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by Muhamad Maulana Azimatun Nur

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Enhancement of C-phycocyanin productivity by *Arthrospira platensis* when growing on palm oil mill effluent in a two-stage semi-continuous cultivation mode

M. M. Azimatun Nur[1,2] · G. M. Garcia[2] · P. Boelen[2] · A. G. J. Buma[2]

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Abstract

Palm oil mill effluent (POME) is well known as agricultural wastewater that has a high potential as a medium for microalgal growth due to its high macro- and micronutrient content. The cyanobacterium *Arthrospira platensis* is considered as a species with a high C-phycocyanin (C-PC) content which is important for fine chemical and pharmaceutical applications. However, cultivation of *A. platensis* on POME to produce economically feasible amounts of C-PC has not been well explored. For this, environmental, nutritional, and cultivation modes (batch, semi-continuous) were varied to optimize C-PC productivity when cultivated at various POME concentrations. *Arthrospira platensis* was found to grow well on POME. Highest biomass and C-PC concentrations were found on 30–100% POME. Central composite rotatable design (CCRD) response surface methodology demonstrated that C-PC productivity was influenced by urea addition at the optimum salinity. The highest C-PC productivity was found on 100% POME during semi-continuous cultivation, while the addition of phosphorus and urea did not significantly improve C-PC productivity. By applying semi-continuous cultivation with 50% POME at the first stage and 100% POME at the second stage, a similarly high C-PC productivity (4.08 ± 1.3 mg L^{-1} day^{-1}) was achieved as compared with (artificial) Zarrouk medium during batch cultivation. We conclude that, when using a two-stage semi-continuous cultivation process, *A. platensis* can produce economically feasible amounts of C-PC when cultivated on 100% POME.

Keywords Wastewater · Light intensity · Urea · C-PC · *Arthrospira platensis*, semi-continuous

Introduction

Cyanobacteria, including *Arthrospira platensis*, have an enormous commercial interest due to their high protein content (up to 70%), essential amino acids, fatty acids (palmitic, linoleic, and oleic acids), and pigments (Abed et al. 2009; de la Jara et al. 2018). Their main pigments are chlorophyll-*a*, phycobiliproteins, and β-carotene (Soni et al. 2017). Phycobiliprotein, mainly consisting of C-phycocyanin (C-PC), is a pigment well known for its antioxidant, anti-inflammatory, and anticarcinogenic functions (Wu et al. 2016; de la Jara et al. 2018). C-PC is one of the major light harvesting cyanobacterial pigments but it functions also as a storage protein in *A. platensis* (Boussiba and Richmond 1980; Hemlata and Fatma 2009). Furthermore, the bulk biomass of *A. platensis* contains high potential sources of various end products such as bioethanol derived from carbohydrates, food, and feed supplements due to its high protein and fatty acids content, apart from cosmetic products such as skin health lotion (Nur and Hadiyanto 2013; Raja et al. 2016). However, large amounts of water are required for cultivation and the high costs of synthetic fertilizers for mass cultivation of the algae are still the main issue (Zhai et al. 2017). Regarding this problem, wastewater which contains high nutrient levels has been proposed as the solution to achieve economically feasible cultivation conditions (Nur et al., 2019a).

One of the promising wastewaters to be used as a growth medium for algae is palm oil mill effluent (POME) which is generated from oil palm factories. POME contains high

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✉ M. M. Azimatun Nur
m.m.azimatun.nur@rug.nl; lanaazim@upnyk.ac.id

[1] Department of Chemical Engineering, Faculty of Industrial Technology, Universitas Pembangunan Nasional "Veteran", Yogyakarta, Indonesia

[2] Department of Ocean Ecosystems, Energy and Sustainability Research Institute, Faculty of Science and Engineering, University of Groningen, Groningen, The Netherlands

amounts of phosphorus, nitrogen, and micronutrients (Mohd Udaiyappan et al. 2017; Nur and Buma 2018). As reported earlier, POME addition promotes the growth of the fucoxanthin and sulfated exopolysaccharide producing marine diatom *Phaeodactylum tricornutum* (Nur et al. 2019a, b). Other researchers found that about 1% of raw POME supplemented to a commercial medium could promote the growth of *A. platensis* resulting in 12% dw of C-PC by applying fed-batch cultivation (Sukumaran et al. 2014). Nevertheless, this small fraction of raw POME would not be sustainable when used in a large-scale industry since the high cost of the commercial fertilizer used in the cultivation as well as high demand of clean freshwater would compete with human consumption. Another study showed that 90% of POME could be used as a growth medium for *A. platensis* using continuous cultivation (Suharyanto et al. 2014). Conversely, continuous cultivation is not easily applicable in large-scale systems since the operation and the maintenance need special equipment and skilled labor, and the cost of the construction is still high (Lehr and Posten 2009; Fernandes et al. 2015). Finally, several studies demonstrated the utilization of POME by using dilution and or synthetic fertilizer supplementation (Sari et al. 2012; Nur et al. 2016). However, these reports were not focused on the optimization of C-PC productivity.

Nutritional and environmental factors such as salinity, irradiance, nitrogen availability, and cultivation mode (batch, semi-continuous, or continuous) regulate pigment productivity (Bezerra et al. 2011; Liu et al. 2016; Ho et al. 2018). The biomass (expressed as dry weight) productivity of *A. platensis* could be enhanced by employing semi-continuous batch cultivation to prevent nutrient limitation and self-shading (Radmann et al. 2007; Moreira et al. 2016). With respect to wastewater utilization, Chaiklahan et al. (2010) reported that cultivation of *A. platensis* on pig wastewater supplemented with bicarbonate and urea by employing a semi-continuous cultivation mode could press the cost of commercial medium up to 4.4 times compared to modified Zarrouk medium. However, based on our knowledge, this cultivation mode has not been tested for *A. platensis* grown on POME medium.

Recently, Benvenuti et al. (2016) showed that the biomass productivity of *Nannochloropsis* sp. could be increased by enriching commercial medium with nitrogen in a semi-continuous cultivation mode. For the present study, it was therefore hypothesized that semi-continuous cultivation could enhance the biomass and C-PC productivity of *A. platensis* cultured on POME medium after optimizing nutrient and other environmental conditions. Semi-continuous cultivation employs two-stage cultivations. In the first stage, microalgae are cultivated in batch mode until they reach ideal growth conditions during the exponential phase. In the second stage, a fraction of the culture is replaced by a new medium at the constant time and volume intervals (Radmann et al. 2007). The objective of this study was to optimize the productivity of C-PC from *A. platensis* cultivated on POME medium by employing semi-continuous cultivation at its optimal nutritional and environmental conditions.

Material and methods

Wastewater preparation

POME was obtained from a small factory in Sumatra, Indonesia, after it had been released from an aerobic open pond lagoon. The wastewater was stored in the freezer (−20 °C) until use, to avoid nutrient degradation over time. Prior to experimental use, POME was thawed and filtered (GF/C glass fiber filter, Whatman) for removal of suspended solids and autoclaved at 121 °C for 15 mins. The wastewater contained 1245 mg L^{-1} COD, 72.4 mg L^{-1} total N, and 7.93 mg L^{-1} PO_4^{3-}P, as estimated previously using appropriate assay kits LCK349 and LCK138 (Hach Lange) (Nur et al. 2019b).

Experimental setup

Arthrospira platensis (SAG 21.99) was obtained from the Algal Culture Collection of the University of Göttingen (Sammlung von Algenkulturen der Universität Göttingen, SAG). Growth and maintenance of the culture were done in Zarrouk medium (Zarrouk 1966), which has a salinity of 12 PSU, in an illuminated U-shaped water basin at 27 °C in a 16:8 h light:dark cycle, at an irradiance of 150 μmol photons m^{-2} s^{-1}. The cultures were acclimated to the experimental conditions for at least 1 week prior to experimentation. In total, five experiments were done using a stepwise approach (Table 1). First, we investigated the effect of irradiance and nitrogen concentration on *A. platensis* biomass and C-PC productivity, grown in standard growth medium (experiment 1). Secondly, *A. platensis* was grown on different dilutions of POME to determine the optimal POME concentration for biomass productivity and C-PC concentration (experiment 2). Environmental and nutritional conditions were further investigated to determine the interactive effects of light intensity, nitrogen, salinity, and POME concentration on C-PC concentration by using full factorial design (experiment 3). As the nitrogen source, we first chose nitrate; however, given the much lower cost as a nitrogen source and the proven capability to support the growth of *A. platensis*, urea was added in the subsequent experiments (Cost et al. 2001). The optimum urea concentration as promising nitrogen source was investigated given the possible toxic effects at higher urea concentrations. Furthermore, salinity was optimized since POME contains a relatively low salinity (experiment 4). Finally, *A. platensis* was cultured in a semi-continuous mode at varying nutrient conditions by adding urea or phosphorus, in order to unravel the

Table 1 Type of experimental setups, factors, and responses. n/a means not applicable

Type of experiment	Factors	Mode of cultivation	Design of experiment	Responses
Experiment 1	Light intensity, nitrate	Batch	n/a	$P_{biomass}$, C-PC, $P_{C\text{-}PC}$
Experiment 2	POME	Batch	n/a	$P_{biomass}$, C-PC
Experiment 3	Light intensity, nitrate, salinity, POME	Batch	Full factorial design	C-PC
Experiment 4	Urea, salinity	Batch	CCRD	Growth rate, $P_{biomass}$, C-PC, $P_{C\text{-}PC}$
Experiment 5	POME, urea, phosphate	Batch, semi-continuous	n/a	$P_{biomass}$, $P_{C\text{-}PC}$

impact of N:P ratio on biomass and C-PC productivity during semi-continuous cultivation (experiment 5). Experimental conditions for each experiment are further described below.

Experiment 1: effect of nitrogen concentration and light intensity on biomass and C-PC productivity (no POME addition)

Cultures of *A. platensis* were grown in triplicate on Zarrouk medium in plastic 40-mL cell culture flasks (Greiner Bio-One, ref. 690160). The flasks were placed in a photosynthetron equipped with a 250-W lamp (MHN-TD power tone, Philips) (Kulk et al. 2011). The cultivation was done at two conditions: (i) Zarrouk medium as a standard medium containing replete nitrate at a concentration of 2.5 g L^{-1} (HN) and (ii) modified Zarrouk medium using 0.1 g L^{-1} nitrate as a source of nitrogen (LN). The photosynthetron allowed for exposure to 10 different light intensities ($I = 8$–800 μmol photons m^{-2} s^{-1}) and was controlled by a water bath (27 ± 0.1 °C). At the end of the exponential phase, samples (4 mL) were taken for immediate biomass measurements using spectrophotometry at 750 nm. Dry biomass was calculated from the optical density as described below. The cultures were harvested for pigment analysis at the end of the exponential growth phase (4–7 days).

Experiment 2: effect of POME on biomass productivity and C-PC concentration

The algae were cultured in 75 mL working volume in 100 mL sterilized Erlenmeyer flasks placed in a U-shaped water bath (Lauda C 6 CS, B03008, Edition 2000 Constant Temp Immersion Heating circulating Water Bath) at 27 °C illuminated by a steady light source (Osram Biolux L 36 W/965) in a 16:8 h light:dark cycle (Van de Poll et al. 2007). Five percent (*v*/*v*) of inoculum was used for the initial cultivation. Different dilutions of POME in ultrapure water provided by a Milli-Q purification system, further referred to as ultrapure water (Milli-Q), were used (5–100% *v*/*v*). Final salinity was set to 4 PSU by using NaCl since natural 100% POME has 4 PSU salinity. Initial pH was set to 9.0 ± 0.2 by using 2 N HCl or 2 N NaOH. Light intensity was set to 200 μmol photons m^{-2} s^{-1} as measured in the center of the culture flask by using a spherical light sensor (Biospherical Instrument QSL2101, USA) which is small enough to be placed inside the culture flasks. At the end of the exponential phase (6 days), the optical density of the cells was measured by spectrophotometry at 750 nm. Samples (1.5 mL) were taken and centrifuged at 10000 rpm for 15 min to separate the algal biomass from the POME medium because of the possible interference of the color of POME in the spectrophotometry measurements. The pellet was washed twice using 0.75% NaCl and resuspended in 1.5 mL ultrapure water (Milli-Q) at the experimental salinity of 4 PSU. Dry biomass was calculated based on the optical density as described below. The cultures were harvested for pigment analysis at the end of the exponential growth phase (6 days).

Experiment 3: effects of nutritional and environmental conditions on the concentration of C-phycocyanin

Arthrospira platensis was cultured in 75 mL working volume in 100-mL sterilized Erlenmeyer flasks in a water bath as described above. Five percent (*v*/*v*) of *A. platensis* culture was used as inoculate to autoclaved and filtered medium. A full factorial design with four variables (irradiance, salinity, nitrate, POME) was performed to reveal the influencing factors and the possible interaction between these factors with respect to C-PC productivity (Table 2). The experiments were carried out at 27 °C; the initial pH was adjusted to 9.0 ± 0.2 by using 2 N HCl or 2 N NaOH. At the end of the exponential growth phase (7–10 days), the cultures were harvested for pigment analysis.

Experiment 4: optimization of C-phycocyanin productivity

Urea is considered a potential source of nitrogen for optimized cultivation of *A. platensis* on POME medium. However, high concentrations of urea are considered toxic for *A. platensis* due to the excess production of ammonium derived from microbial urea conversion. Furthermore, the optimal salinity for C-PC productivity is important to investigate for large-scale applications since it determines the water source (freshwater, seawater) that can be used for the dilution of POME.

Table 2 Experimental factors of full factorial design and their levels to determine significant factors and their interactions on P-biomass (mg L^{-1} day^{-1}) and C-PC concentration (mg L^{-1}). Mean values are based on two replicates ($n = 2$). Standard deviation is shown after ± symbol

Run	Variables				C-PC (mg L^{-1})	
	POME (%)	Salinity (PSU)	Light intensity (μmol photons m^{-2} s^{-1})	Nitrate (mg L^{-1})		
1	50	15	50	0	10.13	±4.80
2	50	15	50	73	13.10	±5.26
3	50	30	50	0	9.13	±0.55
4	50	30	50	73	24.35	±16.73
5	100	15	50	0	15.75	±2.90
6	100	15	50	73	14.41	±1.35
7	100	30	50	0	14.62	±3.17
8	100	30	50	73	11.06	±0.53
9	50	15	200	0	7.04	±0.36
10	50	15	200	73	11.71	±2.21
11	50	30	200	0	5.07	±0.31
12	50	30	200	73	12.87	±8.67
13	100	15	200	0	10.69	±2.05
14	100	15	200	73	8.05	±0.57
15	100	30	200	0	6.74	±0.62
16	100	30	200	73	12.16	±2.50

Therefore, it is important to obtain optimal values of salinity and urea addition, which were all expected to show optimal values with respect to growth, biomass, and C-PC productivity. The optimum growth conditions, significance and the interactive effects of salinity and urea addition on C-PC production, and biomass productivity by *A. platensis* were studied using central composite rotatable design (CCRD) response surface methodology (RSM). To this end, a total of 13 experimental runs were executed (Table 3). The ranges used for these experiments were 36, 150, 425, 700, and 813 mg L^{-1} for urea concentration (x_1), and 5, 10, 22.5, 35, and 40 PSU for salinity (x_2). The empirical form of the second order polynomial model (Eq. 1) can be described as follows:

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where y is the predicted value; β_0, β_i, β_{ii}, and β_{ij} are a constant, linear, quadratic, and the interaction coefficient, respectively; and x_i and x_j are independent variables of the model.

Cultivation was carried out in the same setup as experiment 3. Fifty percent of POME was used and mixed with ultrapure

Table 3 Design of the experiments generated from RSM and the responses. Mean values are based on two replicates ($n = 2$). Standard deviation is shown after ± symbol

Run	Point type	Block	Urea addition (mg L^{-1})	Salinity (PSU)	Growth rate (day^{-1})	$P_{biomass}$ (mg L^{-1} day^{-1})	C-PC (mg L^{-1})	$P_{C\text{-}PC}$ (mg L^{-1} day^{-1})
1	0	1	425	22.5	0.24 ± 0.04	24.79 ± 0.77	19.56 ± 0.18	2.79 ± 0.04
2	-1	1	814	22.5	0.27 ± 0.04	32.41 ± 0.24	22.23 ± 0.09	3.18 ± 0.02
3	1	1	150	10	0.15 ± 0.05	12.46 ± 1.19	14.11 ± 0.37	2.02 ± 0.08
4	-1	1	425	40	0.18 ± 0.04	12.63 ± 0.00	12.31 ± 0.66	1.76 ± 0.13
5	1	1	150	35	0.19 ± 0.03	10.90 ± 3.75	11.42 ± 0.38	1.63 ± 0.08
6	1	1	700	10	0.18 ± 0.07	26.60 ± 0.60	17.20 ± 0.81	2.46 ± 0.16
7	0	1	425	22.5	0.29 ± 0.04	36.66 ± 2.92	21.81 ± 0.74	3.12 ± 0.15
8	0	1	425	22.5	0.23 ± 0.04	33.84 ± 2.14	22.67 ± 0.58	3.24 ± 0.12
9	0	1	425	22.5	0.27 ± 0.05	22.94 ± 0.77	17.18 ± 0.24	2.45 ± 0.05
10	-1	1	36	22.5	0.26 ± 0.07	25.32 ± 3.72	17.11 ± 0.50	2.44 ± 0.10
11	1	1	700	35	0.26 ± 0.02	19.49 ± 1.96	16.57 ± 0.30	2.37 ± 0.06
12	0	1	425	22.5	0.30 ± 0.07	32.20 ± 7.44	20.20 ± 1.47	2.89 ± 0.30
13	-1	1	425	5	0.06 ± 0.02	4.67 ± 0.54	7.28 ± 0.10	1.04 ± 0.02

water (Milli-Q), NaCl, or natural filter sterilized seawater to adjust the salinity. Light intensity was set inside the culture media at 175 μmol photons m^{-2} s^{-1}, and the initial pH was adjusted to 9.0 ± 0.2 by using 2 N HCl or 2 N NaOH. Every day, 1.5 mL of the culture was measured by spectrophotometry to determine the growth rate. At the end of the exponential phase (7 days), the cultures were harvested to measure dry biomass and C-PC content.

Experiment 5: semi-continuous cultivation

For the semi-continuous cultivation, two serial cultivation modes were applied. At the first stage, *A. platensis* was cultivated in batch in 50% POME with or without urea, until the end of the exponential phase was reached (4 days). In the second stage, 30% of the cultures were replaced with fresh medium daily, using different medium compositions (Table 5). Cultivation was carried out in the same setup as experiment 3. The initial salinity at the first cultivation stage was adjusted to 22.5 PSU by using natural seawater, following the outcome of experiment 4. In experiment 5, phosphate (3 mg L^{-1}, run 4) or urea (800 mg L^{-1}, run 5) was added at the second stage. Initial light intensity was set inside the culture media at 175 μmol photons m^{-2} s^{-1}, and the initial pH was adjusted to 9.0 ± 0.2 by using 2 N HCl or 2 N NaOH. Every day, 1 mL of the sample was taken to determine the growth profile. The cultures were harvested at the pseudo-steady-state conditions to measure C-PC and dry biomass. The pseudo-steady-state conditions were reached when the cell concentration was almost constant in two consecutive measurements of semi-continuous cultivation (Bezerra et al. 2011).

Analysis

Growth rate

The growth rate was calculated from the linear regression of the natural logarithm of optical density at 750 nm, which correspond to the dry biomass versus time (Eq. 2).

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (2)$$

where μ is growth rate (day^{-1}), X_2 is the optical density at time t_2 (d), and X_1 the optical density at time t_1 (d). Conversion of OD_{750} to dry biomass for *A. platensis* was done as described previously (Griffiths et al. 2011; Lari et al. 2018) using a *A. platensis* suspension obtained from the end of exponential growth. The suspension was diluted by five serial dilutions which resulted in different cell concentrations. Determination of cell dry weight of *A. platensis* was done using the gravimetric method. The culture sample (30 mL) was harvested by filtering over pre-dried and pre-weighed GF/C filters. The filters were washed with 0.5 M NH_3HCO_3 according to Zhu and Lee (1997). Then, the filter was dried at 75 °C until a constant weight was reached. Equation 3 was used as a regression between the biomass dry weight and the optical density at 750 nm.

$$y = 0.59(OD_{750}) - 0.03 \qquad R2 = 0.98 \quad (3)$$

where y is biomass dry weight (g L^{-1}) and OD_{750} is optical density at 750 nm (Supplementary 3).

C-PC analysis

Ten milliliter of the sample was centrifuged at 4500 rpm for 30 mins. The pellet obtained was stored at − 20 °C until analysis. Extraction was conducted by adding 3 mL of cold buffer phosphate (pH = 6.8) followed by two times freezing and thawing, and sonication at 50% amplitude for 2 min using an ultrasound probe (Vibra Cell, VC 130 PB, USA) following Sarada et al. (1999) and Tavanandi et al. (2018). The filtrate was separated from the pellet by centrifugation (4500 rpm, 4 °C, 30 mins). The concentration of C-PC was determined using a spectrophotometer (Hach DR 3400), by measuring the optical density at 620 nm and 652 nm (Moraes et al. 2011). The concentration of C-PC was determined as

$$\text{C-PC} = \frac{OD_{620} - 0.474(OD_{652})}{5.34} \quad (4)$$

and volumetric C-PC productivity (Eq. 5) was determined by the biomass productivity and the specific pigment content in the biomass (Eriksen 2008; Nur et al. 2019a).

$$P_{\text{C-PC}} = \frac{(N_h - N_0).C_p}{t} \quad (5)$$

where C-PC is the concentration of C-PC (mg mL^{-1}), $P_{\text{C-PC}}$ is C-PC productivity (mg L^{-1} day^{-1}), N_h is final biomass (mg L^{-1}), N_0 is initial biomass (mg L^{-1}), C_p is pigment content (% *w*/*w*), and t is the total duration of the cultivation (d).

For the volumetric C-PC productivity in semi-continuous cultivation, Eq. 6 was employed based on Bezerra et al. (2011).

$$P_{\text{C-PC}} = D.X_s.C_p \quad (6)$$

where $P_{\text{C-PC}}$ is C-PC productivity (mg L^{-1} day^{-1}), D is dilution rate (d^{-1}), X_s is biomass concentration at pseudo-steady-state condition (mg L^{-1}), and C_p is pigment content (% *w*/*w*).

Statistical analysis

Minitab ver. 18 (demo version) was employed for statistical analysis and evaluation for full factorial and CCRD design. Differences between treatments were analyzed with two-way

analysis of variance (ANOVA) at a P value of 0.05 or 0.01. Post hoc tests (Tukey HSD) were performed for pair-wise comparisons. The experimental results were recorded based on at least two replicates and expressed as the means and standard deviations (± SD).

Results

Effect of irradiance and nitrogen concentration on biomass and C-PC productivity

Biomass productivity of *A. platensis* growing on Zarrouk medium without POME (experiment 1) varied significantly with initial nitrogen (nitrate) concentration and irradiance (Fig. 1). Three types of light responses were distinguished: low light (LL, ≤ 100 μmol photons m^{-2} s^{-1}), medium light (ML, 100–300 μmol photons m^{-2} s^{-1}), and high light (HL, ≥ 300 μmol photons m^{-2} s^{-1}). While the initial nitrate in the medium was varied as HN (1.8 g L^{-1} nitrate) and LN (73 mg L^{-1} nitrate). At LL, biomass productivity was not influenced by nitrate availability (HN/LN) ($P > 0.05$). At HL, however, the biomass productivity was affected by nitrate availability ($P < 0.01$). The highest biomass productivity was found at standard (HN) Zarrouk medium at 300 μmol photons m^{-2} s^{-1} (Fig. 1a). C-PC content (percentage of C-PC, when normalized to calculated dry weight) was found to be both irradiance and nitrate dependent (Fig. 1b). The C-PC content was found to be significantly lower at HL compared to ML ($P < 0.01$). Both C-PC content and productivity were significantly higher at ML and HN (around 130 μmol photons m^{-2} s^{-1}) compared to LN ($P < 0.05$). With respect to C-PC concentration (final mg C-PC per liter of culture, Fig. 1c), the highest value was found at around 150–200 μmol photons m^{-2} s^{-1} for both nitrate conditions. Finally, C-PC content and productivity (Fig. 1d) were significantly different when comparing the two nutrient conditions ($P < 0.05$).

Effect of different POME fractions

In experiment 2, between 5 and 30% POME on *A. platensis* biomass productivity was significantly enhanced ($P < 0.05$) (Fig. 2). At 5% POME, the lowest biomass productivity (3.95 ± 3.1 mg L^{-1} day^{-1}) was recorded, while at 100% POME, the biomass productivity reached up to 23.08 ± 3.6 mg L^{-1} day^{-1}. Increasing POME concentrations also significantly enhanced the concentration of C-PC from 4.75 ± 0.5 mg L^{-1} at 5% compared to 12.96 ± 2.8 mg L^{-1} at 100% POME ($P < 0.05$). However, increasing POME from 30 to 100% did not significantly enhance biomass productivity and C-PC concentration ($P > 0.05$) (Fig. 2).

Effect of environmental and nutritional conditions on C-PC concentration

Full factorial design in experiment 3 was employed to reveal the most influencing factors for C-PC concentration of *A. platensis* cultivated under different environmental and nutritional conditions (Table 2). The most influencing factor for C-PC concentration was irradiance, followed by the interaction of POME concentration and nitrate addition (Fig. 3). At low POME and low nitrate addition, C-PC concentration was recorded to be around 8 mg L^{-1}. When 100 mg L^{-1} of nitrate was added to 50% POME, C-PC concentration increased up to 14.8 mg L^{-1}. Addition of 100 mg L^{-1} nitrate to 100% POME resulted in a slightly lower C-PC concentration of 12.4 mg L^{-1} (Fig. 4). Based on this finding, nitrogen addition was further investigated in the next experiment, by utilizing urea as a nitrogen source. Furthermore, salinity did not seem to significantly affect the C-PC concentration, perhaps due to the low range used (15–35 PSU). In the next experiment, the range was therefore expanded to find the optimal salinity.

Effect of urea addition and salinity on C-PC productivity

In experiment 4, 50% *v*/*v* POME was used as culture medium for *A. platensis* while varying salinity and urea addition. Based on CCRD RSM optimization urea both in the linear and quadratic form, salinity in the linear form and the interaction of urea and salinity did not significantly influence growth rate and biomass productivity. Optimal salinity for growth and biomass productivity was recorded at 20–23 PSU (Fig. 5a and b). As for C-PC concentration, salinity in the linear form and the interaction of urea and salinity did not significantly enhance the production (Table 4, Fig. 5c). However, salinity in the quadratic form and urea addition in the linear form were found to be significantly related to C-PC productivity (Fig. 5d). This indicated that the highest salinity level did not always result in the highest C-PC productivity, while the addition of urea above 813 mg L^{-1} could still improve C-PC productivity. The optimal C-PC concentration was recorded as 22.7 mg L^{-1} at 813 mg L^{-1} urea and 22.7 PSU salinity (Fig. 5c).

Effect of nutrient condition and cultivation mode on biomass and C-PC productivity

In experiment 5, the effects of POME, urea, and phosphate addition on biomass and C-PC productivity were investigated during the semi-continuous cultivation of *A. platensis*. Biomass and C-PC productivity were significantly enhanced in run 5 (growth medium containing 50% POME at the first stage and 100% POME supplemented with 800 mg L^{-1} of urea at the second stage), resulting in 70.0 ±

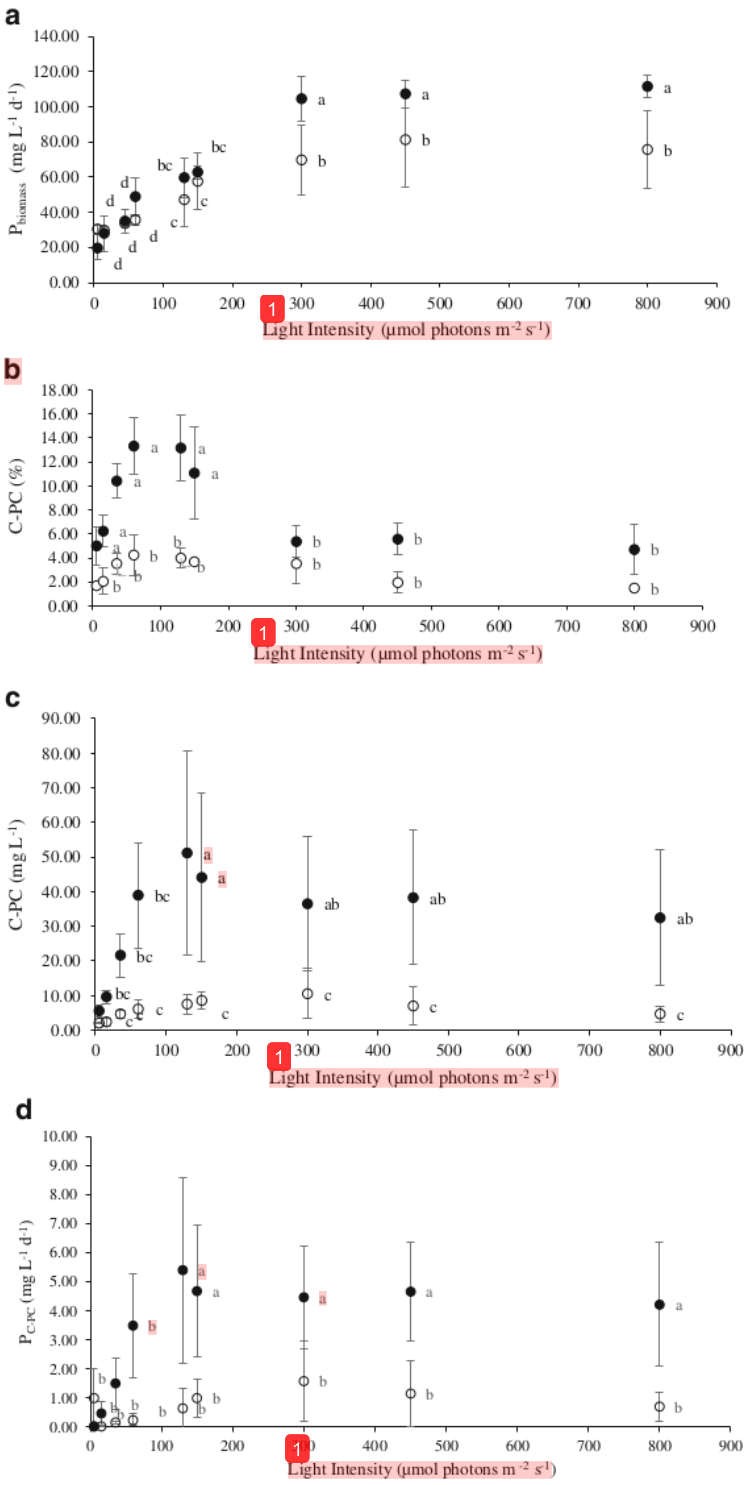

Fig. 1 Effect of irradiance and nitrate availability on **a** biomass productivity, **b** C-phycocyanin content, **c** C-PC concentration, **d** C-PC productivity. Closed circle (●) is standard Zarrouk medium using 1.8 g L^{-1} nitrate (HN), open circle (○) is modified Zarrouk medium using 73 mg L^{-1} nitrate (LN). Average values of triplicate cultures are shown. Error bars indicate the SD of the mean ($n = 3$). Values that do not share letters are significantly different ($P < 0.05$)

1.6 mg L^{-1} day^{-1} and 4.43 ± 0.22 mg L^{-1} day^{-1} of biomass and C-PC productivity, respectively, compared to run 2 ($P < 0.05$). Run 2, (50% POME at both the first and second stage, no addition of urea), showed the lowest biomass and C-

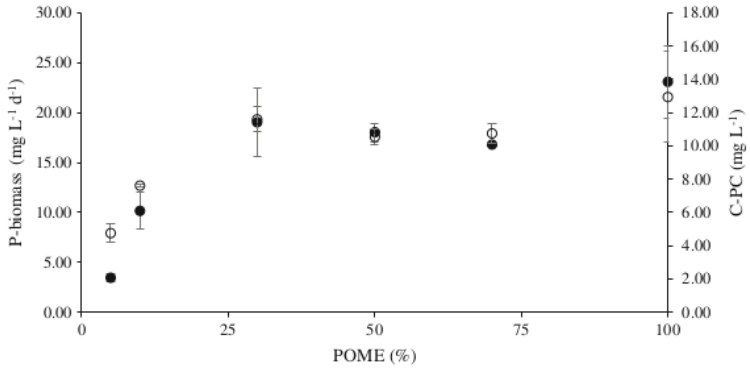

Fig. 2 Biomass productivity (P-biomass) and C-PC concentration of *A. platensis* cultivated on different POME fractions in ultrapure water (Milli-Q) at 4 PSU salinity and 200 μmol photons m^{-2} s^{-1}. Average values of duplicate cultures are shown. Error bars indicate the SD of the mean. Closed circle is biomass productivity. Open circle is C-PC production

PC productivity compared to run 1 and 5 ($P < 0.05$) (Table 5). In run 4, the addition of phosphate during the second stage did not significantly influence biomass and C-PC productivity. The C-PC productivity in run 5 reached 62.76 ± 3.67 mg L^{-1} day^{-1} and 5.76 ± 1.85 mg L^{-1} day^{-1} for biomass and C-PC productivity, respectively, which was comparable to control Zarrouk medium carried out in batch cultivation mode (Fig. 1). Finally, these values were 2.2-fold higher compared to the batch cultivation mode that had 50% POME and 800 mg L^{-1} urea (Fig. 5, Table 5).

Discussion

When developing large-scale production systems, it is vital to know how irradiance and nitrogen availability affect C-PC productivity (Ho et al. 2018). C-PC is a major light harvesting pigment that can also serve as a storage protein in blue-green algae, including *A. platensis* (Boussiba and Richmond 1980; Vonshak et al. 1982; Rastogi et al. 2015). This study showed that cellular C-PC content is strongly dependent on irradiance and nitrate availability (Fig. 1b). This implies that supra-optimal irradiance and lack of nitrogen availability levels in large-scale cultivation system would not benefit biomass and C-PC productivity.

However, biomass productivity was not dependent on nitrogen availability below saturating irradiance levels ($P > 0.05$) (Fig. 1a). A similar result was found in previous studies in which it was demonstrated that biomass production was unchanged in nitrogen-deficient medium compared to Zarrouk medium at low light, while protein production and C-PC content were decreased (Olguín et al. 2001; Sala et al. 2018). When *A. platensis* was cultivated at high light, growth increased and resulted in a higher nitrogen demand. Furthermore, when nitrogen availability was not high enough to support growth, *A. platensis* produced lower biomass levels, resulting in lower C-PC concentrations (Fig. 1a and b). In order to maintain metabolic functions, C-PC may be used as an intracellular nitrogen storage compound by *A. platensis* anticipating nitrogen-limiting conditions (Boussiba and Richmond 1980; Eriksen 2008). Therefore, to guarantee high C-PC productivity, cultivation conditions should carefully be maintained at optimal irradiance and nitrogen levels.

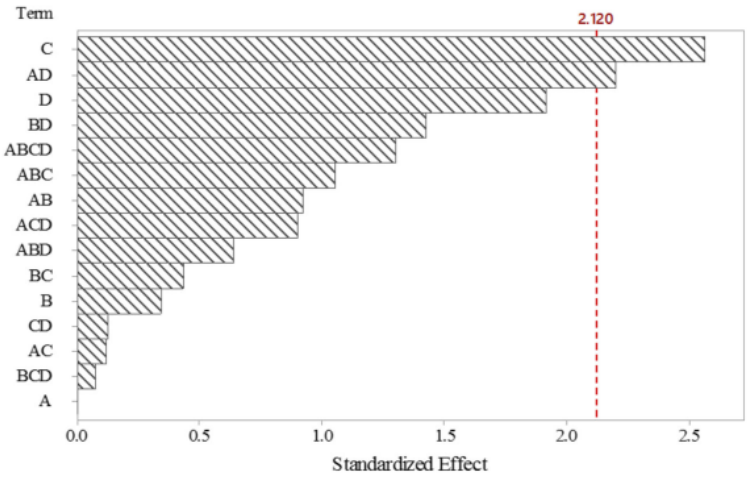

Fig. 3 Pareto chart showing the effects of (combinations of) parameters on the C-PC concentration of *A. platensis*. The vertical line indicates the significance of the effects at 95% confidence level. A is POME, B is salinity, C is irradiance, and D is nitrate

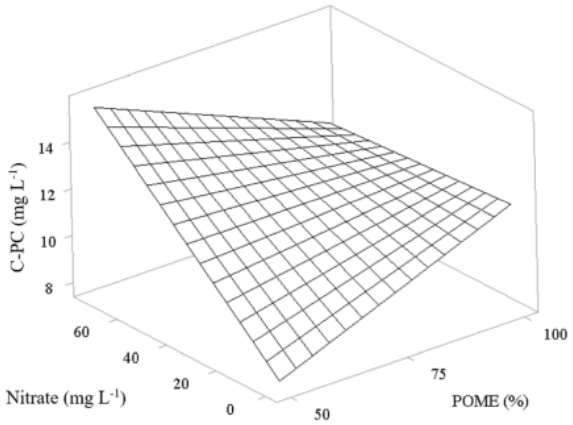

Fig. 4 Response surface plot (3D) of **a** C-PC concentration as a function of nitrate addition and POME concentration at salinity 22.5 PSU and irradiance 125 μmol photons m^{-2} s^{-1}

In our study, biomass productivity and C-PC concentration were also dependent on POME fraction (Fig. 2). *Arthrospira platensis* grew well on 30–100% *v*/*v* POME at a saturating irradiance level. This implies that *A. platensis* can tolerate the high ammonia levels present in the POME, which may reach concentrations up to 100 mg L^{-1} (Sasongko et al. 2015). In support of this, Carvalho et al. (2004) reported that *A. platensis* can tolerate an ammonia concentration of 6.4 mM (109 mg L^{-1}) whereas growth was totally inhibited at 26 mM. In contrast, previous research had shown that the growth of other algal species was inhibited when using more than 30 and 50% *v*/*v* of digested POME (Cheirsilp et al. 2017; Cheah et al. 2018; Nur et al. 2019b). This underlines the tolerance of *Arthrospira* to the high ammonia levels or other potentially toxic substances (e.g., phenolic compounds) present in POME.

Based on experiment 2, the highest influencing factor was irradiance, in accordance with the results of experiment 1 (Fig. 1). The second most influencing factor was the interaction of nitrate addition and POME concentration (Fig. 3). As stressed before, C-PC functions primarily as the main light harvesting pigment, but it has a second role as a storage compound. The highest C-PC concentration was found at 50% POME with the addition of nitrate compared to other

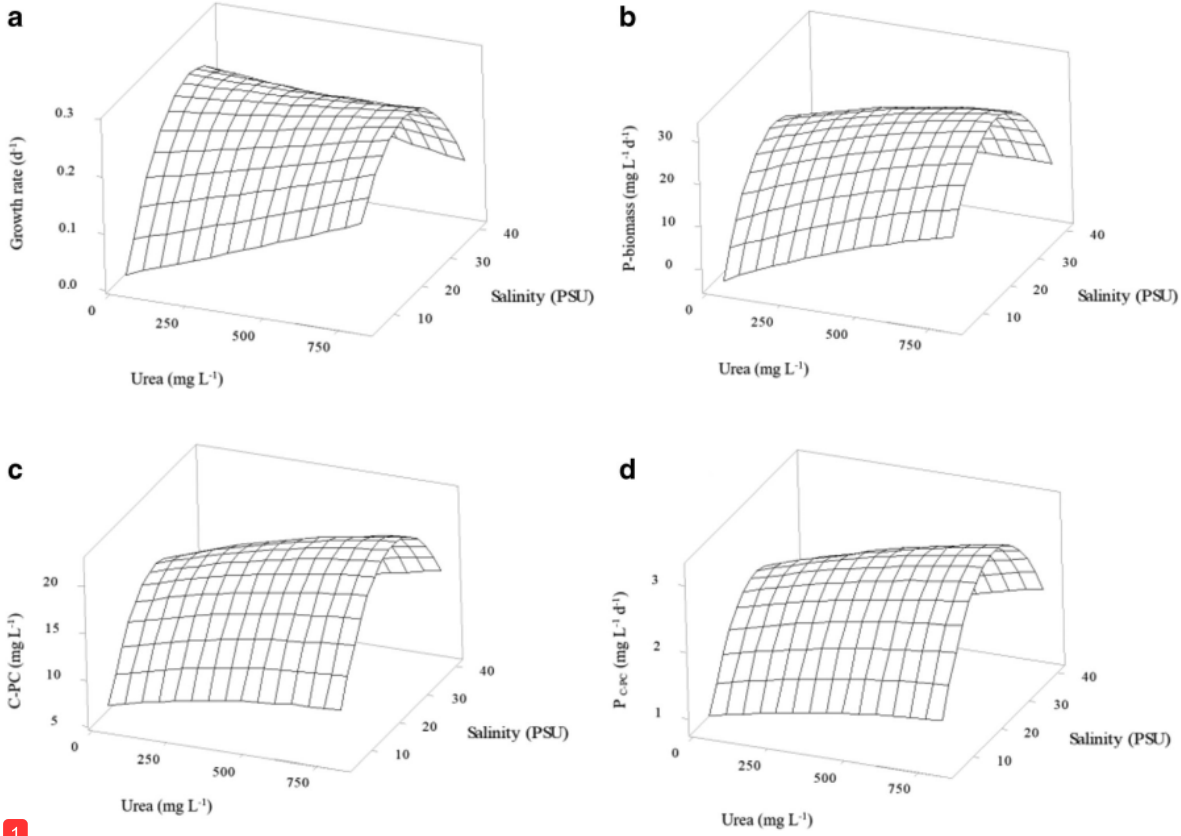

Fig. 5 Response surface plots (3D) showing the effects of salinity (PSU), and urea (mg L^{-1}) on **a** growth rate (day^{-1}), **b** biomass productivity (P-biomass, mg L^{-1} day^{-1}), **c** C-PC concentration (C-PC, mg L^{-1}), and **d** C-PC productivity ($P_{C\text{-}PC}$, mg L^{-1} day^{-1}) generated by *A. platensis* cultivated on 50% POME at 175 μmol photons m^{-2} s^{-1}

Table 4 ANOVA of C-PC concentration generated from CCRD. Values are significant at $P < 0.01$

Source	DF	Adj SS	Adj MS	*F* value	*P* Value	Remarks
Model	5	401.487	80.297	17.55	< 0.01	Significant
Linear	2	69.988	34.994	7.65	0.003	
Urea (mg L^{-1})	1	59.957	59.957	13.11	< 0.01	Significant
Salinity (PSU)	1	10.031	10.031	2.19	0.154	
Square	2	329.407	164.704	36.00	0.000	
Urea (mg L^{-1}) × urea (mg L^{-1})	1	1.907	1.907	0.42	0.526	
Salinity (PSU) × salinity (PSU)	1	328.443	328.443	71.80	< 0.01	Significant
2-way interaction	1	2.119	2.119	0.46	0.504	
Urea (mg L^{-1}) × salinity (PSU)	1	2.119	2.119	0.46	0.504	
Error	20	91.489	4.574			
Lack-of-fit	3	42.888	14.296	5.00	0.011	Not significant
Pure error	17	48.602	2.859			
Total	25	492.976				

treatments (Fig. 4). As reported before, the nitrogen to phosphorus ratio in the medium is important for pigment production (McClure et al. 2018). It seems that when nitrate was added to 100% POME, excess nitrogen was generated. Based on previous results, cultivation of *A. platensis* above the optimal nitrogen concentration in the medium could not enhance the C-PC concentration, since the excess nitrogen was not completely stored as C-PC pigment in *A. platensis* (Setyoningrum and Nur 2015). Based on this, 50% of POME was used for subsequent experiments, while nitrate was replaced with urea as a relatively inexpensive alternative nitrogen source.

Salinity optimization may also be considered important with respect to large-scale cultivation. For example, to make large-scale cultivation sustainable, seawater might be preferred over drinking water or other freshwater sources, when diluting POME to the 50% level. Based on CCRD RSM, the optimal salinity with respect to growth rate, biomass, and C-PC productivity was around 22–24 PSU, which is much higher than POME alone. In support of this, Liu et al. (2016) showed that the production of phycocyanin and carotene by *A. platensis* was optimal when using growth media supplemented with 200–400 mM NaCl, equaling 11.6–23.3 PSU, compared to control Zarrouk medium. The addition of urea significantly increased C-PC productivity and did not show inhibition up to 813 mg L^{-1} ($P < 0.05$).

Semi-continuous cultivation generally stimulated biomass and C-PC productivity (Table 5). When 100% of POME was provided at the second stage of cultivation, the nutrient concentration was increased accordingly, while light intensity in the culture became lower due to the high turbidity of POME: from 175 to 90 μmol photons m^{-2} s^{-1}. The combination of nutrients and irradiance significantly affected biomass and C-PC productivity in accordance with experiment 1 (Fig. 1). By adding extra urea at the second stage of cultivation, the biomass and C-PC productivity slightly increased but this difference was not significant, probably due to the enhanced availability of nitrogen for photosynthesis. This research is in agreement with Benvenuti et al. (2016) who found that the addition of 140 mg L^{-1} nitrogen enhanced biomass production of *Nannochloropsis* sp. when cultivated in semi-continuous mode compared to the 70 mg L^{-1} nitrogen concentration in the medium. Another study reported that semi-continuous cultivation could enhance biomass productivity

Table 5 Biomass and C-PC productivity of *A. platensis* cultivated on POME medium using consecutive two-stage cultivations. Mean values are based on 4 replicates. SD is shown after the ± symbol. The same sharing letters represent no significant difference ($P > 0.05$)

Run	Stage 1 (batch)			Stage 2 (semi-continuous)			$P_{biomass}$ (mg L^{-1} day^{-1})		$P_{C\text{-}PC}$ (mg L^{-1} day^{-1})	
	Medium	External nutrient (mg L^{-1})	Sources	Medium	External nutrient (mg L^{-1})	Sources				
1	50% POME	800	urea	100% POME	–	–	69.59	± 16.9[a]	4.38	± 1.0[a]
2	50% POME	–	–	50% POME	–	–	44.05	± 5.5[b]	2.58	± 0.5[b]
3	50% POME	–	–	100% POME	–	–	65.57	± 17.8[ab]	4.08	± 1.3[ab]
4	50% POME	–	–	100% POME	3	PO_4^-	60.27	± 0.9[ab]	3.74	± 0.3[ab]
5	50% POME	–	–	100% POME	800	urea	70.01	± 1.6[a]	4.43	± 0.2[a]

Table 6 Biomass, C-PC productivity, and final C-PC concentration of *A. platensis* cultivated on POME under different conditions. *n.a.*, not analyzed; *n.ap*, not applicable

Type of cultivation	Media		Total cultivation time	$P_{biomass}$ (mg L^{-1} day^{-1})	$P_{C\text{-}PC}$ (mg L^{-1} day^{-1})	C-PC (mg L^{-1})	References
	Media composition	External nutrient					
Semi-continuous	50% *v/v* POME + 50% *v/v* natural seawater (first stage) 100% *v/v* POME (second stage)	800 mg L^{-1} urea	8 day	69.59	4.08	13.80	This study
Batch	50% *v/v* POME + 50% natural seawater	800 mg L^{-1} urea	7 day	33.64	2.05	22.69	This study
Batch	100% *v/v* POME	n.ap	6 day	23.08	1.29	12.96	This study
Continuous	90% *v/v* POME + 10% *v/v* commercial medium	Commercial medium	14 day	65.00	n.a	n.a	Suharyanto et al. 2014
Batch	30% *v/v* POME + 70% *v/v* distilled water	n.ap	13 day	16.69	n.a	n.a	Nur et al. 2016
Batch	20% *v/v* POME + 80% *v/v* commercial medium	Commercial medium	7 day	28.50	n.a	n.a	Sari et al. 2012

and phycocyanin content of *A. platensis* cultivated on pig wastewater by supplementing the wastewater with sodium bicarbonate and urea. In their study, C-PC productivity under these conditions was similar to the control Zarrouk medium (Chaiklahan et al. 2010). Due to of the high phosphate levels present in the wastewater, the addition of urea could increase the nitrogen to phosphorus ratio, making phosphate potentially limiting and resulting in higher biomass and C-PC production.

In the present study, the addition of a phosphate at the second-stage cultivation did not significantly increase the biomass and C-PC productivity compared to the other treatments (experiment 5) ($P > 0.05$). This indicates that the enrichment of phosphorus, which resulted in a lower N:P ratio, did not significantly affect C-PC production. Another interesting result was shown in run 1 (growth medium containing 50% POME supplemented with 800 mg L^{-1} urea at the first stage and 100% POME at the second stage) where biomass and C-PC productivity were significantly enhanced compared to run 2 (growth medium containing 50% POME only at the first and second stage) ($P < 0.05$). This indicated that the addition of nitrogen at the first stage enhanced the initial biomass for semi-continuous cultivation (Supplementary 1). When 100% POME was added to the second stage, the nutrient could promote the growth of *A. platensis* which already had higher initial biomass compared to the other treatments.

To summarize, the best option for semi-continuous cultivation might be when based on run 3, which used 50% POME at the first stage, and 100% of POME at the second stage without adding external nutrients (Table 5). This cultivation resulted in 65.6 ± 17.9 mg L^{-1} day^{-1} and 4.1 ± 1.3 mg L^{-1} day^{-1} for biomass and C-PC productivity, respectively. The result from run 3 was also higher compared to the batch cultivation systems used in previous research (Table 6). Our results are similar compared to a continuous cultivation system that used 90% of POME and resulted in 65 mg L^{-1} day^{-1} biomass productivity (Suharyanto et al. 2014). By employing the semi-continuous system, inhibitory factors such as self-shading and excess secondary products secreted by the cells could be avoided, thereby resulting in higher biomass productivity (Radmann et al. 2007; Moreira et al. 2016). By using seawater for cultivation, a dual benefit was achieved: the salinity could be increased at the first stage and the demand of freshwater or drinking water could be lowered, overall reducing the cost of cultivation. Furthermore, by utilizing a higher POME concentration at the second stage, large-scale cultivation would reach higher cost-effectiveness and feasibility due to inexpensive fertilizer derived from POME compared to the batch method which utilized low POME concentrations blended with commercial nutrients.

Conclusion

Irradiance and nitrogen concentration were the main factors driving C-PC productivity. Based on CCRD RSM, the optimal salinity was found to be 22.5 PSU, and no inhibition was found up to 813 mg L^{-1} of urea. Biomass and C-PC productivity of *A. platensis* cultivated on POME medium were successfully enhanced using a semi-continuous cultivation mode at 175 μmol photons m^{-2} s^{-1} with 50% POME at the first stage and 100% POME at the second stage. This resulted in the highest C-PC productivity (4.08 ± 1.3 mg L^{-1} day^{-1}), similar to the artificial control Zarrouk medium during batch cultivation.

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Enhancement of C-phycocyanin productivity by Arthrospira platensis when growing on palm oil mill effluent in a two-stage semi-continuous cultivation mode

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