CPC2-4) increased to over 30 mg/g cell. Previous studies reported expression of exogenous *psy* gene from *Dunaliella salina* [14] or *Chlorella zofingiensis* [15] driven by Rbc S2 and Hsp70A promoters, and the transformants constitutively expressed *psy* in *Chlamydomonas reinhardtii* with heterotrophic cultivation in TAP medium, achieving a β-carotene production of 1.1 mg/g cell and 7.8 mg/g, respectively. This is the first report of enhanced expression of β-carotene in *Scenedesmus* CPC2 hosts. With expression of exogeneous *psy*, the β-carotene content was enhanced up to 15.5 mg/g (21.8 mg/L) on TAP medium (a 50% increase over the wild-type strain) with the

CPC2-4 strain. The β-carotene content dramatically increased to more than 30 mg/g cell after 7-day cultivation on BG11 medium, while that of the wild-type strain was significantly lower than the recombinant strain CPC2-4 (**Fig. 2B**). The β-carotene contents produced from other *Scenedesmus* sp. strains reported in the literature were quite low. The content could be improved to 0.05 mg/g cell when grown on swine wastewater [22], but this content is still much lower in contrast to our results. Recently, a new *Scenedesmus* sp. was obtained when screened for high carotenoid productivity (max. up to 20 mg/L/d) [23]. One the other hand, under stressful conditions such as UV irradiation, nutrition depletion or light strategy, some microalgae would accumulate carotenoids through gene regulations [24]. The phytoene desaturase (PDS) has been proven as an essential protein involved in carotenoid synthesis in microalgae such as *Scenedesmus* sp. [8]. To the best of our knowledge, this study is the first attempt to present that exogeneous *psy* gene expression could directly enhance carotenoid production, especially in autotrophic culture of *Scenedesmus* sp. CPC2 with BG11 medium.

3.3 Effect of organic carbon source addition on growth and β-carotene production of the recombinant CPC2 strain

The effects of organic carbon sources on the production of β-carotene by recombinant CPC2 expressing *psy* gene was evaluated using glucose and sodium acetate as organic carbon sources. Our preliminary study showed that addition of 1 g/L acetate (containing 24.3 mM C equivalent) in outdoor cultivation of *Scenedesmus* sp. CPC2 resulted in marked improvement in the biomass productivity (data not shown). Therefore, the CPC2-4 strain was cultivated under mixotrophic conditions with the addition of 1 g/L of acetate and 0.73 g/L of glucose (also containing 24.3 mM C

equivalent) into BG11 medium, respectively. The autotrophic growth that is free of organic carbon source was also conducted as the control. As shown in **Fig. 3**, the addition of glucose and acetate did not significant affect the cell growth of CPC2-4 strain and the maximum biomass concentration remained similar (i.e., 3.4 g/L) for the tests with or without organic carbon sources (**Fig. 3A**). However, the β-carotene content of CPC2-4 transformant significantly decreased when glucose or acetate was added (**Fig. 3B**). Therefore, addition of glucose or acetate as extra carbon source did not enhance the β-carotene production, but rather caused a negative effect on β-carotene accumulation in the cells. This suggests that autotrophic conditions seem to be beneficial to β-carotene production in this specific recombinant microalgal strain. It was reported that sodium acetate could serves as an inducer for the production of β-carotene in fungi (e.g., *Blakeslea trispora*) [21] and microalgae (e.g., *Scenedesmus* sp.) [25], but apparently that was not the case with our recombinant microalgal strain. (**Fig. 3B**). These results indicate that the mixotrophic growth strategy, which could improve microalgal growth and target products formation in other microalgal strains, could not work with our recombinant *Scenedesmus* sp. strains.

3.4 Effect of $MgSO_4$ on β–carotene production in the recombinant CPC2 strain

Some metal ions, in particular magnesium [26-28], are also known as crucial components for the culture of green algae under autotrophic conditions. As shown in **Fig. 4A**, using higher $MgSO_4$ concentration could dramatically enhance cell growth of CPC2 strain. The biomass concentration increased with an increase in the concentration of $MgSO_4$ in the medium and reached a maximum of up to 4.05 g/L at 14 days when 0.15 g/L of $MgSO_4$ was added. The optimized β-carotene production with the recombinant CPC2 strain occurred when the $MgSO_4$ concentration was 0.075 g with a

maximum β-carotene content of as high as 31.8 mg/g cell (73.7 mg/L) (**Fig. 4B**). Thus, $MgSO_4$ seems to be a cost effective inducer for recombinant CPC2 to produce high titer β-carotene. As shown in **Table 1**, although *Dunaliella bardawil* [16] and *Dunaliella salina* [29] could accumulate higher β-carotene content (i.e., 80 mg/g-DCW) than that of wild-type *Scenedesmus* sp. CPC2, the productivity of *Dunaliella* sp. was lower than that of CPC2 strain. In addition, the highest β-carotene content (31.8 mg/g cell) obtained from our recombinant strain is also much higher than that obtained from other genetically engineered microalgae (e.g., recombinant *Chlamydomonas* sp. [14-15, 30]).

4. Conclusion

This work demonstrated an effective way to improve β-carotene productivity in microalgae. A synthetic phytoene synthase gene that regulates β-carotene accumulation level in microalgae was constructed and expressed exogenously in *Scenedesmus* sp. CPC2. The transformants were able to produce β-carotene at a concentration of 15.5 mg/g cell on TAP medium, which is a 50% increase when compared with the performance of the wild-type strain. Addition of $MgSO_4$ as an inducer and switching the growth medium from TAP to BG11 medium could further enhance the β-carotene content to up to 31.8 mg/g cell, which is superior to most reported values in the related studied. The results obtained from this work seem to suggest the high potential of using the recombinant *Scenedesmus* sp. CPC2 strain for purpose of commercial production of β-carotene.

Acknowledgement

The authors acknowledge the financial support provided by China Petroleum Corporation, Taiwan (CPC Co., Taiwan).

References

[1] Chen, C.Y., Yeh, K.L., Aisyah, R., Lee, D.J., Chang, J.S., Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. Bioresour. Technol. 2011, 102, 71-81.

[2] Eduardo, C., de Farias, S., Bertucco, A., Bioethanol from microalgae and cyanobacteria: A review and technological outlook. Proc. Biochem. 2016, 11, 1833–1842.

[3] Takaichi, S., Carotenoids in Algae: Distributions, Biosyntheses and Functions. Mar. Drugs 2011, 9, 1101-1118.

[4] Xie, Y., Ho, S.H., Chen, C.N.N., Chen, C.Y., Ng, I.S., Jing, K., Chang, J.S., Lu, Y., Phototrophic cultivation of a thermo-tolerant *Desmodesmus* sp. for lutein production: Effects of nitrate concentration, light intensity and fed-batch operation. Bioresour. Technol. 2013, 144, 435–444.

[5] Guedes, A.C., Amaro, H.M., Malcata, F.X., Microalgae as sources of carotenoids. Mar. Drugs 2011, 9, 625-644.

[6] Ling, X., Guo, J., Liu, X., Zhang, X., Wang, N., Lu, Y., Ng, I.S., Impact of carbon and nitrogen feeding strategy on high production of biomass and docosahexaenoic acid (DHA) by *Schizochytrium* sp. LU310. Bioresour. Technol. 2015, 184,139-147.

[7] Chang, H.L., Kang, C.Y., Lee, T.M., Hydrogen peroxide production protects *Chlamydomonas reinhardtii* against light-induced cell death by preventing

singlet oxygen accumulation through enhanced carotenoid synthesis. J. Plant. Physiol. 2013, 170, 9769-9786.

[8] Norris, S.R., Barrette, T.R., DellaPenna, D., Genetic dissection of carotenoid synthesis in arabidopsis defines plastoquinone as an essential component of phytoene desaturation. Plant Cell 1995, 7, 2139-2149.

[9] Steinbrenner, J., Linden, H., Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. Plant Physiol. 2001, 125, 810-817.

[10] Toledo-Ortiz, G., Huq, E., Rodríguez-Concepción, M., Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome-interacting factors. Proc. Natl. Acad. Sci. USA, 2010, 22, 11626-11631.

[11] Busch, M., Seuter, A., Hain, R., Functional analysis of the early steps of carotenoid biosynthsis in tobacco. Plant Physiol. 2002, 128, 439-453.

[12] Aluru, M., Xu, Y., Guo, R., Generation of transgenic maize with enhanced provitamin A content. J. Exp. Botany, 2008, 59, 3551-3562.

[13] Huang, J.C., Zhong, Y.J., Liu, J., Sandmann, G., Chen, F., Metabolic engineering of tomato for high-yield production of astaxanthin. Metab. Eng. 2013, 17, 59-67.

[14] Couso, I., Vila, M., Rodriguez, H., Vargas, M.A., León, R., Overexpression of an exogenous phytoene synthase gene in the unicellular alga *Chlamydomonas reinhardtii* leads to an increase in the content of carotenoids. Biotechnol. Prog. 2011, 27, 54-60.

[15] Cordero, B.F., Couso, I., León, R., Rodríguez, H., Vargas, M.A., Enhancement

of carotenoids biosynthesis in *Chlamydomonas reinhardtii* by nuclear transformation using a phytoene synthase gene isolated from *Chlorella zofingiensis*. Appl. Microbiol. Biotechnol. 2011, 91, 341-351.

[16] Ben-Amotz, A., Katz, A., Avron, M., Accumulation of β-carotene in halotolerant algae: Purification and characterization of β-carotene-rich globules from *Dunaliella bardawil (Chlorophyceae).* J. Phycol. 1982, 18, 529–537.

[17] Wichuk, K., Brynjólfsson, S., Fu, W., Biotechnological production of value-added carotenoids from microalgae. Bioengineered. 2014, 5, 204–208.

[18] Chen, C.Y., Kao, A. L., Tsai Z.C., Chow, T.J., Chang, H.Y., Zhao, X.Q., Chen, P. T., Su, H.Y., Chang, J.S., Expression of type 2 diacylglycerol acyltransferase gene DGTT1 from *Chlamydomonas reinhardtii* enhances lipid production in *Scenedesmus obliquus*. Biotechnol. J. 2016, 11, 336-344.

[19] Ho, S.H., Nakanishi, A., Ye, X., Chang, J.S., Hara, K., Hasunuma, T., Kondo, A., Optimizing biodiesel production in marine *Chlamydomonas* sp. JSC4 through metabolic profiling and an innovative salinity-gradient strategy. Biotechnol. Biofuels 2014, 7, 97-105.

[20] Ho, S.H., Chan, M.C., Liu, C.C., Chen, C.Y., Lee, W.L., Lee, D.J., Chang, J.S., Enhancing lutein productivity of an indigenous microalga *Scenedesmus obliquus* FSP-3 using light-related strategies. Bioresour Technol. 2014, 152, 275-82.

[21] Jing, K., He, S., Chen, T., Lu, Y., Ng, I.S., Enhancing beta-carotene biosynthesis and gene transcriptional regulation in *Blakeslea trispora* with sodium acetate. 2016, 114, 10-17.

[22] Kim, M.K., Park, J.W., Park, C.S., Kim, S.J., Jeune, K.H., Chang, M.U., Acreman, J., Enhanced production of *Scenedesmus* spp. (green microalgae)

using a new medium containing fermented swine wastewater. Bioresour. Technol. 2007, 98, 2220-2228.

[23] Přibyl, P., Cepák, V., Kaštánek, P., Zachleder, V., Elevated production of carotenoids by a new isolate of *Scenedesmus* sp. Algal Research 2015, 11, 22-27.

[24] McCarthy, S.S., Kobayashi, M.C., Niyogi, K.K., White mutants of *Chlamydomonas reinhardtii* are defective in phytoene synthase. Genetics 2004, 168, 1249-1257.

[25] Pirastru, L., Darwish, M., Chu, F.L., Perreault, F., Sirois, L., Sleno, L., Popovic, R., Carotenoid production and change of photosynthetic functions in *Scenedesmus* sp. exposed to nitrogen limitation and acetate treatment. J. Appl. Phycology 2012, 24, 117-124.

[26] Gorain, P.C., Bagchi, S.K., Mallick, N., Effects of calcium, magnesium and sodium chloride in enhancing lipid accumulation in two green microalgae. Environ. Technol. 2013, 34, 1887-1894.

[27] Ren, H.Y., Liu, B.F., Kong, F., Zhao, L., Xie, G.J., Ren, N.Q., Enhanced lipid accumulation of green microalga *Scenedesmus* sp. by metal ions and EDTA addition. Bioresour. Technol. 2014, 169, 763-767.

[28] Amor-Ben Ayed, H.B., Taidi, B., Ayadi, H., Pareau, D., Stambouli, M., Effect of magnesium ion concentration in autotrophic cultures of *Chlorella vulgaris*. Algal Research 2015, 9, 291-296.

[29] García-González, M.; Moreno, J.; Manzano, J.C.; Florêncio, F.J.; Guerrero, M.G., Production of *Dunaliella salina* biomass rich in 9-cis-β-carotene and lutein in a closed tubular photobioreactor. J. Biotechnol. 2005, 115, 81–90.

[30] Liu, J., Sun, Z., Gerken, H., Huang, J., Jiang, Y., Chen, F., Genetic engineering of

the green alga *Chlorella zofingiensis*: a modified norflurozon-resistance phytoene desaturease gene as a dominant selectable marker. Appl. Microbiol. Biotechnol. 2014, 98, 5069-5079.

Table 1. Comparison of β-carotene production performance of wild type and recombinant microalgae

Microalgae species	Vector and gene	β-carotene content (mg/g-DCW)	Productivity (mg/L/d)	References
Dunaliella bardawil	Wild type	80	NA	[16]
Dunaliella salina	Wild type	80	2.45*	[29]
Scenedesmus sp. CPC2	Wild type	10.8	27.0	This study
Chlamydomonas reinhardtii	pSI105-psy	1.2	NA	[14]
Chlamydomonas reinhardtii	pSI105-CrPsy	7.8	NA	[15]
Chlamydomonas zofingiensis	pCZT-CrPDS*	4.6	NA	[30]
Scenedesmus sp. CPC2-4	pHSN401-psy	31.8	95.5**	This study

*unit: $mg/m^2/d$

**calculated when the β-carotene content reached its maximum (i.e., 31.8 mg/g)

DCW: dry cell weight

NA: Not available

pCZT-CrPDS* produced astaxanthin.

Figure legends:

Figure 1 (A) Schematic of the expression vector pHSN401-psy in this study. Synthesized *PSY* gene was inserted in pHSN401 between the sites *Xba*I /*Eco*RI. (B) Colonies of hygromycin-resistant transformants plated on TAP with 5 μg/mL Hygromycin agar medium which over-expressed vector pHSN401-psy for 10 days after incubation. (Left) first *psy* transgenic strains of CPC2, (right) Selected 6 recombinants for second transformed. (C) Verification of *psy* gene in six CPC2 second transformants by DNA analysis. PCR-amplification with primers psy-F2/psy-R2 from genomic DNA of the pHSN401-psy transformants. The target size of amplified fragment is 820 bp. Lane P is positive control of PCR for pHSN401-psy, while lane 1 to 6 are PCR for six recombinants. M is DNA ladder marker.

Figure 2 Time course profile of (A) microalgal biomass concentration (black circle: wild type CPC2; open circle: transformant pHSN401-Psy-CPC2-4 (or CPC2-4)) and (B) β-carotene content (black bar) of wild-type CPC2 and CPC2-4 (open bar) when the microalgae were grown on BG11 medium under the condition of 2% CO_2 aeration at an aeration rate of 0.1 vvm, 300 rpm agitation, light intensity of ca. 150 μmol/m^2/s and a temperature of 25°C.

Figure 3 Time course profile of (A) microalgal biomass concentration (black circle: with glucose (0.73 g/L) addition; open circle: with sodium acetate (1.0

g/L) addition; black square: without glucose and acetate addition) and (B) β-carotene content (black bar: with glucose (0.73 g/L or 24.3 mM C equivalent) addition; white bar: with sodium acetate (1.0 g/L or 24.3 mM C equivalent) addition; italic bar: without glucose and acetate addition) when the recombinant pHSN401-Psy-CPC2-4 strain (or CPC2-4 strain) was grown on BG11 medium under the condition of 2% CO_2 aeration at an aeration rate of 0.1 vvm, 300 rpm agitation, light intensity of ca. 150 μmol/m^2/s and a temperature of 25°C.

Figure 4 Time course profile of (A) microalgal biomass concentration with the addition of different $MgSO_4$ concentrations in the medium (black circle: 0.0375 g; open circle: 0.075g; black square: 0.15 g) and (B) β-carotene content with the addition of different $MgSO_4$ concentrations (black bar: 0.0375 g; white bar: 0.075 g; italic bar: 0.15 g) when the recombinant pHSN401-Psy-CPC2-4 strain (or CPC2-4 strain) was grown on BG11 medium under the condition of 2% CO_2 aeration at an aeration rate of 0.1 vvm, 300 rpm agitation, light intensity of ca. 150 μmol/m^2/s and a temperature of 25°C.

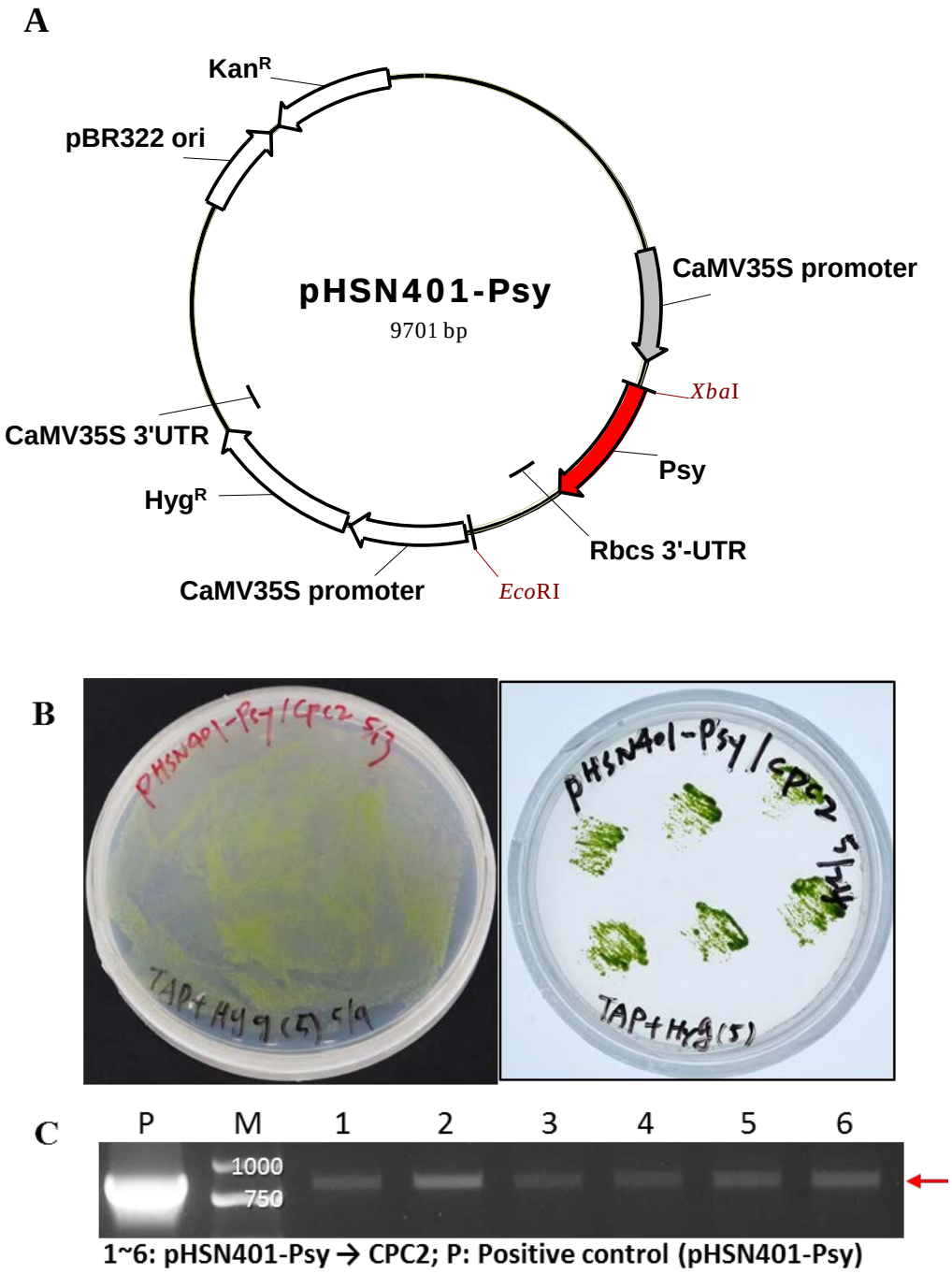

Figure 1.

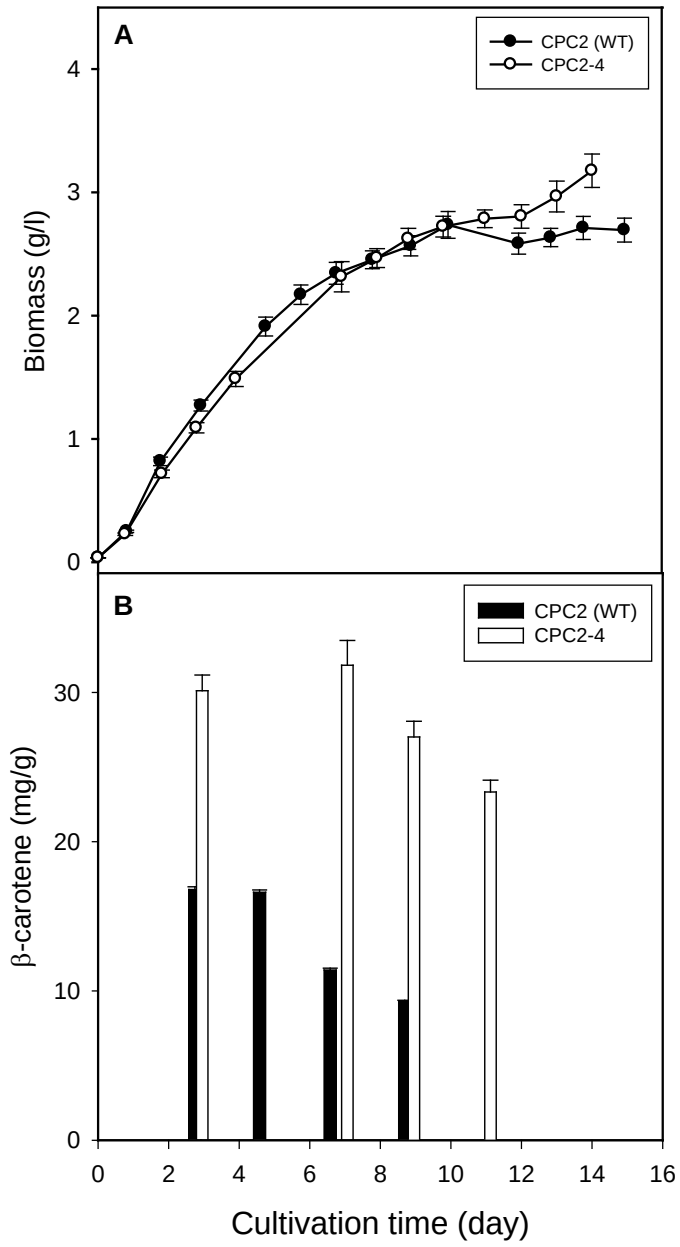

Figure 2.

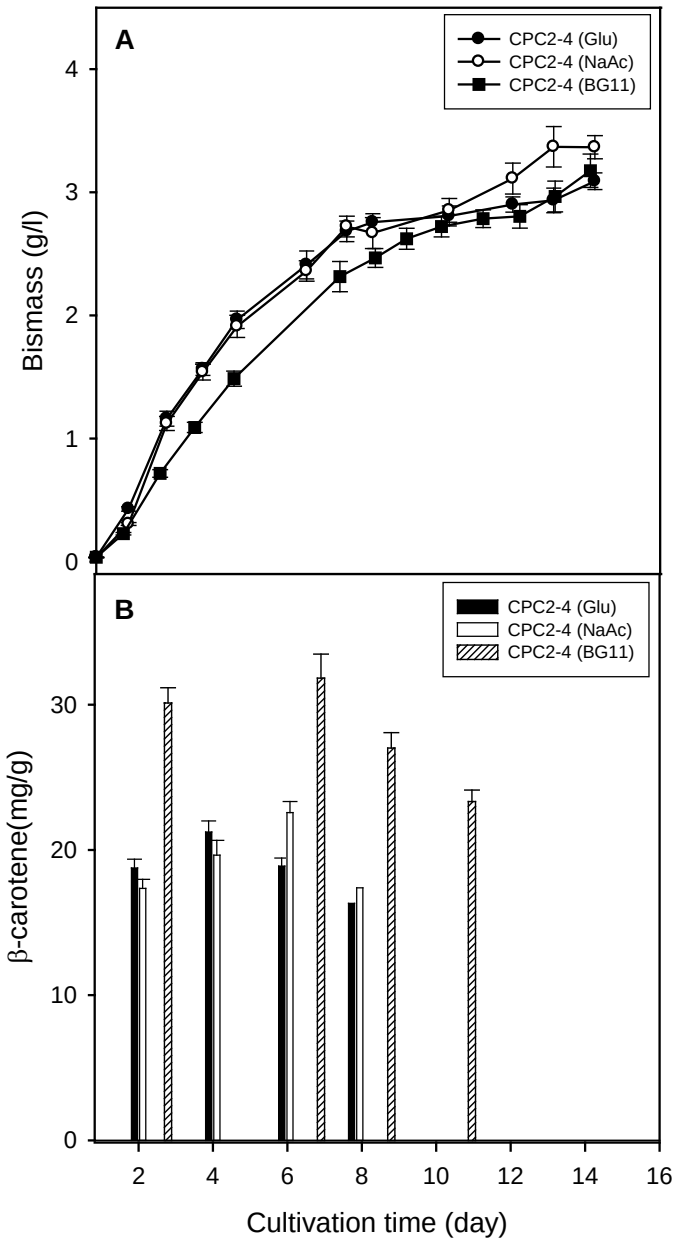

Figure 3.

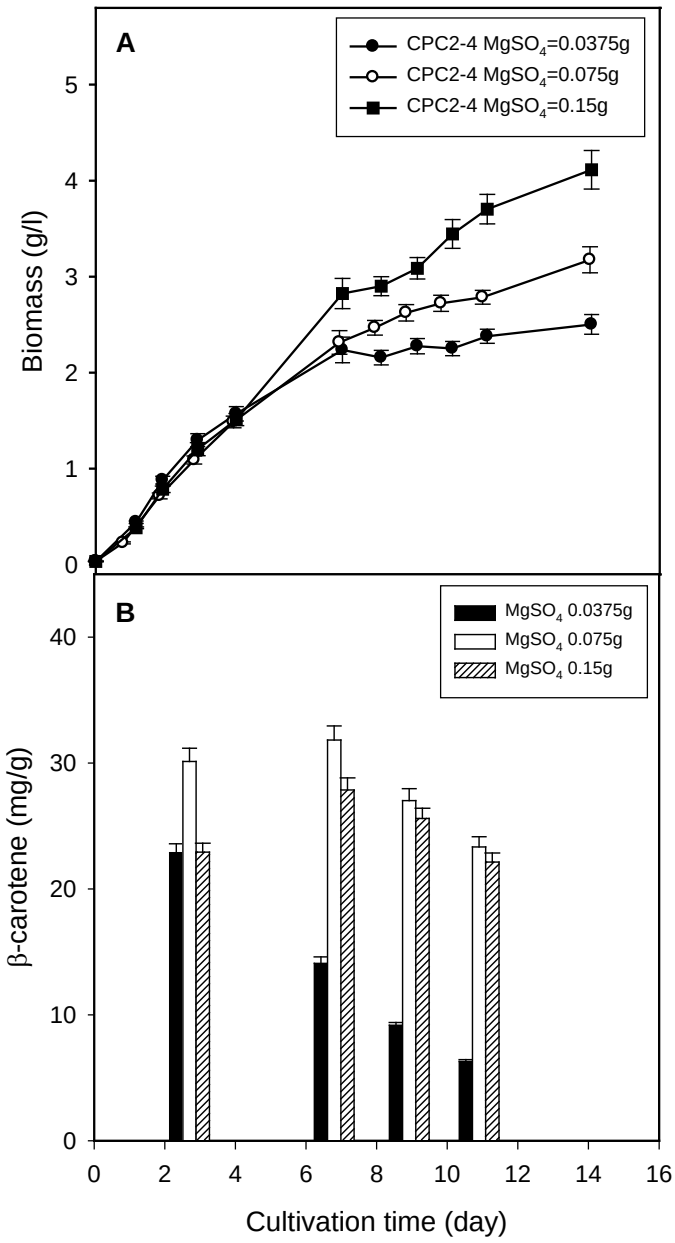

Figure 4.