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Growth rate measurements of *Chlorella vulgaris* in a photobioreactor by Neubauer-improved counting chamber and densitometer

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Abstract. The depletion of fossil fuel sources and increased CO_2 emissions has triggered intensive research to discover renewable energy sources. With the Low Carbon Scenario, the role of renewable energy will increase to 58% in 2050. As a renewable energy source, bioethanol is environmentally friendly which can substitute gasoline. Currently, the third-generation bioethanol production technology from microalgae is still being developed. *Chlorella vulgaris* is one of the microalgae types with high carbohydrate content and is easy and fast to cultivate. This study aims to evaluate the growth rate of *C. vulgaris* cultivated in a bubble column photobioreactor using artificial seawater aerated with pure CO_2. Two LED tube lights were used with 12 h light and 12 dark cycles for 12 – 13 days. Microalgae culture population was measured every 24 h using a Neubauer-improved counting chamber and a microscope equipped with a digital camera. The results showed that the maximum specific growth rate, μ_{max}, was found to be 0.344 d^{-1}, and the highest concentration of 1.88 $\times 10^7$ cells mL^{-1} occurred on day 7. Moreover, the microalgae populations were also measured using a densitometer. Since the calculation of the cell population used secondary data from the literature, the results were less accurate than those of the counting chamber.

1. Introduction

Fossil energy still dominates the Primary Energy Mix in Indonesia (91%), while renewable energy contributes to the rest [1]. The high consumption of fossil energy in the world has led to an increase in CO_2 emissions, which reached 37 Gt in 2017 [2, 3], which resulted in the global warming problem. Meanwhile, based on the supply chain, biomass-based energy can reduce the CO_2 emissions [4], even from the Life Cycle Assessment (LCA), which shows the negative CO_2 emissions [5]. Some researchers have investigated to find renewable energy sources with low CO_2 emissions [6–13].

Bioethanol, an alternative fuel, has promising prospects to reduce dependence on gasoline. Indonesia has targeted to have E-20, i.e., 20% of bioethanol in gasoline, in 2025. Bioethanol is produced from the fermentation of glucose. The first and second generations of bioethanol originate from food crops, agricultural waste, and plantations, while the third generation is produced from algae. Meanwhile, the fourth generation of bioethanol is produced by advanced biochemistry techniques and petroleum-like hydroprocessing. However, research on the third generation of bioethanol is still being carried out intensively to obtain the optimum process [14].

Sugar cane, maize, and cassava are the primary ingredients in the manufacture of first-generation bioethanol. Therefore, it requires large agricultural areas and high planting costs and can increase food prices. For the second generation, bioethanol is produced from biomass. However, the production of

this second-generation bioethanol requires high technology to convert lignocellulose into sugar and high energy for initial processing of biomass and low conversion rate. With the problems encountered in producing the first- and second-generation bioethanol, it is necessary to continue developing more efficient and economical bioethanol. For example, finding biomass that contains high carbohydrates and is easy in the pretreatment process.

Macro/microalgae are low-level plants that have the potential to become raw materials for bioethanol. Algae offers some advantages. They can overgrow in waters and seas. They do not require agricultural land, do not compete with food, do not need fertilizers, and produce bioethanol with high productivity, such as *Nannochloropsis*, *Botryococcus braunii*, *C. vulgaris*, and *Spirulina* [15]. Cultivation of microalgae can be conducted in both open-air and closed photobioreactors (PBR). Open-air PBR consists of either natural or artificial ponds, raceway ponds, and inclined surface systems driven by paddle wheels [16], while the closed ones comprise a horizontal or serpentine tube, flat-plate, bubble column, airlift column, and stirred tank [17]. A good PBR should have an optimal gas-liquid transfer rate, an effective illumination area, easy operation, low potential for contamination, low capital and production costs, and minimal land area requirements [18].

Chlorella vulgaris is one of the microalgae types with high carbohydrate content. Besides, this species is easy to cultivate and superior in growth [19]. The growth rate of microalgae can be measured either based on optical density or the number of cells. Ho *et al*. [20] determined the cell concentration of the culture by measuring optical density at a wavelength of 688 nm (OD688) using a UV/VIS spectrophotometer. A different wavelength (OD540) was used by Liang *et al*. [21] and Undurraga *et al*. [22]. Duygu *et al*. [23] used a Thoma cell counting chamber to calculate the cell concentration by counting the number of cells in a 16-square grid having a total volume of 10^{-4} mL. Other counting chamber methods used to calculate the cell concentration are Sedgewick-Rafter, Palmer-Maloney, and Hemocytometer counting chambers [24]. The hemocytometer, also used for counting blood cells, is a counting slide method to determine extremely high concentrations of cells of small-sized organisms (< 30 μm). The hemocytometer slides have either a 0.1-or 0.2-mm depth and may possess different grid subdivisions. The most common slide is 0.1 mm deep with Improved Neubauer ruling with a nine-square grid having a total volume of 9×10^{-4} mL.

This study aims to evaluate the growth rate of *C. vulgaris* cultivated in a bubble column PBR using artificial seawater aerated with pure CO_2. The cell growth rate was calculated based on the microalgae population counted by a Neubauer-improved counting chamber and a densitometer. In addition, the Monod equation was also used to express the exponential growth of cell culture.

2. Materials and method

2.1. Materials

Microalgae *C. vulgaris* was obtained from a microalgae cultivation center in Sidoarjo, East Java. The artificial Seawater Media (ASWM) with a composition of NaCl 40 g/L, Ca^{2+} 17 ppm, and Mg^{2+} 105 ppm was used as a cultivating media with some nutrient's addition (Na_2HPO_4 2 g/L and $NaNO_3$ 16 g/L) and was obtained from Monsterlaut Indonesia Ltd. Carbon dioxide (99.9 mol %) used as a carbon source was obtained from AGI Ltd.

2.2. Apparatus and method

Microalgae cultivation was conducted in a bubble column PBR made of borosilicate glass. As seen in figure 1, the reactor has an inside diameter of 5.5 cm and a height of 98 cm. The PBR operated at an average temperature of 29 °C, acid condition at pH 5.2, and illumination by two units of 9W white light LED lamp (6500K, 900Lm) with 12 h light and 12 dark cycles for 12 – 13 days. The microalgae culture was aerated with pure CO_2 at a flowrate of 105 mL min^{-1}. The culture samples were taken every 24 h to analyze their population by the Neubauer-improved counting chamber. The counting chamber was then placed under a microscope (Binocular XSZ 107BN Oregon) equipped with a 5-MP digital camera (HDCE-X5N) with a 40x objective to determine the number of microalgae cells. Moreover, the number of cells was also determined by using a densitometer for a comparison. The

culture analyzed by the densitometer was run at a similar operating condition except for the CO_2 flowrate (120 mL min^{-1}).

At the end of the experiment, the cultivated microalgae were centrifugated at 10,000 rpm for 10 minutes to separate the microalgae and ASWM. The concentrated microalgae were then dried in an oven at 65 °C for two days. The dried microalgae were analyzed for their carbohydrate content using Fourier Transform Infrared (Nicolet™ iS™ 5 FTIR) spectroscopy.

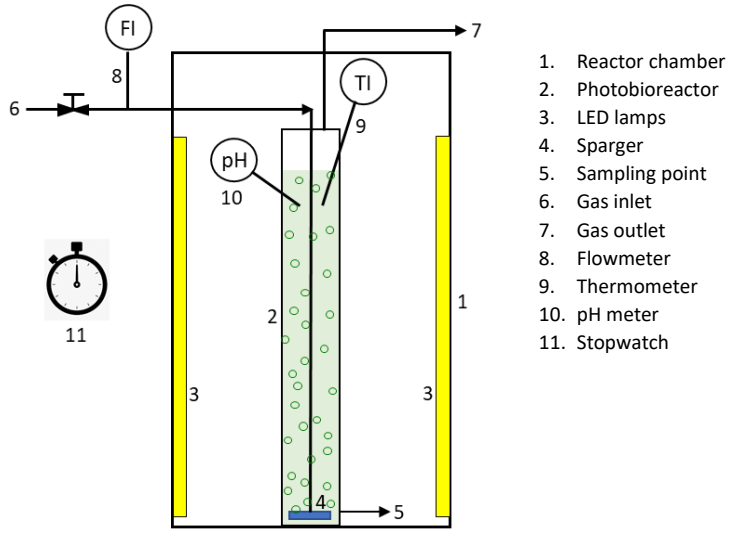

Figure 1. Bubble column PBR for microalgae cultivation

3. Result and discussion

Chlorella vulgaris has the potential to become a raw material for bioethanol production. Not only has high carbohydrate content, but this microalgae species is also easy and fast to cultivate. *Chlorella vulgaris* was cultivated in a bubble column PBR using artificial seawater aerated by pure CO_2 with a certain flowrate. The cultivation was run for up to 13 days. The Neubauer-improved counting chamber and the densitometer were used to determine the cell population every 24 h. The Monod equation was applied to express the exponential growth of cell culture.

Table 1. The population of cells during 12 days of cultivation measured by the Neubauer-improved counting chamber.

Time, day	Number of cells for each measurement, cells						The average number of cells, cells	STD (σ)
	1	2	3	4	5	6		
0.00	53	46	37	49	28	51	44.0	9.6
1.05	46	56	52	35	63	25	46.2	14.0
2.05	55	39	62	63	34	44	49.5	12.2
3.06	55	51	67	63	40	59	55.8	9.6
4.07	65	65	68	59	66	64	64.5	3.0
5.06	85	52	59	55	81	89	70.2	16.6
6.06	79	94	56	64	91	62	74.3	16.0
7.06	65	66	103	69	75	73	75.2	14.2
8.06	54	84	60	75	101	75	74.8	16.9
9.06	77	87	69	68	67	71	73.2	7.7
10.06	73	73	55	88	52	70	68.5	13.2
11.07	67	76	60	46	58	52	59.8	10.7
11.84	60	53	32	50	61	55	51.8	10.6

3.1. Determination of cell population

The microalgae cell concentration was carried out by counting the number of cells in a 16-square small grid of the Neubauer-improved counting chamber with an area of 0.2 mm × 0.2 mm and a depth of 0.1 mm (4×10^{-6} mL), as shown in figure 2. The cell concentration can, therefore, be calculated by equation (1). Due to a random cell distribution, the measurements were repeated six times with the standard deviations, σ, between 4.7 and 30%. The results were presented in table 1. The average cell concentrations during 12 days of cultivation were shown in figure 3.

$$C_c = \frac{\text{Number of cells counted}}{4 \times 10^{-6}} \quad \left(\text{cells mL}^{-1}\right) \tag{1}$$

It was observed that the diameter of cells ranged from 2 – 5 µm, as shown in figure 2. The range of *C. vulgaris* cell diameter from this study agrees with the results observed by de Grooth *et al*. [25]. They found that the cell diameters of *C. vulgaris* are between 2 and 4 µm. Moreover, Vander Wiel *et al*. [26] also reported that the average diameter of *C. vulgaris* is around 3 µm.

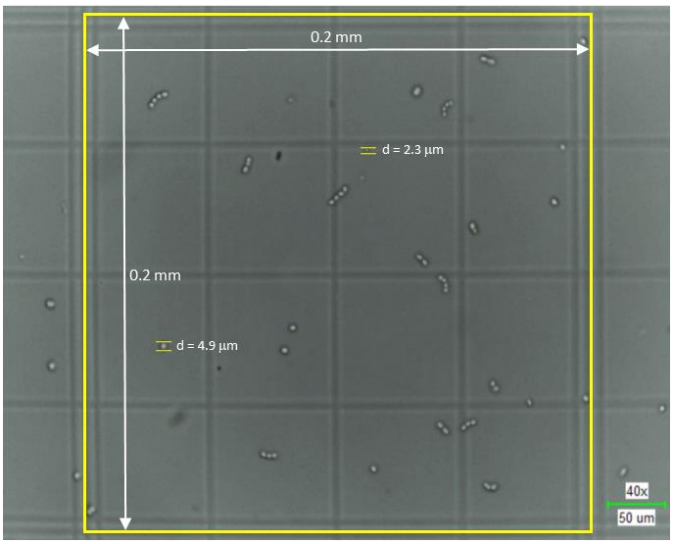

Figure 2. Determination of cell population using the Neubauer-improved counting chamber

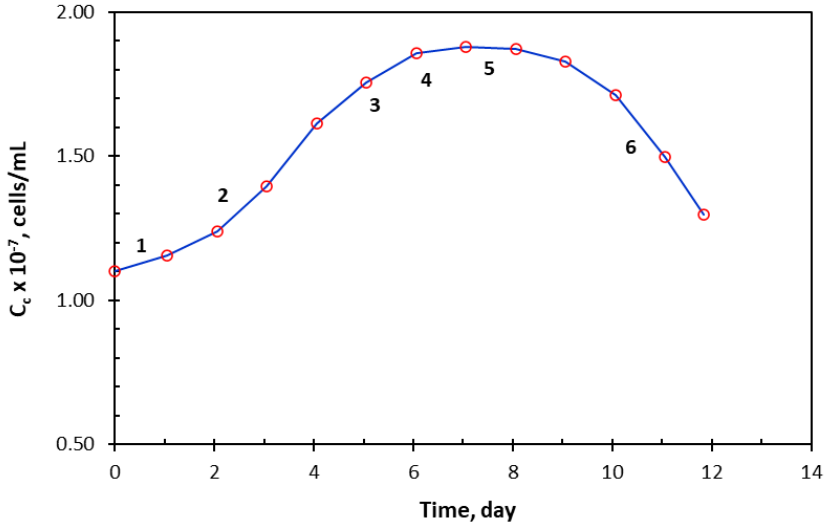

Figure 3. The cell growth of *C. vulgaris* for a 12-day cultivation period with growth phases: (1) lag phase; (2) exponential phase; (3) linear phase; (4) declining growth phase; (5) stationary phase; and (6) death phase

3.2. Cell growth rate

As shown in figure 3, the cell growth curve was similar to the growth curve of microalgae in general. It can be seen from the figure that the cell growth can be divided into six growth phases, i.e., lag phase, exponential phase, linear phase, declining growth phase, stationary phase, and death phase. These growth phases agree with those reported by Mata *et al*. [27] and Lee *et al*. [28]. On the first day of cultivation, an adjustment phase was observed. In this phase, the suspension has a bright green color. Furthermore, the growth of microalgae cells increased exponentially from day 2 to day 4 and started to decrease up to day 6. In this phase, the color of the suspension changes to dark green. From day 7 to day 8, the curve began to a stationary phase, and from day 9, the death phase began to occur. In this phase, the microalgae start to die so that the color of the suspension fades. The highest microalgae concentration was 1.88 $\times 10^7$ cells mL^{-1} which occurred on day 7.

During the exponential growth phase, the cell growth rate, r_g, can be represented by a Monod equation [29] as written in equation (2) as follows:

$$r_g = \frac{\mu_{max} C_s C_c}{K_s + C_s} \tag{2}$$

where μ_{max} denotes a maximum specific growth rate, K_s denotes the Monod constant, while C_s and C_c are the substrate and cell concentrations, respectively. In most cases, the constant K_s is small; thus, the denominator $K_s + C_s$ can be approximated by C_s. Equation (2) can, therefore, be reduced to:

$$r_g = \mu_{max} C_c \tag{3}$$

Using linear regression of r_g vs. C_c, the maximum specific growth rate, μ_{max}, was 0.344 d^{-1}. The maximum specific growth rate obtained in this study is lower than that obtained by Novak and Brune [30], μ_{max} = 1.68 d^{-1}. This situation occurred due to a lack of nutrients (only Na_2HPO_4 and $NaNO_3$) and acid conditions (pH 5.2) while they used complete nutrients consisting of both inorganic and microelements. In addition, they also used carbon from CO_2 as a limiting nutrient; therefore, the culture pH increased up to ~10. The culture acidity, however, affects cell growth. Qiu *et al.* [31] reported that the highest rate for *C. sorokiniana* occurred at pH 7, while Bartley *et al*. [32] reported at pH 8 for *Nannochloropsis salina*.

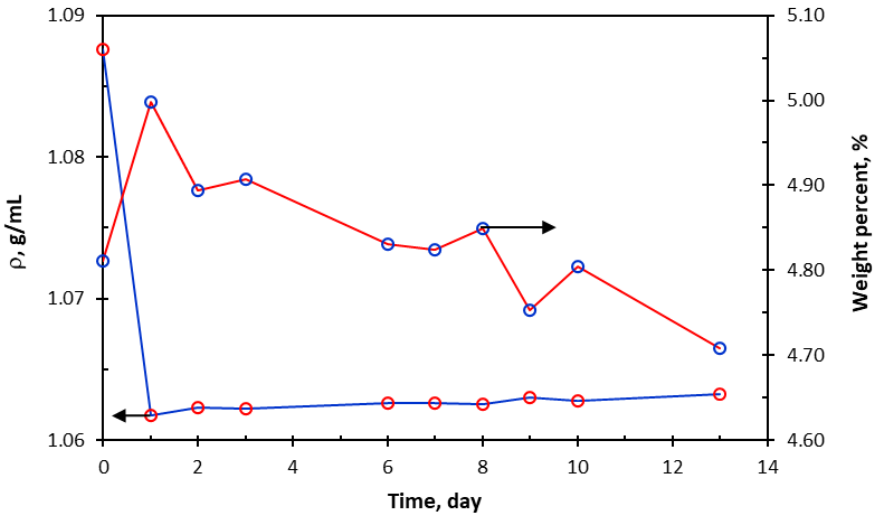

Figure 4. *Chlorella vulgaris* cell growth for 13 days cultivation period

Moreover, the growth curve of microalgae was also evaluated using a densitometer (DMA 4100 M Anton Paar). The *C. vulgaris* culture was cultivated in the same PBR for 13 days with a CO_2 flowrate of 120 mL min^{-1}. The density of the microalgae suspension is the average of three measurements where each sample was taken from different positions (bottom, middle, and top) at the reactor.

Furthermore, a standard curve is needed to determine the mass fraction of microalgae (wt%) in the suspension. By two known density values, i.e., the density values of the cultivation media (1.0876 g mL^{-1} at 30 °C) and the density of dry *C. vulgaris* reported by Hu [33] (0.57 g mL^{-1}), a linear plot can be obtained. During the cultivation stage, it is assumed that the density of the solution represents all changes of dissolved substances in the suspension. The results can be seen in figure 4.

Figure 4 shows that the exponential phase occurred on the first day. Afterward, the weight percent of the cells tends to fluctuate until the end of cultivation (day 13). However, the phases of cell growth are not seen as those evaluated by the counting chamber. On day 4, the color of the suspension started to turn green and began to fade on day 10. This condition happened because there was a decrease in the pH of the suspension from 7.5 to 5.2.

3.3. FTIR spectroscopy

Harvested dried *C. vulgaris* has a deep green color. The results of the qualitative analysis of the FTIR Spectroscopy spectra can be seen in figure 5. From the figure, it can be seen that *C. vulgaris* has a high carbohydrate and protein content. According to Sigee *et al*. [34], the carbohydrate and protein functional groups lie in the wavenumber ranging from 2837 – 3024 and 3026 – 3646 cm^{-1}, respectively. There is a spectral peak in those wavenumber ranges at a value of 3371.98 cm^{-1} with an absorbance of 0.203 and the other four weak peaks.

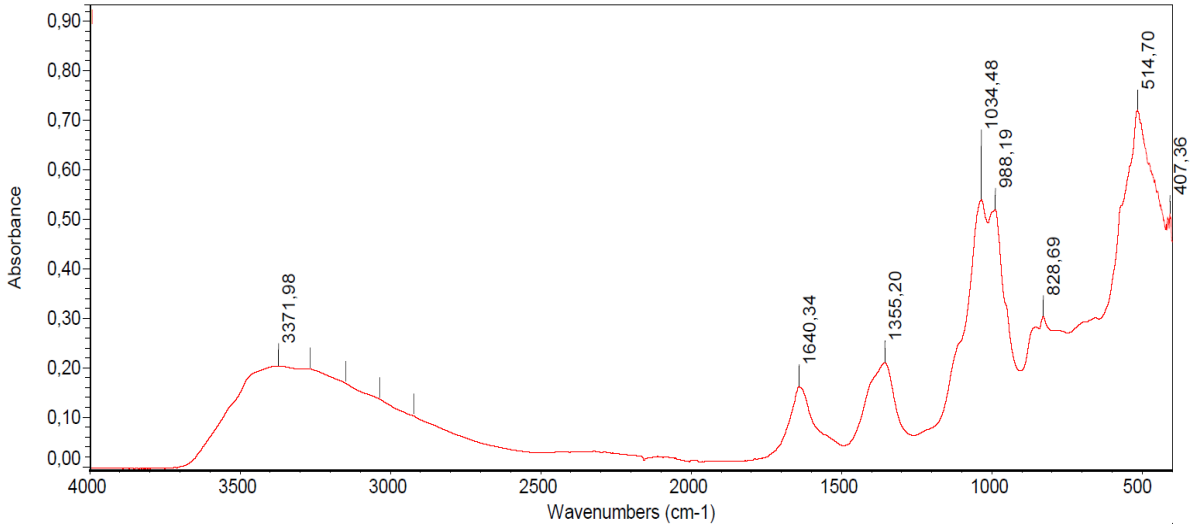

Figure 5. FTIR spectra of *C. vulgaris* after 12 days cultivation period.

4. Conclusion

The growth rate of the microalgae *C. vulgaris* in the bubble column PBR was evaluated using the Neubauer-improved counting chamber and densitometer. The results show that the counting chamber can give the cell population number; therefore, cell concentration can be determined accurately. In addition, the cell growth phases can also be observed. On the other hand, the cell concentrations measured by the densitometer are less accurate to show the cell growth phases compared to those measured by the counting chamber. The maximum specific growth rate was found to be 0.344 d^{-1} with the highest cell concentration of 1.88 $\times 10^7$ cells mL^{-1} from the kinetic analysis of cell growth.

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