Artificial produced water as a medium to grow *Chlorella* sp. for biodiesel production

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Abstract: *Increased interest in renewable, carbon-neutral energy sources makes processing biodiesel from microalgae has become the objective for many researchers and companies. Some kind of wastewater including municipal, industrial and agricultural wastewaters have been identified as alternate growth mediums. Produced water is the largest byproduct of the oil and natural gas extraction process which constitutes of high concentration of pollutants, such as dissolved nitrogen, phosphorus, dissolved organic carbon, heavy metal and monocyclic aromatic compound like BTEX. The purpose of this study is to identify Chlorella* sp. *potential for producing lipid in artificial produced water. Variations made in this study consist of 0%, 25%, 50%, 75% and 100% volume of artificial produced water to the control Walne medium. The highest specific growth rate and biomass productivity of Chlorella* sp. achieved *by culture grown in 25% wastewater with a value of 0.225 day^{-1} and 0.175 g L^{-1}day^{-1}, respectively. The highest lipid yield and productivity in mixed culture of artificial produced water and Walne medium achieved by culture in 25% artificial produced water with value of 0.231 and 40.48 mg.L^{-1}.day^{-1}. C16 and C18 fatty acids which dominated the lipids of Chlorella* sp. *in all culture variations indicated that the lipid of Chlorella* sp. *were suitable for producing high quality biodiesel.*

1 Introduction

Over-consumption of petroleum based-energy has led to environmental problem such as global climate change and energy crisis. Due to that, there is a great demand for alternative resources of petroleum-based energy. Biofuels, produced from biomass, are promising alternatives to fossil-derived fuels due to several distinct advantages including carbon neutrality, reduced emissions of gaseous pollutants (e.g., carbon monoxide, CO_2, and sulfur oxides), continuous availability of biomass feed stocks, and their safety of production by farming [1]. One type of biofuels, biodiesel is a mixture of fatty acid methyl esters (FAME) which is conventionally produced by transesterification of vegetable oils or animal fats [2]. Microalgae have been recognized as promising oil feedstock for biodiesel production due to their rapid growth rate, high lipid content and their ability to grow in non-arable area without competing with food crops for land. Microalgae convert solar energy into chemical energy via CO_2 fixation.

However, biodiesel production from microalgae is expensive due to large amount of water and inorganic nutrients (nitrogen, phosphate, and CO_2) needed. In order to reduce production cost, many researcher has used wastewater as alternative nutrient source to grow microalgae and the wastewater also can be bioremediated prior to discharge. By this means, microalgae provides mutual benefit of producing biofuels while removing nitrogen, phosphorus, and organic carbon from wastewater [3]. Wastewater that had been used as nutrient sources for growing microalgae were agricultural waste, domestic waste and industrial waste.

Oil and gas industry produces a significant amount of industrial wastewater which trapped in underground formations that is brought to the surface along with oil or gas. This water has been in contact with hydrocarbon-bearing formation for centuries, it contains some of the chemical characteristics of the formation and the hydrocarbon itself. It may include water from the formation, water injected into the formation, and any chemicals added during the production and treatment processes. These varied sources of water reach the production well, and is known as produced water (PW) [4]. In general, PW contains a various concentration of hydrocarbons, phenols, BTEX (benzene, toluene, ethylbenzene, and xylene), heavy metals and many inorganic salts [5]. These complex constituents exhibit toxicity to environment and the toxicity increased with the increasing amount of produced water generation. Recent studies used microalgae to remove produced water pollutants and produce biomass [6,7,8]. *Chlorella*

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sp. removed 58. 89%, 73.38%, 30.75% of total nitrogen, total phosphorus and total organic carbon respectively [6]. *Chlorella* sp. also obtained dry biomass up to 0.2 g/L in 100% PW [6]. In other studies, *Nannochloropsis oculata* was able to remove carbon as chemical oxygen demand (COD) up to 54% and produced biomass up to 0.311 g/L in 50% PW loading [7].

Due to the presence of organic constituent in produced water, cultivating microalgae in PW is mixotrophic cultivation. Organic carbon and light energy are simultaneously supplied to algae in this type of cultivation. This strategy of fixing CO_2 and assimilating organic carbon supplied concomitantly promises to greatly increase the cell concentration and lipid content of microalgae [9]. Green microalgae, *Chlorella* which commonly applied for human food, animal feed and bioactive compounds, recently reported as the potential biodiesel feedstock due to their high lipid productivity and environmental adaptation. Mixotrophic grown *Chlorella sorokiniana* had a total lipid content of 34.7%, higher than the photoautotrophically grown cells (9%) and heterotrophically grown cells (17.6%) in 8 g/L initial glucose [9]. In other studies, *Chlorella vulgaris* obtained total lipid content of 25.4% in 1/800 monosodium glutamate wastewater (MSGW) concentration, higher than photoautotrophically grown cells (22.65%) [3]. The FAME composition of *Chlorella vulgaris* grown in domestic wastewater consisted of C16:1 (Methyl Palmitoleate) C17:0 (Methyl Heptadecanoate), C18:0 (Methyl Stearate), C18:1 (Methyl Oleate) and C18:2 (Methyl Linoleate). These fatty acids are usually found in other oil bearing crops such as soybean, sunflower and palm oil. which have been confirmed to be suitable for biodiesel production [10].

There have been many works on producing biodiesel from mixotrophically grown *Chlorella* sp. and few works on cultivating microalgae in PW, but there is still no report investigates biodiesel production in PW grown microalgae. More details on total lipid content and distribution of fatty acids in PW grown microalgae are still required. To meet this call, this study explored the feasibility of growing *Chlorella* sp. in artificial produced water and its potential as biodiesel feedstock.

2 Methodology

The studies were conducted with the marine microalgae *Chlorella* sp. from the culture collection of Biochemistry Laboratory of Chemistry Department, Institut Teknologi Bandung. Five cultivation experiment were carried out to evaluate the growth conditions, lipid content and fatty acid content of *Chlorella* sp. in five different concentrations of artificial produced water (from 0 to 100%). Cell densities, dry biomass weight, organic content and lipid content of microalgae were analyzed every day. At the end of cultivation, *Chlorella* sp. was collected to be dried and extracted for its lipid. The lipid then was transesterified to obtain fatty acid methyl esters (FAMEs) or biodiesel. Analysis of the fatty acids was done to determine the potential of *Chlorella* sp. as a biodiesel feedstock.

2.1 Making growth media and artificial produced water

Nutrients media used for growth of *Chlorella* sp. was Walne media. The artificial produced water used in this study refered to the characteristics of produced water from an Indonesian oil field, located in Balikpapan [11]. The chemicals used to make artificial produced wastewater were crude oil, NH_4Cl, phenol, KNO_3, KH_2PO4 and Na_2S. Artificial produced water used for cultivation was prior filtered to remove suspended solids and dispersed oils which could block the entry of light into the culture. Walne medium and artificial produced water were also sterilized by autoclaves to eliminate contaminants from other microorganisms that could disrupt growth. Table 1 shows the characteristics of artificial produced wastewater.

Table 1. Characteristics of artificial produced water

No	Parameter	Unit	Artificial produced water
1	pH	-	8.19
2	Temperature	^oC	24.7
3	COD	mg/L	4100
4	Oil and grease	mg/L	48.08
5	Nitrate	mg/L	2.544
6	Nitrite	mg/L	0.26
7	Total Nitrogen	mg/L	29.74
8	Total Phosphate	mg/L	0.22
9	Ortophosphate	mg/L	0.057
10	TDS	mg/L	10730
11	Ammonia	mg/L	26.572
12	Phenol Total	mg/L	323.42
13	Total Sulfide	mg/L	0.778

2.2 Cultivation procedure

2.2.1 Preliminary cultivation

As a preliminary cultivation and in order to attain a sufficient initial inoculums cell density. *Chlorella* sp. was cultured by adding 10 mL inoculate in 1 L glass flask containing 810 mL of Walne nutrient media, aerated with ambient air with surrounding temperature ranging from 25 to 28 ^oC and illuminated with 4000 lux light intensity under 12:12 h light/dark cycle. This inoculation was lasted until four days or until *Chlorella* sp. reaching the exponential phase. The contents of Walne were as follows: $FeCl_3$ 0.87 mg/L, $MnCl_2.4H_2O$ 0.36 mg/L, Na_2EDTA 54.24 mg/L, $NaH_2PO_4.2H_2O$ 17.66 mg/L, $NaNO_3$ 100.264 mg/L, $ZnCl_2$ 0.021 mg/L, $CoCl_2.6H_2O$ 0.011 mg/L, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.009 mg/L, $CuSO_4.5H_2O$ 0.02 mg/L, Vitamin B1 (Thiamine HCl) 10×10^{-5} mg/L and Vitamin B12 (Cyanocobalamin) 5×10^{-6} mg/L.

2.2.1 Cultivation in artificial PW

Chlorella sp. was grown in five different concentrations of artificial PW media (from 0 to 100%) using 8 L working volume. Microalgae grown in 0% artificial produced water (Variation 0%) used as control in this study. Microalgae that had grown in preliminary cultivation was re-inoculated in each artificial PW media concentration. Table 2 demonstrates the cultivation experiment in PW media.

Table 2. Five variations of cultivation performed in 8 L total working volume

Variation	Volume of Walne medium (%)	Volume of artificial PW (%)	Volume of Walne medium (L)	Volume of artificial PW (L)
0%	100	0	8	0
25%	75	25	6	2
50%	50	50	4	4
75%	25	75	2	6
100%	0	100	0	8

All cultivation experiments were carried out in a cylindrical plastic container with 10 L capacity placed within closed incubator with dimensions of length × width × height of (1.5 × 0.6 × 0.6) m^3, made from plywood and coated with aluminum foil. Microalgae cultivation was carried out in batch system at 25 to 28 °C and 4000 lux light intensity under 12:12 h light/dark cycle. Continuous filtered air with constant flow was introduced into the reactor using aquarium air pumps with 1 LPM output flow. All the batch experiments were carried out in duplicate

Cultivation was carried out in 13 days. Sterile pipette was used for sampling microalgae culture to prevent contamination from other microorganisms.

2.3 Determination of *Chlorella* sp. growth

Microalgae growth was measured everyday by cells count in a Neubauer Hematocytometer using light microscope with 40× final magnification [1] and biomass concentration as dry weight (g/L) and organic content (g/L).

Dry weight of microalgae was determined by separation from the media using centrifugation. 200 mL cell suspension was centrifuged at 4000 rpm for 30 minutes and then dried at 105 °C for an hour and subsequently cooled to room temperature in a desiccator before weighting [1]. Organic content of microalgae was determined by putting dry biomass obtained prior into furnace at 600 °C for an hour and subsequently cooled to room temperature in a desiccator before weighting. Standard deviations of the average values are presented on diagrams.

The specific growth rate (μ) at the exponential phase was calculated by

$$\mu = \frac{(\ln X_2 - \ln X_1)}{t_2 - t_1} \quad (1)$$

where X_2 and X_1 were cell density/ dry biomass/ organic content of microalgae (g/L) at time of t_2 and t_1, respectively. The relationship between specific growth rate and carbon concentration as substrate was fitted to a Monod growth kinetic model using the equation:

$$\mu = \mu_{max} \frac{S}{K_\mu + S} \quad (2)$$

where μ was the specific growth rate (day^{-1}) calculated during the linear portion of exponential phase growth (Equation 1), μ_m was the maximum specific growth rate (day^{-1}), S was the C concentration (mg/L C), and K_μ (half saturation constant mg/L C) was the C concentration at $\mu_m/2$ [12]. The cells yield was determined by subtracting the highest cell abundance (X_{max}) form the minium value (X_0) [8].

$$X = X_{max} - X_0 \quad (3)$$

Biomass productivity (P) was determined by using equation:

$$P = \frac{(X_2 - X_1)}{t_2 - t_1} \quad (4)$$

2.4 Lipid extraction

The total lipid content of *Chlorella* sp. was analyzed gravimetrically everyday by the soxhlet extraction method. The solvent used in this extraction was n-hexane. An aliquot (200 mL) of the sample was collected and the algal pellets were then dried using an oven (105 °C, 1 hour). The dried algal pellets were extracted with 150 mL of n-hexane for 4 hours of heating. After the extraction process finished, the results lipids were distilled to separate the lipids and n-hexane solvent. After that, the lipid was weighed. Lipid yield (Y) and productivity (L_p) was calculated by

$$Y = \frac{(L_f - L_0)}{(X_f - X_0)} \quad (5)$$

$$L_P = \frac{X_f \times LC}{Vol \times \Delta t} \quad (6)$$

where L_f and L_0 were the final and initial lipid concentration (g/L). X_f and X_0 were the final and initial biomass concentration (g/L). LC was lipid content (%). Vol was working volume and Δt was cultivation time (13 days).

2.5 Preparation of FAME

The lipid sample (100 mg) was added to an erlenmeyer followed by $NaOCH_3$ (2 mL). The erlenmeyer was the covered with aluminium foil paper and then placed at the shaker with a speed of 150 rpm, at 60°C for ± 24 hours. After cooling to room temperature, 9.5 mL methanol and 238 μL 98% H_2SO_4 were added. The reaction continued in a shaker again with a speed of 150 rpm, at 60°C for 1 hour. After cooling to room temperature, the mixture

was transferred to a centrifuge tube (50 mL) followed by 19 mL NaCl (5% w/v) and 11.9 mL n-hexane. The esters formed were extracted in hexane. The tubes were vortexed and centrifuged (4500 rpm for 10 minutes), resulting in two immiscible phases. The upper phase which was the hexane layer with FAMEs was dried over anhydrous sodium sulphate. The FAMEs was recovered into a vial and subjected to gas chromatography-mass spectrometry (GC-MS) analysis.

2.6 Fatty acid methyl esters analysis

The fatty acid composition of biodiesel was performed in a Shimadzu QP2010 Ultra gas chromatograph (GC). The GC condition were as follows: coloumn DB-5, a column temperature program that specified an injector temperature of 280 ºC, an oven temperature program of 60-280 ºC (4 ºC/min), use of N_2 as a carrier gas and velocity in the coloumn was controlled at 41,7 cm/s.

3 Result and discussions

3.1 Microalgae growth

The growth of *Chlorella* sp. in different concentrations of produced water was indicated by cell densities, dry weight, organic content and total lipid content.

3.1.1 Cell density of Chlorella sp.

To Examine the possibility of utilization of the mixture of artificial produced water (PW) and Walne medium to cultivate *Chlorella* sp., the algal cells were cultured in five different concentrations of artificial produced water. Artificial produced water made in this study had high concentration of organic carbon (see. Methodology) due to the presence of high concentration phenol and oil and grease. The result of growth experiments are shown in Fig.1. Growth result indicated an increased cell density for *Chlorella* sp. in all different concentration of artificial PW. The initial cell abundance used in all cultivation was 0.5 $\times 10^6$ cell/mL. Initial growth period for *Chlorella* sp. had an unnoticeable difference lag phase within all different artificial PW concentrations. The lag phase was three days in all variations.

Maximum day growth and maximum cell numbers of *Chlorella* sp. are shown in Table 3. The highest microalgae cell density was reached on 10-11th day of cultivation in all variations, except for the one grown in 100% artificial PW. *Nannochloropsis oculata* reached highest cell density on the 9th day in pure PW, while in 0% PW highest cell density was reached on 12th day [8]. Therefore, the dilution of produced water had an effect on the growth of microalgae. The highest cell yield of *Chlorella* sp. was achieved in 75% artificial PW and the smallest cell yield was achieved in 50% artificial PW (Table 3).

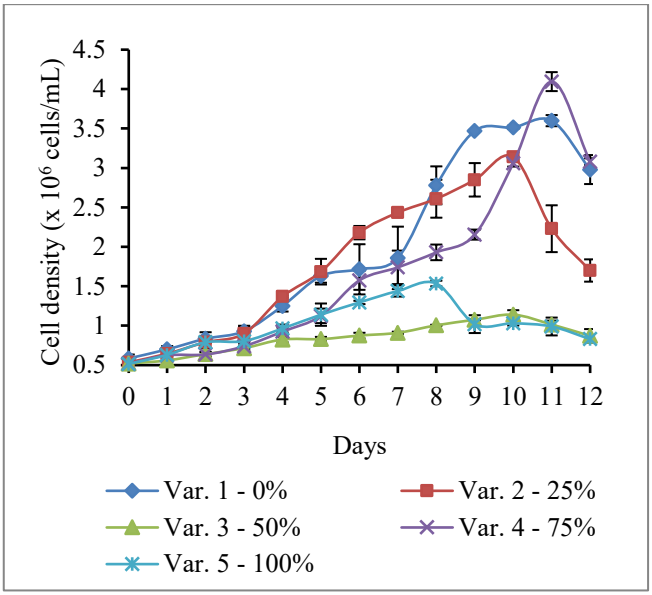

Figure 1. Growth curves of *Chlorella* sp. in artificial produced water with different concentrations

Table 3. Growth parameters of *Chlorella* sp. in five different concentrations

Variation	Max. day growth (d)	Max. cell number ($\times 10^6$ cells/mL)	Cell yield ($\times 10^6$ cells/mL)
0% (control)	11	3.6 ± 0.07	3.02 ± 0.021
25%	10	3.14 ± 0.057	2.61 ± 0.035
50%	10	1.14 ± 0.057	0.62 ± 0.071
75%	11	4.09 ± 0.12	3.58 ± 0.134
100%	8	1.53 ± 0.035	1.02 ± 0.028

It is very likely that the physiological stress caused by the compounds present in artificial produced water generated the lower results achieved in these experiments [8]. The cell yield of *Chlorella* sp. in 100% artificial PW was also smaller than that achieved by *Nannochloropsis oculata* (2.71×10^6 cells/mL) which was also grown in 100% produced water [8].

The specific growth rate at artificial PW loadings of 0%, 25%, 50%, 75% and 100% are shown in Table 4. Comparing the five different concentrations of artificial PW media, the specific growth rates of microalgae tends to be decreased with the increasing of artificial PW concentrations. The specific growth rate of *Chlorella* sp. in 100% artificial produced water was higher than that in *Nannochloropsis oculata* which achieved 0.09 day^{-1} using 100% produced water [8].

Table 4. Specific growth rate (μ) and cell yield of *Chlorella* sp. in five different concentrations

Variation	μ (day^{-1})
0% (control)	0.192 ± 0.021
25%	0.189 ± 0.001
50%	0.076 ± 0.001
75%	0.183 ± 0.004
100%	0.136 ± 0.005

Calculation of maximum growth rate (μ_{max}) and substrate constant (Ks) that achieved by microalgae *Chlorella* sp. was done with carbon substrate because carbon plays an important role in microalgae growth. Based on the calculation, it was obtained that μ_{max} was 0.1205 and Ks was 122.8. This showed that if *Chlorella* sp. was grown in cultures with carbon below 122,8 mg/L, the maximum growth rate will not be achieved.

3.1.2 Dry biomass of Chlorella sp.

Dry biomass and organic content were results of microalgae cell growth by assimilating nutrients in medium into their cells. Based on experiments, the results of microalgae dry biomass and organic content were shown in Figure 2 and Figure 3 below.

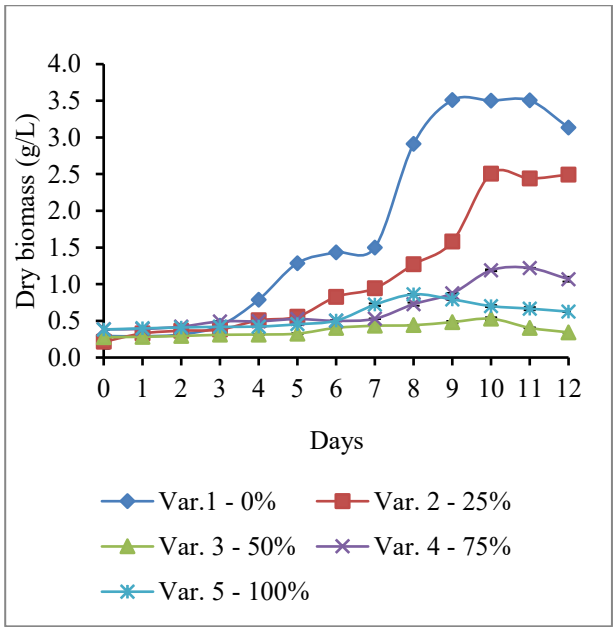

Figure 2. Dry biomass of *Chlorella* sp.

The growth of *Chlorella* sp. in control (Walne media) obtained the highest biomass concentration where after three days of lag period biomass growth up to 3.509 g/L. While *Chlorella* sp. grown in 25% and 50% artificial PW had five days of lag period. *Chlorella* sp. grown in 75% and 100% artificial PW had six days of lag period (Fig. 2). A different result was observed in *Chlorella* sp. which was grown in 100% produced water. had only 2 day of lag period [6]. This difference was probably due to different concentration of compounds present in produced water.

Table 5 shows maximum dry biomass concentration and biomass productivity achieved by *Chlorella* sp. in all five different concentrations of artificial PW. Highest biomass concentration was achieved by *Chlorella* sp. in 25% artificial PW. The biomass concentration dropped from 2.506 g/L to 0.86 g/L when the artificial PW concentration increased from 25% to 100% suggesting an inhibition of microalgal growth in high artificial PW concentration. Contaminates such as metals, nutrients and other organic compounds present in PW may justifies the lower biomass production in treatments where PW was increased.

Table 5. Growth parameters of *Chlorella* sp. in five different concentrations

Variation	Max. dry biomass (g/L)	Biomass productivity (g/L/day)
0% (control)	3.509 ± 0.002	0.219 ± 0.001
25%	2.506 ± 0.002	0.175 ± 0.007
50%	0.53 ± 0.018	0.005 ± 0.0002
75%	1.22 ± 0.002	0.052 ± 0.002
100%	0.86 ± 0.003	0.019 ± 0.0004

However, the maximum dry weight achieved by *Chlorella* sp. in 50% artificial PW was higher than those achieved by *Nannochloropsis oculata* (0.31 g/L) and *Isochrysis galbana* (0.314 g/L) in 50% produced water [7]. The maximum dry weight achieved by *Chlorella* sp. in 100% artificial PW was also higher than that achieved by *Chlorella* sp. (0.2 g/L) grown in 100% PW [6]

The highest biomass productivity achieved by *Chlorella* sp. in 25% artificial PW (Table 5). The biomass productivity decreases with increasing artificial PW suggesting an inhibition of microalgal growth in high artificial PW concentrations. Biomass productivity achived by *Chlorella vulgaris* in mixotrophic cultivation suppplied by bold basal media (0.180) was higher than highest biomass productivity of *Chlorella* sp. in this study. This may be attributed by some compounds in this artificial wastewater. But the biomass productivity of *Chlorella* sp. in 25% artificial PW was still higher than other cultivations of Chlorella grown in wastewater media. *Chlorella vulgaris* achieved biomass productivity of 0.0409 g/L/day and 0.0615 g/L/day in 0.02 v/v domestic water and 100- fold diluted MSG (monosodium glutamate wastewater), respectively [3,10]. Based on that, artificial PW seems becoming a better alternative wastewater medium to grow Chlorella.

Table 6. Specific growth rate of *Chlorella* sp. in artificial produced water

Variation	μ (day^{-1})
0% (control)	0.304 ± 0.0007
25%	0.225 ± 0.019
50%	0.068 ± 0.001
75%	0.104 ± 0.001
100%	0.092 ± 0.001

The cultivation of *Chlorella* sp. in the medium containing 25% artificial PW also recorded the highest specific growth rate (0.225 day^{-1}) (Table 6). This value was not as different compared with *Chlorella vulgaris* grown in 0.02 v/v domestic wastewater (0.3 day^{-1}) and in bold basal medium (0.29 day^{-1}) [10,13].

3.1.3 Lipid content of Chlorella sp.

Chemical solvent is the most reliable method in lipid extraction to determine total microalgae lipid content due to the high polarity of fatty acids [14]. In the present study, hexane was used as chemical solvent in extracting lipid of microalgae. Total lipid content of algal biomass for each variation was shown in Figure 3.

Based on measurements, the highest lipid content was achieved by the culture with 25% artificial PW (0.5695 g/L) on the 11th day. While the lowest lipid content was achieved by culture with 50% artificial PW, with value of 0.0638 g/L.

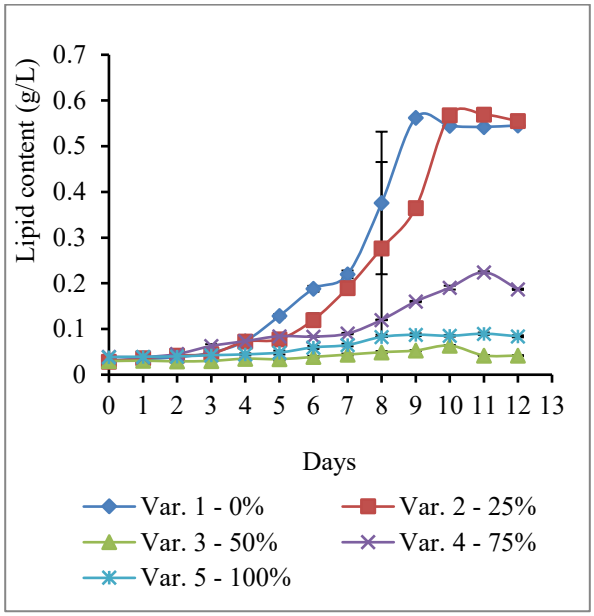

Figure 3. Lipid content of *Chlorella* sp.

The total lipid percentage of algal biomass in this study were between 12.04% and 23.33% under different artificial PW concentrations. These values were similar with *Chlorella vulgaris* (between 13.5% and 25.4%) which was cultivated under different MSGW concentrations [3]. Lipid percentage from the five culture variations was obtained by dividing the highest weight of lipids produced with the weight of dry biomass on that day. The highest lipid percentage was achieved by culture with 25% artificial PW, with value of 23.33%, then followed by 75% artificial PW, with value of 18.35% and the smallest lipid percentage was achieved by culture with 50% artificial PW with value of 12.04%. Whereas, lipid percentage of *Chlorella* sp. in 100% artificial PW was 13.47%. Lipid percentage of *Chlorella* sp. in 25% artificial PW which containing 80.86 mg / L phenol was close to that achieved by *Dunaliella salina* (21%) which was also cultivated in 100 mg / L phenol [15]. The highest lipid content of *Chlorella* sp. which was achieved in 25% artificial PW was also higher than those achieved by *C.pyrenoidosa* which was cultivated in glycerol (17.3%) and acetate (13.4%).

Lipid yield was calculated to determine lipid productivity in each gram of dry biomass (gram lipid / gram biomass). Yield lipid and lipid productivity of *Chlorella* sp. in each variation shown in Table 7.

Table 7. *Yield* and lipid productivity of *Chlorella* sp.

Variation	Yield Lipid	Productivity (mg/L.day)
0% (control)	0.181 ± 0.0003	39.596
25%	0.231 ± 0.01	40.481
50%	0.18 ± 0.006	2.614
75%	0.217 ± 0.013	11.385
100%	0.186 ± 0.004	3.5

Lipid productivity of *Chlorella* sp. in artificial PW was in the range between 2.614 and 40.481 mg/L/day. The largest lipid yield and lipid productivity were achieved by culture in 25% artificial PW. Comparing those five values of lipid productivity, the lipid productivity tends to be decreased with the decreasing of phosphate in cultures. The more concentration of artificial water, the less phosphate in media. This was consistent with lipid productivity achieved in *Chlorella zofingiensis*. Lipid productivity of *C. zofingiensis* when the amount of nitrogen and phosphate was sufficient in media, was 68.1 mg / L. day. However, when cultivated in cultures with phosphate deficiency, the productivity was reduced to 44.7 mg / L. days [16].

Highest lipid productivity achieved by *Chlorella* sp. in 25% artificial PW (40.481 mg/L/day) was higher than the highest lipid productivity achieved by *Chlorella vulgaris* (12.50 mg/L/day) which was cultivated in 1/100 monosodium glutamate wastewater [3]

3.2 Identification of Lipid *Chlorella* sp.

Fatty acid methyl ester (FAME) is the main component of biodiesel and therefore, the chemical composition of distinctive FAME profile plays a critical role in determining the properties of biodiesel produced [10]. A transesterification / alcoholysis process was carried out to convert triacylglycerol to FAME.

Identification of the types of lipids produced by microalgae *Chlorella* sp. performed by GC-MS (Gas Chromatography - Mass Spectometry) using fatty acid standards. Lipids analyzed were lipids produced from microalgae *Chlorella* sp. in all variations of culture. FAME composition of *Chlorella* sp. in those five different concentration of artificial PW was shown in Table 8.

Table 8. Fatty acids methyl esters of artificial produced water grown *Chlorella* sp. by GC-MS

Fatty acid	FAME composition (%)				
	0%	25%	50%	75%	100%
C14:0	0.9	0.6	-	-	-
C16:0	17.78	15.59	22.94	23.01	75.65
C16:1	4	7.91	-	6.08	-
C16:2	11.8	-	-	-	-
C18:0	2.66	1.75	-	5.59	-
C18:1	-	17.19	-	50.5	-
C18:2	17.48	44.68	54.55	13.85	12.24
C18:3	7.57	-	-	-	-
C20:0	2.4	-	-	-	-

Fatty acid	FAME composition (%)				
	0%	25%	50%	75%	100%
TFA	64.59	87.72	77.49	99.03	87.89
SFA	23.74	17.94	22.94	28.6	75.65
MUFA	4	25.1	-	56.58	-
PUFA	36.85	44.68	54.55	13.85	12.24

Note: SFA = Saturated Fatty Acid, MUFA= Monounsaturated Fatty Acid, PUFA = Polyunsaturated Fatty Acid

The overall FAME composition of *Chlorella* sp. was mostly consisted of palmitic acid (C16:0) and linoleic acid (C18:2). The amount of C16:0 in *Chlorella* sp. ranged between 15.59% and 75.65%. The amount of C16:0 was increased with increasing amount of artificial PW and it was the dominant fatty acid in the culture with 0% and 100% artificial PW. Whereas, the amount of C18:2 in *Chlorella* sp. ranged between 12.24% and 54.55%. Palmitoleate acid (C16:1) and stearic acid (C18:0) were also found in almost all culture variations. Myristic acid (C14:0) was only found in cultures with 0% and 25% artificial PW with value of 0.9% and 0.6%, respectively. Arachidic acid (C20:0) was only found in 0% artificial PW with value of 2.4%. Myristic acid (C14:0) and arachidic acid (C20:0) was found in small amounts, compared to other C16 and C18 fatty acids. The abundance of C16 and C18 fatty acids in 0%, 25%, 50%, 75% and 100% artificial PW were 61.29%, 87.12%, 77.49%, 99.03% and 87.89%, respectively. Palmitoleate acid (16:1) was found in the culture with 0%, 25% and 75% artificial PW. Stearic acid was found in culture with 0%, 25% and 75% artificial PW. Hexadecadienoic acid (C16:2) and linolenic acid (C18:3) were only found in the lipids of *Chlorella* sp. grown in 0% artificial PW. Oleic acid (C18:1) was present in culture of 25% and 75% artificial PW.

Based on Table 8 it can be seen that palmitic acid (C16:0) and linoleic acid (C18:2) are the dominant fatty acids found in the culture of *Chlorella* sp. in all variations. Bagul et al., 2017 states that microalgae oil which contains high levels of palmitic acid and linoleic acid can meet European standards on biodiesel [17]. The quality requirements of biodiesel in Indonesia itself are regulated by SNI 7182: 2015 with the test parameters of density at 40^{o}C, kinematic viscosity at 40^{o}C, cetane numbers, flash points, fog points, copper plate corrosion, carbon residues, water and sediment, 90% distillation temperature sulfuric ash, phosphorus, acid number, free glycerol, total glycerol, methyl ester content, iodine number, monoglyceride level and oxidation stability. From the nineteen parameters, parameters that can be assessed qualitatively based on the percentage of methyl esters were kinematic viscosity (KV), cetane number (CN), oxidative stability and methyl ester content. One parameter that is also important to determine the characteristics of biodiesel is the high heating value (HHV).

Based on kinematic viscosity (KV), cetane number (CN), oxidative stability, methyl ester content and high heating value (HHV), each culture variation was given a value of 1 to 5. A value of 1 indicates the composition of the FAME in culture was able to give the best results in those biodiesel parameters, while a value of 5 indicates the composition of FAME in culture could caused the worst results in those parameters. The value of the five parameters was determined by the composition of FAME contained in the lipids of *Chlorella* sp., so that a qualitative assessment can be made. Therefore, the percentage of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) was calculated in each culture (Table 8).

The cetane number (CN) is a dimensionless number showing the combustion quality of a diesel engine and is a leading indicator of biodiesel quality. The value of cetane numbers greatly influences engine performance parameters such as combustion and exhaust emissions and the higher value of cetane numbers is also associated with lower nitrogen oxide exhaust emissions. The branching and length of the molecular chains affect the value of cetane numbers, with their value decreased by decreasing the length of the chain and increasing branches in the molecular chain. Unbranched and long chain fatty acids produced good conventional biodiesel oil, which represents fast fuel combustion. A low cetane number indicates a long ignition delay which is a delay in fuel injection and the start of combustion. Higher CNs promote faster automatic ignition of fuel, and often lead to lower NOx emissions. So that the assessment of cetane numbers on biodiesel *Chlorella* sp. based on the percentage of C18 and C16 fatty acids in culture, because all fatty acids observed in the culture of *Chlorella* sp. in all variations had no branches, both the saturated and unsaturated fatty acids. The culture with the highest percentage of C18 and C16 fatty acids received a value of 1 in weighting, while the culture with the percentage of C18 and C16 fatty acids had the least value of 5 (Table 9).

Table 9. Weighting calculation for biodiesels in each culture variation

Parameter	Weighting value of biodiesel in each culture variation				
	0%	**25%**	**50%**	**75%**	**100%**
Cetane number	5	3	4	1	2
Kinematic Viscocity	5	3	4	1	2
Oxidative stability	4	3	5	2	1
High Heating Value	2	5	3	4	1
Methyl esters percentage	5	3	4	1	2
Total	21	17	20	9	8

High viscosity of biodiesel can cause operational problems, such as the formation of deposits on the engine. In addition, higher density and viscosity values caused poor atomization and poor mixing of fuel and air

which causes poor combustion and ultimately reduces engine efficiency. The viscosity value of a biodiesel is known to increased with increasing fatty acid chain length. So the weighting values for all cultures in this kinematic viscosity parameter are same as those in the cetane number parameter (Table 9).

The oxidative stability index is measured within a few hours and is related to the amount and position of unsaturation of fatty acids, which when damaged caused biodiesel oxidation. Oxidative stability decreased with increasing content of polyunsaturated fatty acids. So the weighting value of 1 is given to the culture with the smallest amount of polyunsaturated fatty acids (PUFA) (Table 9).

HHV is a unit of measurement of energy that is produced when a fuel has complete combustion. When HHV increased, fuel consumption will decrease [18]. HHV increased with increasing fatty acid chain length and decreased with increasing unsaturation of fatty acids. So the weighting value of 1 is given to the culture with the smallest amount of polyunsaturated fatty acids (Table 9).

Based on the weighting value, it can be seen that the *Chlorella* sp. in culture of 100% PW get the smallest weighting value, which means the composition of FAME contained in the culture of 100% artificial PW were capable in producing biodiesel with the best characteristics. Based on Table 9, it can also be concluded that the characteristics of biodiesel obtained by *Chlorella* sp. starting from the best were culture with 100%, 75%, 25%, 50% and 0% artificial. This shows the lipid of *Chlorella* sp. cultivated in 100% artificial produced water and in those mixtures of Walne medium and artificial produced water are able to produce biodiesel with better quality than that grown in Walne medium only.

4 Conclusions

Based on the results of the study, microalgae *Chlorella* sp. was able to grow in the mixture of Walne medium and artificial produced water. The highest specific growth rate and biomass productivity of *Chlorella* sp. achieved by culture grown in 25% wastewater with a value of 0.225 day^{-1} and 0.175 g $L^{-1}day^{-1}$, respectively. The highest lipid yield and productivity in culture of mixed produced water and Walne medium achieved by culture in 25% produced water with value of 0.231 and 40.48 $mg.L^{-1}.day^{-1}$. C16 and C18 fatty acids which dominated the lipids of *Chlorella* sp. in all culture variations indicated that the lipid of *Chlorella* sp. were suitable for producing high quality biodiesel. Based on qualitative calculations, biodiesel produced by *Chlorella* sp. in 100% produced water has the best biodiesel quality, based on parameters of cetane number, kinematic viscosity, oxidative stability, methyl ester content and high heating value.

5 References

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