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Enhancement in phycobiliprotein accumulation in *Aphanothece* sp. using different carbon sources and flashing frequency

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ABSTRACT

Cultivation of microalgae for the production of biomass and high value products is critically ties to the economic feasibility of the algal production system. In this study, we proposed the usage of exogenous carbon sources to activate the photoheterotrophic growth of the *Aphanothece* sp. Based on our findings, supplementation of the BG-11 media with 2 % glucose or 4 % sucrose can increase biomass production by 74.15 % (3.11 ± 0.35 g/L) and 59 % (2.83 ± 0.27 g/L), respectively. Under the photoheterotrophic growth using 2 % glucose, the production of C-PC and APC from *Aphanothece* sp. was increased up to 6.72 ± 0.13 mg/g fresh weight and 0.13 ± 0.01 mg/g fresh weight, respectively (chlorophyll *a* content up to 66.3 ± 3.2 μg/g fresh weight). Our findings also showed that, the usage of flashing frequency at the stationary growth phase of *Aphanothece* sp. (photoautotrophic) upregulated the concentration of C-PC and APC by 64.51 % and 46.34 %, respectively. A positive stimulus of flashing frequency on the production of the C-PC and APC from photoheterotrophic culture of *Aphanothece* sp. was observed. The economic analysis of supplementation of the *Aphanothece* sp. media with 2 % glucose can decrease the production cost of the system (g/L) by 62.81 % due to higher biomass generated by photoheterotrophic growth of *Aphanothece* sp. in compared to the photoautotrophic condition.

1. Introduction

Cyanobacteria (Cyanophyta, blue-green algae) are a diverse morphologically group of the oldest photosynthetic microorganisms, and over 2000 species of cyanobacteria have been identified in freshwater, marine, and terrestrial ecosystems. Cyanobacteria produce spectra of bioactive molecules that enables them to survive under biotic and abiotic conditions [1,2]. These bioactive metabolites are of interest since they are demonstrating a wide range of pharmacological properties (i.e. antioxidant, anticancer, antibacterial, antiprotozoals, and immunomodulators) [3,4]. C-phycocyanin (C-PC) and allophycocyanin (APC) are two phycobiliproteins (pigment-protein complexes) isolated from the light harvesting antenna systems (phycobilisome) in cyanobacterial species, such as *Aphanothece* sp., *Nostoc* sp., *Calothrix* sp., and *Spirulina platensis*. C-PC and APC have various applications in the pharmaceutical and nutraceutical industries as antioxidants, anti-inflammator, neuroprotectors, hepatoprotectors, and food colorants [5,6]. In 2013, the United States Food and Drug Administration (FDA) has approved the usage of phycocyanin (PC) in the pharmaceutical and nutraceutical industries and has unlocked lots of opportunities for its market [7]. Statistics show that, the PC market will reach to 1 Billion $ (US) by 2028 [8]. Thus, due to the PC pharmaceutical, nutraceutical values, and the PC market demands, the researches that influence the improvements in biomass and PC production have immediate impacts.

Among of different cyanobacterial species capable to produce C-PC, and APC less studies have been performed on *Aphanothece* genius mainly because of low biomass yield and its long period of cultivation [9]. To address the low biomass yield in *Aphanothece* production systems, photoautotrophic or photoheterotrophic cultivations supported by an exogenous carbon source are a potential approach for producing commercially important metabolites like C-PC and APC, since *Aphanothece* metabolism supports photoautotrophic and photoheterotrophic growth. Nevertheless, the choice of economic inputs for formulation of the media is essential for the overall bioeconomy of every microalgal production system [10–12]. Carbon sources are usually the most critical factors for the growth and metabolism of cyanobacterial species, and the

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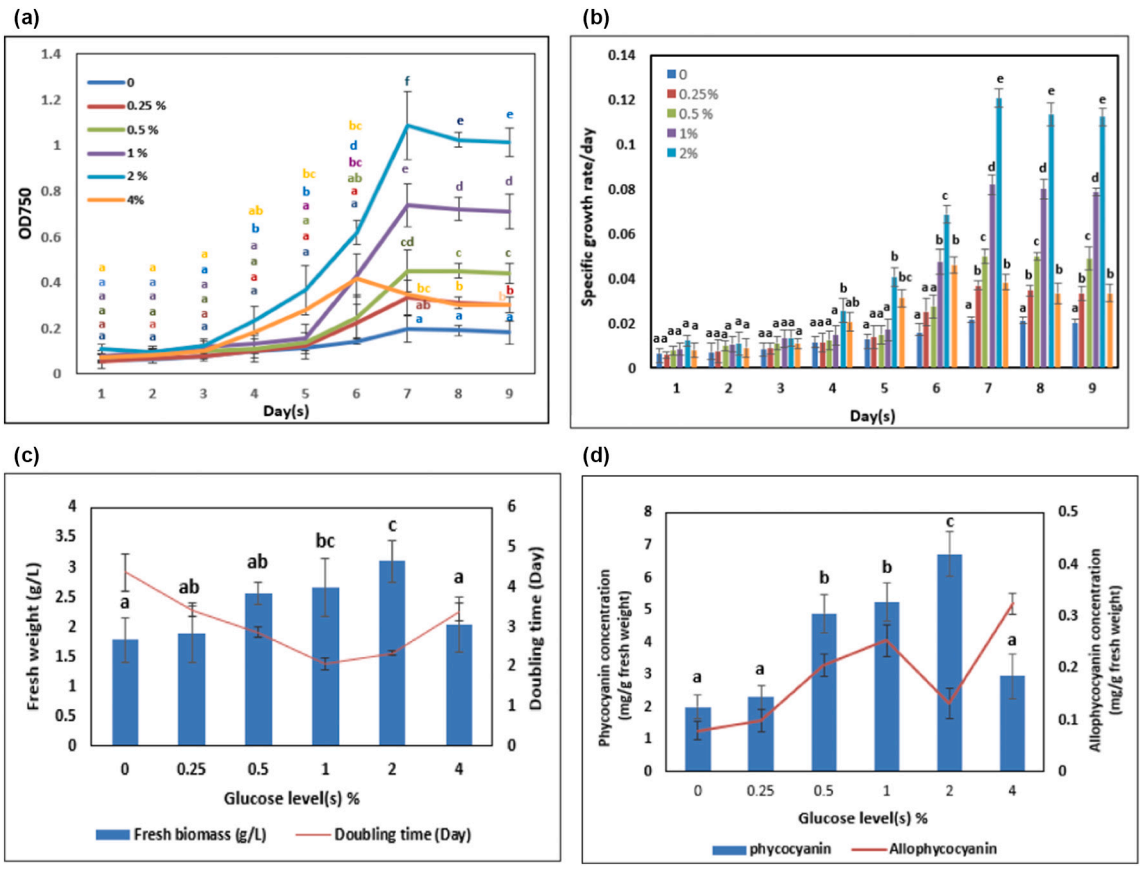

Fig. 1. (a–d) are describing the growth parameter of the *Aphanothece* sp. growth under photoautotrophic and photoheterotrophic conditions using glucose as an exogenous organic carbon. (a) An OD $_{750}$ was used to analysis the growth of *Aphanothece*, the highest OD_{750} of 1.08 ± 0.14 at 7th day was recorded in 2 % glucose (3.11 ± 0.35 g/L fresh weight) while the lowest was recorded from photoautotrophic condition (1.78 ± 0.67 g/L fresh weight). (b) The highest specific growth rate per day (0.120 ± 0.2 day) was recorded from 2 % glucose at the day 7th. (c) The lowest doubling growth time was recorded on 1 and 2 % sucrose respectively. (d) The highest phycocyanin production was recorded on 2 % glucose, while the highest Allophycocyanin was recorded from 4 % glucose. Data is presented based on the Mean ± SD (n = 4), bars labeled with different letters are significantly different from each other at $p < 0.05$.

organic carbon is mainly up-taken by the Pentose Phosphate and Embden-Meyerhof-Parnas Pathways, while the inorganic carbon sources are primarily up taken by CO2-concentrating mechanisms [10–14]. In this regard, study on the usages of different exogenous organic carbon source on the batch cultivation of the *Phormidium* sp. revealed the maltodextrin in comparison to fructose and mannose has the potential to boost the biomass production up to 1.07 g biomass/L/day, while the mannose was the carbon source with the uppermost substrate consumption rate (3.18 g/L/day) [13]. In another study by Schwarz et al. [14] the *Trichocoleus sociatus* medium supplemented with raffinose (2.53 g/L) resulted in production of 3.77 g/ L dry biomass, whereas glucose was the best option for the *Nostoc muscorum* leading to a biomass production of 2.46 g / L.

In general, the cyanobacterial species are considered autotrophic, whereas if supported by the cyanobacterial metabolism, the photoheterotrophic and heterotrophic cultivation could result in a higher biomass and yield of the production system [12]. Optimization of culture media that include various levels of organic carbons have been extensively analyzed for cyanobacterial cultivation, however the feasibility of the using different carbon sources is considerably related to the cost of the carbon sources utilized in the cyanobacterial production system to activate the photoheterotrophic or heterotrophic. In this regard, the glucose, sucrose, glycerol, and sodium acetate are considered as the cost-effective exogenous carbon source to be used for activating the photoheterotrophic and heterotrophic growth of algal production system, however, to our knowledge, no published study has used glucose, sucrose, glycerol, and sodium acetate as exogenous carbon source to address the low biomass of *Aphanothece* sp. The objective of this study was to determine the effects of different exogenous carbon sources on photoheterotrophic growth of *Aphanothece* sp., the effect of flashing frequency on the biomass yield, production of C-PC and APC by this species, and lastly the economic feasibility of this production system.

2. Materials and methods

2.1. Microorganism cultivation and maintenance

The cyanobacteria *Aphanothece* sp. (strain SP25) was obtained from UTEX (UTEX, Austin, TX, USA). The stock cultures were propagated and maintained at 20 ± 1 °C with continuous light at 80 μmol photons m^{-2} s^{-1} on the BG- 11 media with a composition as follow, NaNO3 (1.5 g /L), K_2HPO_4 (40 mg /L), $CaCl_2{\cdot}2\,H_2O$ (36 mg/ L), $MgSO_4{\cdot}7\,H_2O$ (75 mg/ L), Citric Acid·H_2O (6 mg /L), $C_6H_8FeNO_7$ (6 mg /L), $Na_2EDTA{\cdot}2\,H_2O$ (1 mg /L), Na_2CO_3 (20 mg /L), H_3BO_3 (2.86 mg /L), $MnCl_2{\cdot}4\,H_2O$ (1.81 mg /L), $ZnSO_4{\cdot}7\,H_2O$ (0.22 mg/ L), $Na_2MoO_4{\cdot}2\,H_2O$ (0.39 mg/ L), $CuSO_4{\cdot}5\,H_2O$ (0.079 mg/ L) and $Co(NO_3)_2{\cdot}6\,H_2O$ (0.04 mg/ L).

To evaluate the influence of exogenous organic carbon on the photoheterotrophic growth and C-PC and APC productions by *Aphanothece* sp., different concentration of glucose (0, 1, 2, 4, 8, and 12 %), sucrose (0, 0.25, 0.5, 1, 2, and 4 %), glycerol (0, 0.25, 0.5, 1, 2, and 3 %), and sodium acetate (0, 0.25, 0.5, 1, 2, and 3 %) were added to the BG11 medium (pH = 7.5). These concentrations of the exogenous carbon sources were determined based on our pervious unpublished studies. A

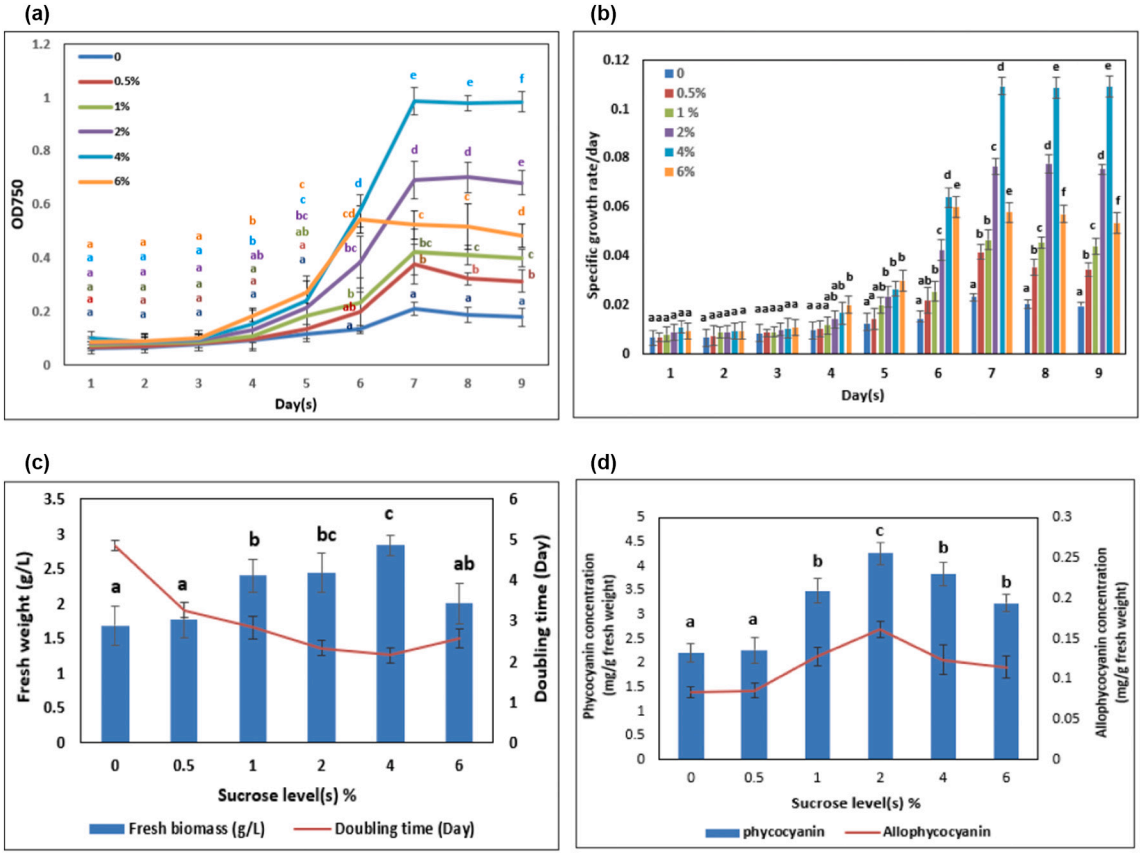

Fig. 2. (a–d) are describing the growth parameter of the *Aphanothece* sp. growth under photoautotrophic and photoheterotrophic conditions using sucrose as an exogenous organic carbon. Using 4 % of sucrose increased the biomass to 2.83 ± 0.27 g/L (68.45 % increase in comparison to photoautotrophic condition) with the doubling time of 2.15 ± 0.18 day, and the highest specific growth rate of 0.1 ± 0.003 day (at the 7th day). At 2 % sucrose 4.26 ± 0.25 mg/g fresh weight for C-PC and 0.162 ± 0.03 mg/g fresh weight for allophycocyanin APC were obtained. Data is presented based on the Mean ± SD (n = 4), bars labeled with different letters are significantly different from each other at $p < 0.05$.

BG11 media without carbon source was used for photoautotrophic growth of *Aphanothece* sp.

2.2. Biomass production studies

Cell growth was determined using a method described by Incharoensakdi and Karnchanatat [15] by measuring the optical density (OD) of the cell cultures at 750 nm using a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). Cell growth was measured daily in triplicate. The total fresh weight was measured at the 3th day passed from the stationary phase. The specific growth rate of the *Aphanothece* sp. was calculated using the equation

$\mu = \ln(N_2 / N_1) / (t_2 - t_1)$, where μ is the specific growth rate, and N_1 and N_2 are the biomass at time 1 (t_1) and time 2 (t_2), respectively. Doubling times of the *Aphanothece* sp. growth were calculated from the following formula:

Doubling time $G = \ln 2 / \mu$, where μ is the specific growth rate of the *Aphanothece* sp.

2.3. Phycobiliprotein quantification

The biomass obtained at the stationary phase was extracted with sodium phosphate buffer 0.2 M (pH = 6.85) followed by cycles of freezing and thawing at 24 h intervals. The phycobiliprotein quantification was calculated as described by Bennett and Bogorad [16] by measuring against sodium phosphate buffer blanks at 615 and 652 nm using the following formulas:

C-PC = (A615 − 0.474 × A652) / 5.34; APC = (A652 − 0.208 × A615) / 5.09, and the data was expressed as mg/mL.

2.4. Flashing frequency treatment

A UTEX RGB-LED Lighting Platform was used to evaluate, whether the production of C-PC and APC by *Aphanothece* sp. can be improved by flashing frequencies at the stationary growth phase. In this regard, a flashing frequency of 50 Hz, Duty cycle = 0.5, 80 μmol photons m^{-2} s^{-1} was selected based on our previous unpublished studies. The *Aphanothece* sp. cultivated in BG- 11 media - supplemented with different organic carbon sources were treated by the flashing frequency at the initial stationary growth phase (7th day) of *Aphanothece* for three days. Subsequently, the samples were analyzed for further biomass production and C-PC and APC content.

2.5. Cost analysis calculation

The price of each organic carbon source used in this study was obtained from Sigma Aldrich, USA. The economic feasibility for the usage of applied exogenous organic carbon sources was calculated according to the following formula, introduced by Park and colleagues [17].

$$\text{Cost (USD)} : (\text{g/biomass}) = ((A \times B) + C)/D$$

$$\text{Cost (USD)} : (\text{g/C} - \text{PC})\ ((A \times B) + C)/E$$

$$\text{Cost (USD)} : (\text{g/APC})\ ((A \times B) + C)/E$$

where, A is the quantity of each exogenous organic carbon sources

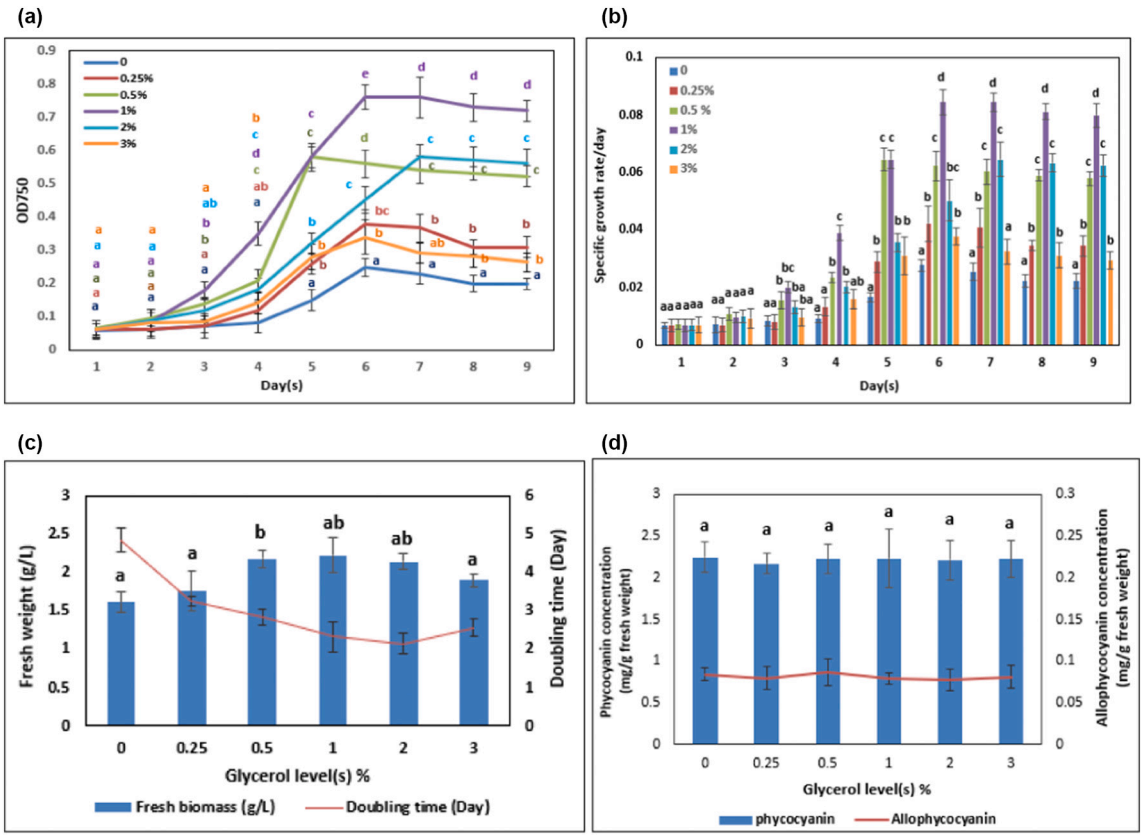

Fig. 3. (a–d) are describing the growth parameter of the *Aphanothece* sp. growth under photoautotrophic and Photoheterotrophic conditions using glycerol as an exogenous organic carbon. Note addition of glycerol at 1 % increased the biomass to 2.25 ± 0.22 g/L (doubling time: 2.32 ± 0.03 day; specific growth rate = 0.08 ± 0.002 day), however, increasing the glycerol from 1 % to 3 % decreased the biomass production by 16 % (Fresh weight:1.89 ± 0.3 g/L; doubling time: 2.56 ± 0.03 day). The production of C-PC and APC remained stable at 2.2 ± 0.02 mg/g fresh weight, and 0.08 ± 0.01 mg/g fresh weight, respectively, at all the applied concentrations of glycerol. Data is presented based on the Mean ± SD (n = 4), bars labeled with different letters are significantly different from each other at $p < 0.05$.

(glucose, sucrose, glycerol, and sodium acetate), B is the price of the organic carbon (USD/ mg), C is the price of the medium (USD/L), D is the produced biomass (g/L), C-PC is the price of C-phycocyanin (g/g Fresh weight), and APC is the price of allophycocyanin (g/g Fresh weight).

2.6. Experimental replication and statistics

The experiment was set up based on an entirely randomized design test, and the data obtained were expressed as the average of three replicates. Statistical analysis was made using IBM SPSS Statistics 23 software., and a Tukey HSD test was performed to get the homogeneous subsets with a significance of 0.05 p-value. The graphs and figures were made using a Microsoft Excel software.

3. Results and discussions

3.1. Glucose as an ideal organic carbon source for Aphanothece sp. photoheterotrophic growth

The influence of different exogenous types of organic carbons on growth, biomass and C-PC and APC content of *Aphanothece* sp. was studied after nine days of cultivation (Inoculum OD_{750} = 0.05) under photoheterotrophic conditions (Figs. 1–4). A lag phase within four days of cultivation followed by a logarithmic growth phase was observed for *Aphanothece* sp. under photoautotrophic condition, and the stationary growth phase of *Aphanothece* sp. was recorded at the day 7th (OD_{750} = 0.19 ± 0.02, Fresh weigh = 1.78 ± 0.67 g/L, Doubling time: 4.38 ± 0.46 day). Under the photoheterotrophic condition, different exogenous organic carbon sources and their concentrations affected the lag phase of *Aphanothece* sp. growth. Among the selected organic carbon sources 1 % glucose followed by 4 % sucrose and 1 % glycerol had a significant positive influence ($p < 0.05$) on the *Aphanothece* sp. growth, while sodium acetate didn't have any significant stimulus at any of its applied concentrations on the *Aphanothece* sp. growth. The highest significant increase ($p < 0.05$) up to 74.7 % in biomass production (Fresh weight: 3.11 ± 0.77; highest specific growth rate: 0.12 ± 0.004 at the day 7th; Doubling time: 2.07 ± 0.04 day) was achieved at photoheterotrophic condition using 2 % glucose. An inhibitory effect on the *Aphanothece* sp. growth was observed by increasing the concentration of glucose from 2 % to 4 % (34.7 % decrease) (Fig. 1a–c). Using 4 % of sucrose significantly increased ($p < 0.05$) the biomass to 2.83 ± 0.27 g/L (68.45 % increase in comparison to photoautotrophic condition) with the doubling time of 2.15 ± 0.18 day, and the highest specific growth rate of 0.1 ± 0.003 day (at the 7th day). Increasing sucrose to 6 % in BG-11 media decreased the biomass to 1.9 ± 0.28 g/L (doubling time = 2.56 ± 0.22 day; specific growth rate = 0.05 ± 0.003 day 7th) (Fig. 2(a–c)).

The addition of glycerol to the BG-11 media had a stimulatory effect on the biomass production of *Aphanothece* sp. No significant differences ($p > 0.05$) were observed on the biomass production by using higher than 0.5 % glycerol, and raising the glycerol concentration to 3 % had decreased the biomass production by 16 % (Fresh weight:1.89 ± 0.3 g/L; doubling time: 2.56 ± 0.03 day). However, using 1 % glycerol biomass increased to 2.25 ± 0.22 g/L (doubling time: 2.32 ± 0.03 day; specific growth rate = 0.08 ± 0.002 day).

In our study, we found sodium acetate is not an ideal exogenous

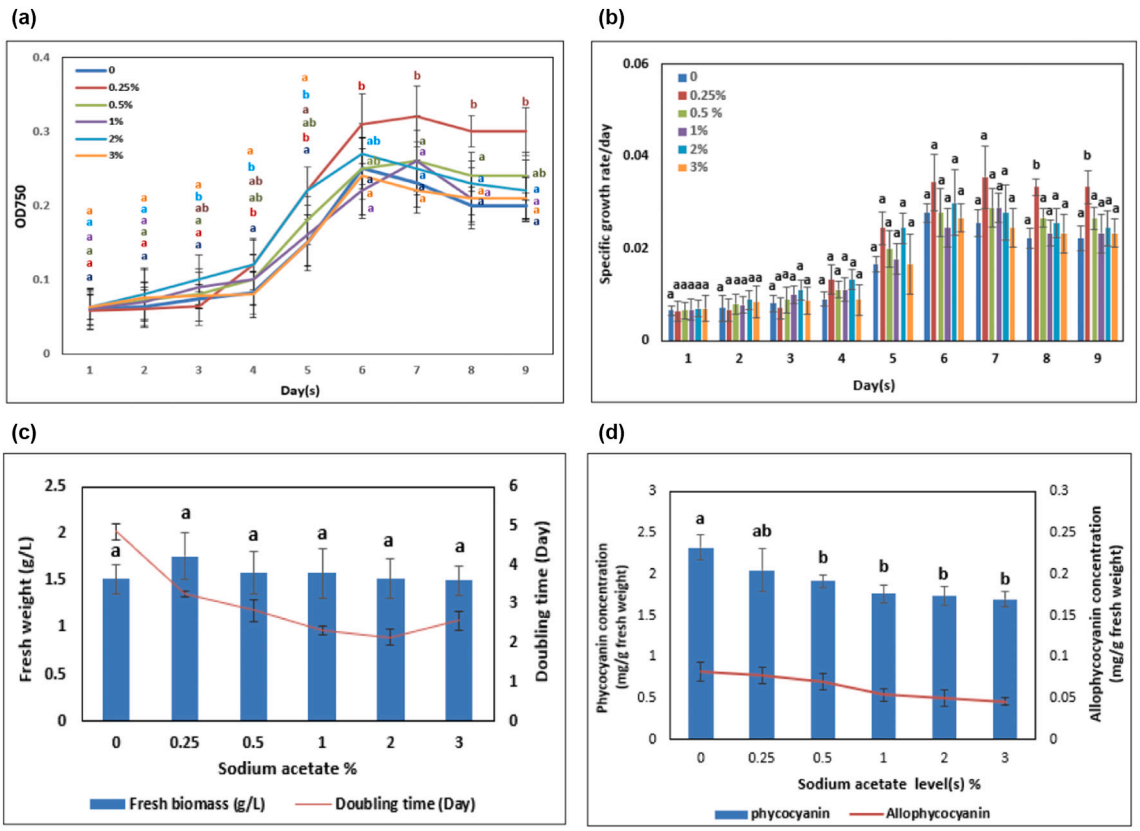

Fig. 4. (a–d) are describing the growth parameter of the *Aphanothece* sp. growth under photoautotrophic and Photoheterotrophic conditions using sodium acetate as an exogenous organic carbon. Sodium acetate didn't have any significant stimulus at any of its applied concentrations on the *Aphanothece* sp. growth. Sodium acetate negatively impacted on the production of the C-PC and APC, as by increasing the concentration to 3 % the C-PC and allophycocyanin APC contents decreased to 1.7 ± 0.09 mg/g fresh weight, and 0.046 ± 0.02 mg/g fresh weight, respectively (27.15 % of decrease to the photoautotrophic condition). Data is presented based on the Mean ± SD (n = 4), bars labeled with different letters are significantly different from each other at $p < 0.05$.

Table 1
Chlorophyll *a* content (μg/g fresh weight) under photoautotrophic, and photoheterotrophic growth of *Aphanothece* sp.

Treatments	A	B	C	D	E	F
Glucose	36 ± 2.6^{a}	37.3 ± 3.05^{a}	58.3 ± 3.5^{b}	61.3 ± 3^{bc}	66.3 ± 3.2^{c}	35.3 ± 4.1^{a}
Sucrose	34 ± 1.5^{a}	33 ± 3.05^{a}	54.6 ± 3.5^{b}	62 ± 2.5^{c}	52.6 ± 1.5^{b}	51.33 ± 3.5^{b}
Glycerol	36.3 ± 2.4^{a}	34.26 ± 2.3^{a}	34.5 ± 2.8^{a}	33.6 ± 3.4^{a}	35 ± 3.2^{a}	34.6 ± 3.2^{a}
Sodium acetate	35 ± 1.2^{a}	33.6 ± 1.5^{a}	32.3 ± 2.3^{a}	25.6 ± 2.5^{b}	25.3 ± 1.6^{b}	25.6 ± 3.2^{b}

Note: A = 0 % organic carbon source, and the A letter is demonstrating the photoautotrophic condition. The B, C and D letters are demonstrating the applied concentration for each treatment for the photoheterotrophic growth of *Aphanothece* as follow, Glucose: B = 0.25 %, C = 0.5 %, D = 1 %, E = 2 %, F = 4 %; Sucrose: B = 0.5 %, C = 1 %, D = 2 %, E = 4 %, F = 6 %; Glycerol; Sodium acetate: B = 0.25 %, C = 0.5 %, D = 1 %, E = 2 %, F = 3 %. Data is presented based on the Mean ± SD (n = 4). Data labeled with different letters are significantly different from each at the same class of treatment at $p < 0.05$.

carbon source for the photoheterotrophic culture of *Aphanothece* sp., since no significant differences ($p > 0.05$) in biomass production were observed among the applied concentrations (0.25 %–3 %) and the photoautotrophic growth of *Aphanothece* sp. (Fresh weight: 1.51 ± 0.15 g/L; doubling time 4.84 ± 0.2 day) (Fig. 4a–c).

Photoheterotrophic cultivation is a system that bonds the heterotrophic and autotrophic advantages while diminishing the disadvantages from both cultivation systems. During the photoheterotrophic cultivation, the microalgae use the light, CO_2, and organic carbon sources for the growth. The variability in growth among different microalgal species during photoheterotrophic cultivation has been widely reported, and the most exogenous carbon sources used have been glucose and acetate [18]. The study of *Galdieria sulphuraria* (an acidophilic microalga) in photoautotrophic and photoheterotrophic conditions concluded that, the biomass productivity is 1.8 times higher under the photoheterotrophic cultivation [19]. In another comparative cultivation study using *N. flagelliforme*, the cell density under photoheterotrophic cultivation obtained 4.98 times higher of that of photoautotrophic and 2.28 times of that of heterotrophic growth [20]. Menegol et al. [21] have reported the photoheterotrophic cultivation of *Nannochloropsis gaditana*. The alga produced 30 % more biomass under photoheterotrophic condition using glucose (5 g /L) and glycerol (1 g/L), but not acetate. The CO_2-refixation through the aerobic respiration in the presence of glucose during the photosynthesis reported to be critical for biomass production under photoheterotrophic cultivation [22]. The sucrose reported to be efficient during photoheterotrophic cultivation of *Chlorococcum* sp. and the biomass reached to 3.5 g/L. Sucrose can be easily transform to glucose and fructose molecules, thus, microalgae can uptake sucrose [23]. During the photoheterotrophic metabolism, glucose converts to glucose 6- phosphate and later to pyruvate through the glycolysis, and it enters the TCA cycle followed by mitochondrial oxidative phosphorylation for ATP production [24]. Cell density and biomass are the major factor for the microalgal large-scale productions and are significantly link to the cost of microalgae utilization, in this regard; the biomass production can increase under photoheterotrophic cultivation. Organic carbon sources are broadly used for microalgae cultivation, nevertheless the usages of organic carbon sources can be

Table 2
The effect of the flashing frequency on the C-phycocyanin and allophycocyanin production (mg/g fresh weight) by *Aphanothece* sp.

Treatments		A	B	C	D	E	F
	C-PC	3.82 ± 0.33^{a}	–	–	–	–	–
	APC	0.12 ± 0.08^{A}	–	–	–	–	–
Glucose	C-PC	–	4.26 ± 0.28^{a}	5.73 ± 0.1^{b}	6.1 ± 0.26^{b}	7.9 ± 0.36^{c}	4.57 ± 0.11^{a}
	APC	–	0.15 ± 0.02^{A}	0.45 ± 0.07^{B}	0.56 ± 0.06^{C}	0.64 ± 0.05^{D}	0.46 ± 0.05^{B}
Sucrose	C-PC	–	3.25 ± 0.15^{a}	4.18 ± 0.14^{b}	5.1 ± 0.25^{c}	4.8 ± 0.22^{cd}	4.56 ± 0.17^{d}
	APC	–	0.14 ± 0.21^{A}	0.16 ± 0.31^{B}	0.27 ± 0.02^{B}	0.24 ± 0.02^{BC}	0.21 ± 0.017^{C}
Glycerol	C-PC	–	3.12 ± 0.07^{a}	3.52 ± 0.06^{b}	3.53 ± 0.61^{b}	3.54 ± 0.14^{b}	3.24 ± 0.28^{a}
	APC	–	0.14 ± 0.02^{A}	0.14 ± 0.01^{A}	0.15 ± 0.02^{A}	0.14 ± 0.15^{A}	0.14 ± 0.02 ^{A}
Sodium acetate	C-PC	–	2.26 ± 0.15^{a}	2.33 ± 0.3^{a}	2.3 ± 0.15^{a}	2.4 ± 0.2^{a}	2.38 ± 0.15^{a}
	APC	–	0.14 ± 0.02^{A}	0.1 ± 0.01^{A}	0.07 ± 0.15^{AB}	0.06 ± 0.01^{B}	0.05 ± 0.1^{AB}

Note: The A = 0 % organic carbon source, and A letter is demonstrating the photoautotrophic condition. The B, C and D letters are demonstrating the applied concentration for each treatment for the photoheterotrophic growth of *Aphanothece* as follow, Glucose: B = 0.25 %, C = 0.5 %, D = 1 %, E = 2 %, F = 4 %; Sucrose: B = 0.5 %, C = 1 %, D = 2 %, E = 4 %, F = 6 %; Glycerol; Sodium acetate: B = 0.25 %, C = 0.5 %, D = 1 %, E = 2 %, F = 3 %. The C-PC and APC productions were measured at the day 9th at 3 days passed from the initial stationary growth phase of *Aphanothece* sp. Data is presented based on the Mean ± SD ($n = 4$). Data labeled with different letters are significantly different from each other at the same class of treatment at $p < 0.05$.

challenging, as not all the microalgal strains have the TCA (tricarboxylic acid cycle) or the Krebs cycle – enzymes to use the organic carbon sources (i.e., glucose, acetate, and glycerol) [25].

3.2. Stimulatory influence of carbon sources on the chlorophyll a and phycobiliprotein contents

The selected exogenous organic carbon sources had effectiveness on the chlorophyll *a* content in *Aphanothece* sp. among them the 2 % glucose, and 2 % sucrose significantly improved ($p < 0.05$) the chlorophyll *a* content up to 66.3 ± 3.2 μg/g fresh weight and 62 ± 2.5 μg/g fresh weight, respectively, which was 83.3 % and 72.2 % of increase to the photoautotrophic condition (Chlorophyll *a* = 36 ± 2.6 μg/g fresh weight). In contrast, glycerol and in particular sodium acetate had a negative influence on the chlorophyll *a* content in *Aphanothece* sp. and the chlorophyll *a* content decreased to 25.6 ± 3.2 by increasing the concentrations of the sodium acetate to 3 % (Table 1.).

We observed a positive concentration - dependent stimulatory effect on the production of C-PC and APC by *Aphanothece* sp. under the photoheterotrophic growth of *Aphanothece* sp. using glucose and sucrose, and by increasing the glucose concentration to 2 % the C-PC significantly ($p < 0.05$) increased to 6.72 ± 0.13 mg/g fresh weight (APC = 0.13 ± 0.01 mg/g fresh weight) demonstrating 236 % of increase to the photoautotrophic condition. This data was recorded 4.26 ± 0.25 mg/g fresh weight for C-PC and 0.162 ± 0.03 mg/g fresh weight for APC using sucrose at 2 %. The production of C-PC and APC remained stable at 2.2 ± 0.02 mg/g fresh weight, and 0.08 ± 0.01 mg/g fresh weight, respectively, at all the applied concentrations of glycerol, signifying no difference ($p > 0.05$) to the photoautotrophic growth condition of *Aphanothece* sp. In our study we observed that, the sodium acetate had a negative impact on the production of the C-PC and APC, as by increasing the concentration to 3 % the C-PC and APC contents decreased to 1.7 ± 0.09 mg/g fresh weight, and 0.046 ± 0.02 mg/g fresh weight, respectively (27.15 % of decrease to the photoautotrophic condition). Previous studies also show that, the positive effectiveness of glucose as an organic carbon source during photoheterotrophic cultivation of *Chlorella pyrenoidosa* on the chlorophyll *a* concentration and cell density [25]. Photoheterotrophic culture of *Anabaena* sp. (PCC 7120) resulted in an increased energy distribution in PSII system, and higher concentration of biomass and chlorophyll *a* content in comparison to autotrophic condition [26]. It is reported that, the increase in *Chlorophyll a* synthesis could be associated with the cells need to increase the light energy capture because of high cellular density and limitation of light for photosynthesis because of cell-shading effects [27].

Chlamydomonas reinhardtii as an algal study-model supports the photoautotrophic, heterotrophic, and photoheterotrophic growth. During the photoheterotrophic growth of *C. reinhardtii* using acetate the photosynthetic CO_2 fixation and the net O_2 evolution were reduced [28]. In photoheterotrophic cultivation of *Platymonas subcordiformis*, acetate had a negative influence on the chlorophyll *a* and *b* content [29]. Previous studies show, acetate may have negative influence on the photosynthesis and uptake of the inorganic carbons, and potentially can down-regulate the PSII system through quenching of Chl fluorescence and inhibit the carbon fixation by reducing the synthesis of Ribulose-1, 5-bisphosphate carboxylase-oxygenase (RuBPCase) [30–32].

3.3. Flashing frequency increases the C-PC and APC productions by Aphanothece sp.

In most of microalgae production systems, as soon as the cells reached to the highest concentration level, the ratio of light to dark zones reaches to the lowest level, this triggers an increase in cells respiration metabolism, and ultimately leads to biomass reduction. A promising approach for increasing the efficiency of light-based

Table 3
The economic feasibility analysis of the used exogenous organic carbon for the *Aphanothece* sp. production system.

Carbon source	Additive (g/L)	Price (USD)			Maximum dry weight (g/L)	Cost (USD/ g biomass)
		Carbon source (USD/g)	BG11 (USD/L)	Total (USD/L)		
Control	–	–	45	45	1.78 ± 0.67^{a}	25.28
Glucose	200	4.24	45	49.24	3.1 ± 0.35^{b}	15.88
Sucrose	400	12.68	45	57.68	2.83 ± 0.27^{b}	20.38
Glycerol	100	10.5	45	55.5	2.25 ± 0.22^{a}	25

Note: Control (The photoautotrophic growth condition); Glucose, Sucrose, and glycerol (The photoheterotrophic growth condition). The cost was analyzed by obtaining the price of the supplemented chemicals from the Sigma Aldrich, USA, 2022. Data is presented based on the Mean ± SD (n = 4). Data labeled with different letters are significantly different from each other at the same class of treatment at $p < 0.05$.

Table 4
The economic feasibility analysis of the used exogenous organic carbon to produce C-phycocyanin and allophycocyanin from *Aphanothece* sp.

Carbon source	Concentration (g/L)	Maximum Biomass (g/L)	Maximum C-PC production (mg/g fresh weight)	Maximum APC production (mg/g fresh weight)	Media Cost (USD/L)	Cost C-PC (USD/mg)	Cost APC (USD/μg)
0	0	1.78 ± 0.67^{a}	3.82 ± 0.33^{a}	0.12 ± 0.08^{a}	45	11.78	37.5
Glucose	200	3.1 ± 0.35^{b}	7.9 ± 0.36^{b}	0.64 ± 0.05^{b}	49.24	6.23	7.7
Sucrose	400	2.83 ± 0.27^{b}	4.8 ± 0.22^{c}	0.24 ± 0.02^{c}	57.68	12.01	24.03
Glycerol	100	2.25 ± 0.22^{a}	3.53 ± 0.61^{a}	0.15 ± 0.02^{a}	55.5	15.58	37

Note: The maximum C-PC and APC in this table is presented after flashing frequencies (50 Hz, Duty cycle = 0.5, 80 μmol photons m^{-2} s^{-1}) treatment. The cost was analyzed by obtaining the price of the supplemented chemicals from the Sigma Aldrich, USA, 2022. Data is presented based on the Mean ± SD (n = 4). Data labeled with different letters are significantly different from each other at the same class of treatment at $p < 0.05$.

microalgae production to deliver light deep inside the culture is through flashing frequencies (high intensity light flashes) [33–35]]. At the next stage of our study, we hypothesized if the flashing frequencies (50 Hz, Duty cycle = 0.5, 80 μmol photons m^{-2} s^{-1}) can increase the production of C-PC and APC at the stationary phase of *Aphanothece* sp. growth (day 7th). Our results showed, using flashing frequencies at the stationary growth phase of *Aphanothece* sp. (photoautotrophic) significantly increases ($p < 0.05$) the concentration of C-PC and APC by 64.51 % and 46.34 %, respectively. A significant positive influence ($p < 0.05$) of flashing frequency on the production of C-PC and APC from photoheterotrophic culture of *Aphanothece* sp. was observed, and the highest production of C-PC (7.9 ± 0.36 mg/g fresh weight) and APC (0.74 ± 0.05 mg/g fresh weight) were recorded from 2 % glucose, demonstrating 17.55 % and 433.3 % of increase for APC and C-PC (Table 2.). We also observed, the flashing frequency (50 Hz, 80 μmol photons m^{-2} s^{-1}) had no significant positive influence ($p < 0.05$) on the *Aphanothece* sp. C-PC and APC production using different concentrations of glycerol and sodium acetate.

The flashing frequencies have been effective in production of high value metabolites (i.e. polyunsaturated fatty acids, chlorophyll, lutein, *β*-carotene, neoxanthin, and violaxanthin) in microalgae *Tetraselmis chui*, *Koliella Antarctica*, and *Nannochloropsis gaditana.* A three-fold higher level of eicosapentaenoic acid (EPA) was obtained from flashing frequencies compared to continuous light treatment [34]. The usage of flashing frequencies also reported as an efficient strategy to decrease the energy consumption in microalgae production system. For example, in *Haematococcus pluvialis* the astaxanthin and biomass production were significantly higher under flashing frequencies (25–200 Hz, Duty cycle = 0.17, 0.33, 0.67), and flashing frequencies reduced the energy consumption for astaxanthin production by up to 70 % [36].

3.4. Economic feasibility analysis of applied carbon sources

Photoheterotrophic cultivation is a metabolic approach that an organism uses the photoautotrophic and heterotrophic metabolism simultaneously. The main benefits of photoheterotrophic cultivation of microalgae include higher growth and biomass productivity, prolonged exponential phase, and possibility of manipulation of algal- derived metabolites [36].

Economic analysis is a necessary step for evaluation the feasibility and sustainability of algae-based production system. The economic analysis shown in Tables 3 and 4. explains the supplementation of media with the exogenous organic carbon source, including glucose and sucrose is economically feasible due to higher biomass generated by photoheterotrophic growth of *Aphanothece* sp. in compared to the photoautotrophic condition. These organic carbon sources can increase biomass by 74.15 % and 59 % for glucose and sucrose, respectively. These results are interesting when compared to the cost of additional media for production of more biomass, as supplementation of the media with 2 % glucose can decrease the production cost of the system (g/L) by 62.81 %. Moreover, the economic analysis shown in Table 4. clearly explains that, among the studied exogenous Carbon sources, supplementation of the media with glucose at the concentration of 200 g/L has reduced the cost of the C-PC, and APC production by 47 % and 80 %, respectively. Photoheterotrophic cultivation has been utilized as an alternative approach for large-scale algal biomass production system since more reliable biomass productivity is achievable compared to photoautotrophic growth because of constant accessibility of the energy source in the form of organic carbon. While further costs would arise for the carbon sources, since the carbon cost contributes nearly 80 % of the medium cost. In this light, many researchers have been studying the using of the waste/crude organic carbon sources for reducing the overall cost of the algal cultivation systems. In our paper, glycerol had less usages feasibility due to higher cost and lower biomass production in our production system, nevertheless the waste/crude glycerol can be alternatively used to reduce the cost of the production system because of its low price (~0.5$ per 400 g).

4. Conclusion

At the time of writing, the microalgal production system still is facing challenges on the economical production feasibility, because of low yield of biomass. In this study, we proposed using glucose or sucrose as the exogenous organic carbon sources in *Aphanothece* sp. to increase the biomass and the C-PC and APC productions due the photoheterotrophic growth. Based on our findings, supplementation of the BG-11 media with 2 % glucose or 4 % sucrose to activate the photoheterotrophic growth of *Aphanothece* sp. can increase biomass production by 74.15 % and 59 %, respectively. We observed a positive concentration-dependent stimulatory effect on the production of C-PC and APC by *Aphanothece* sp. under the photoheterotrophic conditions of *Aphanothece* sp. using glucose and sucrose, and by increasing the glucose concentration to 2 % the C-PC increased to 6.72 ± 0.13 mg/g fresh weight (APC = 0.13 ± 0.01 mg/g fresh weight) demonstrating 236 % of increase to the photoautotrophic condition. This strategy might be considered as an opportunity for the simultaneous production of high-value compounds like C-PC and APC and the biomass for biofuel production. The economic analysis showed that the activation of photoheterotrophic growth of *Aphanothece* sp. using 2 % glucose or 4 % sucrose as the exogenous organic carbon source offers a rational approach to improve the biomass, and C-PC and APC due to their positive cost-benefit based on their effects on biomass and C-PC and APC production.

CRediT authorship contribution statement

A.P designed and carried out the experiments, analyzed the data, and wrote the manuscript. I·I-A and M.L-K·D helped in maintaining and culturing the *Aphanothece* sp., and data collection. B·H and G.Z. revised and edited the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Further Reading

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