Unlocking Microalgal Treasures

Utilization of palm oil mill effluent as growth medium for the production of value-added microalgal compounds

Muhamad Maulana Azimatun Nur



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university of groningen

Unlocking microalgal treasures

Utilization of palm oil mill effluent as growth medium for the production of value-added microalgal compounds

PhD thesis

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To my family, my friends, my teachers,

"Hope is a good thing, maybe the best of things, and no good thing ever dies."
-Stephen King in The shawshank redemption

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CHAPTER 1



Palm oil mill effluent with different fractions

Introduction and Thesis Outline

Opportunities and Challenges of Microalgal Cultivation on Wastewater, with Special Focus on Palm Oil Mill Effluent and the Production of High Value Compounds

M.M.Azimatun Nur,
Anita G.J. Buma

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1. Palm Oil Mill Effluent

South East Asia is the region with the highest production of palm oil worldwide. Based on FAOSTAT (2016), South East Asian coconut palm oil (CPO) production shares 88.6% of the total world production of 54.38 million ton, increasing from 40.33 to 48.12 million ton between 2010 and 2013. Within this region, Indonesia is currently known as the largest CPO producer, followed by Malaysia and Thailand (FAOSTAT, 2016). Badan Pusat Statistik (BPS, 2015) recorded that CPO production in Indonesia rose at 30.14% between 2010 and 2014.

Table 1. POME characteristic before and after treatment by conventional ponding system (Sasongko et al. 2015). For explanation of abbreviations see text.

Parameters	Cooling pond	After conventional treatment
Temperature (°C)	70-80	30-40
pH	4.0-5.0	7.0
Total COD (mg L^{-1})	40,000-90,000	350-1,300
Total BOD_5 (mg L^{-1})	15,000-30,000	100-700
TSS (mg L^{-1})	20,000-40,000	700
TDS (mg L^{-1})	15,000-30,000	-
VSS (mg L^{-1})	15,000-35,000	-
Total Nitrogen (TN) (mg L^{-1})	1494.66	456-750
Total Phosphorus (PO_4-P) (TP) (mg L^{-1})	315.36	68.40-180
Kalium (mg L^{-1})	1,000-2,500	110-924
Magnesium (mg L^{-1})	250-1,000	17-152

During CPO production, a high amount of wastewater is produced, which poses a challenging environmental problem. Palm oil milling by wet processing is commonly used in Indonesia and Malaysia (Hosseini and Wahid, 2015; Wu et al., 2010). About 1 ton of so-called fresh fruit bunch (FFB) produces 0.66 ton palm oil mill effluent (POME) (Indriyati, 2008). Sasongko et al. (2015) reported, based on a palm mill with a capacity of 45 tons FFB h^{-1}, located in a 20,000 ha plantation, the production of POME could reach up to 360,000 m^3 per year. Furthermore, wastewater treatment plants at two particular ponding sites (PTPN V Riau province, and PTPN VII Lampung province, Indonesia) had an average palm oil production of 21,454 m^3 $month^{-1}$, and an effluent rate of 20,000 m^3 $month^{-1}$. Upon discharge, the raw POME is a brownish liquid with temperatures ranging between 80 and 90°C. Further characteristics include: pH ranging from 4.0 to 5.0, biological oxygen demand (BOD) and chemical oxygen demand (COD) ranging from 15,000-30,000 mg L^{-1} and 40,000-90,000 mg L^{-1} respectively, high total suspended solids (TSS) between 20,000-40,000 mg L^{-1}, total dissolved solid (TDS) between 15,000-30,000 mg L^{-1}, and volatile suspended solids (VSS) between 15,000-35,000 mg L^{-1} (Table 1). Rupani et al. (2010) suggested

that further treatment should be done to meet standard regulations before POME can be released into the environment (i.e. rivers, lakes) (Sasongko et al., 2015; Tabassum et al., 2015).

To treat POME, several mechanical, chemical or biological methods have been developed, as reviewed by Rupani et al. (2010) and Liew et al. (2015). These methods include filtration, sedimentation and flocculation. However, to date, the conventional ponding system is the most common method for treating POME (Wu et al. 2010; Liew et al. 2015). In Malaysia, more than 85% of palm oil mills employ the ponding system for POME treatment due to its low capital cost (Tong and Bakar Jaafar, 2004). This system employs a series of anaerobic, facultative anaerobic, and aerobic ponds in an open lagoon (Lam and Lee, 2011). The conventional ponding system has various limitations such as a long HRT (Hydraulic Retention Time), a strong smell, greenhouse gas (GHG) emission, and the large area required for treatment (Indriyati, 2008, Tabassum et al., 2015). Furthermore, its high COD and nutrient content, as well as its dark brown color which is derived from phenolic compounds could also threaten living organisms if it is directly discharged to surrounding areas (lakes, rivers). Therefore, more effective POME treatment systems are urgently needed.

In recent years, new concepts of utilizing waste into more useful products are developed to meet industrial demands. Valorization is the process of converting waste materials into valuable products. Several researchers reported the valorization of POME into bioenergy, yeast biomass, and enzymes. Louhasakul et al. (2016) reported the fermentation by yeast of POME medium thereby accumulating > 33% of lipid. Iwuagwu and Ugwuanyi (2014) utilized POME as a source of carbon and nitrogen for food grade yeast biomass production. Hasanudin et al. (2015) reported that the utilization of treated POME as liquid fertilizer increased fresh fruit bunch (FFB) production up to 13%, while Md-Din et al. (2014) showed that POME can be utilized as substrate for bacteria to produce poly-β-hydroxyalkanoates. Finally, POME was found to be a suitable substrate for bacteria to produce biomethane (Ahmad et al., 2014). Based on the recent literature, the utilization of POME by microalgae was so far mainly focused on lipid production, bulk biomass and wastewater treatment.

2. Microalgae for value added products

Microalgae, including diatoms, have a great potential to produce food, feed, fuel, fine chemicals and fertilizers on a commercial scale. Apart from biofuel production, algae serve as a potential renewable source for other commercial applications (Milledge, 2011): a) Environmental applications such as wastewater treatment and CO_2 mitigation; b) Human nutrition; c) Animal and aquatic feed; d) Cosmetics; e) High-value molecules such as fatty acids; f) Pigments such as beta-carotene,

astaxanthin, phycobiliproteins; g) Biochemical products such as bioplastics and exopolysaccharides; h) Biofertilizer; i) Drug synthesis for antimicrobial, antiviral, antibacterial and anticancer activities.

To date, the value added products from microalgae still face high production costs compared to other sources (Wijffels et al., 2010). For example, it was reported that the production of high-purity Eicosapentaenoic acid (EPA) from *Phaeodactylum tricornutum* requires total production costs of US$ 4,602 kg^{-1}, with 60% of the cost arising from the recovery process and 40% from biomass production. It is reported that the costs need to be reduced by 80% to be economically viable (Milledge 2011; Molina-Grima et al. 2003). At the same time, the annual worldwide demand of EPA is 300 t (Singh et al. 2005).

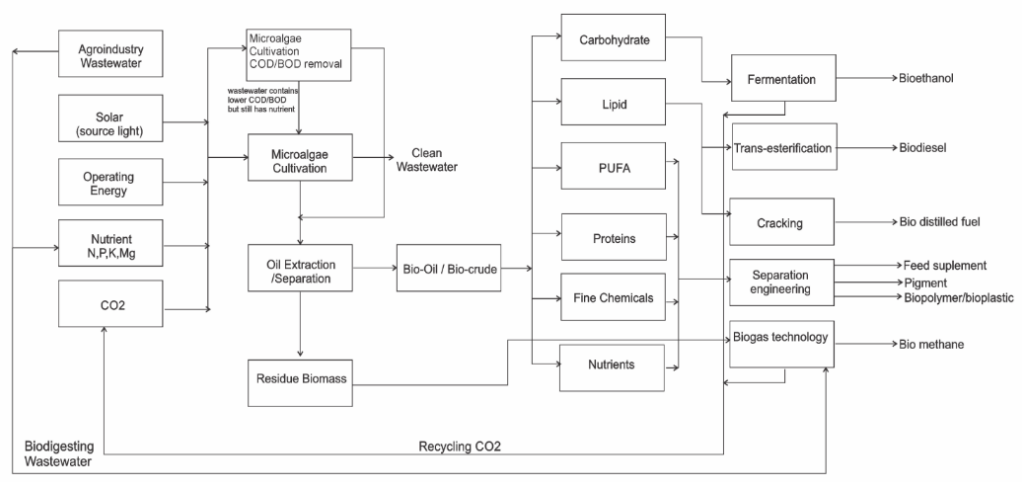

Figure 1. Integrated Process of Wastewater Treatment through Biorefinery by Microalgae Modified from Singh and Gu (2010)

Microalgae are considered to be promising for future raw feedstock because of their potency of producing derivative products in the biorefinery process (Singh and Gu, 2010). Biorefinery techniques are necessary to exploit all products from microalgae after cultivation. The main problem is to separate the different fractions without damaging each of them (Vanthoor-Koopmans et al., 2013). A biorefinery concept of microalgae growing on wastewater is described in Figure 1. Microalgae are mainly composed of carbohydrates, lipids and protein, and other components such as pigments. The combination of the biorefinery concept in the downstream process and the utilization of wastewater for microalgal growth could reduce the production cost in the microalgal industry. This is because the whole cell can be utilized for food, feed, fuel, and fertilizer products, while the wastewater could replace the costs of synthetic nutrients. Yet, screening of microalgae based on

their biochemical composition is essential for efficient biomass production from wastewater towards commercially viable applications (Abinandan and Shanthakumar, 2015).

3. Cultivation of microalgae on POME

Generally, microalgae are cultivated on POME to obtain bulk biomass, carbohydrates, lipids, and proteins, mainly for fuel and animal feed purposes. Table 2 summarizes the presently available studies of microalgal cultivation on POME resulting in value-added products. Sukumaran et al. (2014) found 12% phycocyanin from *A. platensis* cultivated on 1% raw POME. Furthermore, Vairappan and Yen (2008), and Shah et al. (2016) reported the production of PUFA from *Isochrysis* sp, *Arthrospira platensis*, and *Nannochloropsis oculata* cultivated on POME medium.

Table 2. Summary of POME medium for microalgae growth and production

Microalga	Medium	Product/ Output	References
Isochrysis sp.	5% POME (anaerobic digested) + 0.075% NPK + sea water	Biomass; lipid	Vairappan and Yen (2008)
Arthrospira platensis	1% fresh raw POME + commercial nutrient	Biomass; C-phycocyanin, carotenoid, chlorophyll	Sukumaran et al., (2014)
Arthrospira platensis	90% POME + 10% commercial medium	Bulk biomass	Suharyanto et al. (2014)
Chlorella sp.	20% digested POME + 40% synthetic nutrient+ water	Biomass, lipid	Hadiyanto and Nur (2014)
Nannochloropsis oculata	10% raw POME + sea water	Biomass, lipid	Shah et al., (2016)
Tetraselmis suecica		Biomass, lipid	
Chlorella vulgaris	30% digested POME	Biomass, lipid, carbohydrate	Nur et al. (2016)

Although several studies have focused on value-added production using POME wastewater, more work is urgently needed, especially using species from other taxonomic groups and utilizing higher POME fractions for high value-added compounds. For example, the application of diatoms in cultivation studies using POME was not addressed so far. Diatoms may contain much higher levels of the fish fatty acids (LC-PUFAs) EPA and DHA as compared with the most common species used for cultivation (as given in Table 2). Boelen et al. (2013) reported that EPA and DHA production rates were highly species specific. Moreover, diatoms contain high levels of the carotenoid fucoxanthin, which may have significant health benefits.

Based on Table 2, the average of the POME fraction applied for microalgal cultivation is around 30-50%. Suharyanto et al. (2014) cultivated *Arthrospira platensis* on POME medium to produce bulk biomass by applying continuous

cultivation. It was reported that 24% BOD was removed and 0.267 g L^{-1} d^{-1} biomass was produced in this cultivation mode, being higher compared to batch mode. However, the continuous cultivation mode is difficult to implement in large scale cultivation systems. In addition, the utilization of sea water needs to be optimized to replace freshwater in the dilution of POME fractions. Therefore, to optimize the utilization of POME and remove the polluting compounds, several factors should be considered: mode of cultivation, nutritional requirements and environmental conditions.

3.1 Microalgal Cultivation Conditions

Microalgae commonly grow under photo-autotrophic conditions. Several species are able to switch between photo-autotrophic and heterotrophic growth, while during mixotrophic growth both metabolic life styles occur simultaneously (Perez-Garcia and Bashan, 2015). Cultivation of microalgae on POME is challenging since the chemical oxygen demand (COD), biological oxygen demand (BOD) and macronutrient levels are high. In addition, the often dark color of POME inhibits light penetration potentially causing light limitation for microalgal photosynthesis and growth.

3.1.1. Heterotrophic growth

Heterotrophic growth is a process where microalgae utilize organic substrates through aerobic respiration thereby generating energy without light. Under heterotrophic conditions, the cultivation of microalgae has been shown to be successful for commercialization of high-value chemicals, such as cosmetics, pharmaceuticals and food supplements (Perez-Garcia and Bashan, 2015). The economic advantages of heterotrophic growth over photo-autotrophic growth using large-scale microalgae cultivation were summarized earlier by Chen (1996) and Borowitzka (1999). They reported that high cell population and biomass densities (between 20 and 100 g L^{-1}) can be achieved in darkness under heterotrophic cultivation in fermenters. In addition, compared to photo-autotrophic conditions, heterotrophic conditions showed enhanced ammonium and phosphate uptake in synthetic wastewater by *Chlorella vulgaris* (Perez-Garcia et al., 2011). Moreover, Ummalyma and Sukumaran (2014) reported increased lipid production by *Chlorococcum* sp. when cultivated on dairy effluent wastewater under heterotrophic compared to mixotrophic conditions. The utilization of POME as the growth medium for heterotrophic microalgae is not clear. Sukumaran et al. (2014) reported that the addition of 4% raw POME to the medium of *Arthrospira platensis* changed the condition into heterotrophic growth due to the dark color that inhibited irradiance exposure. However, specific growth rate, pigments and biomass were higher during the addition of a high fraction of POME, which

inhibited the penetration of light, compared to autotrophic cultivation that utilized commercial fertilizer without POME addition. It was also demonstrated that micronutrients derived from POME promoted microalgal growth.

3.1.2. Mixotrophic growth

Mixotrophic growth is the process where microalgae use inorganic as well as organic carbon sources in the presence of light. Under this condition, photo-autotrophic and heterotrophic processes may occur simultaneously (Wang et al. 2014; Kang et al. 2004). Inorganic carbon (i.e. CO_2, HCO_3^-) and macronutrients are utilized through photosynthesis, which is influenced by irradiance quality, quantity and dynamics, whereas organic compounds (i.e. glucose, carboxylic acid, glycerol) are assimilated through aerobic respiration, which is affected by the availability and type of organic substrate.

Mixotrophic conditions for wastewater treatment were described by Salla et al. (2016) and Li et al. (2014), showing increased biomass, carbohydrates, lipids, and growth rate for *Arthrospira platensis* and *Chodatella* sp.. The utilization of wastewater by microalgae under mixotrophic conditions was reviewed by Wang et al. (2014). Overall, the efficiency of nutrient removal (N, P) from wastewater by microalgae seems higher under heterotrophic and mixotrophic conditions than under photo-autotrophic conditions (Li et al., 2014; Perez-Garcia et al., 2010). Nur and Hadiyanto (2015) documented that the biomass and growth rate of *C. vulgaris* were lower when POME was added to the medium as organic carbon source and $NaHCO_3$ as inorganic carbon source compared to the addition of organic carbon alone (i.e. D-glucose and glycerol). Furthermore, Sukumaran et al. (2014) stated that specific growth rate, biomass, and pigment content of *A. platensis* were higher under mixotrophic conditions compared to heterotrophic conditions.

Apart from the cultivation under mixotrophic or heterotrophic conditions, microalgal growth, biomass composition, production, and nutrient uptake using wastewater are also influenced by biotic factors such as the presence of competitors (zooplankton, bacteria, viruses) (Noue et al., 1992). Finally, cultivation strategies of microalgae (batch, fed-batch, and continuous culturing) also affect growth, biomass accumulation, composition and nutrient uptake as reported by several researchers (Graverholt and Eriksen, 2007; Coelho et al. 2014; Kumar et al. 2016).

3.2. Microalgae Cultivation Mode

3.2.1. Fed-batch cultivation mode

The fed-batch cultivation mode is common in the bioprocess industry. This semi-batch strategy is used to avoid the limitation or inhibition of substrate and the accumulation of toxic compounds (catabolic respiration) during cultivation. The medium is replaced periodically during the process after which the biomass is harvested during and at the end of the process.

Several researchers reported the advantages of the fed-batch mode in microalgal cultivation. Xie et al. (2015) and García-Cañedo et al. (2016) reported that by applying autotrophic fed-batch cultivation mode, pigment content of *Arthrospira platensis* and *Scenedesmus incrassatulus* were increased as a result of nutrient addition. Under mixotrophic fed-batch conditions, the cellular content of the long chain poly unsaturated fatty acid EPA of *Nannochloropsis* sp was enhanced (Xu et al. 2004). Moreover, under heterotrophic fed-batch mode, carbohydrate and protein content were found to be higher for *Neochloris oleoabundans* as compared to batch mode (Morales-Sánchez et al. 2013).

With respect to wastewater treatment, Ji et al. (2015) reported that nutrient removal from wastewater by *Desmodesmus* sp. was higher in fed-batch compared to batch mode, lowering Total Nitrogen (TN) by 94.2 and PO_4-P 88.7%, while generating 25 mg L^{-1} d^{-1} biomass and 6.52 mg L^{-1} d^{-1} lipid after 40 days with 2 days pulse feed. Under this condition, the alga was cultivated on digested pig manure that contained high levels of ammonium which could negatively impact algal growth. Similar results were found by Markou (2015) who reported that ammonia and phosphorus removal were more than 95% in anaerobically digested poultry litter that contained high levels of ammonia. When cultivated in fed-batch mode, *Arthrospira platensis* produced 126.5 mg L^{-1} d^{-1} biomass, 66.53 mg L^{-1} d^{-1} protein and 15.3 mg L^{-1} d^{-1} phycocyanin. By employing fed-batch cultivation, the inhibition by excess nutrients in the wastewater could therefore remain limited.

Hongyang et al. (2011) reported that the biomass and lipid production of *Chlorella pyrenoidosa* on soybean wastewater that contained high amounts of COD could be enhanced by applying fed-batch mode, resulting in 1070 mg L^{-1} d^{-1} biomass accumulation, and 400 mg L^{-1} d^{-1} of lipid productivity. At the same time, the nutrient content was lowered by 77.8% for COD, 88.8% for TN, and 70.3% for TP. Espinosa-Gonzalez et al. (2014) found that the utilization of high concentrations of glucose and galactose from dairy industry wastewater could be optimized by applying fed-batch cultivation, which resulted in 1720 mg L^{-1} d^{-1} of biomass and 352.6 mg L^{-1} d^{-1} of lipid production. In addition, Park et al. (2017) found that the biomass productivity of *Micractinium inermum* was higher by applying fed-batch cultivation (950 mg L^{-1} d^{-1}), which was supplemented with external nutrients at day

2, compared to the batch control (800 mg L^{-1} d^{-1}). The cultivation was run in an airlift photobioreactor by using a mixture of digested pig wastewater as well as domestic wastewater. This study demonstrated that the fed-batch process may avoid high COD and strong color that could otherwise limit light penetration in the wastewater medium.

3.2.2. Continuous cultivation mode

In continuous mode, the outflow of harvested biomass and inflow of the fresh medium are maintained in an equal, steady state. Typically, this mode is started after a batch cultivation phase, to obtain a certain initial cell density. Several researchers employed a continuous cultivation mode to increase the chosen value added product. Sloth et al. (2006) and Van-Wagenen et al. (2015) employed continuous culturing under mixotrophic conditions to enhance lutein yield. The phycocyanin content of *Galdieria sulphuraria* was higher in a continuous culture set-up compared to a fed- batch set-up (Graverholt and Eriksen, 2007). Coelho et al. (2014) compared cultivation modes of *Chlorella* sp and found a higher biomass accumulation rate in continuous compared to fed-batch mode. Furthermore, Kumar et al. (2016) found a higher biomass and lipid productivity in continuous cultures of *Chlorella sorokiniana* as compared with fed-batch mode. Similarly, carbohydrate productivity was found to be high under mixotrophic conditions using continuous culturing (Wang et al., 2016a) and EPA yield was found to be higher during continuous culturing (Wen and Chen, 2002) as compared with the fed-batch mode (Xu et al. 2004). Beta carotene yield was also increased in continuous mode (Zhu and Jiang, 2008) compared to fed-batch (Yamaoka et al., 1994) when growing *Dunaliella salina* under autotrophic conditions.

With respect to wastewater treatment applications, recent studies showed that continuous cultivation can be used to increase the value added content in microalgae while simultaneously lowering the nutrient content in wastewater. Dickinson et al. (2013) utilized *Scenedesmus* sp. grown in municipal wastewater to produce carbohydrate (130 mg L^{-1} d^{-1}) and protein (120 mg L^{-1} d^{-1}). Furthermore, Jebali et al. (2015) utilized industrial wastewater for *Scenedesmus* sp. cultivation, resulting in 506.7 mg L^{-1} d^{-1} carbohydrate and 171.9 mg L^{-1} d^{-1} protein, while nitrogen and phosphorus were removed at a rate of 34.6 and 12.7 mg L^{-1} d^{-1}, respectively. In addition, Ruiz et al. (2013) applied the same species and cultivation system on urban wastewater, resulting in 105 mg L^{-1} d^{-1} lipid accumulation rate, removing 86.8% TN, and 97.7% TP from the wastewater. McGinn (2012) found that biomass productivity from the algae cultivated on municipal wastewater was almost 2-fold higher in continuous mode compared to batch mode, while both TN and TP could be lowered up to 99%. The product accumulation rates of *Chlorella pyreonoidosa* growing on digested anaerobic starch, which contained high levels of

organic carbon and which had a strong white color, was also enhanced by applying continuous cultivation: the microalga produced 43.37 mg L^{-1} d^{-1} lipid, and lowered organic carbon content by 61.9% and total nitrogen and phosphorus by 78.7 and 97.2% respectively (Chu et al., 2015). Similarly, Gao et al (2016) found that the biomass productivity of *Chlorella vulgaris* cultivated in a continuous cultivation on nutrient rich aquaculture wastewater, was 5.8-fold higher compared to batch cultivation. The microalga produced 42.6 mg L^{-1} d^{-1} biomass and removed 86.1% TN and 82.7% TP. Furthermore, Suharyanto et al. (2014) cultivated *Arthrospira platensis* on POME medium to produce bulk biomass by applying continuous cultivation. It was reported that 24% BOD was removed and 0.267 g L^{-1} d^{-1} biomass was produced in this cultivation mode, being higher compared to batch mode.

Overall, continuous microalgal cultivation may have several advantages, but at the same time, it faces multiple challenges during the process as reviewed by Fernandes et al. (2015). However, continuous cultivation strategies seem to be promising in wastewater treatment, since the inhibition and limitation of substrates from wastewater can be avoided. Clearly, biomass and high value product yield benefit from a continuous supply of relatively low levels of nutrients but relatively high, constant irradiance levels, as compared with fed batch mode.

3.3. Environmental factors affecting microalgal cultivation

Several environmental factors influence microalgal cultivation. In large scale cultivation systems, these factors are ideally manipulated and controlled to obtain the desired product in the most optimal way,

3.3.1. Light

Photoautotrophic microalgae utilize light as the energy source but during the photosynthetic process 50% of the energy may be lost in the conversion of solar energy to chemical energy (Perrine et al., 2012). Algae containing chlorophyll *a* and *b*, which are the major light harvesting pigments for green algae, primarily absorb blue and red light. Therefore, green algae are found to grow better in blue and red light (Singh and Singh, 2015).

Light energy can be stored in the form of carbohydrates or lipids. Several researchers demonstrated the light dependency of carbohydrate storage in algal cells (Ho et al. 2012; Carvalho et al., 2009). Subramanian et al. (2013) found that storing energy as carbohydrates under high light is energetically less favorable than triacylglycerol (TAG) on a per carbon basis. He et al. (2015) also reported that under high light (400 μmol photon m^{-2} s^{-1}), carbohydrates decreased whereas lipids increased.

Under nutrient saturated conditions, light is the critical factor for photosynthetic activity. Microalgae require a specific light level to reach their

maximum growth rate, which is referred to as the saturating light level. If the light intensity is far above the saturation level, it will inhibit growth (photo-inhibition). On the other hand, if the light intensity is far below the saturation level, growth will be light limited (light-limitation) (Lee et al., 2015). Lee and Lee (2001) found that the specific removal rate of organic carbon from artificial wastewater under light-dark cycles was higher than under continuous illumination. Yan et al. (2013) reported that the nutrient removal from domestic wastewater by *Chlorella vulgaris* was influenced by irradiance wavelength as well as irradiance intensity when applying a light emitting diode (LED). In addition, the dependency of the irradiance responses to remove nutrients from wastewater varied with irradiance level and irradiance period depending on the algal strain (Gonçalves et al. 2014; Li et al. 2012). Furthermore, Olguín et al. (2001) found that light intensity influenced both the lipid accumulation rate and the lipid profile of *Arthrospira platensis* grown on digested pig waste. Marcilhac et al. (2014) found that light intensity affected microalgae-bacteria interactions when grown on urban wastewater. In addition, Jiang et al.(2016) reported that different light intensities influenced lipid, protein, and carbohydrate content of *Chlorella vulgaris* cultivated on monosodium glutamate wastewater. Several researchers reported the cultivation of microalgae on POME medium by varying light parameters to obtain optimal yields of the desired products. Kamyab et al. (2016) stated that the light cycle plays an important role in the lipid production of *Chlorella pyrenoidosa*, cultivated on diluted POME, and continuous irradiance exposure gave the highest growth rate and lipid content compared to exposure to light dark cycles. Furthermore, regular dilution was employed to enhance irradiance in the cultures when cell densities were getting high, as reported by Hadiyanto and Nur (2015). In addition, Takriff et al. (2016) increased light penetration by adding activated carbon as adsorbent on the POME pre-treatment, thereby decreasing light attenuation.

3.3.2. Temperature

The effect of temperature on microalgal growth makes it one of the most important environmental factors influencing growth rate and biochemical composition of algae. In one study (Ras et al., 2013), optimal growth rates for mesophilic species growing outdoors in a wastewater treatment plant were found between 20 and 25°C, increasing up to 40°C for thermophilic strains (*Chaetoceros* sp., *Anacystis nidulans*) or decreasing to 17 °C for psychrophilic strains (*Asterionella formosa*). Furthermore, Delgadillo-Mirquez et al. (2016) found optimum conditions for *Chlorella* cultivated on wastewater at 25^{0}C. Zhang et al. (2016) reported optimal temperatures between 18-25°C for *Chlorella* sp. to produce biodiesel in a wastewater treatment plant.

Decreasing the temperature below the optimal level may increase the unsaturation of lipids as reported by Wang et al. (2016b). *Chlorella sorokiniana* LS-2 grown at a suboptimal temperature of 18°C showed enhanced lipid content mainly containing unsaturated fatty acids. However, absolute lipid productivity was decreased since lower temperatures prolonged the exponential phase and decreased growth rate compared to 26°C. In addition, sub optimal cultivation temperatures may affect pigments and growth rate. The total carotenoid production of *Chlorococcum* sp. almost doubled when growth temperature increased from 20^0C to 35^0C (Liu and Lee, 2000). Also, astaxanthin production in *Haematococcus* sp. increased threefold when growth temperature increased from 20^0C to 30^0C (Tjahjono et al., 1994). Vairappan and Yen (2008) reported that growth rate and biomass of *Nannochloropsis* sp grown on POME medium was affected by cultivation temperature and light. The culture conditions were compared using a 1L photobioreactor (Temp: 23°C, illumination: 180 ~ 200 µmol photons m^{-2} s^{-1}) and a 10L outdoor system (Temp: 26–29°C, illumination: 50 ~ 180 µmol photons m^{-2} s^{-1}). Growth rates were higher in the photobioreactor but biomass production was higher in the outdoor culture.

3.3.3. Carbon

For autotrophic growth the supply of inorganic carbon (CO_2 and HCO_3^-) is most important. The CO_2 -H_2CO_3-HCO_3^-–$CO_3^{(2-)}$ system serves as important buffer in freshwater cultivation systems and may control and maintain specific pH levels that are suitable for large scale cultivation (Grobbelaar, 2004). Under mixotrophic and heterotrophic cultivation conditions, organic carbon plays an important role in microalgal growth, final yield and lipid accumulation. In general, energy storage molecules, such as lipids and carbohydrates (starch and glycogen) are accumulated under heterotrophic and mixotrophic conditions; therefore, the cellular content of these compounds may be higher than under photo-autotrophic conditions (Choix et al., 2014).

Various types of wastewater already contain carbon that can be utilized by microalgae (Abdel-Raouf et al., 2012). However, a high organic content may limit the growth of certain microalgae in raw wastewater, resulting in a higher retention time (Nur and Hadiyanto, 2015). Several researchers reported the supplementation of external carbon in mixotrophic and heterotrophic wastewater cultivation systems to increase biomass, growth rate and nutrient uptake. Perez-Garcia (2010) found the highest growth rate and ammonium uptake of *Chlorella vulgaris* growing on sterilized municipal wastewater when adding sodium acetate under heterotrophic conditions, while the absence of external carbon supplementation resulted in reduced growth. Gupta et al. (2016) reported that the addition of glycerol enhanced biomass productivity and nutrient uptake of *Chlorella vulgaris* and *Nannochloropsis oculata*, when cultivated on municipal wastewater. Similar

results were reported by Ma et al. (2016) who reported that nutrient uptake and lipid yield was increased when synthetic wastewater was supplemented with waste glycerol. The addition of inorganic carbon (CO_2) could also improve the lipid accumulation of microalgae cultivated on domestic wastewater under mixotrophic conditions (Devi and Mohan, 2012).

Raw POME contains 12.75% crude protein, 10.21% crude lipid, and 29.55% carbohydrate (Habib et al., 1997). In most cases, researchers utilized the wastewater at low concentrations to avoid growth inhibition due to toxic levels of a variety of components. Shah et al. (2014) utilized 1-20% v/v of raw wastewater, which contained 148–2833 mg L^{-1} COD, to promote the growth of *Isochrysis galbana* during which 76.4-74.8%. of the COD could be removed. In addition, Sukumaran et al. (2014) utilized 1–4 % v/v fresh raw POME in commercial fertilizer by applying fed-batch cultivation to avoid inhibition during the cultivation of *Arthrospira platensis*.

It is possible to lower the organic carbon of raw POME by applying anaerobic fermentation processes. During this process, complex carbohydrates, lipids and proteins are degraded. This degradation leads to the formation of CH_4, CO_2 and carboxylic acid (i.e. acetic acid, propionic acid and butyric acids) (Mumtaz et al., 2008). It is reported that digested POME contains acetic acid at concentration ranging between 1170 and 3540 mg L^{-1} (Poh et al., 2010). Several researchers reported that microalgae can be cultivated on higher concentrations of digested POME as compared with undigested POME. Zainal et al. (2012) utilized 100% v v^{-1} of anaerobically digested POME, which was demonstrated to be rich in organic acid, as growth medium for *A. platensis*. Here, 90% of COD was removed. Rajkumar and Takriff, (2015) also reported that *Arthrospira platensis* and *Scenedesmus dimorphus* could be cultivated on 90% v v^{-1} anaerobically digested POME in an open pond system.

3.3.4. Nitrogen

In general, microalgae have a limited ability to accumulate nitrogen storage materials when growing under nitrogen-sufficient conditions. Conversely, cyanobacteria produce sufficient nitrogen storage under high nitrogen concentrations in the form of pigments such as phycocyanin (Boussiba and Richmond, 1980). Recently, growth and biomass yield of microalgae were found to be influenced by the supplementation of different nitrogen compounds and concentrations (Ramanna et al., 2014). Beuckels et al. (2015) showed that nitrogen availability affected phosphorus removal of microalgae during wastewater treatment.

It is reported that digested POME contains high total Kjeldahl nitrogen (TKN), but low concentrations of inorganic nitrogen, indicating that it contains high levels of organic nitrogen (Sasongko et al., 2015; Poh et al., 2010). Therefore

addition of external inorganic nitrogen is recommended to support algal growth, of which the recommended inorganic N:P ratio should be around 6.8-10:1 (Olguin et al. 2012). Mutanda et al. (2011) reported that the addition of 5 mM $NaNO_3$ on post-chlorinated wastewater could increase the biomass productivity of *Chlorella* sp. Hadiyanto et al. (2012) supplemented digested POME wastewater with urea fertilizer in order to obtain higher growth rates of *Chlorella* sp by modifying the nitrogen to phosphorus ratio. Furthermore, Halim et al. (2016) reported that the addition of sodium nitrate and dihydrogen phosphate to digested POME, which resulted in 10:1 N ($NaNO_3$) / P (NaH_2PO_4) molar ratio, could increase the biomass production of *Nannochloropsis* sp. and remove 90% TN and 83% TP, compared to the control (digested POME medium without external nutrient addition). The nutrient removal was higher compared to the study by Shah et al. (2016), who reported that *Nannochloropsis oculata* removed 64–75% of total nitrogen (TN) from raw POME at low concentrations (1-15%).

3.3.5. Phosphorus

Phosphorus is another major macronutrient that influences cellular metabolic processes by forming structural and functional components required for maintenance, growth and survival (Fan et al., 2014). Most algal species show a rather consistent phosphorus content, averaging 0.03-3% of their dry weight (Reynolds, 2006). This implies that limiting phosphorus concentrations in the medium results in the repression of photosynthesis (Belotti et al., 2014). However, previous research reported that microalgae can only utilize phosphate as an inorganic phosphorus source, while phosphite and organic phosphate cannot be used (Loera-Quezada et al., 2015). Therefore, supplementation of phosphate in lagoon wastewater increased the chlorophyll-a content and lipid productivity of consortium microalgae, while alteration of the nitrogen to phosphorus ratio also increased nutrient removal efficiency (Lee et al., 2013). In addition, Zhang et al. (2014) reported that the supplementation of phosphorus and iron positively influenced biomass yield, lipid yield, and nutrient uptake of *Scenedesmus obliquus* cultivated on municipal wastewater. Poh et al. (2010) showed that digested POME contains a high total phosphorus load, mostly consistingested anaerobic POME wastewater.

3.3.6. Silicon

Especially for diatoms, silicon is an important macronutrient. Brzezinski (1985) reported that marine diatoms, mainly from the genera *Thalassiosira* and *Chaetoceros*, have a silicon to nitrogen atomic ratio around 1. Silicate plays a vital role in diatom cell wall formation and deoxyribonucleic acid (DNA) synthesis during the metabolic process (Darley and Volcani, 1969). Roessler (1988) reported

that silicon deficiencies influenced the accumulation of neutral lipids in *Cyclotella cryptica*, and silicon limitation also resulted in a 5 % shift in lipid chain length, from C18 to C16 fatty acids (Adams and Bugbee, 2014). In addition, lipid accumulation was found to be influenced by silicate depletion both in autotroph (Wilhelm et al. 2006) and heterotroph cultures (Wen and Chen 2000). Graham et al (2012) concluded that the presence of silicon was important when freshwater diatoms (genera *Cyclotella*, *Aulacoseira, Fragilaria, Synedra*) were cultivated on wastewater. The amount of silicon present or added to the medium for diatoms should be 17.5 times the mass of phosphorus for optimal utilization of phosphorus and nitrogen in wastewater medium.

3.3.7. Micronutrients

Several micronutrients are important for microalgal growth and composition. Iron plays a vital role in cellular biochemical composition because of its redox properties and function in fundamental processes such as photosynthesis, respiration, nitrogen assimilation and DNA synthesis (Marchetti and Maldonado, 2016). To date, Fe is supplied in mass cultivation systems in a chelated form such as ferric citrate ($C_6H_5FeO_7$), ferric EDTA ($FeCl_3$-EDTA) or ferrous ascorbate (Botebol et al., 2014). Several researchers reported the effect of iron on microalgal pigments such as astaxanthin, beta-carotene, and fucoxanthin (Wang et al., 2013a; Cai et al., 2009; Mojaat et al., 2008; Erdoğan et al., 2016). Recent studies showed that the addition of iron to wastewater enhanced biomass yield and nutrient uptake of *Chlorella* sp. (Zhao et al., 2016). Similar results were obtained by Zhang et al. (2014) who found that the addition of $FeCl_3 \cdot 6H_2O$ to municipal wastewater influenced biomass yield, lipid yield, and nutrient uptake of *Scenedesmus obliquus*. However, Habib et al (2003) measured the micronutrient concentration of digested POME and concluded that POME contains sufficient levels of micronutrients such as potassium (963 mg L^{-1}), calcium (531 mg L^{-1}), aluminum (136 mg L^{-1}), magnesium (87 mg L^{-1}), iron (79 mg L^{-1}), sodium (69 mg L^{-1}), and zinc (48 mg L^{-1}). These micronutrients influenced the poly unsaturated fatty acid (PUFA) profile of *Chlorella vulgaris* growing on different POME concentrations.

3.3.8. Salinity

Salinity affects the production of pigments in microalgae. For example, highest chlorophyll-a and total carotenoid productivity was found at a salinity of 2 ppt for *Dunaliella viridis* (Ilkhur et al., 2008). Xia et al. (2014) compared four types of salts (NaCl, $NaHCO_3$, NaS_2O_3 and Na-Acetic acid) with respect to lipid production in *Desmodesmus abundans, and the highest production was obtained for NaCl*. In addition, in *Dunaliella tertiolecta* ATCC 30929, a high salinity increased its lipid content up to 70% (Takagi et al., 2006). In the freshwater alga *Scenedesmus sp.*, lipid

production was stimulated by NaCl (Salama et al., 2013). However, excess salinity stress in the cultivation medium inhibits photosynthesis which subsequently reduces biomass and net lipid productivity. As discussed by Minhas et al. (2016), salinity stress tends to affect the fatty acid profile of microalgae.

With respect to wastewater utilization, Salama et al. (2014) documented that *Chlamydomonas mexicana* exhibited a higher specific growth rate when 100 and 200 mmol L^{-1} NaCl was supplemented to municipal wastewater, whereas nitrogen was completely removed and 38% of lipid was obtained when 400 mmol L^{-1} NaCl was added.

3.3.9. pH

The efficient growth of microalgae on wastewater depends on critical variables, including pH, temperature, and the availability of nutrients (Pitman et al., 2011). It is well known that digested POME and other wastewaters contain nitrogen mostly in the form of ammonia (Sasongko et al., 2015; Poh et al. 2010; Markou, 2015). Yet, the equilibrium between ammonia (NH_3) and ammonium (NH_4^+) is dependent on pH and temperature (Korner et al., 2001). It is reported that the ratio of ammonia to ammonium increases 10-fold for each unit increase in pH (Erickson, 1985).

Some researchers reported the negative effects of ammonia on the growth of microalgae related with pH. Abeliovich and Azov (1976) found that ammonia at a concentration over 2 mM and a pH over 8 in a high-rate sewage oxidation pond inhibited the growth of *Scenedesmus obliquus*. Furthermore, Belkin and Khoo et al. (2017) reported that the growth of *Chlorella vulgaris,* which was cultivated on municipal wastewater, was highly influenced by initial pH. Under acidic (pH 2) or alkaline (9 or 11) pH, growth rates were low. In contrast, highest biomass productivity and nutrient removal efficiency were recorded at pH 3. Furthermore, Hodaifa et al. (2009) reported that the specific growth rate, protein, and chlorophyll content of *Scenedesmus obliquus* were high when the medium (olive-mill wastewater) was maintained at a constant pH value of 7.0. Yet, PUFAs and essential fatty acids increased when the pH was set at 9.

4. Pretreatment of wastewater for microalgal growth

To optimize the utilization of wastewater for microalgal growth, several pretreatment methods have been proposed. These pretreatments were done to lower COD, BOD, turbidity, suspended solids, and microorganisms. Hadiyanto et al. (2013) utilized water lily in the process to lower the COD and BOD content of POME as the medium for *Arthrospira platensis* cultivation. Takriff et al. (2016) reported an increase in *Scenedesmus dimorphus* biomass in pretreated POME medium by using activated carbon to increase the light penetration in the medium. Nwuche et al (2014) and Cho et al. (2011a) used a filtration method to remove contaminants

from wastewater which resulted in the increase of lipid productivity in *Chlorella* sp.. Autoclaving was applied by Shi et al. (2016) and Li et al. (2011); this resulted in an increase in microalgal biomass yield and productivity. In contrast, Wang et al. (2015) obtained lower microalgal biomass when the wastewater was pretreated using autoclaving. Here, autoclaving was not found to be effective for lowering the turbidity in the medium. A similar pretreatment was employed by Shah et al. (2014) by combining autoclaving, centrifugation and filtration. Following this procedure, marine microalgae were found to accumulate high lipid levels when cultivated on the treated POME. Other pretreatment processes include electrolysis (Wang et al., 2013b; Wang et al., 2015), centrifugation (Shi et al., 2016; Wang et al., 2015) and sparging with air (Cheng et al., 2013).

Researchers also reported the pretreatment process by adding chemical agents or activated carbon to coagulate and adsorb the color. Activated carbon addition was found to be more effective to adsorb the dark color compared to the addition of starch and rice powder as a coagulator. Mutanda et al. (2011) described the application of NaOCl in wastewater medium to lower turbidity. Markou et al. (2012) utilized 12.5 g L^{-1} NaOCl as pretreatment of olive oil mill effluent, which resulted in no negative impact on microalgae. Finally, Qin et al. (2014) showed that the addition of 30 ppm NaOCl gave a better pretreatment for dairy manure rather than UV irradiation. Overall, any pretreatment process of wastewater is highly recommended and is likely to enhance microalgal growth and production.

5. Potency of microalgae as a source of bioactive compounds growing on POME

Microalgae have a great potential to produce food, feed, fuel, fine chemicals and fertilizers on a commercial scale, in spite of facing several issues (Ruiz et al., 2016; Fu et al., 2015; Milledge, 2011). Apart from biofuel production, microalgae may serve as a potential renewable source for other commercial applications (Milledge, 2011) as described earlier. Despite the high nutrient levels, the utilization of POME as growth medium for microalgae at industrial scales is still challenging. First, the high organic compounds, consisting of tannins, lignin, and phenolic compounds could negatively affect growth (Habib et al., 2003; Neoh et al., 2013; Nur et al., 2016). The dark coloration due to high concentrations of suspended solids could inhibit light penetration, which is a critical factor for photosynthetic growth (Pacheco et al., 2015; Nur et al., 2017).

Moreover, the presence of heterotrophic bacteria may affect biomass productivity (Cho et al., 2011b; Li et al., 2011). The low pH and salinity of the wastewater needs to be adjusted before it can be used as growth medium for alkaline microalgae such as *A. platensis* or marine microalgae, such as *Phaeodactylum tricornutum* which contains high levels of bioactive compounds. In addition, in

some cases, the heavy metals in POME could prevent utilization of the bioactive compounds from the algae as pharmaceutical, cosmetic or human consumption unless the metals are carefully removed (Ahmad et al., 2017).

However, the conditions as mentioned above could be prevented by employing some pretreatment processes to lower COD, color, and heavy metals from the POME as described previously in section 4. To increase the salinity, the cultivation might be relocated to seashore areas. Furthermore, the wastewater could be blended with hypersaline wastewater generated from industrial activities, such as chemical manufacturing, and oil production (Woolard and Irvine, 1995), to make the cultivation become more feasible for marine microalgae.

6. Thesis Outline

As explained above, the utilization of POME as cultivation medium for microalgae to produce high value-added compounds with an outlook towards large scale cultivation was not well explored at the start of this PhD project. Therefore, the goal of this thesis was to fill the gaps in this knowledge by:

I. investigating the potential of growing a range of microalgal species on POME and to monitor the production of their associated high value products.
I. investigating a range of environmental and nutritional parameters and their interrelation, to optimize the utilization of POME as growth medium for key microalgae and their associated value added products.
I. investigating the potential of microalgal cultivation to improve POME quality by color and phenolic compound removal.

Addressing the specific aims given above would not only give basic insight in POME utilization by microalgae, but would also provide essential knowledge with respect to the interrelation between parameters, in order to optimize the production of value added compounds (II.). With respect to III, microalgae could be used to remove color since the costs to remove color from POME when using membrane filtration are very high (Ahmad et al., 2006; Amat et al., 2015). Furthermore, microalgae could be used to remove phenolic compounds from POME since the maximum toxicity concentrations for phenolic compounds is between 10–24 mg L^{-1} for humans and between 9–25 mg L^{-1} for fish (Kulkarni and Kaware 2013), while conventionally treated aerobic POME contains 280–680 mg L^{-1} of phenolic compounds (Chantho et al., 2016).

To address the points given above, four experimental studies were performed under different selected parameters, using several (freshwater, brackish, marine) algal species.

In **Chapter 2**, growth of the marine diatom *Phaeodactylum tricornutum* on POME was followed as a function of light intensity, temperature, salinity, nutrient

enrichment and POME fraction to produce the high value added compound fucoxanthin. This brown pigment exhibits anti-cancer, anti-obesity, and anti-diabetic activity (Kim et al., 2012).

The main research question of this chapter was:
What are the optimal POME fraction and environmental and nutritional conditions for the marine diatom *Phaeodactylum tricornutum* cultivated on POME, in order to optimize fucoxanthin productivity?

A series of experiments was done where *P. tricornutum* was grown on a range of POME fractions, during which light intensity, temperature, salinity and nutritional conditions (via nitrate, urea, phosphate additions) were varied. Box-behnken response surface methodology (RSM) statistical tools were used to evaluate the impacts of the above described parameters on algal biomass and fucoxanthin productivity.

In **Chapter 3**, the effects of POME fraction, salinity, irradiance, nutrient addition and temperature on growth and biomass composition of *P. tricornutum* were studied, with special focus on sulfated exopolysaccharide (sEPS) and carbohydrate content. Sulfated exopolysaccharide (sEPS) is a specific group of polysaccharide substances generated from secondary metabolic processes within algae, excreted under normal as well as under unfavorable conditions (Raposo et al., 2013; Ates, 2015). sEPS is associated with internal carbohydrate production. Therefore, it is important to study both external and internal polysaccharide production. Several applications have been widely implemented in agricultural fields to improve soil properties, and thereby to enhance plant growth (Painter, 1993). In the pharmaceutical field, sEPS from *P. tricornutum* is promising as anti-inflammatory, antiviral, antiparasitic, anti-tumor, and hypocholesterolemic product, as reported before (Guzmán et al., 2003; Raposo et al., 2013; Delattre et al., 2016).

The main research question of this chapter was:
What are the optimal conditions with respect to POME fraction, urea addition, salinity, and temperature, for the growth, nutrient removal, sEPS, carbohydrate and biomass production of *P. tricornutum*?

To address the question, a series of experiments was done by varying POME fractions, environmental and nutritional conditions (the latter via urea additions) after which sEPS production and intracellular carbohydrate content were measured. Then, general full factorial design and Box-behnken RSM were performed to evaluate the most influencing factors and their interactive effects on growth rate, biomass, and sulfated exopolysaccharide released from the cells. The effect of nutrient enrichment on nutrient removal efficiency of POME was studied in a last experiment of the series.

In **chapter 4**, the brackish cyanobacterium *Arthrospira platensis* was used to unravel the utilization of POME at high concentrations, as well as to explore the production of C-phycocyanin under different cultivation modes, environmental and nutritional conditions. Phycobiliproteins are pigments mainly consisting of C-Phycocyanin (C-PC). It is well known for its antioxidant, anti-inflammatory, and anti-carcinogenic functions (Wu et al., 2016; de la Jara et al., 2018).

The main question for this chapter was:
What are the optimal conditions for nutritional, environmental, and cultivation mode to optimize C-PC productivity by *A. platensis* grown on high POME fractions?

To answer this question, a series of experiments was done which involved the effect of nutritional, environmental, and cultivation mode. Biomass and C-PC productivity (no POME) were studied as a function of nutrient availability and light intensity after which the same type of experiments were done, including different POME fractions. POME, salinity, light intensity and nitrogen addition were varied using full factorial design, followed by the optimization of urea concentration and salinity for C-PC production. Different nutritional (nitrogen and/ or phosphorus addition) and cultivation modes (batch or semi-continuous) were evaluated to understand and optimize the utilization of high POME fractions on biomass and C-phycocyanin productivity of *A. platensis*.

In **Chapter 5**, *A platensis* was employed to study biomass productivity, biodegradation of color, COD and total phenolic compounds of POME as a function of environmental and nutritional conditions. This was done, since in treated conventional POME, both color and phenolic compounds still exceed maximum regulatory allowed levels. Thus, these levels need to be reduced before POME can be released into the environment.

The main question for this chapter was:
What are the optimal cultivation conditions for *A. platensis* to degrade color and phenolic compounds of POME?

A series of experiments was done involving the effect of initial phenolic compounds, nutritional and environmental factors. The effect of POME fractions was studied on *A. platensis* growth rate, final biomass, POME color, phenolic compounds and COD removal, without adding external nutrients. Then, general full factorial design was employed to study the most influencing factor (POME fraction, salinity, irradiance level and nitrate concentration) as well as their possible interactive effects on biomass productivity and total POME color removal. In the last experiment, central composite design (CCD) RSM was employed to unravel the interactive effects of irradiance level and initial phenol concentration on growth, final biomass and phenol removal by *A. platensis*. Additional control experiments (without *A. platenisis*) were executed to understand the effect of irradiance exposure on the degradation of color and total phenolic compounds.



CHAPTER 2

Phaeodactylum tricornutum

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

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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 feedstock diatom producing bioactive compounds such as the carotenoid fucoxanthin, which is 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*. Furthermore, 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.

1. 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 (De Francisci et al., 2017; Ravindran et al., 2016). In this respect, the marine diatom species *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 allegedly exhibits anti-cancer, anti-obesity, and anti-diabetic activity (Kim et al., 2012). It is 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 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* was not explored so far.

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, supra optimal temperatures could be an extra stress factor on the growth of *P. tricornutum*. 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.

2. Material and Methods

2.1. 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 over a long time period. Prior to the experiments, the wastewater was thawed and filtered over GF/C glass fiber filters (Whatmann, 47 mm) to remove particulate matter. Subsequently, the filtrate was sterilized at 120°C for 15 minutes. 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 et al. 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.

Table 1. POME wastewater characteristic for different treatments. COD is chemical oxygen demand, TN is total nitrogen, TP is total phosphorus.

	Raw POME (Unautoclaved)	Sample POME (Unfiltered autoclaved)	Sample POME (GF/C filtration + Autoclaved)
COD (mg L^{-1})	1554	1418	1245
NO_3^- N (mg L^{-1})	17.5	16.1	10.3
TN (mg L^{-1})	117.4	108.5	72.4
PO_4-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

2.2. 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 (Guillard 1975) supplemented with silicate (100 µM) and $NaHCO_3$ (2.38 mM). The culture was acclimated to the experimental conditions for at least one 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.

1. Effect of temperature and light intensity on P.tricornutum 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 by using a hemocytometer. Growth rates was 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).

2. Effect of different POME concentrations on P. tricornutum growth and fucoxanthin productivity

P. 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 2 N HCl or 2 N 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.

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

P. 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 (six biolux and six 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 as inoculate to 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°C and 25°C, initial pH was adjusted to 8.0±0.2 by using 2 N HCl or 2 N NaOH, and salinity was adjusted to 35 ppt using NaCl. The light intensity was measured using a light sensor (Biospherical Instrument QSL2101, California, 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 by using a hemocytometer. At the end of the exponential growth phase (7-15 days cultivation), the cultures were harvested for pigment analysis.

4. 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

Table 2. Fucoxanthin productivity of *P. tricornutum* cultivated at 300 µmol photons m^{-2} s^{-1}, on 30% POME supplemented with different nutrients. Mean values are from 2 replicates. SD are shown after the ± symbol. Note: 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.

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 10^6	Fucoxanthin productivity (µg L^{-1} d^{-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	3	Nitrate	2.6	15	4:1	17.96±0.10	23.02±5.68
NP	20	30% POME	3	Nitrate	2.6	15	4:1	18.18±1.03	20.40±4.94
UP	20	30% POME	3	Urea	2.6	15	4:1	16.74±0.39	16.37±1.44
U	20	30% POME	23	Urea	-	7	108:1	7.02±6.66	25.44±21.80
Control	20	30% POME	-	-	-	7	13:1	1.05±0.27	4.01±3.57
P-Low	20	30% POME	-	-	0.3	7	8:1	2.09±0.02	5.81±0.75
P-High	20	30% POME	-	-	1.6	7	3:1	2.36±0.19	9.36±0.02
f/2	25	f/2	12.3	Nitrate	1.1	15	24:1	14.91±2.42	23.07±0.82
NPT	25	30% POME	3	Nitrate	2.6	15	4:1	16.60±0.49	18.41±8.00
NP	25	30% POME	3	Nitrate	2.6	15	4:1	16.06±2.11	18.90±8.49
UP	25	30% POME	3	Urea	2.6	15	4:1	13.60±3.32	16.30±3.78
Control	25	30% POME	-	-	-	7	13:1	0.56±0.01	2.60±0.12

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 (X_1), 5, 20, and 35 ppt for salinity (X_2), and 0, 50, 100 mg L^{-1} for urea addition (X_3). The empirical form of the second order polynomial model (Eq.1) can be described as:

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

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.

Table 3. Design of the experiment of BBD with the results. Mean values of the experimental results are from 2 replicates. SD is shown after the ± symbol.

Run	Temperature (°C)	Salinity (ppt)	Urea addition (mg L^{-1})	Final cell density (cells L^{-1}) x 10^6		Fucoxanthin productivity (µg L^{-1} d^{-1})	
				Experimental	Predicted	Experimental	Predicted
1	20	5	50	5.00±0.20	5.54	10.07±0.81	13.20
2	20	35	50	7.02±6.66	5.61	25.44±21.80	20.46
3	20	20	0	1.32±0.14	2.03	9.16±1.21	8.11
4	20	20	100	7.56±0.42	7.84	17.09±0.21	20.72
5	22.5	5	0	1.22±0.03	0.81	6.88±0.28	5.18
6	22.5	35	0	1.30±0.33	2.02	6.68±0.95	13.78
7	22.5	5	100	6.72±0.11	5.68	25.19±6.23	18.02
8	22.5	35	100	6.25±0.29	7.49	34.74±1.61	36.46
9	22.5	20	50	6.49±2.58	6.69	32.75±19.86	32.79
10	25	5	50	0.13±0.02	0.17	0.23±0.16	5.97
11	25	35	50	6.41±0.31	5.44	29.69±4.31	20.46
12	25	20	0	1.04±0.01	0.33	6.42±0.10	1.99
13	25	20	100	6.08±0.03	5.59	23.09±0.33	24.90

Cultivation was carried out in the same set-up 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.

2.3. Analyses

2.3.1. Wastewater composition

Chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP), were analysed by using the assay kit LCK514, LCK349, and LCK138, respectively, provided by Hach Lange on a 3900 DR spectrophotometer, following the protocol listed in the assay product kits. Wastewater sample was collected from different sources, raw POME, autoclaved, and autoclaved + filtered (GF/C). The sample was hydrolyzed according to the protocol to measure TN and TP.

2.3.2. Growth rate

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

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

where μ is growth rate (day^{-1}) X_2 is the cell density (cell mL^{-1}) at time t_2 (day) and X_1 the cell density (cell mL^{-1}) at time t_1 (day)

2.3.3. 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°C until analysis. Before analysis, the filters were freeze-dried at -50°C for 48 hours and a pressure of 30 x 10^{-3} mbar. Extraction was conducted by adding 5 mL of 90% cold acetone into the sample at 4°C for 48 hours. After extraction, the sample was analysed 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.

$$P_p = \frac{(N_h - N_0) \times C_p}{t} \qquad (3)$$

where P_p is pigment productivity (µg L^{-1} day^{-1}), N_h is final cell density (cells L^{-1}), N_0 is initial cell density (cells L^{-1}), C_p is pigment content (µg cell^{-1}), and t is total duration of the cultivation (d).

2.3.4. 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 analysed with a one-way analysis of variance (ANOVA) with a p-value of 0.05. Post hoc tests (Tukey HSD) were performed for pair-wise 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.

3. Results

3.1. 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 (1st experimental series) varied significantly with temperature and irradiance (Figure 1). Three types of light responses were distinguished, Low Light (LL, < 100 µmol photons m^{-2} s^{-1}), Medium Light (ML, 100–200 µmol photons m^{-2} s^{-1}) and High Light (HL, > 200 µmol photons m^{-2} s^{-1}).

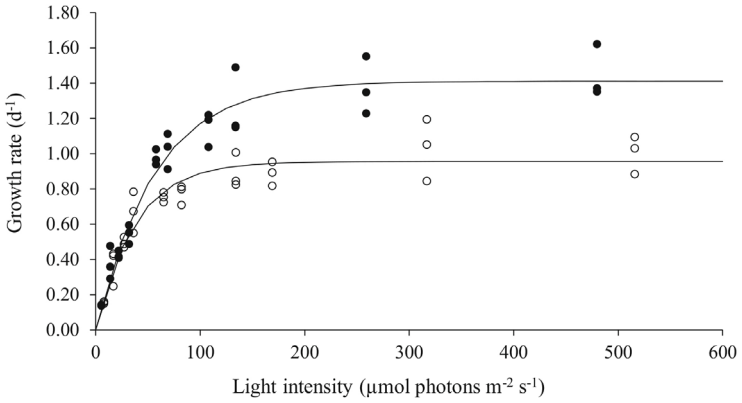

Figure 1. Growth rate of *P. tricornutum* cultivated on f/2 medium as a function of light intensity and temperature Symbols: 20°C (○) and 25°C (●)

At ML and HL, the growth rate was influenced by temperature ($p < 0.01$). At 25°C, the growth rate was significantly ($p < 0.01$) increased as compared with the 20°C cultures. Light was found to be saturating at 100 µmol photons m^{-2} s^{-1} for 20°C with the maximum growth rate of 0.95 day^{-1}. At 25°C growth was saturated at 175 µmol photons m^{-2} s^{-1} with a maximum growth rate of 1.41 day^{-1}. For both temperatures, no photoinhibition was found up until 500 µmol photons m^{-2} s^{-1} (Figure 1).

Fucoxanthin and chlorophyll-a content were found to be both irradiance and temperature dependent (Figure 2). Fucoxanthin content was significantly lower at ML and HL compared to LL for each temperature ($p < 0.01$).

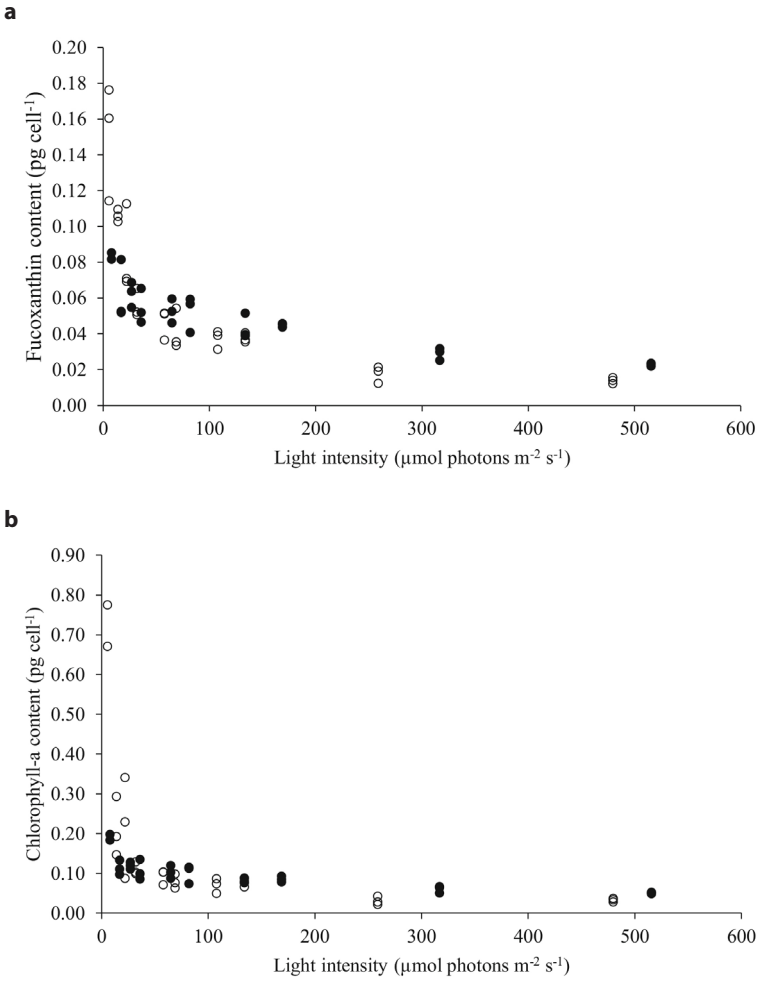

Figure 2. Fucoxanthin (a), and chlorophyll-a (b) content of *P. tricornutum* cultivated on f/2 medium at 20°C (○) and 25°C (●) at different irradiances.

However, when comparing both temperatures, the fucoxanthin content significantly differed only at ML and HL ($p < 0.05$). For fucoxanthin and chlorophyll-a productivity (Figure 3), the highest value was found at around 100-125 µmol photons m^{-2} s^{-1} for both temperatures. The pigment productivity was not significantly different when comparing both temperatures ($p > 0.05$).

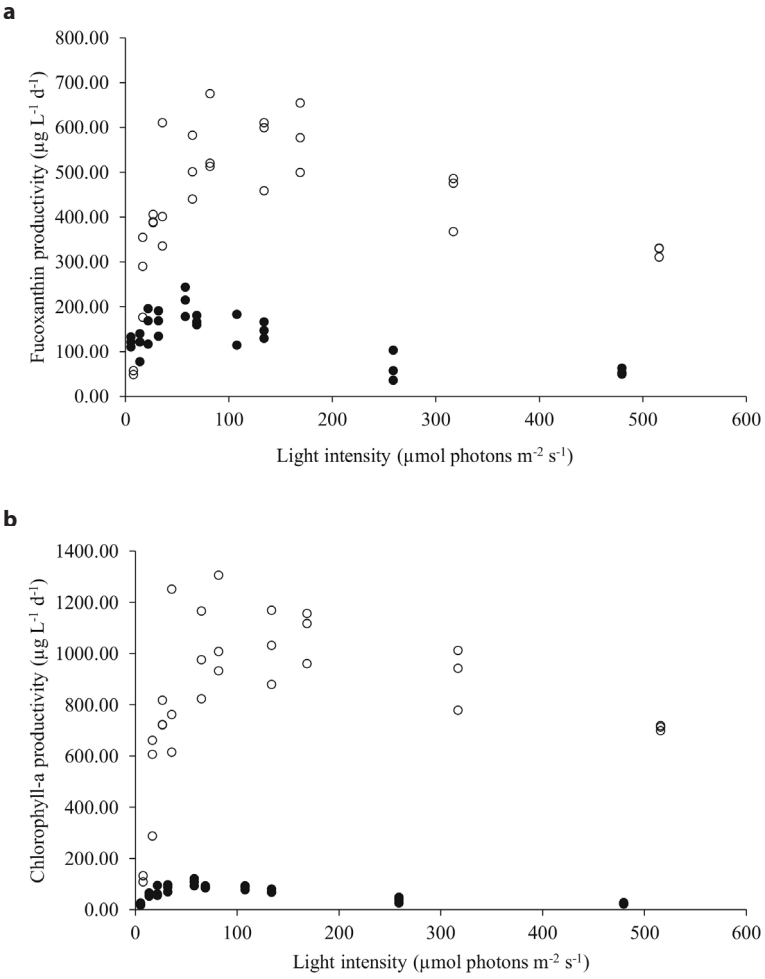

Figure 3. Fucoxanthin (a), and chlorophyll-a (b) productivity of *Phaeodactylum tricornutum* cultivated under different light intensities at 20°C (○) and 25°C (●)

3.2. Effect of different POME fractions

When cultivating *P. tricornutum* on different sterilized-unfiltered POME concentrations (Figure 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 (Figure 4). However, the growth rate at 50% v/v POME did not significantly differ from the 30% v/v 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.

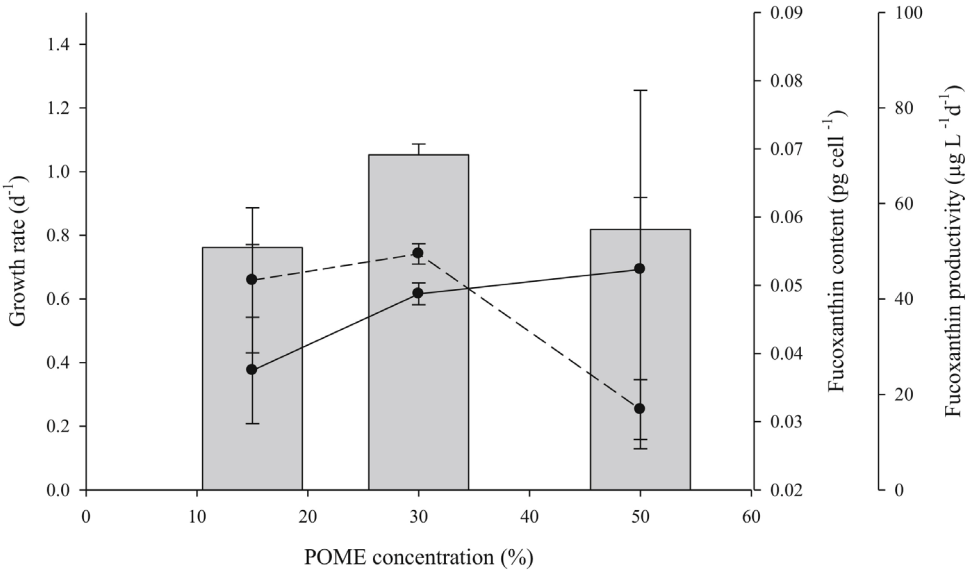

Figure 4. Fucoxanthin productivity (dashed line), fucoxanthin content (full line) and growth rate (bar) of *P. tricornutum* cultivated on the mixture of sea water and unfiltered POME concentrations at 23°C and 100 µmol photons m^{-2} s^{-1}. Average values of duplicate cultures are shown. Error bars indicate the SD of the mean.

3.3. 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 NPT, UP and 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).

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

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

$$Pp\ (\mu g\ L^{-1}\ d^{-1}) = -32.79 + 0.49\ X_1 - 6.76\ X_2 - 8.88\ X_3 - 10.44\ X_1^2 - 6.01\ X_2^2 - 8.42\ X_3^2 + 3.13\ X_1.X_2 + 2.58\ X_1.X_3 + 2.46\ X_2.X_3 \quad \text{Eq. 4}$$

where X_1, X_2, X_3 are temperature, salinity, and urea, respectively.

The analysis of variance (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 (Figure 5), temperature, urea and salinity were important factors to obtain the optimal fucoxanthin productivity. Furthermore, all tested 2-way interactive effects between the factors were found to be not significant ($p>0.05$). The optimum condition for fucoxanthin productivity was found 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 (Figure 6). Overall, the optimum combination of environmental conditions with respect to cell density (8.13×10^6 cells mL^{-1}) was found to be 21°C, 23 ppt salinity and 90 mg L^{-1} urea addition.

4. 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 (Figure 2). This implies that supraoptimal irradiance levels in large scale cultivation set-ups would not benefit fucoxanthin productivity, even when growth rates are high (Figure 1).

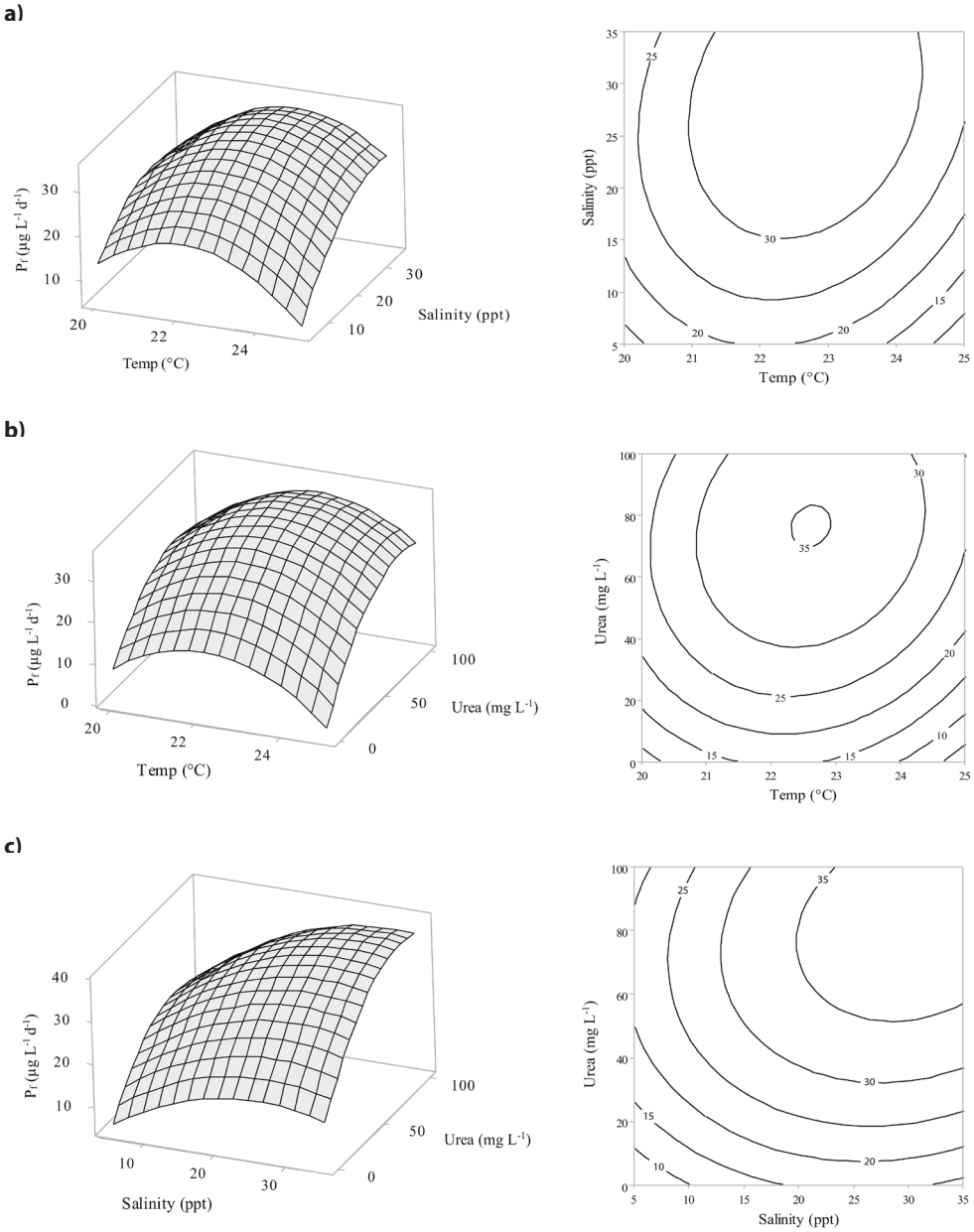

Figure 5. Response surface plots (3D) showing the effects of temperature (°C), urea (mg L^{-1}), and salinity (ppt) on fucoxanthin productivity (P_f) (µg L^{-1} day^{-1}) generated by *P. tricornutum* at hold value 50 mg L^{-1} (a), 20 ppt (b), and 22.5°C (c). The optimal fucoxanthin productivity (38 µg L^{-1} day^{-1}) was found at 23 °C, 31 ppt salinity and 85 mg L^{-1} urea addition.

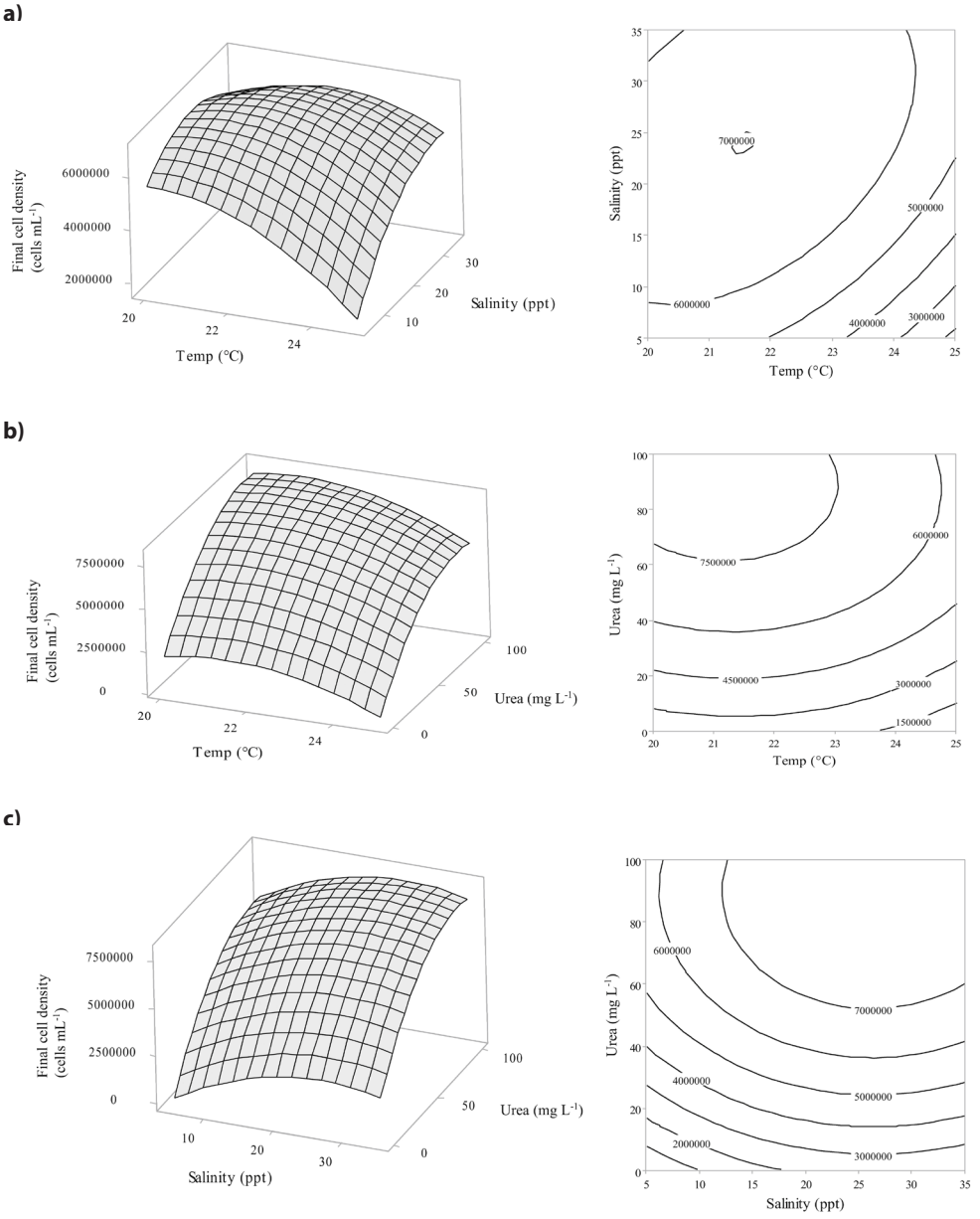

Figure 6. Response surface plots (3D) and contour (2D) plots showing the effects of temperature (°C), urea (mg L^{-1}), and salinity (ppt) on final cell density (cells mL^{-1}) by *P. tricornutum* at hold value 50 mg L^{-1} (a), 20 ppt (b), and 22.5°C (c) . The optimal final cell density (8.13 x 10^6 cells mL^{-1}) was found at 21°C, 23 ppt salinity and 90 mg L^{-1} urea addition

However, by adjusting the cell density, the average irradiance received by the cells can be adjusted. The optimum light intensity for pigment productivity was found at around 80-125 µmol photons m^{-2} s^{-1} (Figure 3). In addition temperature was found to influence 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 would 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, the 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 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 (Figure 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* as reported before (Ayre et al., 2017). Furthermore, the presence of potentially toxic phenolic compounds in digested POME could also lower the growth rate of *P. tricornutum* as found before (Limkhuansuwan 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 found to be 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 Refield 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 *Chlorella 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 f2 and a modified L1 medium, respectively (McClure et al., 2018; Xia et al., 2013). 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 sole urea instead of other nitrogen sources (e.g. nitrate) or phosphorus could therefore significantly save production costs, and therefore 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 could assimilate urea as a source of nitrogen by converting it to NH_4^+ and CO_2 through urease activity or ammonium and bicarbonate via ATP-urea amidolyase (Solomon and Glibert, 2008; Bekheet and Syrett, 1977). This might lead to the high ammonium or ammonia concentrations, which tend to be toxic for diatoms such as *Phaeodactylum tricornutum,* as well as other algae such as chlorophytes (Fidalgo, et al., 1995; Collos and Harrison, 2014). This was supported by Setyoningrum and Nur (2015) who 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 (Figure 5, Table 3). Thus, the strategy by maintaining the factors at optimum conditions could enhance the fucoxanthin productivity.

5. 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 with care. 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.

Acknowledgment

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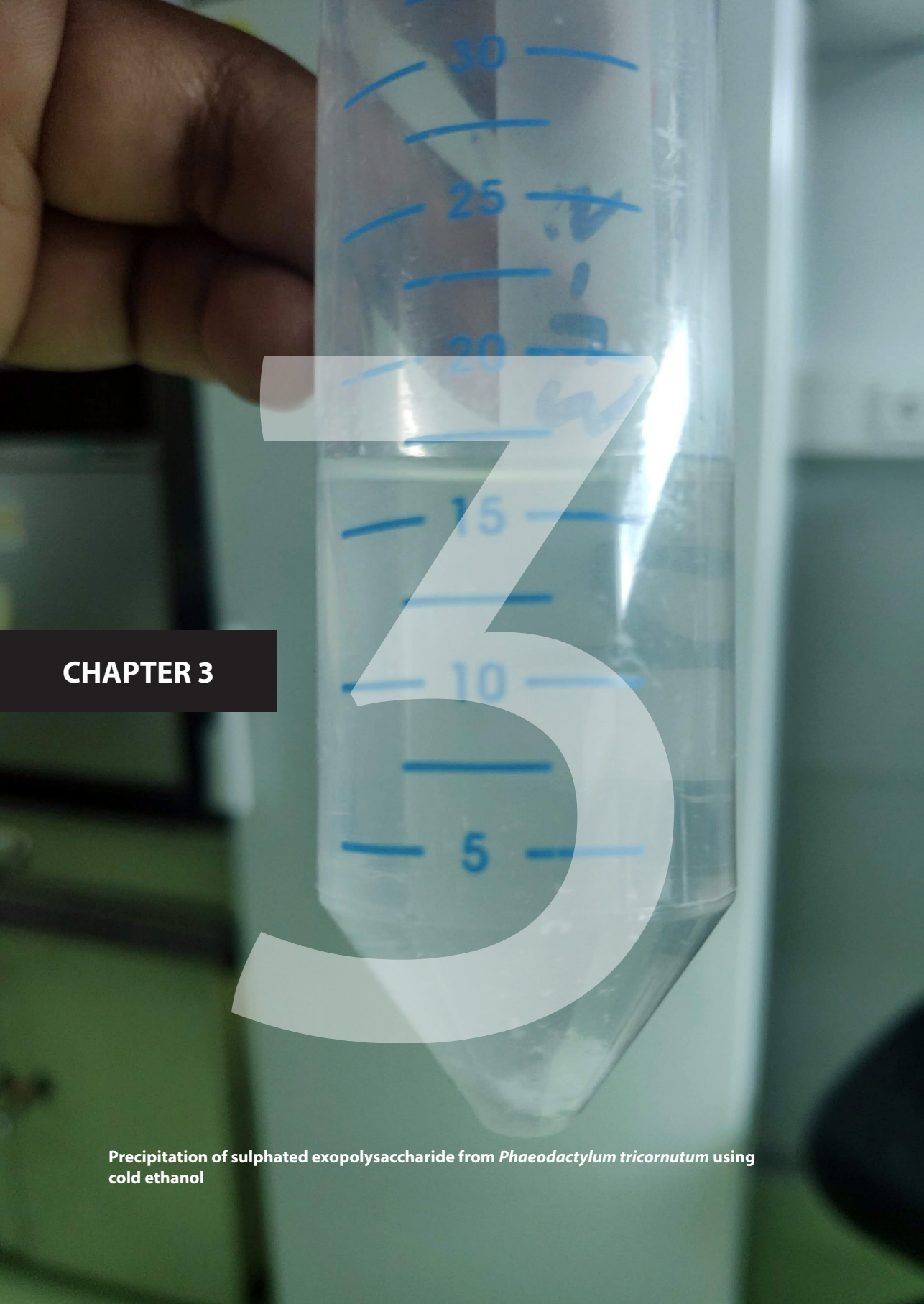
CHAPTER 3



Precipitation of sulphated exopolysaccharide from *Phaeodactylum tricornutum* using cold ethanol

Sulfated exopolysaccharide production and nutrient removal by the marine diatom *Phaeodactylum tricornutum* growing on palm oil mill effluent

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Abstract

Palm Oil Mill Effluent (POME) is the major source of environmental hazard in palm oil industry. Yet, due to its high nutrient content, POME may offer opportunities for the growth of algae as a source of value-added compounds such as sulfated extracellular polysaccharide (sEPS) while simultaneously removing valuable nutrients such as phosphate. The aim of this paper was to evaluate growth, total sEPS production and nutrient removal by *Phaeodactylum tricornutum* grown on POME under a range of experimental conditions (temperature, salinity, supplementation of extra nutrients). *P. tricornutum* was found to grow well on a range of POME concentrations, with 30% POME as optimum concentration. Nitrate and urea addition enhanced both growth rate and final biomass, whereas phosphate significantly stimulated growth only at low temperature. Box-Behnken response surface methodology revealed that interactions between temperature and salinity, and between temperature and urea influenced sEPS production. The highest total sEPS (140 mg L^{-1}) concentration was recorded at 25°C, 2.6 % salinity, and 100 mg L^{-1} urea addition. Our study shows that POME wastewater, supplemented with urea at relatively high temperatures, can be considered as a potential medium for *P. tricornutum* to replace commercial nutrients while producing high amounts of sEPS and removing almost 90% of phosphorous from the wastewater.

1. Introduction

Palm oil mill effluent (POME) is agricultural wastewater generated from palm oil processing. Presently, Asian coconut palm oil (CPO) production comprises 87.8% of the total world production of 57.32 million ton. Within this region, Indonesia is currently known as the largest CPO producer, followed by Malaysia (Faostat, 2018). Direct discharge of POME into the environment causes severe pollution due to its high organic matter content, high chemical oxygen demand (COD), biochemical oxygen demand (BOD), high nutrient levels, dark color and strong smell (Nur et al., 2017). To date, the conventional ponding system is the most common method for treating POME (Liew et al. 2015). In Malaysia, more than 85% of palm oil mills have adopted the ponding system due to its low capital cost (Tong and Bakar, 2004). Nevertheless, the conventional ponding system has various limitations such as low nutrient removal efficiency, long retention times, and enhanced greenhouse gas emissions (Tabassum et al., 2015). The strategy to decrease adverse characteristics of POME by using microalgae while simultaneously producing value added products could be promising due to the fact that POME contains high micro- and macronutrient levels (Nur and Buma, 2018). However, the nutrient removal efficiency by microalgae will also depend on cultivation conditions as well as on nutrient composition in terms of speciation and initial N/P ratio (Beuckels et al., 2015). Given the worldwide phosphorus shortage, nutrient conditions within cultivation systems should be optimal or otherwise optimized such that complete phosphate removal from the wastewater is achieved.

Extracellular polysaccharide or exopolysaccharide (EPS) is a group of polysaccharide substances generated from secondary metabolic processes within algae, excreted under normal as well as under unfavorable conditions (Raposo et al., 2013; Ates, 2015). EPS may contain xylose, galactose or glucose. In addition, sulfated exopolysaccharides (sEPS) are formed, which may be attached to lipids or proteins, depending on the cultivated algal species (Delattre et al., 2016). In general, the overall composition of sEPS is species specific. For example, sEPS produced by *Phaeodactylum tricornutum* contains 7.5-13.3% sulphate, and 1.4-6.3 % uronic acids, while sEPS from *Chlorella stigmatophora* contains 7.8-9.4% sulphate and 3.7-9.0 uronic acid (Guzmán et al., 2003).

Recently, the interest in utilizing EPS from microalgae has increased. Several applications have been widely implemented in agricultural fields to improve soil properties, and thereby to enhance plant growth (Painter, 1993). In the pharmaceutical field, sEPS from *P. tricornutum* is promising as anti-inflammatory, antiviral, antiparasitic, anti-tumor, hypocholesterolemic, as repored before (Guzmán et al., 2003; Raposo et al., 2013; Delattre et al., 2016). However, the production of microbial sEPS at a large scale, especially from microalgae, is limited due to the high production cost (Ates, 2015). Alternatively, the cultivation of microalgae on

POME with the aim to produce sEPS and carbohydrates could be promising, given the relatively high nutrient content of the wastewater, thereby making commercial nutrients redundant. Furthermore, the treated, sEPS containing POME might be utilized for soil improvement in the vicinity of the palm oil plants.

It has been suggested that culture conditions and nutrient composition of the medium influence the sEPS production of microalgae (Guzmán-Murillo et al., 2007; Ekelhof and Melkonian, 2017). Microalgae tend to produce higher sEPS under stressed conditions such as excess irradiance and supra-optimal temperatures, as a means to prevent cell damage via the action of exopolymers (Kumar et al., 2007; Delattre et al., 2016). Other factors such as nutritional conditions and salinity were also reported to influence EPS production and composition (Abdullahi et al., 2006; Díaz et al., 2014). However, based on our knowledge, the influence of temperature on EPS production was not yet explored. Furthermore, the interaction between these parameters was not well reported so far. Therefore, it can be assumed that total biomass, total sEPS, and intracellular carbohydrate productivity by *P. tricornutum* cultivated on POME would be influenced by environmental conditions such as salinity, nutrient availability and temperature. The aim of this research was to study the utilization of nutrient enriched POME and the interaction of urea addition, salinity, and temperature on growth, nutrient removal, sEPS and biomass production of *P. tricornutum*. We chose urea addition because it guarantees complete phosphorus removal (Nur et al, 2019a). Furthermore, excess nutrients from the wastewater that cause eutrophication could be treated by using microalgae since it is cost effective compared to chemical wastewater treatment (Smith et al., 1998; Delrue. et al., 2016). In order to find optimal cultivation conditions, we employed response surface methodology (RSM) based from box-behnken design (BBD).

We chose the marine diatom *P. tricornutum* as test alga since *P. tricornutum* was shown before to grow on POME to produce bioactive compounds (Nur et al. 2019a). Furthermore, sEPS composition of *P. tricornutum,* which mainly consist of glucose, mannose, and galactose, was also affected by environmental conditions such as salinity and nutrient addition (Abdullahi et al., 2006). Finally, in contrast to most diatoms, *P. tricornutum* has a cell wall that is rich in sulfated polysaccharides and poor in silica (Le Costaouëca et al., 2017). Therefore, *P. tricornutum* could be a promising candidate as a source of sEPS, when growing on POME in an optimum condition.

2. Material and Methods

2.1 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 at -19°C to prevent degradation over a long time period. The wastewater was thawed and filtered over GF/C glass fiber filters (Whatmann, 47 mm) to remove particulate matter. Subsequently, the filtrate was sterilized at 121°C for 15 minutes. This treated POME contained 1245 mg L^{-1} COD, 72.4 mg L^{-1} total dissolved nitrogen (TDN), and 7.93 mg L^{-1} total dissolved phosphorus (TDP), as estimated using the assay kits LCK349 and LCK138 (Hach Lange).

2.2. Experimental setup

Precultures 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 3.5 % (w/v) with demineralized water) by the protocol of Guillard (Guillard 1975) supplemented with silicate (100 µM). The algae were cultivated in a 16:8h light dark cycle at a temperature of 25°C. The cultures were illuminated with fluorescent lamps (see below) providing 300 µmol photons m^{-2} s^{-1}. The cultures were diluted with fresh Guillard medium if growth reached the stationary phase. The cultures were acclimated to the experimental conditions for at least one week prior to experimentation. In total four experiments were done.

2.2.1. Determination of the optimal POME concentration for P. tricornutum growth

Six concentrations of POME (5%–100% v/v) and one control (f/2) were prepared based on appropriate dilutions in sterilized oligotrophic seawater. The pH of each concentration was adjusted to 8 by using 2N NaOH or HCl and the salinity was set to 3.5 % (w/v) by using commercially available artificial sea salt (Reef Salt, Aqua Medic, Germany). All media were supplemented with silicate (100 µM). Two replicates (each 75ml, in 100 ml Erlenmeyer flasks) were made for each concentration. *Phaeodactylum tricornutum* (2%v/v, which equals an initial cell density of around 15 x 10^4 cells mL^{-1}) was inoculated into each flask and the cultures were incubated in a water bath at 25°C and a light: dark cycle of 16h: 8h. The water bath was equipped with a temperature controller and a U shaped lamp that contained 12 fluorescent lamps (six Biolux and six skywhite lamps, Osram) coupled to reflectors (Doublelux) and connected to dimmers (Osram) and set as described by Van de Poll et al. (2007). The culture flask was closed with a cotton stopper and gently shaken manually every day to resuspend the cells and to provide aeration to the cells. For all experiments, a saturating light intensity (300 µmol photons m^{-2} s^{-1}) was used as

determined earlier (Nur et al., 2019a). Due to the variable irradiance penetration in the culture flasks as a result of the different POME fractions, the light intensity was adjusted using neutral density screens. The light intensity was measured in the center of culture flask by using a spherical light sensor (Biospherical Instrument QSL2101, California, USA) which is small enough to be placed inside the culture flasks. Every 24 hrs, 1ml of sample was taken from each culture flask and direct cell counting was performed using a haemocytometer under a light microscope. At least 200 cells were counted using a hand counter. The cultivation was stopped at the end of the exponential phase (4-7 days).

2.2.2. Effects of different nutrient additions on P. tricornutum biomass and sEPS production when growing on 30% POME

P. tricornutum was cultured in 75 mL working volume in 100 mL sterilized Erlenmeyer flasks and placed in a water bath as described above. About 2% (v/v) of *P. tricornutum* culture was used as inoculate to 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°C and 25°C, initial pH was adjusted to 8.0±0.2 by using 2 N HCl or 2 N NaOH, and salinity was adjusted to 3.5 % w/v using artificial sea salt (Reef Salt, Aqua Medic, Germany). The light intensity was set to 300 µmol photons m^{-2} s^{-1} inside the cultivation medium. Every 24 hrs, 1ml of sample was taken from each culture flask and direct cell counting was performed using a haemocytometer under a light microscope. At the end of the exponential growth phase (7-15 days cultivation), the cultures were harvested for sEPS, biomass (dry weight) and intracellular carbohydrate.

2.3.3. Effect of salinity, temperature, and urea addition on growth rate, biomass and sEPS production of P. tricornutum growing on 30% POME

General full factorial design (GFFD) with three variables was performed to determine the factor most affecting sEPS and biomass production as well as their interactions with respect to the growth rate. A further experiment based on GFFD was employed using Box behnken design (BBD) response surface methodology (RSM) to study the optimum value of the variables for biomass and sEPS production. The empirical form of the second order polynomial model for BBD can be described as (Eq. 1)

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

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.

The experimental design, based on two replicates, consisted of three factors, temperature (20, 22.5, 25°C), salinity (0.5, 2.0, 3.5 % w/v), and urea addition (0, 50, 100 mg L^{-1}). The alga was cultured in 75 mL working volume in a 100 mL sterilized Erlenmeyer flask and placed in the water bath equipped with U shaped lamps and temperature controller as described above. About 2% (v/v) of *P. tricornutum* culture was used as inoculate to the medium consisting of 30% v/v POME + 70% v/v sea water. Salinity was adjusted by using sea water and milli Q. All media were supplemented with silicate (100 µM), and pH was adjusted to 8±0.2 by using 2 N HCl or 2 N NaOH at the beginning of the cultivation. The light intensity was set to 300 µmol photons m^{-2} s^{-1} inside the cultivation medium. About 1 mL of the sample was taken daily and preserved using 1% v/v lugol. The cell density was counted by using a haemocytometer until the cultures reached the end of the exponential phase (7 days). Then, cultures were harvested for analyses of final biomass (dry weight) and sEPS.

2.2.4. Effect of nutrient enrichment on nutrient removal efficiency of POME

P. tricornutum was cultured in 75 mL working volume in 100 mL sterilized Erlenmeyer flasks and placed in a water bath as described above. About 2% (v/v) of *P. tricornutum* culture was used as inoculate to 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 control. Different nutrient enrichments were employed, 50 mg L^{-1} of urea (U), and 3 mg L^{-1} of phosphate (P). All media were supplemented with silicate (100 µM). The experiments were carried out at 20°C, initial pH was adjusted to 8.0±0.2 by using 2 N HCl or 2 N NaOH, and salinity was adjusted to 3.5 % (w/v) using artificial sea salt (Reef Salt, Aqua Medic, Germany). The light intensity was set to 300 µmol photons m^{-2} s^{-1} inside the cultivation medium. At the beginning of cultivation and the end of the exponential growth phase (7-9 days), the culture was filtered using GF/F glassfiber filters and nutrients were analyzed in the filtrate to obtain nutrient removal efficiency (for analysis, see description below).

2.3. Analyses

2.3.1 Growth rate

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

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

where μ is growth rate (day^{-1}) and X_2 and X_1 are the cell densities (cells mL^{-1}) at time 2 (t_2, day) and time 1 (t_1, day) respectively.

2.3.2. Biomass based on dry weight

Determination of cell dry weight of *P. tricornutum* was done using the gravimetric method. About 30 mL of the sample 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 95 °C until a constant weight was reached.

2.3.3. Carbohydrate and sEPS determination

To analyze the intracellular carbohydrate content of *P.tricornutum*, 1 mL of the sample was centrifuged at 13,000 rpm for 5 min. The supernatant was kept for sEPS determination. The pellet was washed twice by using cold Milli Q to remove remaining supernatant. The collected pellet was stored at -20°C until later analysis. Carbohydrate extraction was carried out by using 1 mL double distilled water (Milli-Q) at 80°C for 1 h (Van Oijen et al., 2005). The sample was then centrifuged at 5000 rpm for 5 min at 22°C in a temperature controlled centrifuge. The supernatant was collected to analyze total carbohydrates, using the total carbohydrate assay kit (Sigma, MAK104-1KT) at 490 nm on a Multilabel counter (Victor 1420, Perkin Elmer) according to the Dubois method (Dubois et al., 1956). D-glucose was used as a standard curve.

The determination of sEPS was done by colorimetry as explained by Ramus (1977) and Guzmán -Murillo et al. (2004) with slight modifications. Briefly, 1 mL of cell-free culture supernatant was gently mixed with 4 mL 0.5 M acetic acid and 500 µL Alcian blue 8GX (1 mg ml^{-1} in acetic acid 0.5M, pH 2.5), vortexed at 3000 rpm for 20 sec and then incubated overnight at room temperature (22°C). The samples were centrifuged at 3000 g for 30 min at 22°C. The sample was read at 610 nm on a Hach DR 3900, which did not interfere with the maximum peak of wastewater absorption at 320-330 nm. The difference in absorbance between the blank medium (30% v/v POME or Milli Q) and the samples was taken as proportional polyanion concentration. Dextran sulfate was used as a standard curve.

The volumetric carbohydrate productivity was calculated based on Eq 3.

$$P_c = \frac{(X_t - X_0).C_c}{t} \qquad (3)$$

where P_c is carbohydrate productivity (mg L^{-1} day^{-1}), X_t is final biomass concentration (mg L^{-1}), X_0 is initial biomass concentration(mg L^{-1}), C_c is carbohydrate content per total biomass dry weight (w/w), and t is total duration of the cultivation (d).

2.3.4. Nutrient analysis

For nutrient analysis, about 5-10 mL of the sample was filtered using GF/F glassfiber filter (Whatman), after which the filtrate was used for dissolved nutrient analyses as described below. Total phosphorus and orthophosphate were analyzed using the assay kit LCK349 (0.05–1.50 mg L^{-1} orthophosphate, 0.15–4.50 mg L^{-1} TDP, provided by Hach, Netherland). Samples were diluted using Milli-Q if necessary. For TDP analysis, 2 mL of the sample was carefully pipetted to the tube containing reagent LCK394 and hydrolyzed to a temperature block (Hach LT 200) at 100 °C for 60 min. After cooling down to room temperature, the tube was shaken vigorously and 0.2 mL of reagent was added. The orthophosphate determination procedure was identical to TDP analysis except for the hydrolysis step. For total dissolved nitrogen analysis, samples were diluted with MilliQ if necessary due to the range of TDN provided by the assay kit (1-16 mg L^{-1}) from LCK138 (Hach, Netherland). Briefly, 1.3 mL of the sample and reagents were added to a reaction tube and hydrolyzed at 120°C for 30 min in the heater block. After cooling down, the kit reagent was added to the tube and shaken. Then 0.5 mL of the sample was pipetted in a tube containing kit reagents (LCK138). For nitrate analysis, LCK339 (Hach, Netherland) was used (0.23-13.5 mg L^{-1} NO_3-N) and the sample was diluted with MilliQ if necessary. About 1 mL of the sample was pipetted to the tube containing reagents (LCK339) after which 0.2 mL of kit reagent was added. After 15 min, the sample was measured using spectrophotometry. Measurements were done using Hach Lange on a 3900 DR spectrophotometer following the programs listed in the machine.

The efficiency of nutrient removal was calculated based on Eq. 4

$$E = \frac{n_0 - n_t}{n_0} \times 100 \qquad (4)$$

where E is nutrient removal efficiency (%), n_0 is nutrient concentration (mg L^{-1}) of the sample at the beginning of cultivation and n_t is the nutrient concentration (mg L^{-1}) of the sample at the end of cultivation.

2.3.5. Statistical analysis

Minitab ver. 18. (Demo version) was used for statistical tool analysis, GFFD and BBD design and the evaluation. Differences between treatments were analysed with one-way or two-way analysis of variance (ANOVA) with p-value of 0.05. Post hoc tests (Tukey HSD) were performed for pair-wise comparisons. The experimental results were obtained based on at least two replicates as expressed in the standard deviations (±SD).

3. Results

3.1 Effect of POME fraction on the growth rate of *P. tricornutum*

The highest growth rate (1.10 d^{-1}) was recorded for *P. tricornutum* growing on f/2 medium, thus artificial medium without POME.

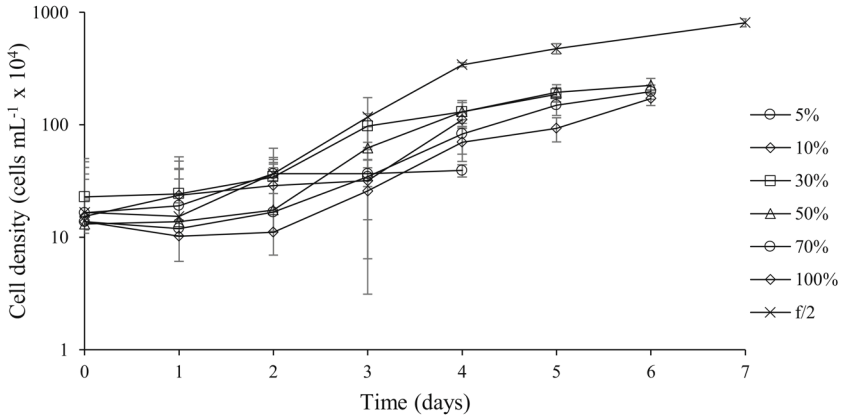


Figure 1. Growth profile of *P. tricornutum* cultivated at 25°C and 300 µmol photons m^{-2} s^{-1} under different POME concentration.

All POME fractions showed substantially lower growth rates, ranging between 0.30 and 0.66 d^{-1} (Table 1, Figure 1). Maximum growth was observed at 30% POME: above this fraction, growth rates decreased slightly. Therefore, 30% v/v of POME was used for all subsequent experiments.

Table 1. Growth rate of *P. tricornutum* cultivated on f/2 and on the mixture of sea water (SW) and filtered POME concentrations at 25°C and 300 µmol photons m^{-2} s^{-1} (measured inside the culture). Average values of duplicate cultures are shown. SD is shown after ± symbol. *) The molar ratio is calculated from dissolved inorganic nitrogen as nitrate and dissolved phosphorus as phosphate in the media. Values that do not share a common superscript letter in the same column are significantly different (P<0.05)

Media	NO_3^- N (µM)	PO_4-P (µM)	N:P molar ratio*	Growth rate (day^{-1})	
f/2	880.00	36.90	24:1	1.10	±0.04[a]
5% POME + 95% SW	36.79	2.89	13:1	0.30	±0.06[b]
10% POME + 90% SW	73.57	5.78	13:1	0.48	±0.02[bc]
30% POME + 70% SW	220.71	17.33	13:1	0.66	±0.12[c]
50% POME + 50% SW	367.86	28.88	13:1	0.63	±0.05[c]
70% POME + 30% SW	515.00	40.44	13:1	0.61	±0.01[c]
100% POME	735.71	57.77	13:1	0.61	±0.01[c]

3.2 Effects of nutrient enrichment on growth rate, total biomass, carbohydrate productivity, and sEPS.

The highest temperature (25°C) resulted in overall higher growth rates as compared with the 20°C cultures ($P<0.05$) (Table 2). At both temperatures, the growth rates after nutrient enrichment (both nitrogen and phosphorus) on 30% POME was not significantly different compared to f/2 medium. The lowest growth rate was found in the control cultures based on 30% POME without nutrient addition (C) for both temperatures (Table 2) ($P<0.05$). At 25°C, POME with urea addition (U) alone (no phosphate addition) resulted in a growth rate averaging 0.88 ± 0.04 day^{-1}, which was significantly higher compared to C (0.41 ± 0.07 day^{-1}). Furthermore, the growth rate at 25°C was not significantly different between urea addition alone and nitrate/urea plus phosphate addition (NPT, NP, UP) ($P>0.05$). However, at 20°C , the growth rate after urea addition alone was significantly different compared to f/2, NPT, NP, and UP medium ($P<0.05$). Based on this case, the addition of phosphorus alone could also influenced growth rate at low temperature. In our research, the addition of 1 mg L^{-1} phosphate alone to POME resulted at 20°C in a growth rate as high as the urea addition alone (data not shown).

With respect to total dry biomass production, the addition of nitrogen and phosphorus resulted in significantly different values compared to the POME control C at both temperatures ($P>0.05$) (Table 2). Furthermore, the total biomass production after the addition of urea alone (U) was not significantly different compared to NPT, NP, UP at both temperatures ($P>0.05$). Also no significant difference was found between both temperatures (Table 2) ($P>0.05$).

Temperature significantly influenced carbohydrate content and total sEPS production ($P<0.05$), while nutrient enrichment on 30% POME did not significantly influence total sEPS production at both temperatures (Table 2). Total sEPS normalized to biomass was found to be significantly higher with the addition of urea-only (U) compared to combined nutrient additions (NPT, NP, UP) at 25°C. Furthermore, the addition of urea-only (U) resulted in no significantly different carbohydrate productivity compared to the other nutrient additions (NPT, NP, UP) at the high temperature ($P<0.05$), although a trend was observed of elevated sEPS levels for the Control and Urea-only additions.

Since the urea-only conditions (U) showed positive results (growth, biomass, and sEPS higher than other nutrient additions) as compared with the 30% POME alone cultures, this addition was chosen for the last experimental series. In this way, phosphate addition was avoided.

Table 2. Growth rate, total biomass, total sEPS, carbohydrate productivity and carbohydrate content of *P. tricornutum* cultivated at 300 µmol photons m^{-2} s^{-1}, on f/2 only and on 30% POME supplemented with different nutrients. Mean values are from 2 replicates. SD are shown after the ± symbol. Note: F2 = f/2 medium no POME, NPT = POME, nitrate, phosphate and trace elements, NP = POME, nitrate, and phosphate, UP = POME, urea, and phosphate, U = POME and urea, C = POME only. Values that do not share a common superscript letter in the same column are significantly different (P<0.05). *) The ratio is calculated from the molar of total dissolved inorganic nitrogen (TDIN) from nitrate or urea and dissolved phosphorus as phosphate from external nutrient addition and/or POME

Medium	Temp (°C)	External nitrogen source	TDIN (µM)	PO_4-P (µM)	N:P molar ratio *	µ (day^{-1})		Total Biomass (g L^{-1})		Carbohydrate productivity (mg L^{-1} d^{-1})		Carbohydrate content (%)		Total sEPS production (mg L^{-1})		Total sEPS normalized to biomass (g g^{-1})	
F2	20	NO_3^-N	880.00	36.90	24:1	0.84	±0.04^{a}	0.35	±0.06^{a}	5.45	±0.13^{a}	24.06	±3.88^{a}	78.89	±11.21^{a}	0.23	±0.01^{a}
NPT	20	NO_3^-N	435.00	101.67	4:1	0.87	±0.01^{a}	0.25	±0.04^{a}	5.60	±1.65^{ab}	32.66	±5.07^{a}	56.06	±0.50^{b}	0.22	±0.03^{a}
NP	20	NO_3^-N	435.00	101.67	4:1	0.88	±0.02^{a}	0.27	±0.00^{a}	5.15	±0.17^{a}	28.32	±0.64^{a}	61.56	±2.59^{b}	0.23	±0.01^{a}
UP	20	urea	435.00	101.67	4:1	0.86	±0.01^{a}	0.21	±0.10^{a}	5.48	±0.69^{a}	41.86	±14.61^{a}	59.97	±3.68^{b}	0.31	±0.13^{a}
U	20	urea	1863.57	17.33	108:1	0.69	±0.04^{b}	0.18	±0.05^{ab}	8.63	±1.19^{bcd}	34.38	±4.37^{a}	71.94	±2.68^{ab}	0.43	±0.09^{ab}
C	20	-	220.71	17.33	13:1	0.29	±0.04^{c}	0.10	±0.0^{b}	5.87	±1.80^{abc}	41.90	±0.24^{a}	78.93	±6.44^{a}	0.83	±0.23^{c}
F2	25	NO_3^-N	880.00	36.90	24:1	1.10	±0.04^{d}	0.29	±0.02^{a}	9.41	±0.10^{d}	48.49	±2.17^{b}	101.25	±7.03^{cd}	0.35	±0.04^{d}
NPT	25	NO_3^-N	435.00	101.67	4:1	1.09	±0.06^{de}	0.31	±0.09^{a}	9.53	±0.07^{d}	46.61	±12.75^{b}	94.64	±1.00^{d}	0.31	±0.09^{d}
NP	25	NO_3^-N	435.00	101.67	4:1	1.07	±0.09^{de}	0.28	±0.07^{a}	8.82	±0.13^{cd}	49.42	±13.51^{b}	96.89	±4.18^{d}	0.36	±0.08^{d}
UP	25	urea	435.00	101.67	4:1	1.02	±0.00^{de}	0.26	±0.00^{a}	8.03	±0.33^{abcd}	46.81	±1.41^{b}	95.65	±8.12^{d}	0.37	±0.03^{d}
U	25	urea	1863.57	17.33	108:1	0.88^{e}	±0.01	0.17	±0.03^{ab}	9.17	±1.00^{d}	38.95	±2.15^{b}	131.18	±0.75^{c}	0.80	±0.13^{ef}
C	25	-	220.71	17.33	13:1	0.41^{f}	±0.07	0.11	±0.04^{b}	7.43	±0.07^{abcd}	52.68	±22.07^{b}	100.52	±29.20^{cd}	0.96	±0.12^{f}

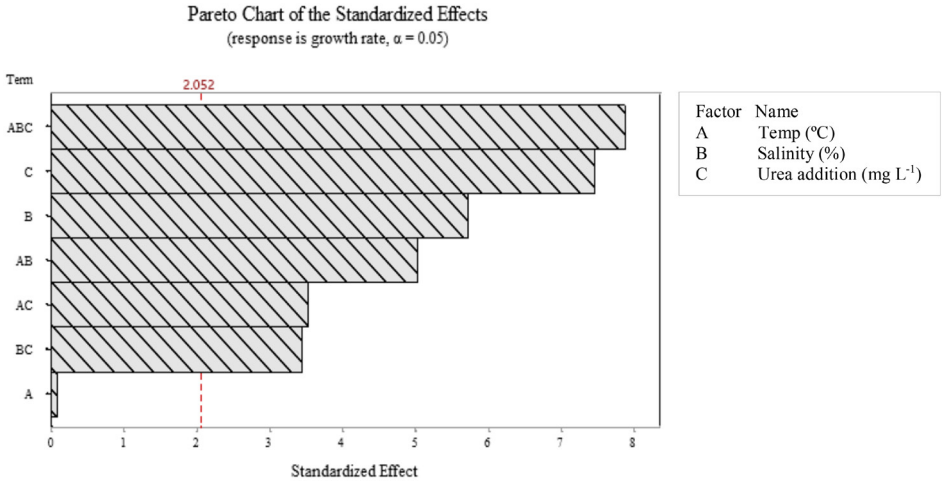


Figure 2. Pareto chart showing the effects of parameters on the growth rate of *P. tricornutum*. The vertical line indicates the significance of the effects at 95% confidence level.

3.3 Optimization of total biomass and total sEPS as a function of salinity, temperature, and urea addition.

Based on the GFFD statistical analysis, all interactive variables influenced the growth rate of *P. tricornutum* ($P<0.05$) (Figure 2). Increased salinity or urea concentration enhanced the growth rate and the interactive effect of temperature-urea and temperature-salinity also enhanced the growth rate (Figure 3). Based on the Pareto chart (Figure 2), the interactive effect of temperature, salinity and urea was the most influencing factor with respect to the growth rate, followed by urea addition and salinity. Therefore, temperature, urea, and salinity were chosen for the optimization of the total biomass and total sEPS based on BBM RSM (Table 3).

BBD showed that higher temperatures increased total sEPS ($P<0.05$), while the addition of urea alone had no effect on total sEPS ($P>0.05$) (Table 4, Figure 4). The optimal salinity was recorded at 2.0-2.5 % (w/v) ($P<0.05$). The interactive effect of temperature-salinity and the interactive effect of temperature-urea influenced the total sEPS ($P<0.05$) (Table 4, Figure 4).

As for total biomass, urea levels exceeding 40-80 mg L^{-1}, temperatures above 22-23 °C and a salinity exceeding 2.0-3.5 % (w/v) lowered total biomass ($P<0.05$) (Figure 5). Furthermore, the interactive effect between variables did not significantly influence biomass ($P>0.05$).

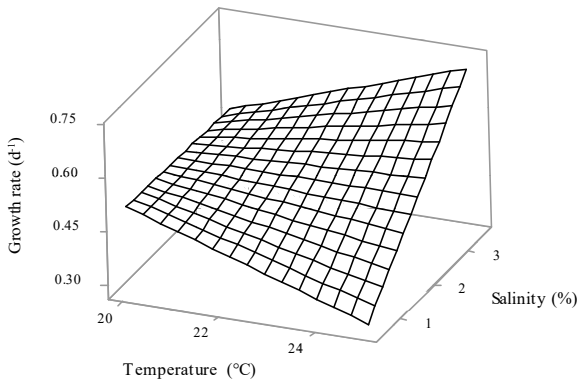
Table 3. Design of the experiment of BBD with the results. Mean values of the experimental results are from 2 replicates. SD is shown after the ± symbol.

Temperature (°C)	Salinity (%)	Urea addition (mg L^{-1})	Total Biomass (g L^{-1})			Total sEPS (mg L^{-1})		
			Experimental		Predicted	Experimental		Predicted
20	0.5	50	0.06	±0.00	0.07	43.48	±1.14	48.18
20	3.5	50	0.18	±0.05	0.18	71.94	±2.68	62.44
20	2.0	0	0.08	±0.01	0.11	96.88	±8.92	103.87
20	2.0	100	0.13	±0.00	0.13	87.17	±0.69	84.98
22.5	0.5	0	0.08	±0.01	0.02	41.18	±1.26	30.68
22.5	3.5	0	0.12	±0.00	0.14	87.33	±1.17	85.44
22.5	0.5	100	0.11	±0.01	0.07	50.41	±2.95	46.40
22.5	3.5	100	0.19	±0.02	0.17	65.44	±0.08	78.38
22.5	2.0	50	0.24	±0.11	0.27	99.31	±3.43	101.65
25	0.5	50	0.02	±0.00	0.02	33.74	±1.68	46.83
25	3.5	50	0.17	±0.03	0.14	131.71	±0.75	119.31
25	2.0	0	0.05	±0.02	0.05	94.45	±26.77	108.41
25	2.0	100	0.12	±0.01	0.11	141.06	±0.69	135.97

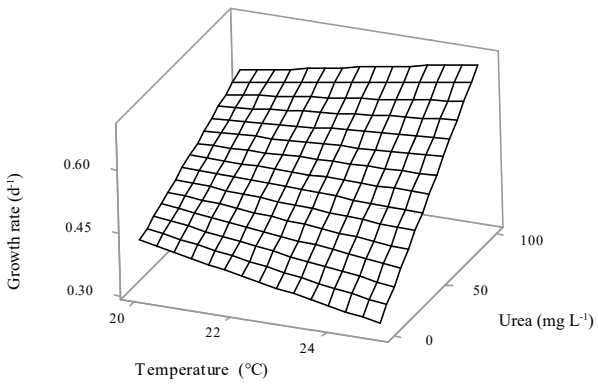
3.4 Nutrient removal efficiency

TDP and TDN removal efficiency were highly dependent on the nutrient enrichment used (Figure 6). The highest TDP removal efficiency (88.2±2.3 %) was recorded for POME supplemented with urea (U) alone (no phosphate addition). This value was significantly different from the control C (POME without nutrient: 68.8±3.07%) and from the P (phosphate addition) (54.3±5.1%) ($P<0.05$). For TDN removal efficiency, the addition of nutrients was nowhere significantly different compared to the control ($P>0.05$). The highest TDN removal was found for POME enriched with urea (45.2±3.1 %), followed by the control (18.4±12.2 %), and P addition (13.7±11.4%).

a)



b)



c)

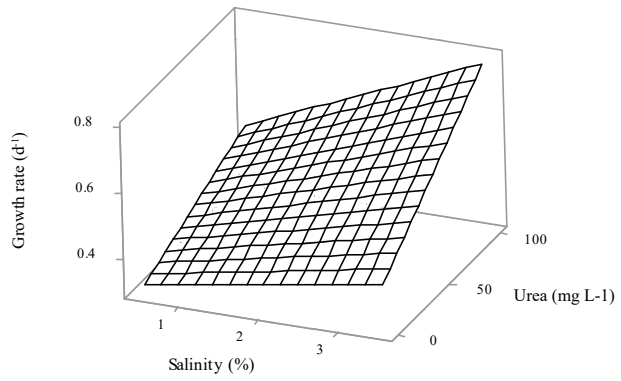


Figure 3. Response surface plot (3D) of growth rate as a function of temperature, salinity, and urea addition at hold value a) 50 mg L^{-1} urea addition, b) 3.5 % (w/v) salinity, and c) temperature 22.5°C.

4. Discussion

The present study shows that optimal growth of *Phaeodactylum tricornutum* is achieved when growing on 30% POME supplemented with urea. Without nutrient addition, the observed higher growth rate at 30% POME (v/v) as compared with 5 and 10% POME (v/v) could be due to the relatively higher nutrient concentrations in the 30% POME cultures (Table 1). As reported previously, POME contains high levels of micro nutrients such as iron, zinc, potassium, and magnesium, which could boost algal growth (Habib et al., 2003). Furthermore, POME may also contain acetic acid which could be utilized by *P. tricornutum* by applying mixotrophic growth (Nur et al., 2019a; Villanova et al., 2017).

Table 4. Estimated regression coded coefficient of Box behnken design RSM for total sEPS production (mg L^{-1})

Variables	Coefficient	P-value	Remarks
Constant	101.65	<0.01	significant
Temp	13.88	<0.01	significant
Salinity (%)	21.69	<0.01	significant
Urea (mg L^{-1})	2.17	0.534	
Temp (°C).Temp (°C)	7.81	0.228	
Salinity (%).Salinity (%)	40.27	<0.01	significant
Urea (mg L^{-1}).Urea (mg L^{-1})	-1.15	0.856	
Temp (°C). Salinity (%)	14.56	<0.05	significant
Temp (°C).Urea (mg L^{-1})	11.61	<0.05	significant
Salinity (%).Urea (mg L^{-1})	-5.69	0.242	

In contrast, at the highest POME fractions (> 30% v/v) the slightly lower growth rates could not be explained by lower light penetration due to high turbidity in POME, as irradiance was set to saturating levels in all cultures (300 µmol photons m^{-2} s^{-1}) (Nur et al., 2019a). Instead, the presence of potentially toxic phenolic compounds could provide a valid explanation, since these are still contained in digested POME (Chantho et al., 2016). The total concentration of phenolic compounds in POME can reach up to 303 mg L^{-1} as demonstrated before (Neoh et al., 2013), yet unfortunately these compounds were not measured during our experiments. Marine microalgae such as *Dunaliella salina* and *P. tricornutum* were described to be more sensitive to phenolic compounds from digested POME than freshwater algae such as *Chlorella vulgaris* (Nur et al., 2016; Duan et al. 2017). Overall, a 30% POME fraction was found to be most suitable to be used as growth medium for *P. tricornutum* for all subsequent experiments in this study. However, the optimal fraction is determined by the quality and the nutritional content of POME (COD, BOD, nitrogen, phosphorus, lignin) which may vary depending on

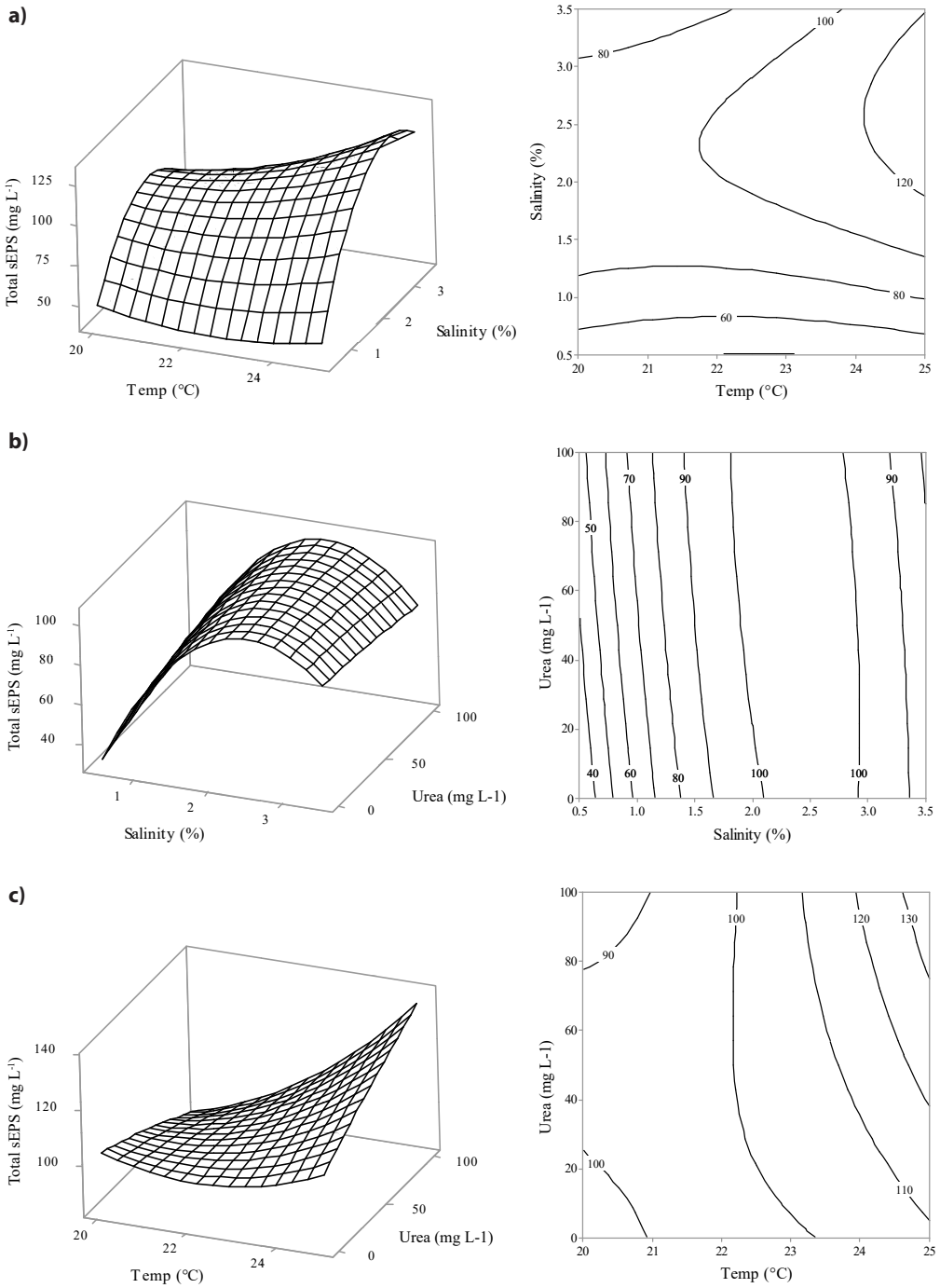


Figure 4. Response surface plots (3D) showing the effects of temperature (°C), urea (mg L^{-1}), and salinity (%) on total sEPS generated by *P. tricornutum* at hold value 22.5°C (a) 50 mg L^{-1} (b), and 2.0 % (c)

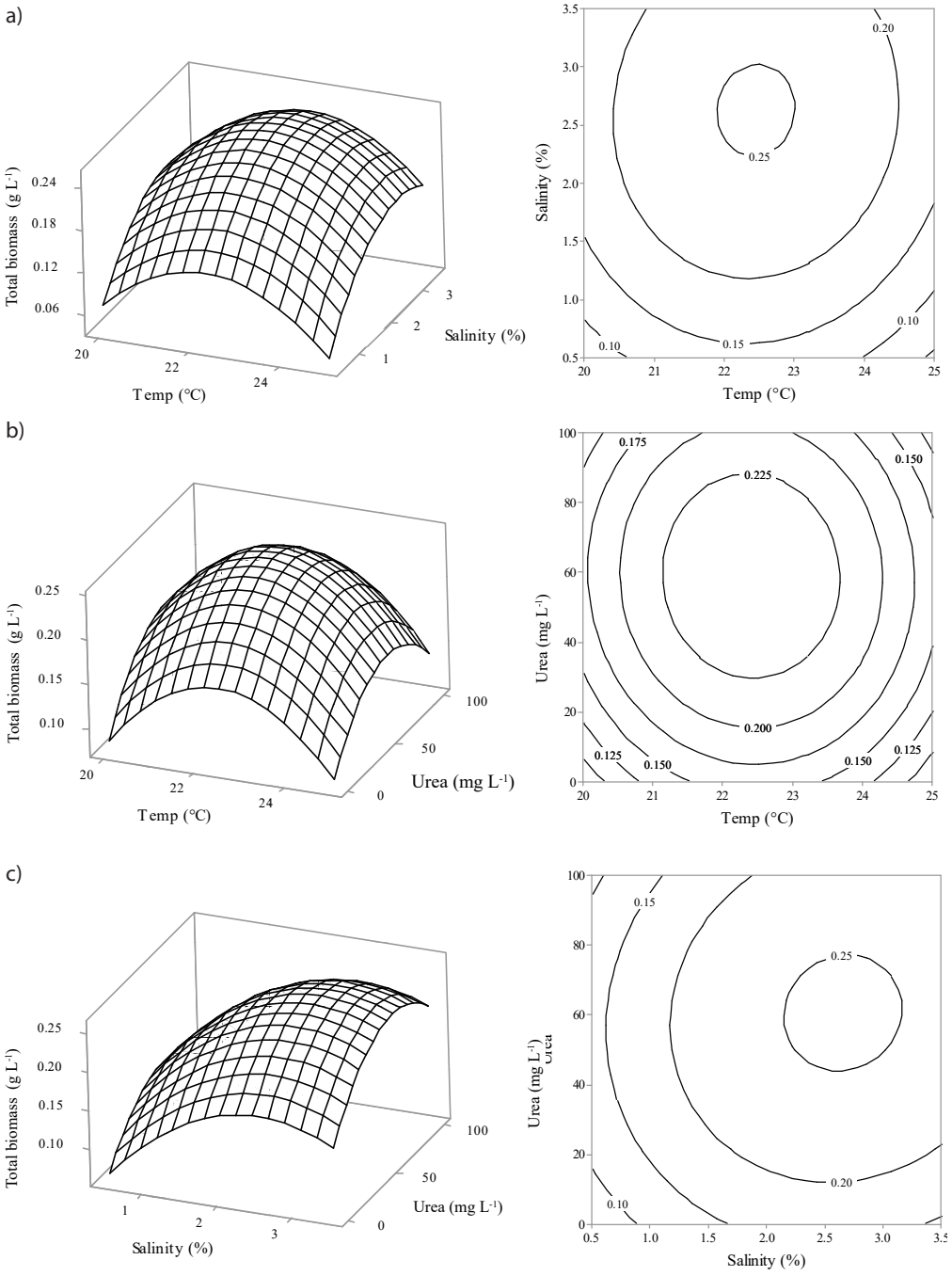


Figure 5. Response surface plots (3D) showing the effects of temperature (°C), urea (mg L^{-1}), and salinity (%) on total biomass (g L^{-1}) generated by *P. tricornutum* at hold value 50 mg L^{-1} (a), 22.5 °C (b), and 2.0 % (c)

salinity, *P. tricornutum* showed a lower growth rate compared to 2.0 and 3.5 % (w/v) Furthermore, the addition of urea did not promote growth, when salinity was low and temperature was high, possibly due to low salinity stress in *P. tricornutum*. Liang et al (2014) reported that optimum growth and photosynthetic efficiency for *P. tricornutum* was recorded at 2.0-4.0 % (w/v) salinity. This condition was also supported by our finding that the optimum condition for total biomass production was found at 2.0 – 3.0 % (w/v) salinity, 40-80 mg L^{-1} urea addition, at 22-23 °C (Figure 5).

Total sEPS production and sEPS content were found to be influenced by temperature (Table 2, Figure 4). When temperature was relatively low, total sEPS was significantly reduced. An explanation for this could be that *P. tricornutum* accumulates sEPS when the culture is in a stressed condition to protect cells from damage (Delattre et al., 2016). Previous studies using *Graesiella* revealed that total EPS production increased with temperature (Mezhoud et al., 2014).

In the present study, cultivation at 25 °C also resulted in an elevated carbohydrate content. This matches an earlier study where carbohydrate content was enhanced when *P. tricornutum* was grown at high irradiance, high temperature, around 27°C, and low nitrogen availability, reflecting unfavorable conditions (Buono et al., 2016). In our experiments, carbohydrate content and total sEPS content in *P. tricornutum* seemed linked. Previous studies showed that the intracellular carbohydrate production of green algae was in line with the EPS production, as a response to nutrients, toxic compounds, temperature and light intensity (García-Cubero et al., 2018; El-Sheekh et al., 2012).

Total sEPS production was also influenced by nutrient availability. Although not significant, the addition of nitrogen and phosphate to 30% POME seemed to result in lower total sEPS production compared to control (C) cultures for both temperatures (Table 2). This implies that phosphate limitation could stimulate sEPS production. The availability of nutrients with the addition of nitrogen increased total biomass, but decreased total sEPS normalized to biomass (Table 2). At control C (POME without nutrients), low growth rates and total biomass were found, indicating that the cultures was nitrogen limitated, which could result in a higher total sEPS normalized to biomass: sEPS levels were 0.83 ± 0.23 g g^{-1} algal biomass at low temperature and 0.96 ± 0.12 g g^{-1} at high temperature. These values were 2-fold and 1.2-fold higher compared to the urea-only cultures (Table 2). This result was supported by previous studies who found that the total exopolysaccharide (normalized to biomass) increased when *Cylindrotheca closterium* and *Porphyridium marinum* were grown in nitrogen limited medium (Soanen et al., 2016; Staats et al., 2000).

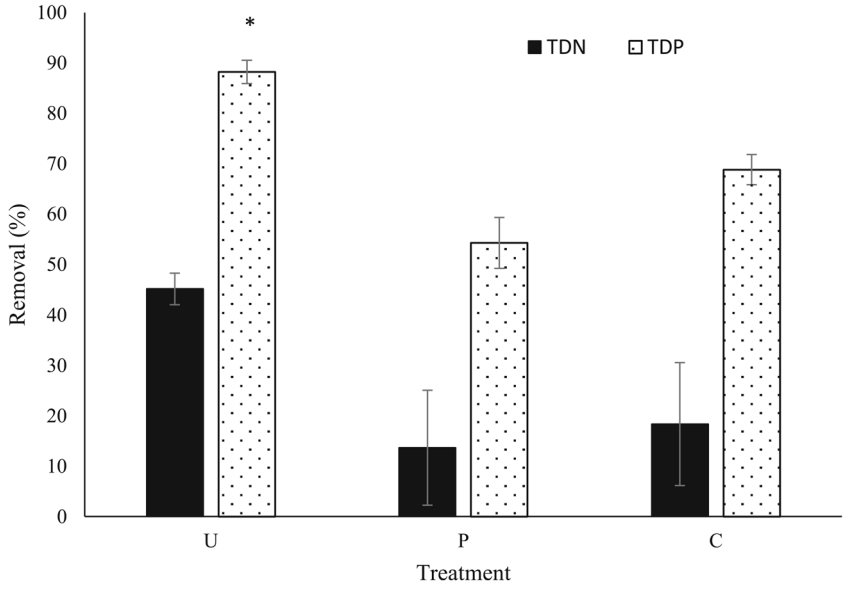


Figure 6. Nutrient removal by *P. tricornutum* (at 20 °C) growing on 30% POME with phosphate or urea addition. P = 3 mg L^{-1} phosphate addition, U = 50 mg L^{-1} urea addition, C = control POME without nutrient enrichment. Asterisk(*) indicates significantly different from other values (P<0.05).

In this work, the interactive effect of temperature and urea influenced total sEPS production. The addition of urea at high temperature promoted total sEPS production (Figure 4). It seems that total sEPS at high temperature with the addition of urea was more dependent on the increase in total biomass production rather than total sEPS normalized to biomass. This is in accordance with earlier studies who reported that media enriched with nitrogen resulted in higher total EPS production (Ekelhof and Melkonian, 2017; Magaletti et al., 2004; Guerrini, et al., 2000; Lupi et al., 1994), although the total sEPS normalized to biomass was slightly lower compared to the control (POME only) (Table 2). Furthermore, the higher total sEPS normalized to biomass as found at the higher temperature (Table 2), might be explained by the interaction of temperature and urea that may become toxic at high temperatures, possibly due to the rapid hydrolysis of urea to ammonium by the cellular enzyme urease (Ni, 2014; Glibert et al., 2008; Lomas et al., 1999). In support of this, EPS production by algae was increased when toxic compounds were added to the medium to protect cells from damage (El-Sheekh et al., 2012). In summary, in order to obtain optimal conditions for total sEPS production by *P. tricornutum*, cultivation conditions are proposed to be 25 °C, 2.6 % salinity, and 100 mg L^{-1} urea addition. In this way, 140 mg L^{-1} sEPS can be obtained based on the RSM approach (Figure 4, Table 4). However, to make sEPS production more sustainable and cheap, the biomass that contained value added products

(fucoxanthin; carbohydrate) could also be used as the source of fine chemicals and bioethanol by utilizing the bio-refinery concept. In this case, the optimum biomass and sEPS could be produced by the diatom for 7 days cultivation by adding 50 mg L^{-1} urea on 30% POME. By using wastewater and adding 50 mg L of urea, the cost of fertilizer for the growth could be greatly reduced.

5. Conclusion

In conclusion, the present study has shown that *P. tricornutum* is a suitable candidate for effective cultivation on 30% POME while producing sEPS at the large scale, when salinity requirements are met at 2.0-3.5 % (w/v). To obtain nearly complete phosphorus removal, high growth rates and total sEPS, 25 °C cultivation temperature needs to be accompanied by urea addition.

Acknowledgment

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Supplementary data

S1. Analysis of Variance Total sEPS production using BBM RSM. Value significant at P <0.01

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	26676.30	2964.03	15.11	0.00
Linear	3	11701.70	3900.56	19.88	0.00
Temp (°C)	1	3240.60	3240.58	16.52	0.00
Salinity (%)	1	7910.10	7910.06	40.31	0.00
Urea (mg/L)	1	79.00	78.95	0.40	0.53
Square	3	11539.90	3846.64	19.60	0.00
Temp (°C)*Temp (°C)	1	305.30	305.31	1.56	0.23
Salinity (%)*Salinity (%)	1	8122.70	8122.66	41.40	0.00
Urea (mg/L)*Urea (mg/L)	1	6.70	6.67	0.03	0.86
2-Way Interaction	3	3115.30	1038.44	5.29	0.01
Temp (°C)*Salinity (%)	1	1878.50	1878.53	9.57	0.01
Temp (°C)*Urea (mg/L)	1	1195.00	1194.97	6.09	0.02
Salinity (%)*Urea (mg/L)	1	287.30	287.32	1.46	0.24
Error	18	3531.90	196.22		
Lack-of-Fit	4	1846.20	461.56	3.83	0.03
Pure Error	14	1685.70	120.40		
Total	27	30208.20			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
14.0078	88.31%	82.46%	73.55%

S2. Analysis of Variance biomass production

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	0.08	0.01	6.59	0.00
Linear	3	0.02	0.01	3.75	0.03
Temp (°C)	1	0.00	0.00	0.26	0.62
Salinity (%)	1	0.01	0.01	10.34	0.01
Urea (mg/L)	1	0.00	0.00	0.66	0.43
Square	3	0.04	0.01	8.43	0.00
Temp (°C)*Temp (°C)	1	0.03	0.03	21.57	0.00
Salinity (%)*Salinity (%)	1	0.02	0.02	11.10	0.00
Urea (mg/L)*Urea (mg/L)	1	0.02	0.02	11.95	0.00
2-Way Interaction	3	0.00	0.00	0.28	0.84
Temp (°C)*Salinity (%)	1	0.00	0.00	0.30	0.59
Temp (°C)*Urea (mg/L)	1	0.00	0.00	0.09	0.77
Salinity (%)*Urea (mg/L)	1	0.00	0.00	0.46	0.51
Error	16	0.02	0.00		
Lack-of-Fit	3	0.01	0.00	1.80	0.20
Pure Error	13	0.02	0.00		
Total	25	0.10			

Model Summary

S	R-sq	R-sq(adj)
0.0372735	78.76%	66.81%

S3. ANOVA for the growth rate generated from full factorial design

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	26	3.57	0.14	12.52	0.00
Linear	6	1.18	0.20	17.89	0.00
Temp (°C)	2	0.00	0.00	0.08	0.93
Salinity (%)	2	0.44	0.22	20.20	0.00
Urea (mg L^{-1})	2	0.73	0.37	33.41	0.00
2-Way Interactions	12	0.97	0.08	7.38	0.00
Temp (°C)*Salinity (%)	4	0.46	0.12	10.55	0.00
Temp (°C)*Urea (mg L^{-1})	4	0.26	0.06	5.90	0.00
Salinity *Urea (mg L^{-1})	4	0.25	0.06	5.70	0.00
3-Way Interactions	8	1.42	0.18	16.19	0.00
Temp (°C)*Salinity ((%)*Urea (mg L^{-1})	8	1.42	0.18	16.19	0.00
Error	27	0.30	0.01		
Total	53	3.87			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.104783	92.34%	84.96%	69.36%



CHAPTER 4

Arthrospira platensis under microscope

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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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 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. *A. 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} d^{-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.

1. 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 beta 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 et al., 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 is still a 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 growth medium for algae is palm oil mill effluent (POME) which is generated from oil palm factories. POME contains high amounts of phosphorus, nitrogen, and micronutrients (Mohd Udaiyappan et al., 2017; Nur et al., 2018). As reported earlier, POME addition promotes the growth of the fucoxanthin and sulphated exopolysaccharide producing marine diatom *Phaeodactylum tricornutum* (Nur et al., 2019a; Nur et al., 2019b). 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). However, 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 growth medium for *A. platensis* using continuous cultivation (Suharyanto et al., 2014). However, 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 (Fernandes et al., 2015; Lehr and Posten, 2009). 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 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 new medium at 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.

2. Material and methods

2.1. Wastewater preparation

Palm oil mill effluent (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 (-19°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 minutes. 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).

2.2. 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).

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	$P_{biomass}$, $P_{C\text{-}PC}$

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 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.

2.2.1. 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 690 160). 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 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).

2.2.2. Experiment 2: Effect of POME on biomass and C-PC productivity

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 36W/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 contains 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, California, 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 10.000 rpm for 15 minutes 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).

2.2.3. Experiment 3: Effects of nutritional and environmental conditions on the concentration of C-Phycocyanin

A. 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. Full factorial design with four variables (irradiance, salinity, nitrate, POME etc) 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.

Table 2. Experimental factors of full factorial design and their levels to determine significant factors and their interactions on P-biomass (mg L^{-1} d^{-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	100	13.10	±5.26
3	50	30	50	0	9.13	±0.55
4	50	30	50	100	24.35	±16.73
5	100	15	50	0	15.75	±2.90
6	100	15	50	100	14.41	±1.35
7	100	30	50	0	14.62	±3.17
8	100	30	50	100	11.06	±0.53
9	50	15	200	0	7.04	±0.36
10	50	15	200	100	11.71	±2.21
11	50	30	200	0	5.07	±0.31
12	50	30	200	100	12.87	±8.67
13	100	15	200	0	10.69	±2.05
14	100	15	200	100	8.05	±0.57
15	100	30	200	0	6.74	±0.62
16	100	30	200	100	12.16	±2.50

2.2.4. 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 (fresh water, sea water) that can be used for the dilution of POME. 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, 23, 35, and 40 PSU for salinity (x_2). The empirical form of the second order polynomial model (Eq.1) can be described as:

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

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, x_j are independent variables of the model.

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	Salinity	Growth rate		$P_{biomass}$		C-PC		$P_{C\text{-}PC}$	
			(mg L^{-1})	(PSU)	(d^{-1})		(mg L^{-1} d^{-1})		(mg L^{-1})		(mg $L^{-1}d^{-1}$)	
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

Cultivation was carried out in the same set-up as Experiment 3. Fifty percent of POME was used and mixed with ultrapure water (Milli-Q), NaCl, or natural filter

sterilized sea water 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.

2.2.5 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 set-up as Experiment 3. The initial salinity at the first cultivation stage was adjusted to 22.5 PSU by using natural sea water, following the outcome of Experiment 4. In Experiment 5, phosphate (3 mg L^{-1}, run 4) or urea (800 mg L^{-1}, run 5) were 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 (Bezzera et al., 2011).

2.3. Analysis

2.3.1 Growth rate

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

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

where µ is growth rate (day^{-1}) X_2 is the optical density at time t_2 (day) and X_1 the optical density at time t_1 (day). Conversion of OD750 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. Thirty mL of sample 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.

$$y = 0.59(OD_{750nm}) - 0.03 \; R^2 = 0.98 \quad \text{Eq. 3}$$

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

2.3.2. C-PC analysis

10 mL of sample was centrifuged at 4500 rpm for 30 min. The pellet obtained was stored at -20°C until analysis. Extraction was conducted by adding 3 mL of cold buffer phosphate (pH=6.8) into the sample followed by two times freezing and thawing, and sonication at 50% amplitude for 2 min using an ultrasound probe (Vibra Cell, VC 130PB, Newton 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 min). The concentration of C-PC was determined using a spectrophotometer (Hach DR 3900), by measuring the optical density at 620 nm, and 652 nm (Moraes et al., 2011). The concentration of C-PC was determined as

$$\text{C-PC (mg mL}^{-1}) = \frac{(OD_{620} - 0.474(OD_{652}))}{5.34} \quad \text{Eq. 4}$$

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).

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

where $P_{C\text{-}PC}$ 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 total duration of the cultivation (d).

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

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

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

2.4. Statistical analysis

Minitab ver. 18. (Demo version) was employed for statistical analysis and evaluation for full factorial and CCD design. Differences between treatments were analysed with two-way analysis of variance (ANOVA) at a p-value of 0.05. Post hoc tests (Tukey HSD) were performed for pair-wise comparisons. The experimental results were recorded based on at least two replicates as expressed in the averages and standard deviations (±SD).

3. Results

3.1. 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 (Figure 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} (Figure 1a).

C-PC content (percentage of C-PC, when normalized to calculated dry weight) was found to be both irradiance and nitrate dependent (Figure 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, Figure 1c), the highest value was found at around 150-200 µmol photons m^{-2} s^{-1} for both nitrate conditions. Finally, C-PC content as well as productivity (Figure 1d) were significantly different when comparing the two nutrient conditions ($P < 0.05$).

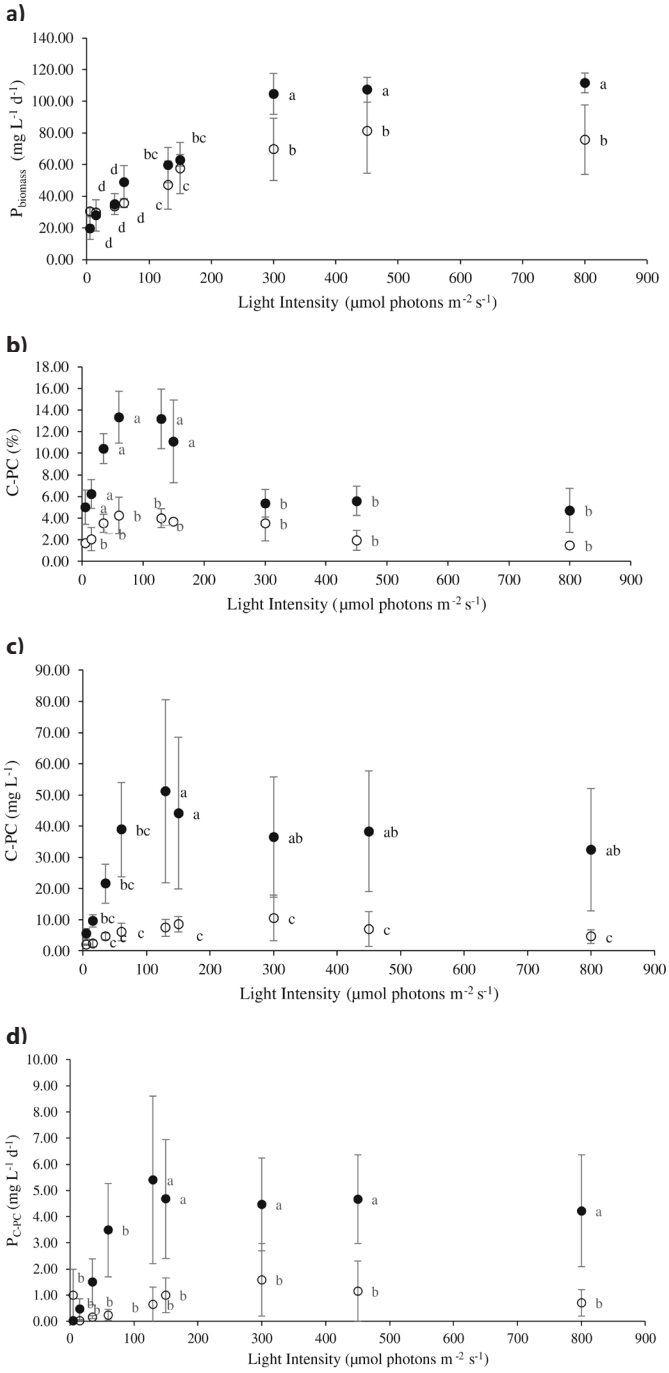

Figure 1. Effect of irradiance and nitrate availability on a) biomass productivity, b) C-Phycocyanin content, c) C-PC concentration , d) C-PC productivity. Closed circle is standard Zarrouk medium using 2.5 g L^{-1} $NaNO_3$ (HN), open circle is Modified Zarrouk medium using 0.1 g L^{-1} $NaNO_3$ (LN). Average values of triplicate cultures are shown. Error bars indicate the SD of the mean (n=3).

3.2. Effect of different POME fractions

In Experiment 2, between 5% and 30% POME on *A. platensis* biomass productivity was significantly enhanced (P<0.05) (Figure 2).

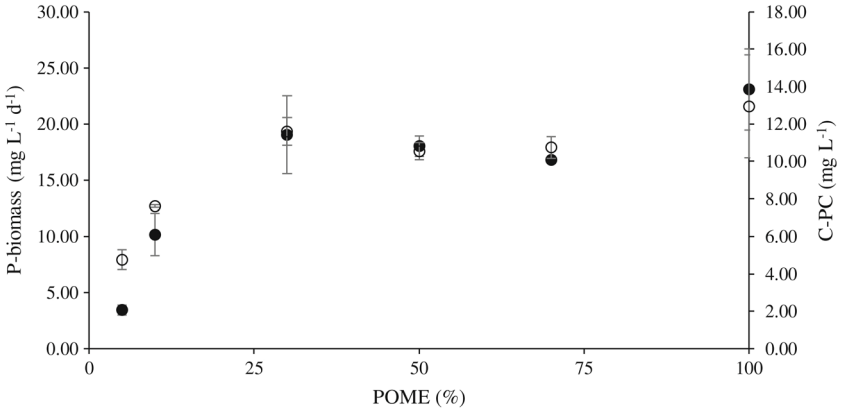

Figure 2. Biomass productivity and C-PC concentration of *S. platensis* cultivated on different POME fractions in Milli-Q at 4 PSU salinity and 200 µmol photons m^{-2} s^{-1}. Average values of duplicate cultures are shown. Error bars indicate the SD of the mean. Closed circle is biomass productivity.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

At 5% POME, the lowest biomass productivity (3.95±3.1 mg L^{-1} d^{-1}) was recorded, while at 100% POME, the biomass productivity reached up to 23.08±3.6 mg L^{-1} d^{-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% until 100% did not significantly enhance biomass productivity and C-PC concentration (P>0.05) (Figure 2).

3.3. 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 (Figure 3). At low POME and low nitrate addition, C-PC concentration was recorded to be around 8 mg L^{-1}.

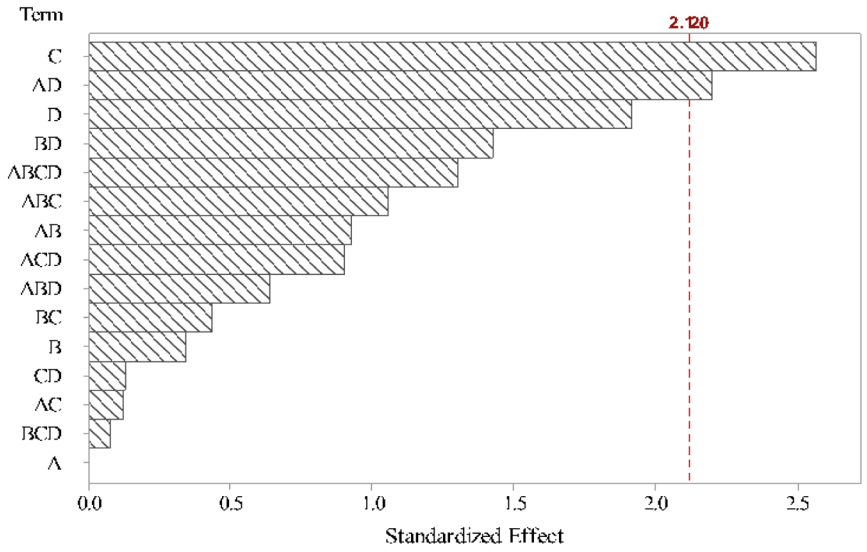

Figure 3. Pareto chart showing the effects of (combinations of) parameters on the C-PC concentration of *A. platensis*. The vertical line indicates the significance of the effects at 95% confidence level. A is POME, B is salinity, C is irradiance, and D is nitrate

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} (Figure 4). Based on this finding, nitrogen addition was further investigated in the next experiment, however utilizing urea as 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.

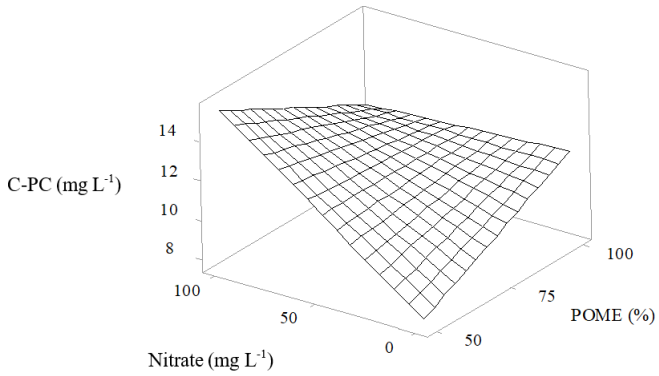

Figure 4. Response surface plot (3D) of C-PC concentration as a function of nitrate addition and POME concentration at salinity 22.5 PSU and irradiance 125 µmol photons m^{-2} s^{-1}

3.4. 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 (Figure 5a, 5b). 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, Figure 5c). However, salinity in the quadratic form and urea addition in the linear form were found to be significantly related with C-PC productivity (Figure 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 (Figure 5c).

3.5. 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 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±1.6 mg L^{-1} d^{-1} and 4.43±0.22 mg L^{-1} d^{-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-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} d^{-1} and 5.76±1.85 mg L^{-1} d^{-1} for biomass and C-PC productivity, respectively, which was comparable to control Zarrouk medium carried out in batch cultivation mode (Figure 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 (Figure 5, Table 5).

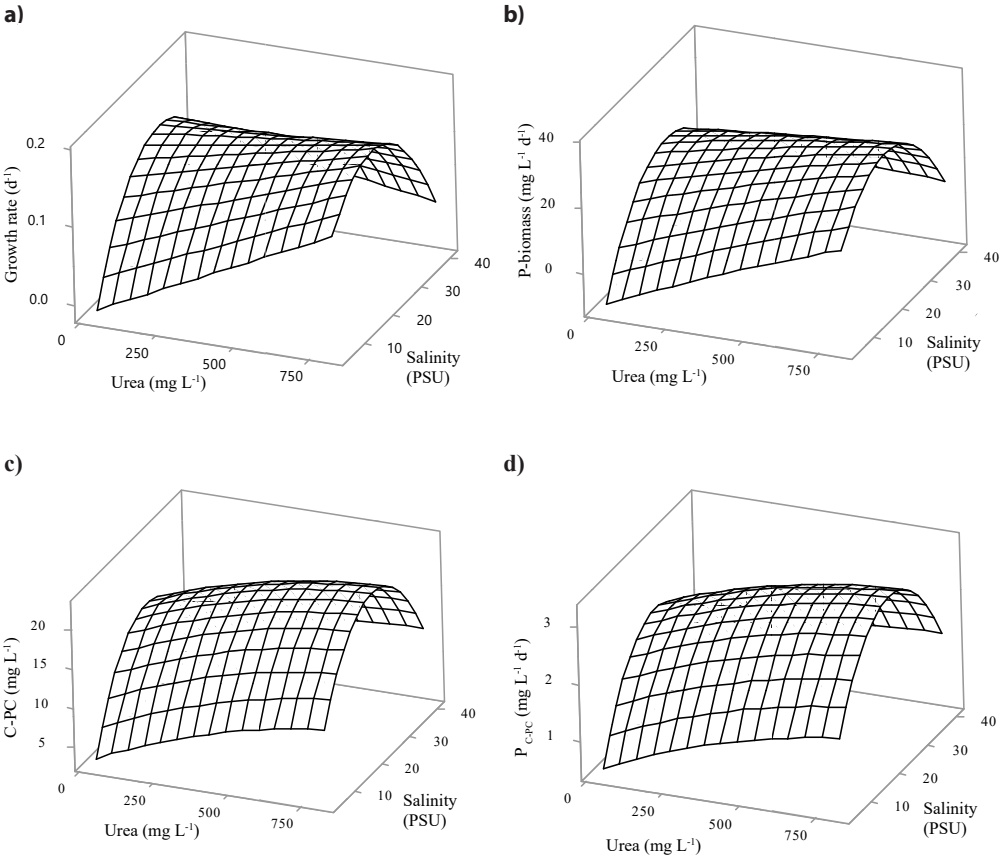

Figure 5. Response surface plots (3D) showing the effects of salinity (PSU), and urea (mg L^{-1}) on a) growth rate (d^{-1}), b) biomass productivity ($P_{biomass}$, mg L^{-1} d^{-1}), c) C-PC concentration (C-PC, mg L^{-1}), and d) C-PC productivity ($P_{C\text{-}PC}$, mg L^{-1} d^{-1}) generated by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

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 (Figure 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.

Table 4. ANOVA from the full factorial design 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	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)*Urea (mg/L)	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)*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				

However, biomass productivity was not dependent on nitrogen availability below saturating irradiance levels (P>0.05) (Figure 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 (Figure 1 a, 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 a high C-PC productivity, cultivation conditions should carefully be maintained at optimal irradiance and nitrogen levels.

In our study, biomass productivity and C-PC concentration were also dependent on POME fraction (Figure 2). *A. 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; Nur et al., 2019b; Cheah et

al., 2018). This underlines the tolerance of *Arthrospira* to the high ammonia levels or other potentially toxic substances (e.g. phenolic compounds) present in POME.

Table 5. Biomass and C-PC productivity of *A. platensis* cultivated on POME medium using a consecutive two stage cultivation. Mean values are based on 4 replicates. SD are 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} d^{-1})		$P_{C\text{-}PC}$ (mg L^{-1} d^{-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}

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

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

Salinity optimization may also be considered important with respect to large scale cultivation. For example, to make large scale cultivation sustainable, sea water might be preferred over drinking water or other fresh water 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 (Figure 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 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 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 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 a higher initial biomass compared to the other treatments.

Summarizing, 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 $L^{-1}d^{-1}$ and 4.1±1.3 mg L^{-1} d^{-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} d^{-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 sea water 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 a 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.

4. 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} d^{-1}), similar to the artificial control Zarrouk medium during batch cultivation.

Acknowledgment

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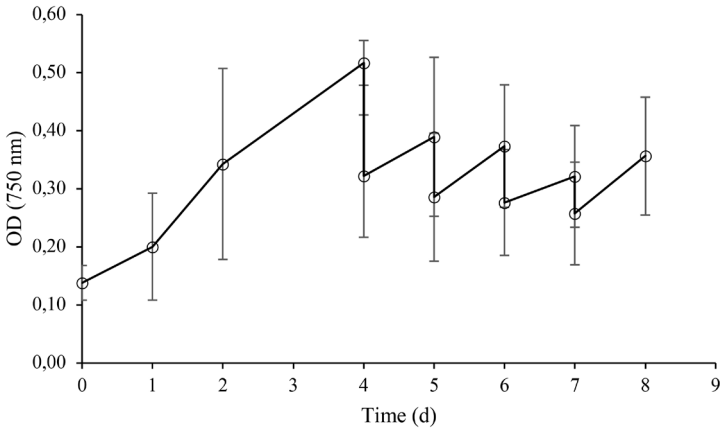
Supplementary data

a)



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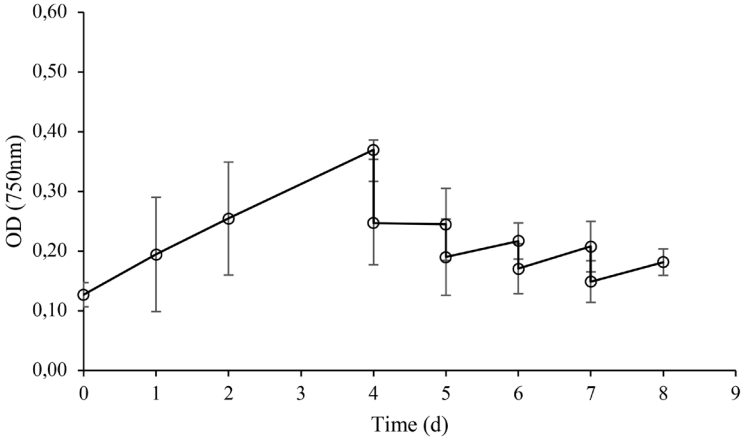

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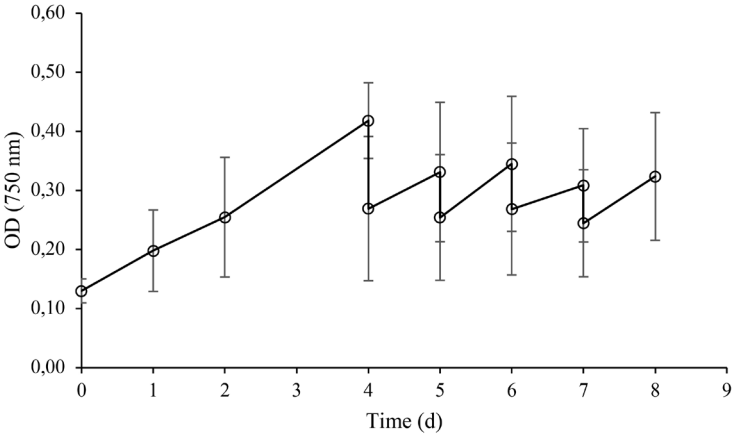
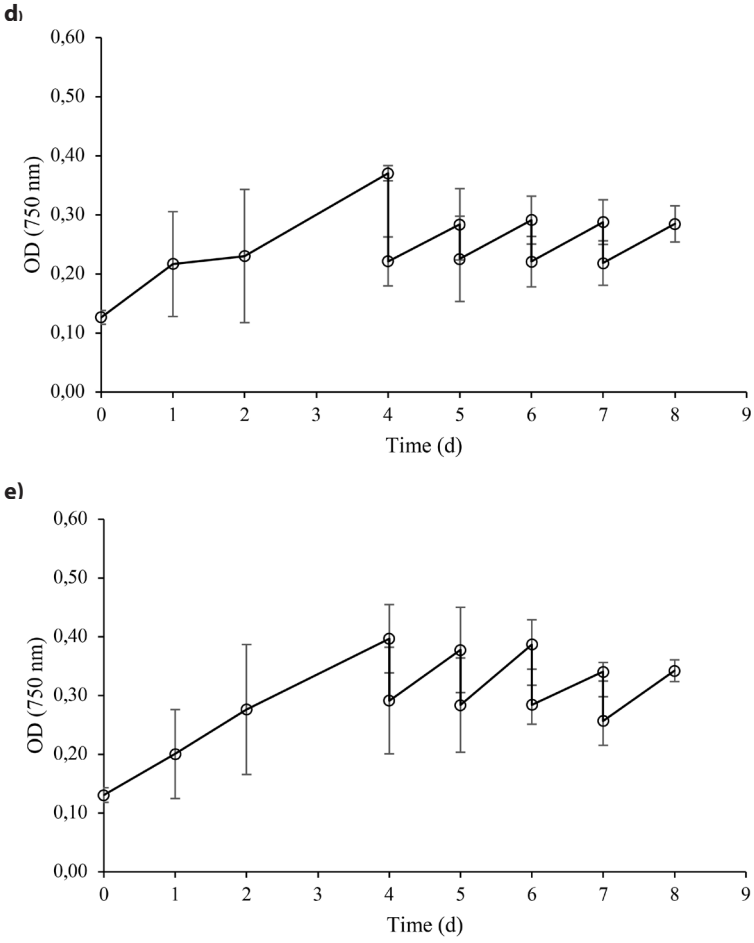




S1. Growth profile of *A. platensis* using semi-continuous cultivation under different conditions (a) 50% + 800 mg L^{-1} urea at the first stage, 100% POME at the second stage; b) 50% POME at the first stage, 50% POME at the second stage; c) 50% POME at the first stage, 100% POME at the second stage; d) 50% POME at the first stage, 100% POME + 3 mg L^{-1} PO_4^{3-} at the second stage; e) 50% POME at the first stage, 100% POME + 800 mg L^{-1} urea at the second stage. Average values of four replication cultures are shown. Error bars indicate the SD of the mean.

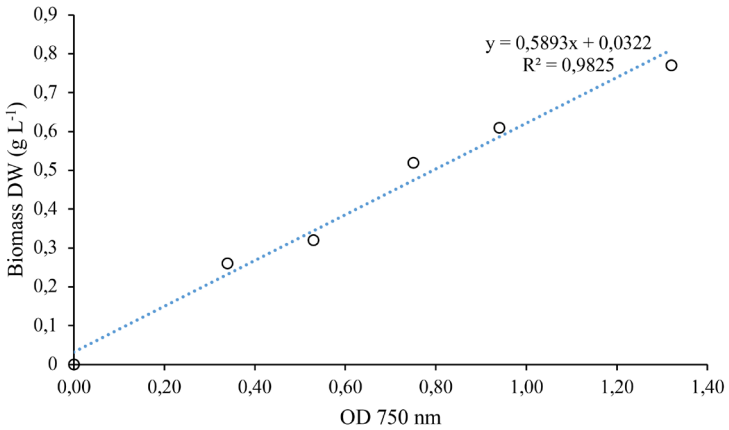
S2. Analysis of Variance for the Growth rate of *A. platensis* cultivated on 50% POME at different urea and salinity conditions

Analysis of Variance

Total	**25**	**0.134022**			
Model	5	0.086243	0.017249	7.22	0.001
Linear	2	0.007730	0.003865	1.62	0.223
Urea (mg/L)	1	0.003031	0.003031	1.27	0.273
Salinity (PSU)	1	0.004699	0.004699	1.97	0.176
Square	2	0.071644	0.035822	14.99	0.000
Urea (mg/L)*Urea (mg/L)	1	0.000018	0.000018	0.01	0.932
Salinity (PSU)*Salinity (PSU)	1	0.070128	0.070128	29.36	0.000
2-Way Interaction	1	0.006878	0.006878	2.88	0.105
Urea (mg/L)*Salinity (PSU)	1	0.006878	0.006878	2.88	0.105
Error	20	0.047779	0.002389		
Lack-of-Fit	3	0.012233	0.004078	1.95	0.160
Pure Error	17	0.035546	0.002091		

Model Summary

S	**R-sq**	**R-sq(adj)**	**R-sq(pred)**
0.0488768	64.35%	55.44%	37.19%



S3. Standard linear regression between biomass dry weight and optical density at 750 nm.



CHAPTER 5

Arthrospira platensis culture

Removal of color and phenolic compounds from palm oil mill effluent by *Arthrospira platensis*

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This chapter is to be submitted in Waste and Biomass Valorization

Abstract

Palm oil mill effluent (POME) released from conventional treatment systems poses severe environmental problems due to its dark color, its high Chemical Oxygen Demand (COD) and high content of phenolic compounds. Compared to other techniques, biological treatment is potentially more environmental friendly, less expensive and energy efficient. However, the possible biodegradation of phenolic compounds and color by microalgae was not well explored. This research aimed to reveal optimal conditions for pollutant removal through biodegradation by the cyanobacterium *Arthrospira platensis*. This species was grown under a range of POME fractions and environmental conditions (irradiance, salinity, nutrients) during which growth, final biomass, color, COD and phenolic compound levels were followed. Although photodegradation (without algae) caused most of the phenolic compounds and color removal, *A. platensis* contributed to degradation by 10-15% after 5 days of incubation. POME fractions influenced *A. platensis* growth rate, final biomass, COD and color removal. The optimization of phenolic compound removal by using central composite design (CCD) response surface methodology (RSM) showed that the highest phenolic compound removal (94%) was found at 200 µmol photons m^{-2} s^{-1} and 400 mg L^{-1} initial phenolic compound concentration. The combination of high initial phenolic compounds and high light intensity increased the growth rate up to 0.45 d^{-1} and final biomass up to 400 mg L^{-1} while total phenolic compounds were almost completely (94%) removed. Finally, this study showed that phenolic compounds and color degradation from POME were dominated by the activity of photodegradation at high irradiance, while the activity of *A. platensis* dominated at low light intensity.

1. Introduction

Indonesia is the largest palm oil producer in the world. However, along with the production of 1 ton of fresh fruit bunch, 600-870 kg of palm oil mill effluent (POME) wastewater is generated. Direct discharge of untreated POME into aquatic environments may cause problems. When POME is released from the palm oil mills, its characteristics are the following: temperature ranges between 70 and 80°C, pH is low (between 4 and 5), it is highly colloidal, it has high chemical and biological oxygen demand, and a high concentration of phenolic compounds which give it a particular deep reddish to brown color (Chaijak, et al., 2017). Regulatory standards for POME aim to reduce the wastewater discharges taking chemical oxygen demand (COD) and biological oxygen demand (BOD) as key parameters. Yet, even when the standard is reached, the effluent still has a strong smell, high levels of phenolic compounds, and a dark color that could discomfort the local community and the environment (Igwe et al., 2007; Poh et al., 2010). Physical treatment using membrane systems such as ultrafiltration and nanofiltration are able to remove the color completely (Ahmad et al., 2006; Amat et al., 2015). However, the investment cost for the membrane filtration system could be too high for a medium to small industry. Furthermore, in conventionally treated aerobic POME, the phenolic compounds can reach concentrations between 280 and 680 mg L^{-1} which is dominated by gallic acid, followed by p-hydroxybenzoic acid, protocatechuic acid, caffeic acid, syringic acid, and vanillic acid (Chantho et al., 2016). Previous research has shown that the maximum toxicity concentrations for phenolic compounds range between 10–24 mg L^{-1} for humans and between 9–25 mg L^{-1} for fish (Kulkarni and Kaware 2013). Discharging traditionally treated POME into rivers could thus pose a high environmental risk for the surrounding area. Therefore, additional treatments are needed to eliminate the phenols.

In general, physiochemical and biological methods could be applied to remove phenolic compounds from the wastewater (Mohammadi et al., 2015; Pradeep et al., 2015). Compared to other techniques, biological treatment is potentially more environmental friendly, less expensive as well as energy saving. Moreover, it may lead to complete mineralization of toxic compounds (Al-Khalid and El-Naas 2012, Villegas et al., 2016). Nowadays, wastewater treatment using microalgae is more preferable compared to yeast and bacteria since the biomass produced has higher economic value (Surkatti and Al-Zuhair, 2018; Nur et al., 2018). Some strains of microalgae have the capability to utilize phenols as energy sources as reviewed recently (Surkatti and Al-Zuhair, 2018; Lindner and Pleissner, 2019). Some species such as *Anabaena variabilis*, *Spirulina maxima*, *Chlorella* sp., and *Scenedesmus obliquus* have shown good efficiency in removing phenolic compounds from wastewater by utilizing it as organic carbon source in the presence of light (Naoki et al. 1979; Klekner and Kosaric, 1992; Scragg, 2006; Lee et al., 2015; Papazi et al.,

2019). Some factors such as the type and concentration of the phenolic compounds could influence the rate and efficiency of the removal (Surkatti and Al-Zuhair, 2018; Priyadharshini and Bakthavatsalam, 2016; Hirooka et al., 2003). Yet, earlier results did not consider phenol removal by photodegradation, although it was shown before that irradiance could contribute to phenolic compound degradation (Lika and Papadakis 2009). In contrast, earlier research found that photodegradation activity also contributed to the antibiotic tetracycline in a domestic wastewater treatment system, using microalgae in the presence of light (Norvill et al., 2017).

To the best of our knowledge, information on color and phenolic compound removal from POME using microalgae was so far lacking. Previous research has demonstrated the removal of COD and color by *Chlorella sorokiniana* by varying POME fractions (Haruna et al., 2018). However, these results did not include the potential role of photodegradation. In the present study, factors affecting phenolic compound and POME color removal were considered by including photodegradation and microalgal activity by varying the initial phenol concentration in POME, irradiance, POME fractions, external nitrogen addition, and salinity. Gallic acid was varied and chosen as external phenolic compound since it is the most abundant phenolic compound in POME (Chantho et al., 2016). *Arthrospira platensis* was chosen since it can be easily cultured in outdoor and large scale facilities. At the same time, this species synthesizes high value-added compounds such as phycocyanin (Sukumaran al., 2018; Nur et al., 2019c). The aim of this research was therefore to explore and optimize the utilization of *A. platensis* to remove color and phenolic compounds from POME by varying nutritional and environmental conditions. A range of control experiments (without microalgae) was done, to unravel the contribution of photodegradation to the removal processes under consideration.

2. Materials and Methods

2.1. Wastewater preparation

Treated POME was obtained from a small factory in Sumatra, Indonesia as used previously (Nur et al., 2019a). POME was stored at -20°C to avoid degradation over time. Prior to experimental use, POME was thawed and filtered using GF/C glass fiber filter (Whatman) to remove suspended solids followed by autoclaving (121°C,15 minutes). Filtered and autoclaved POME contained 1425 mg L^{-1} COD, 56 mg L^{-1} total dissolved nitrogen, 6.93 mg L^{-1} dissolved PO_4^{3-}P, 240 mg L^{-1} total phenolic compounds, and 3600 ptco color as estimated by spectrophotometry using appropriate assay kits LCK 318, LCK349, LCK138 (Hach Lange) (Nur et al., 2019b), and Folin-ciocalteu reagent (Sigma Aldrich, Netherland) (see analysis section below).

2.2. Experimental setup

Arthrospira platensis (SAG 21.99) was purchased from the Culture Collection of Algae of the University of Göttingen (SAG, Germany). The culture was grown and maintained in Zarrouk medium (Zarrouk, 1966), at a fixed temperature of 27°C and an irradiance level of 150 µmol photons m^{-2} s^{-1} in a 16:8 h light: dark cycle. Three step-wise experiments were executed: the first experiment was aimed to unravel the effect of POME fraction (in ultrapure water, MilliQ) on *A. platensis* growth rate, final biomass, POME color, phenolic compounds, and chemical oxygen demand (COD) removal, without adding external nutrients; the second experiment was done to study the most influencing factor (POME fraction, salinity, irradiance level and nitrate concentration) as well as their possible interactive effects on biomass productivity and total POME color removal by using factorial design; the third experiment was aimed to unravel the interactive effect of irradiance level and initial phenol concentration on growth, final biomass and phenol removal by *A. platensis* using central composite design (CCD) response surface methodology (RSM). The cultures were acclimated to the experimental conditions for at least one week prior to experimentation.

2.2.1. Effect of POME on total biomass and COD removal

A. platensis was cultured in duplicate in 100 mL sterilized Erlenmeyer flasks with 75 mL working volume. The flasks was placed in a U-shaped water bath (Lauda C 6 CS, B03008, Edition 2000 Constant Temp Immersion Heating circulating Water Bath) as described previously (Nur et al., 2019b). The experimental temperature was set at 27°C and cultures were illuminated by a steady light source (Osram Biolux L 36W/965) in a 16:8 h light: dark cycle.

Five percent (v/v) of culture inoculum was added to five different dilutions of POME in ultrapure water (10-100%v/v), in triplicate. Since natural 100% POME contains 4 PSU salinity, the final salinity for each POME dilution below 100% was set to 4 PSU by adding NaCl. Furthermore, the initial pH was set to 9.0±0.2 by using 2 N HCl or 2 N NaOH. Initial light intensity was set to a saturating level of 200 µmol photons m^{-2} s^{-1} for each POME condition, as measured in the center of 100 mL sterilized Erlenmeyer culture flask (75 mL working volume), using a spherical light sensor (Biospherical Instrument QSL2101, California, USA). The light penetrated into the flask was adjusted by using 1-4 layers of black neutral density screen since the turbidity of POME was different for each fraction. Every day, the optical density of the cells was measured by spectrophotometry at 750 nm to determine the growth rate (see analysis section below). At the beginning of the incubations, the control (without *A. platensis*) was analyzed to determine the initial phenol, color, and COD concentration (see analysis section below). At the end of the incubation (6 days), final biomass in the culture was determined by centrifugation followed

by spectrophotometric detection (see below), whereas COD concentration, color, and phenolic compounds were determined in the supernatant (see analysis section below). To follow photodegradation of POME over time, triplicate control experiments were done by incubating 10-100 % POME without adding *A. platensis* in the same experimental setup.

Table 1. Factorial design experiment

Run	POME (%)	Salinity (PSU)	Light intensity (µmol photons m^{-2} s^{-1})	Nitrate (mg L^{-1})
1	50	15	50	0
2	50	15	50	100
3	50	30	50	0
4	50	30	50	100
5	100	15	50	0
6	100	15	50	100
7	100	30	50	0
8	100	30	50	100
9	50	15	200	0
10	50	15	200	100
11	50	30	200	0
12	50	30	200	100
13	100	15	200	0
14	100	15	200	100
15	100	30	200	0
16	100	30	200	100

2.2.2. Effects of nutritional and environmental conditions on POME color removal

A. 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 inoculum to the medium as described above. Full factorial design with four variables (POME, salinity, irradiance, nitrate) was performed to reveal the influencing factors and the possible interaction between these factors with respect to POME color removal by *A. platensis* (Table 1). 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. Salinity was adjusted using ultrapure water (MilliQ), NaCl, or natural filter sterilized sea water. At the beginning of the experiment, the control (without *A. platensis* inoculum) was analyzed to determine the initial color (see analysis section below). At end of the exponential growth phase (7-10 days), the cultures were centrifuged and the supernatant was kept to determine color (see analysis

section below). The biomass measurement was done at the beginning and the end of cultivation by using spectrophotometry (see analysis section below). An additional control experiment was done by incubating POME without adding *A. platensis* in order to follow photodegradation of POME over time within the same experiMental setup. Two replicates were done for the experiment.

2.2.3. Effect of light and phenol addition on growth rate, and biomass

To understand the interaction between light intensity and phenol addition on growth, final biomass, and phenol removal, different light intensities and concentrations of external gallic acid were applied following the CCD RSM approach. A fixed POME fraction of 50% was used in this experimental series since, based on experiment 1, the final biomass was not significantly lower than 100% POME. A total of 13 experimental runs was performed (Table 3). The ranges used for these runs were 8.6–290 mg L^{-1} for gallic acid (x_1) and 80- 220 µmol photons m^{-2} s^{-1} for initial light intensity (x_2). The empirical form of the second order polynomial model (Eq.1) can be described as:

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \qquad \text{Eq. 1}$$

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, x_j are independent variables of the model.

Cultivation was carried out in the same set-up as the first experimental series. Fifty percent of POME (diluted with ultrapure MilliQ) was mixed with gallic acid, stirred for 4 h at 1500 rpm at room temperature on a magnetic stirrer (Stuart SD162, England). The initial pH was adjusted to 9.0±0.2 by using 2 N HCl or 2 N NaOH. Salinity (20 psu) was adjusted by using NaCl. External urea (800 mg L^{-1}) was added to the medium to avoid nitrogen limitation based on previous experiments (Nur et al., 2019c). Every day, the optical density of the culture was measured at 750 nm to determine the growth rate (see analysis section below). At the end of the exponential phase (5 days), the cultures were harvested and the filtrate was stored to determine phenol removal efficiency. Additional measurements were done to determine the correlation of initial total phenols and initial total color in POME (Supplementary 1). Two additional control experiments were executed: in the first control experiment 50% POME was incubated without adding *A. platensis* (Table 3), in order to follow photodegradation of POME over time under the experimental conditions applied (initial and final sampling only). A second control experiment was done to understand the evolution of color degradation over time for a range of initial phenolic compounds, by performing daily sampling (Supplementary 2). Two replicates were done for the experiment.

2.3. Analytical Methods

2.3.1. Growth Rate

Every day, 3 ml of sample was taken and centrifuged at 10.000 rpm during 15 minutes, after which the pellet was resuspended in 3 ml of ultrapure water (salinity 4 PSU). This process was repeated twice to avoid color interferences between POME and *A. platensis* absorption. Absorbance of the resuspended cells was measured at 750 nm. Growth rate was determined by plotting the relationship between time (days) versus cell absorbance of the natural logarithm of OD_{750nm}, Growth rate was determined by the slope of the linear regression analysis.

2.3.2. Dry biomass measurement

Dry biomass was calculated based on optical density as described previously (Nur et al., 2019c). Since POME may strongly interfere with the *A. platensis* absorption measurements, samples (3 mL) were taken and centrifuged at 10.000 rpm for 15 minutes to separate the algal biomass from the POME medium. The pellet was washed twice using 0.75% NaCl and resuspended in 3 mL ultrapure water at the experimental salinity of 4 PSU. The optical density of the suspension was measured at 750 nm and the dry biomass was calculated following Eq. 2.

$$y = 0.59(\mathrm{OD}_{750\mathrm{nm}}) - 0.03 \; R^2 = 0.98 \qquad \text{Eq. 2}$$

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

Biomass productivity was calculated as described previously (Nur et al., 2019a) following (Eq. 3)

$$P_x = \frac{X_t - X_0}{t} \qquad \text{Eq. 3}$$

where P_x is biomass productivity mg L^{-1} d^{-1}, X_t is final biomass (mg L^{-1}), X_0 is initial biomass (mg L^{-1}), and t is total duration of cultivation (day).

2.3.3. Determination of COD

For COD analysis, samples (3 mL) were taken and centrifuged at 10.000 rpm during 15 minutes to remove *A. platensis* cells. Two mL of the sample (diluted with ultrapure water if necessary) was carefully pipetted to a tube containing reagent LCK314 (Hach, Netherland). The tube was gently inverted, then hydrolyzed to a temperature block (Hach LT 200) at 148 °C for 120 min. After cooling down to room temperature, the COD value in the tube was read based on spectrophotometry (Hach, DR3900).

COD removal efficiency was calculated using Eq. 4.

$$\mathrm{TCOD}\ (\%) = \frac{\mathrm{COD_0} - \mathrm{COD_t}}{\mathrm{COD_0}} \mathrm{x}\ 100 \qquad \text{Eq. 4}$$

where TCOD is total COD removal (%), COD_0 is COD of the sample at day 0 of cultivation (mg L^{-1}), and COD_t is COD of the sample at the end of cultivation (mg L^{-1}). The calculation of COD removal by *A. platensis* (COD_s) and the control (COD_p) were also derived from Eq. 4.

COD removal by *A. platensis* was corrected by subtracting total COD removal (TCOD) with removal of the COD from control as stated in Eq. 5

$$COD_s = TCOD - COD_p \quad \text{Eq. 5}$$

where COD_s is COD removal efficiency as a result of activity from *A. platensis* (%), and COD_p is COD removal efficiency from the control expressing photodegradation activity (%).

COD removal in absolute value was calculated from Eq. 6.

$$ACOD = COD_0 - COD_t \quad \text{Eq. 6}$$

where ACOD is absolute value of COD removed by total activity (mg L^{-1}), COD_0 is COD at the first day of cultivation (mg L^{-1}), and COD_t is COD at the end of cultivation (mg L^{-1}). Eq. 6 was also applied for control as expressed of photodegradation activity and *A. platensis* activity.

Absolute value of COD removed by *A. platensis* was corrected by subtracting the absolute total value of COD from control expressing photodegradation, following Eq. 7.

$$ACODs = ACOD - ACODp \quad \text{Eq. 7}$$

where ACODs is concentration of COD removed by *A. platensis* activity (mg L^{-1}), ACOD is concentration COD removed by total activity (mg L^{-1}), and ACODp is the concentration of COD from control expressing photodegradation activity (mg L^{-1}).

2.3.4. Determination of color removal

Color measurements were done following Hach, (2014) which is suitable for water, wastewater, and seawater samples. Samples (2 mL) were taken and centrifuged at 10.000 rpm during 15 minutes to remove *A. platensis* cells. The supernatant was then diluted in a ratio 1:3 by using ultrapure water and measured using a spectrophotometer (Hach Lange DR 3900) at 455 nm. A platinum cobalt standard solution (PtCo) was used to prepare a standard curve of measurements at 455 nm (spectrophotometer, program 120). Measurements were recorded in PtCo units.

Color removal efficiency was calculated using (Eq. 8):

$$TC = \frac{C_0 - C_t}{C_0} x\ 100 \quad \text{Eq. 8}$$

where TC is total color removal (%), C_0 is color of the sample at day 0 of cultivation, and C_t is color of the sample at the end of cultivation. The calculation of color

removal by *A. platensis* (TC_s) and the control, representing photodegradation only (TC_p) were also applied from Eq. 8.
Color removal by *A. platensis* was corrected by subtracting total removal with removal of the color from photodegradation activity following (Eq. 9):

$$TC_s = TC - TC_p \quad \text{Eq. 9}$$

where TC_s is color removal efficiency as a result of activity from *A. platensis* (%), and TC_p is color removal efficiency from the control as a result of photodegradation activity (%).
Color removal in absolute values was calculated from Eq. 10.

$$ATC = TC_0 - TC_t \quad \text{Eq. 10}$$

where ATC is absolute value of color removed by total activity (ptco), TC_0 is color at the first day of cultivation (ptco), and TC_t is color at the end of cultivation (mg L^{-1}). Eq. 10 was also applied for control expressing photodegradation activity and *A. platensis* activity.
The absolute value of color removed by *A. platensis* was corrected by subtracting the absolute total value of color from control expressing photodegradation, following Eq. 11.

$$ATCs = ATC - ATCp \quad \text{Eq. 11}$$

where ATCs is concentration of color removed by *A. platensis* activity (ptco), ATC is concentration color removed by total activity (ptco), and ATCp is the concentration of color removed from control expressing photodegradation activity (ptco).

2.3.5. Determination of phenolic compounds

Total phenolic compounds were determined by applying minor modifications of the Folin-Ciocalteau method as described previously (Ergül et al., 2011; Chantho et al., 2016). First, samples were centrifuged at 10.000 rpm for 15 min to eliminate *A. platensis*. Then, 200 µL of the sample (diluted with ultrapure water if necessary) was placed in a 15 mL dark conical tube and mixed with 1 mL Foline Ciocalteau phenol reagents (Sigma Aldrich, Netherlands) (4-fold-dilution with ultrapure water). After 5 min, 1 mL of a saturated sodium carbonate solution (200 g L^{-1}) was added, vortexed at 2500 for 10 s and left for 30 min at room temperature. The absorbance was measured spectrophotometrically (Hach, DR 3900, Netherlands) at 725 nm. A calibration curve of gallic acid was used as a standard, with 5 set points in the range 0-850 mg L^{-1}.
Phenol removal efficiency (TP) was calculated using Eq. 12

$$TP = \frac{P_0 - P_t}{P_0} x100 \quad \text{Eq. 12}$$

where TP is total phenol removal (%), P_0 is phenol of the sample at day 0 of cultivation (mg L^{-1}), and P_t is the concentration of phenol in the sample at the end of cultivation (mg L^{-1}).

Phenol removal by *A. platensis* was corrected by subtracting total phenol removal with phenol removal from the control expressing photodegradation activity as stated in Eq. 13.

$$TP_s = TP - TP_p \quad \text{Eq. 13}$$

where TP_s is phenol removal efficiency as a result of activity from *A. platensis* (%), and TP_P is phenol removal efficiency from the control as a result of photodegradation (%).

The absolute value of total phenolic compounds removed was calculated by Eq. 14

$$ATP = TP_0 - TP_t \quad \text{Eq. 14}$$

where ATP is the absolute value of phenolic compounds removed by total activity (mg L^{-1}), TP_0 is total phenolic compounds at the first day of cultivation (mg L^{-1}), and TP_t is total phenolic compounds at the end of cultivation (mg L^{-1}). Eq. 10 was also applied for photodegradation activity and *A. platensis* activity.

Concentration of phenol removed by *A. platensis* was corrected by subtracting the absolute total value of phenolic compounds by the absolute concentration of phenol removed from the control expressing photodegradation activity, following Eq. 15.

$$ATPs = ATP - ATPp \quad \text{Eq. 15}$$

where ATPs is the absolute concentration of phenolic compounds removed by *A. platensis* activity (mg L^{-1}), ATP is the concentration of phenolic compounds removed by total activity (mg L^{-1}), and ATPp is the concentration of phenolic compounds removed from the control expressing photodegradation activity (mg L^{-1}).

2.4. Data analysis

Experimental design and statistical analyses were done using ANOVA Factorial Design and Response Surface Methodology, run in Minitab 18. ANOVA analysis was done with $\alpha = 0.05$ for attribution of sources of variation. Optimization experiments were generated as per CCD model. Pareto Charts and contour plots were used to understand the most influencing factors and the correlations between parameters.

3. Results

3.1. Effect of POME fraction

Arthrospira platensis was found to grow well on all POME fractions, including 100% POME. Between 10% and 100% POME, final biomass increased almost 1.5 fold, whereas POME fractions above 30% POME showed no significant differences (P>0.05) (Figure 1).

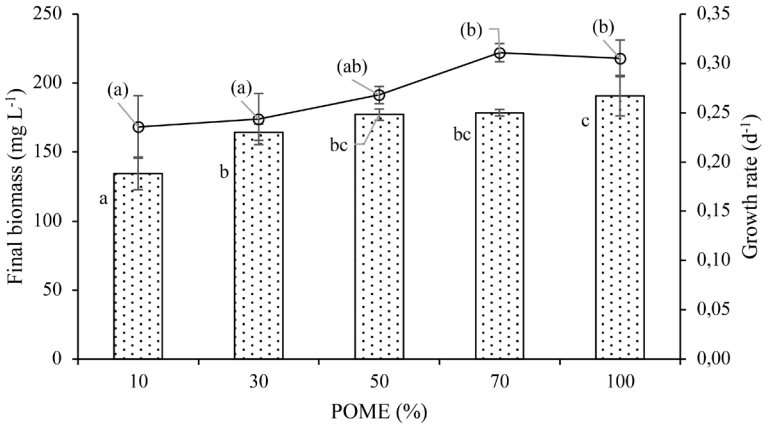

Figure 1. Final biomass (calculated dry weight in mg.L^{-1}) and growth rate of *A. platensis* cultivated on different POME fractions. Average values of triplicate cultures are shown. Error bars indicate the SD of the mean. Bar is final biomass; line is growth rate. Values that do not share letters are significantly different (P<0.05).

The lowest final biomass was found on 10% POME (135 mg L^{-1}) (P<0.05) whereas at 100% POME a final biomass of 190 mg L^{-1} was recorded. For growth rate, POME fractions above 30% showed significantly increased growth rates compared with the lower fractions(P<0.05). No significant differences in growth rate were found between 50% and 100% POME (P>0.05).

Increasing the POME fraction from 10% to 20% significantly enhanced COD removal in absolute value by photodegradation (ACODp), from 28 to 72 mg L^{-1}. Above 30% POME no significant increase in ACODp removal was recorded (125 to 245 mg L^{-1}, Table 2). Higher POME fractions also significantly influenced COD removal by *A. platensis* (ACODs). The highest ACODs was recorded at 100% POME which removed 317 mg L^{-1} (P<0.05) and the activity of *A. platensis*, ACODs, was significantly higher compared to photodegradation activity ACODp (P<0.05). The interaction of POME fractions and total activity significantly influenced COD removal in absolute value. By combining photodegradation and *A. platensis* activity (total activity), the highest absolute value for COD removal was found at 100% POME (P<0.05) (Table 2).

Table 2. COD removal, phenolic removal, and color removal in absolute values at different POME fractions and activities. Average values are shown (n=3). Value after ± symbol indicates standard deviation. Value does not share letters indicated significantly different (P<0.05)

POME	Activity	COD removal (mg L^{-1})		Color removal (ptco)		Phenolic compounds removal (mg L^{-1})	
10	photodegradation	28.63^{a}	±1.48	105.67^{a}	±20.31	11.46^{a}	±3.03
30	Photodegradation	72.33^{bc}	±6.81	389.67^{b}	±22.59	33.65^{b}	±1.17
50	Photodegradation	125.00^{cd}	±36.50	737.67^{c}	±53.16	69.32^{c}	±7.95
70	Photodegradation	152.33^{cd}	±33.86	1377.33^{d}	±35.57	108.21^{d}	±7.20
100	Photodegradation	246.33^{d}	±20.50	2239.33^{e}	±54.50	171.25^{e}	±1.75
10	*A. platensis*	17.03$^{(a)}$	±2.70	37.33$^{(a)}$	±9.24	0.00$^{(a)}$	±0.00
30	*A. platensis*	55.67$^{(a)}$	±8.14	163.67$^{(b)}$	±8.96	10.74$^{(a)}$	±2.23
50	*A. platensis*	147.00$^{(b)}$	±19.29	225.67$^{(b)}$	±54.37	21.17$^{(a)}$	±16.22
70	*A. platensis*	217.67$^{(c)}$	±6.03	166.00$^{(b)}$	±28.84	12.76$^{(a)}$	±10.48
100	*A. platensis*	317.00$^{(d)}$	±41.07	365.67$^{(c)}$	±56.75	4.28$^{(a)}$	±6.29
10	Total	45.67^{A}	±4.04	143.00^{A}	±18.52	5.48^{A}	±1.09
30	Total	128.00^{A}	±6.93	553.33^{B}	±15.28	44.39^{B}	±1.09
50	Total	272.00^{B}	±30.20	963.33^{C}	±75.06	90.49^{C}	±8.27
70	Total	370.00^{C}	±30.00	1543.33^{D}	±56.86	120.97^{D}	±3.30
100	Total	563.33^{D}	±61.10	2605.00^{E}	±10.00	175.03^{E}	±5.15

Absolute phenolic compound removal (ATPp) and color removal (ATCp) were significantly influenced by POME fraction when considering photodegradation activity. Above 50%, POME fractions significantly influenced the removal of ATPp and ATCp. For *A. platensis* activity, the removal of phenolic compounds (ATPs) and color (ATCs) were not significantly influenced by POME fractions (P<0.05). At 100% POME, color was removed up to 2239, 365, and 2605 ptco by photodegradation, *A. platensis* activity, and total activity, respectively. The highest removal of phenolic compounds and color were found at 100% POME considering total activity (Table 2). The interaction of total activity and POME fractions significantly influenced the removal of ATC and ATP (P<0.05). The lowest activity was found for *A. platensis* activity both for phenolic compounds (ATPs) and color removal (ATCs) (P<0.05).

For COD removal (in percentage value) by photodegradation (CODp), the value was not significantly affected by POME fraction (P>0.05). POME fraction significantly affected COD removal efficiencies by *A. platensis* activity (CODs) (P<0.05): increasing POME fractions from 10% to 50% enhanced COD removal efficiencies with almost 3-fold (Figure 2). For POME fractions above 50 %, CODs removal was not significantly different. When considering total COD removal (TCOD), the lowest removal was found at 10% POME.

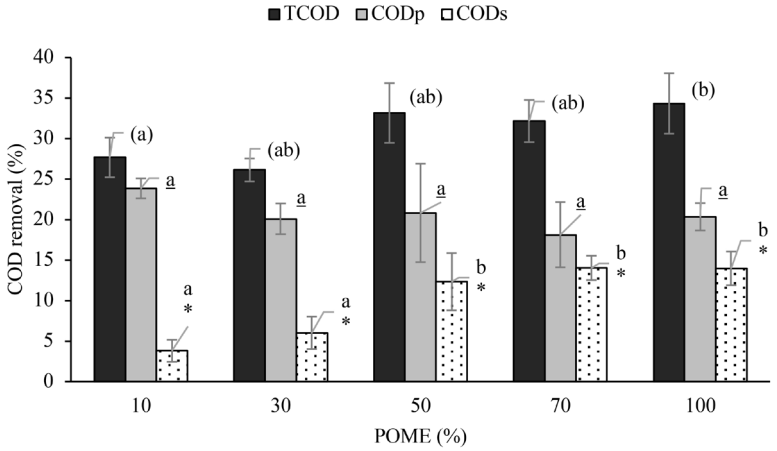

Figure 2. Total COD removal (TCOD), COD removal by photodegradation (CODp), and by *A. platensis* activity (CODs), as a function of POME fraction. Average values of triplicate incubations are shown. Error bars indicate the SD of the mean. Values that do not share letters are significantly different (P<0.05).

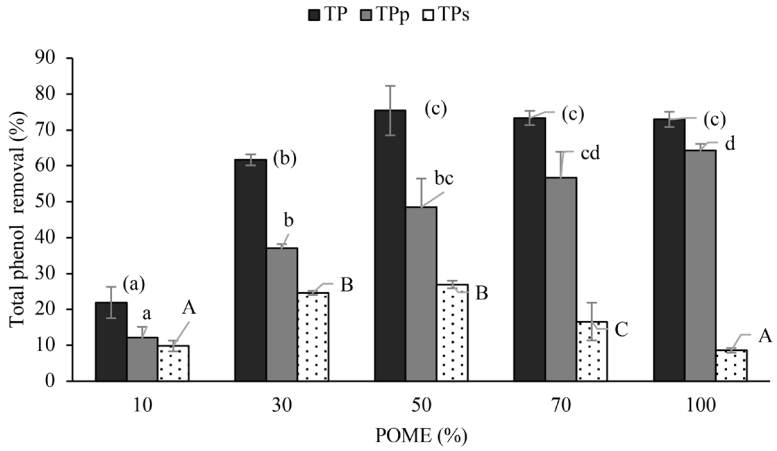

Figure 3. Total phenol removal (TP), phenol removal by photodegradation (TPp), and by *A. platensis* activity (TPs). Average values of triplicate cultures are shown. Error bars indicate the SD of the mean. Values that do not share letters are significantly different (P<0.05).

POME fractions influenced the removal efficiency of phenolic compound, both through photodegradation (TPp) and through the activity of *A. platensis* (TPs). TPp was found to be significantly higher at 100% POME compared to 50% POME and lower (P<0.05). However, no significant difference was found between 70 and 100% POME (P>0.05). Low phenolic compound removal by *A. platensis* activity (TPs) was found for 10% and 100% POME, while high TPs removal was found around 30-50% POME (Figure 3). For total phenolic compound removal (TP), increasing

POME fraction from 10 to 50% significantly increased removal efficiency (P<0.05). Above 50% POME fraction, the efficiencies were not significantly different from each other (P>0.05).

Increasing POME fractions from 10 to 100% also influenced color removal efficiencies by photodegradation (TCp) as well as total activity (TC) (P<0.05) (Figure 4). However, the POME fraction did not significantly influence color removal based on *A. platensis* activity (TCs). The highest color removal efficiency was found at 100% POME both for TCp and TC (P<0.05).

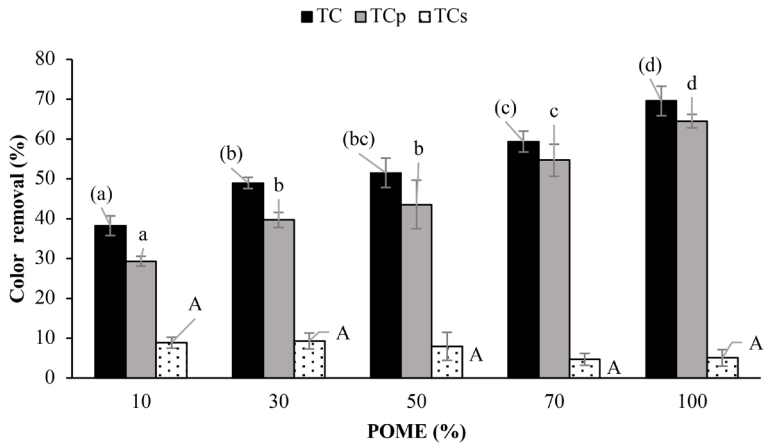

Figure 4. Total color removal (TC), color removal by photodegradation (TCp), and by *A. platensis* (TCs) Activity. Average values of triplicate cultures are shown. Error bars indicate the SD of the mean. Values that do not share letters are significantly different (P<0.05).

3.2. Effect of environmental and nutritional conditions on biomass productivity, and total POME color removal

The most influencing factor for biomass productivity was found to be light intensity alone (Figure 5a, 6a). Increasing light intensities, from 50 to 200 µmol photons m^{-2} s^{-1} significantly enhanced *A. platensis* biomass productivity (P<0.05) (Figure 5a,b, Figure 6a). Increasing salinity, from 15 to 30 PSU, significantly decreased the final biomass (P<0.05) (Figure 5c). The presence of nitrate at high POME also significantly enhanced biomass productivity when the irradiance level was high (Figure 5b). However, based on Pareto chart, the addition of nitrate alone, POME alone, and salinity alone did not enhance biomass productivity (P>0.05)(Figure 6a).

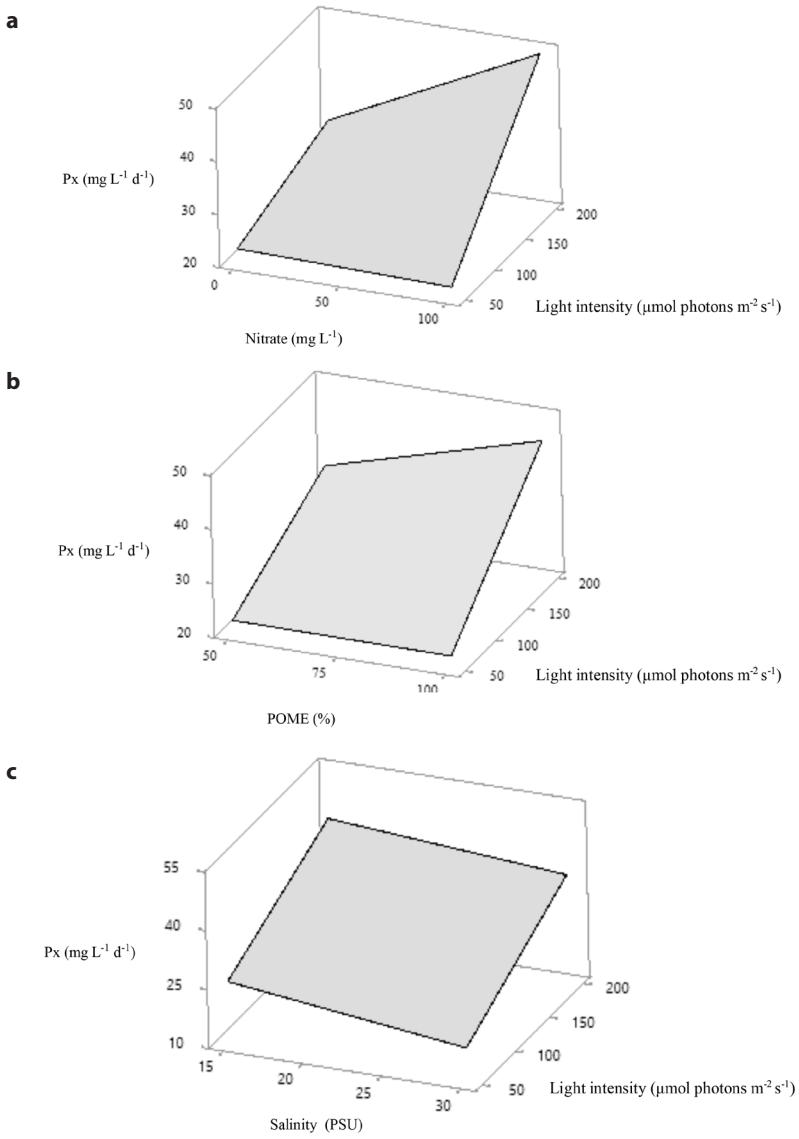

Figure 5. Selected response surface (3D) of biomass productivity (P_x) as a result of the factorial design experiment a) light intensity vs nitrate addition at a fixed value of 75% POME and 22.5 PSU, b) light intensity vs POME fraction at fixed values of 22.5 PSU and 50 mg L^{-1} nitrate addition, and c) salinity vs POME fraction at fixed values of 125 µmol photons m^{-2} s^{-1} and 50 mg L^{-1} nitrate addition

For total color removal (TC), the most influencing factor was found to be irradiance only (Figure 6b), while the other factors were not significantly influential. When considering the activity of *A. platensis* alone (TCs), none of the factors significantly influenced the color removal (Figure 6c). Based on these results, light intensity

was optimized to explore phenolic compound removal as the activity of *A. platensis* normalized to photodegradation (third experimental series).

3.3. Effect of light intensity and initial phenol concentration on phenol and color removal efficiency

Since light intensity was found to be the most influencing factor for color removal (second experimental series, Figure 6b), the third experimental series was done to reveal the effect of photodegradation on phenolic compound removal during the cultivation of *A. platensis*. Based on a preliminary experiment, color and phenolic compounds showed a strong correlation (R^2=0.95) (supplementary material 1).

Therefore, different initial phenolic concentrations in POME combined with different light intensities were applied to understand the interaction of these factors. Then, we plotted the effect of light intensity and initial phenol concentration on phenol removal using POME only (without *A. platensis*, TPp). Phenolic compound removal was significantly influenced by light intensity and initial phenol concentration (Figure 7a). Low initial phenol concentration, and increasing light intensity (from 50 to 200 µmol photons m^{-2} s^{-1}) resulted in increasing phenolic compound removal from 30 to 70%. At a higher initial phenol concentration, the phenol removal increased from 60 up to 80%. Both the increasing initial phenol concentration and light intensity resulted in enhanced phenol removal efficiency.

For total phenol removal by *A. platensis* (TPs) which included the activity of *A. platensis* alone, increasing light intensity decreased phenolic compound removal efficiency (Figure 7b). At low light (LL) (< 100 µmol photons m^{-2} s^{-1}), phenol degradation reached up to 30-40% for all initial phenol concentrations. When the light level exceeded 150 µmol photons m^{-2} s^{-1}, TPs was significantly lower, at around 10-15%.

For total phenol removal (TP), the highest phenol removal (>90%) was recorded when a medium irradiance level (ML) (100-150 µmol photons m^{-2} s^{-1}) and high initial phenol concentration were employed. The lowest phenol removal (< 75%) was recorded at low light and low initial phenol concentration (Figure 7c).

For the absolute concentration of total phenolic compounds removed by *A. platensis* activity (CTPs), increasing light intensity decreased phenolic compound removal but these differences were not significant (P=0.052). However, when initial total phenolic compounds were increased at lower light intensity, the concentration of phenolic compounds removed by *A. platensis* was found to be higher (Figure 7d, Table 3). The interaction of light intensity and initial total phenolic compounds significantly influenced CTPs (P<0.05). Based on RSM calculation, the highest concentration of total phenolic compounds removed by *A. platensis* was found at 80 µmol photons m^{-2} s^{-1} and 411 mg L^{-1} of initial phenolic compounds which resulted in 136 mg L^{-1} CTPs.

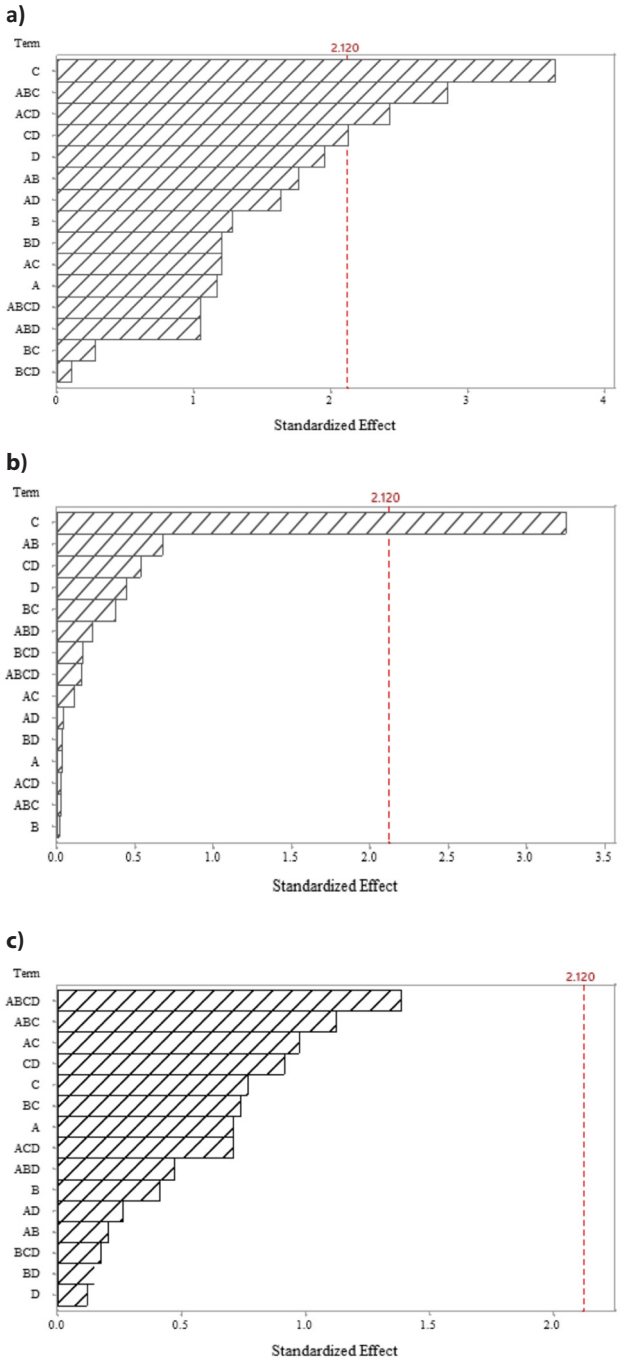

Figure 6. Pareto chart showing the selected interaction effects of parameters on a) biomass productivity, b) total color removal (TC) c) color removal by *A. platensis* (TCs). A is POME, B is salinity, C is light intensity, and D is nitrate addition. The vertical line indicates the significance of the effects at 95% confidence level.

Table 3. Experimental design of CCD RSM and the responses of phenolic removal in absolute values. Average values of the response are shown from duplicate experiments (n=2). SD is shown after the ± symbol. ATP is the concentration of phenolic compounds removed by total activity (mg L^{-1}), ATPp is concentration of phenolic compounds removed by photodegradation activity (mg L^{-1}), and ATPs is concentration of phenolic compounds removed by *A. platensis* activity (mg L^{-1}). Star symbol (*) is the middle point in CCD RSM design

Run	Initial total phenolic (coded)	Initial light intensity (coded)	External GA addition (mg L^{-1})	Initial total phenolic (mg L^{-1})	Initial light intensity (µmol photons m^{-2} s^{-1})	ATP (mg L^{-1})		ATPp (mg L^{-1})		ATPs (mg L^{-1})	
1	-1.4145	0	8.58	128.58	150.00	96.68	±3.93	81.03	±0.49	15.65	±4.43
2	-1	-1	50.00	170.00	100.00	129.36	±1.97	71.99	±0.49	57.38	±1.48
3	-1	1	50.00	170.00	200.00	138.75	±2.46	117.19	±0.49	21.56	±2.95
4	0	0	150.00*	270.00*	150.00*	231.60	±0.98	207.26	±0.98	24.34	±1.97
5	0	1.4145	150.00	270.00	220.71	232.30	±0.00	209.00	±3.44	23.30	±3.44
6	0	0	150.00*	270.00*	150.00*	231.60	±0.98	207.26	±0.98	24.34	±1.97
7	1	-1.4145	150.00	270.00	79.29	235.78	±5.90	182.22	±36.39	53.55	±30.49
8	0	0	150.00*	270.00*	150.00*	231.60	±0.98	207.26	±0.98	24.34	±1.97
9	1	-1	250.00	370.00	100.00	324.46	±0.49	207.96	±1.97	116.50	±2.46
10	1	1	250.00	370.00	200.00	339.06	±7.38	311.59	±3.93	27.47	±3.44
11	0	0	150.00*	270.00*	150.00*	231.60	±0.98	207.26	±0.98	24.34	±1.97
12	1.4145	0	291.42	411.42	150.00	374.18	±11.80	338.71	±6.89	35.47	±4.92
13	0	0	150.00*	270.00*	150.00*	231.60	±0.98	207.26	±0.98	24.34	±1.97

3.4. Effect of light intensity and initial phenol concentration on growth rate and final biomass

The highest growth rate (>0.45 d^{-1}) was also recorded at HL and high initial phenol concentration. At HL, the increase of initial phenol concentration significantly boosted the growth rate, while at LL, the increase of initial phenol concentration decreased the growth rate. Growth rate was significantly influenced by the interaction of light intensity and initial phenol concentration (Figure 8a).

The highest final *A. platensis* biomass (>400 mg L^{-1}) was recorded at high irradiance (HL) (>150 µmol photons m^{-2} s^{-1}) and high initial phenol concentration. The interaction of light intensity and initial phenol concentration significantly influenced the final biomass. When the light was set to HL, the final biomass was significantly influenced by initial phenol concentration (Figure 8b). However, at LL, the increase of initial phenol concentration did not significantly influence final biomass production.

4. Discussion

The degradation of phenolic compounds and color of POME was significantly influenced by the activity of photodegradation. It is possible that the presence of hydroxyl radicals (OH^-), which are generated from irradiated fulvic acid-like substances in POME (Kongnoo et al., 2012), could degrade the phenolic compounds. Faust and Hoigne (1987) found that the combination of fulvic acid and sunlight could enhance the degradation of 2,4,6-trimethylphenol in natural waters, while Jacobs et al (2012) found that the degradation of phenolic compound occurred through reaction with the hydroxyl radical (OH^-) generated by irradiated fulvic acids. Possibly, at POME fractions above 50% photodegradation became more pronounced, due to higher fulvic-acid like concentrations, resulting in higher absolute phenolic compound removal (ATPp). However, at very low POME values, it seems that color and phenol could not be degraded by the combined action of fulvic-acid and light (See Supplementary 2). This result is in agreement with a previous study which showed that the presence of low organic material in POME could not be degraded by simple physical-chemical methods (Kongnoo et al., 2012).

Compared to photodegradation, the activity of *A. platensis* to remove phenolic compounds (ATPs) and color (ATCs) on POME fractions was lower. At experiment 1, with high irradiance levels (Table 2), *A. platensis* only contributed around 10-25% (Figure 3-4).

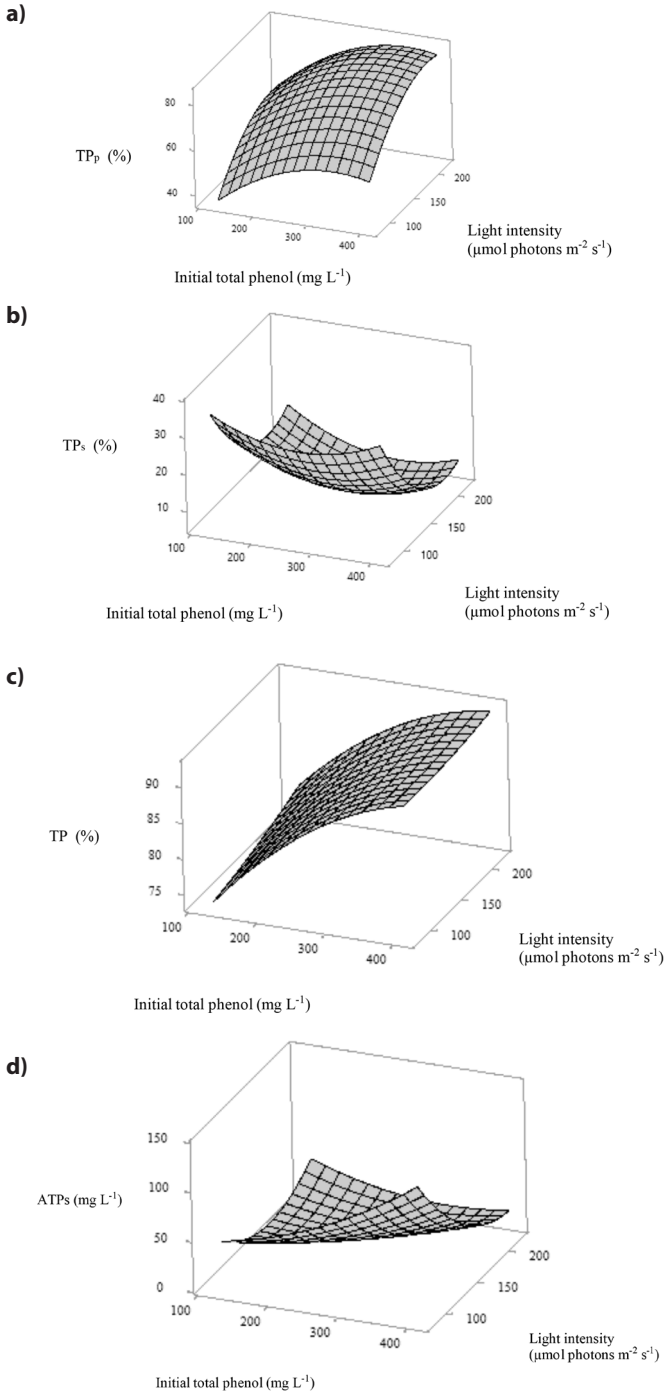

Figure 7. Response surface plot (3D) of (a) total phenolic compound removal by photodegradation (TPp), (b) total phenolic compound removal by *A. platensis* (TPs), c) total phenolic compound removal (TP), and d) concentration of total phenolic compounds removed by *A. platensis* activity (ATPs) as a function of initial light intensity and initial total phenolic compounds

This indicated that *A. platensis* activity to remove phenolic compounds on POME was depended on the activity of photodegradation. At high light intensity, hydroxyl radical can be more produced by fulvic acid and resulted higher photodegradation activity. This result was supported by experiment 3 which showed that the activity of *A. platensis* to remove phenolic compounds in absolute value was lower compared to photodegradation at high irradiance, and vice verse (Table 3).

In this study, a combination of fulvic acid presented in POME and light intensity also influenced absolute COD (ACODp) (Figure 2). At 100% POME, 246.33 mg L^{-1} of COD was removed by photodegradation. At 10% POME, only 28.63 mg L^{-1} was removed. Previous research showed that total organic carbon in POME, which is associated with COD, was degraded in the presence of the hydroxyl radical and metals such as Fe^{2+} (Gamaralalage et al., 2019). It seems that trace metals contained in POME, such as iron, zinc, and copper, could also contribute to COD degradation after reacting with the hydroxyl radical (Safarzadeh-Amiri, et al., 1996; Hurtado et al., 2016; Ding et al., 2016). Increasing the POME fraction might therefore result in higher iron and hydroxyl levels, thereby enhancing ACODp through photodegradation. However, the value of ACODp was significantly lower compared to COD degradation by *A. platensis* activity (ACODs). This result is also in agreement with a previous study which demonstrated that the degradation of total organic carbon in POME, mainly detected as acetic acid, by hydroxyl radicals and iron, resulted in a low removal efficiency (Gamaralalage et al., 2019). In the presence of *A. platensis*, ACODs and total biomass was higher at increasing POME fractions. It is possible that higher POME fractions result in higher organic carbon levels which can be utilized by *A. platensis* for growth, resulting in higher biomass and higher breakdown of ACOD. This is supported by an earlier study, which demonstrated that organic carbon in treated POME such as acetic acid could be utilized as substrate by *A. platensis* (Zainal et al., 2012).

With respect to the environmental factors, light intensity significantly enhanced the removal of total color (TC). Previous study found that at higher light intensities, phenol oxidation was more enhanced (Blaková et al, 1998), thus resulting higher color removal. While for color removal by *A. platensis*, the activity was not significantly affected by the selected environmental factors (Figure 6c), even though the biomass productivity of *A. platensis* was significantly affected by light intensity and the interaction of light intensity and nitrate, the interaction of POME fractions and salinity, and the interaction of POME fractions and nitrate addition (Figure 5, 6a). It seems that a very high microalgal biomass is required to eliminate all color of POME. Baldev et al (2013) reported that the removal of a synthetic dye was influenced by the initial inoculum concentration of *Coelastrella* sp. At the higher initial inoculum concentration, a higher number of the cells was exposed to the medium, thereby enhancing dye removal. Furthermore, Stephen et

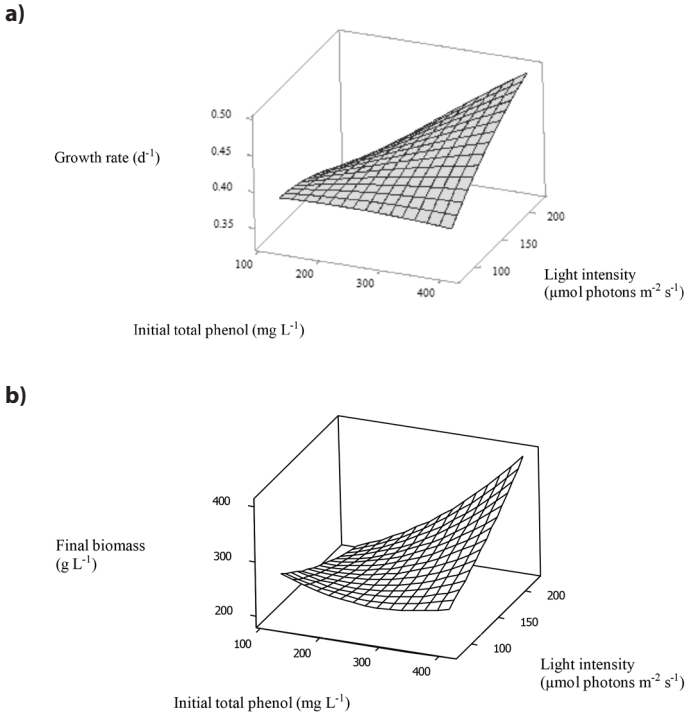

Figure 8. Response surface plot (3D) of a) growth rate and b) final biomass expressed as dry weight (g L^{-1}) as a function of initial light intensity and initial total phenolic compounds

al (2017) found that *Chlorella pyrenoidosa* at an initial inoculum of around 3–4 g L^{-1} could remove phenol up to 95% when supplemented with nutrients.

CCD RSM revealed that increasing initial phenolic compounds and light intensity significantly enhanced the phenol removal efficiency by photodegradation. It seems that gallic acid as the external phenolic compound was easily degraded by photodegradation. The higher gallic acid concentrations were applied, the higher removal efficiency was obtained. On the long run, however, the remaining phenolic compounds are probably more difficult to degrade, and the remaining phenolic compounds could not be removed (See Supplementary 2). Previous study reported that simple phenolic compounds which contained lower hydroxyl group bonded to the aromatic ring such as gallic acid was more easily degraded by hydroxyl radicals compared to more complex phenolic compounds such as caffeic acid, and protocatechuic, which the compounds can also be found on POME (Chanto et al., 2016; Sroka et al., 2003).

Previous work demonstrated that microalgae could degrade phenolic compounds in several ways; by utilizing it as an organic carbon source, or by oxidizing it due to the binding of dissolved oxygen produced by microalgae (Lika and Papakadis, 2009). In our study, at low light intensity, photodegradation

activity was low. It is possible that the hydroxyl radical produced from fulvic-acid in POME was low at low light, thereby causing lower phenolic degradation rates. On the other hand, the degradation of phenolic compounds by *A. platensis* was high at low light intensity. This might imply that the phenolic compounds were assimilated by *A. platensis*, which would be in agreement with previous work demonstrating phenolic compound uptake by the cyanobacterium *Spirulina maxima*, as organic carbon source (Lee et al., 2015). When the irradiance level was high, photodegradation became more pronounced compared with *A. platensis* activity (Table 2, Figure 7b, c), even though *A. platensis* still contributed to phenolic compound removal at around 10-20%. By combining high light intensity and high initial phenolic compound level, the final biomass and growth rate also increased. A possible explanation for this is that the degradation of phenol at high light intensity resulted in lower molecular weight acids which could be easily utilized by *A. platensis* (Vlyssides et al., 2011; Golmakani et al., 2012; Gamaralalage et al., 2019).

5. Conclusion

This study revealed the capability of *A. platensis* to degrade COD, color and phenolic compounds in POME wastewater. The initial fraction of POME that was present influenced growth rate, final biomass, COD removal absolute value and absolute color removal by *A. platensis*. Based on a factorial design approach it was shown that salinity, nitrogen addition, and initial POME concentration did not influence total color removal. The addition of gallic acid as phenolic compounds to POME at high light intensity could increase the growth rate up to 0.45 d^{-1} and final biomass up to 400 g L^{-1} while on the other hand total phenolic compounds were removed almost completely (94%). Photodegradation activity significantly contributed to POME, COD, color and phenolic compound removal. Phenolic compounds that are present in POME could be removed by *A. platensis* when cultivated on high POME fractions at low irradiance conditions. High phenolic compound removal can be achieved by combining *A. platensis* activity and photodegradation. Overall, this study showed that phenolic compounds and color degradation from POME were dominated by the activity of photodegradation at high irradiance, while the activity of *A. platensis* dominated at low light intensity.

Acknowledgment

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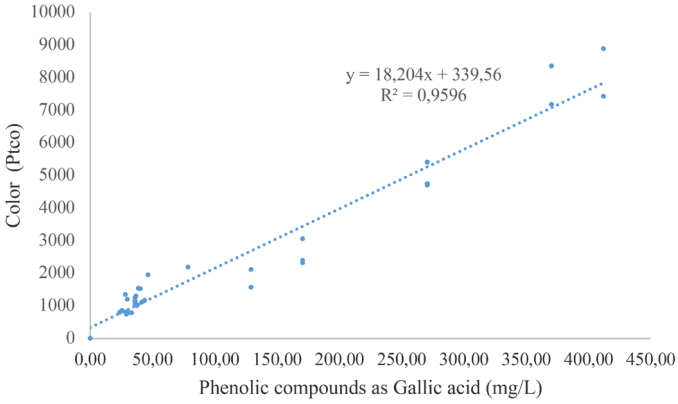
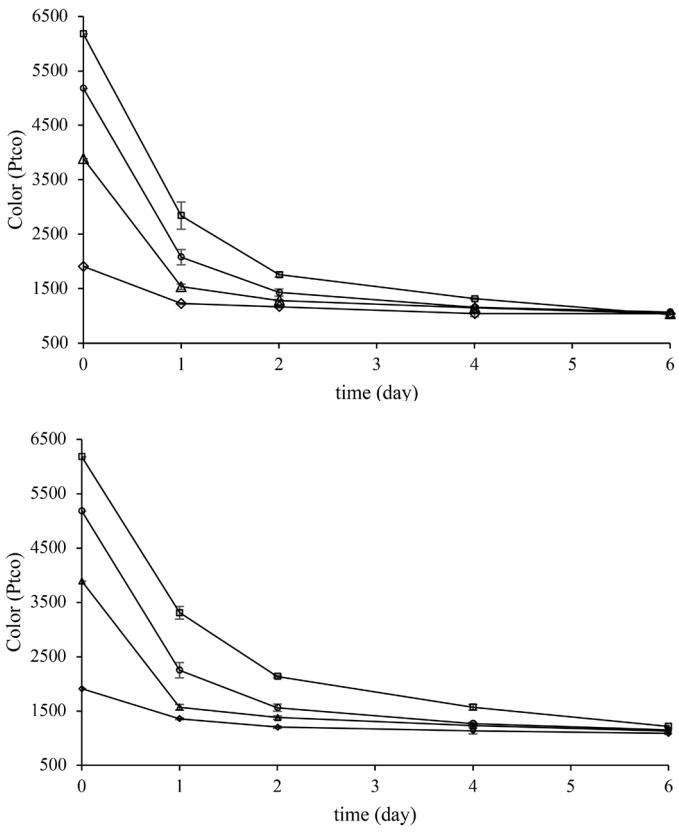
Supplementary



S1. Correlation of total color and total phenol (internal + external)



S2. Profile of color degradation by a) total activity and b) activity by photodegradation at 200 µmol photons m^{-2} s^{-1} and 50% POME at different initial phenolic compounds. Average values are shown (n=3). Bar lines indicate standard deviation.

Summary

1. Summary

Currently, Indonesia is known as the largest CPO producer in the world, followed by Malaysia and Thailand. During the production, about 1 ton of fresh fruit bunch (FFB) produces 0.66 ton palm oil mill effluent (POME). The pH of raw POME ranges between 4.0 and 5.0, and its chemical oxygen demand (COD) and biological oxygen demand (BOD) are extremely high. Furthermore, POME contains a high amount of total suspended solids (TSS), total dissolved solids (TDS) and volatile suspended solids (VSS). It is therefore evident that comprehensive treatment should be done to meet standard regulations before POME can be released into the environment (i.e. rivers, lakes). To treat POME, several mechanical, chemical or biological methods have been developed. However, compared to other techniques, biological treatment is more environmental friendly, less expensive as well as energy saving. Over the past decades, the utilization of agricultural waste(water) with the aim to meet industrial demands and to generate useful products has gained increasing interest. Valorization in this context is the process of converting waste materials into valuable products by using microorganism such as yeast, bacteria, and microalgae. In general, the utilization of microalgae to treat wastewater is more preferable since the substances produced by these organisms may have a high economic value. At the start of this PhD project, the utilization of POME by microalgae was so far mainly focused on lipid production, bulk biomass and wastewater treatment: the production of high value-added compounds with an eye towards large scale cultivation was hardly considered. Therefore, the goal of this thesis was to fill the gaps in this knowledge by: i) investigating the potential of growing several microalgal species on POME and to monitor the production of their associated high value products, ii) investigating a range of environmental and nutritional parameters and their interrelation, to optimize the utilization of POME as growth medium for key microalgae and their associated value added products, iii) investigating the potential of microalgal cultivation to improve POME quality through color and phenolic compound removal. These research aims were translated into specific goals, and subsequently addressed in four research chapters.

In **chapter 2**, we explored the utilization of POME by the marine diatom *Phaeodactylum tricornutum* and investigated a variety of environmental conditions in order to optimize fucoxanthin productivity at different irradiance, temperature and nutrient conditions. Fucoxanthin produced by diatoms such as *P. tricornutum*

can exhibit anti-cancer, anti-obesity, and anti-diabetic activity. I hypothesized that the fucoxanthin productivity of *P. triconutum* grown on POME depended on (a combination of) irradiance level, nutritional ratios, salinity and temperature. First, optimum irradiance conditions for fucoxanthin productivity were determined for *P. tricornutum* growing on artificial medium. This optimum irradiance was applied in all subsequent experiments involving POME fractions. Then, the impacts of various POME fractions on growth rate and fucoxanthin productivity were investigated and selected nutrient additions were done to optimize fucoxanthin productivity. 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. Through this stepwise experiments, we found that *P. tricornutum* may be used for large scale cultivation on 30% v/v POME with the aim to produce fucoxanthin. BBD RSM revealed that 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. Furthermore, optimal urea (85 mg L^{-1}), temperature (23 °C) and salinity (22-23 PSU) conditions were determined from our final experimental series.

Since recently, the interest in utilizing exopolysaccharide (EPS) from microalgae has increased. In **chapter 3**, the production of sulfated exopolysaccharide (sEPS), a specified EPS byproduct synthesized by *P. tricornutum*, was studied by using POME as growth medium. In the pharmaceutical field, sEPS from *P. tricornutum* is promising as anti-inflammatory, antiviral, antiparasitic, anti-tumor, and hypocholesterolemic substance. However, the production of sEPS at a large scale is limited due to the high production costs. Alternatively, the cultivation of microalgae on POME with the aim to produce sEPS could be promising, given the relatively high nutrient content of the wastewater, thereby making commercial nutrients partly or completely redundant. Furthermore, the treated, sEPS containing POME might be utilized for soil improvement in the vicinity of the palm oil plants. Microalgae tend to produce higher sEPS under stressed conditions such as excess irradiance and supra-optimal temperatures, as a means to prevent cell damage via the action of exopolymers. Other factors such as nutritional conditions and salinity were also reported to influence EPS production and composition. It was therefore hypothesized that the production of sEPS by *P. tricornutum* grown on POME would be influenced by environmental and nutritional conditions. To confirm this, a series of four step-wise experiments was done by: I: determining the optimal POME concentration for *P. tricornutum* growth, II:studying the effect of nutrient additions on *P. tricornutum* biomass and sEPS production when growing on 30% POME, III: applying BBD RSM to unravel the

optimal conditions for temperature, salinity, and urea addition, and IV: studying the effect of nutrient enrichment on nutrient removal efficiency of POME. The results showed that *P. tricornutum* is a suitable candidate for effective cultivation on 30% POME while producing sEPS at the large scale, when salinity requirements are met at 20-35 PSU. By using BBD RSM, we found that the interaction of high urea (100 mg L^{-1}) and relatively high temperature (25°C) stimulated both growth rate and sEPS production. Furthermore, the addition of urea to the POME medium was found to stimulate phosphorus removal from POME by *P. tricornutum*.

In **chapter 4**, the brackish cyanobacterium *Arthrospira platensis* was cultivated on high POME fractions after which the production of the value-added product, the pigment C-phycocyanin, was explored as a function of different cultivation modes as well as environmental and nutritional conditions. As reported earlier, a high POME fraction can be utilized by *A. platensis* when grown in continuous cultivation mode. However, continuous cultivation is not easily applicable in large scale systems. 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. To confirm this hypothesis, a stepwise approach was followed: first we investigated the interactive effect of irradiance and nitrogen concentration on *A. platensis* biomass and C-PC productivity, grown on standard growth medium. Secondly, growth and C-PC productivity of *A. platensis* were studied on different POME fractions. Then, environmental and nutritional conditions were further investigated to determine the interactive effects of light intensity, urea, salinity, and POME concentration on C-PC productivity. 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. Finally, *A. platensis* was cultured in a semi-continuous mode at varying nutrient conditions by adding urea or phosphorus, in order to unravel the impact of N:P ratio on biomass and C-PC production during semi-continuous cultivation. In this study, we found that irradiance and nitrogen concentration were the main factors driving C-PC productivity. Based on central composite design (CCD) RSM, the optimal salinity was found to be 22.5 PSU, and no inhibition was found up to 813 mg L^{-1} of urea when employing batch cultivation. By applying semi continuous cultivation with 50% POME at the first stage and 100% POME at the second stage, C-PC productivity was higher compared to batch cultivation with the addition of urea, and could reach productivity levels as high as the artificial control Zarrouk medium during batch cultivation.

In **chapter 5**, we investigated the removal of phenolic compounds and color on POME by using *A. platensis*. To the best of our knowledge, information on color and phenolic compound removal from POME using microalgae was so far lacking.

In the present study, factors affecting phenolic compound and POME color removal were considered by including photodegradation and microalgal activity by varying the initial phenol concentration in POME, irradiance, POME fractions, external nitrogen addition, and salinity. A range of control experiments (without microalgae) was done, to unravel the contribution of photodegradation to the removal processes under consideration. In this study, we found that POME fractions influenced growth rate, final biomass, absolute COD removal, and absolute color removal by the activity of *A. platensis*. Based on factorial design, salinity, nitrogen addition, and initial POME concentration did not influence total color removal. Based on CCD RSM, the addition of phenolic compounds as gallic acid in POME at high light intensity could increase the growth rate up to 0.45 d^{-1} and final biomass up to 400 g L^{-1} while on the other hand total phenolic compounds were removed almost completely (94%). Photodegradation activity contributed significantly to phenolic compound removal. However, *A. platensis* activity was higher compared to photodegradation when removing phenolic compounds at low light condition. High phenolic compound removal can thus be achieved by the combination of *A. platensis* activity and photodegration.

2. Implications and recommendations

Palm oil plays a primary role in the oil crops worldwide. It is still the most economical source of vegetable oil due to its high productivity (volume/ area/ time) and its relatively low requirement of productive land to grow compared to other crops. This industry has a huge contribution on the revenue in Indonesia as well as other countries in the region of South East Asia. However, the palm oil industry faces serious issues with respect to sustainability, including the many negative impacts to the environment.

Since 2014, more strict regulatory standards have been set for POME quality before it can be discharged into the environment. However, the POME discharge by medium to small factories, which commonly use cheap and conventional ponding systems to treat the wastewater, does not meet the regulatory standard due to the ineffectiveness of the treatment technology. Some factories mix POME with empty fruit bunch (EFB) for compost production. However, the high ratio of EFB to POME, long composting period, and land requirements to process and store it, are still not answering the problems. Other options are also available such as installing membrane filtration, evaporating POME to minimise discharge of the wastewater effluent, and digesting POME using bioreactor digesters. Among other management wastewater treatments, installing bioreactor digesters seems promising to make the industry more sustainable, since not only the pollutants can be lowered, but also the biogas produced from this system can be utilized by the factory itself.

Generally, microalgae are cultivated on wastewater to obtain carbohydrates, lipids, and proteins which are mainly focused on fuel and animal feed. However, microalgae are known to produce high value bioactive compounds, which may have different pharmaceutical and cosmetic applications. In this thesis, we have successfully grown the marine diatom *Phaeodactylum tricornutum* on POME during which the brown pigment fucoxanthin, and the byproduct sulphated exopolysaccharide, were produced. C-Phycocyanin, an antioxidant produced by *A. platensis*, was also enhanced by applying semi-continuous cultivation mode on high POME fractions. By utilizing POME as growth medium for microalgae, the cost of the bioactive compounds produced by microalgae might be reduced, since synthetic media can be replaced by suitable POME fractions. Furthermore, the treated, sEPS containing POME might be utilized for soil improvement in palm tree plantations.

Despite its high nutrient levels, the utilization of POME as growth medium for microalgae at industrial scale is still challenging. First, the high levels of organic compounds, consisting of tannins, lignin, and phenolic compounds could negatively affect growth. The dark coloration due to the high concentrations of suspended solids could inhibit light penetration, which is a critical factor for photosynthetic growth. Moreover, the presence of heterotrophic bacteria may affect biomass productivity. The pH and salinity of the wastewater needs to be adjusted before it can be used as growth medium for alkaline microalgae such as *A. platensis* or marine microalgae, such as *Phaeodactylum tricornutum*. Heavy metals contained in POME might be lowering the quality of the microalgal product, and might be harmful for food and pharmaceutical applications.

However, the conditions as mentioned above could be prevented by employing some pretreatment process to lower COD, color, and heavy metals from the POME as described previously. To increase the salinity, cultivation might be relocated to seashore areas, to allow for the utilization of natural seawater. Furthermore, the wastewater could be blended with hypersaline wastewater generated from industrial activities, such as chemical manufacturing and oil production, to make the cultivation become more feasible for marine microalgae. Finally, it should be considered that the type of cultivation system such as closed cultivation using photo-bioreactors versus open (pond) cultivation could also influence the growth, biomass, and the value-added products. It may be clear that more research must be done to optimize POME utilization for high-value product generation by microalgae. The optimization should be done based on a combination of approaches such as appropriate pretreatments, choice of species, cultivation modes, cultivation conditions, nutrient ratio's, before the valorization of POME becomes economically feasible.

3. Concluding remarks (Future outlook)

It is predicted that bioenergy and high-value biochemical production from microalgae cultivated on palm oil mill effluent become more feasible in the future if the owners implement a wastewater treatment system based on bioreactor digesters. Microalgae can be more easily cultured on the digested POME which contains lower COD and toxic substances. The cultivation can be integrated with the biogas purification system where CO_2 can be utilized for photo-autotrophic cultivation. By integrating this, a higher heating value biogas can be obtained, and high value-added pigments from microalgae can be obtained at a lower price compared to common cultivation based on commercial fertilizers. Some modifications such as nitrogen limitation and light saturation can be used to enhance some carotenoid pigments such as beta carotene and astaxanthin.

A biorefinery concept could also be applied in the future, by applying specified separation techniques to produce derivative products from microalgae. For example, a single cell of microalgae could produce lipid, protein, carbohydrate, and pigments, which can be converted to fuel, feed, polymers (bioplastics, nano carbon composite), and antioxidants (fucoxanthin, phycocyanin, astaxanthin). By implementing this concept, the production cost could be further minimized, thus lowering the price of the products.

Samenvatting

1. Samenvatting

Indonesië staat momenteel bekend als de grootste producent van ruwe palmolie ter wereld, gevolgd door Maleisië en Thailand. Tijdens de productie genereert 1 ton verse fruittrossen (FFB) 0,66 ton palmolie effluent (POME). De pH van onbewerkte POME varieert tussen 4,0 en 5,0 en de chemische zuurstofbehoefte en biologische zuurstofbehoefte zijn extreem hoog. Bovendien bevat POME een grote hoeveelheid totaal gesuspendeerde vaste stoffen, totaal opgeloste vaste stoffen en vluchtige gesuspendeerde vaste stoffen. Het is daarom duidelijk dat uitgebreide behandelingen moeten worden uitgevoerd om aan de standaardvoorschriften te voldoen voordat POME in het milieu (dwz rivieren, meren) kan worden geloosd. Om POME te behandelen, zijn verschillende mechanische, chemische of biologische methoden ontwikkeld. In vergelijking met andere technieken is biologische behandeling echter milieuvriendelijker, goedkoper en energiebesparend. In de afgelopen decennia geniet het gebruik van landbouwafval (water) met het doel om aan industriële eisen te voldoen en nuttige producten te genereren, toenemende belangstelling. Valorisatie is in dit verband het proces van het omzetten van afvalstoffen in waardevolle producten met behulp van micro-organismen zoals gisten, bacteriën en microalgen. Over het algemeen heeft het gebruik van microalgen voor de behandeling van afvalwater de voorkeur, omdat de stoffen die door deze organismen worden geproduceerd een hoge economische waarde kunnen hebben. Bij het begin van dit promotieproject was het gebruik van POME door microalgen tot dan toe vooral gericht op de productie van lipiden, bulkbiomassa en afvalwaterbehandeling: de productie van hoogwaardige verbindingen met het oog op grootschalige kweek was nog nauwelijks onderzocht. Daarom was het doel van dit promotieproject om de leemten in deze kennis op te vullen door: i) het potentieel van het kweken van verschillende microalgensoorten op POME te onderzoeken en de productie van hun bijbehorende hoogwaardige producten te volgen, ii) een reeks milieu- en nutrientencondities te onderzoeken , om het gebruik van POME als groeimedium voor belangrijke microalgen en hun bijbehorende producten te kunnen optimaliseren, iii) het potentieel van microalgenkweek te onderzoeken om de POME-kwaliteit te verbeteren door kleur- en fenolverbindingen te verwijderen. Deze onderzoeksdoelen werden vertaald in specifieke doelen en vervolgens behandeld in vier onderzoekshoofdstukken. In hoofdstuk 2 hebben we het gebruik van POME door de mariene diatomee *Phaeodactylum tricornutum* onderzocht met

als doel om de productiviteit van fucoxanthine te optimaliseren bij verschillende licht-, temperatuur- en nutriënt-omstandigheden. Fucoxanthine kan een anti-kanker, anti-obesitas en anti-diabetische werking hebben. Ik veronderstelde dat de fucoxanthine-productiviteit van *P. triconutum* gekweekt op POME afhing van (een combinatie van) lichtintensiteit, nutriëntverhoudingen, zoutgehalte en temperatuur. Eerst werden optimale lichtomstandigheden voor de fucoxanthine-productiviteit bepaald door *P. tricornutum* te kweken op kunstmatig medium. De gevonden optimale lichtintensiteit werd vervolgens toegepast in alle volgende experimenten waarbij op verschillende POME fracties werd gekweekt. Vervolgens werden de effecten van verschillende POME-fracties op de groeisnelheid en de productiviteit van fucoxanthine onderzocht en werden geselecteerde voedingsstoffen toegevoegd om de productiviteit van fucoxanthine te optimaliseren. Box-Behnken design (BBD) –response surface methodology (RSM) werd gebruikt om de optimale combinatie van omgevingscondities te vinden en om de interactie tussen zoutgehalte, temperatuur en nutriënt-concentratie met betrekking tot de fucoxanthine-productiviteit te begrijpen. Door deze stapsgewijze experimenten vonden we dat *P. tricornutum* kan worden gebruikt voor grootschalige fucoxanthine-productie door te kweken op 30% v / v POME. BBD RSM analyse liet zien dat de optimale productiviteit van fucoxanthine werd beïnvloed door temperatuur, zoutgehalte en de toevoeging van ureum. Verrijking van voedingsstoffen door fosfor verhoogde de celdichtheid en de productiviteit van fucoxanthine niet, terwijl ureumtoevoeging beide stimuleerde. Verder werden optimale omstandigheden voor ureum (85 mg L^{-1}), temperatuur (23 ° C) en zoutgehalte (22-23 PSU) bepaald op basis van een laatste experimentele serie. Sinds kort is de belangstelling voor de toepassing van exopolysacharide (EPS) uit microalgen toegenomen. In hoofdstuk 3 werd de productie van gesulfateerd exopolysacharide (sEPS), een gespecificeerd EPS-bijproduct gesynthetiseerd door *P. tricornutum*, bestudeerd met behulp van POME als groeimedium. Op farmaceutisch gebied is sEPS van *P. tricornutum* veelbelovend als ontstekingsremmende, antivirale, antiparasitaire, antitumor en hypocholesterolemische stof. De productie van grootschalige sEPS is echter beperkt vanwege de hoge productiekosten. Als alternatief kan de teelt van microalgen op POME met het doel sEPS te produceren veelbelovend zijn, gezien het relatief hoge gehalte aan voedingsstoffen in het afvalwater, waardoor commerciële voedingsstoffen geheel of gedeeltelijk overbodig worden. Verder kunnen de behandelde, sEPS bevattende POME worden gebruikt voor bodemverbetering in de buurt van de palmolieplantages. Microalgen hebben de neiging om hogere sEPS te produceren onder stressvolle omstandigheden zoals overmatige bestraling en supra-optimale temperaturen, dit als een methode om celbeschadiging te voorkomen. Andere factoren zoals nutriënten-omstandigheden

en zoutgehalte leken ook de EPS-productie en samenstelling te beïnvloeden. Daarom werd verondersteld dat de productie van sEPS door *P. tricornutum* gekweekt op POME zou worden beïnvloed door milieu- en nutriënt-omstandigheden. Om dit te bevestigen, werd een reeks van vier stapsgewijze experimenten uitgevoerd door: I: het bepalen van de optimale POME-concentratie voor de groei van *P. tricornutum*, II: het bestuderen van het effect van nutriënt-toevoegingen op de biomassa van *P. tricornutum* en de productie van sEPS bij groei op 30% POME, III: toepassing van BBD RSM om de optimale omstandigheden voor temperatuur, zoutgehalte en ureumtoevoeging te ontrafelen, en IV: onderzoek naar het effect van nutriënt-verrijking op de efficiëntie van de verwijdering van voedingsstoffen uit POME. De resultaten toonden aan dat *P. tricornutum* een geschikte kandidaat is voor effectieve sEPS productie op 30% POME en een zoutgehalte tussen 20-35 PSU. Met behulp van BBD RSM hebben we vastgesteld dat de interactie tussen hoog ureum (100 mg L^{-1}) en relatief hoge temperaturen (25 ° C) zowel de groeisnelheid als de sEPS-productie stimuleerde. Bovendien bleek de toevoeging van ureum aan het POME-medium de fosforverwijdering uit POME door *P. tricornutum* te stimuleren. In hoofdstuk 4 werd de brakwater cyanobacterie *Arthrospira platensis* gekweekt op hoge POME-fracties waarna de productie van het pigment C-phycocyanine (C-PC) werd onderzocht als een functie van verschillende kweekomstandigheden. Zoals eerder onderzoek had aangetoond kan een hoge POME-fractie door *A. platensis* worden gebruikt wanneer deze wordt gekweekt in continue kweekmodus. Continue teelt is echter niet gemakkelijk toepasbaar in grootschalige systemen. Voor de huidige experimenten werd daarom verondersteld dat semi-continue kweek de biomassa en C-PC-productiviteit van *A. platensis* gekweekt op POME-medium zou kunnen verbeteren na het optimaliseren van nutriënt- en andere omgevingscondities. Om deze hypothese te bevestigen, werd een stapsgewijze aanpak gevolgd: eerst onderzochten we het interactieve effect van lichtintensiteit en stikstofconcentratie op de biomassa en C-PC-productiviteit van *A. platensis*-, wanneer deze werd gekweekt op artificieel groeimedium. Ten tweede werden groei en C-PC-productiviteit van *A. platensis* onderzocht op verschillende POME-fracties. Vervolgens werden de omgevings- en nutriënt-omstandigheden verder onderzocht door de interactieve effecten van lichtintensiteit, ureum, zoutgehalte en POME-concentratie op de productiviteit van C-PC te bepalen. De optimale ureumconcentratie als veelbelovende stikstofbron werd onderzocht in verband met mogelijke toxische effecten bij hogere ureumconcentraties. Bovendien werd het zoutgehalte geoptimaliseerd omdat POME een relatief laag zoutgehalte bevat. Ten slotte werd *A. platensis* gekweekt in een semi-continue modus bij verschillende nutriënt-omstandigheden door ureum of fosfor toe te voegen, om de impact van de N: P-verhouding op biomassa en C-PC-productie tijdens semi-continue teelt te ontrafelen. In deze studie hebben we vastgesteld dat

lichtintensiteit en stikstofconcentratie de belangrijkste factoren zijn voor de productie van C-PC. Gebaseerd op CCD-RSM bleek het optimale zoutgehalte 22,5 PSU te zijn en werd geen remming gevonden tot 813 mg L^{-1} ureum bij toepassing van batchkweek. Door het toepassen van semi-continue teelt met 50% POME in de eerste fase en 100% POME in de tweede fase was de C-PC-productiviteit hoger in vergelijking met batchkweek met de toevoeging van ureum, en kon een productiviteitsniveau worden bereikt dat even hoog was als het kunstmatige controle-medium Zarrouk. In hoofdstuk 5 hebben we de verwijdering van fenolverbindingen en kleur van POME onderzocht met behulp van *A. platensis*. Voor zover ons bekend ontbrak tot dan toe informatie over de verwijdering van kleur en fenolverbindingen uit POME met behulp van microalgen. In de huidige studie werden factoren die de fenol- en kleurverwijdering beïnvloeden, bestudeerd waarbij ook het mogelijk belangrijke proces van fotodegradatie werd meegenomen. Hierbij werd de initiële fenolconcentratie in POME, lichtintensiteit, POME-fracties, externe stikstoftoevoeging en zoutgehalte gevarieerd. Een reeks controle-experimenten (zonder microalgen) werd uitgevoerd om de bijdrage van fotodegradatie te ontrafelen. In deze studie vonden we dat POME-fracties de groeisnelheid, uiteindelijke biomassa, absolute fenol- en kleurverwijdering door *A. platensis* beïnvloeden. Gebaseerd op Factorial Design werd gevonden dat zoutgehalte, stikstoftoevoeging en initiële POME-concentratie geen invloed hebben op de totale kleurverwijdering. Gebaseerd op CCD RSM zou de toevoeging van fenolverbindingen als gallic-zuur in POME bij hoge lichtintensiteit de groeisnelheid tot 0,45 d^{-1} en de uiteindelijke biomassa tot 400 g L^{-1} kunnen verhogen, terwijl aan de andere kant de totale fenolverbindingen bijna volledig (94%) werden verwijderd. Verder droeg fotodegradatie-activiteit aanzienlijk bij aan de verwijdering van fenolverbindingen. De activiteit van *A. platensis* was echter relatief hoger ten opzichte van fotodegradatie bij lage lichtintensiteiten. Een hoge verwijdering van fenolverbindingen kan dus worden bereikt door de combinatie van *A. platensis*-activiteit en fotodegradatie.

2. Implicaties en aanbevelingen

Palmolie speelt een primaire rol in de wereldwijde productie van plantaardige olie. In vergelijking met andere gewassen is het nog steeds de meest economische bron van plantaardige olie vanwege de hoge productiviteit (volume / oppervlakte / tijd) en de relatief lage behoefte aan productief land. De palmolie-industrie levert een enorme bijdrage aan de omzet in Indonesië en andere landen in Zuidoost-Azië. De palmolie-industrie genereert echter ernstige problemen met betrekking tot duurzaamheid, waaronder de vele negatieve gevolgen voor het milieu. Sinds 2014 zijn er strengere wettelijke normen vastgesteld voor POME-kwaliteit voordat deze in het milieu kan worden geloosd. De POME-lozing door kleine tot middelgrote

fabrieken, die gewoonlijk goedkope en conventionele vijverinstallaties gebruiken om het afvalwater te behandelen, voldoet echter niet aan de wettelijke norm vanwege de ineffectiviteit van de behandel-technologie. Sommige fabrieken mengen POME met lege fruittrossen (EFB) voor de productie van compost. Het hoge gehalte van EFB ten opzichte van POME, de lange composteringsperiode en de landvereisten om het te verwerken en op te slaan, lossen echter nog steeds de problemen niet op. Er zijn ook andere opties beschikbaar, zoals het installeren van membraanfiltratie, het verdampen van POME om de afvoer van het afvalwaterafval te minimaliseren en het vergisten van POME met behulp van bioreactor-vergisters. In vergelijking met andere afvalwaterbehandelingen lijkt het installeren van bioreactorvergisters veelbelovend om de industrie duurzamer te maken, omdat niet alleen de hoeveelheid verontreinigende stoffen kan worden verlaagd, maar ook omdat het biogas dat uit dit systeem wordt geproduceerd door de fabriek zelf kan worden gebruikt. Over het algemeen worden microalgen gekweekt op afvalwater om koolhydraten, lipiden en eiwitten te verkrijgen ten behoeve van brandstof en veevoer. Het is echter bekend dat microalgen hoogwaardige bioactieve verbindingen produceren, die verschillende farmaceutische en cosmetische toepassingen kunnen hebben. In dit proefschrift hebben we met succes de mariene diatomee *Phaeodactylum tricornutum* op POME gekweekt, waarbij het bruine pigment fucoxanthine en het bijproduct gesulfateerde exopolysacharide werden geproduceerd. De productie van C-Phycocyanin, een antioxidant geproduceerd door *A. platensis*, werd ook geoptimaliseerd door het toepassen van een semi-continue kweekmodus bij gebruik van hoge POME-gehaltes. Door POME te gebruiken als groeimedium voor microalgen, kunnen de kosten van de bioactieve verbindingen die door microalgen worden geproduceerd, worden verlaagd, omdat synthetische media kunnen worden vervangen door geschikte POME-fracties. Bovendien kan de behandelde, sEPS bevattende POME worden gebruikt voor bodemverbetering in palmboomplantages. Ondanks de hoge nutriëntenniveaus is het gebruik van POME als groeimedium voor microalgen op industriële schaal nog steeds een uitdaging. Ten eerste kunnen de hoge niveaus van organische verbindingen, bestaande uit tannines, lignine en fenol-verbindingen de groei negatief beïnvloeden. De donkere kleur als gevolg van de hoge concentraties gesuspendeerde vaste stoffen kan de lichtpenetratie remmen, wat een kritische factor is voor autotrofe, fotosynthetische groei. Bovendien kan de aanwezigheid van heterotrofe bacteriën de productiviteit beïnvloeden. De pH en het zoutgehalte van het afvalwater moeten worden aangepast voordat het kan worden gebruikt als groeimedium voor alkalische microalgen zoals *A. platensis* of mariene microalgen, zoals *Phaeodactylum tricornutum*. Zware metalen in POME kunnen de kwaliteit van het microalgenproduct verlagen en kunnen schadelijk zijn voor voedsel- en farmaceutische toepassingen. De hierboven genoemde omstandigheden kunnen

echter worden voorkomen door een voorbehandelingsproces toe te passen om fenolen, kleur en zware metalen in de POME te verlagen. Om het zoutgehalte te verhogen, kan de teelt worden verplaatst naar kustgebieden, om het gebruik van natuurlijk zeewater mogelijk te maken. Bovendien zou het afvalwater kunnen worden gemengd met hypersalien afvalwater zoals dat wordt gegenereerd door industriële activiteiten, zoals chemische productie en olieproductie. Ten slotte moet worden meegenomen dat het type kweeksysteem, zoals gesloten teelt met foto-bioreactoren versus open (vijver) teelt ook de groei, biomassa en het gehalte van hoogwaardige producten kan beïnvloeden. Het moge duidelijk zijn dat meer onderzoek nodig is om het gebruik van POME te optimaliseren voor de productie van hoogwaardige stoffen door microalgen. De optimalisatie moet worden gedaan op basis van een combinatie van benaderingen zoals geschikte voorbehandelingen, keuze van soorten, kweekwijzen, kweekomstandigheden, of nutriënt-verhoudingen, voordat de valorisatie van POME economisch haalbaar wordt.

3. Slotopmerkingen (toekomstperspectief)

Er wordt voorspeld dat bio-energie en hoogwaardige biochemische productie door microalgen gekweekt op POME in de toekomst haalbaarder wordt wanneer de plantage-eigenaren een afvalwaterbehandelingssysteem op basis van bioreactor-vergisters implementeren. Microalgen kunnen gemakkelijker worden gekweekt op de verteerde POME die minder fenolen en giftige stoffen bevatten. De kweek kan worden geïntegreerd met het biogaszuiveringssysteem waarbij CO_2 kan worden gebruikt voor foto-autotrofe kweek. Door dit te integreren, kan biogas met een hogere stookwaarde worden verkregen en kunnen pigmenten met een hoge toegevoegde waarde uit microalgen worden verkregen tegen een lagere prijs in vergelijking met gewone kweek op basis van commerciële meststoffen. Sommige modificaties zoals stikstofbeperking en lichtverzadiging kunnen worden gebruikt om sommige carotenoïde pigmenten zoals bètacaroteen en astaxanthine te verbeteren. Een bioraffinage-concept zou in de toekomst ook kunnen worden toegepast, door specifieke scheidingstechnieken toe te passen om afgeleide producten van microalgen te produceren. Een enkele microalgen-cel kan bijvoorbeeld lipiden, eiwitten, koolhydraten en pigmenten produceren, die kunnen worden omgezet in brandstof, voer, polymeren (bioplastics, nanokoolstofcomposiet) en antioxidanten (fucoxanthine, phycocyanine, astaxanthine). Door dit concept te implementeren, kunnen de productiekosten verder worden geminimaliseerd, waardoor de prijs van de producten wordt verlaagd.

Ringkasan

1. Ringkasan

Akhir-akhir ini Indonesia dikenal sebagai produsen minyak kelapa sawit terbesar di dunia, diikuti oleh Malaysia dan Thailand. Selama proses produksinya, sekitar 1 ton tandan buah segar (TBS) menghasilkan 0.66 ton limbah cair kelapa sawit (LCKS). Nilai pH dari LCKS curah berkisar 4,0 – 5,0, sementara nilai *chemical oxygen demand* (COD) dan *biological oxygen demand* (BOD) sangat tinggi. Di samping itu, LCKS mengandung nilai total padatan tersuspensi (TSS), total padatan terlarut (TDS) dan padatan tersuspensi volatile (VSS) yang tinggi. Sehingga diperlukan penanganan khusus untuk memenuhi standar baku limbah sebelum LCKS dibuang ke lingkungan. Untuk mengolah limbah ini, beberapa metode kimia, mekanik, dan biologis telah dikembangkan. Namun, jika dibandingkan dengan metode lain, metode biologis lebih ramah lingkungan, lebih murah, dan efisien dari sisi energi.

Selama beberapa dekade, terdapat kecenderungan pemanfaatan limbah cair perkebunan untuk menghasilkan nilai produk yang memiliki ekonomis yang tinggi. Valorisasi adalah proses pemanfaatan material bahan sisa / limbah menjadi produk bernilai tinggi dengan memanfaatkan yeast, bakteri ataupun mikroalga. Secara umum, pemanfaatan mikroalga untuk mengolah limbah cair lebih menguntungkan karena produk mikroalga yang dihasilkan memiliki nilai ekonomis yang lebih tinggi dibanding yeast atau bakteri. Pada awal project studi PhD, pemanfaatan LCKS untuk media tumbuh mikroalga lebih banyak ditekankan pada biomassa, lipid, dan limbah cair kelapa sawit. Fokus pada produk bernilai tinggi dengan orientasi skala massal masih minim. Sehingga, tujuan dari thesis ini adalah mengisi kekosongan dari pengetahuan ini dengan i) menginvestigasi potensi pertumbuhan beberapa jenis mikroalga pada LCKS dan memonitor produksi produk bernilai tinggi, ii) menginvestigasi beberapa parameter yang berkaitan dengan kondisi lingkungan dan nutrisi dan hubungannya, serta mengoptimasi pemanfaatan LCKS sebagai media tumbuh mikroalga dan produk bernilai tinggi dari mikroalga, iii) menginvestigasi potensi kultivasi mikroalga untuk meningkatkan kualitas LCKS melalui pendekatan warna limbah dan kandungan total fenol. Tujuan-tujuan dari penelitian ini dijabarkan lebih lanjut menjadi beberapa tujuan yang lebih spesifik dalam empat bab.

Pada bab 2, kami mengeksplore pemanfaatan LCKS sebagai media tumbuh diatom air laut, *Phaeodactylum tricornutum*, dan menginvestigasi variasi kondisi lingkungan untuk mengoptimalkan produktivitas fucoxanthin pada penyinaran, temperatur, dan kondisi nutrisi yang berbeda. Fucoxanthin yang diproduksi oleh

diatom dapat menghambat kanker, obesitas, dan diabetes. Dalam hipotesis kami, produktivitas fucoxanthin dari *P. tricornutum* yang dibiakkan dalam LCKS tergantung pada kombinasi penyinaran, rasio nutrisi, salinitas, dan temperatur. Pada eksperimen pertama, telah ditentukan level penyinaran pada kondisi optimum di mana diatom dibiakkan pada media sintesis. Hasil penyinaran optimal ini nantinya akan diaplikasikan pada semua eksperimen yang berkaitan dengan pemanfaatan LCKS. Pada eksperimen selanjutnya, fraksi LCKS dan penambahan nutrisi divariasi guna mengetahui laju pertumbuhan dan produktivitas fucoxanthin yang paling optimal. Box-behnken design (BBD) response surface methodology (RSM) digunakan untuk mengoptimasi kombinasi antar kondisi lingkungan dan mengetahui interaksi antara salinitas, temperatur, dan konsentrasi nutrisi pada produktivitas fucoxanthin. Melalui eksperimen ini, kami menemukan bahwa *P. tricornutum* dapat digunakan untuk produksi fucoxanthin skala massal pada 30% LCKS. Dari hasil perhitungan BBD RSM, produktivitas fucoxanthin dipengaruhi oleh temperatur, salinitas, dan penambahan urea. Penambahan fosfor tidak meningkatkan densitas sel dan produktivitas fucoxanthin, sementara penambahan urea mempengaruhi densitas sel dan fucoxanthin. Lebih jauh, kondisi optimal dari produktivitas yakni 85 mg L^{-1}, temperatur 23°C dan salinitas 22-23 PSU.

Pada bab tiga, produksi *sulfated exopolysaccharide* (sEPS), produk samping spesifik yang diproduksi *P. tricornutum*, dipelajari dengan memanfaatkan LCKS sebagai media tumbuh. Pada bidang farmasi, sEPS dari *P. tricornutum* merupakan produk anti pendarahan, antivirus, anti parasit, antitumor, dan hypo kolesterol. Namun, produksi sEPS pada skala besar sangat terbatas karena biaya produksinya yang tinggi. Alternatifnya, kultur mikroalga dalam LCKS untuk produksi sEPS menjanjikan untuk diterapkan karena dapat menurunkan biaya produksi. Lebih lanjut, LCKS yang digunakan sebagai media kultur mikroalga, dapat dimanfaatkan untuk meningkatkan kualitas tanah di perkebunan kelapa sawit karena mengandung sEPS. Mikroalga cenderung memproduksi sEPS pada kondisi stress seperti radiasi berlebih dan suhu yang tinggi sebagai upaya mikroalga menghambat kerusakan sel. Faktor lain seperti kondisi nutrisi dan salinitas juga dilaporkan mempengaruhi produksi sEPS. Sehingga diduga bahwa produksi sEPS oleh *P. tricornutum* yang dibiakkan dalam LCKS akan dipengaruhi oleh kondisi lingkungan dan nutrisi. Untuk mengkonfirmasi ini, empat tahap eksperimen telah dilakukan, yakni i) menentukan konsentrasi LCKS optimal untuk pertumbuhan *P. tricornutum*. ii) mempelajari efek penambahan nutrisi terhadap biomassa *P. tricornutum* dan sEPS ketika dibiakkan dalam 30% LCKS, iii) mengaplikasikan BBD RSM untuk mengetahui kondisi optimal dari temperatur, salinitas, dan penambahan urea, serta iv) mempelajari efek penambahan nutrisi terhadap efisiensi penurunan polutan dalam LCKS. Hasil dari penelitian ini menunjukkan bahwa *P. tricornutum* merupakan kandidat yang cocok dalam memproduksi sEPS skala besar dengan memanfaatkan 30% LCKS. Dengan

menggunakan BBD RSM, kami menemukan bahwa interaksi urea tinggi (100 mg L^{-1}) dan temperatur tinggi (25 °C) dapat meningkatkan laju pertumbuhan dan produksi sEPS. Lebih jauh, penambahan urea ke dalam LCKS dapat menurunkan fosfor dengan bantuan *P. tricornutum*.

Pada bab empat, cyanobacterium air payau, *Arthrospira platensis*, dikultur dalam LCKS fraksi tinggi dengan tujuan memperoleh produk pigmen C-phycocyanin yang tinggi yang dipengaruhi oleh metode kultivasi, kondisi lingkungan, dan nutrisi. Seperti yang dilaporkan sebelumnya, LCKS fraksi tinggi dapat digunakan oleh *A. platensis* jika menggunakan mode kultivasi kontinyu. Namun demikian, mode kultivasi tersebut relatif lebih sukar diaplikasikan dalam skala besar. Pada studi ini kami menduga bahwa dengan menggunakan mode kultivasi semi-kontinyu, produktivitas biomassa dan C-phycocyanin dari *A. platensis* menjadi lebih tinggi jika dibiakkan dalam LCKS fraksi tinggi pada kondisi lingkungan dan nutrisi optimum. Untuk mengkonfirmasi hipotesis ini, pendekatan penelitian terstruktur telah dilakukan: pertama, kami menginvestigasi efek interaksi penyinaran dan konsentrasi nitrogen terhadap produktivitas biomassa dan C-PC, dengan menggunakan media standar. Kedua, dilakukan studi tentang laju pertumbuhan dan produktivitas C-PC dari *A. platensis* yang dibiakkan dalam LCKS dengan fraksi yang berbeda. Kemudian, kondisi nutrisi dan lingkungan diinvestigasi lebih lanjut untuk menentukan interaksi antara penyinaran, urea, salinitas, dan konsentrasi LCKS terhadap produktivitas C-PC. Penambahan urea juga dioptimasi seiring kemungkinan terbentuknya racun pada konsentrasi tinggi. Salinitas juga dipotimasi karena LCKS relatif mengandung nilai salinitas yang rendah. Pada eksperimen terakhir, *A. platensis* dikultur dengan menggunakan mode semi-kontinyu dengan mengubah kondisi nutrisi. Pada penelitian ini kami menemukan bahwa penyinaran dan konsentrasi nitrogen adalah faktor utama dalam produktivitas C-PC. Berdasarkan central composite design (CCD) RSM, salinitas optimal diperoleh pada 22.5 PSU, tidak ada inhibisi dengan penambahan urea sampai 813 mg L^{-1} jika menggunakan mode batch. Dengan menggunakan semi-kontinyu, di mana pada stage pertama digunakan 50% LCKS dan 100% POME pada stage kedua, produktivitas C-PC menjadi lebih tinggi jika dibandingkan dengan mode batch dengan penambahan urea, dan hampir sama dengan menggunakan media Zarrouk pada mode batch.

Pada bab lima, kami menginvestigasi pengurangan kandungan fenol dan warna dari LCKS menggunakan *A. platensis*. Berdasarkan pengetahuan kami, informasi mengenai penghilangan warna dan fenol dalam LCKS menggunakan mikroalga sangatlah minim. Pada studi ini, faktor-faktor yang mempengaruhi pengurangan warna dan fenol dari LCKS diinvestigasi, termasuk pengaruhi fotodegradasi dan aktivitas mikroalga dengan mengubah variasi konsentrasi fenol awal dari LCKS, penyinaran, fraksi LCKS, penambahan nitrogen eksternal, dan salinitas. Eksperimen kontrol tanpa mikroalga juga telah dilakukan untuk mengetahui

kontribusi fotodegradasi terhadap mengurangan warna dan fenol dalam LCKS. Dari eksperimen ini, kami menemukan bahwa fraksi LCKS mempengaruhi laju pertumbuhan, biomassa akhir, pengurangan COD absolute akhir, dan warna absolute dengan aktivitas *A. platensis*. Berdasarkan factorial design, salinitas, penambahan nitrogen, dan konsentrasi awal LCKS tidak mempengaruhi perubahan warna dari LCKS. Berdasarkan CCD RSM, nilai awal fenol dari LCKS pada penyinaran tinggi dapat meningkatkan laju pertumbuhan sampai 0.45 d^{-1} dan biomassa akhir 400 mg L^{-1}, sementara fenol dapat dihilangkan dengan efisiensi 94%. Aktivitas fotodegradasi berkontribusi secara signifitkan terhadap pengurangan fenol dalam LCKS. Namun, aktivitas *A. platensis* untuk mengurangi fenol hanya tinggi jika penyinaran dalam kondisi rendah. Pengurangan kandungan fenol dalam LCKS dapat dimaksimalkan dengan menggunakan kombinasi aktivitas *A. platensis* dan fotodegradasi.

2. Implikasi dan rekomendasi

Minyak kelapa sawit memiliki peran penting dalam industri makanan secara global. Minyak kelapa sawit merupakan minyak dengan nilai ekonomis tertinggi karena produktivitasnya, membutuhkan lahan yang lebih minim, dibanding tumbuhan penghasil minyak lain. Industri perkebunan kelapa sawit memiliki kontribusi sangat besar terhadap pendapatan penduduk di Indonesia, dan juga negara di Asia Tenggara lainnya. Namun, industri ini memiliki beberapa isu yang berkaitan dengan keberlanjutannya, termasuk imbas negatif terhadap lingkungan.

Sejak 2014, baku mutu limbah cair kelapa sawit yang lebih ketat telah dikeluarkan oleh pemerintah untuk mengurangi dampak polusi lingkungan dari industri kelapa sawit. Namun, beberapa industri skala kecil dan menengah masih menggunakan metode pengolahan limbah yang murah dan konvensional, sehingga parameter LCKS tidak memenuhi standar yang telah ditetapkan. Beberapa industri perkebunan kelapa sawit telah memanfaatkan LCKS dengan tandan buah kosong (TBK) untuk produksi pupuk kompos. Namun, volume LCKS yang terlalu besar tidak sebanding dengan tandan buah kosong yang kecil. Selain itu lamanya waktu pembuatan kompos, dan kebutuhan lahan, masih menjadi kendala dalam pemanfaatan LCKS ini. Beberapa pilihan lain dalam mengolahan limbah ini antara lain dengan menggunakan membran filtrasi, evaporasi, dan digestasi menggunakan digester anaerob. Dari beberapa pilihan itu, pengolahan LCKS dengan digester cukup menjanjikan karena selain baku mutu limbah yang lebih baik, juga diperoleh biogas yang dapat dimanfaatkan di industri pengolahan kelapa sawit.

Secara umum, mikroalga dibiakkan dalam limbah cair untuk mendapatkan produk karbohidrat, lipid, dan protein, dengan fokus energi dan pakan. Namun, mikroalga dikenal sebagai produsen komponen bioaktif dengan nilai ekonomis yang tinggi dengan aplikasi yang luas. Dalam thesis ini, kami telah berhasil

mengembangkan diatom air laut, P. tricornutum, dalam LCKS untuk memproduksi pigmen fucoxanthin dan produk samping sulfated exopolysaccharida. Produksi C-phycocyanin, antioksidan yang diproduksi dari *A. platensis*, juga dapat ditingkatkan dengan menggunakan mode semi-kontinyu pada konsentrasi LCKS yang tinggi. Dengan memanfaatkan LCKS sebagai media kultur mikroalga, biaya produksi bioaktif dari mikroalga dapat diturunkan karena media sintesis dapat diganti dengan fraksi LCKS. Lebih lanjut, LCKS yang telah digunakan sebagai media kultur P. tricornutum, juga mengandung sEPS yang berguna untuk meningkatkan mutu tanah di area penanaman kelapa sawit.

Pemanfaatan LCKS untuk media tumbuh mikroalga masih memiliki beberapa kendala. Pertama, kandungan organik dalam LCKS seperti tanin, lignin, dan fenol dapat berdampak negatif terhadap laju pertumbuhan mikroalga. Warna gelap dari LCKS dapat menghambat penetrasi cahaya, yang sangat penting dalam pertumbuhan mikroalga. Lebih lanjut, keberadaan bakteri dapat mempengaruhi produktivitas biomassa dari mikroalga. pH dan salinitas dari LCKS juga perlu diatur sebelum dapat dimanfaatkan sebagai media tumbuh mikroalga jenis tertentu seperti *A. platensis* dan *P. tricornutum.* Logam berat yang terkandung di dalam LCKS juga dapat menurunkan kualitas produk sehingga kurang aman dikonsumsi dan digunakan sebagai produk farmasi.

Namun demikian, beberapa kondisi yang telah disebutkan dapat ditanggulangi dengan menggunakan beberapa pendekatan preventif seperti penurunan kandungan COD, warna, logam berat, yang telah dijelaskan pada penelitian pendahulu. Untuk meningkatkan salinitas, kultivasi dapat direlokasi di area yang berdekatan dengan laut. Dan juga limbah LCKS dapat diblending dengan limbah industri yang menghasilkan limbah cair dengan kandungan garam tinggi seperti industri kimia. Lebih jauh lagi, perlu ditentukan tipe kultivasi yang sesuai seperti kultur terbuka atau tertutup. Jelas masih banyak pekerjaan yang perlu dilakukan untuk mengoptimasi produk bernilai tinggi dari mikroalga yang dibiakkan dalam LCKS. Optimisasi dapat dilakukan dengan mengkombinasikan beberapa parameter seperti pretreatment, pemilihan spesies, mode kultur, kondisi kultur, rasio nutrisi, sehingga volarisasi LCKS menjadi benar-benar fisibel.

3. Kesimpulan

Dapat diprediksi bahwa produk berbasis bioenergi dan biokimia dengan nilai ekonomis yang tinggi dari mikroalga yang dibiakkan dalam LCKS akan semakin fisibel di masa depan jika para pengelola industri kelapa sawit mengimplementasikan pengolahan limbah cair menggunakan sistem biodigester. Mikroalga akan lebih mudah dibiakkan dalam LCKS yang telah diolah dengan digester, di mana kandungan COD dan toksiknya menjadi lebih rendah. Kultivasi dapat diintegrasikan dengan purifikasi biogas di mana CO_2 dapat dimanfaatkan oleh mikroalga pada kondisi

autotrofik. Dengan mengintegrasikan ini, biogas dengan heating value yang lebih tinggi akan diperoleh, dan pigmen dengan nilai ekonomis yang lebih tinggi dapat dicapai dengan biaya yang lebih murah dibanding dengan kultur berbasis pupuk komersial. Beberapa modifikasi seperti pembatasan nitrogen dan pencahayaan jenuh dapat digunakan untuk meningkatkan pigmen karotenoid seperti beta karoten dan astaxanthin yang memiliki nilai komersial tinggi.

Konsep biorefinery juga mungkin dapat digunakan ke depan, dengan mengimplementasikan teknik separasi tertentu guna menghasilkan produk turunan dari mikroalga. Sebagai contoh, dari satu sel mikroalga, dapat menghasilkan lipid, protein, karbohidrat, dan pigment, di mana produk ini dapat diolah menjadi bahan bakar nabati, pakan, polimer (bioplastik, nano karbon), dan antioksidan. Dengan mengimplementasikan konsep ini, biaya produksi dapat lebih diturunkan lagi, sehingga menurunkan harga produk.

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Pigment mixture, painted by Azkiya Syauqina Azim

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About the author

M.M. Azimatun Nur was born in Semarang, Jawa Tengah, Indonesia. After graduating from a bachelor's degree from chemical engineering UNDIP Semarang, he involved in a project dealing with palm oil mill effluent for *Spirulina* growth at large scale cultivation, in Lampung,Sumatra, with a collaboration between some institutions in The Netherland and Indonesia. During his master's degree, he was working in the center of biomass and renewable energy, UNDIP, for three years with his main project in the utilization of palm oil mill effluent as medium growth of microalgae for value-added products. He obtained fundings from Lembaga Pengelola Dana Pendidikan (LPDP) Pertamina Foundation for the project. After graduating from his master's degree, he works in the chemical engineering department, Universitas Pembangunan Nasional Veteran Yogyakarta. In the late of 2015, he obtained a scholarship from LPDP for his Ph.D. study at The University of Groningen. After finishing his Ph.D., he will back to Indonesia and starting his career as a researcher and lecturer in microalgal applications.

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