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Published in:
Journal of Applied Phycology

DOI:
10.1007/s10811-018-1563-6

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Nur, M. M. A., Muizelaar, W., Boelen, P., & Buma, A. G. J. (2019). Environmental and nutrient conditions influence fucoxanthin productivity of the marine diatom Phaeodactylum tricornutum grown on palm oil mill effluent. Journal of Applied Phycology, 31(1), 111-122. https://doi.org/10.1007/s10811-018-1563-6

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Environmental and nutrient conditions influence fucoxanthin productivity of the marine diatom *Phaeodactylum tricornutum* grown on palm oil mill effluent

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Received: 23 April 2018 / Revised and accepted: 21 June 2018 / Published online: 16 July 2018 © The Author(s) 2018

Abstract
Palm oil mill effluent (POME) is a type of wastewater posing large problems when discharged in the environment. Yet, due to its high nutrient content, POME may offer opportunities for algal growth and subsequent harvesting of high-value products. The marine diatom *Phaeodactylum tricornutum* is a potential feedstock diatom for bioactive compounds such as the carotenoid fucoxanthin, which has been shown to have pharmaceutical applications. The aim of this paper was to evaluate the growth and fucoxanthin productivity of *P. tricornutum* grown on POME, as a function of light intensity, temperature, salinity, and nutrient enrichment. High-saturating irradiance (300 μmol photons m⁻² s⁻¹) levels at 25 °C showed highest growth rates but decreased the fucoxanthin productivity of *P. tricornutum*. Box-Behnken response surface methodology revealed that the optimum fucoxanthin productivity was influenced by temperature, salinity, and the addition of urea. Nutrient enrichment by phosphorus did not enhance cell density and fucoxanthin productivity, while urea addition was found to stimulate both. We conclude that POME wastewater, supplemented with urea, can be considered as the potential medium for *P. tricornutum* to replace commercial nutrients while producing high amounts of fucoxanthin.

Keywords POME wastewater · Light intensity · Temperature · Fucoxanthin · *Phaeodactylum tricornutum*

Introduction
The possibility of utilizing wastewater as growth medium for microalgae with the aim to produce value-added products receives increasing attention due to its high economic sustainability (Ravindran et al. 2016; De Francisci et al. 2018). In this respect, the marine diatom *Phaeodactylum tricornutum* has gained much attention in the last decades. This is due to its ability to grow in large-scale facilities, while producing bioactive compounds such as pigments which might benefit human health (Leu and Boussiba 2014). The carotenoid fucoxanthin contained in diatoms as well as in brown seaweeds and other microalgae such as dinoflagellates and coccolithophorids exhibits anti-cancer, anti-obesity, and anti-diabetic activity (Kim et al. 2012). It has been suggested that the production of value-added products from microalgae on the large scale becomes more economically feasible when artificial growth media are replaced by low-cost nutrients such as those derived from agricultural or domestic wastewater (Pittman et al. 2011). Palm oil mill effluent (POME) is agricultural wastewater generated from palm oil processing that could have the potential to fuel microalgal growth due to the high concentrations of micro and macronutrients (Nur and Hadiyanto 2013). However, to the best of our knowledge, the utilization of POME wastewater for the cultivation of the marine diatom *P. tricornutum* has not been explored.

Key environmental factors such as light intensity, temperature, salinity, and nutrient composition, regulate algal growth in large-scale facilities (Borowitzka 2016; Nur and Buma 2018). Simultaneously, apart from affecting growth, irradiance level might also affect the fucoxanthin content of *P. tricornutum* since it is a light harvesting pigment (Gómez-Loredo et al. 2016). Furthermore, supraoptimal temperatures could be an extra stress factor on the growth of *P. tricornutum*. 

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In addition, the biomass production, growth, and fucoxanthin productivity of *P. tricornutum* depend on nutrient concentration and the balance between relevant nitrogen and phosphorus (N/P) species in the media (Choi and Lee 2015; Whitton et al. 2016; McClure et al. 2018).

The aim of the present paper was to utilize POME as growth medium for *P. tricornutum* and to find optimal environmental conditions for maximal fucoxanthin productivity by applying different irradiance, temperature and nutrient conditions. To this end, optimal irradiance and temperature dependent fucoxanthin productivity was investigated first based on artificial medium. Then, the impacts of various POME fractions on the growth and fucoxanthin productivity were investigated and selected nutrient additions were done to optimize the fucoxanthin productivity. POME consists of a highly complex mixture of both organic and inorganic N and P species (Poh et al. 2010). As a result, organic and inorganic N/P ratios differ strongly, whereas organic N- and P-containing compounds might be affected by irradiance exposure or bacterial activity. Therefore, both N and P limitation might eventually occur when algae are cultivated on POME alone. Furthermore, different POME pretreatments may lead to N/P ratios above or below the expected optimal Redfield ratio of 16:1. Due to this, the impacts of various nitrogen and phosphorus additions on possible fucoxanthin enhancement were tested. In addition, urea was added since this N source might serve as a cheap alternative to nitrate additions, leading to a nitrogen surplus and thus to complete phosphorus removal from the wastewater. The Box-Behnken design (BBD) response surface methodology (RSM) was employed to reveal the optimum combination of environmental conditions and to understand the interaction of salinity, temperature, and nutrient concentration with respect to fucoxanthin productivity.

**Material and methods**

**Wastewater preparation**

POME wastewater was collected from a small factory in Sumatra, Indonesia, after it had been released from an aerobic open pond lagoon. The wastewater was stored at −19 °C to prevent degradation. Prior to the experiments, the wastewater was thawed and filtered through GF/C glass fiber filters (Whatman, 47 mm) to remove particulate matter. Subsequently, the filtrate was sterilized at 120 °C for 15 min. POME composition was analyzed before and after these treatments. Based on these results (Table 1), relevant nutrient additions were chosen: POME treatment (filtration, autoclaving) changed its characteristics in terms of nutrients, pH, and color (Table 1). At the same time, the inorganic N/P ratio of the effluent might theoretically lead to either N or P limitation, depending on the type of treatment (Table 1), given the deviation from the ideal Redfield ratio of 16:1 (Table 1) (Olguin 2012). Furthermore, the potential photochemical or bacterial breakdown of organic N and P species during cultivation might alter these initial molar ratios, thereby creating unforeseen changes in the availability of inorganic nutrients for phytoplankton growth. Therefore, in order to meet cellular nutrient requirements and optimize productivity, some external inorganic nitrogen and phosphorous addition could be necessary. In particular, addition of a cheap nitrogen source such as urea might typically install P limitation at the end of the growth phase, thereby causing almost complete P removal from the wastewater.

**Experimental setup**

Stock cultures of *P. tricornutum* Bohlin (CCMP2558, NCMA, Maine, USA) were grown on a standard f/2 medium based on filtered natural oligotrophic seawater obtained from NIOZ, Netherlands (adjusted to a salinity of 35 ppt with demineralized water) using the protocol of Guillard (1975) supplemented with silicate (100 μM) and NaHCO3 (2.38 mM). The culture was acclimated to the experimental conditions for at least 1 week. The algae were cultivated in a 16:8 light/dark cycle. The culture was diluted with fresh medium if growth reached the stationary phase. The research questions were addressed by using a stepwise experimental approach. Four experiments were carried out, as listed below.

**Effect of temperature and light intensity on growth and fucoxanthin productivity (no POME addition)**

Cultures of *P. tricornutum* were grown in triplicate on f/2 medium in plastic 60-mL cell culture flasks (Greiner Bio-One, ref. 690,160). The flasks were placed in a photosynthetron illuminated by a 250 W lamp (MHN-TD power tone, Philips) as described by Kulk et al. (2011) at two temperatures (T = 20 and 25 °C) controlled by a water bath (± 0.1 °C) at 10 different light intensities (I = 8–500 μmol photons m−2 s−1). Samples for cell counts (2 ml) were taken daily and immediately counted using a hemocytometer. Growth rates were calculated from cell counts as described below. The cultures were harvested for pigment analysis at the end of the exponential growth phase (7–14 days).

**Effect of different POME concentrations on growth and fucoxanthin productivity**

*Phaeodactylum tricornutum* was cultured in duplicate on an autoclaved mixture of unfiltered POME and sterile
natural sea water (no nutrient additions) at different unfiltered POME dilutions (15, 30, and 50% v/v) with a working volume of 75 mL in sterilized 100-mL Erlenmeyer flasks. An inoculum of 2% (v/v) of the culture was used to start the experiments. The flasks were placed in a climatized room equipped with fluorescent lamps (Biolux Osram). The temperature of the room was set at 23 °C, initial pH was adjusted to 8.2 with 2N HCl or 2N NaOH and light intensity was adjusted outside the cultivation medium at 100 μmol photons m⁻² s⁻¹, resulting in different light penetrations for each flask. The samples for cell counts (2 ml) were taken daily and immediately counted by using a hemocytometer. Growth rates was calculated from cell counts as described below. At the end of the exponential growth phase (8 days), the cultures were harvested for pigment analysis.

**Table 1** POME wastewater characteristic for different treatments

|                          | Raw POME (unautoclaved) | Sample POME (unfiltered autoclaved) | Sample POME (GF/C filtration + autoclaved) |
|--------------------------|-------------------------|------------------------------------|------------------------------------------|
| COD (mg L⁻¹)             | 1554                    | 1418                               | 1245                                     |
| NO₃⁻--N (mg L⁻¹)         | 17.5                    | 16.1                               | 10.3                                     |
| TN (mg L⁻¹)              | 117.4                   | 108.5                              | 72.4                                     |
| PO₄−P (dissolved) (mg L⁻¹) | 2.0                     | 2.0                                | 1.7                                      |
| TP (mg L⁻¹)              | 10.7                    | 8.3                                | 7.9                                      |
| Color (PtCo)             | 5630                    | 5040                               | 4370                                     |
| pH                       | 7.8                     | 8.9                                | 8.8                                      |
| Inorganic N/P ratio (molar) | 18.5:1                  | 18:1                               | 13:1                                     |

COD chemical oxygen demand, TN total nitrogen, TP total phosphorus

**Effect of different nutrient enrichments on fucoxanthin productivity of *P. tricornutum* growing on 30% POME**

*Phaeodactylum tricornutum* was cultured in 75-mL working volume in 100-mL sterilized Erlenmeyer flasks and placed in a water bath equipped with temperature controller and a U-shaped lamp that contained 12 fluorescent lamps (6 biolux and 6 skywhite lamps, Osram) coupled with reflectors (Doublelux) and connected to dimmers (Osram) and set as described by Van de Poll et al. (2007). About 2% (v/v) of *P. tricornutum* culture was used to inoculate autoclaved and filtered medium consisting of 30% v/v POME + 70% v/v filtered natural oligotrophic sea water. Cultures growing on 30% POME alone (no nutrient additions) served as controls. Nitrogen and phosphorus were added to the media to generate different N/P molar ratios (Table 2). All media were supplemented with silicate (100 μM). The experiments were carried out at 20 and 25 °C, initial pH was adjusted to 8.0 ± 0.2 by using 2N HCl or 2N NaOH, and salinity was adjusted to 35 ppt using NaCl. The light intensity was measured using a light sensor (Biospherical Instrument QSL2101, USA) and set to 300 μmol photons m⁻² s⁻¹ inside the cultivation medium and, if necessary, adjusted by using neutral density screens. The samples for cell counts (2 mL) were taken daily and immediately counted using a hemocytometer. At the end of the exponential growth phase (7–15 days cultivation), the cultures were harvested for pigment analysis.

**Effect of salinity, temperature, and urea addition on fucoxanthin productivity**

A high concentration of urea is considered toxic for *P. tricornutum* due to the excess production of ammonium derived from urea conversion. Therefore, urea concentration, salinity, and temperature were all expected to show optima with respect to growth and fucoxanthin productivity. Therefore, the optimum growth condition, significance and the interaction effects of salinity, temperature and urea addition on fucoxanthin productivity and cell density were studied using the Box-Behnken design (BBD) response surface methodology (RSM) with 13 total experimental runs (Table 3). The ranges used for these experiments were 20, 22.5, and 25 °C for temperature (X¹); 5, 20, and 35 ppt for salinity (X²); and 0, 50, and 100 mg L⁻¹ for urea addition (X³). The empirical form of the second-order polynomial model (Eq. 1) can be described as:

\[
y = \beta_0 + \sum \beta_i x_i + \sum \beta_i x_i^2 + \sum \beta_{ij} x_i x_j
\]  

where y is the predicted value; \(\beta_0, \beta_i, \beta_{ii}, \text{and } \beta_{ij}\) are a constant, linear, quadratic, and interaction coefficient, respectively.

Cultivation was carried out in the same setup as the third experimental series. Salinity was adjusted by using...
a mixture of Milli-Q and natural sea water, light intensity was set inside the media at 300 μmol photons m⁻² s⁻¹, and the initial pH was adjusted to 8.0 ± 0.2 by using 2 N HCl or 2 N NaOH. The medium was supplemented with 100-μM silicate. After 7 days of cultivation, the cultures were harvested to measure the cell density and pigments.

Analyses

**Wastewater composition**

Chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP), were analyzed by using the assay kit

| Table 2 | Fucoxanthin productivity of *P. tricornutum* cultivated at 300 μmol photons m⁻² s⁻¹, on 30% POME supplemented with different nutrients. The fucoxanthin was harvested at the end of exponential phase. Mean values are from two replicates. SD are shown after the ± symbol |
|---------|----------------------------------------------------------------------------------------------------------|
| Medium | Temp (°C) | Nutrients | Nitrogen addition (mg L⁻¹) | Nitrogen source | Phosphorus addition (mg L⁻¹) | Cultivation time (day) | N/P ratio* | Final cell density (cells L⁻¹) x 10⁶ | Fucoxanthin productivity (μg L⁻¹ day⁻¹) |
| f/2    | 20       | f/2       | 12.3 | Nitrate | 1.1 | 15 | 24:1 | 19.39 ± 3.45 | 40.40 ± 4.52 |
| NPT    | 20       | 30% POME + trace elements | 3 | Nitrate | 2.6 | 15 | 4:1 | 18.18 ± 1.03 | 20.40 ± 4.94 |
| NP     | 20       | 30% POME | 3 | Nitrate | 2.6 | 15 | 4:1 | 16.74 ± 0.39 | 16.37 ± 1.44 |
| UP     | 20       | 30% POME | 3 | Urea | 2.6 | 15 | 4:1 | 7.02 ± 6.66 | 25.44 ± 21.80 |
| U      | 20       | 30% POME | 23 | Urea | – | 7 | 108:1 | 1.05 ± 0.27 | 4.01 ± 3.57 |
| Control | 20     | 30% POME | – | – | – | 7 | 13:1 | 2.09 ± 0.02 | 5.81 ± 0.75 |
| P-Low  | 20       | 30% POME | – | – | 0.3 | 7 | 8:1 | 2.36 ± 0.19 | 9.36 ± 0.02 |
| P-High | 20       | 30% POME | – | – | 1.6 | 7 | 3:1 | 14.91 ± 2.42 | 23.07 ± 0.82 |
| f/2    | 25       | f/2       | 12.3 | Nitrate | 1.1 | 15 | 24:1 | 16.06 ± 0.49 | 18.41 ± 8.00 |
| NPT    | 25       | 30% POME + trace elements | 3 | Nitrate | 2.6 | 15 | 4:1 | 16.06 ± 2.11 | 18.90 ± 8.49 |
| NP     | 25       | 30% POME | 3 | Nitrate | 2.6 | 15 | 4:1 | 16.06 ± 1.32 | 16.30 ± 3.78 |
| UP     | 25       | 30% POME | 3 | Urea | 2.6 | 15 | 4:1 | 16.06 ± 0.49 | 18.41 ± 8.00 |
| Control | 25     | 30% POME | – | – | – | 7 | 13:1 | 0.56 ± 0.01 | 2.60 ± 0.12 |

**f/2, f/2 medium; NPT POME, low nitrate, high phosphate and trace elements; NP POME, low nitrate, and high phosphate; UP POME, low urea, and high phosphate; U POME and high urea; control POME alone; P-Low POME and low phosphate; P-High POME and high phosphate**

*The ratio is calculated from the molar of inorganic nitrogen and phosphorus from the wastewater and external nutrient addition

**Table 3** Design of the experiment of BBD with the results. Mean ± Std Dev. (n = 2)

| Run | Temperature (°C) | Salinity (ppt) | Urea addition (mg L⁻¹) | Final cell density (cells L⁻¹) x 10⁶ | Fucoxanthin productivity (μg L⁻¹ day⁻¹) |
|-----|-----------------|----------------|------------------------|--------------------------------------|----------------------------------------|
|     | Experimental    | Predicted      |                        |                                      |                                        |
|     | Experimental    | Predicted      |                        |                                      |                                        |
| 1   | 20              | 5              | 50                     | 5.00 ± 0.20                          | 10.07 ± 0.81                           |
| 2   | 20              | 35             | 50                     | 7.02 ± 6.66                          | 25.44 ± 21.80                          |
| 3   | 20              | 0              | 0                      | 1.32 ± 0.14                          | 9.16 ± 1.21                            |
| 4   | 20              | 20             | 100                    | 7.56 ± 0.42                          | 17.09 ± 0.21                           |
| 5   | 22.5            | 5              | 0                      | 1.22 ± 0.03                          | 6.88 ± 0.28                            |
| 6   | 22.5            | 35             | 0                      | 1.30 ± 0.33                          | 6.68 ± 0.95                            |
| 7   | 22.5            | 5              | 100                    | 6.72 ± 0.11                          | 25.19 ± 6.23                           |
| 8   | 22.5            | 35             | 100                    | 6.25 ± 0.29                          | 34.74 ± 1.61                           |
| 9   | 22.5            | 20             | 50                     | 6.49 ± 2.58                          | 32.75 ± 19.86                          |
| 10  | 25              | 5              | 50                     | 0.13 ± 0.02                          | 0.23 ± 0.16                            |
| 11  | 25              | 35             | 50                     | 6.41 ± 0.31                          | 29.69 ± 4.31                           |
| 12  | 25              | 0              | 0                      | 1.04 ± 0.01                          | 6.42 ± 0.10                            |
| 13  | 25              | 20             | 100                    | 6.08 ± 0.03                          | 23.09 ± 0.33                           |
Growth rate

The growth rate was calculated from the linear regression of the natural logarithm of cell density versus time (Eq. 2)

\[
\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1}
\]

where \( \mu \) is the growth rate (day\(^{-1} \)), \( X_2 \) is the cell density (cells mL\(^{-1} \)) at time \( t_2 \) (day), and \( X_1 \) is the cell density (cells mL\(^{-1} \)) at time \( t_1 \) (day).

Pigment analysis

Between 5 and 25 mL of sample was filtered through a GF/F filter (25 mm, max pressure ~ 0.2 bar). The filter obtained was folded in aluminum foil and immersed immediately in liquid nitrogen, and then stored at \(-80^\circ C\) until analysis. Before analysis, the filters were freeze-dried at \(-50^\circ C\) for 48 h and a pressure of \(30 \times 10^{-3}\) mbar. Extraction was conducted by adding 5 mL of 90% cold acetone into the sample at \(4^\circ C\) for 48 h. After extraction, the sample was analyzed using HPLC using a Waters (Model 2695) system, a cooled auto-sampler (4 °C), and a Waters 996 diode-array detector. The freeze-drying, extraction, and HPLC analysis methods are based on van Leeuwe et al. (2006). The volumetric fucoxanthin or chlorophyll-\(a\) productivity was calculated based on Eq. 3.

\[
P_p = \frac{(N_f - N_0) \times C_p}{t}
\]

where \( P_p \) is pigment productivity (\(\mu g \ L^{-1} \ day^{-1} \)), \( N_f \) is final cell density (cells L\(^{-1} \)), \( N_0 \) is initial cell density (cells L\(^{-1} \)), \( C_p \) is pigment content (\(\mu g \ cell^{-1} \)), and \( t \) is total duration of the cultivation (days).

Statistical analysis

IBM SPSS Statistics 24 was used for all statistical analyses. Minitab ver. 18. (Demo version) was used for BBD design and evaluation. Difference between treatments were analyzed with analysis of variance (ANOVA) with a \( p \) value of 0.05. Post hoc tests (Tukey HSD) were performed for pairwise comparisons. The experimental results were obtained for a minimum of two replicates and expressed using averages and standard deviations (±SD), except for wastewater characteristic analysis.

Results

Effect of light and temperature on biomass, growth rate, and fucoxanthin productivity

The growth rate of \(P. \) tricornutum growing on f/2 medium without POME (first experimental series) varied significantly with temperature and irradiance (Fig. 1). Three types of light responses were distinguished: low light (LL, < 100 \(\mu mol \) photons m\(^{-2} \) s\(^{-1} \)); medium light (ML, 100–200 \(\mu mol \) photons m\(^{-2} \) s\(^{-1} \)); and high light (HL, > 200 \(\mu mol \) photons m\(^{-2} \) s\(^{-1} \)). At ML and HL, the growth rate was influenced by temperature (\( p < 0.01 \)). At \(25^\circ C\), the growth rate was significantly (\( p < 0.01 \)) increased compared with the \(20^\circ C\) cultures. Light was found to be saturating at 100 \(\mu mol \) photons m\(^{-2} \) s\(^{-1} \) for \(20^\circ C\) with the maximum growth rate of \(0.95 \ day^{-1} \). At \(25^\circ C\), growth was saturated at 175 \(\mu mol \) photons m\(^{-2} \) s\(^{-1} \) with a maximum growth rate of \(1.41 \ day^{-1} \). For both temperatures, no photoinhibition was found up to 500 \(\mu mol \) photons m\(^{-2} \) s\(^{-1} \) (Fig. 1). Fucoxanthin and chlorophyll-\(a\) content were both irradiance and temperature-dependent (Fig. 2). Fucoxanthin content was significantly lower at ML and HL compared to LL for each temperature (\( p < 0.01 \)). However, when comparing both temperatures, the fucoxanthin content significantly differed only at ML and HL (\( p < 0.05 \)). For fucoxanthin and chlorophyll-\(a\) productivity (Fig. 3), the highest value was found at around 100–125 \(\mu mol \) photons m\(^{-2} \) s\(^{-1} \) for both temperatures. The pigment productivity was not significantly different when comparing both temperatures (\( p > 0.05 \)).

Effect of different POME fractions

When cultivating \(P. \) tricornutum on different sterilized-unfiltered POME concentrations (Fig. 4) increasing POME concentrations slightly promoted the cellular fucoxanthin content, from 0.04 ± 0.01 for the 15% to 0.05 ±
0.03 pg cell\(^{-1}\) for the 50% POME culture but these results were not significant. An optimum growth rate was recorded at 30% \(v/v\), followed by 50% \(v/v\), and 15% \(v/v\) of POME (Fig. 4). However, the growth rate at 50% \(v/v\) POME did not significantly differ from the 30 and 15% \(v/v\) POME conditions. In addition, the fucoxanthin productivity was significantly higher at 30% \(v/v\) POME compared to 50% \(v/v\) POME (\(p < 0.05\)) Given this result, 30% POME was used for all subsequent experiments.

**Effect of nutrient enrichment on the cell density and fucoxanthin productivity**

The addition of external nitrogen and/or phosphorus resulted in different N/P molar ratios in the media as well as final cell densities and fucoxanthin productivity (Table 2). At 20 and 25 °C, the highest cell density was found when *P. tricornutum* was grown on f/2 medium, followed by POME, low nitrate, high phosphate, and trace elements (NPT); POME, low urea and high phosphate (UP); and POME, low nitrate and high phosphate (NP). Lowest final cell densities were found in the cultures grown on POME supplemented with phosphorus (P-High, P-Low) followed by control medium (30% POME, no nutrient addition). The addition of nitrogen in the form of urea (U) resulted in significantly higher final cell densities compared to the control medium (30% POME, no nutrient addition) and phosphorus additions (P-High, P-Low). The addition of trace elements (NPT) did not significantly influence the cell density compared to NP and UP (\(p > 0.05\)).

With respect to fucoxanthin, the highest productivity was found in the f/2 medium, followed by NPT medium (\(p < 0.05\)). The addition of both nitrogen and phosphorus to the 30% POME medium enhanced fucoxanthin productivity compared to the control (30% POME, no nutrient addition) (\(p < 0.05\)). Furthermore, the addition of urea alone increased fucoxanthin productivity 6.3-fold compared to the control, while the addition of phosphorus alone (P-Low and P-High) was not significantly different from the control (\(p > 0.05\)). The addition of urea only (U) resulted in a high fucoxanthin productivity, similar to NP and UP cultures which had access to external phosphorus as well as external nitrogen (\(p > 0.05\)) (Table 2).
Fig. 4 Fucoxanthin productivity (dashed line), fucoxanthin content (full line) and growth rate (bar) of P. tricornutum cultivated on the mixture of seawater and unfiltered POME concentrations at 23 °C and 100 μmol photons m⁻² s⁻¹. Average values of duplicate cultures are shown. Error bars indicate the SD of the mean.

Optimization of fucoxanthin productivity as a function of salinity, temperature, and urea addition.

A second-order polynomial equation in uncoded units was found to explain the fucoxanthin productivity (Eq. 4):

\[
\text{Fucoxanthin productivity (μg L}^{-1}\text{d}^{-1}) = -781 + 72.3X_1 - 0.520X_2 - 0.015X_3
- 1.670X_1^2 - 0.027X_2^2 - 0.003X_3^2
+ 0.084X_1X_2 + 0.021X_1X_3
+ 0.003X_2X_3
\]  

(4)

where \( X_1, X_2, \) and \( X_3 \) are temperature (°C), salinity (ppt), and urea (mg L⁻¹), respectively.

ANOVA was employed to test the significance of the fit of the proposed model. A \( p \) value < 0.05 was used to demonstrate the significance of the factors and the interactions. A linear effect indicates that fucoxanthin productivity was positively correlated with the tested condition. On the other hand, a quadratic effect indicates that the factor interacts itself and has an optimum value inside the tested range. As shown (Fig. 5), temperature, urea, and salinity were important factors to obtain the optimal fucoxanthin productivity. Furthermore, all tested two-way interactive effects between the factors were not significant (\( p > 0.05 \)). The optimum condition for fucoxanthin productivity was at a urea addition ranging between 80 and 85 mg L⁻¹, a salinity between 25 and 31 ppt, and a temperature between 21 and 23 °C.

As for final cell density, urea and temperature were found to be the factors mostly influencing cell density, while salinity only slightly influenced cell density (\( p = 0.057 \)). For final cell density, it was also shown that a urea level exceeding 60–100 mg L⁻¹ and a salinity exceeding 20–30 ppt resulted in lower cell densities. Finally, the optimum temperature in terms of cell density was estimated at 21.5–22 °C (Fig. 6). Overall, the optimum combination of environmental conditions with respect to cell density (8.13 × 10⁶ cells mL⁻¹) was found to be 21 °C, 23 ppt salinity, and 90 mg L⁻¹ urea addition.

Discussion

When focusing on pigments as value-added products at large-scale cultivation, it is vital to study the effects of light intensity on pigment productivity. In diatoms, including P. tricornutum, chlorophyll-\( a \) and fucoxanthin are the major light harvesting pigments (Gómez-Loredo et al. 2016). As a result, cellular contents of both pigments showed a classical strong irradiance relationship when considering cellular content (Fig. 2). This implies that supraoptimal irradiance levels in large-scale cultivation setups would not benefit fucoxanthin productivity, even when growth rates are high (Fig. 1). However, by adjusting the cell density, the average irradiance received by the cells can be adjusted (Richmond 2013). The optimum light intensity for pigment productivity was at around 80–125 μmol photons m⁻² s⁻¹ (Fig. 3). In addition, temperature influenced the pigment content and productivity only at the high light intensities (HL). Therefore, the productivity of fucoxanthin of P. tricornutum in a bioreactor and outdoor cultivation could be optimized by controlling the light intensity at a relatively low level or adjusting the cell density.

Previous studies showed that in order to reduce contaminants and turbidity, a pretreatment process of wastewater is important for microalgal growth. Wang et al. (2015) found that centrifugation of digested poultry manure effluent gave higher biomass of Chlorella vulgaris compared to autoclaving (120 °C, 30 min). Cho et al. (2011) found that filtration (0.2 μm) was more efficient
Fig. 5 Response surface plots (3D) showing the effects of temperature (°C), urea (mg L\(^{-1}\)), and salinity (ppt) on fucoxanthin productivity (\(P_f\)) (µg L\(^{-1}\) day\(^{-1}\)) generated by *P. tricornutum* at hold value 50 mg l\(^{-1}\) (a), 20 ppt (b), and 22.5 °C (c). The optimal fucoxanthin productivity (38 µg L\(^{-1}\) day\(^{-1}\)) was found at 23 °C, 31 ppt salinity, and 85 mg L\(^{-1}\) urea addition.
compared to UV-B irradiation to treat secondary municipal wastewater as growth medium for Chlorella sp. with the aim to produce lipids. Shah et al. (2016) reported that different dilutions of raw POME wastewater mixed with sea water influenced the dry weight of Tetraselmis suecica and Nannochloropsis oculata due to the changes in nutrient
composition and light penetration in the medium. However, at high treated POME concentrations, the high organic substrate together with high turbidity inhibited the growth rate of microalgae (Nur et al. 2017). In our experiments, growth rate was higher at 30% POME (v/v) than at 15% POME (v/v) which might be due to enhanced nutrient concentrations in the 30% POME cultures. On the other hand, when 50% v/v POME was used, the growth rate was lower, likely related with enhanced turbidity in the bottles, lowering light availability and thus increasing the fucoxanthin content as found in the first experimental series (Fig. 2). This finding was supported by Hadiyanto et al. (2017) who reported that the optimum growth rate and biomass production for the marine alga *Nannochloropsis* sp. was found on 30% digested and filtered POME. On the other hand, the cellular fucoxanthin content at the 50% POME condition was only slightly enhanced and did not significantly differ from the lower POME concentrations. This indicates that other factors might explain the observed lower growth rates in the 50% POME cultures. High ammonia concentrations in the wastewater are considered inhibitory for *P. tricornutum* (Ayre et al. 2017). Furthermore, the presence of potentially toxic phenolic compounds in digested POME could also lower the growth rate of *P. tricornutum* (Limkhansuwan and Chaiprasert 2010; Duan et al. 2017). Overall, a 30% POME fraction was found to be the most suitable medium to optimize fucoxanthin productivity in *P. tricornutum*. In our experiments, *P. tricornutum* was found to grow on 30% POME alone (control, no nutrient addition) at both temperatures. The N/P inorganic nutrient ratio (13:1) contained in this POME medium was close to the Redfield ratio (16:1). Yet, the addition of nitrogen species (nitrate, urea) strongly increased growth rate and fucoxanthin productivity. Apart from enhancing growth rate and pigment productivity, the addition of external inorganic nitrogen beyond the Redfield ratio would guarantee efficient phosphorous removal from the wastewater. The result was also supported by Halim et al. (2016) who reported that the addition of inorganic sodium nitrate and dihydrogen phosphate into digested POME increased the biomass production of *Nannochloropsis* sp. and *C. vulgaris*, compared to the control POME medium. However, in our finding, the addition of urea was found to be a good alternative to nitrate addition.

Our experiments show that the addition of sole urea enhanced the fucoxanthin productivity and cell density as compared with cultivation on 30% (v/v) POME alone. At the same time, addition of phosphate alone did not result in a significantly higher fucoxanthin productivity and final cell densities. This implies that the availability of phosphorous in 30% v/v POME does not limit the growth of *P. tricornutum* under our experimental conditions. This finding was supported by a previous study who reported that a high amount of external nitrate increased the fucoxanthin productivity, growth rate, and biomass production of *P. tricornutum* and *Odontella aurita* cultivated on a modified f/2 and a modified L1 medium, respectively (Xia et al. 2013; McClure et al. 2018). Furthermore, early nitrogen limitation reduced the fucoxanthin productivity of *P. tricornutum* cultivated on a modified f/2 medium (Alipanah et al. 2015).

The present study shows that the replacement of nitrate with urea did not give a significantly different fucoxanthin productivity. This is in accordance with a previous study showing that urea is a suitable nitrogen source for *P. tricornutum* growth and cell productivity (Guzmán-Murillo et al. 2007). The strategy of adding only urea instead of other nitrogen sources (e.g., nitrate) or phosphorus could therefore significantly save production costs as it is a readily available and cheap nutrient source. However, the addition of urea needs to be more explored and optimized due to the potential toxic effects of high ammonium or ammonia concentrations derived from urea conversion (Tuantet et al. 2014; Agwa and Abu 2016).

To apply cultivation at a large scale, the environmental factors that are associated with the production cost should be taken into account. Salinity, temperature, and urea addition were therefore evaluated with respect to the optimal fucoxanthin productivity. In this final experimental series, increasing urea and salinity were found to have a positive effect on the fucoxanthin productivity. The optimum temperature when considering fucoxanthin productivity was found at around 21–22 °C. Previous studies showed that the optimum temperature for biomass production of *P. tricornutum* was 21.5 °C, at a salinity range between 20 and 30 psu (Yongmanitchai and Ward 1991; Liang et al. 2014), which is in agreement with our results. Furthermore, the optimal urea addition was found at around 80–85 mg L⁻¹. Possibly, high urea concentrations might lead to growth reduction through toxicity effects. Previous researchers demonstrated that microalgae can assimilate urea as a source of nitrogen by converting it to NH₄⁺ and CO₂ through urease activity or ammonium and bicarbonate via ATP-urea amidolyase (Bekheet and Syrett 1977; Solomon and Gilibert 2008). This might lead to high ammonium or ammonia concentrations, which tend to be toxic for diatoms such as *P. tricornutum*, as well as other algae such as chlorophytes (Fidalgo et al. 1995; Collos and Harrison 2014). Setyoningrum and Nur (2015) also found that optimum urea addition for the production of C-phycocyanin by *Spirulina platensis* was 115 mg L⁻¹. Moreover, our study showed that the interactive effect between variables was not significantly influencing the fucoxanthin productivity. It revealed that the combined factors did not influence each other significantly during cultivation (Fig. 5, Table 3). Thus, the strategy by maintaining the factors at optimum conditions could enhance the fucoxanthin productivity.
Conclusion

The present study has shown that *P. tricornutum* may be used for large-scale cultivation on 30% v/v POME with the aim to produce a value-added compound such as fucoxanthin. However, some issues need to be addressed. These include optimal irradiance levels, which are likely much lower than outdoor incident solar irradiance levels, especially in the (sub)tropics. Furthermore, optimal temperature and salinity conditions need to be addressed as shown in our final experimental series. It is considered that 30% of filtered POME is the potential medium for *P. tricornutum* to replace commercial nutrients and produce high amounts of fucoxanthin productivity. However, the addition of urea greatly enhances the biomass and fucoxanthin productivity while at the same time ensuring the removal of phosphorous from the wastewater. E-supplementary data of this work can be found in online version of the paper.

Acknowledgments This project was funded by Lembaga Pengelola Dana Pendidikan (LPDP), Kementerian Keuangan, Republik Indonesia. Reference no. PRJ-72/LPDP.3/2016.

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References

Agwa OK, Abu GO (2016) Influence of various nitrogen sources on biomass and lipid production by *Chlorella vulgaris*. Brit Biotechnol J 15:1–13

Alipanah L, Kholf J, Winge P, Bones AM, Brembu T (2015) Whole-cell response to nitrogen deprivation in the diatom *Phaeodactylum tricornutum*. J Exp Bot 66:6281–6296

Ayre JM, Moheimani NR, Borowitzka MA (2017) Growth of microalgae on undiluted anaerobic digestate of piggery effluent with high ammonium concentrations. Algal Res 24:218–226

Bekheet IA, Syrett PJ (1977) Urea-degrading enzymes in algae. Br Phycol J 12:137–143

Borowitzka MA (2016) Algal physiology and large-scale outdoor cultures of microalgae. In: Borowitzka M, Beardall J, Raven J (eds) The physiology of microalgae. Developments in applied phycology, vol 6. Springer, Cham, pp 601–652

Cho S, Luong TT, Lee D, Oh Y, Lee T (2011) Reuse of effluent water from a municipal wastewater treatment plant in microalgae cultivation for biofuel production. Bioresour Technol 102:8639–8645

Choi HJ, Lee SM (2015) Effect of the N/P ratio on biomass productivity and nutrient removal from municipal wastewater. Bioprocess Biosyst Eng 38:761–766

Collos Y, Harrison PJ (2014) Acclimation and toxicity of high ammonium concentrations to unicellular algae. Mar Pollut Bull 80:8–23

De Francisci D, Su Y, Itali A, Angelidaki I (2018) Evaluation of microalgae production coupled with wastewater treatment. Environ Technol 39:581–592

Duan W, Meng F, Lin Y, Wang G (2017) Toxicological effects of phenol on four marine microalgae. Environ Toxicol Pharmacol 52:170–176

Fidalgo JP, Cid A, Abalde J, Herrero C (1995) Culture of the marine diatom *Phaeodactylum tricornutum* with different nitrogen sources: growth, nutrient conversion and biochemical composition. Cah Biol Mar 36:165–173

Gómez-Loredo A, Benavides J, Rito-Palomas M (2016) Growth kinetics and fucoxanthin production of *Phaeodactylum tricornutum* and *Isochrysis galiana* cultures at different light and agitation conditions. J Appl Phycol 28:849–860

Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Press, New York, pp 26–60

Guzmán-Murillo MA, López-Bolaños CC, Ledesma-Verdejo T, Gabriela R-L, Cadena-Roa MA, Ascencio F (2007) Effects of fertilizer-based culture media on the production of exocellular polysaccharides and cellular superoxide dismutase by *Phaeodactylum tricornutum* (Bohlin). J Appl Phycol 19:33–41

Hadiyanto H, Soetrisnanto D, Silviana S, Mahdi MZ, Titisari YN (2017) Evaluation of growth and biomass productivity of marine microalgae Nannochloropsis sp. cultured in palm oil mill effluent (POME). Philippine J Sci 146:355–360

Halim FTA, Guo X, Su G, Ngee HL, Zeng X, He N, Lin L, Danquah MK (2016) Sustainable microalgae-based palm oil mill effluent treatment process with simultaneous biomass production. Can J Chem Eng 94:1848–1854

Kim SM, Jung YJ, Kwon ON, Cha KH, Um BH, Chung D, Pan CH (2012) A commercial source of fucoxanthin extracted from the microalgae *Phaeodactylum tricornutum*. Appl Biochem Biotechnol 166:1843–1855

Kulk G, Van de Poll WH, Visser RJW, Buma AGJ (2011) Distinct differences in photoacclimation potential between prokaryotic and eukaryotic oceanic phytoplankton. J Exp Mar Biol Ecol 298:63–72

Leu S, Boussiba S (2014) Advances in the production of high-value products by microalgae. Ind Biotechnol 10:169–183

Liang Y, Sun M, Tian C, Cao C, Li Z (2014) Effects of salinity stress on *P. tricornutum* cell response to nitrogen deprivation in the diatom *Chaetoceros gracilis* (Bacillariophyceae). Bot Mar 57:469–476

Limkhansuwam V, Chaiprasert P (2010) Decolorization of molasses melanoids and palm oil mill effluent phenolic compounds by fermentative lactic acid bacteria. J Environ Sci 22:1209–1217

McClure DD, Luiz A, Gerber B, Barton GW, Kavanagh JM (2018) An investigation into the effect of culture conditions on fucoxanthin production using the marine microalgae *Phaeodactylum tricornutum*. Algal Res 29:41–48

Nur MMA, Buma AGJ (2018) Opportunities and challenges of microalgal cultivation on wastewater, with special focus on palm oil mill effluent and the production of high value compounds. Waste Biomass Valor. https://doi.org/10.1007/s12649-018-0256-3

Nur MMA, Hadiyanto H (2013) Utilization of agroindustry wastewater as growth medium for microalgae based bioenergy feedstock in Indonesia (an overview). J Sustain 1:3–7

Nur MMA, Setyoningrum TM, Budiaman IGS (2017) Potency of *Botryococcus braunii* cultivated on palm oil mill effluent (POME) wastewater as the source of biofuel. Environ Eng Res 22:417–425

Olguin EJ (2012) Dual purpose microalgae–bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a biorefinery. Biotechnol Adv 30:1031–1046

Pittman JK, Andrew PD, Osundeò O (2011) The potential of sustainable algal biofuel production using wastewater resources. Bioresour Technol 102:17–25

Poh PE, Yong W-J, Chong MF (2010) Palm oil mill effluent (POME) characteristic in high crop season and the applicability of high-rate anaerobic bioreactors for the treatment of POME. Ind Eng Chem Res 49:11732–11740
Ravindran B, Gupta SK, Cho WM, Kim JK, Lee SR, Jeong KH, Lee DJ, Choi HC (2016) Microalgae potential and multiple roles-current progress and future prospects—an overview. Sustainability 8:1215–1230
Richmond A (2013) Biological principles of mass cultivation of photo-autotrophic microalgae. In: Richmond A, Hu Q (eds) Handbook of microalgal culture: applied phycology and biotechnology, 2nd edn. John Wiley & Sons, Chichester, pp 171–204
Setyoningrum TM, Nur MMA (2015) Optimization of C-phycocyanin production from *Spirulina platensis* cultivated on mixotrophic condition by using response surface methodology. Biocatal Agric Biotechnol 4:603–607
Shah SMU, Ahmad A, Othman MF, Abdullah MA (2016) Effects of palm oil mill effluent media on cell growth and lipid content of *Nannochloropsis oculata* and *Tetraselmis suecica*. Int J Green Energy 13:200–207
Solomon CM, Glibert PM (2008) Urease activity in five phytoplankton species. Aquat Microb Ecol 52:149–157
Tuantet K, Janssen M, Temmink H, Zeeman G, Wijffels RH, Buisman CJN (2014) Microalgae growth on concentrated human urine. J Appl Phycol 26:287–297
Van de Poll WH, Visser RJW, Buma AGJ (2007) Acclimation to a dynamic irradiance regime changes excessive irradiance sensitivity of *Emiliania huxleyi* and *Thalassiosira weissflogii*. Limnol Oceanogr 52:1430–1438
Van Leeuwe MA, Villerius LA, Roggeveld J, Visser RJW, Stelfs J (2006) An optimized method for automated analysis of algal pigments by HPLC. Mar Chem 102:267–275
Wang M, Wu Y, Li B, Dong R, Lu H, Zhou H, Cao W (2015) Pretreatment of poultry manure anaerobic-digested effluents by electrolysis, centrifugation and autoclaving process for *Chlorella vulgaris* growth and pollutants removal. Environ Technol 36:837–843
Whitton R, Mével AL, Pidou M, Ometto F, Villa R, Jefferson B (2016) Influence of microalgal N and P composition on wastewater nutrient remediation. Water Res 91:371–378
Xia S, Wang K, Wan L, Li A, Hu Q, Zhang C (2013) Production, characterization, and antioxidant activity of fucoxanthin from the marine diatom *Odontella aurita*. Mar Drugs 11:2667–2681
Yongmanitchai W, Ward OP (1991) Growth of and omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions. Appl Environ Microbiol 57:419–425