

# Cultivation of *Chlorella pyrenoidosa* as a raw material for the production of biofuels in palm oil mill effluent medium with the addition of urea and triple super phosphate

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## Abstract

**Background:** The utilization of microalgae as a renewable energy is an important aspect in solving shortage of future oil reserve in 15 years. One of the renewable energy sources is microalgae biodiesel. Palm oil mill effluent (POME) is a wastewater that has a high content of organic materials. These organic materials can be used as growth nutrients for microalgae. *Chlorella pyrenoidosa* is one of the most potential microalgae used as a raw material for the production of biodiesel since it contains lipids (8%-35%).

**Methods:** *Chlorella pyrenoidosa* was cultured on the POME medium with concentrations of 0%, 25%, 50%, 75%, and 100%v with addition of synthetic nutrients (urea: TSP) at the ratios of 2:1 ; 1:2 ; 0.5:1 in a 500 mL Erlenmeyer flask, at pH 6-8, aeration using aquarium pumps, and using LED lights (3000 lux).

**Results:** It was revealed that at POME concentration of 25% and with addition of urea: TSP at a ratio of 2:1, the optimum specific growth rate (0.306/day) with the highest number of cells was  $3.530 \times 10^7$  cells/mL and the highest lipid content was 36% of its dry weight. The removal efficiency of POME could be obtained from the removal efficiency of chemical oxygen demand (COD), total nitrogen, and orthophosphate (P-PO<sub>4</sub>), which was 70, 90.42, and 81.12%, respectively.

**Conclusion:** According to the results, under appropriate culture conditions, *C. pyrenoidosa* can produce lipids with good use of nutrients contained in the POME medium.

**Keywords:** *Chlorella pyrenoidosa*, POME, Lipid, Urea: TSP

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## Introduction

Microalgae have recently become a worldwide concern as a new raw material for the biofuel production (1). It is an organism that is considered as an ideal and potential raw material for bioenergy production (2). Microalgae is a photosynthetic microorganism that has the ability to use sunlight and carbon dioxide to produce algal biomass (3). It is a potential raw material that can fulfill the global demand for transportation fuels in the future, since microalgae can be produced in a short time and can produce oil 10 times more than oil derived from food crops (4).

Biofuels production from microalgae biomass can provide a pollution-free and environmentally friendly energy source (5). The idea of using microalgae as a source of

fuel has become a serious discussion due to the increase of fuel prices and environmental problems related to global warming due to burning fossil fuels (6). The increase in fuel prices is due to the increasing dependence of the society on fuel, which causes a reduction in oil reserves (7). Recently, biodiesel (green fuel) as a renewable and non-toxic energy which produces low amounts of pollution has been proposed as a replacement for fossil fuels. The use of this fuel leads to a decrease in the harmful emissions of carbon monoxide, hydrocarbons, and particulate matter, as well as the elimination of SO<sub>x</sub> emissions, and as a result, a decrease in the negative effects of greenhouse gases (8). Microalgae can produce high enough lipids ( $\pm 65\%$  in its dry weight) to be converted into biodiesel (9). Microalgae are chosen because of its ability to grow quickly, live in



freshwater, seawater, and wastewater, while it does not require a large area for its production activities. Besides, it can absorb carbon dioxide, so that it can reduce the negative effects of greenhouse gases (10). Economically, microalgae are chosen because of its considerable availability and low production costs (11,12). One of the potential microalgae that can be developed into bioenergy raw material is *Chlorella pyrenoidosa*.

*Chlorella pyrenoidosa* is a unicellular green algae that lives in freshwater, seawater, and wet areas. Moreover, *C. pyrenoidosa* can grow and develop very quickly (13). In Riau, especially in PT Perkebunan Nusantara (PTPN V) Riau province, *C. pyrenoidosa* grows abundantly in sewage treatment ponds. *C. pyrenoidosa* has a lipid content ranging from 8%-35% (14). The growth rate, cell density, and lipid content of the microalgae are essential parameters contributing to lipid production (8). To obtain the high amount of biomass with optimum lipid content, it is necessary to do the cultivation process. In this study, *C. pyrenoidosa* was cultivated using a medium of palm oil mill effluent (POME) containing organic materials, which can be used as a carbon source for the cultivation media (15,16). Cultivation of algae in the POME waste is an effective and efficient alternative. POME is a high-strength effluent rich in minerals, such as N, P, K, and other minerals suitable for growing media for algae. By utilizing POME for microalgae cultivation, palm oil mills can apply the principle of reduction because there will be a reduction in the volume of waste. It is because the treated POME contains nutrients with a mass ratio of C: N: P (34: 5: 1) (17), whereas microalgae requires nutrients that are large enough with the mass ratio of C: N: P (56: 9: 1) (18). Studies have shown that the quality and quantity of the microalgae lipid content can be changed as a result of changes in growth conditions (temperature, light intensity) or medium composition (nitrogen, phosphate, and iron concentration) (19). Some studies are already done on the methods of microalgae extraction from POME medium by centrifugation using *Chlorella* (20). In recent studies, only microalgae *Chlorella* sp or *Chlorella vulgaris* were used to investigate the effect of medium cultivation with a variation in the growth conditions (20). The purpose of this study was to fulfill the nutritional needs of microalgae for lipid production extracted from microalgae, as well as the utilization of algae blooms in sewage treatment ponds in PTPN V, Riau province. So, it is necessary to manipulate the availability of nutrients

## Materials and Methods

The medium used for the cultivation process was POME from PTPN V Riau province. The additional nutrients were added in the form of urea and TSP. *C. pyrenoidosa* was obtained from the cultivation using a chamber coated with aluminum foil, which serves to keep the resulting light intensity quantized so that the microalgae in the Erlenmeyer flask get maximum light. *C. pyrenoidosa* was

collected from BBPBAP Jepara, which was cultivated using Bold Basal Medium (BBM).

## Calculation of cell density and specific growth rate

Microalgae cell number was calculated using a thomacytometer during the data collection period of 0, 1, 3, 5, 7, 9, 11, and 13 days. The microalgae cell density was calculated using the following formula:

$$N = n \times 10^4 \quad (1)$$

Where  $N$  is *C. pyrenoidosa* cell density (cell/mL) and  $n$  is the total number of cells calculated for each sample (cells). The specific growth rate of microalgae was calculated using the following formula:

$$\mu_{\max} = \frac{\ln(xi) - \ln(xo)}{(ti - to)} \quad (2)$$

Where  $Xo$  is microalgae cell density at the beginning of cultivation,  $Xi$  is microalgae cell density at the end of cultivation, and  $ti-to$  is duration of cultivation time.

## Calculation of lipid extraction

Lipid extraction was carried out by the Bligh and Dyer method (21). The lipids were extracted with chloroform-methanol solution (2:1, v/v), and then separated into a liquid layer of chloroform and methanol. Methanol and water were added to produce the final solvent from the mixture of chloroform: methanol: water (1: 1: 0.9). The chloroform layer was washed with 1 drop of HCl 6N solution and evaporated to dry (22). The total lipids were determined gravimetrically (23).

$$\% \text{ Total lipid} = \frac{Lw}{Bw} \times 100 \quad (3)$$

Where  $Lw$  is lipid weight (g) and  $Bw$  is microalgae biomass weight (g).

## Calculation of removal efficiency of COD, total nitrogen, and orthophosphate (P-PO<sub>4</sub>)

To calculate the percentage of removal efficiency of chemical oxygen demand (COD), TN, and P-PO<sub>4</sub>, the following equation was used:

$$\text{Efficiency (\%)} = \left[ \left( \frac{Cin - Cef}{Cin} \right) \right] \times 100\% \quad (4)$$

Where  $Cin$  is influent concentration (mg/L) and  $Cef$  is effluent concentration (mg/L) (24).

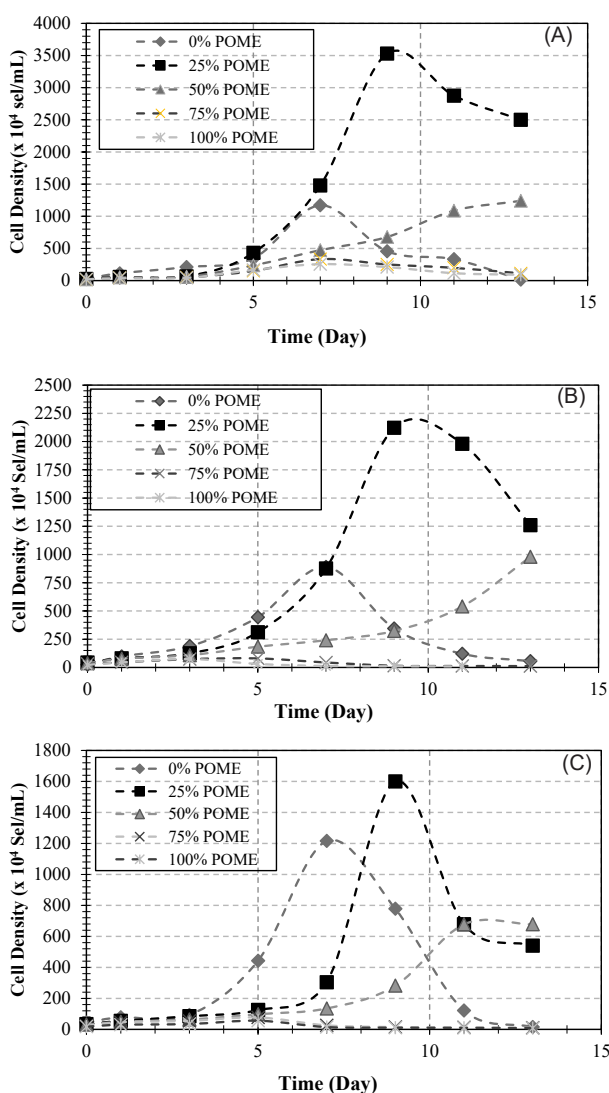
## Results

During the cultivation periods, microalgae were observed for 13 days with sampling time on days 0, 1, 3, 5, 7, 9, 11, and 13 using a thomacytometer with the variables used including POME concentration and nutrients ratio of urea: TSP.

### Cell density and specific growth rate of *Chlorella pyrenoidosa*

Cell density and specific growth rate of *C. pyrenoidosa* were obtained in a medium containing POME of 25% v with the addition of urea: TSP (2:1), which were equal to  $3530 \times 10^4$  cells/mL (Figure 1A) and 0.530/day, respectively (Figure 2). As shown in Figure 1, the higher the medium POME concentration, the lower the microalgae cell density in the cultivation process.

As shown in Figure 1, the cell density of each treatment was different. At a concentration of 0% (control), the growth rate of *C. pyrenoidosa* increased rapidly followed by a 25% POME concentration, while at a concentration of 50%, 75%, and 100%, the growth rate of *C. pyrenoidosa* decreased. The highest cell density values were obtained in a medium containing 25% POME with the addition of urea: TSP (2:1), which was equal to  $3530 \times 10^4$  cells/mL (Figure 1A). This value was obtained on the 9<sup>th</sup> day



**Figure 1.** Cell density of *C. pyrenoidosa* with a variation in the nutrients ratio of urea: TSP. (A) 2:1, (B) 1:2, (C) 0.5:1.

of cultivation, which was in the exponential phase. The increase of cell density could be seen in the magnitude of the average growth rate of cell in each phase. The growth rate of microalgae in each treatment can be seen in the specific growth rate graph in Figure 2.

As shown in Figure 2, the specific growth rate of each treatment was different. The highest growth rate (0.530/day) was obtained in the medium of 25% POME and with the addition of nutrients with a ratio of 2:1, while the lowest one (0.306/day) was obtained at 100% POME concentration.

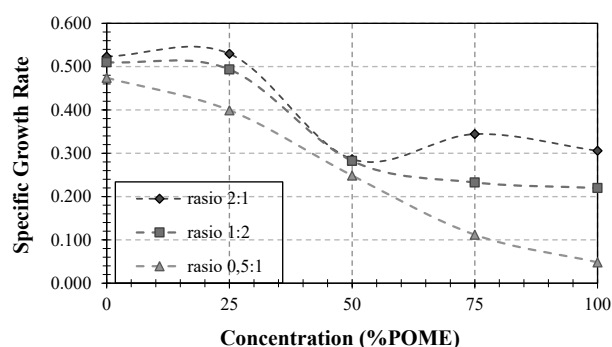
### Lipid production

Lipid content was strongly influenced by several factors, such as medium composition, light intensity, nutrition, and pH (25). Changes in these factors caused differences in lipid content in cells. In this study, the highest lipid content of *C. pyrenoidosa* was 36% (dry weight) on the 11<sup>th</sup> day of cultivation. This value was obtained in the variation of 25%v POME medium and with the addition of urea: TSP (2:1). The lipid content of *C. pyrenoidosa* can be seen in Figure 3.

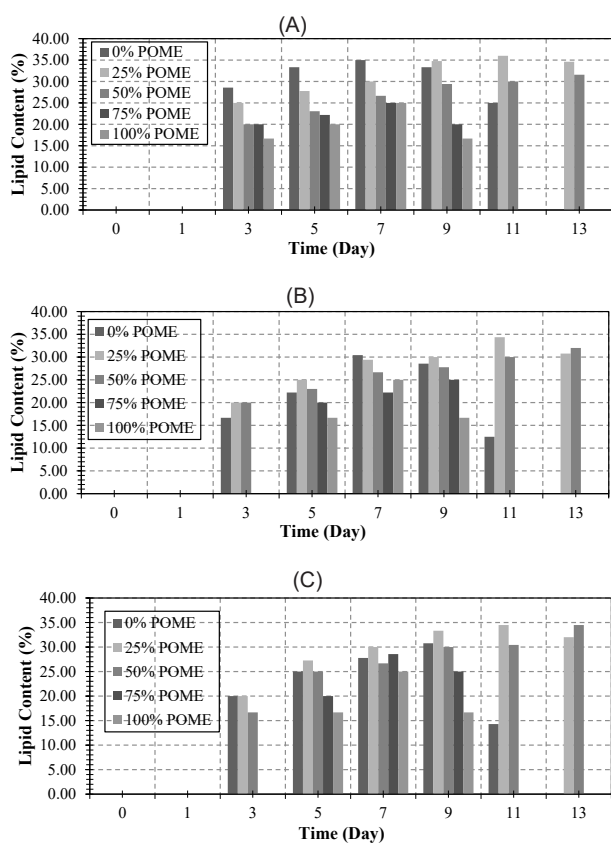
As shown in Figure 3, the highest lipid content was found after the addition of urea: TSP (2:1). It can be seen that the addition of nutrients in the form of urea and TSP resulted in the increase of lipid content, while nitrogen and phosphate derived from urea and TSP had important functions in the process of cell metabolism, including chlorophyll synthesis, because high chlorophyll content would cause the photosynthesis process to run well (26). The relationship between cell density and lipid content can be seen in Figure 4.

### Allowance for COD, nitrogen, and orthophosphate

In the process of cultivation carried out for 13 days, the removal efficiency of COD, nitrogen, and phosphate were obtained to be 90.42, 70, and 81.12%, respectively, in the medium of 25% POME with the addition of urea: TSP (2:1). Microalgae can reduce the pollutant compounds found in POME, where organic compounds can also be utilized by microalgae as a source of energy and carbon



**Figure 2.** The specific growth rate of *C. pyrenoidosa* with a variation in the ratio of urea:TSP.

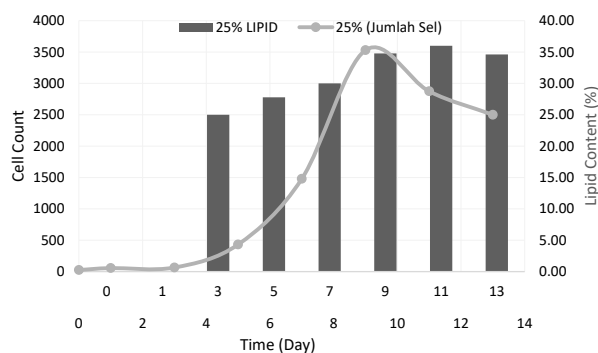


**Figure 3.** The relationship between waste concentrations and addition of nutrients with lipid content. (A) 2:1, (B) 1:2, (C) 0.5:1.

resources, which reduces the need for the additional nutrients for microalgae. Nutrients play an important role in the microalgae growth as a source of energy to produce biomass (26).

### Discussion

The results show that *C. pyrenoidosa* showed high specific growth rate and lipid content in some conditions. At high concentrations of POME, microalgae were not able to digest nutrients in the excess POME, so that it could reduce the digestibility and there was the possible production of toxic metabolites (27). At 0% concentration (control), the growth rate of *C. pyrenoidosa* was fast followed at 25% concentration, while at concentrations of 50, 75, and 100%, the growth of *C. pyrenoidosa* decreased. High POME concentrations can be a shortened phase of algae cell adaptation and takes a long time to reach the stationary phase, so produce in lower biomass due to the duration of the adaptation phase (20). The highest cell density was obtained to be  $3,530 \times 10^4$  cell/mL in the medium of 25% POME with the addition of urea: TSP (2:1). This value was obtained on the 9th day of cultivation in the exponential phase. In the exponential phase, the cell division started and marked by rising growth rate so that the population density increased (28). The addition of nutrients with a ratio of 2:1 produced the highest cell density in almost all variations of the medium concentration. This was because



**Figure 4.** The relationship between microalgae cell density and lipid content over time with optimum variables.

the addition of nutrients at a ratio of 2:1 contained suitable nutrients for the growth of *C. pyrenoidosa*. Consequently, it resulted in a high increase in cell growth rate as well. This result is consistent with the results reported by Lee (29), who found that in addition to low cell density, microalgae lacking nitrogen and phosphate (0 ppm) also had a less green color compared to those with higher concentrations of nitrogen and other phosphates. At the beginning of the phase (day 0 to day 5), microalgae cells would accumulate large amounts of chlorophyll molecules when nitrogen and phosphate sources were still abundant, then, when the extracellular nitrogen source completely consumed, the cells would start to use chlorophyll as a source of intracellular nitrogen, which resulted in a reduction in chlorophyll concentration in the cell. The increase of cell density could be seen from the magnitude of the average growth rate of cell in each phase (30). When urea and TSP were added to the POME medium, microalgae growth would become faster, but the lipid content at the beginning of cultivation tended to be lower. This happened because, in the early phases of growth, the microalgae synthesized more proteins for cell growth and propagation (31). When the microalgae reached the stationary phase, the lipid content in the microalgae would increase. This happened because in this phase, the nutrient content in the culture media started to run out and more accumulated of microalgae through photosynthesis resulted in the production of lipids (32). In the process of cultivation carried out for 13 days, removal efficiency of COD, nitrogen, and phosphate was obtained to be 90.42, 70, and 81.12%, respectively, in the medium of 25% POME with the addition of urea: TSP (2:1). Microalgae can reduce the pollutant compounds found in POME, where organic compounds can also be utilized by microalgae as a source of energy and carbon resources, which reduces the need for the additional nutrients for microalgae (33). Nutrients plays an important role in the microalgae growth as a source of energy to produce biomass. Microalgae cell growth is inversely proportional to the content of nutritional parameters in the POME. In other words, by increasing the microalgae amount, the COD, nitrogen, and phosphate decrease. This is because



microalgae cells grow by utilizing organic ingredients in the waste as nutrients for growth (25).

### Conclusion

This study examined the growth of *C. pyrenoidosa* with a variation in the POME concentration and addition of urea and TSP. At 25% POME concentration and with addition of urea: TSP (2:1), a better medium for the growth of microalgae with the highest number of cells ( $3.530 \times 10^7$  cells/mL) and the highest lipid content of 36% in dry weight was obtained. The removal efficiency of COD, nitrogen, and orthophosphate ( $P-PO_4$ ) were 90.42%, 70%, and 81.12%, respectively.

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### Ethical issues

The authors hereby certify that all data collected during the study are as stated in the manuscript, and no data from the study has been or will be published separated elsewhere.

### Competing interests

The authors declare that they have no conflict of interests.

### Author's contributions

The authors contributed and were involved in the problem suggestion, experiments design, data collection, and manuscript approval.

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