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ABSTRACT

Recently, the mass scale production of fucoxanthin and fatty acid from microalgae has increased. However, a new and low cost cultivation strategy is needed to enhance the production. This work investigated the potency of mixed diatom and green algae isolated from Morosari Bay, Java Sea, Indonesia, to enhance the production of fucoxanthin and lipid by employing mixotrophic condition. A four stepwise experiment was done to study the environmental and nutritional factors, as well as to optimize the selected parameters by employing Box Behnken response surface methodology. The mixed algae was found to grow well on palm oil mill effluent (POME). Changing condition from autotrophic to mixotrophic decreased the pigment content. The highest fucoxanthin concentration (6 mg L^{-1}) was found on the glycerol addition compared to other organic carbon sources. The optimal condition to produce 160 mg L^{-1} d^{-1} biomass and 60 mg L^{-1} d^{-1} lipid was found on 30% POME, supplemented with 3 g L^{-1} glycerol and 200 mg L^{-1} urea at 30 °C. Mono unsaturated fatty acid (MUFA) was the most dominant fatty acid class found in the mixed algae.

1. Introduction

Recently, the production of high valuable compounds from microalgae has increased. Microalgae could produce several products from one single cells such as pigments, protein, lipid, and carbohydrate, which is well known as biorefinery process (Gifuni et al., 2019). Some pigments such as fucoxanthin and phycocyanin could lower the risk of diabetes, obesity, chronic inflammation, and hypertension (Gammone and D'Orazio, 2015). Meanwhile fatty acid composition of microalgae contains rich of saturated fatty acid (SFA), mono unsaturated fatty acid (MUFA) and poly unsaturated fatty acid (PUFA) which is potential as biodiesel source, bioplastic source, and replacing fish fatty acid (Stansell et al., 2012; Nur and Buma, 2019). However, to make the production become more sustainable and feasible, some environmental and nutritional factors need to be investigated.

Indonesia is known as the largest palm oil mill effluent producer worldwide. Along 1 ton of palm oil production, about 50% of wastewater was generated. In the previous research, microalgae could utilize digested palm oil mill effluent (POME) for value added product since POME contains rich of macro and micronutrient (Nur et al., 2019). By using POME, high cost of fertilizer and water supply during the cultivation could be pressed. Nur et al. (Shah et al., 2016) succesfully utilized POME supplemented with urea as medium growth for marine diatom *Phaeodactylum tricornutum* to produce fucoxanthin. Shah et al. (Adarme-Vega et al., 2012) used

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

Type of experiment	Factors	Design of experiment	Responses
Experiment 1	Light intensity, temperature	n/a	Growth rate, P-biomass, P-lipid, lipid content
Experiment 2	POME	n/a	Growth rate, lipid productivity
Experiment 3	Organic carbon sources	n/a	Growth rate, P-biomass, P-lipid, lipid content, pigment production
Experiment 4	Glycerol, temperature, urea	Box-behnken	Growth rate, P-biomass, P-lipid

marine microalgae *Nannochloropsis oculata*, and *Tetraselmis suecica* to produce lipid and remove nutrients from POME. However, to make the production become more sustainable, the biomass production need to be enhanced before it can be applied in the biorefinery process to valorize a whole biomass into several valuable products (Moreno-Garcia et al., 2017).

Previous research has succesfully enhanced the production of biomass from microalgae growing on POME by using mixotrophic condition. Nur and Hadiyanto (2015) utilized 40% POME and added D-glucose to enhace the production of lipid from *Chlorella vulgaris*. Cheah et al. (2018) reported that the biomass and lipid production were enhanced in POME medium supplemented with urea, glucose and glycerol. Yang and Wei (2020) reported that mixotrophic condition could enhance the production of fucoxanthin from *P. tricornutum*. In mixotrophic condition, organic carbon is introduced as a source of energy along with inorganic carbon with the presence of light (Subramanian et al., 2016; Gupta et al., 2016). Therefore, it is important to explore the source of organic carbon for the best and inexpensive cost in large scale cultivation (Cheah et al., 2018). However, the cultivation on mixotrophic condition has not well explored to produce co-production, fucoxanthin and lipid growing on POME. Here, a mixture of diatom and green marine algae collected from Morosari Bay, in Java Sea, Indonesia, were employed to obtain high fucoxanthin and lipid production. The objective of this research was to investigate the effect of environmental and nutritional conditions to the production of fucoxanthin and lipid, as well as optimizing the selected parameters by using Box Behnken response surface methodology.

2. Materials and methods

2.1. Experimental setup

A mixed culture was obtained near the mangrove park at 6°54′56.4″S 110°28′58.0″E, Morosari Bay, Demak, Central Java, Indonesia. The sampling was done in 1 m depth. A microscope observation indicated that the mixed culture mostly contained *Nannochloropsis* sp. and *Thalassiosira* sp. Based on Chemtax pigment analysis, the culture contained 40% of diatom and 60% green algae. The culture was maintained at 27 °C in a 16:8 h light: dark cycle, and at an irradiance of 150 μmol m^{-2} s^{-1} using f/2 medium supplemented with silicate (100 μM) and $NaHCO_3$ (2.38 mM). The culture was acclimated at least two weeks prior to experiments.

A stepwise experiment consisted of four experiments were done (Table 1). Firstly, the effect of irradiance and temperature were investigated on growth rate, lipid content, biomass, and lipid productivity, grown on standard f/2 medium (Experiment 1). Secondly, the mixed culture was grown on different POME fractions to determine the optimal POME concentration for lipid productivity and the growth (Experiment 2). Different organic carbon sources were also investigated to obtain the best organic carbon candidate for the growth, biomass, lipid, and pigments production (Experiment 3). Since in the previous experiment 3, the best organic carbon source was found on glycerol, then glycerol concentration was optimized along with temperature, and urea concentration by using response surface methodology to unravel the best condition for the lipid productivity (Experiment 4). Experimental conditions for each of the experiment are described below.

2.1.1. Experiment 1: effect of temperature and light intensity

In Experiment 1, the mixed culture was grown using f/2 medium. A 50 mL plastic culture flask (Grenier Bio-One) was used with 50 mL working volume. An inoculum of 2% (v v^{-1}) of the culture was used to start the experiments. The flasks were placed in a photosynthetron illuminated by 250 W lamp and equipped with a water bath (±0.1 °C). Light intensity was varied at 10 different levels ranging from 45 up to 800 μmol m^{-2} s^{-1}. Temperature was varied at 25 and 30 °C. Samples (2 mL) were taken daily and immediately measured by using spectrophotometer Hach DR3400 to obtain the optical density at 750 nm. Growth rates were calculated from the optical density as described in Eq (1). The cultures were harvested for lipid analysis at the end of the exponential growth phase (7–14 days).

2.1.2. Experiment 2: effect of different POME fractions on growth and lipid productivity

POME was obtained from a small factory in Indonesia as described previously after it was treated in a traditional anaerobic pond followed by aerobic pond (Nur et al., 2019). POME was filtered using GF/C filter paper to remove contaminants and the autoclaved at 121 °C for 15 min.

The mixed culture was grown in a 100 mL Erlenmeyer flask medium with working volume of 70 mL. Four different POME fractions were applied ranging from 10 to 70 v v^{-1}. An inoculum of 2% (v v^{-1}) of the culture was used to start the experiments. Sterile natural sea water was mixed to the autoclaved POME fractions. Salinity was adjusted to 35 PSU by using NaCl, temperature was set to 25 °C and initial pH was adjusted to 8 by using HCl or NaOH. The flask was placed in a U shaped water bath which was equipped with lamps. Initial light intensity was set to 200 μmol m^{-2} s^{-1} at the center of the flask. Since different POME fractions resulted different light penetration, light intensity was adjusted by using a shading cloth. Samples were taken daily to determine the growth rate by monitoring the optical density using spectrophotometer as described below. The cultures were harvested for lipid analysis at the end of the exponential growth phase.

2.1.3. Experiment 3: effect of organic carbon source on growth, pigment, and lipid production

The mixed culture was grown in a 100 mL Erlenmeyer flask with a working volume of 70 mL containing sterilized f/2 medium. An inoculum of 2% (v v^{-1}) of the culture was used to start the experiments. The flasks were placed in a U shape water bath as described above. Initial light intensity was set to 200 μmol m^{-2} s^{-1} at the center of the flask. In the beginning of the cultivation, salinity was set to 35 PSU by using NaCl, initial pH was set to 8 by using 0.1 M NaOH or HCl, and temperature was set to 25 °C. Different source of organic carbons were varied. For each the flask, 1 g L^{-1} of organic carbon source was introduced to the medium. The organic carbon sources were glucose (Glu), glycerol (Gly), sodium acetate (AC), and sucrose (S). Each day, the sample (2 mL) was taken immediately and measured the optical density to determine the growth rate. At the end of exponential phase (3 days), the culture was harvested and measured the pigment and lipid production as described in Eq. (1).

2.1.4. Experiment 4: optimization of urea, POME, and temperature on growth, and lipid productivity

Based on the previous finding, urea was considered toxic for microalgal growth when adding at high concentration (Shah et al., 2016). In addition, based on experiment 3, the best organic source was found on glycerol. Therefore, urea concentration, glycerol concentration, and temperature were all expected to show optima with respect to growth and lipid productivity. Thus, the optimum growth condition, the interaction effects, and the significance of urea, glycerol concentration and temperature on the growth, biomass, and lipid productivity were studied using the Box Behnken design (BBD) response surface methodology (RSM) with 13 total experimental runs. The ranges used for these experiments were 1, 2.5, and 4 g L^{-1} for glycerol (×1); 25, 30, and 35 °C for temperature (×3); and 50, 275, and 500 mg L^{-1} for urea addition (×3). An additional cultivation using f/2 medium was employed as a control medium.

The experiment was carried out in a U shaped water bath equipped with lamps. A 10% of inoculate was cultured in a 100 mL Erlenmeyer flask with working volume of 60 mL. The media was consisted of 30% autoclaved and filtered POME and diluted with sterile sea water. Initial light intensity was set to 200 μmol m^{-2} s^{-1} in the center of the flask. Initial pH was set to 8 by using 0.1 NaOH or HCl. Samples (2 mL) were taken daily to obtain the optical density by using spectrophotometer. At the end of exponential phase (4–6 days), the cultures were harvested for lipid analysis as described in Eq (1).

2.2. Analysis

2.2.1. Growth rate

The growth rate was calculated from the linear regression of the natural logarithm of optical density versus time. The optical density of the culture showed a strong relationship to the dry biomass (n = 6) as shown in Eq. 1

$$y = 0.95x + 0.01 \; R2 = 0.98 \tag{1}$$

where y is dry biomass (g L^{-1}), and x is the optical density of the culture at 750 nm. The biomass was harvested from 50 mL of the culture by using GF/C filter paper and washed twice by using 0.5 M NH_3HCO_3. Then, the filter was dried at 95 °C until a constant weight was reached.

Biomass and lipid determination were calculated based on Eq (2).

$$Px = \frac{X_t - X_0}{t_x - t_0} \tag{2}$$

where Px is biomass productivity (mg L^{-1} d^{-1}), X_t is biomass production (mg L^{-1}) at day t (t_x, day), X_0 is biomass production (mg L^{-1}) at day zero (t_0, day).

2.2.2. Lipid determination

Lipid concentration was determined gravimetrically as modified by previous report (Axelsson and Gentili, 2014). A volume of the sample (35 mL) which was reached at 0.7 optical density (680 nm) was centrifuged at 10,000 rpm for 15 min. The supernatant was discharged, the remaining pellet was freeze dried by using liquid nitrogen and then stored in a −80 °C freezer until further analysis. The pellet was extracted by a mixture of methanol and chloroform (2:1), followed by sonication for 5 min. The mixtures was then left overnight at room temperature. The pallet was separated from the liquid phase by using centrifuge at 10,000 rpm for 5 min. A 0.7% NaCl solution was added to the liquid mixtures to separated the layers. The lower layer, which contained lipid and chloroform, was transferred in a foil cup. The remaining chloroform was evaporated by drying the foil cup at 100 °C for 3 h, and then weighted gravimetrically. The content of the lipid was calculated by dividing the lipid concentration to the biomass production in dry weight, while the lipid productivity was calculated based on Eq. (2).

2.2.3. Pigments determination

A known volume and optical density of the sample was filtered through a GF/F filter. The filter obtained was freezed dried in a freeze-dryer (Unicryo MC 2 L freeze dryer, Germany) at − 60 °C for 24 h by using liquid nitrogen, and then stored at − 80 °C until analysis. Before analysis, the filters were freeze-dried at − 50 °C for 48 h and a pressure of 30 × 10^{-3} mbar. The sample was extracted by adding 5 mL of 90% cold acetone into the sample at 4 °C for 48 h. The extract was analyzed by using HPLC (Waters Model 2695), equipped with a cooled auto-sampler (4 °C), a Waters 996 diode-array detector, and a Zorbax C8 3.5 μm column as following the previous protocol (Van Leeuwe et al., 2006). The standard pigments were obtained from South Product Co. Ltd. (Okinawa, Japan).

2.2.4. Fatty acid class determination

Fatty acid class analysis was performed based on previous metod by transesterifying the lipid to fatty acid methyl esther (FAME)

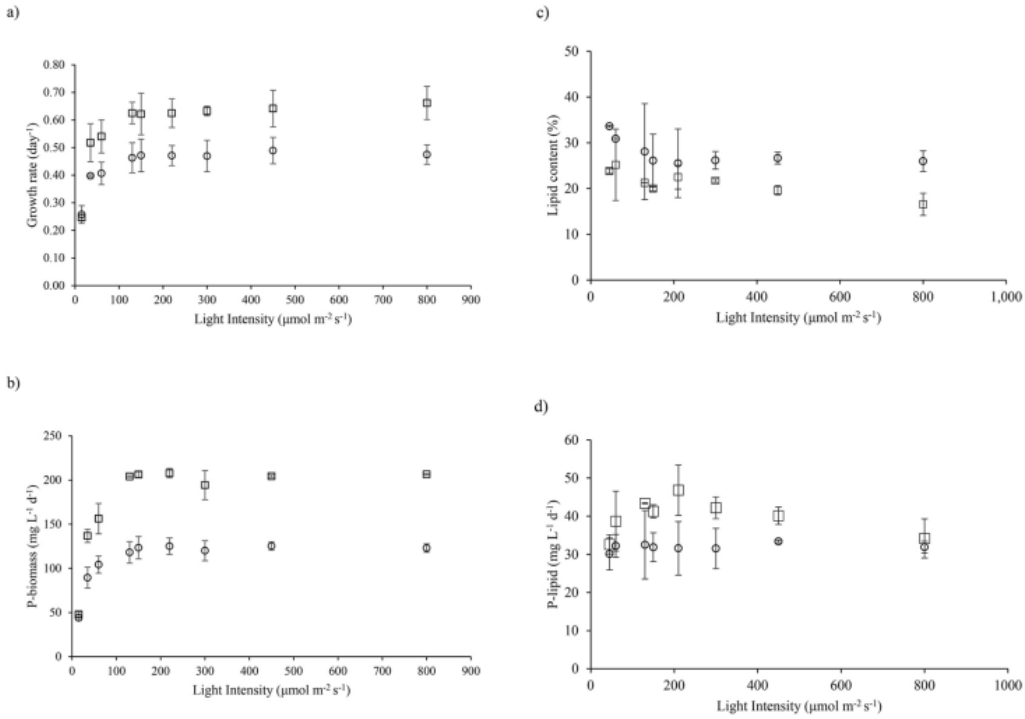

Fig. 1. Effect of light intensity (μmol m^{-2} s^{-1}) and temperature (°C) on a) growth (d^{-1}), b) biomass productivity (mg L^{-1} d^{-1}), c) lipid content (%), and d) lipid productivity (mg L^{-1} d^{-1}) of the mixed microalgae grown at ○ (25°C) and □ (30°C). Average values of triplicate cultures are shown. Error bars indicate the SD of the mean.

using mild reaction (Boelen et al., 2017a). The remaining pigment in the FAME sample was separated by using small Al_2O_3 column using dichloromethane (DCM) as eluent. FAME injected in a gas chromatograph (Hewlett-Packard 5890 series II) equipped with a flame-ionization detector (FID) using a Hewlett-Packard 7673 GC-SFC injector. Restek RTX-2330 (30 m × 0.25 mm x 0.020 μm) was employed as the column. The injection temperature was set to 250 °C, and the detector temperature was set to 250 °C. The column was kept at 120 °C for 2 min, then the temperature was increased by 3 °C min^{-1} until 220 °C and maintained at 220 °C for 10 min. Peaks were identified by comparing their retention times to the polyunsaturated fatty acid No. 1 standard mixture (Matreya, USA) and quantified by comparing to the internal standard.

2.3. Statistical analysis

Chemtax software (open access, v.195) was used to analyze the taxonomy group of microalgae based on pigment markers in the mixed algae. Minitab ver. 18. was used for all statistical analyses, including BBD RSM design and evaluation. Difference between treatments were analyzed using analysis of variance (ANOVA) with a p value of 0.05. Post hoc tests were performed for pairwise comparisons. The experimental results were obtained for a minimum of two replicates and expressed using averages and standard deviations (±SD).

3. Results and discussion

3.1. Effect of light intensity and temperature on the growth rate and lipid productivity

In the first experiment, the mixed algae consisted of green algae and diatom was cultivated on f/2 medium at different light intensities and two temperatures. Light intensity was divided to three levels; low light (LL, 15–100 μmol m^{-2} s^{-1}), medium light (ML, 100–300 μmol m^{-2} s^{-1}), and high light (HL, 300–800 μmol m^{-2} s^{-1}). In this experiment, growth rate was significantly influenced by temperature ($p < 0.05$) except at very low light intensity (5 μmol m^{-2} s^{-1}). Light intensity significantly inflenced the growth rate from LL to ML ($p < 0.05$). The optimum light intensity was found at 130 μmol m^{-2} s^{-1} for both temperatures. Biomass productivity was also influenced by light intensity and temperature. Higher temperature resulted higher biomass productivity. At above 130 μmol m^{-2} s^{-1}, the biomass productivity was not significantly influenced by light intensity (Fig. 1a and b). This result was in agreement to the previous research which stated that temperature and light intensity significantly inflenced the growth rate and biomass productivity of diatom *P. tricornutum* grown on f/2 medium (Nur et al., 2019).

In this experiment, the increasing of light intensity from LL to ML did not decrease the lipid content ($p > 0.05$). However, the interaction of temperature and light intensity significantly influenced the lipid content (Fig. 1c). It is found that at ML to HL, the

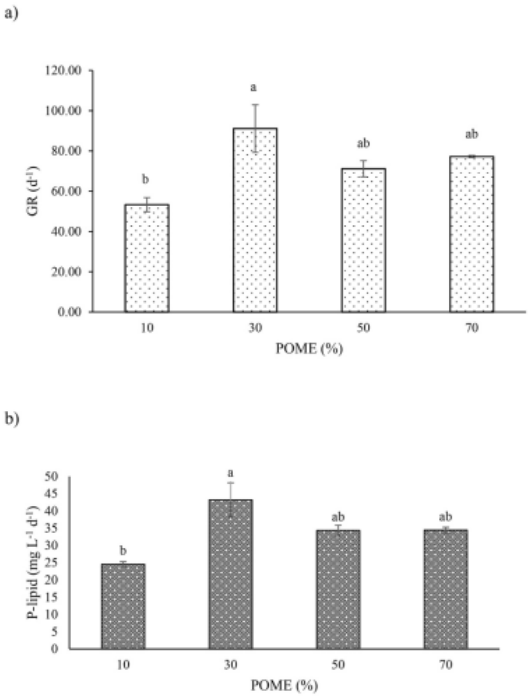

Fig. 2. Effect of POME (%) on a) growth rate (d^{-1}) and b) lipid productivity (mg L^{-1} d^{-1}). Average values of duplicate cultures are shown. Error bars indicate the SD of the mean.

Table 2
Growth rate, biomass productivity, lipid productivity, and lipid content per biomass dw (%) of mixed algae cultivated at 200 μmol m^{-2} s^{-1} with different media. Duplicate values are shown. Value after symbol ± shows standard deviation. Values do not share letters are significantly different ($p < 0.05$). GR is growth rate (d^{-1}), P_x is biomass productivity (mg L^{-1} d^{-1}), P_L is lipid productivity (mg L^{-1} d^{-1}), L is lipid content (%). Glucose (Glu), glycerol (Gly), sodium acetate (AC), sucrose (S), f/2 medium (Control).

Source	GR		P_x		P_L		L	
Glu	1.03	±0.02[a]	320.84	±1.12[a]	119.17	±1.18[a]	35.1	±0.30[a]
Gly	1.05	±0.01[a]	299.16	±0.90[a]	104.17	±9.19[a]	32.8	±2.73[a]
S	1.02	±0.01[a]	345.69	±8.06[a]	119.33	±5.66[a]	32.6	±0.81[a]
AC	1.04	±0.01[a]	321.47	±4.25[a]	116.67	±4.71[a]	34.3	±0.98[a]
Control	0.69	±0.03[b]	101.30	±5.37[b]	31.42	±1.77[b]	26.2	±0.54[b]

increasing of temperature decreased lipid content per dry weight of biomass from 25 to 16% ($p < 0.05$). Previous researcher recorded that lipid content of *Nannochloropsis* sp. and *Chlorella* sp decreased with the increasing of light intensity (Cheirsilp and Torpee, 2012). As in accordance to the lower growth rate found at low light, microalgae tend to accumulate higher lipid content due to unfavourable conditions. When high light is introduced, microalgae start to synthesize energy to produce biomass rather than accumulate it as lipid.

In this experiment, the highest lipid productivity was found at saturated light intensity (13–200 μmol m^{-2} s^{-1}) at 30 °C. At LL and HL, the increasing of temperature did not significantly influence the lipid productivity. This indicated that lipid productivity was influenced by biomass productivity and lipid content. In the previous result, it was found that lipid content was increased at HL, while the biomass productivity was found at saturated light intensity. To obtain high lipid productivity, ML was selected as the optimum light intensity for further experiment.

3.2. Effect of POME on the growth lipid content, biomass and lipid productivity

The increasing of POME fraction from 10 to 30% (v v^{-1}) significantly influenced growth rate and lipid productivity (Fig. 2). At above 30% POME, the increasing of the fraction was not significantly influenced the growth and the productivity ($p > 0.05$). POME fractions was not influencing the lipid content ($p < 0.05$), but the biomass production ($p < 0.05$). Since the nitrogen to phosphorus ratio in all POME fractions were not changing, lipid content was not significantly influenced by the dilution of POME. In addition, light intensity was maintained 200 μmol m^{-2} s^{-1} for all the dilutions, thus, different POME fractions would not affected the penetration of the light. However, higher POME fraction resulted lower biomass production due to phenolic compounds (Nur et al., 2019). Therefore, 30% POME was chosen for next experiment since it gave the highest biomass and lipid productivity.

Table 3
Pigment content (% w w^{-1}) of mixed algae cultivated at 200 μmol m^{-2} s^{-1} with different organic carbon source additions. Duplicate values are shown. Value after symbol ± shows SD. Values do not share letters are significantly different ($p < 0.05$). Glucose (Glu), glycerol (Gly), sodium acetate (AC), sucrose (S), f/2 medium (Control).

Carbon source	Fucoxanthin		Neoxanthin		Violaxanthin		Lutein		Chlorophyll-b		Chlorophyll-a	
Glu	0.16±	0.07^{a}	0.08±	0.01^{a}	0.01±	0.00^{a}	0.18±	0.05^{a}	0.18±	0.06^{a}	1.11±	0.32^{a}
S	0.20±	0.04^{a}	0.08±	0.01^{a}	0.01±	0.00^{a}	0.20±	0.02^{a}	0.20±	0.03^{a}	1.26±	0.28^{a}
AC	0.33±	0.06^{ab}	0.18±	0.05^{a}	0.13±	0.03^{b}	0.33±	0.07^{a}	0.29±	0.05^{ab}	1.81±	0.01^{ab}
Gly	0.59±	0.08^{b}	0.23±	0.03^{a}	0.19±	0.03^{b}	0.44±	0.08^{a}	0.36±	0.02^{ab}	2.84±	0.29^{ab}
Control	1.05±	0.03^{c}	0.20±	0.06^{a}	0.16±	0.04^{b}	0.64±	0.24^{a}	0.95±	0.39^{b}	5.96±	2.32^{b}

Table 4
Pigment production (mg L^{-1}) of mixed algae cultivated at 200 μmol m^{-2} s^{-1} with different organic carbon source additions. Duplicate values are shown. Value after symbol ± shows SD. Values do not share letters are significantly different ($p < 0.05$). Glucose (Glu), glycerol (Gly), sodium acetate (AC), sucrose (S), f/2 medium (Control).

Carbon source	Fucoxanthin		Neoxanthin		Violaxanthin		Lutein		Chlorophyll-b		Chlorophyll-a	
Glu	1.61±	0.68^{a}	0.84±	0.09^{b}	0.10±	0.00^{a}	1.78±	0.53^{a}	1.77±	0.59^{a}	11.19±	3.20^{a}
S	2.22±	0.49^{a}	0.86±	0.12^{b}	0.12±	0.01^{a}	2.18±	0.15^{a}	2.19±	0.40^{a}	13.74±	3.37^{a}
AC	3.36±	0.67^{ab}	1.87±	0.50^{ab}	1.29±	0.29^{bc}	3.29±	0.76^{a}	2.93±	0.42^{a}	18.33±	0.32^{a}
Gly	5.59±	0.72^{b}	2.15±	0.28^{a}	1.81±	0.31^{b}	4.12±	0.74^{a}	3.40±	0.17^{a}	26.75±	2.60^{a}
Control	3.65±	0.04^{ab}	0.69±	0.23^{b}	0.56±	0.14^{ac}	2.24±	0.92^{a}	3.34±	1.47^{a}	20.89±	8.84^{a}

3.3. Effect of different organic carbon source on the growth rate, biomass productivity, lipid productivity, and lipid content

Mixed algae was cultured on f/2 medium with the addition of external organic carbon to reach mixotrophic condition. Table 2 showed that the addition of organic carbon significantly influenced growth rate, biomass productivity, lipid content, and lipid productivity compared to control f/2 medium ($p < 0.05$). Growth rate and biomass productivity were enhanced up to 1.5 and 3-fold compared to f/2 medium only, while lipid productivity and lipid content were enhanced to 4-fold and 1.5-fold, respectively. However, different source of organic carbon did not significantly influence the growth and the productivity ($p > 0.05$). This implies that the mixed algae which contains green algae and diatom could utilize a wider variety of organic carbon source as a source of energy and store it in the form of lipid.

3.4. Effect of different organic carbon source on the pigment profile

A mixture of algae in this experiment was collected from Morosari Bay, Demak, Indonesia. By analyzing the profile of the pigments, a dominant strain could be distinguished. In our preliminary study, the mixed algae mostly contained diatom and green algae. This result is confirmed in Table 3 which the mixed algae growing on control f/2 medium contains violaxanthin, neoxanthin, and lutein, and chlorophyll-b as pigment markers for green algae, while fucoxanthin can be used as a pigment marker for diatom. Previous research reported that diatom such as *Phaeodactylum tricornutum* growing on a standard f/2 medium using 200 μmol m^{-2} s^{-1} could accumulate fucoxanthin up to 5 mg L^{-1} (Nur et al., 2019). In this research, fucoxanthin was found on 3.6 mg L^{-1} which indicated that diatom in the mixture could dominate up to 70% of the population.

Table 3 showed that the addition of organic carbon influenced the pigment content (normalized to dry biomass) compared to control which cultured in autotrophic condition ($p < 0.05$). The highest fucoxanthin was found on control f/2 medium, while the addition of organic carbon decreased the fucoxanthin content. The lowest fucoxanthin content (0.16%) was found when glucose was added to the medium followed by sucrose (0.2%), while the addition of glycerol resulted the highest fucoxanthin content (0.6%) in mixotrophic condition. The addition of organic carbon did not significantly influenced neoxanthin and lutein content ($p > 0.05$). For violaxanthin content, the addition of glucose and glycerol significantly lower the content ($p < 0.05$). The addition of glucose and sucrose also decreased chlorophyll *a* and *b* significantly compared to control medium ($p < 0.05$). In autotrophic condition, pigment is used to capture light as a source of energy. In mixotrophic condition, microalgae decreased the production of pigments since abundant energy source was introduced as organic carbon. Lewitus et al. (1991) reported that the addition of organic carbon could increase the biomass production of *Pyrenomonas salina* but decreased the photosynthetic activity.

As compared to other organic carbon sources, the addition of glycerol significantly increased the fucoxanthin, neoxanthin, and violaxanthin production up to 5.59 mg L^{-1}, compared to glucose and sucrose ($p < 0.05$) (Table 4). The addition of sodium acetate slightly reduced fucoxanthin, neoxanthin, and violaxanthin concentration compared to glycerol but not significant ($p > 0.05$). The addition of all organic carbon sources did not promote chlorophyll *a*, chlorophyll *b*, and lutein concentration ($p > 0.05$). Since glycerol is considered as a byproduct and relatively cheap, this organic carbon source was used in the next experiment to optimize the concentration.

In this experiment, the effect of organic carbon sources did not influence the ratio of diatom and green microalgae. Based on the pigment marker result, the ratio of fucoxanthin to chlorophyll-a, and the ratio of chlorophyll-b to chlorophyll-a were not significantly influenced by the organic carbon sources (Table 5). The ratio of fucoxanthin to chlorophyll-a was the pigment marker for diatom, while chlorophyll-b to chlorophyll-a was the pigment marker for green algae, as reported previously (Lee et al., 2020).

Table 5
Fucoxanthin to chlorophyll-a (Fuco/Chl-a) and chlorophyll-b to chlorophyll-a (Chl-b/Chl-a) ratio of mixed culture at different organic carbon sources. Duplicate values are shown. Value after ± symbol shows SD. Values do not share letters are significantly different ($p < 0.05$). Glucose (Glu), glycerol (Gly), sodium acetate (AC), sucrose (S), f/2 medium (Control).

Organic carbon source	Fuco/Chl-a	SD	Chl-b/Chl-a	SD
Glu	0.14	±0.01[a]	0.14	±0.03[a]
Gly	0.21	±0.01[a]	0.15	±0.00[a]
Control	0.19	±0.06[a]	0.16	±0.02[a]
AC	0.18	±0.02[a]	0.17	±0.00[a]
S	0.16	±0.00[a]	0.15	±0.00[a]

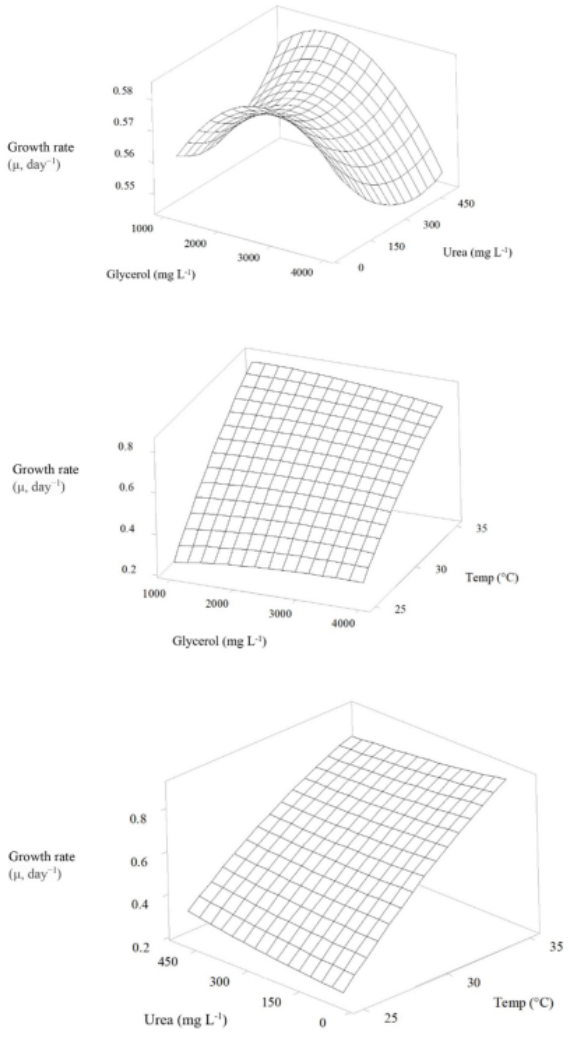

Fig. 3. Response surface plots (3D) showing the effects of temperature (°C), urea (mg L^{-1}), and glycerol (mg L^{-1}) on growth rate (μ, day^{-1}) of mixed algae.

3.5. Optimization of urea, glycerol and temperature on the growth, biomass and lipid productivity

In this experiment, the concentration glycerol, urea and temperature were further investigated and optimized by using Box Behnken response surface methodology. The result revealed that the increasing of glycerol and urea concentration did not significantly enhanced the growth rate independently ($p > 0.05$) (Fig. 3), while the increasing temperature from 25 to 35 °C significantly enhanced the growth rate ($p < 0.05$). The highest growth rate was found optimum at 30 °C. The interaction of glycerol and temperature slightly influenced the growth rate ($p = 0.058$), while the interaction of temperature and urea influenced the growth rate ($p < 0.05$).

For biomass productivity, the increasing of glycerol concentration alone, and temperature alone significantly enhanced the

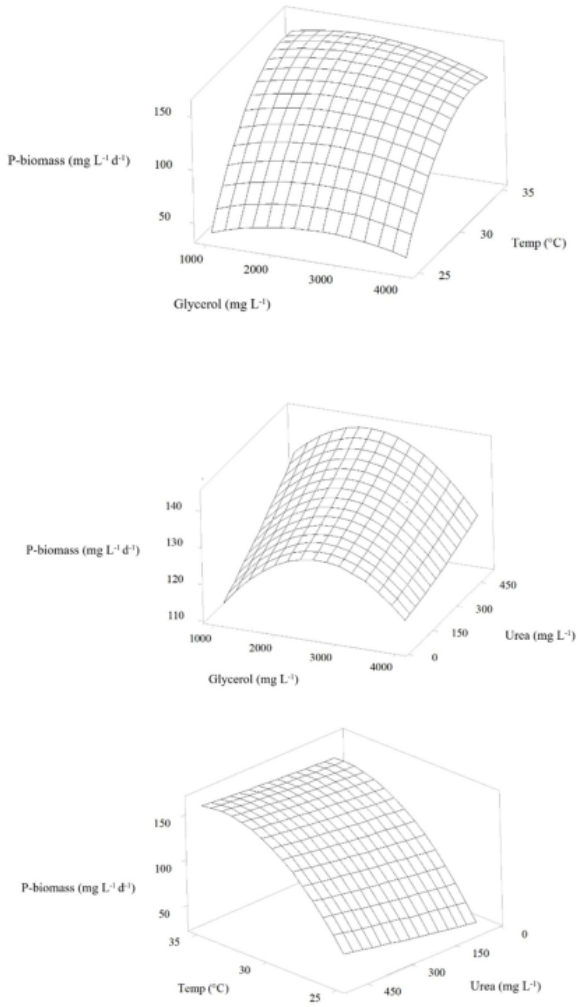

Fig. 4. Response surface plots (3D) showing the effects of temperature (°C), urea (mg L^{-1}), and glycerol (mg L^{-1}) on P-biomass (mg L^{-1} d^{-1}) of mixed algae.

productivity ($p < 0.05$) (Supplementary 2). The optimum glycerol concentration was found at 3 g L^{-1} and temperature at 30 °C. None of the interactions significantly influenced the biomass productivity ($p > 0.05$). The increasing of biomass is important for the fucoxanthin production since the pigment is linked to the biomass (Nur et al., 2019; Ishika et al., 2017). In mixotrophic condition using glycerol, the fucoxanthin production could reach up to 0.59% (Table 3) or around 1960 μg L^{-1} d^{-1} if the biomass productivity reached 299 mg L^{-1} d^{-1} (Table 1). This productivity is higher compared to previous research which resulted 400 μg L^{-1} d^{-1} fucoxanthin from *P. tricornutum* (Wang et al., 2018). The fucoxanthin productivity could be increased if light intensity is decreased below 100 μmol m^{-2} s^{-1} since fucoxanthin accumulate higher content at lower light intensity (Nur et al., 2019).

The increasing of temperature and urea independently influenced lipid content (Supplementary 3). The decreasing of urea concentration increased lipid content ($p < 0.05$), and the increasing of temperature decreased lipid content ($p < 0.05$). This result is in agreement in experiment 1 which showed that the increasing temperature 25–30 °C decreased lipid content. This indicated that at lower temperature, below 30 °C, the growth and biomass productivity was interupted, and resulted higher lipid content as shown in Fig. 3 and 4. This research was in line to the previous researchers who found that the lipid content of tropical microalgae, *Amphiprora* sp., was increased when the temperature was lowered from 32 to 28 °C (Teoh et al., 2013). Thus, the mixed algae seems accumulate higher energy storage as a form of lipid when temperature was set to below 30 °C, as an adaptive mechanism for enhancing the chance of survival in the lower temperature/cold environment.

Fig. 5 showed the effect of urea, glycerol and temperature on lipid productivity of mixed algae. The addition of glucose alone did not significantly enhance the productivity ($p > 0.05$), while the addition of temperature alone and urea alone could influence the productivity ($p < 0.05$). The optimum temperature was found at 30 °C ($p < 0.05$). The interaction of glycerol and urea significantly influenced the lipid productivity ($p < 0.05$). At low glycerol concentration, the addition of urea decreased the productivity, while at

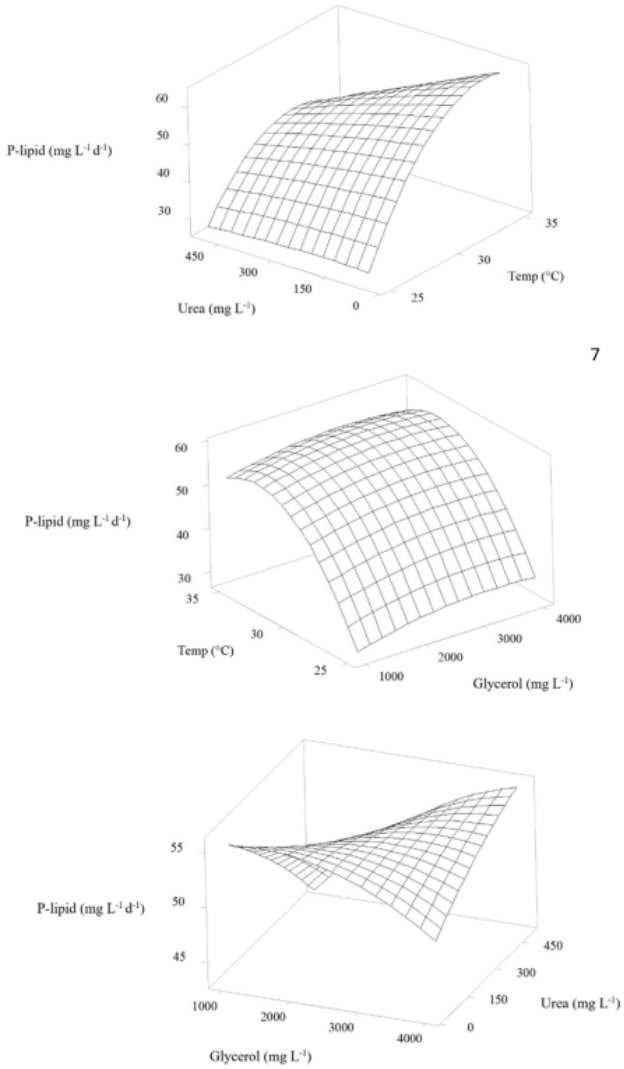

Fig. 5. Response surface plots (3D) showing the effects of temperature (°C), urea (mg L^{-1}), and glycerol (mg L^{-1}) on P-lipid (mg L^{-1} d^{-1}) of mixed algae.

high glycerol concentration, the addition of urea was not significantly enhance the productivity. This indicated that carbon to nitrogen ratio in the medium plays important role in the lipid productivity. At abundant glycerol concentration and low urea addition, carbon to nitrogen ratio is high, thus, nitrogen become limited, and microalgae produce higher lipid. Previous research reported that lipid content of *Chlorella vulgaris* growing on mixotrophic condition was increased from 7.6–11.3%–23.9% when C/N ratio was increased from 50 to 92.7 (Li et al., 2016).

The interaction of temperature and urea significantly influenced the lipid productivity ($p < 0.05$). At low temperature, the addition of urea seems not significantly enhance the productivity, while at very high temperature, the addition of urea decreased the lipid productivity drastically. Therefore, to obtain the highest lipid productivity, the mixed algae could be cultured at 30% POME, using 3 g L^{-1} glycerol at around 25–30 °C without the addition of urea. However, to obtain the highest biomass and lipid productivity, urea needs to be introduced to 200 mg L^{-1}.

3.6. Effect of temperature and organic carbon on the fatty acid profile

The effect of temperature and culture condition (mixotropy autotrophy) were also investigated for fatty acid profile (Table 6). In this experiment, the increasing of temperature from 25 to 35 °C signficantly decreased SFA, MUFA, and PUFA production ($p < 0.05$) (Table 6). Changing cultivation mode from autotrophic to mixotropic significantly increased SFA, MUFA and PUFA production ($p < 0.05$) (Table 6). For the composition of the lipid, changing cultivation model did not significantly influenced SFA, MUFA, and PUFA content normalized to dry biomass ($p > 0.05$). Similar result was recorded for *Thalassiosira pseudonana* which the fatty acid profile

Table 6
Fatty acids (mg L^{-1}) and composition (%) of mixed algae at different temperature and culture condition. Control is control f/2 medium at 25 °C. A is 30% POME with the addition of 2.5 g L^{-1} glycerol at 25 °C, B is 30% POME with the addition of 2.5 g L^{-1} glycerol at 35 °C. Duplicate values are shown. Value after ± symbol shows SD. Values do not share letters are significantly different ($p < 0.05$).

Lipid class	Fatty acid profile	Temp (°C)					
		25				35	
		Control		A		B	
SFA	14:0	0.23	±0.02	0.74	±0.02	0.27	±0.04
SFA	16:0	0.21	±0.05	1.10	±0.03	0.12	±0.17
MUFA	16:1n-7	0.11	±0.15	2.20	±1.81	ND	ND
MUFA	18:1n-7	0.10	±0.14	3.54	±1.69	0.16	±0.23
MUFA	20:1n-7	0.18	±0.25	3.75	±0.19	2.90	±2.99
MUFA	20:1n-9	2.09	±1.98	1.68	±1.09	3.71	±0.19
PUFA	18:3n-6	0.26	±0.01	0.91	±0.11	0.25	±0.05
PUFA	22:6n-3	0.59	±0.06	0.71	±0.02	0.39	±0.05
∑SFA	Total	0.45	±0.07[a]	1.84	±0.02[b]	0.39	±0.20[a]
∑MUFA	Total	2.47	±1.72[a]	11.16	±1.40[b]	6.78	±2.95[a]
∑PUFA	Total	0.40	±0.02[a]	1.64	±0.09[b]	0.38	±0.06[a]
SFA/∑ lipid	(%)	16.20	±10.31[a]	12.67	±1.18[a]	5.03	±0.56[b]
MUFA/∑ lipid	(%)	71.14	±16.10[a]	76.06	±1.75[a]	89.56	±0.94[a]
PUFA/∑ lipid	(%)	12.66	±5.79[a]	11.27	±0.56[a]	5.41	±1.50[a]

content was unchanged when the culture was shifted from autotrophic to mixotrophic condition (Baldisserotto et al., 2021). When high biomass production was enhanced on mixotrophic condition, the production of SFA, MUFA, and PUFA were proportional to the biomass.

Shifting temperature from 25 to 35 °C significantly decreasing SFA content from 12 to 5% ($p < 0.05$). The increasing of SFA is associated to cell membrane which is rigid in an unvaforable environment (Hixson and Arts, 2016). This result was in line with the previous study who found that the increasing temperature could lowering SFA content of tropical microalgae. It was found that the increasing temperature from 28 to 33 °C decreased SFA content from 36 to 33% in tropical *Chlorella* sp, while for tropical diatom, *Amphiprora* sp, the increasing temperature from 28 to 33 °C decreased SFA content from 51 to 44% (Baldisserotto et al., 2021). The dominant fatty acid production was found in MUFA at around 70–90% for all conditions, while PUFA only contributed around 5–13% from total fatty acid for all conditions (Table 6). Previous research reported that PUFA content of *Thalassiosira* sp grown in outdoor tropical condition was around 5–13%, while MUFA content was 36–40% (Kusumaningtyas et al., 2017). Based on previous study, the dominant fatty acids present in *Nannochloropsis* sp were palmitic acid (16:0), palmitoleic acid (16:1n-7) and eicosapentaenoic acid (20:5n-3). Oleic acid (18:1n-9) typically remained at low abundance (~0.1% cell dry weight) (Hulatt et al., 2017). In this study, γ-linolenic acid (18:3n-6) was present (Table 6). This result was in line with the previous studies who reported that γ-linolenic acid (18:3n-6) was present both in *Thalassiosira* and *Nannochloropsis* (Nwoba et al., 2020; Bhattacharjya et al., 2020).

In the present study, the presence of EPA was not detected, while DHA concentration was recorded at around 0.4–0.7 mg L^{-1}. It seems that the absence of EPA was influenced by the cultivation conditions. The ratio of EPA to DHA, which is associated to the conversion of EPA into DHA, was influenced by the culture conditions in microalgae. Previous researchers mentioned that EPA might be converted into DHA by Δ5-elongase and a Δ4-desaturase enzyme (Meyer et al., 2004). Boelen et al. (2017b) mentioned that the ratio of DHA to EPA in marine microalgae was high in the end of exponential phase culture. Patel et al. (2019) reported that DHA/EPA ratio increased when cultivation was changed from photoautotrophic to mixotrophic condition. While DHA to EPA ratio was found lower in diatom when the culture temperature was shifted from 15 to 25 °C (Qiao et al., 2016). Previous research reported that EPA was not detected in tropical marine diatom, *Chaetoceros gracilis*, which was cultured at low temperature (16–19 °C) since EPA could act as the substrate to form DHA in metabolic pathway (Pratiwi et al., 2009).

4. Conclusion

This work revealed that a mixture of algae obtained from Morosari Bay, Demak, is a promising source of fucoxanthin and MUFA. The optimum POME fraction was found on 30% while light intensity was found at 200 μmol m^{-2} s^{-1}. BBD RSM showed that the optimum condition to produce high fucoxanthin and lipid was found at 3 g L^{-1} glycerol, temperature of 25–30 °C, supplemented with 200 mg L^{-1} urea. Switching autotrophic to mixotrophic cultivation condition enhanced SFA, MUFA, and PUFA production. Mixotrophic cultivation condition is a promising strategy to enhance fucoxanthin and lipid production.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2021.102228.

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