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GROWTH RATE AND BIOCHEMICAL CHARACTERIZATION OF *CHLORELLA PYRENOIDOSA* CULTIVATED IN SUGARCANE VINASSE MEDIUM

Megawati*, Astrilia Damayanti, Zuhriyan Ash Shiddieqy Bahlawan, Erma Nurunia, Fatkhulil Jannah Eva Agustina, Fidyawati, Hanifah

Chemical Engineering Department, Engineering Faculty, Universitas Negeri Semarang, Semarang, Indonesia

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*Corresponding author
megawati@mail.unnes.ac.id
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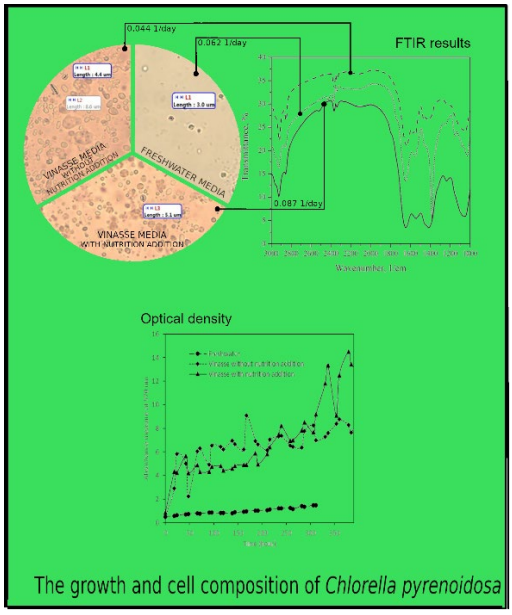
Graphical abstract



Abstract

Chlorella pyrenoidosa is a microalgae species that contains proteins, carbohydrates, amino acids, carotenoids, vitamins, and minerals. Due to its compounds, the researchers have attempted to make bioethanol using *C. pyrenoidosa* through a biorefinery approach. However, the ratio of bioethanol production towards the raw material needs of *C. pyrenoidosa* is still small because of its low carbohydrate content. Thus, in this research, vinasse is used as its growth medium to increase the carbohydrate content. The research objective is to study the effect of vinasse volume ratio and nutrient addition towards the size, optical density, carbohydrate composition, growth rate of the *C. pyrenoidosa*, and its evaluation as a biorefinery raw material. *C. pyrenoidosa* was cultivated in freshwater and vinasse (20 and 30% v/v) in mini ponds, equipped with lighting using 3280 lumens lamp, aeration with air, and Guillard as nutrient. In vinasse, the cultivation was done with and without periodic nutrient additions. The microalgae cell size was increased if cultivated in vinasse and given Guillard addition, which is 3.0-3.6 μm (in freshwater), 4.1-8.6 μm (in vinasse with nutrient every 2 days), 4.8-6.3 μm (in vinasse without nutrient every 2 days). The microalgae carbohydrate composition cultivated in vinasse was sharply increased compared to in freshwater. Thus, *C. pyrenoidosa* cultivated in vinasse is very potential for bioethanol production. Specific growth of *C. pyrenoidosa* in vinasse with nutrient is faster (0.087 day^{-1}) than without nutrient (0.023 day^{-1}) and in freshwater (0.062 day^{-1}). Cultivated *C. pyrenoidosa* contains proteins, lipids, and carbohydrates, so it has the potential of becoming a biorefinery raw material.

Keywords: *Chlorella pyrenoidosa*, freshwater, growth rate, nutrient, vinasse

1.0 INTRODUCTION

Chlorella pyrenoidosa is one of the microalga species that live in freshwater, which belongs to the *Chlorophyta* division and *Chlorella* genus. This microalga species can grow well in climates with warm temperatures like the area of Java Island, therefore many cultivation areas are developed [1]. *Chlorella* species can grow into 10,000 cells in 24 h and the life phase of *C. pyrenoidosa* is between 11-15 h. Chemical compounds that can be found in *Chlorella* are proteins, carbohydrates, amino acids, carotenoids, vitamins, and minerals [2,3]. In the last decade, *Chlorella* has been extensively explored to be used in many applications including pharmacy, food and animal feedstock, natural dyes, and cosmetics [4]. Even recently *Chlorella* has been tried to be utilized for biofuel production, one of which is bioethanol [5-7].

All this time, bioethanol is produced from sugar-based feedstocks (sugar beets, sugarcane), starch-based feedstocks (corn, wheat, barley, etc.), and lignocellulosic materials (bagasse, corn, rice straw, rice husk, switchgrass, and so on). Material based on sugar and starch was still very expensive and competing over interest with food material and feed. Meanwhile, material based on lignocellulose is very cheap and easy to be handled [8]. However, the production cost of

lignocellulosic ethanol is still very expensive. Therefore, a more profitable feedstock is needed to be developed. Microalgae could become one of the alternatives [5-7, 9, 10]. Lignin contents in microalgae, especially *Chlorella* are very low, so it becomes easy to be hydrolyzed and then fermented into bioethanol [11]. Several species of microalgae contain many carbohydrates (26, 37-55%) [12,13]. In addition, carbohydrates are often found in the microalgae cell wall (cellulose, hemicellulose, and pectin) [10].

Bioethanol from microalgae is often called third-generation bioethanol [9, 14]. Although there have been many attempts to develop it, the ratio of bioethanol production towards feedstock need of *C. pyrenoidosa* is still small due to the low content of cellulose and hemicellulose in microalgae [14]. Following the development of biodiesel, those kinds of the problem need to be resolved and microalgae growth engineering needs to be done [9]. The purpose is to increase the carbohydrate content in microalgae, so its conversion level to bioethanol will be increased. This engineering process is categorized into fourth-generation bioethanol [7]. One engineering method of microalgae growth is using vinasse as a mixture of its growth medium [12, 15]. Besides species of microalgae, carbohydrate content in microalgae were also affected by cultivation condition (medium, CO_2, nitrogen, temperature, pH, light intensity, and photobioreactor types) [10, 12]. Vinasse is an ethanol waste product from molasses/sugarcane waste that contains many useful chemical compounds, namely organics carbon, nitrogen, phosphorus, and other compounds, which makes them suitable for microalgae cultivation [3, 12, 16-18]. Therefore, in this study, vinasse was chosen as the cultivation medium for *C. pyrenoidosa*.

According to Kendirlioglu, the photosynthesis process on *Chlorella* could not be transpired in the absence of lights [19]. Because of that, lights from lamps or sunlight are very important as it used as the energy source for the photosynthesis process. In addition, microalgae can grow very well at lamp lighting condition 8000 lux [19], aeration using CO_2 with a rate of 200 mL/min, pH range of 5.7-8.1, and temperature of 25-28 ^oC [19-21]. Fresh air containing 0.03% of CO_2 can be utilized for *Chlorella* aeration. The usage of air as a substitute for pure CO_2 is also for the process efficiency when applied on an industrial scale. Microalgae growth using fresh air (0.03% CO_2) for aeration has been done by Tang et al. [21]. The result showed that the maximum concentration of *C. pyredoinosa* was about 0.87 g/L. Meanwhile, *Chlorella* growth using 5, 10, 20, 30, and 50% CO_2 at a rate of 200 L/h resulting in biomass concentrations of 1.4, 1.48, 1.15, 0.9, and 0.6 g/L, respectively. That means aeration using air as a CO_2 supply could be applied. Moreover, the previous researcher showed that microalgal growth could be inhibited by the concentration of CO_2 aeration above 5% which is considered harmful to microalgal cells [20]. In this study, the source of CO_2 is taken from the air to make it more economical.

Besides condition that needs to be kept, *Chlorella* also needs nutrients on its growth. One of nutrient that is widely used is Guillard solution. This solution is one of microalgae nutrient that consists of $NaNO_3$ 75 g/L dH_2O, $NaH_2PO_4H_2O$ 75 g/L dH_2O, $NaSi_3.9H_2O$ 75 g/L dH_2O, trace metal solution, and vitamin solution [22]. According to Ong et al., Guillard nutrient has been utilized for growth of *Chlorella sp.* [23-24]. In summary, this research studies the growth of *C. pyrenoidosa* in vinasse medium in economic circumstances; using air to supply CO_2 and Guillard as a nutrient. The influence of vinasse volume ratio and Guillard addition as nutrient periodically toward cell sizes, cell composition, and growth rate are also discussed.

2.0 METHODOLOGY

C. pyrenoidosa seeds were obtained from Ugo plankton shop, Purworejo Regency, Central Java. These seeds were grown in an aqueous medium at pH 8. The seed was not too thick and have a light green color. When the seeds have arrived at the Laboratory of Biomass, Chemical Engineering, Universitas Negeri Semarang, the seeds were immediately aerated and lighted, so they could adapt to the environment. Aeration used an AC/DC air pump (Amara, mini AC/DC AA 6603) with an air velocity of 2x3 L/min and a pressure of 2x0.015 Mpa for 2 days. As for lighting, a lamp (Philips, 52 W) was used. Before being cultured, the seed was analyzed for its optical density using UV-Vis spectrometry (Thermo Scientific, Genesys 10UV) and for the shape and size of the cells using a digital microscope (Camlab, BA210).

Cultivation and harvesting of *C. pyrenoidosa* in freshwater and vinasse mediums

Cultivation of *C. pyrenoidosa* was carried out in two mini-open ponds (dia. 8xlength 31 cm) that were made from transparent plastic. The *Chlorella* seed was mixed with distilled water at a ratio of 1:1 in the ponds and then Guillard nutrient (2 mL) was added. Guillard is a nutrient that contains $NaNO_3$ 75 g/L dH_2O, $NaH_2PO_4H_2O$ 75 g/L dH_2O, $NaSi_3.9H_2O$ 75 g/L dH_2O, trace metal solution, and vitamin solution. Air for CO_2 supply is fed through a hose (dia. 5 mm) using an air pump. This pump has 2 outputs with a speed of 3 L/min each. The lamp used as a light source has 3280 lumens. This lamp was placed outside the pond at a distance of about 15 cm. During growth, every day at 8:30 a.m. and 2:00 p.m., the temperature and light strength of each pond was measured using an optical thermometer (Xueliee, GM 320) and luxmeter (Benetech, GM 1010), and also 1 mL samples were taken and then diluted into 5 mL to observe its optical density using UV-Vis spectrometry at a wavelength of 570 nm. This cultivation was carried out for 14 days.

After 14 days, *C. pyrenoidosa* was harvested using a procedure taken from a published article [25]. *C. pyrenoidosa* was turned off by adding NaOH solution (Merck KGaA, 1.06498.1000) and then precipitated for 24 h, then filtered using a chamois cloth with a size of 60 m and the microalgae were taken using an iron spatula. The microalgae were washed with distilled water three times to remove the other solid components and then dried using an oven step by step (Memmert, UN 160 161 Liter). At the first step, the microalgae were heated at 30 ^oC for 12 h and then weighed. For the second step, the microalgae were heated again for 10 min and weighed. If the weight was not constant, then it was heated again for 10 min and the process was repeated until the constant weight was obtained. The *C. pyrenoidosa* obtained were stored inside a sealed plastic bag. The *C. pyrenoidosa* was analyzed by their functional groups using Fourier Transform Infrared-FTIR (Shimadzu Scientific Instruments IR-Prestige-21) at Universitas Gadjah Mada.

Vinasse waste used as cultivation medium of *C. pyrenoidosa* was obtained from Madubaru sugarcane factory, Yogyakarta.

Before being used, vinasse waste was pretreated to remove its impurities and inhibitor [26]. The vinasse was also analyzed for the number of COD, BOD, and proximate content of ammonia and inorganic phosphate. The pretreatment was performed by filtering the vinasse twice using a centrifuge (Zentrifugen Rotofix, 32A) using 4000 rpm speed. After that, vinasse was diluted by distillate water to get a 20 and 30% volume ratio. The next stage was sterilization, which was done by putting vinasse in an autoclave (TOMY Autoclave, ES-315) at 121 ^{o}C for 15 min and after it was cooled, the vinasse was stored in a freezer at 4 ^{o}C. Cultivation was done by the same method as using the freshwater medium. The cultivation in vinasse was done in 2 conditions, with nutrient dosing in every 2 days and with the nutrient dosing only at the initial stage (without nutrient dosing every 2 days). These cultivations were carried out for 19 days. The harvest method for cultivation using vinasse is almost the same as using a freshwater medium. After 19 days, *C. pyrenoidosa* from the cultivation result was ready to be harvested.

The growth rate of *C. Pyrenoidosa*

Specific growth is calculated on exponential growth rate phase condition and expressed by Equation (1), in which μ is specific growth (A/h), X_0 is the initial optical density of the exponential phase (A), and X_t is the optical density at any time t of exponential phase (A), t is time (h).

$$\mu = \frac{ln(X_t - X_0)}{t_t - t_0} \quad (1)$$

$$Error = \frac{[X_t - X_0]}{X_0} 100\% \quad (2)$$

$$t_d = \frac{0.693}{\mu} \quad (3)$$

Equation (1) could be solved by doing linear regression between $ln(X_t)$ as y-axis towards t as the x-axis. The slope value from the regression result equation is the value of μ (specific growth) and the intercepts result is the value of $ln(X_0)$. The average error value is the ratio of X_0 value from regression result toward experimental data (Equation (2)). Multiplication time or generation time (t_d) is the time needed by *C. pyrenoidosa* to multiply. For calculation of multiplication time (t_d), Equation (3) could be used.

3.0 RESULTS AND DISCUSSION

Effect of vinasse volume ratio on size of *C. pyrenoidosa* cell

The measurements result of *C. pyrenoidosa* cells used in this study is presented in Figures 1a and 1b. *C. pyrenoidosa* has cells with a size of 3.0-3.6 μm. According to Hadiyanto et al., the shape of *C. pyrenoidosa* cells is round and based on Takahashi, the size of the *Chlorella* cell is around 4.1-4.8 μm [1, 27]. Meanwhile, microalgae cells range in size from 5 to 50 μm [3]. That means the size of *C. pyrenoidosa* cell used in this research is smaller than the *Chlorella* species' cell size in general. Condition of medium and growth affect the cell size of microalgae [27].

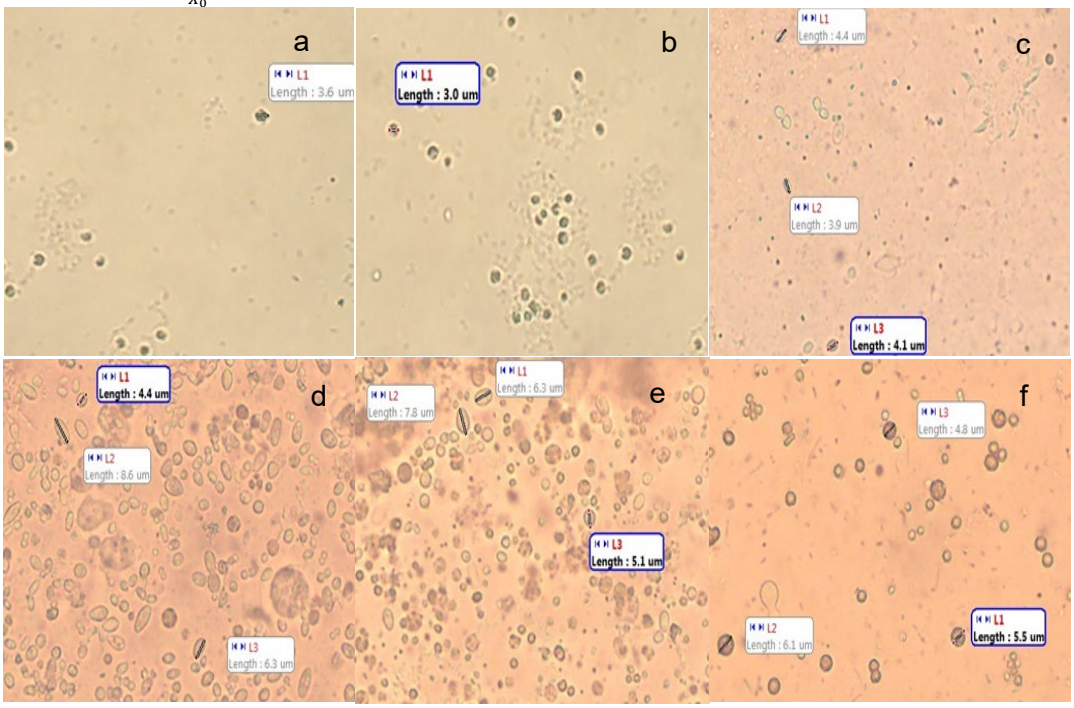


Figure 1 The measurement result of *C. pyrenoidosa* cell cultivated using magnification by 400x in the freshwater medium for samples A (a) and sample B (b); and in 20% vinasse with nutrient dosing every 2 days (c); in 30% vinasse with nutrient dosing every 2 days (d); in 20% vinasse without nutrient dosing every 2 days (e); in 30% vinasse without nutrient dosing every 2 days (f)

Meanwhile, Figures 1c and 1d show the size of *C. pyrenoidosa* cell from cultivation result in vinasse with nutrient addition every 2 days. It appears that the number and size of *C. pyrenoidosa* cells in the vinasse are more and bigger than in the freshwater medium. The sizes of *C. pyrenoidosa* cells are 4.1-4.4 and 4.4-8.6 μm, for 20 and 30% vinasse volume ratio, respectively. Whereas, the number of cells in a 30% vinasse volume ratio is also more concentrated than the 20%. In contrast, Figures 1e and 1f show that without nutrient dosing every 2 days, the size of *C. pyrenoidosa* cell in 30% vinasse (4.8-6.1 μm) is smaller than 20% vinasse (5.1-6.3 μm). In addition, the number of cells in a 30% vinasse ratio is less than 20%. This indicates that nutrient must be given periodically when *C. pyrenoidosa* is grown in a more concentrated vinasse medium.

Effect of vinasse medium on growth of *C. pyrenoidosa*

C. pyrenoidosa cultivation in the freshwater was carried out in 2 ponds (Sample A and B). The growth profile can be seen in Figure 2a. Both ponds show almost similar results. The growth of *C. pyrenoidosa*, in the beginning, is very fast, then ramps briefly at 100 to 150 h (4-6 days). After that, its growth returned quickly until 312 h (13 days), and then the experiment was stopped at 14 days. It means that *C. pyrenoidosa* is a type of microalgae that is easily adaptable [3]. According to Ribeiro et al, the growth of *C. sorokiniana* in mixed mediums (nitrogenated, Bold, and NPK mediums) presented two defined growth phases. The first growth phase took place until day 3 or day 4 of cultivation, followed by a short lag phase, and then the second growth phase. These two growth phases occur due to differences in carbon sources [28]. The results show that the growth still occurred until the 14th day; this means that the difference in the medium greatly affected the growth curve.

The optical density of *C. pyrenoidosa* in freshwater shows a range between 0.512 to 1.494 A. If assumed that correlation between optical density (X) with microalgae biomass concentration (Y) is Y = 0.215X-0.0239 (R^2 = 0.9946) [20]. This correlation can be used because the microalgae used is the same (*C. pyrenoidosa*). Hence, the highest biomass concentrations of *C. pyrenoidosa* cultivated in freshwater on samples A and B are 0.29 and 0.30 g/L, respectively. According to Gunawan et al., the biomass concentration of *C. pyrenoidosa* cultivated at the operation condition of: light intensity 2000 lux/h, 84 h, CO_2 rate 0.034%, and G15 Deptan nutrient (5% NO_3, 18% K_2O, 3% MgO, 8% S, 0.35% Fe, 0.02% Mn, 0.02% Zn, and 0.015% Boron) could achieve around 0.288 g/L [20]. The growth of *C. pyrenoidosa* in the freshwater medium was also studied by Asuthkar et al. with variations in lamp types as a source of energy [29]. The results show that the optical density range of *C. pyrenoidosa* is between 0.3 to 0.65 A. This density was achieved after 240 h and 1986 lux irradiation. In this study, the lamp used had 3280 lumens and was installed at a distance of 0.15 m (resulting in an irradiation strength of 11600 lux). Therefore, the higher the irradiation power, the faster the growth of *C. pyrenoidosa* [29].

Meanwhile, the growth of *C. pyrenoidosa* in vinasse was carried out in 4 conditions: a volume ratio of 20 and 30% vinasse, without and with the dosing of Guillard every 2 days. The used vinasse has a COD number of 33.3 mg/L and pH of 2.53. The optical density measurement results are presented in Figures 2b and 2c. These figures show that the constant periods *C. pyrenoidosa* growth were found to be earlier and longer, which is before 50 h (2 days), and up to 200 h (8 days). This indicates that *C. pyrenoidosa* requires earlier and longer adaptations in vinasse than in freshwater medium.

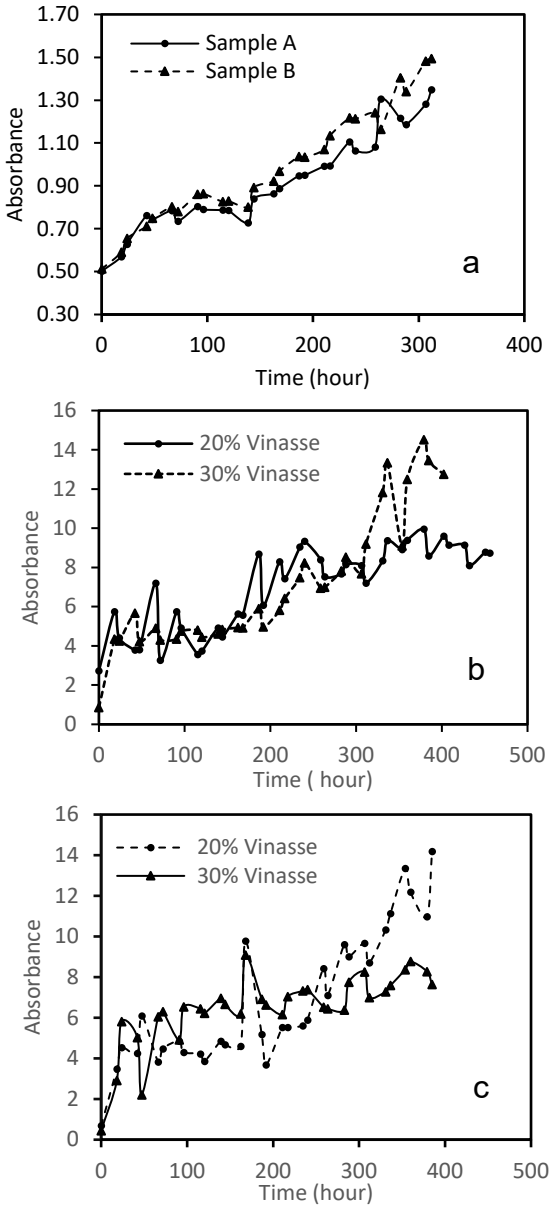


Figure 2 The optical density of C. pyrenoidosa cultivated in freshwater medium (a), in vinasse medium with nutrient dosing every 2 days (b), and without nutrient dosing every 2 days (c)

The vinasse used is different from its normal medium to grow (freshwater) which makes microalgae in adaptation phase more sensitive to nutrients and conditions changes. Thus, a longer adaptation period is needed. After a constant phase, *C. pyrenoidosa* grows again, even sharply in 30% vinasse medium with nutrient dosing every 2 days. The optical density of *C. pyrenoidosa* in the vinasse medium is higher than in the freshwater one. As has been explained before, vinasse still contains many chemical compounds, which are ammonia and organic compounds. These compounds can be used for supporting the growth of *Chlorella* [16, 28].

Table 1 Functional Groups of FTIR Analysis

Wavelength (cm^{-1})	Functional Groups	Domenighini and Giordano [30]	Xin and Yu [31]
3600 – 3700	O-H		
3300 – 3500	N-H stretching		
2960 – 2875	C-H	2800 – 3000 (Lipid)	
2850 – 2970	C-H		
1740 – 1610	C=O	1740, 1650 (amide I)	1610-1670 (STCHO)
1400 – 1600	C=C	1540 (amide II)	1560-1600 (STCHO), 1210-1483 (CHO
1200 – 950	C-O	950-1200 (Carbohydrate)	1184-1200 (CHO)
1200 – 900	C-O-C		
1200 – 800	C-C		

FTIR spectra of *C. pyrenoidosa* cultivated in freshwater and vinasse mediums

Theoretically, identification of *C. pyrenoidosa* cells functional group was done by using Table 1. In this table, three regions showing lipid, protein, and carbohydrate existence are shown. Apart from that, a specific region special for carbohydrate type, i.e. structure carbohydrate (CHO) and total carbohydrate (STCHO) is also provided [30, 31]. Accordingly, three main compounds can be found in *C. pyrenoidosa*, which are protein, carbohydrate, and lipid. These compounds are analyzed using FTIR and the analysis result was similar to the traditional chemical methods [2, 3, 32, 33]. Due to its insignificant changes in correlation with lipid content variations, the band around 1740 cm^{-1} is not used for lipid content estimation in the present study. The band around 3000–2800 cm^{-1} could better display the quantitative changes of lipid content; therefore it is used instead.

In this research, the result of FTIR of *C. pyrenoidosa* cultivated can be seen in Figure 3. The carbohydrates are observed at wavenumbers of 1195.87-1002.98 and 1396.46-1211.30 cm^{-1}, which is indicated by the CHO carbohydrates. Therefore, *C. pyrenoidosa* is promising as a raw material for making bioethanol because it contains carbohydrates [12]. Then, the *C. pyrenoidosa* carbohydrate content could be used as a reference and its value is small, so it needed to be improved if it will be converted into bioethanol. One of the efforts to improve it, as has been done in this research, is using vinasse as a growth medium. The presence of the protein C=O stretching vibrations, N-H stretching, and N-H bending are shown by the protein compound wavelength of 1735.93-1666.50 cm^{-1} (amide I) and 1597.06-1404.18 cm^{-1} (amide II), respectively. The vibrating of C-H stretching in acyl chains is indicated by the 2954.95-2877.80 cm^{-1} wavelength of the lipid compound.

Figure 3 shows that in the medium variation, the absorption curve shape of *C. pyrenoidosa* cultivated is almost the same, but the transmission is different. This indicates that the contents of proteins, lipids, and carbohydrates of *C. pyrenoidosa* cultivated is not equal [31]. Figure 3 shows also that the transmission of *C. pyrenoidosa* cultivated in vinasse with nutrient dosing every 2 days decreases if compared to *C. pyrenoidosa* cultivated in the freshwater medium. This indicates that in the vinasse with nutrient dosing every 2 days the higher the protein, lipid, and carbohydrate content will be found in the *C. pyrenoidosa* cell. On contrary, the transmission of *C. pyrenoidosa* cultivated in vinasse without nutrient dosing every 2 days increases, compared to the one cultivated in the freshwater medium. It means the cells of *C. pyrenoidosa* that cultivated in vinasse without nutrient dosing contain the lowest protein, lipid, and carbohydrate content. Without nutrient, its contents are the smallest. More clearly, the transmittance values can be seen in Table 2. Nutrient gives a very strong effect on the carbohydrates content of *C. pyrenoidosa* cultivated in vinasse. Domenighini and Giordano reported that FTIR results from the algal cell are greatly affected by the nutrient given [30]. That means, *C. pyrenoidosa* that cultivated in vinasse will produce biomass with high carbohydrates if given by nutrient every 2 days.

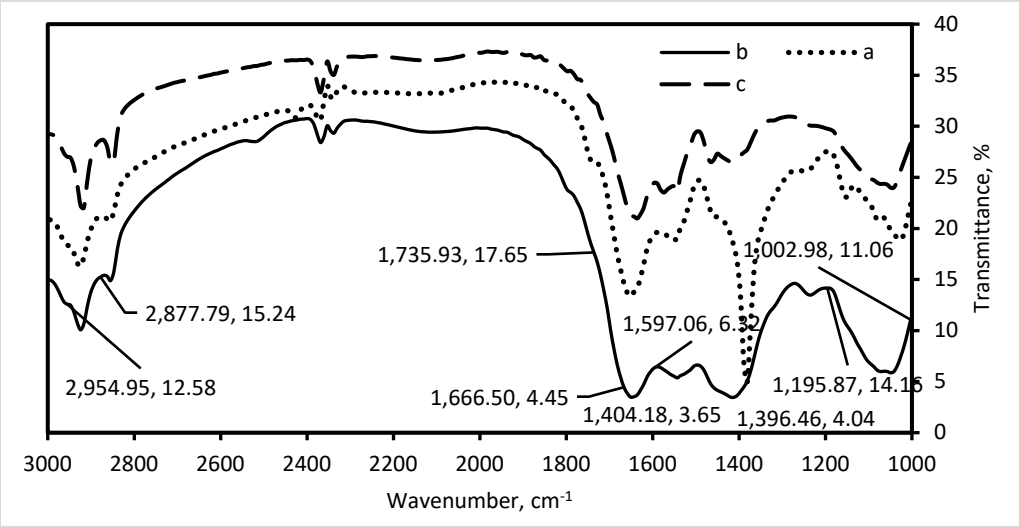


Figure 3 Infrared spectrum absorption graph of *C. pyrenoidosa* cultivated in freshwater medium (a); in vinasse with nutrient dosing every 2 days (b), in vinasse without nutrient dosing every 2 days (c)

Table 2 Peak intensity of FTIR analysis of *C. pyrenoidosa* cultivated in freshwater and vinasse mediums

Biochemical Compound	Wavenumber (cm^{-1})	Transmittance (%)		
		Freshwater	Vinasse (30% v/v)	
			with nutrient	without nutrient
Carbohydrate	1002.98	22.36	11.06	28.31
	1195.87	27.57	14.16	29.76
	1211.30	27.10	14.06	29.96
	1396.46	13.20	04.04	27.15
Amide II	1404.18	16.68	03.65	26.75
	1597.06	19.40	06.32	24.45
Amide I	1658.78	13.70	03.86	22.02
	1735.93	26.53	17.65	32.37
Lipid	2877.79	21.17	15.24	28.62
	2954.95	18.36	12.58	27.01

In addition, its protein composition is high (about 28.1% dry weight). Before being processed into bioethanol, it will be more useful if the protein were extracted first. Protein can be used for human nutrient, animal feed, and aquaculture nutrient. Protein extraction in *Chlorella* can be done by enzymatic hydrolysis and chemical extraction [34]. Meanwhile, its lipid composition (11.5% dry weight) likes protein extraction, it is far better if the lipid is also extracted. Lipid extraction can be done using non-polar solvent [35]. Step of biomass utilization into various chemical products is often called biorefinery, which is massively developed so that the process becomes efficient and economic [11, 12, 35, 37].

The Growth Rate of *C. pyrenoidosa*

The natural logarithm of optical density of *C. pyrenoidosa* plotted against time gives curves (Figures 4A-C), the slope of which is the specific growth rate. Values of specific growth rates were reported in Table 3. The specific growth rate of *C. pyrenoidosa* that cultivated in 30% vinasse with nutrient dosing every 2 days (0.087 day^{-1}) is faster than in freshwater medium (0.062 day^{-1}) and also than cultivated in 20% vinasse medium (0.074 day^{-1}). On the contrary, for the cultivation of *C. pyrenoidosa* in vinasse that not given nutrient every 2 days, but only given on early cultivation, the growth of *C. pyrenoidosa* become slower, which is 0.044 and 0.023 day^{-1}, for 20 dan 30% vinasse, respectively. It means, vinasse as a medium could accelerate the growth of *C. pyrenoidosa* if given by nutrient regularly and the more concentrated vinasse that is used, the faster its growth. Nutrient will help microalgae to adapt to medium, other than light [30].

Values of specific growth of *C. pyrenoidosa* in this research is smaller than previous research. In previous research, its value is 0.69 day^{-1}, 8 times higher than in this research [20]. The previous research used different nutrient, which was: 1 ppm Na_2MG EDTA, 36 ppm $CaCl_2.2H_2O$, 75 ppm $MgSO_4.7H_2O$, 40 ppm $K_2HPO_4.3H_2O$, 2.86 ppm H_3BO_3, 1.81 ppm $MnCl_2.4H_2O$, 0.222 ppm $ZnSO_4.7H_2O$, 0.079 ppm $CuSO_4.5H_2O$, 0.05 ppm $CoCl_2.6H_2O$, 0.391 ppm $NaMoO_4.2H_2O$, and 1500 ppm $NaNO_3$. Guillard that used on this research was cheap, so deeper economic analysis is needed when it going to be utilized for industrial-scale [22]. Medium is indeed very influential toward the growth of microalgae. There is research that specifically investigates the differences of medium (Bristol, Chu, Bold 3N, TAP(-), and BG-11) towards the specific growth rate of *S. obliquus* the result are as follows 0.172; 0.170; 0.186; 0.179; and 0.175 day^{-1}, respectively.

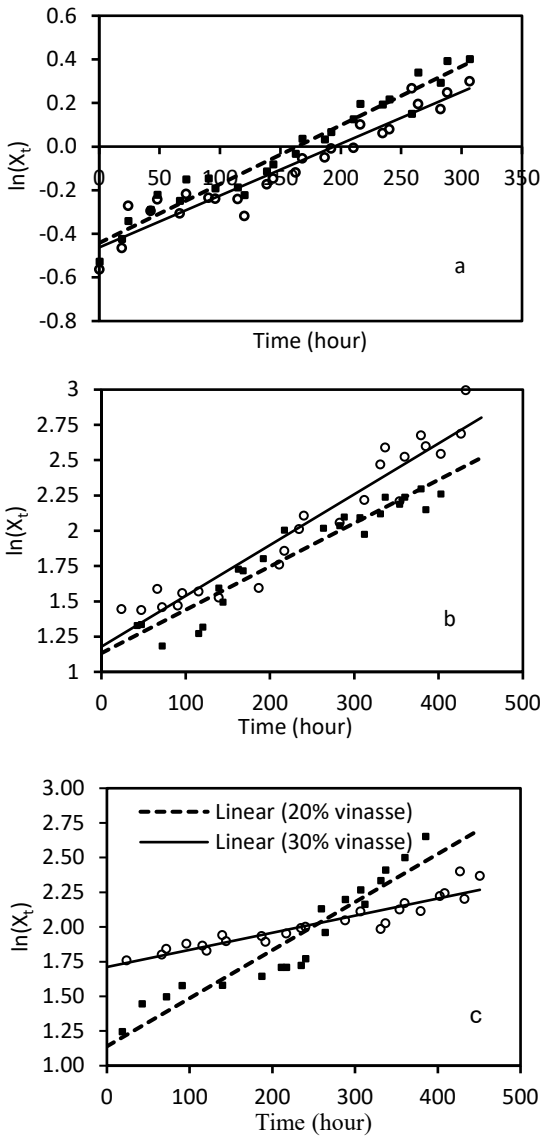


Figure 4 Growth rate curve in terms of optical density of *C. pyrenoidosa* cultivated in various mediums: freshwater medium (a), vinasse medium with nutrient dosing every 2 days (b), and vinasse medium without nutrient dosing every 2 days (c)

Table 3 Growth rate parameter of *C. pyrenoidosa* cultivated in various mediums (freshwater and vinasse mediums)

Medium	Specific growth rate (day^{-1})	Doubling time (day)
Freshwater	0.062	11.1
20% vinasse with nutrient every 2 days	0.074	09.3
30% vinasse with nutrient every 2 days	0.087	08.3
20% vinasse without nutrient every 2 days	0.044	18.0
30% vinasse without nutrient every 2 days	0.023	24.0

Research that gives the value of specific growth and doubling time that is almost similar with this research is the cultivation of *C. minutissima* in BBM medium (Bold's Basal Medium) by Şerbetçioğlu Sert et al., which are 0.0879 day^{-1} dan 7.8 days [38]. The doubling time of microalgae is supposed to be less than 7 days. Excessive aeration could give stress so that microalgae need more time to multiply [39]. According to Lim et al., the doubling time of microalgae in 100 mL of the flask is usually about 6-7 days [40]. The research that proofs that type of medium is affecting the growth rate of microalgae was revealed by Riberio et al. [28]. They used *C. sorokiniana* and 250 mL Erlenmeyer flask as its photobioreactor, with 200 mL working volume. The result shows that *C. Sorokiniana* could grow faster in a mixed medium, which is a mixture between nitrogenated, NPK, and Bold Basal. Each specific growth and doubling times in nitrogenated, NPK, Bold Basal, and mixed are 0.09 and 7.8, 0.065 and 10.7, 0.067 and 10.3, 0.94 day^{-1} and 0.7 days, respectively. Table 3 shows that *C. pyrenoidosa* cultivated in 30% vinasse with nutrient addition every 2 days has the fastest generation time, i.e. every 8.3 days.

4.0 CONCLUSION

The size of *C. pyrenoidosa* cell was bigger when cultivated in vinasse and given nutrient every 2 days compared with without nutrient and also than cultivated in freshwater, which is 3.0-3.6 μm (in freshwater medium), 4.1-8.6 μm (in vinasse with nutrient every 2 days), 4.8-6.3 μm (in vinasse without nutrient every 2 days). Specific growth of *C. pyrenoidosa* in vinasse with nutrient is faster (0.087 day^{-1}) than without nutrient (0.023 day^{-1}) and in freshwater medium (0.062 day^{-1}). Carbohydrate composition in *C. pyrenoidosa* that cultivated in vinasse which given by nutrient every 2 days is higher than *C. pyrenoidosa* that cultivated in vinasse without nutrient given every 2 days and which cultivated in the freshwater medium. Therefore, *C. pyrenoidosa* that cultivated in vinasse must be given by nutrient to increase the carbohydrates, so that it can become a prospect as raw material for bioethanol production.

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