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Lipid production by microalgae *Chlorella pyrenoidosa* cultivated in palm oil mill effluent (POME) using hybrid photo bioreactor (HPBR)

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ABSTRACT

Palm oil mill effluent (POME) as high organic wastewater is a promising substrate in the scenario of algae bloom, by enhancing its lipid production to be further used in biofuel manufacturing. In this research, effect of POME as high nutritional substrate, different cultivation scales such as flask or hybrid photo bioreactor (HPBR), carbon-to-total nitrogen (C:TN) ratio, various light and dark cycles, and diverse organic loading rates (OLR) on the lipid productivity of microalgae *Chlorella pyrenoidosa* was assessed. Results demonstrated high microalgae growth rate (1.80 d^{-1}) at 250 mg COD/L of substrate, while moderate increase (1.37 d^{-1}) and growth inhibition (0.80 d^{-1}) were recorded at 500 mg COD/L and 1,000 mg COD/L of substrate concentration, respectively. Furthermore, a result proved that low-volume cultivation of microalgae in a flask with lipid productivity at 1.78 mg/L d significantly restricted microalgae production compared with larger scale such as HPBR with lipid productivity at 230 mg/L d. Moreover, highest lipid production at 44.5, 114.9, and 100.5 mg/L d, C:TN ratio at 100:6 and OLR at 36 kg COD/m^3 d, respectively, were documented for continuous illuminaion (24 h). The combination of above conditions can be optimal setting to reach the highest lipid productivity by microalgae *C. pyrenoidosa*. In addition, the results of this study can be further considered in microalgae lipid production using other wastewaters in order to enhance the lipid production as well as wastewater treating functions.

Keywords: *Chlorella pyrenoidosa*; Hybrid photo bioreactor (HPBR); Lipid; Microalgae; Palm oil mill effluent (POME)

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1. Introduction

Bioenergy from waste is recognized as an important aspect to mitigate the demand of conventional petroleum, such as oil fuel from hydrocarbon. Currently, the prices hike a great tremendous effect to the global economy making a disastrous impact on many countries. The subsidized petrol and diesel for the country has increased continuously because they solely dependent on the transportation, energy consumption, and industrialization. Continuous use of petroleum source fuel is now widely recognized as unsustainable to environment since it can accelerate the climatic changes. Besides, the major disadvantage of using petroleum-based fuel is atmospheric pollution due to incomplete burning processes, as well as generation of by-products from secondary pollution (e.g. CO_2 and H_2S from nature). Many researchers focus on developing alternative energy resources such as solar, wind, hybrid electricity, and biomass conversions. The study focuses on culturing the substrate media from palm oil mill effluent (POME) as a source of fatty acid. The selection of algae as a sole microorganism growth has emerged as one of the most promising sources for biodiesel (alternative energy) production. In addition, palm oil industry is growing rapidly among potential tropical countries, especially in Malaysia [1]. It was estimated that for each tonne of crude palm oil produced, 5–7.5 tonnes of water is required, and more than 50% of the water will end up as POME. The wastes are in the form of high organic matters concentration, such as cellulosic wastes with a mixture of carbohydrates and oils. As a consequence, discharge of untreated POME creates adverse impact on the environment [1]. Compared to the conventional wastewater treatment process, which introduces activated sludge, biological floc, to degrade organic carbonaceous matter to CO_2, algae can assimilate organic pollutants into cellular constituents such as lipid and carbohydrate, thus achieving pollutant reduction in a more environmental friendly way. In fact, microalgae have become the focus of attention for both wastewater treatment and biomass production as early as the 1950s [2]. Microalgae are generally photosynthetic, eukaryotic microorganisms in the form of unicellular or multicellular that can grow well in aquatic environment but are also found on the surface of all types of soils. Their simple structures enabled them to have high growth rates and photosynthetic efficiencies [3]. Microalgae capture light energy and the energy is used for cell synthesis, and convert inorganic molecule into simpler sugar as a source of energy for cell metabolism [4]. While the mechanism of photosynthesis in microalgae is similar to that of higher plants, they are generally more efficient converters of solar energy because of their simple cellular structure. They were categorized into 11 divisions as follows: Cyanophyta, Prochlorophyta, Glaucophyta, Rhodophyta, Heterokonta, Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorarachniophyta, and Chlorophyta. The largest groups are Chlorophyceae (green algae), Phaeophyceae (brown algae), Pyrrophyceae (dinoflagellates), Chrysophyceae (golden-brown algae), Bacillariophyceae (diatoms), and Rhodophyceae (red algae) [4]. Photoautotrophic [5–9], Heterotrophic [5,6,8,10–13], Mixotrophic [9], and Photo-heterotrophic [5] are various microalgae cultivation methods used by several researchers.

Many studies have shown that microalgae are able to remove nitrogen and phosphorus from wastewater [14]. Microalgae grow rapidly; they are able to divide once every 3–4 h, but mostly divide every 1–2 d under favorable growing conditions [6,15]. The average lipid content of algal cells varies between 1 and 70%, but can reach 90% of dry weight under certain conditions [16]. Moreover, view of factors such as light, temperature, pH, salinity, irradiance, and, most markedly, nutrient availability such as carbon [17–20], nitrogen [21,22], phosphorus [23,24], and metal elements [25–27] has been shown to affect both lipids composition and lipids content in many microalgae [28]. Owing to the diminishing of petroleum reserves and environmental consequences of exhausted gases from fossil diesel, seeking for lipid-rich biological materials to produce biodiesel effectively has attracted much renewed interest. Much interest was on the development of an integrated algae oil and wastewater treatment process. This process would simultaneously create algae feedstock for biogas or biodiesel and remove nutrients from wastewater. However, POME has an extremely high content of degradable organic matter due to the presence of uncovered palm oil [29]. The discharge of improperly treated POME though has adverse impacts on the environment. However, the substances in POME were able to support the growth of microbial as well as microalgae. Microalgae naturally exist in many palm oil mill processes, phenomena known as algae bloom, hence declining water quality. The study provides an alternative way to reduce the pollution load (chemical oxygen demand [COD] basis) due to the carbon uptake to produce lipid. In addition, a biological process was developed to enhance the lipid production from microalgae in POME. Hence, screening and isolating the dominant strains of microalgae in collected POME from algae pond along with the determination of selected operational condition, which are pretested from the

carbon-to-nitrogen (C/N) ratio, photo periods, and organic loading rate (OLR), which preferably maximize both lipid and biomass production of microalgae, were performed thoroughly. Moreover, the suitability of lipid production from microalgae in a fabricated hybrid photo-bioreactor (HPBR) was assessed.

2. Materials and method

2.1. Microalgae cultivation

Microalgae *Chlorella pyrenoidosa* was cultivated in 0, 250, 500, and 1,000 mg COD/L of POME in flask scale.

2.2. Synthetic culture preparation of modified Bold's Basal Medium

Microalgae *C. pyrenoidosa*, which was isolated from algae pond in Kulai Besar, was cultured in modified Bold's Basal Medium (BBM) as a control. The modified culture media were introduced as an effective medium for enhancing the growth rate of *C. pyrenoidosa* [30]. All solutions were sterilized using autoclave at 121˚C for 15 min to eliminate any contamination prior to the experiment. In addition, the pH was adjusted to 6.8 before autoclaving [30]. The stock solution used for maintenance of microalgae was same as that used for growth (modified BBM). A 2 mL suspension including *C. pyrenoidosa* was inoculated using capped conical flask containing 18 mL of culture media. The flasks were later incubated in the presence of light (100 $\mu mols^{-1} m^{-2}$) and room temperature. The inoculated cultures were maintained for 14 d. Furthermore, cultures were often streaked on the agar plates for constant purity check.

Moreover, POME samples were freshly collected from facultative ponds at FELDA palm oil mill in Kulai, Johor, Malaysia and preserved at 4˚C in a cool room. Then, they were stored in 5-L plastic containers with proper label. Sampling was done every three months, and collections were refrigerated at 4˚C in order to prevent any possible contamination and limit the biodegradation process. Prior to the cultivation of microalgae, the collected POME was left to stand for at least 1 h at ambient temperature to ensure that its temperature reached the room temperature. Furthermore, the duration was prepared as sufficient to enable both settling and separation of the sludge and other hazardous materials (e.g. glass) contained within the POME. The characteristics of applied POME are as shown in Table 1.

2.3. Inhibition model

In order to determine the quality of microbial growth at different initial concentrations of POME and to know the effect of initial substrate concentration on the process quality, the kinetic model was applied to the obtained experimental results. In this study, inhibition model was found to be suitable as it had the behavior that fits well to the experimental process being carried out. Some of the most widely used inhibition models for describing cell growth are Haldane, Linear, Exponential, Teissier Models, etc. which are adapted from modified Monod equations for substrate inhibition of enzymatic reactions. According to the inhibition model, the microbial growth could be accounted by Eq. (1) as follows:

$$Y = \mu_m X/(K_m + (1 + K_s/K_i)) \quad (1)$$

where μ_m is the maximum specific growth rate, K_m is the Michaelis–Menten constant, and K_i is the dissociation constant with the same unit as X. All the values of μ_m, K_s, and K_i were obtained by using Graph Pad Prism Software (evaluation).

2.4. HPBR preparation

The cultured microalgae *C. pyrenoidosa* was later transferred into a 5-L batch mode HPBR. All experiments were performed around 28 ± 1˚C and pH between 7.5 and 8.5. The reactor was programmed to agitate at 100 rpm. Instead of constant illumination, the culture was exposed to 16 h light and 8 h darkness cycle. In the influent and effluents of the reactors, COD nitrate, total nitrate, phosphate, total phosphate, total suspended solids (TSS), MLVSS, MVSS, soluble COD, and volatile suspended solids (VSS) were measured daily. All analyses were carried out in accordance with APHA standard methods.

The bioreactor was equipped with the nutrient supply system including fresh medium reservoir (a 10-L built-in tank in the reactor rack), which was connected to a peristaltic pump to feed nutrient to the culture vessel. In addition, a drainage system was incorporated with the HPBR to remove the spent nutrient. The outflow medium was drained into a 5-L tank. Furthermore, the temperature in the culture vessel was controlled and maintained by constant circulating water of the required temperature from the built-in water bath. The culture was illuminated by four 32 W white fluorescence lights (Philip, Germany). Moreover, lights were attached vertically to the four pillars of the metal rack of the culture vessel. In addition, Photo synthetically active radiance at the surface of the reactor was measured using a Lux meter.

Moreover, inoculums were taken from the conical flasks and transferred into the culture vessel, and samples were taken directly via the sampling tube.

Table 1
Characteristic of the sampled POME

No.	Parameter*	Concentration range (mg/L)	Average (mg/L)
1	pH	4.15–4.4.5	4.25
2	COD	1,350–2,120	1,600
3	Soluble COD	20,500–24,500	22,000
4	BOD	300–400	330
5	Total volatile solid	27,300–30,150	28,100
6	Total suspended solid	15,660–23,560	18,900
7	Total phosphorus	200–600	350
8	Total nitrogen	500–800	500

*All parameters are in units of mg/L except pH.

Sampling was done at a regular time interval. Based on Calvin Benson cycle, Photo-period is considered to be necessary, during which micro-algae produces organic compounds from CO_2 and H_2O by using high-energy ATP and NADPH that are created by the photo-system of algae during the process of photosynthesis. This cycle is distinct from the so-called light reaction that takes place in the photosystem, which produces energy. For cyclic light-autotrophic/dark-heterotrophic cultivation, cells were cultured under continuous illumination following the scheduled light and dark regime as presented in Table 2. In addition, samples were cultured in 14 d of variation in nutrient (COD: total nitrogen [TN]) ratios at 100:6, 100:17, and 100:32, respectively. All various ratios were maintained at fixed concentration (500 mg COD/L) of POME. Finally, the effect of OLR (provided by constant influent COD but increasing influent flow rate) on the performance of the HPBR was assessed.

2.5. Organic loading rate

The effect of OLR on lipid production and microalgae growth was investigated using laboratory-scale HPBR. The reactor was operated at different rate at 96, 72, 48, and 36 kg COD/m^3 d. The reactor was initially fed with diluted POME. The effect of different ratios of OLR on the performance of the HPBR was assessed.

Table 2
Light/dark cycle duration

Experiment No.	Photo duration (hour) light	Dark
1	24	0
2	16	8
3	8	16

2.6. Chemical oxygen demand

The aim of COD estimation is to study the potential of *C. pyrenoidosa* in reducing the different nutrients in POME wastewater. The microalgae cells were homogenized thoroughly using vortex and then added into the COD vials. The reactor block was preheated to ensure an accurate digestion at 150 °C for 2 h. After 2 h, the samples were allowed to cool to reach the room temperature. Measurements were taken using Hach DR spectrophotometer according to method 5220 B [31].

2.7. Total nitrogen

An aliquot of 2 mL microalgae *C. pyrenoidosa* suspension was collected from the reactor for nutrient consumption analysis at the beginning of inoculation period. The samples were initially centrifuged at 4,000 rpm for 10 min. Then, the supernatants were appropriately diluted and analyzed for TN according to the HACH DR 5000 spectrophotometer manual.

2.8. MLSS and mixed liquor volatile suspended solids (VSS)

Microalgae biomass was obtained by filtering a sample through a pre-dried 0.45 μm Whatman filter paper. The residue left on the filter paper was dried in oven for 1 h at temperature 103 °C. Volatile suspended solids (VSS) assessment was performed by incinerating the dried solids at 550 °C in furnace for 20 min. The weight lost on ignition of the solids represents the volatile solids in the sample. All analyses were measured by standard methods 2540 D, E for TSS and VSS, respectively [31].

$$\mathrm{TSS}\left(\frac{\mathrm{mg}}{\mathrm{L}}\right) = \frac{(A-B)\times 1{,}000}{\text{sample volume}} \quad (2)$$

where A is the weight of filter plus dried residue (mg) and B is the weight of filter (mg).

$$\mathrm{VSS}\left(\frac{\mathrm{mg}}{\mathrm{L}}\right) = (C - D) \times \frac{1,000}{\text{sample volume (mL)}} \quad (3)$$

where C is the weight of residue plus dish before ignition (mg) and D is the weight of residue plus dish or filter after ignition (mg).

2.9. Cell dry weight

The dry weight of microalgae biomass was determined via gravimetric method, and the growth factor was expressed as mg/L of dry biomass [32]. An aliquot of sample was collected and harvested by centrifugation at 4,000 rpm for 15 min with temperature at 4˚C [32]. Then, cells were dried at 60˚C for 24 h.

2.9.1. Kinetics and parameters

The specific growth rate (μ) and biomass productivity are calculated by Eqs. (4) and (5), respectively.

$$\mu = 1/\mathrm{tln}(X_m/X_0) \quad (4)$$

where X_m is the concentration of biomass at the end of batch run, X_0 is the concentration of biomass at the beginning of a batch run, and t is the duration of the batch run (hour, day). In addition, lipid productivity is achieved through Eq. (5) as:

$$P_{\mathrm{lipid}} = \frac{C_l}{t} \quad (5)$$

where C_l is the concentration of lipids at the end of the batch run and t is the duration of the run (hour, day).

2.10. Lipid extraction and quantification (Nile Red method)

Dry extraction procedure [33] as a modification of the wet extraction method [34] was used to extract lipids of microalgal cells. Typically, cells were harvested by centrifugation at 4,000 rpm for 15 min and washed once with distilled water. After drying the samples using freeze drier, the samples were pulverized in a mortar and extracted using a mixture of chloroform: methanol (2:1, v/v).

About 50 mL of solvent was used for per gram of dried sample in each extraction step. After stirring the sample using magnetic stirrer bar for 5 h and ultrasonication for 30 min, samples were centrifuged at 3,000 rpm for 10 min. The solid phase was separated carefully using filter paper. The solvent phase was evaporated in a rotary evaporator at 60˚C. The procedure was repeated three times until the entire lipid was extracted.

Dye Nile Red, 9-diethylamino-5H-benzo [α] phenoxazine-5-one, was reported as an excellent stain for the detection of intracellular lipid droplets by spectrofluorometer [35]. The quantification of cellular neutral lipid with fluorescent spectrophotometer was carried out based on the method previously utilized by other researchers [35,36]. Cultures were initially mixed with Nile Red solution (0.1 mg mL^{-1} in acetone; 100/1, v/v) for 7 min prior to running the fluorescence spectrophotometer (HITACHI F-4500). The relative fluorescence of Nile Red for lipid was achieved after deduction of both the auto-fluorescence of algal cells and the self-fluorescence of Nile Red from the gross reading at 580 nm wavelength.

3. Results and discussion

3.1. Effect of substrate

As it is clearly shown in Fig. 1(A) and Table 3, the overall concentration of COD was dropped with time for different concentration of POME as substrate. Result showed that *C. pyrenoidosa* was able to eliminate 71.43%, 71.16%, 69.42%, and 29.81% of CODs in various concentrations of POME at 0, 250, 500, and 1,000 mg/L, respectively. In addition, *C. pyrenoidosa* was reported as an effective agent on organic matter removal, especially at 250 mg COD/L of POME. The COD removal at 500 mg COD/L was comparable to that of 250 mg COD/L, while at 1,000 mg COD/L, the rate was drastically decreased.

3.2. Substrate consumption rate

Interestingly, *C. pyrenoidosa* also reached the optimum growth rate in the absence of POME. This phenomenon might be because of an inhibitory effect of high concentration of POME toward the growth of *C. pyrenoidosa.* Furthermore, results demonstrated that the substrate inhibition model based on Eq. (5) presented in this work could well describe the specific cell growth rate relation with the concentration of a limiting nutrient at both low and high nutrient concentrations. In heterotrophic cultures with dense *C. pyrenoidosa* suspensions, the carbon substance in the medium is depleted very rapidly, and thus a large quantity of carbon source is needed for batch culture to extend the growth period. Therefore, it is necessary

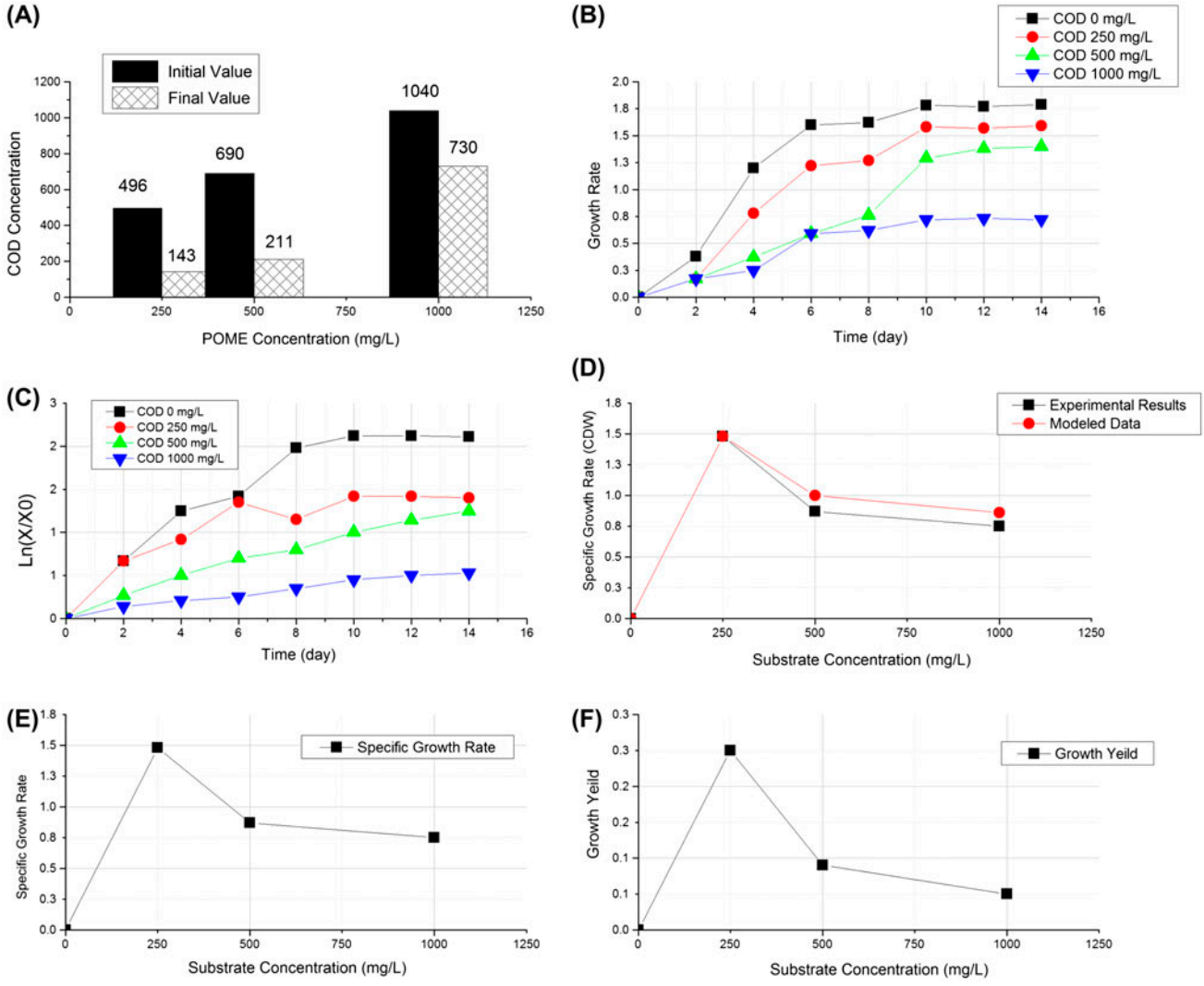

Fig. 1. (A) Initial with final COD removal in different POME concentration for *C. pyrenoidosa*. (B) Growth rate of microalgae under different initial substrate concentration by *C. pyrenoidosa*. (C) Variation in the Napierian logarithm of the quotient between the MLSS/MLVSS concentrations at any time by *C. pyrenoidosa* (D) Comparison of the model values and experimental data, CDW measurement by *C. pyrenoidosa* performance. (E) Effect of COD concentration on the specific growth rate of *C. pyrenoidosa*. (F) Effect of COD concentration on the growth yield of *C. pyrenoidosa*.

Table 3
COD consumption rate by *C. pyrenoidosa* at different concentrations of POME

COD consumption rate (mg/L d)				
Concentration (mg/L) Initial overall	0	250	500	1,000
Concentration (mg/L)	124	396	590	940
Final concentration (mg/L)	33	37	40	480
Rate	4.55	17.95	19.64	23.00

to determine the suitable concentration of substrate, which can be applied to prevent specific cell growth inhibition.

3.3. Growth rate of microalgae

Napierian Logarithm of X/X_0 with the operation time for different substrate concentrations is shown in Fig. 1(B). The X presents the growth rate of microalgae *C. pyrenoidosa* concentration (cell dry weight [CDW]) at the end of experiment and X_0 exhibits the growth rate of microalgae *C. pyrenoidosa* concentration (CDW) at the beginning of the experiments. The CDW concentration increased with the operation time as the substrate concentration increased from 250 to 500 mg COD/L. However, a drastic growth rate reduction was achieved as the substrate concentration was increased to 1,000 mg COD/L.

3.4. Biomass productivity

The initial MLSS concentration was boosted when the initial COD concentration was grown from 250 to 500 mg COD/L. In addition, final MLSS concentration improved as the initial COD concentration increased from 250 to 500 mg COD/L. Moreover, results indicated an inhibition in the growth of *C. pyrenoidosa* (Fig. 1(C)).

3.5. Inhibition model

Fig. 1(D) presents experimental and modeled results of specific growth rate as a function of initial POME concentration. Specific growth rates were determined for each initial POME concentration (S) by plotting Ln (X/Xo) vs. time. These data were then fitted to Eq. (1) by nonlinear least-squares regression techniques for the estimation of bio-kinetic constant (Table 4). As previously mentioned, the critical concentration of POME was 1,000 mg COD/L for the growth of *C. pyrenoidosa*. In addition, the specific growth rate was fallen with rising substrate concentration, which means that substrate could be an inhibitor at higher concentration. Moreover, *C. pyrenoidosa* can perform heterotrophic growth besides the common autotrophic growth. Organic substances may function directly as an essential organic nutrient or act as an accessory growth factor [37]. This phenomenon is presented in Fig. 1(E) and (F) that shows reduction in the production yield and growth rate of *C. pyrenoidosa* when the substrate concentration was increased. However, the growth was gradual with apparent COD inhibition at concentrations above 500 mg COD/L. The COD concentration above 500 mg COD/L greatly affected the efficiency of biomass production.

3.6. Lipid productivity

Lipid content is strongly affected by several factors such as substrate composition, light intensity, and pH [38,39]. Any change on these factors may result in different cellular lipid content. In this study, *C. pyrenoidosa* was found as a high lipid-containing strain, which is about 38.6% of its biomass after 2 d of measurement as well as a high lipid-producing agent at 0.193 mg L^{-1} d^{-1} when the initial COD concentration was set to 500 mg COD/L (Fig. 2).

Table 4
Bio-kinetic constant obtained from substrate inhibition model using GraphPad Prism 5 software

Bio-kinetic constant	Best fit value
Vmax	2.710
K_m	34.85
K_i	314.5
R^2	0.9757

3.7. Comparison between biomass and lipid productivity of C. pyrenoidosa *in flask scale and HPBR*

Cultivation in HPBR was performed in a 5-L stirred tank reactor with turbine impellers. The double vessel has an inner diameter of 145 mm and inner height of 300 mm. On the other hand, the 250-mL Pyrex Erlenmeyer flask with the dimension of 132 × 82 mm was used. A rough estimation of the *C. pyrenoidosa* productivity was performed on the laboratory microalgae culture optimization studies. *C. pyrenoidosa* biomass manufacturing yield was optimized using HPBR in a 5-L batch culture over 14 d, which led to a maximum production at 0.68 g/L d dry cell weight, while the 250-mL flask culture prior to optimization produced around 0.009 g/L d of lipid biomass. Furthermore, light intensity plays a major role in the productivity of microalgae culture [40]. At higher medium depths and cell concentrations, the light intensity must be increased to effectively penetrate through the culture (e.g. 1,000 lux is suitable for Erlenmeyer flasks, and 5,000–10,000 is required for larger volumes). Hence, the increase in the volumetric production of HPBR can be explained by shorter optical path of flask-scale cultivation compared with that of HPBR. Ironically, the result clearly indicates lower

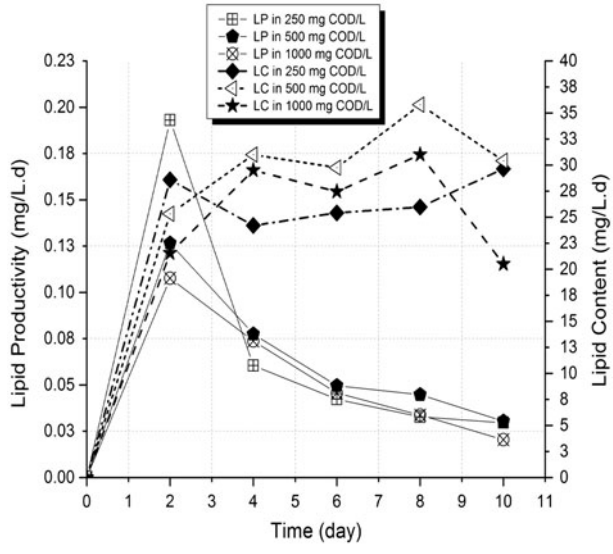

Fig. 2. Lipid productivity of *C. pyrenoidosa* in 250, 500 and 1,000 mg COD/L of POME.

biomass productivity compared with that of reactor scale. Furthermore, mixing of culture is another essential function to suspend the biomass and to enhance the interaction between the liquid nutrient and the cells. Mixing prevents gradient of nutrients, pH, and temperature inside the reactor.

In this research, the feasibility of large-scale cultivation of *C. pyrenoidosa* was determined by growing the microalgae in laboratory-scale bioreactor to examine whether a high biomass concentration and lipid content could be reached. Results using both flask scale and HPBR are demonstrated briefly in Table 5. The process was divided into two sequential stages as flask scale followed by laboratory-scale bioreactor.

In order to maximize the biomass production yield of the culture over a short period of time, *C. pyrenoidosa* was cultured in batch mode. Hence, the culture was started at low density and supplied with sufficient nutrients, which enabled it to proliferate to reach the maximum density, and thus the lipid content was enhanced. The cell culture density was achieved greater than that by continuous culture as it was solely maintained constant for short period of time before the next culture was started. Furthermore, batch cultivation helped the microalgae to be produced at its highest density before the next cultivation.

3.8. Organic carbon substrate and nutrient utilization rate

Fig. 3 demonstrates the utilization rate of organic carbon and nutrient with operation time for three different C:TN ratio (100:6, 100:17, and 100:32). *C. pyrenoidosa* exhibited short adaptation phase for both organic and inorganic compounds in wastewater. After couple of days, both organic carbon and nutrients were dropped gradually proving that microalgae utilized the carbon source not only for its growth but also for lipid production. By the end of the experiment, *C. pyrenoidosa* eliminated 32%, 63%, and 46% of organic carbon of various C:TN ratios at 100:6, 100:17, and 100:32, respectively. It proved that microalgae are able to utilize different organic compounds as the carbon source for its growth or biomass productivity. The rate of organic carbon substrate for various ratios based on TN concentration is depicted in Fig. 4(A). Interestingly, lipid production was significantly increased when the specific growth rate was less than 0.1/d with substrate consumption rate greater than 119 mg/L d. In a typical growth rate factor, any specific growth rate higher than 0.1/d is considered as a nonlimiting condition for the growth of microalgae. Any value less than this specified rate is regarded as the limiting growth factor, which usually suitable for lipid storage, rather than for its growth. In addition, high lipid productivity could be obtained under nutrients and carbon limitation (Fig. 4(B) and (C)). Compared with other C:TN ratios, the ratio at 100:6 demonstrated the highest lipid productivity at 114.9 mg/L d. It is worth noting that nitrogen-limiting conditions relatively increased the lipid fraction by microalgae performance [39].

Table 6 presents the lipid content of *C. pyrenoidosa* and its lipid productivity with different culture conditions. The lipid productivity and its content of the studied species were found comparable to those cultured using BBM at reducing $NaNO_3$ concentration [39]. When *C. pyrenoidosa* was cultured at C/N ratio of 100:6 (N-deficient condition), the lipid productivity was obtained at 114.9 mg/L d.

Table 5
Comparison between flask scale and HPBR in terms of physical operation, biomass productivity, lipid content, and lipid productivity

	Flask scale	HPBR
Average biomass Productivity (mg/L d)	9.5	130
Average lipid content (% of biomass)	21	68
Average lipid Productivity (mg/L d)	1.78	230
Type of operation	Manual	Semi-auto
Volume of reactor (L)	0.25	5
Dimension (mm × mm)	132 × 82	300 × 145
Mixing	Easier	Difficult
Light path	Shorter	Deeper, may reduce light availability to the culture

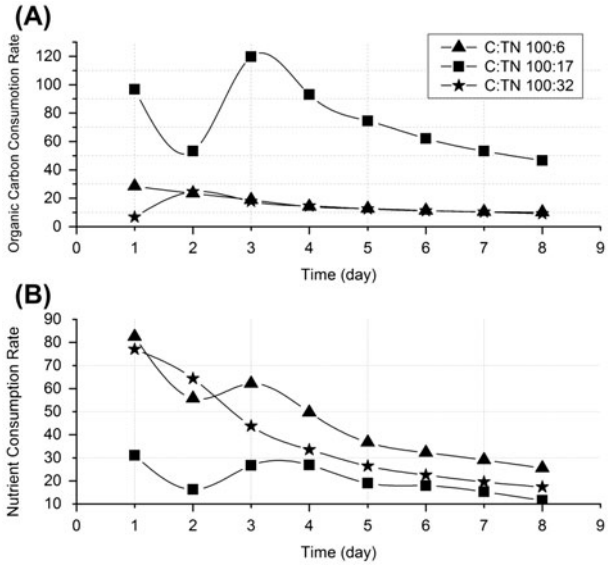

Fig. 3. Utilization rate of organic carbon (A) and nutrients (B) by *C. pyrenoidosa* during the experiment time.

Generally, high C/N ratio favors lipid accumulation usually triggered by nitrogen depletion in the culture. Higher lipid accumulation was found in *C. pyrenoidosa* under C/N ratio at 100:6, which led to lipid production of 39% of its biomass. However, the lipid content was found relatively low in microalgae grown under C/N ratios at 100:32 and 100:17. Under environmental stress (such as nutrient limitation), cell division may cease but the lipid productivity still seems to remain high, leading to an accumulation of lipids in the cells. However, amplified oil content does not lead to improved lipid productivity because the overall rates of lipid productivity are lower during periods of nutrient limitation. Higher level of oil in the cells is more than offset by lower rates of cell growth [41].

Typically, the lipid production is accelerated with the decrease in nitrogen concentration. In addition, reduction in nutrient concentration improves lipid production [39]. High cellular lipid content is promoted by a growth medium, which is either carbon or nitrogen limited. The former is preferred, as a low C/N ratio boosts the proportion of unsaturated acids, especially trienoic acids.

3.9. Effect of photo cycles on biomass and lipid production by C. Pyrenoidosa in POME

With regard to evaluated photoperiods, results of volumetric growth rate were differed statistically ($p < 0.05$). In addition, there was a greater alteration in maximum cell concentrations compared with that in continuous illumination for (16:8) and (8:16) light period, suggesting that higher dark period positively affects the rate of photosynthetic metabolism (Fig. 5(A)).

Microalgae lipid content is presented in Fig. 5(B). For three light and dark cycles, the maximum amount of lipid content was achieved at the end of cultivation. The highest lipid content was achieved when *C. pyrenoidosa* was cultured under continuous light (24:0) at 17.00 mg/mg CDW, followed by 16:8 and 8:16 with ratios at 12.90 and 9.50 mg/mg CDW, respectively. It can be clearly observed that photo light duration has major impact on lipid content productivity as well as biomass growth rate and microalgae photosynthetic activity. Under these light regimes, *C. pyrenoidosa* was unable to store light energy to sustain its growth during the dark period similar to continuous illumination. In the absence of light, microalgae respire. Thus, it is assumed that the defeat of biomass under dark/light cycle was most probably due to photorespiration [42,43]. Furthermore, there is a loss of biomass due to the utilization of oxygen and storage of carbon as an energy source, as well as cell maintenance rather than cell synthesis. However, there are several exceptions in which microalgae concentration was found higher when grew under 12:12 (light/dark period) illumination [44,45].

Normally, microalgae are capable to store considerable amounts of light energy to support its growth in dark periods. However, as light energy is absorbed through photosynthetic pigments in microalgae, solely a small fraction of energy is stored. Over 60% of the energy is dissipated in the form of heat in the reactor and it limits the capacity of microalgae to store light energy [46].

Studies have also shown that both light cycle and mixing turbulence affect the photosynthetic activity as well as microalgae growth rates in a photo-bioreactor [46,47]. In this case, the shortage of light energy was overcome with increasing turbulence or mixing to increase productivity and photosynthesis efficiency. It should be stated that light/dark cycle and turbulence are two separate parameters, but they function synergistically [40].

In addition, the availability of light in a typical photo-bioreactor is another factor, which affects on cell density. High cell density accounts for mutual shadings, which limits the light penetration within the reactor [48,49]. Moreover, in this study, cell concentration was decreased proportionally with the period of time in which microalgae *C. pyrenoidosa* was exposed to intermittent light radiation as compared to continuous illumination. A similar study was revealed that specific light absorption of *Chlorella reinhardtii* was

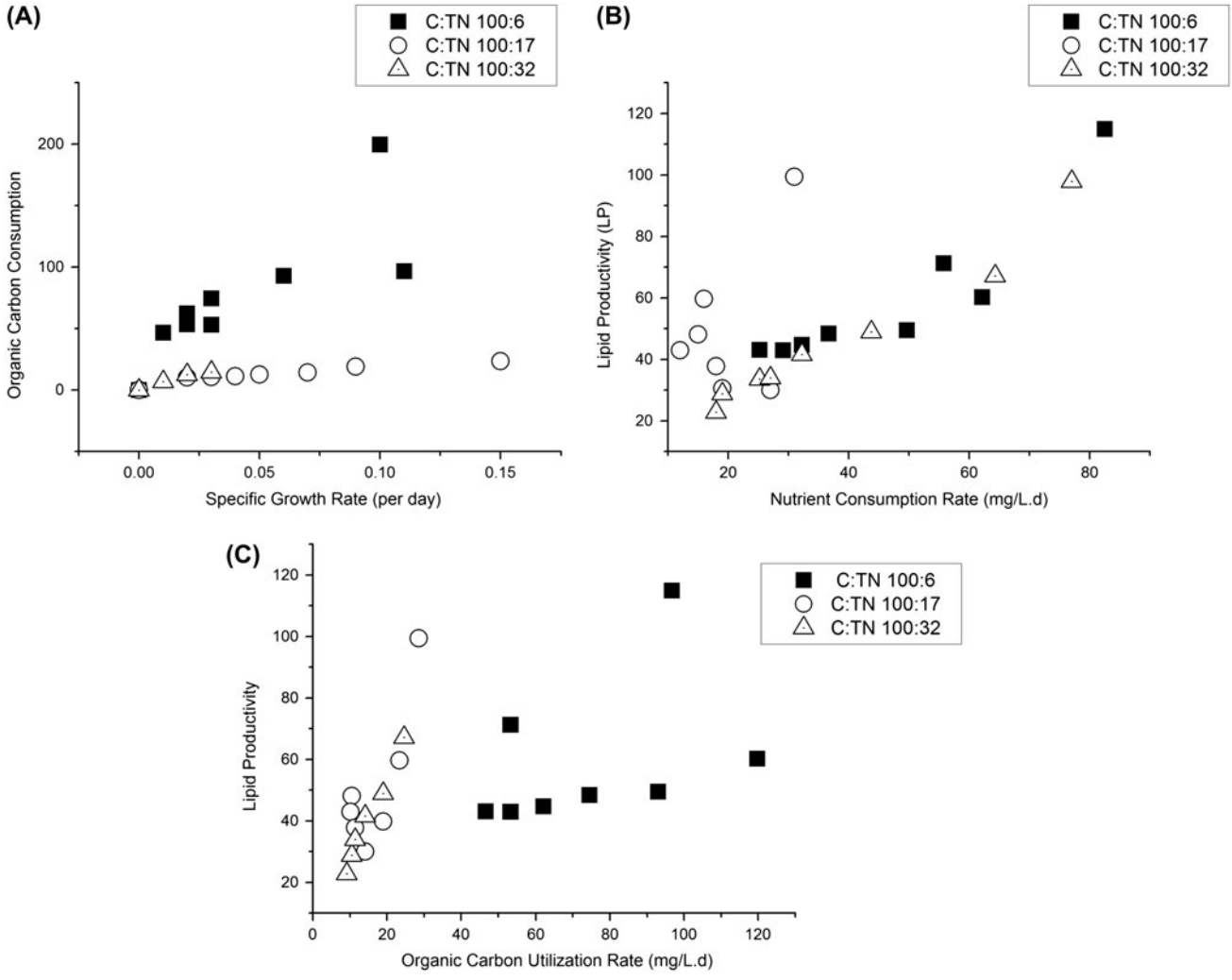

Fig. 4. (A) Relationship between organic carbon consumption rate and microalgae specific growth rate. (B) Relationship between lipid productivity and nutrient utilization rate. (C) Relationship between lipid productivity and organic carbon substrate.

Table 6
Effect of C/TN ratio on lipid content and lipid productivity after 9 d of cultivation

C:TN ratio	Lipid content (% biomass)	Lipid productivity (mg/L d)
100:6	39	114.9
100:17	18	99.4
100:32	19	97.9

augmented due to chlorophyll-*a* content enhancement under intermittent light/dark cycles [46].

3.10. Effect of OLRs on the biomass and lipid production by C. Pyrenoidosa in POME

The system was operated for 14 d for each different OLRs ranging from 36 to 96 kg COD/m^3 d. These values were obtained by regulating the flow rate of the influent. As shown in Fig. 6 and Table 7, the biomass productivity and specific growth rate in all diluted POME samples were amplified with the increase in the OLRs. At the end of experiment, the drastic drop in the lipid productivity of *C. pyrenoidosa* with the OLRs ($R^2 = 0.954$) is presented in Fig. 6, which in turn provides a good correlation

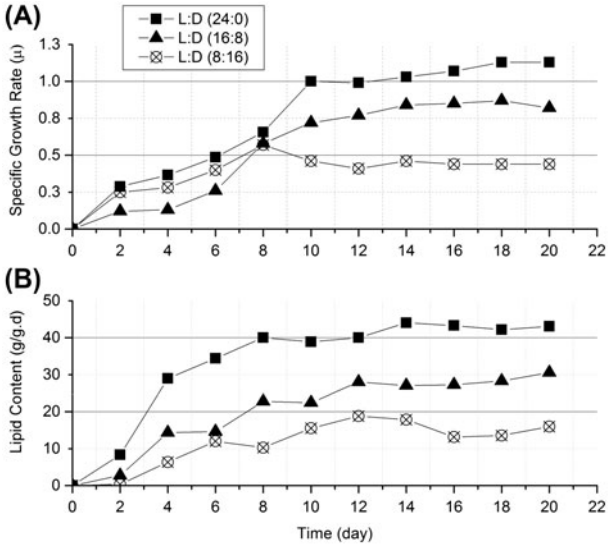

Fig. 5. (A) The effect of light and dark cycles on specific growth rate profiles . (B) Lipid productivities for different light and dark cycles during microalgae cultivation.

between the biomass ($R^2 = 0.9421$) and lipid productivity.

Generally, higher OLR promotes higher biomass productivity with lower lipid content. This confirms the findings that present higher growth rate of microbial substances with higher OLR [50]. High organic load operation as available feeds for microbial cultivation in the reactors allows the microalgae *C. pyrenoidosa* to develop faster. Instead of lipid accumulation, a complex nutrient of high-strength piggery wastewater including organic compounds was used for microalgae growth enhancement in mixotrophic mode by assimilating organic carbon along with fixing the CO_2 in light [51].

In this study, high lipid production by microalgae *C. pyrenoidosa* as local isolated microalgae in POME was obtained under condition of limited nutrient and carbon concentration. In addition, *C. pyrenoidosa* was able to reach relatively high lipid content and lipid productivity when cultured in POME with C:TN ratio at 100:6. Another fact is that nitrogen limitation had significant effect on the microalgae biomass production and lipid productivity in comparison with carbon limitation. Furthermore, proper period of illumination (24 h) was essential for both lipid growth and productivity by *C. pyrenoidosa*. Moreover, comparison between flask and HPBR cultivation of *C. pyrenoidosa* clearly exhibited that highest lipid and biomass productivity of *C. pyrenoidosa* in POME were achieved using HPBR. In addition, lipid and biomass productivity of *C. pyrenoidosa* were restricted through the flask as a reason of low-volume cultivation scale. In short, the *C. pyrenoidosa* as a promising and high potential lipid producer can be applied as a sustainable raw material to produce high amounts of lipid used in biofuel manufacturing.

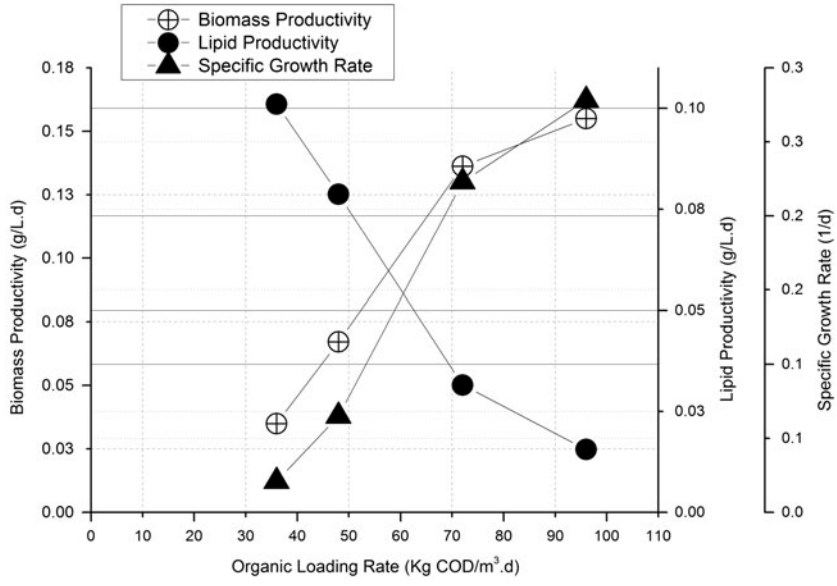

Fig. 6. Specific growth rates (μ), biomass productivity ($P_{biomass}$), and lipid productivity (P_{lipid}) of *C. pyrenoidosa* under different OLRs in POME.

Table 7
Lipid productivity of *C. pyrenoidosa* under different OLRs

Experiment	OLR (kg COD/m^3 d)	Lipid productivity (mg/L d)
1	36	100.5
2	48	78.7
3	72	31.5
4	96	19.7

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