Enhancement of *Chlorella vulgaris* Biomass Cultivated in POME Medium as Biofuel Feedstock under Mixotrophic Conditions

M.M. Azimatun Nur*[1] & H. Hadiyanto[2]

[1]Chemical Engineering Department, Faculty of Industrial Technology
UPN "Veteran" Yogyakarta, Jl. SWK 104, Condongcatur, Sleman,
Yogyakarta 55203, Indonesia
[2]Center of Biomass and Renewable Energy, Diponegoro University,
Jl. Prof. Soedarto S.H., Tembalang, Semarang 50275, Indonesia
*Email: lanaazim@upnyk.ac.id

Abstract. Microalgae cultivated in mixotrophic conditions have received significant attention as a suitable source of biofuel feedstock, based on their high biomass and lipid productivity. POME is one of the wastewaters generated from palm oil mills, containing important nutrients that could be suitable for mixotrophic microalgae growth. The aim of this research was to identify the growth of *Chlorella vulgaris* cultured in POME medium under mixotrophic conditions in relation to a variety of organic carbon sources added to the POME mixture. The research was conducted with 3 different carbon sources (D-glucose, crude glycerol and $NaHCO_3$) in 40% POME, monitored over 6 days, under an illumination of 3000 lux, and with pH = 7. The biomass was harvested using an autoflocculation method and dry biomass was extracted using an ultrasound method in order to obtain the lipid content. The results show that *C. vulgaris* using D-glucose as carbon source gained a lipid productivity of 195 mg/l/d.

Keywords: *Chlorella vulgaris; POME; Mixotrophic cultivation; biofuel feedstock.*

1 Introduction

Indonesia is well known as the largest palm oil producer in the world. Palm oil mill effluent (POME) wastewater is generated as an output of the production of palm oil [1]. About 1 ton of fresh fruit bunch (FFB) can be converted to 0.66 ton of POME and 0.2 of ton crude palm oil [2]. Most of the POME in Indonesia is treated using traditional anaerobic open ponds to reduce COD and BOD contents.

In Indonesia, energy availability will become a serious issue within the next 20 years. The consumption of energy tends to increase over time, while the production of fossil oil decreases (Figure 1). Therefore, searching for new sources of renewable energy is required to face a possible future energy crisis.

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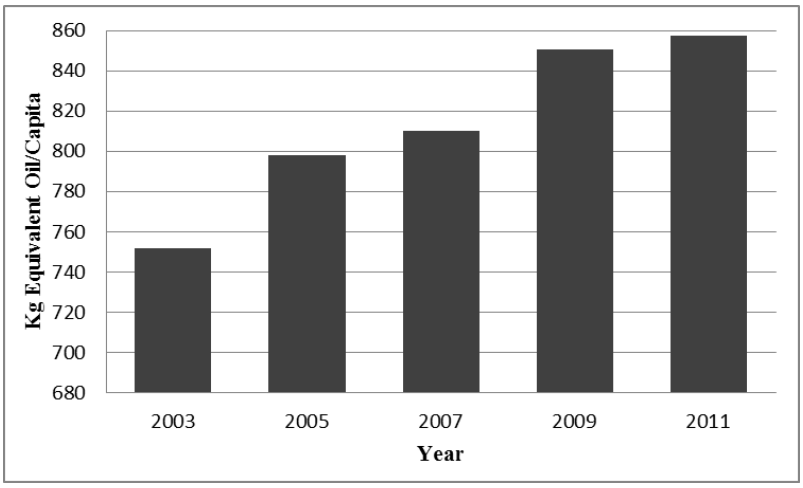

Figure 1 Indonesia energy consumption equivalent per capita [3].

One of the most promising technologies for Indonesia that should be explored is microalgae-based biofuel. Only limited microalgae can produce lipids for biofuel and utilize agro-industrial wastewater as a growth medium [4]. Digested POME is rich in nutrients, such as nitrogen and phosphorus, and may potentially be used as a medium for microalgae growth [5]. This medium could reduce the need for synthetic fertilizers for microalgae cultivation, bearing in mind that the cost of nitrogen and phosphorus fertilizer increases almost every year [6]. However, the main problem of POME is its high turbidity and dark color. These characteristics lead POME as medium for microalgae growth to create mixotrophic conditions.

Several microalgae can grow well under autotrophic conditions by utilizing inorganic material and light, while others grow in heterotrophic conditions by utilizing organic material without light as energy source. Mixotropic conditions are conditions in which microalgae grow under organic and inorganic carbon sources and are more flexible with light utilization. Microalgae consume organic and inorganic carbon sources and light as energy to grow in a complex reaction. Thus, the microalgae require less energy and accumulate more biomass [7-10].

Heredia-Arroyo, *et al*. [11] cultivated *C. vulgaris* under mixotrophic conditions using glucose, glycerol, acetate and a mixture of these organic carbons. The results showed that lipid productivity was increased by adding glucose and glycerol to the medium. Meanwhile, Hadiyanto and Azimatun-Nur [12] revealed that *Chlorella* sp. can grow well in a POME medium, resulting in a 34 mg/L/day lipid productivity, while the lipid profile was suitable for biodiesel

feedstock. The purpose of the present research was to investigate the growth, biomass and lipid accumulation of *Chlorella vulgaris* cultivated in palm oil mill effluent (POME) under mixotrophic conditions and without adding synthetic fertilizer.

2 Materials and Method

2.1 Cultivation Medium

POME was collected from the PTPN VII Lampung (Indonesia) 4th aerobic lagoon. The POME was filtered using 80-mesh filter cloths to reduce the total amount of suspended solids. The POME in this research contained a COD of 1620 ppm and total nitrogen of 284 ppm.

2.2 Culture *Chlorella vulgaris*

Chlorella vulgaris was obtained from the Center of Biomass and Renewable Energy (CBIORE), Diponegoro University, Semarang, Indonesia. The inoculum was cultivated in a modified medium [12,13] consisting of 40 ppm urea, 20 ppm TSP (triple super phosphates), 10 ppm NH_4SO_4, 1 ppm $FeCl_3$ and 25 μg/l vitamin B12. The medium was used as the control variable under autotrophic conditions.

2.3 Cultivation Condition

Chlorella vulgaris was cultivated in 3 different carbon sources: D-glucose, crude glycerol and $NaHCO_3$. The growth rate of the algae was evaluated for each carbon source, with variation of carbon source concentration between 0 and 1200 g/l. In the experiment, inoculums of microalgae 10% v/v (0.7 OD_{680}) and 40% v/v POME wastewater were mixed and diluted with distilled water in a 1L glass flask. The medium was conditioned to have a 3000 lux intensity, pH 6.8-7.2 acidity, 26-28°C temperature, 2 ppt salinity, and was aerated using an aquarium air pump to mix the medium.

2.4 Measurement

The concentration of biomass was measured using a spectrophotometer (Optima sp-300) at 680nm wavelength for 6 days. The optical density was plotted in dry biomass to develop the regression between optical density and dry biomass (gr/l).

The specific growth rate (μ) was calculated using the equation from Hadiyanto and Azimatun-Nur [12] for the logarithmic growth phase (Eq. (1)).

$$\mu = \frac{\ln(OD_1) - \ln(OD_0)}{t - t_0} \quad (1)$$

OD_1 is the optical density on the last day of cultivation, OD_0 is the optical density on the first day of cultivation, t is the end time of cultivation (day), and t_0 is the starting time of cultivation (day).

2.5 Biomass Harvesting

The medium was harvested using an autoflocculation method. NaOH 0.5 M was used to increase the pH to 10.5. Then the microalgae suspension was intensively mixed (1000 rpm) for 10 min, followed by gentler mixing (250 rpm) for an additional 20 min. Subsequently, the suspension was settled for 30 min [14]. Biomass was dried at 55°C on a tray dryer for 2 hours and the total biomass product was weighed.

2.6 Lipid Extraction

Lipid extraction was applied using an ultrasound extraction method [12,15]. Dry biomass was extracted using n-hexane (1:10 gr/ml). A mixture of dry biomass and n-hexane was placed in an ultrasound-assisted Branson 2510-DTH at 40 kHz for 30 minutes with a temperature of 35°C. N-hexane containing lipids was separated using Whatman filter paper, grade 2. The solvent was removed by distillation until no more solvent was collected. The dried residue was extracted again for three more replications. The total amount of lipids was weighed (W_2). The lipid content was calculated using Eq. (2).

$$\text{L}=\frac{W_2}{W_1}.100\% \quad (2)$$

L is lipid content (%), W_1 is the total biomass product in dry form (mg/l), W_2 is the total lipid content (mg/l). Lipid productivity (Y,mg/l/day) was calculated using Eq. (3) [11].

$$Y = \mu \,.\, L \,.\, W \quad (3)$$

where Y is the lipid productivity in mg/l/day, μ is the specific growth rate of cultivation in d^{-1}, L is the lipid content (%), and W is the total dry biomass (gr/l).

3 Results and Discussion

3.1 Growth Rate and Biomass Production

3.1.1 Growth Rate

The highest growth rate according to the type of carbon source was obtained using glucose, followed by glycerol and $NaHCO_3$ (Table 1). Due to different carbon and nitrogen ratios in the medium, the growth rate of microalgae was not constant. A lack of carbon seems to inhibit microalgae growth. Moreover, 40% POME containing 648 ppm COD provided 243 ppm of carbon (calculated from 12/32 COD) and gave a 2.15 C/N ratio. However, the optimum C/N ratio for microalgae growth is approximately 6.22 C/N. Therefore, more carbon was needed for the microalgae to grow. It seems that additional organic carbon sources in POME (i.e. glucose 1000 ppm) influence the C/N ratio that the microalgae require and consequently influence the growth rate. When 1200 ppm of D-glucose was added, the growth rate of *C. vulgaris* decreased due to the toxic content and the very high C/N ratio (56/9), which inhibits growth [16]. D-glucose is preferred to other organic sources (i.e. sugar, sugar alcohol, organic acid) due to its high energy content and its ease in assimilating in cells. However, a high concentration of glucose inhibits growth, as the metabolic transport system in the cell is disturbed [10,17]. Schmidt, *et al*. [18] found that yields of microalgae decreased as glucose or fructose were increased to more than 166 gr/l. Meanwhile, Wen and Chen [19] recorded decreasing yields when the medium was added from 1 to 40g/l. However, the effect from the addition of a high concentration of D-glucose, which inhibits microalgae growth, is not really clear [10].

Table 1 Growth rate (d^{-1}) in 40% POME using addition of different carbon sources.

Addition (ppm)	Growth rate (d^{-1})		
	D-glucose	Glycerol	$NaHCO_3$
200	0.572	0.188	0.317
400	0.679	0.251	0.261
600	0.935	0.328	0.178
800	1.106	0.230	0.146
1000	1.406	0.181	0.110
1200	1.356	0.173	0.074

Addition of $NaHCO_3$ resulted in a lower growth than that of organic sources. Microalgae do not seem suitable to consume inorganic carbon sources in mixotrophic conditions. When inorganic carbon was added, growth rates were

lower due to inhibition factors. On the other hand, for glycerol the highest growth rate was recorded for an addition of 600 ppm. It seems that toxic matter in crude glycerol (i.e. residual methanol, FFA, etc.) could inhibit the growth of *C. vulgaris*.

Several researchers have reported that the addition of organic carbon sources to the wastewater will influence the growth rate of microalgae. Mixotrophic conditions limit growth due to both the consumption of organic carbon and of light as photosynthetic energy at the same time. Moreover, the nitrogen and phosporus content also decreases, due to biomass formation and the high driving force that is supplied by light and organic carbon sources [20-22].

3.1.2 Biomass Production

Microalgae cultivated in the D-glucose additive gave a high biomass production in the first day of cultivation, which gradually decreased (Figure 2). The highest biomass production was produced from 1000 ppm D-glucose addition, at about 1.4 gr/l dry weight, followed by a concentration of 1200 ppm. When using glycerol, the highest recorded biomass production from 600 ppm was about 0.98 gr/l dry weight on the 2nd day with a drop on the 3rd day. This phenomenon reveals that in mixotropic conditions, D-glucose is the simplest carbon that can be utilized by the microalgae as an organic carbon source instead of glycerol [10].

Bhatnagar, *et al*. [21] reported that the addition of 1% w/v glucose and 250mg/l of N as $NaNO_3$ into wastewater provided the highest biomass yield compared to BG11 addition and without any supplementation. As mentioned before, an organic carbon source and light can be assimilated in a metabolic cell to produce biomass. D-glucose provides the highest biomass yield compared to other carbon sources due to its high energy content. According to Figure 2(a), the highest biomass was recorded on day 1. This means that the D-glucose was consumed rather fast and that biomass was produced more rapidly than with addition of glycerol.

When glycerol was supplemented in the medium, see Figure 2(b), the biomass yield increased gradually. Meanwhile, a lag phase (acclimation phase) occurred when glycerol was added as carbon source [10]. It seems that the influence of toxic content in crude glycerol, such as methanol or FFA, had a negative effect on the microalgae. However, this addition provides a better result in yielding biomass compared to addition of $NaHCO_3$, due to the assimilation of energy from light and organic carbon, as reported by Ceron Garcia, *et al*. [23].

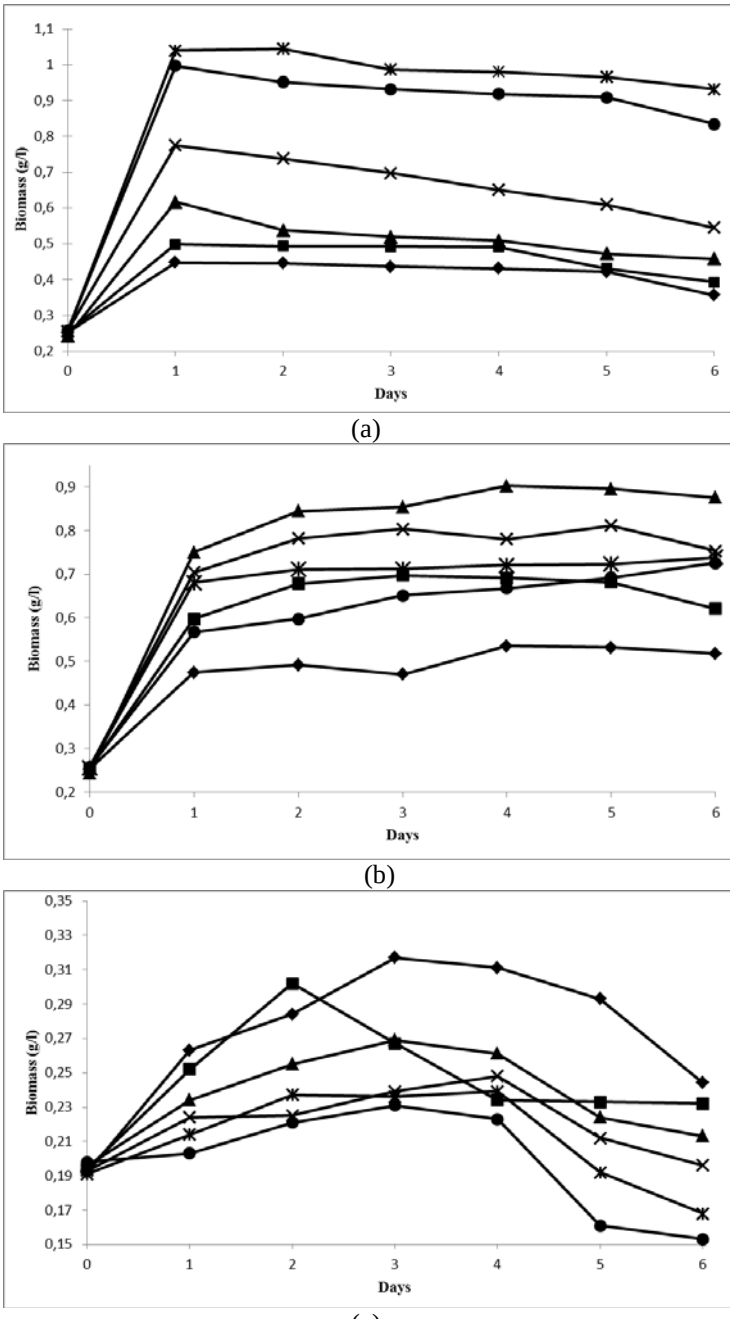

Figure 2 Growth phase of *C. vulgaris* in 40% POME. (a) D-glucose, (b) glycerol, (c) $NaHCO_3$. Note: ◆ = 200, ■ = 400, ▲ = 600, × = 800, ∗ = 1000, ● = 1200 ppm.

When $NaHCO_3$ was used as the source of carbon under mixotrophic conditions, the biomass yield had a negative result due to the limitation as described in the previous section. According to Figure 2(c), the highest biomass yield was recorded on day 3, after which it entered into death phase. It seems that *C. vulgaris* preferred organic carbon in the POME because the medium had changed to mixotrophic as its color inhibited light penetration. Figure 2(c) also describes the negative effect of bicarbonate supplementation to the medium. This means that in mixotophic medium, the inorganic carbon source is not assimilated in the cell body. However, when $NaHCO_3$ was used under autotrophic conditions, biomass production reached up to 0.7 gr/l of dry weight on the 5^{th} day due to photosynthetic reactions.

3.2 Lipid Content and Lipid Productivity

The lipid product of each organic carbon source was extracted from the algae biomass. Lipid productivity obtained in D-glucose was higher than in glycerol (Table 2).

As mentioned before, glucose provides a higher energy source compared to an organic carbon source. When nitrogen content (282 ppm N-total) in the POME medium was depleted, the microalgae tended to accumulate energy from the carbon as a lipid. In normal cultivation conditions, nitrogen is required to synthesize protein content into biomass. A lack of nitrogen will disturb the metabolic reactions of the cells, thus carbon sources that contain energy will tend to be stored in the form of lipid content [10], which occurs due to the imbalances and stress conditions of the cultivation environment [24]. This phenomenon was also recorded in our previous research [12].

Table 2 Comparison of lipid productivity results.

Medium	Growth rate (d^{-1})	Biomass content (g/L)	Lipid content (%)	Lipid productivity (mg/L/d)	Source
Digested Dairy Manure	0.474	1.7	14	112.8	[25]
MSG Wastewater	1.803	1.6	14	403.8	[26]
Artificial Wastewater	0.663	1.7	33	371.9	[27]
2^{nd} Municipal WW	0.458	0.67	31	95	[28]
20%POME	0.749	0.73	6.9	37.7	[12]
40%POME + D-glu	1.406	1.43	9.7	195	This study
40%POME + Gly	0.328	0.98	7.3	23.5	This study

4 Conclusion

Cultivation of *C. vulgaris* in 40% POME with the addition of different carbon sources was performed in this experiment. The carbon concentration and type of carbon source influenced biomass, growth rate and lipid accumulation to a high degree. The addition of 1000 ppm D-glucose to the medium provided a lipid productivity of 195 mg/l/day, while the use of 600 ppm glycerol resulted in 23.5 mg/l/day. Overall, it has been shown that utilizing mixotrophic conditions can be an alternative method for microalgae cultivation in POME medium for the generation of biomass to be used as biofuel feedstock.

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