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Enhanced production of poly-3-hydroxybutyrate and carotenoids by *Arthrospira platensis* under combined glycerol and phosphorus supplementation

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ABSTRACT: *Arthrospira platensis* is one of the most beneficial cyanobacteria due to its high protein content and high-value compounds such as poly-3-hydroxybutyrate (PHB) and carotenoids which can be used in promoting health in many sectors. *A. platensis* cultivation can be performed using urban or industrial wastewater as a source of nutrients, including glycerol and phosphorus. The objective of this study was to enhance biomass, pigments, and partial PHB in *A. platensis* IFRPD1182 grown under different glycerol and phosphorus concentrations. The highest values of biomass, carotenoids, and PHB production were observed in cells grown in the G_3P_3 medium supplemented with 4.6 g/l glycerol and 356 mg/l phosphorus on the fifth day of cultivation with maximal values of 1.90 ± 0.03 mg/ml, 6.30 ± 0.12 mg/l, and 34.76 ± 2.71 mg/l, respectively. In addition, RT-PCR analysis revealed that the cells grown in G_3P_3 medium increased *crtB* and *crtP* transcripts, encoding phytoene synthase and phytoene desaturase, respectively, and involved in carotenoid biosynthesis with 1.47- and 1.50-fold increases, respectively. This indicates that the increased expression of *crtB* and *crtP* genes in the cells can be achieved by a combination of glycerol and phosphorus, where the carotenoids were highly accumulated.

KEYWORDS: *Arthrospira platensis*, carotenoid, glycerol, PHB, phosphorus

INTRODUCTION

Microalgae and cyanobacteria are a promising new source biomass for the production of high-value natural products including photosynthetic pigments and biopolymers [1–3]. Particularly, the cultivation of these species in agro-industrial wastes and wastewater has been widely proposed as a sustainable alternative for biomass production [4]. By using a wastewater medium for growth, the production cost of biomass from microalgae and cyanobacteria could be reduced [5]. The presence of organic wastes in the mixotrophic cultivation stimulated the growth of microalgal species [6]. Glycerol is a waste by-product from the biodiesel industry that has been used as a carbon source for heterotrophic microalgae growth [7]. In addition, the biotransformation of glycerol is an alternative for the generation of various biological products [7]. Phosphorus has been particularly reported to be a major pollutant for water eutrophication as well as an essential nutrient for the lipid biosynthesis of microalgae [8]. Therefore, the recycling of glycerol and phosphorus present in wastewater by microalgae and cyanobacteria should be interesting. Among cyanobacteria, *Arthrospira platensis* is a filamentous and non-nitrogen-fixing strain that can be cultivated and produces various commercialized bioproducts worldwide. It is rich in amino acids and proteins (between 55–70% of its dry weight), vitamins, and minerals, and it has high polyunsaturated fatty acid content [9]. *A. platensis* contains several high-value pigments such as carotenoids and biopolymers such as poly-3-hydroxybutyrate (PHB) when cells are grown in optimal conditions. Carotenoids are the main accessory pigments, and they alter accumulation in response to environmental stimuli such as low-temperature, salinity, and oxidative stress [10]. The biosynthesis of carotenoids is initiated with the combination of 2 molecules of geranyl pyrophosphate by phytoene synthase (encoded by *crtB*). Then, a phytoene molecule is converted into lycopene through desaturation and isomerization steps. In oxygenic phototrophs, phytoene desaturase (encoded by *crtP*) catalyzes the first desaturation to produce 9,15,9′-tri-*cis* ζ-carotene. Then, 15-*cis* ζ-carotene isomerase catalyzes the isomerization of 9,15,9′-tri-*cis* ζ-carotene to form 9,9′-di-*cis* ζ-carotene. Subsequently, ζ-carotene desaturase (encoded by *crtQ*) catalyzes the conversion

of 9,9′-di-*cis* ζ-carotene to 7,9,7′,9′-tetra-*cis* lycopene (pro-lycopene) [11]. During the desaturation reaction, poly-*cis* forms of ζ-carotene and lycopene are isomerized to *trans* forms by carotenoid isomerase (encoded by *crtH*) [11].

Previously, it was demonstrated that the *Paracoccus* sp. strain LL1 could metabolize glycerol as a substrate for the production of high-value carotenoids with concomitant enhancement of polyhydroxyalkanoates (PHAs) [12]. Commonly, poly-3-hydroxybutyrate (PHB) is one of the PHAs that can be extracted from microbial biomasses. The highest accumulation of PHB in *Arthrospira platensis* was observed in cells grown photoautotrophically under nitrogen deprivation with acetate supplementation [13]. PHB is an attractive alternative to common thermoplastics due to its hydrophobicity, biodegradability, and biocompatibility. Furthermore, PHB nanofiber from *Spirulina platensis* has exhibited properties equal to or better than nanofiber made with commercially available PHB [14]. The PHB biosynthesis is initiated with the condensation of 2 acetyl-CoA molecules into acetoacetyl-CoA by β-ketothiolase (encoded by *phaA*). The second reaction is the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (encoded by *phaB*). Finally, the 3-hydroxybutyryl-CoA monomers are polymerized into poly-3-hydroxybutyrate (PHB) by PHA synthase (encoded by *phaC*) [13].

The use of industrial residues (glycerol and phosphorus) as a nutrient source may be a way to make high-value bioproducts from cyanobacteria economically feasible. Recently, it was reported that the addition of glycerol waste to *Spirulina* (*Arthrospira*) sp. LEB 18 culture stimulated cell growth and altered the fatty acid profile [15]. Besides, *Spirulina platensis* could use glycerol for valuable fatty acid and γ-linolenic acid production [16]. The highest carotenoid yield of *Scenedesmus* sp. was obtained at an N/P ratio of 20:1 and crude glycerol concentration of 5 g/l [17], whereas the highest biomass concentrations and PHB contents of *Arthrospira platensis* were achieved by supplying a Zarrouk medium with pure and crude glycerol (6.14 g/l). Moreover, *Synechocystis* sp. PCC 6803 grown under normal photoautotrophic cultivation with an adequate N/P supply produced a high biomass content, but low glycogen and PHB accumulation [18]. A number of challenges in attempting to produce both carotenoids and PHB from mixotrophic culture of cyanobacteria under the availability of nutrients have been reported. However, while increasing the amount of one product, the other will be reduced. Co-production of valuable bioproducts has been proposed to alleviate the overall production cost. Therefore, this study aims to simultaneously enhance the production of both carotenoids and PHB in *Arthrospira platensis* IFRPD 1182 by using a combination of glycerol and phosphorus supplied in a culture medium and monitoring the expression of genes involved in carotenoid biosynthesis.

MATERIALS AND METHODS

Cyanobacterial strain and culture conditions

The *Arthrospira platensis* IFRPD1182 (hereafter referred to as *A. platensis* IFRPD1182) used in this study was obtained from the Institute of Food Research and Product Development, Kasetsart University (IFRPD). The cells were pre-cultured in Zarrouk medium as previously described by Costa et al [19]. Then, the cells were constantly cultivated under fluorescent light intensity at 60 μmol/m^2/s in 12:12 dark/light photoperiod illumination under continuous shaking at 120 rpm at 30 ± 2 °C until the culture reached the late logarithm phase (5 days, optical density at 730 nm (OD_{730}) ∼1–1.2). To study the effect of glycerol, the pre-culture cells were washed with sterilized distilled water (DW) 3 times and resuspended into a fresh Zarrouk medium containing different concentrations of pure glycerol at 0, 0.046, 0.46, and 4.6 g/l. To study the effect of glycerol and phosphorus, the pre-culture cells were washed with DW 3 times and resuspended into a fresh Zarrouk medium containing glycerol at 4.6 g/l combined with different concentrations of phosphorus at 0, 89, 178, and 356 mg/l. The cultures were initiated with an OD_{730} of 0.5 and further cultivated in the above-mentioned condition. Then, cells were harvested at specific time intervals (0, 1, 2, 3, 4, and 5 days) for biomass production, pigment determination, and PHB accumulation. All the experiments were performed in triplicate.

Biomass production

The dry cell weight (DCW) of *A. platensis* IFRPD1182 was analyzed by filtering through preweighed 2.5 μm GF/C membranes (Whatman, USA). After that, the membranes were washed twice with distilled water and dried at 105 °C (Thermo Scientific, USA) until reaching constant weight and then measured using an analytical balance (Sartorius, Germany).

Chlorophyll-*a* (Chl-*a*) and carotenoid (Car) determination

One milliliter of *A. platensis* IFRPD1182 cells was harvested by centrifugation at 8000 × *g* for 7 min under room temperature. Then, the supernatant was discarded, and the dried pellet was added along with 1 ml pre-cooled methanol (99.8%) [20], mixed by a vortex, wrapped in aluminum foil, and kept in the dark at 4 °C for 20 min. After that, the sample was centrifuged at 15 000 × *g*, at 4 °C for 7 min. The supernatant was kept for pigment determination. Chl-*a* and Car production were quantified according to Eqs. (1) and

(2) as previously described [21]:

$$\text{Chl-}a\ (\text{mg/l}) = 12.9447(A_{665} - A_{720}), \quad (1)$$

$$\text{Car}\ (\text{mg/l}) = \frac{1000(A_{470} - A_{720}) - 2.86(\text{Chl-}a)}{221}, \quad (2)$$

where Chl-*a* is chlorophyll-*a* production (mg/l), Car is carotenoid production (mg/l), and A_{470}, A_{665}, and A_{720} are the absorbance at 470, 665, and 720 nm, respectively.

PHB determination

The PHB accumulation in *A. platensis* IFRPD1182 cells was determined by Nile red staining as previously reported by Duangsri et al [13]. Briefly, Nile red (Sigma Aldrich, USA) was dissolved in acetone at 1 mg/ml to make a stock solution. The staining solution proper contained Nile red 0.5 μg/ml. Then, 20 μl of samples were mixed with 10 μl of Nile red staining solution and incubated at 50 °C for 10 min under darkness. The solution was dropped onto a glass slide and closed with a coverslip, and then observed under a fluorescent microscope (Zeiss, USA) at an excitation wavelength of 450–490 nm under 40 × magnification. For the quantitative analysis of the PHB contents, samples (dried cells) were boiled in concentrated H_2SO_4 for 1 h. The PHB-hydrolyzed product (crotonic acid) was analyzed by using High-Performance Liquid Chromatography, HPLC (Water, USA) with 5 μm C18 reverse-phase column (I.D. 4.6 × 150 nm) and supplied with an SPD-20A UV/VIS detector at 210 nm. The condition of HPLC analysis was performed by using a 20 μl sample injection. The solvent system was run at 40% (v/v) of acetonitrile and 60% (v/v) of 0.1% acetic acid with a flow rate of 0.6 ml/min [13]. The commercial PHB (Sigma, USA) was used as the standard and prepared in the same manner as the cell sample. The PHB contents were calculated as the weight of PHB to the dry cell weight (mg/g DCW).

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

One milliliter of the *A. platensis* IFRPD1182 cells was harvested by centrifugation at 9000 × *g* at 25 °C for 10 min, and the pellet cells were then frozen in liquid nitrogen. Then, total RNA was extracted from the pellet cells using TRIZol reagent (Invitrogen, USA) by following the manufacturer's protocol. DNA contamination was removed by RNase-free DNase (Promega, USA). RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), a specific forward primer, and total RNA (200 ng) were used for reverse transcription in a 20 μl reaction volume following the manufacturer's instructions. The RT-PCR condition was run as follows: pre-denaturation at 94 °C for 5 min, followed by 33 cycles of denaturation at 94 °C for 45 sec, annealing at 57 °C for 1 min, extension at 72 °C for 1 min, and post-extension at 72 °C for 10 min. The PCR products, 228-bp of *crtB* gene, 221-bp of *crtP* gene, and 284-bp of *16S rRNA* gene of *A. platensis* IFRPD1182, were amplified using a thermal cycler (SensQuest, Germany) and analyzed by electrophoresis on a 1.5% agarose gel using the specific primers as shown in Table S1. After electrophoresis, the PCR products were quantified using the Gel Imaging System (Omega Fluor, USA). The intensity of the target genes under each condition studied was normalized to the internal housekeeping gene, *16S rRNA*, under the same condition, which was represented as a relative transcript ratio (fold).

Statistical analysis

All data obtained in this study represent the means of 3 independent biological replicates, and the error bars represent the standard deviation (Mean ± SD, $n = 3$). The statistical analysis was analyzed by one way analysis of variance (ANOVA), and the significant difference ($p < 0.05$) were compared by Duncan's multiple range tests using SPSS version 22 (IBM, USA).

RESULTS

Effects of glycerol and phosphorus concentration on biomass production

In the present work, the initial biomass concentration of *A. platensis* IFRPD1182 was fixed at 0.30 ± 0.03 mg/ml, and the cells were then cultivated in medium with varying glycerol concentrations (G_0, G_1, G_2, or G_3) (Fig. 1). It was found that the biomass production of the cells increased sequentially with increasing amounts of glycerol up to 4.6 g/l from the first day to the fifth day (Fig. 1a). The presence of a higher glycerol concentration than 4.6 g/l in the medium resulted in a decrease in biomass production. The maximum biomass production (1.28 ± 0.02 mg/ml) was obtained from cells grown in the G_3 medium for 5 days. In addition, we further determine biomass production of the cells grown in the medium containing glycerol combined with varying phosphorus concentrations (G_3P_1, G_3P_2, or G_3P_3). It was found that biomass production of the cells increased with increasing amounts of phosphorus up to 356 mg/l from the first day to the fifth day (Fig. 1b). With the combination of glycerol and phosphorus in the G_3P_2 or G_3P_3 medium, enhanced biomass was notably obtained from the third day of cultivation. The biomass production of cells grown in the G_3P_3 medium for 5 days reached a maximum of 1.90 ± 0.03 mg/ml, which was significantly increased 6.3-fold compared to the initial biomass concentration at zero-day (Fig. 1b).

Effects of glycerol and phosphorus concentration on PHB accumulation

The PHB accumulation was observed in *A. platensis* IFRPD1182 grown in different media (Z, G_3P_1, G_3P_2, or G_3P_3) for 5 days (Fig. 2). The results showed that

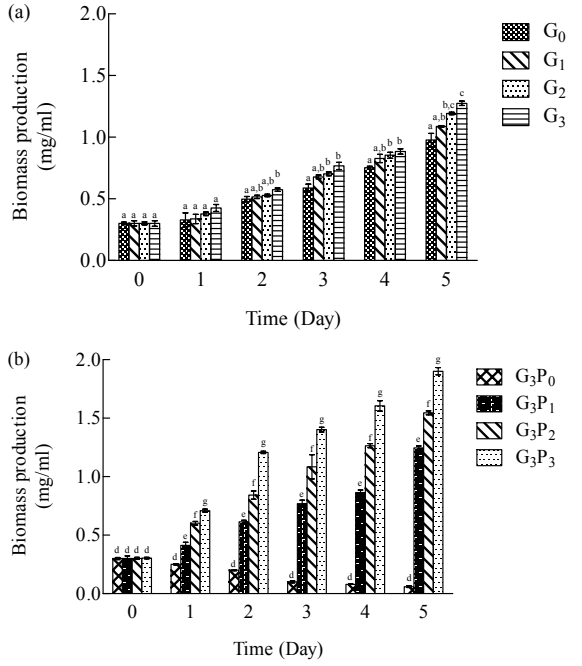

Fig. 1 Effects of glycerol and phosphorus on biomass production in *A. platensis* IFRPD1182. Cells were cultured in Zarrouk (Z) medium containing different glycerol concentrations (0, 0.046, 0.46, and 4.6 mg/l represented as G_0, G_1, G_2, and G_3, respectively) for 5 days (a), and medium containing glycerol 4.6 g/l (G_3) combined with varying phosphorus concentrations (0, 89, 178, and 356 mg/l represented as G_3P_0, G_3P_1, G_3P_2, and G_3P_3, respectively) for 5 days (b). Bar graphs represent mean values (±SD) of 3 independent experiments. Different letters (a, b, or c) indicate significant differences ($p < 0.05$) between the groups obtained from cells cultured in medium without glycerol-added (G_0) and with glycerol-added (G_1, G_2, and G_3), while different letters (d, e, f, or g) indicate significant differences ($p < 0.05$) between the groups obtained from cells cultured in medium containing glycerol without phosphorus-added (G_3P_0) and with phosphorus-added (G_3P_1, G_3P_2, and G_3P_3).

PHB appeared as bright fluorescent orange granules upon Nile-red staining in all conditions with glycerol- and phosphorus-added (Fig. 2a). The highest PHB content was obtained in cells grown in the G_3P_1 medium, which was 24.81±1.00 mg/g DCW. The presence of a higher phosphorus concentration than 89 mg/l in the medium (G_3P_2 or G_3P_3) resulted in a decrease in PHB content (Fig. 2b). On the other hand, the PHB production reached a maximum of 34.76±2.71 mg/l on day 5 in cells grown in the G_3P_3 medium (Fig. 2c). However, PHB production was hardly observed in cells grown in the Z medium.

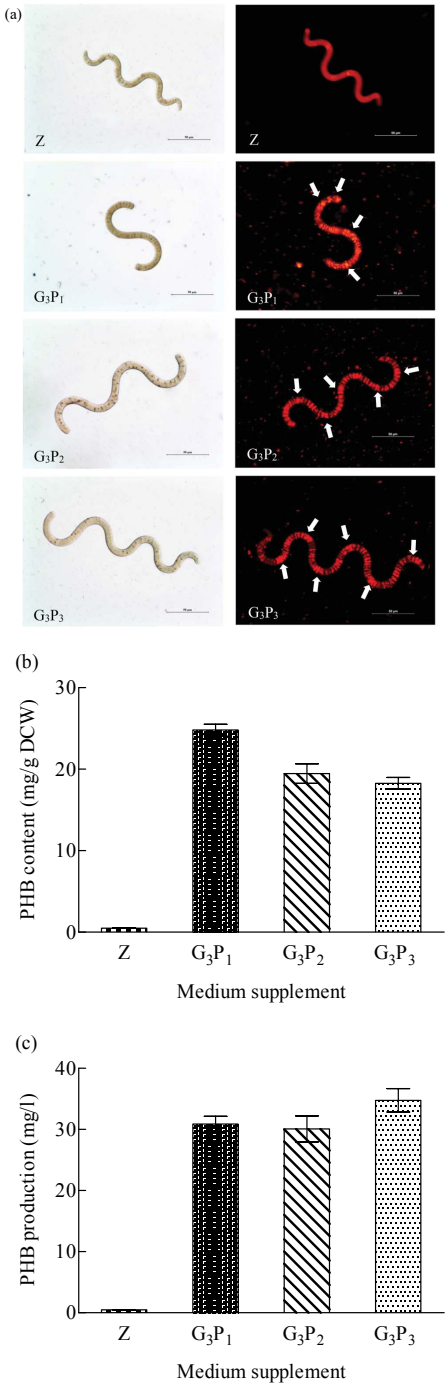

Fig. 2 Effects of glycerol and phosphorus on PHB accumulation in *A. platensis* IFRPD1182. Cells were cultured in different media for 5 days. (a) *A. platensis* IFRPD1182 stained cells were observed under brightfield microscopy (left) and fluorescent microscopy as bright orange granules indicated by white arrows (right). (b) PHB content was represented as mg/g DCW. (c) PHB production was represented as mg/l. Bar graphs represent mean values (±SD) of 3 independent experiments.

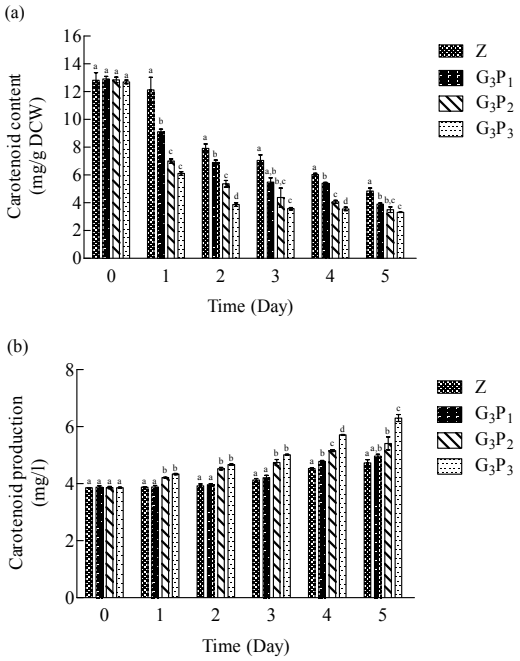

Fig. 3 Effects of glycerol and phosphorus on carotenoid content (a) and production (b) in *A. platensis* IFRPD1182. Cells were cultured in different media for 5 days. Bar graphs represent mean values (± SD) of 3 independent experiments. Different letters (a, b, c, or d) indicate significant differences ($p < 0.05$) between the groups obtained from cells cultured in Z medium and the medium containing different glycerol and phosphorus concentrations.

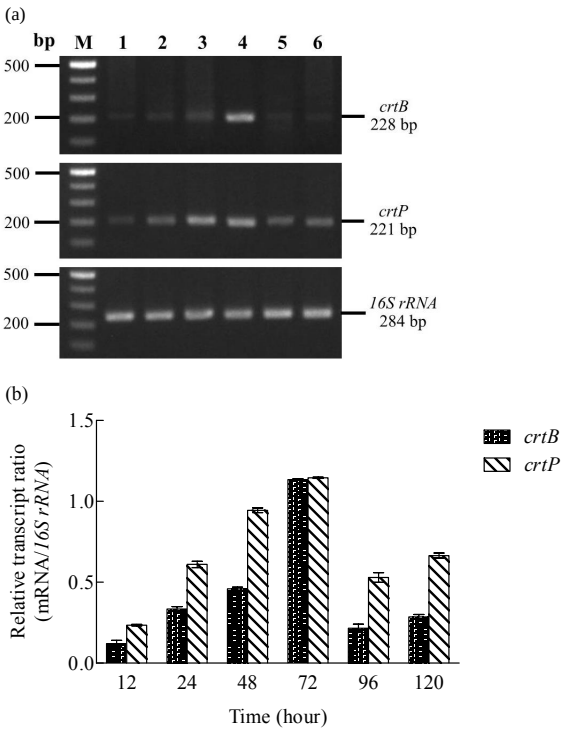

Fig. 4 Changes in transcript levels of *crtB* and *crtP* genes in *A. platensis* IFRPD1182. Cells were cultured in Z medium for 12, 24, 48, 72, 96, and 120 h before the analysis of gene expression by RT-PCR. The upper panel (a) shows a typical example of DNA products resolved on 1.5% agarose gel. Lane M: DNA marker (100 bp plus DNA ladder), lanes 1–6: *crtB* and *crtP* amplified products at the indicated time. The lower panel (b) shows the relative transcript ratio of *crtB* and *crtP* genes (fold) at the indicated time. Bar graphs represent mean values (± SD) of 3 independent experiments.

Effects of glycerol and phosphorus concentration on carotenoid production

The carotenoid content and production were determined in *A. platensis* IFRPD1182 grown in different media (Z, G_3P_1, G_3P_2, or G_3P_3) for 5 days (Fig. 3). The maximum carotenoid content of the cells was observed on the first day when the cells were grown in the Z medium, compared to cells grown in the other medium. After 1 day, the carotenoid content was then gradually decreased from the first day to the fifth day in all conditions (Fig. 3a). On the contrary, the carotenoid production of cells grown in the G_3P_2 and G_3P_3 media increased sequentially after 1 day and reached a maximum of 5.41 ± 0.22 mg/l and 6.30 ± 0.12 mg/l on day 5, respectively, which was significantly increased 1.1- and 1.3-fold ($p < 0.05$) compared to the cells grown in the Z medium (Fig. 3b).

Effects of glycerol and phosphorus on the expression of *crtB* and *crtP* genes

The molecular response of *crtB* and *crtP* genes, encoding phytoene synthase and phytoene desaturase, respectively, of *A. platensis* IFRPD1182 to glycerol and phosphorus was investigated at the transcriptional level using RT-PCR. Three biologically independent replicates were cultivated and analyzed under Z medium for 120 h (Fig. 4). The transcript levels of the *crtB* and *crtP* genes of the cells were upregulated after cultivation for 24 h (Fig. 4a), and the highest transcript levels appeared at 72 h. Moreover, declined transcript levels were observed after 72 h of cultivation (Fig. 4b). Then, the cells were subjected to the medium containing different concentrations of glycerol (G_1, G_2, or G_3) for 72 h (Fig. 5). The results showed the expression of *crtB* and *crtP* genes increased when glycerol in the medium was increased (Fig. 5a). The highest relative expression of *crtB* and *crtP* genes were observed significantly in cells grown in the G_3 medium, representing approximately 1.36- and 1.48-fold increases, respectively ($p < 0.05$) (Fig. 5b), compared with cells grown in the G_1 medium. After that, the cells were subjected to the medium containing

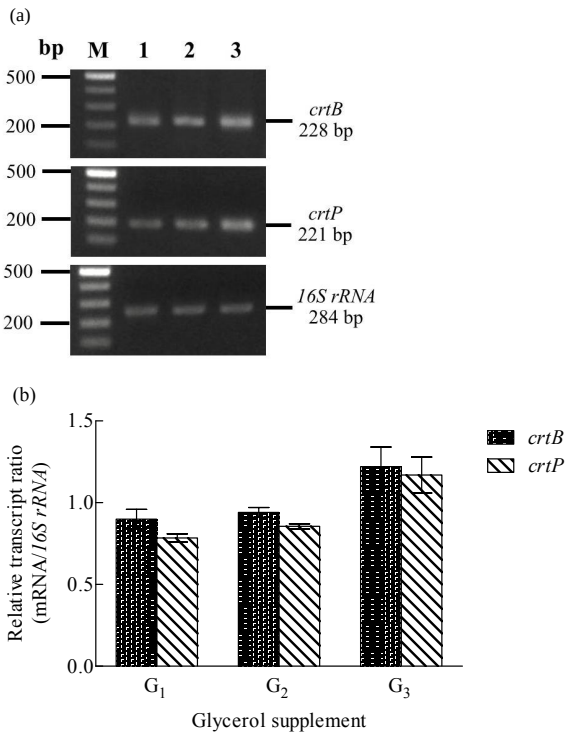

Fig. 5 Effects of glycerol on the expression levels of *crtB* and *crtP* genes in *A. platensis* IFRPD1182. Cells were cultured in the glycerol-added medium for 72 h before the analysis of gene expression by RT-PCR. The upper panel (a) shows a typical example of DNA products resolved on 1.5% agarose gel. Lane M: DNA marker (100 bp plus DNA ladder), lane 1: G_1, lane 2: G_2, and lane 3: G_3. The lower panel (b) shows the relative transcript ratio of *crtB* and *crtP* genes (fold). Bar graphs represent mean values (±SD) of 3 independent experiments.

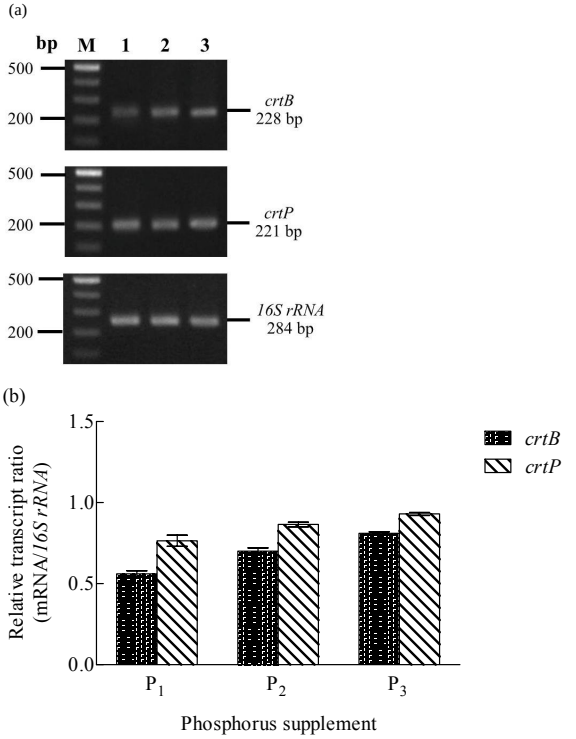

Fig. 6 Effects of phosphorus on the expression levels of *crtB* and *crtP* genes in *A. platensis* IFRPD1182. Cells were cultured in the phosphorus-added medium for 72 h before the analysis of gene expression by RT-PCR. The upper panel (a) shows a typical example of DNA products resolved on 1.5% agarose gel. Lane M: DNA marker (100 bp plus DNA ladder), lane 1: P_1, lane 2: P_2, and lane 3: P_3. The lower panel (b) shows the relative transcript ratio of *crtB* and *crtP* genes (fold). Bar graphs represent mean values (±SD) of 3 independent experiments.

different concentrations of phosphorus (P_1, P_2, or P_3) for 72 h (Fig. 6). The result showed that the expression of *crtB* and *crtP* genes was increased when phosphorus in the medium was increased (Fig. 6a). The highest relative expression levels of *crtB* and *crtP* genes were significantly observed in cells grown in the P_3 medium, which involved approximately 1.45- and 1.21-fold increases, respectively (Fig. 6b), compared to cells grown in the P_1 medium. The cells were also subjected to the G_3P_3 medium for 72 h (Fig. 7). The result showed that the expression of *crtB* and *crtP* genes increased significantly (Fig. 7a) in the G_3P_3 medium with increases which were approximately 1.47- and 1.50-fold, respectively ($p < 0.05$) (Fig. 7b), compared with cells grown in Z medium.

DISCUSSION

Arthrospira platensis is one of the most promising cyanobacteria that can synthesize high-value compounds from light energy and the remaining organic and inorganic carbon sources from wastewater and industrial processes [22]. In addition, cyanobacteria have been used as a source of food, animal feedstock, and fertilizers and can be applied in the cosmetics industry and health sector because they are rich in nutritional value through lipids, proteins, carbohydrates, and pigments under excess or limitation of nutrients in the culture medium [1, 23, 24]. There are several reports of converting crude glycerol into high-added-value products by phototrophic organisms. Glycerol is absorbed by microalgae by simple diffusion into cells and is used as a cell absorption agent [4]. Phototrophic organisms contain various enzymes for converting glycerol to glyceraldehyde-3-phosphate and glycerate [7]. These molecules are intermediates in glycolysis, so glyceraldehyde-3-phosphate may be used as an intermediate of the Calvin-Benson photosynthesis cycle [25] and used as an intermediate in MEP path-

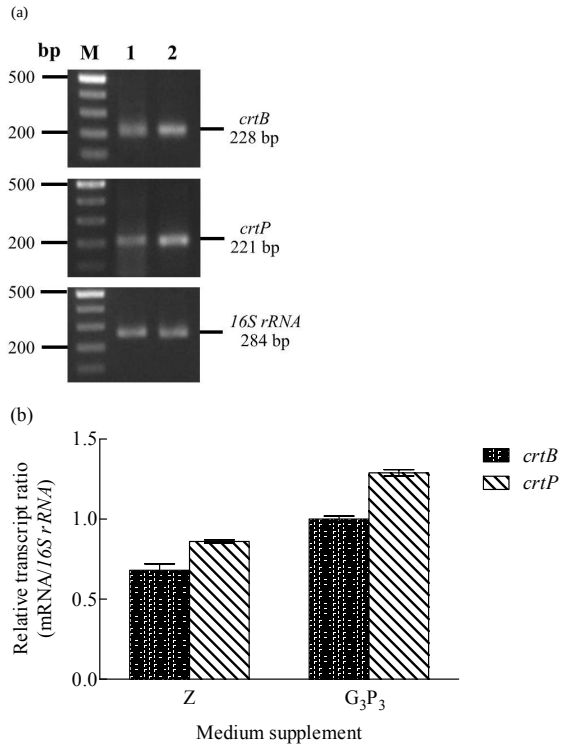

Fig. 7 Effects of glycerol and phosphorus on the expression levels of *crtB* and *crtP* genes in *A. platensis* IFRPD1182. Cells were cultured in different media for 72 h before the analysis of gene expression by RT-PCR. The upper panel (a) shows a typical example of DNA products resolved on 1.5% agarose gel. Lane M: DNA marker (100 bp plus DNA ladder), lane 1: Z and lane 2: G_3P_3. The lower panel (b) shows the relative transcript ratio of *crtB* and *crtP* genes (fold). Bar graphs represent mean values (± SD) of 3 independent experiments.

ways [26]. The rise of glyceraldehyde-3-phosphate accelerates the process of glycolysis and TCA pathways, ultimately increasing cellular metabolism and helping to increase the growth and the production of new cells [27]. Additionally, phosphorus is a key component in the sustainable growth and development of microalgae. In the composite wastewater tests, phosphorus was uptaken by *Chlorella* sp. at the beginning of growth due to the ability of microalgae to use excessive phosphorus as energy storage for biomass growth and biosynthesis [8]. *A. platensis* could remove between 88.8–93.7% of the phosphorus source present in the wastewater [28]. On the other hand, the lack of nitrogen and phosphorus in the medium resulted in decreased photosynthesis and affected the growth of microalgae [29, 30].

In this study, the biomass and carotenoid production of *Arthrospira platensis* IFRPD1182 were enhanced when glycerol and phosphorus concentration in the medium were elevated (Figs. 1 and 3). The maximum biomass and carotenoid production of *A. platensis*, IFRPD1182 were 1.90 ± 0.03 mg/ml and 6.30 ± 0.12 mg/l, respectively, when the cells were grown in Zarrouk medium containing glycerol (4.6 g/l) and phosphorus (356 mg/l). These results suggested that *A. platensis* IFRPD1182 was able to assimilate glycerol and phosphorus towards metabolic processes for biomass and carotenoid production. This was in line with a previous study which reported that the *Spirulina* (*Arthrospira*) sp. LEB 18 [15] and *S. platensis* CFTRI strains [16] could utilize glycerol as an organic substrate to generate higher levels of biomass and pigment production. The addition of glycerol to the *Spirulina* sp. LEB 18 culture stimulated cell growth, providing 3.00 g/l biomass and 0.72 g/l/d maximum productivity. Moreover, Narayan et al demonstrated that a marked decrease was observed in the chlorophyll *a* and C-phycocyanin contents of *S. platensis* grown on glycerol medium when compared to the medium without glycerol [16], which was similar to the present study. Also, *Phaeodactylum tricornutum* grown in a culture medium supplemented with 0.1 M of glycerol under 165 μmol/m^2/s had a 74% increase in growth compared to autotrophic culture [31]. Additionally, *Scenedesmus* sp. showed the highest biomass and lutein yields when cells were grown in the presence of crude glycerol (6 g/l) [17].

In addition, the previous report showed that there are many important enzymes in microalgae involved in carotenoid synthesis, particularly phytoene synthase (encoded by the *crtB* gene) and phytoene desaturase (encoded by the *crtP* gene) [32]. The present study showed that the expression of *crtB* and *crtP* genes of *A. platensis* IFRPD1182 was increased after 24 h and continued to increase until 72 h, after which it declined (Fig. 4), indicating that the *crtB* and *crtP* genes were time dependent. This was comparable to previous information suggesting that the highest transcript levels of the *crtB* gene of *Haematococcus pluvialis* were sustained from 24 to 72 h [32]. Furthermore, it was found that the highest relative expression of *crtB* and *crtP* genes of *A. platensis* IFRPD1182 was obtained in the combined condition of glycerol of 4.6 g/l and phosphorus of 356 mg/l at 72 h of cultivation. It may be possible that *A. platensis* IFRPD1182 metabolized glycerol as a carbon source through oxidative or reductive pathways for the synthesis of carotenoids by the induction of *crtB* and *crtP* genes expression and metabolized phosphorus for the synthesis of cellular components. In addition, Ryu et al exhibited glucose-induced carotenoid gene expressions of *Synechocystis* sp. PCC 6803 were regularly mediated by an increase in cytosolic pH [33]. In addition, the expression of phytoene synthase (*crtB*) and phytoene desaturase (*crtP*) was enhanced in the dark-adapted *Synechocystis* cells upon glucose treatment as a consequence of transcriptional activation [34]. Moreover, photosynthetic

organisms require phosphorus for NADPH production, and NADPH has been reported to be a requirement for the cyclization reaction of carotenoid biosynthesis [35].

The balance of the C/N ratio in the cells and the limitation of N and P in the medium are common strategies for activating PHB accumulation in *A. platensis* [13]. The PHB accumulation was strain-specific but not associated with any particular cyanobacterial morphology [36]. The present study showed that *A. platensis* IFRPD1182 accumulated the highest PHB (34.76 ± 2.71 mg/l) when cells were grown in Zarrouk medium supplied with glycerol (4.6 g/l) combined with phosphorus (356 mg/l) for 5 days. These results suggested that *A. platensis* IFRPD1182 directly utilized glycerol to increase the intracellular acetyl-CoA pool causing a C/N ratio imbalance towards the synthesis of PHB while utilizing phosphorus to generate the NADPH required for the enzymatic activity of the PHB biosynthesis pathway [37]. On the other hand, *A. platensis* grown in Zarrouk medium supplemented with pure glycerol under nutrient depletion (N and P) showed the highest PHA accumulation of 11.06 mg/g [3]. In addition, PHB accumulation in *Nostoc muscorum* increased to 22.7% of dry weight after 4 days of P deficiency, while PHB content in *S. platensis* remained low even after prolonged P starvation [38]. Moreover, a previous report showed that the limitation of both N and P caused a huge effect on decreasing the yields of the C-phycocyanin and PHB by *A. platensis* [2]. Under an adequate supply of N and P in a single-stage photoautotrophic culture, *Synechocystis* sp. PCC 6803 produced a high level of glycogen and PHB, which is probably regulated by a signal of C/N balance affecting PHB metabolism [18]. Furthermore, *Trichosporon oleaginosus* could utilize crude glycerol to produce 43.82 g/l biomass and 21.87 g/l lipids in fed-batch fermentation [39].

CONCLUSION

This study demonstrated that *Arthospira platensis* IFRPD1182 could utilize glycerol and phosphorus for biomass, carotenoids, and PHB production. The results of this study offered a promising utilization of alkaliphilic cyanobacteria for the coproduction of carotenoids and PHB employing glycerol and phosphorus as substrates, which was mediated by the increased expression of *crtB* and *crtP* involved in carotenoid biosynthesis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.072.

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REFERENCES

1. Markou G, Nerantzis E (2013) Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions. *Biotechnol Adv* **31**, 1532–1542.
2. Nematollahi MA, Laird DW, Hughes LJ, Raeisossadati M, Moheimani NR (2020) Effect of organic carbon source and nutrient depletion on the simultaneous production of a high value bioplastic and a specialty pigment by *Arthrospira platensis*. *Algal Res* **47**, ID 101844.
3. Corrêa PS, Teixeira CMLL (2021) Polyhydroxyalkanoates and pigments coproduction by *Arthrospira* (*Spirulina*) *platensis* cultivated in crude glycerol. *J Appl Phycol* **33**, 1487–1500.
4. Markou G, Georgakakis D (2011) Cultivation of filamentous cyanobacteria (blue-green algae) in agro-industrial wastes and wastewaters: a review. *Appl Energy* **88**, 3389–3401.
5. Guldhe A, Kumari S, Ramanna L, Ramsundar P, Singh P, Rawat I, Bux F (2017) Prospects, recent advancements and challenges of different wastewater streams for microalgal cultivation. *J Environ Manage* **203**, 299–315.
6. Vu CHT, Lee H-G, Chang YK, Oh H-M (2018) Axenic cultures for microalgal biotechnology: establishment, assessment, maintenance, and applications. *Biotechnol Adv* **36**, 380–396.
7. de Morais EG, Nunes IL, Druzian JI, de Morais MG, da Rosa APC, Costa JAV (2022) Increase in biomass productivity and protein content of *Spirulina* sp. LEB 18 (*Arthrospira*) cultivated with crude glycerol. *Biomass Convers Biorefin* **12**, 597–605.
8. Zhu S, Wang Y, Xu J, Shang C, Wang Z, Xu J-L, Qi W (2015) Luxury uptake of phosphorus changes the accumulation of starch and lipid in *Chlorella* sp. under nitrogen depletion. *Bioresour Technol* **198**, 165–171.
9. Prates DDF, Radmann EM, Duarte JH, Morais MG, Costa JAV (2018) *Spirulina* cultivated under different light emitting diodes: enhanced cell growth and phycocyanin production. *Bioresour Technol* **256**, 38–43.
10. Takaichi S (2011) Carotenoids in algae: distributions, biosyntheses and functions. *Mar Drugs* **9**, 1101–1118.
11. Kato S, Tanno Y, Takaichi S, Shinomura T (2019) Low temperature stress alters the expression of phytoene desaturase genes (*crtP1* and *crtP2*) and the ζ-carotene desaturase gene (*crtQ*) together with the cellular carotenoid content of *Euglena gracilis*. *Plant Cell Physiol* **60**, 274–284.
12. Kumar P, Jun H-B, Kim BS (2018) Co-production of polyhydroxyalkanoates and carotenoids through bioconversion of glycerol by *Paracoccus* sp. strain LL1. *Int J Biol Macromol* **107**, 2552–2558.
13. Duangsri C, Mudtham N-A, Incharoensakdi A, Raksajit W (2020) Enhanced polyhydroxybutyrate (PHB) accumulation in heterotrophically grown *Arthrospira*

platensis under nitrogen deprivation. *J Appl Phycol* **32**, 3645–3654.

14. Morais MGd, Stillings C, Dersch R, Rudisile M, Pranke P, Costa JAV, Wendorff J (2015) Biofunctionalized nanofibers using *Arthrospira* (*Spirulina*) biomass and biopolymer. *BioMed Res Int* **2015**, ID 967814.
15. Morais EGd, Druzian JI, Nunes IL, Morais MGd, Costa JAV (2019) Glycerol increases growth, protein production and alters the fatty acids profile of *Spirulina* (*Arthrospira*) sp. LEB 18. *Process Biochem* **76**, 40–45.
16. Narayan MS, Pillai M, Vatchravelu K, Neelwarne B, Mahadevaswamy M (2005) Utilization of glycerol as carbon source on the growth, pigment and lipid production in *Spirulina platensis*. *Int J Food Sci Nutr* **56**, 521–528.
17. Lakshmidevi R, Gandhi N, Muthukumar K (2020) Enhanced biomass and lutein production by mixotrophic cultivation of *Scenedesmus* sp. using crude glycerol in an airlift photobioreactor. *Biochem Eng J* **161**, ID 107684.
18. Monshupanee T, Incharoensakdi A (2014) Enhanced accumulation of glycogen, lipids and polyhydroxybutyrate under optimal nutrients and light intensities in the cyanobacterium *Synechocystis* sp. PCC 6803. *J Appl Microbiol* **116**, 830–838.
19. Costa JAV, Colla LM, Duarte Filho P (2004) Improving *Spirulina platensis* biomass yield using a fed-batch process. *Bioresour Technol* **92**, 237–241.
20. Tavanandi H, Vanjari P, Raghavarao K (2019) Synergistic method for extraction of high purity allophycocyanin from dry biomass of *Arthrospira platensis* and utilization of spent biomass for recovery of carotenoids. *Sep Puri Technol* **225**, 97–111.
21. Sinetova MA, Červený J, Zavřel T, Nedbal L (2012) On the dynamics and constraints of batch culture growth of the cyanobacterium *Cyanothece* sp. ATCC 51142. *J Biotechnol* **162**, 148–155.
22. Abad S, Turon X (2012) Valorization of biodiesel derived glycerol as a carbon source to obtain added-value metabolites: focus on polyunsaturated fatty acids. *Biotechnol Adv* **30**, 733–741.
23. Hifney A (2013) Abiotic stress induced production of β-carotene, allophycocyanin and total lipids in *Spirulina* sp. *J Biol Earth Sci* **3**, 54–64.
24. Pimratch S, Butsat S, Kesmala T (2015) Application of blue-green algae and mineral fertilizers to direct seeding lowland rice. *ScienceAsia* **41**, 305–314.
25. Perez-Garcia O, Escalante FME, de-Bashan LE, Bashan Y (2011) Heterotrophic cultures of microalgae: metabolism and potential products. *Water Res* **45**, 11–36.
26. Zhao L, Chang W-C, Xiao Y, Liu H-W, Liu P (2013) Methylerythritol phosphate pathway of isoprenoid biosynthesis. *Annu Rev Biochem* **82**, 497–530.
27. Bhatnagar A, Chinnasamy S, Singh M, Das KC (2011) Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. *Appl Energy* **88**, 3425–3431.
28. Araujo GS, Santiago CS, Moreira RT, Dantas Neto MP, Fernandes FAN (2021) Nutrient removal by *Arthrospira platensis* cyanobacteria in cassava processing wastewater. *J Water Process Eng* **40**, ID 101826.
29. Minhas AK, Hodgson P, Barrow CJ, Adholeya A (2016) A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids. *Front Microbiol* **7**, 546–546.
30. Pongpadung P, Liu J, Yokthongwattana K, Techapinyawat S, Juntawong N (2015) Screening for hydrogen-producing strains of green microalgae in phosphorus or sulfur deprived medium under nitrogen limitation. *ScienceAsia* **41**, 97–107.
31. Cerón Garcí MC, Fernández Sevilla JM, Acién Fernández FG, Molina Grima E, García Camacho F (2000) Mixotrophic growth of *Phaeodactylum tricornutum* on glycerol: growth rate and fatty acid profile. *J Appl Phycol* **12**, 239–248.
32. Steinbrenner J, Linden H (2001) 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* **125**, 810–817.
33. Ryu J-Y, Song J, Lee J, Jeong S, Chow W, Choi S-B, Pogson B, Park Y-I (2004) Glucose-induced expression of carotenoid biosynthesis genes in the dark is mediated by cytosolic pH in the cyanobacterium *Synechocystis* sp. PCC 6803. *J Biol Chem* **279**, 25320–25325.
34. Lagarde D, Beuf L, Vermaas W (2000) Increased production of zeaxanthin and other pigments by application of genetic engineering techniques to *Synechocystis* sp. strain PCC 6803. *Appl Environ Microbiol* **66**, 64–72.
35. Hornero-Méndez D, Britton G (2002) Involvement of NADPH in the cyclization reaction of carotenoid biosynthesis. *FEBS Lett* **515**, 133–136.
36. Kaewbai-ngam A, Incharoensakdi A, Monshupanee T (2016) Increased accumulation of polyhydroxybutyrate in divergent cyanobacteria under nutrient-deprived photoautotrophy: an efficient conversion of solar energy and carbon dioxide to polyhydroxybutyrate by *Calothrix scytonemicola* TISTR 8095. *Bioresour Technol* **212**, 342–347.
37. Li ZJ, Cai L, Wu Q, Chen GQ (2009) Overexpression of NAD kinase in recombinant *Escherichia coli* harboring the *phbCAB* operon improves poly(3-hydroxybutyrate) production. *Appl Microbiol Biotechnol* **83**, 939–947.
38. Panda B, Sharma L, Mallick N (2005) Poly-β-hydroxybutyrate accumulation in *Nostoc muscorum* and *Spirulina platensis* under phosphate limitation. *J Plant Physiol* **162**, 1376–1379.
39. Chen J, Zhang X, Yan S, Tyagi RD, Drogui P (2017) Lipid production from fed-batch fermentation of crude glycerol directed by the kinetic study of batch fermentations. *Fuel* **209**, 1–9.

Appendix A. Supplementary data

Table S1 Gene-specific primer sequences used in this study.

Gene		Sequence	Estimated product size (bp)	T_m^* (°C)
crtB	F1-primer	5′-ACTCTGTCGCCAAATTACCG-3′	228	55.2
	R1-primer	5′-ACATCAACCGGCTCAATAGG-3′		54.8
crtP	F1-primer	5′-TAACCGCTTCCTCCAAGAGA-3′	221	55.4
	R1-primer	5′-CAGCGGTTAGCACTTCTTCC-3′		56.0
16S rRNA	F1-primer	5′-CCTGCAGGCATGGAGAAAAAAATC-3′	284	60.07
	R1-primer	5′-TCTTGGTGAAAGCCGAGAGT-3′		59.99

* T_m = melting temperature.