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COMPARATIVE STUDIES OF CELL GROWTH OF FRESHWATER MICROALGA *CHLORELLA* SP. IN PHOTOAUTOTROPHIC, HETEROTROPHIC AND MIXOTROPHIC CULTURES

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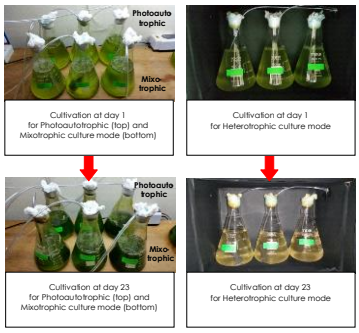
Graphical abstract



Abstract

Lately, research on biodiesel production as a renewable and sustainable energy has become increasingly apparent due to the fact that fossil fuel is decreasing and the concern of global warming issues. The third generation of biofuel, which is microalgae-based biodiesel had gained interest over the last decade. The ability of microalgae to grow in various conditions is one of its advantages as the potential and promising feedstock for biodiesel. Microalgae can be cultivated in three modes such as photoautotrophic, heterotrophic and mixotrophic culture mode. Unlike photoautotrophic mode where light is required, the heterotrophic mode mainly utilized carbon compounds to grow. On the other hand, the mixotrophic mode is the condition where light and carbon compounds are supplied for microalgae culturing. This paper investigates the cell growth of *Chlorella* sp. cultivated in photoautotrophic, heterotrophic and mixotrophic culture mode. It was found that *Chlorella* sp. was capable of producing the highest cell concentration of $6.67 \pm 0.56 \times 10^6$ cell mL^{-1} in the photoautotrophic mode for 23 days of cultivation period. This was 1.3 times and 3.2 times greater than the cell concentration in mixotrophic ($5.02 \pm 0.49 \times 10^6$ cell mL^{-1}) and heterotrophic ($2.03 \pm 0.29 \times 10^6$ cell mL^{-1}) culture, respectively. On the contrary, the highest specific growth rate obtained in the study was from heterotrophic mode (0.32 ± 0.04 day^{-1}) followed by photoautotrophic and mixotrophic mode with 0.26 ± 0.05 day^{-1} and 0.20 ± 0.04 day^{-1}, respectively. *Chlorella* sp. cell grew well under the photoautotrophic and mixotrophic mode. However, the insufficient of glucose level had contributed to lower cells productivity in the heterotrophic culture. Therefore, the mixotrophic mode could also be an alternative pathway in microalgae cultivation for biodiesel production if the glucose supplied was adequate and at the suitable level.

Keywords: Biodiesel, chlorella sp., photoautotrophic, heterotrophic, mixotrophic, cell concentration, growth rate

Abstrak

Sejak kebelakangan ini, penyelidikan terhadap penghasilan biodisel sebagai tenaga yang boleh diperbaharui dan lestari telah menjadi semakin ketara disebabkan oleh hakikat bahawa bahan api fosil semakin berkurangan dan kebimbangan terhadap isu-isu pemanasan global. Generasi ketiga biobahan api, iaitu biodisel berasaskan mikroalga telah mendapat tarikan sepanjang dekad yang lalu. Keupayaan mikroalga untuk tumbuh dalam pelbagai keadaan adalah salah satu kelebihannya sebagai bahan mentah yang berpotensi dan mempunyai masa depan yang cerah untuk

biodisel. Mikroalga boleh dikultur dalam tiga mod pengkulturan seperti fotoautotrofik, heterotrofik dan mixotrofik. Tidak seperti mod fotoautotrofik yang mana sumber cahaya diperlukan, mod heterotrofik menggunakan sebatian karbon untuk pertumbuhan. Sebaliknya, mod mixotropik adalah pengkulturan mikroalga dalam keadaan dimana cahaya dan sebatian karbon dibekalkan. Kertas kajian ini menyiasat pertumbuhan sel *Chlorella* sp. yang dikultur dalam keadaan mod fotoautotrofik, heterotrofik dan mixotrofik. Hasil daripada kajian telah mendapati bahawa, *Chlorella* sp. mampu menghasilkan kepekatan sel yang tertinggi sebanyak 6.67 ± 0.56 x 10^6 sel mL^{-1} menggunakan mod fotoautotrofik dengan tempoh pengkulturan selama 23 hari. Ini adalah 1.3 kali dan 3.2 kali lebih besar daripada kepekatan sel dalam mod pengkulturan mixotrofik (5.02 ± 0.49 x 10^6 sel mL^{-1}) dan heterotrofik (2.03 ± 0.29 x 10^6 sel mL^{-1}) masing-masing. Sebaliknya, kadar pertumbuhan spesifik yang paling tinggi diperolehi dalam kajian ini adalah daripada kaedah pengkulturan mod heterotrofik (0.32 ± 0.04 $hari^{-1}$) diikuti dengan mod fotoautotrofik dan mixotrofik dengan 0.26 ± 0.05 $hari^{-1}$ dan 0.20 ± 0.06 $hari^{-1}$, masing-masing. *Chlorella* sp. tumbuh dengan baik di bawah mod fotoautotrofik dan mixotrofik. Walau bagaimanapun, paras glukosa yang tidak mencukupi telah menyumbang kepada produktiviti sel-sel yang lebih rendah di mod heterotrophic. Oleh itu, mod mixotrofik juga boleh menjadi jalan alternatif dalam pengkulturan mikroalga bagi penghasilan biodiesel sekiranya glukosa yang diberikan adalah mencukupi dan pada tahap yang sesuai

Kata kunci: Biodiesel, *chlorella* sp., fotoautotrofik, heterotrofik, mixotrofik, kepekatan sel, kadar pertumbuhan

1.0 INTRODUCTION

Over recent years, the concern of global warming issues, decreasing of petroleum-based fossil fuel and the inconsistency of fuel price toward the global market had affected the environment, the economic and even to the humankind. Thus, seeking an alternative renewable energy such as biodiesel, which are economical, environmentally friendly and sustainable is highly crucial for future generation.

Biodiesel crops can be categorized into three generations. The first generation of biodiesel is related to edible crops such as maize, soybean, rapeseed and oil palm. Unfortunately, the issue regarding food versus fuel is often debated due to the world hunger problem, especially in Africa and Asia countries [1]. The second generation feedstock is from non-edible crops such as jatropha, tobacco seed, jojoba oil and mahua. However, it consumed large area for large production [2]. In fact, the issue in achieving positive energy is still questionable. In Malaysia specifically, biodiesel is not yet feasible for industrial scale due to high production cost, limitation of the extraction technology and low demands. Moreover, only a few companies that conducted biodiesel research derived from palm oil [3]. The third generation of biodiesel is based on algae crops such as seaweed and microalgae.

Unlike other conventional crops, microalgae provide several advantages for biodiesel feedstock. Microalgae required less freshwater compared to land crops. It has a rapid growth rate, which capable to reproduce twice of their weight within 24 hours [2, 4]. Microalgae are non-seasonal type of production, hence, can be harvested more than twice compared with the first and second generation where time is the limitation [2]. Moreover, the need of a land area for cultivation is lesser. Most importantly, it contained high lipid content [5, 6]. Due to high cell productivity as well as high lipid content, microalga is capable of producing a biodiesel yield twice than oil palm [2] and ten times than best oil like rapeseed [5].

The biodiesel production from microalgae involved four main processes; cultivation, harvesting, lipid extraction and biodiesel conversion [7, 8]. Among them, the cultivation of microalgae plays an important role in order to attain high cell productivity for massive production. The cultivation of microalgae can be conducted in three different modes and they are photoautotrophic, heterotrophic and mixotrophic mode [8].

In photoautotrophic condition, microalgae used sunlight and carbon dioxide (CO_2) for photosynthesis and convert it into energy [9]. This mode is widely used in the laboratory, pilot and industrial scale. To produce a higher biomass production yield it required a massive cultivation, thus photoautotrophic mode is the best option and it is compatible with indoor or outdoor cultivation. This cultivation mode is claimed to be more economic compared to heterotrophic and the mixotrophic mode [5, 7].

Some microalgae species are able to survive and grow well in an absent of light. However, without light microalgae unable to perform photosynthesis process thus, these microalgae are depending on the additional of external sources to grow such as inorganic or organic carbon sources (e.g. glucose, glycerol, acetate and CO_2) [10]. This mode is known as heterotrophic cultivation condition. The heterotrophic mode is more suitable only for laboratory scale and pilot scale because it must be placed in dark condition during the cultivation process. On the other hand, the presence of both light and carbon compound is called mixotrophic condition.

The percentage of lipid content in *Chlorella* sp. cultivated in photoautotrophic and mixotrophic (depending on their culture conditions) can achieve up to 58 % and 55 % of their dry weight, respectively [11]. Mixotrophic cultivation also could be an

alternative pathway to increase the growth rate of microalgae in a short period; however, a similar factor with the heterotrophic mode, mixotrophic also affected by the level of carbon sources provided for the cultures [12]. Mixotrophic culture condition was reported to improve the growth rate and lipid productivity of microalgae [12 – 14].

The objective of the study was to investigate the growth of microalga, measured by its cells concentration and specific growth rate under photoautotrophic, heterotrophic and mixotrophic culture conditions. In this research, a local freshwater microalga *Chlorella* sp. was selected as the potential strain. *Chlorella* sp. is a unicellular green alga with a size range from 3.0 to 8.0 µm and has a spherical shape [11]. The lipid content of *Chlorella* sp. is riched with saturated fatty acids, mainly palmitic acid and stearic acid and are the most commonly fatty acids exist in the biodiesel composition [15]. Higher content of saturated fatty acids may provide higher stability of oxidative [15]. Nevertheless, the lipid productivity produced by microalgae are greatly influenced by the cultivation condition.

2.0 EXPERIMENTAL PROCEDURE

2.1 Microalga Strain

A local freshwater microalga *Chlorella* sp. was selected for the investigation. The strain was obtained from Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah and originated from Tun Fuad Stephens Lake, Kota Kinabalu, Sabah (located at 6°N and 116°E) [16].

2.2 Microalga Culture Medium

The medium used in this experiment was Bold's Basal Medium (BBM). Filtered water (Elga, Purelab Prima) was used for the preparation of the stock solutions. The medium was sterilized in an autoclave at a temperature of 121˚C and a pressure of 15 psi for 15 minutes. The initial volume of microalga culture used was 1000 mL. The cultivation was exposed under the 12 hours light, 12 hours dark cycle for both photoautotrophic and mixotrophic mode while no light was exposed in the heterotrophic mode. All photoautotrophic and mixotrophic cultivations were conducted under a florescent white cool lamp with an intensity of 24 µE/s.m^2 (Lux meter Luteon LX-101) and were aerated at 1.0 L/min (Cole-Parmer flow rate meter). Whereas, the heterotrophic culture sample was placed on a magnetic stirrer. A digital pH meter (Eutech pH700) was used to measure the cultures temperature and pH. The cultures temperature and pH were maintained between 24.5˚C to 27.2˚C and 8.83 to 8.87, respectively. Glucose with the concentration of 0.315 ± 0.001 g/L was added into the heterotrophic and mixotrophic cultures. The microalga cultivation period was employed for 23 days and all the experiments were conducted in triplicates.

2.3 Microalgal Cell Observation and Growth Determination

For every 48 hours, the cells were harvested for observation and enumeration. Cells were viewed under a light microscope (Nikon Eclipse BO1, Light Microscope) with the magnification power of 40 and connected to a computer. Ten milliliters of samples were harvested for enumeration conducted using the Neubauer Improved hemocytometer and a light microscope (Axiolab, Light Microscope) with the magnification power of 10. The optical density (OD_{670}) was also employed to determine the cell concentration of *Chlorella* sp. at a wavelength of 670 nm [15] using Jasco UV-Vis spectrophotometer. The OD analysis was conducted to support the results obtained from the cell counting values.

2.4 Microalga Specific Growth Rate Determination

The specific growth rate of *Chlorella* sp. was calculated using the Equation 1 [4].

$$\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1) \quad (1)$$

N_2 represents the cell concentration (cells/mL) at time t_2 (day) and N_1 represents the cell concentration (cells/mL) at time t_1 (day).

2.5 Glucose Content Determination

The initial amount of glucose added to the heterotrophic and mixotrophic mode was 0.315 ± 0.001 g/L and the glucose was supplied only once during the whole cultivation period. A sample of 10 mL from heterotrophic and mixotrophic culture was taken every 48 hours for glucose content determination. The sample was centrifuged (Sigma 3-18 K Sartorius) at 8000 rpm for 5 min to separate the medium solution and microalga biomass. Benedict's reagent was added into the algae-free media with the ratio of 1:10. The mixtures were heated at 80˚C in a water bath for 30 minutes to allow the glucose to reduce the copper ion from Cu^{2+} to Cu^{+} which is appeared to be in blue and red color, respectively [11, 17]. The glucose concentration was determined by using Jasco UV-Vis spectrophotometer at a wavelength of 550 nm and using the standard curve of $y = 0.0014x + 0.0085$, with the correlation coefficient, R^2 of 0.995. The x represents the glucose concentration and y represents the absorbance value at 550 nm.

2.6 Scanning Electron Microscopy

Approximately 10 mL of *Chlorella* sp. from each culture mode was harvested and centrifuged at 8000 rpm for 5 min. The concentrated cells then lyophilized using a freeze dryer (Heto Drywiner LL1500) at -41˚C under vacuum condition for 24 hours. The surface morphology of the *Chlorella* sp. cell was examined by a Scanning electron microscopy (SEM) (Carl Zeiss EVO MA 10).

3.0 RESULTS AND DISCUSSION

3.1 Cell Growth of *Chlorella* sp.

Figure 1 represents the cell growth of *Chlorella* sp. in photoautotrophic, heterotrophic and mixotrophic condition. It can be seen that the cell concentration in photoautotrophic and mixotrophic cultures increased with time. After 8 days, photoautotrophic culture produced higher cell concentration compared to mixotrophic culture ($p < 0.01$). Detail inspection in Figure 1 shows that mixotrophic culture has a higher cell concentration than that of photoautotrophic culture from day 1 to day 8. This is likely due to the existence of glucose in mixotrophic culture. The additional of an organic carbon source for microalgae cultivation affect the microalgae growth rate as reported from previous literature [13, 18, 19]. Nevertheless, when glucose content is fully consumed by the microalga and the supplied light could not reach further into the bottom layer of the flask [10], this may explain the slightly lower cell concentration in mixotrophic culture as compared with photoautotrophic culture after 9 days onwards. As for heterotrophic culture, from day 1 to day 5 it has similar increase with photoautotrophic and mixotrophic cultures.

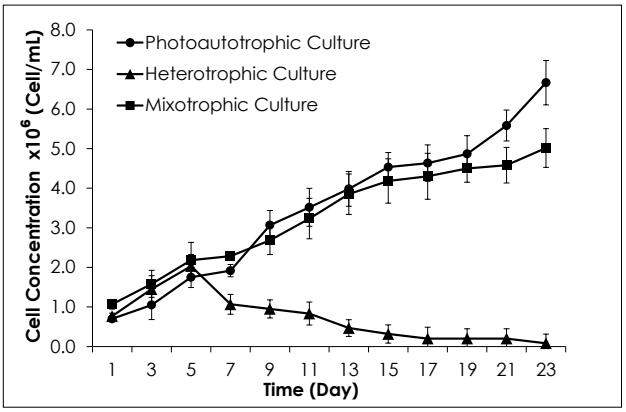


Figure 1 Cell growth of *Chlorella* sp. in photoautotrophic, heterotrophic and mixotrophic condition (results represent: mean ± std.dev, n=3)

However, the cell concentration started to decrease after 6 days onwards. In a heterotrophic culture which was without the light exposure, besides the nutrients contained in the culture medium, heterotrophic microalga was totally relied on the glucose to grow and survive [20]. The microalga cells were only able to reproduce for 5 days, and then due to the limitation of glucose the cells started to decrease afterward. In comparison with other reported literature [11, 13, 18], the initial glucose concentration used in this study was small.

According to Suali and co-workers [11], the highest specific growth rate of *Chlorella* sp. cultivated under mixotrophic mode using Jaworski's medium recorded when the glucose concentration was in a range from 1.0 to 5.0 g/L. However, in a large-scale application by adding chemicals into the culture system will directly increase the production cost [5, 7]. For that reason, in this study, a small amount of glucose (below 1.0 g/L) was used. Therefore, in order to enhance and maintain a higher growth rate of heterotrophic microalgae, the initial glucose content must be adequate and added continuously. Investigating the optimum value of glucose concentration below that 1.0 g/L (range from 0.1 to 0.9 g/L) could be an interesting future work.

Figure 2 and Figure 3 represent the growth profile of photoautotrophic and mixotrophic mode. The graphs show the increasing of both cell concentration and the OD value with time. For heterotrophic culture, OD analysis was not performed because UV-Vis spectrophotometer detects the green pigment (chlorophyll) in the cell as representing the cell concentration. In heterotrophic culture, it was found that the green pigment of the cell decreased due to no photosynthesis occurred.

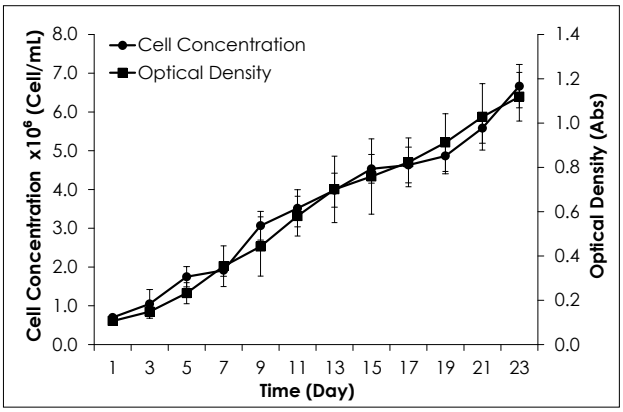


Figure 2 Growth profile in cell concentration and optical density of *Chlorella* sp. under photoautotrophic condition (results represent: mean ± std.dev, n=3)

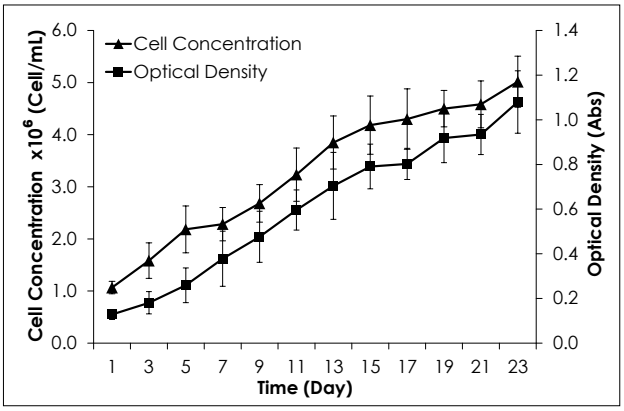


Figure 3 Growth profile in cell concentration and optical density of *Chlorella* sp. under mixotrophic condition (results represent: mean ± std.dev, n=3)

The maximum cell concentration and specific growth rate of *Chlorella* sp. in photoautotrophic, heterotrophic and mixotrophic cultures are shown in Table 1. The cells cultured in photoautotrophic mode produced the highest cell concentration with 6.67 ± 0.56 x 10^6 cell mL^{-1} at day 23, followed by mixotrophic mode with 5.02 ± 0.49 x 10^6 cell mL^{-1} at day 23 and heterotrophic mode with 2.03 ± 0.29 x 10^6 cell mL^{-1} at day 5. Providing a consistent light and carbon

compounds (glucose and CO_2 from the surroundings), *Chlorella* sp. was able to undergo photosynthesis in photoautotrophic and mixotrophic, thus, maintaining the cell production rate. Without light and limitation of glucose content contributed to poor cell production rate in heterotrophic cultivation.

Table 1 Maximum cell concentration and specific growth rate of *Chlorella* sp. in different cultivation mode (results represent: mean ± std.dev, n=3)

Cultivation Mode	x_{max} (x10^6 cells mL^{-1})	μ_{max} (Day^{-1})
Photoautotrophic	6.67 ± 0.56	0.26 ± 0.05
Heterotrophic	2.03 ± 0.29	0.32 ± 0.04
Mixotrophic	5.02 ± 0.49	0.20 ± 0.06

x_{max} is the cell concentration in cells/mL.
μ_{max} is the specific growth rate during the exponential growth phase.

On the contrary, heterotrophic culture showed the highest specific growth rate with 0.32 ± 0.04 day^{-1} (from day 1 to day 3) ($p < 0.05$), followed by photoautotrophic cultivation with 0.26 ± 0.05 day^{-1} calculated from day 3 to day 5. The lowest was recorded in mixotrophic culture with 0.20 ± 0.06 day^{-1} calculated from day 1 to day 3. The insufficient carbon source supplied in heterotrophic culture will increase the competitive rate of the cells to reproduce in a short time [20]. The specific growth rate in photoautotrophic and mixotrophic was not much different ($p > 0.05$) due to the light factor presence in both cultures.

3.2 Glucose Content Reduction in Mixotrophic and Heterotrophic Culture

The main point of measuring the glucose content in both heterotrophic and mixotrophic culture was to support the explanations from Figure 1 towards its growth curve. As for photoautotrophic mode, the glucose concentration effect was irrelevant because only light was provided.

The initial glucose concentration used in this study was 0.315 ± 0.001 g/L for both heterotrophic and mixotrophic cultures. Figure 4 indicates that the glucose concentration decreased from day 1 to day 7 of both cultures ($p < 0.01$). Heterotrophic condition showed the highest reduction of glucose concentration which was 0.242 ± 0.009 g/L at day 3 and decreased dramatically at day 7 with 0.006 ± 0.002 g/L (98 % of reduction). For mixotrophic mode, the glucose concentration was found to be 0.278 ± 0.011 g/L at day 3 and 0.014 ± 0.06 g/L at day 7 (95.4 % of reduction). Without the light exposure, microalga unable to undergo photosynthesis process. In other words, microalga cannot produce its own food for cell reproduction, hence, heterotrophic microalga utilized the carbon from other microorganism or organic waste as its main energy source [7]. For this reason, the decreasing level of glucose had clearly demonstrated that heterotrophic microalga consumes the glucose drastically, thus, resulting the enhancement of cell concentration as shown in Figure 1. When the microalga cell concentration reached to its maximum point with a limited glucose content inside the culture simultaneously, this led to the decreasing of cell productivity of the microalga. Moreover, microalga cultivated in heterotrophic mode was dependent on the initial amount of carbon compound supplied into the culture compared to a mixotrophic condition which was affected by the light intensity and the type of carbon source [18]. Therefore, *Chlorella* sp. was able to consume the glucose faster due to the initial glucose concentration was low [14].

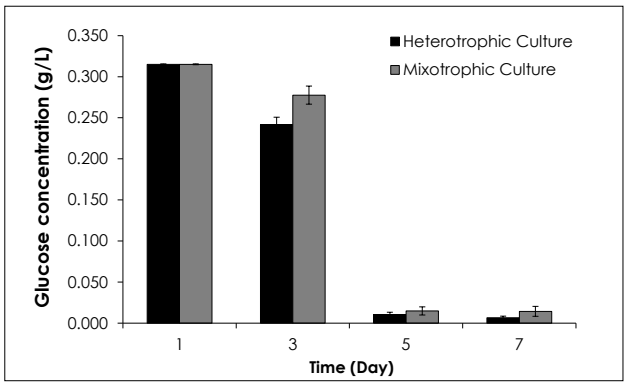


Figure 4 The glucose concentration in heterotrophic and mixotrophic cultures (results represent: mean ± std.dev, n=3)

3.3 Microscopic Observation and Surface Morphology of *Chlorella* sp. Cell

Figure 5a and Figure 5b displayed the cells morphology of *Chlorella* sp. in photoautotrophic mode under two different magnification powers. Based on the SEM images, the cells do not have their typical round shape as reported by Suali *et al*. [11]. This might be due to the lyophilization effect that caused the cells to shrink. Conventionally, lyophilization is used as one of the microalgae cell disruption methods to extract lipid from microalgae. However, the cell clearly displayed a distinct cell envelope and almost spherical in shape. The cell sizes were in the diameter range of 3 µm to 8 µm. For heterotrophic and mixotrophic culture, image not shown due to a similar shape and sizes were also obtained.

The cells of *Chlorella* sp. were also viewed (Figure 6) under a light microscope on the final day (day 23) of cultivation period. The purpose of the cell observation was to visualize any cell changes in terms of shape, size and color. Based on Figure 6a the cells distribution in the photoautotrophic culture was clearly seen to be higher compared to heterotrophic (Figure 6b) and mixotrophic culture (Figure 6c). The cells distribution in the heterotrophic was the lowest and it was also observed that the cells were less green due to loss of its chlorophylls (Figure 6b).

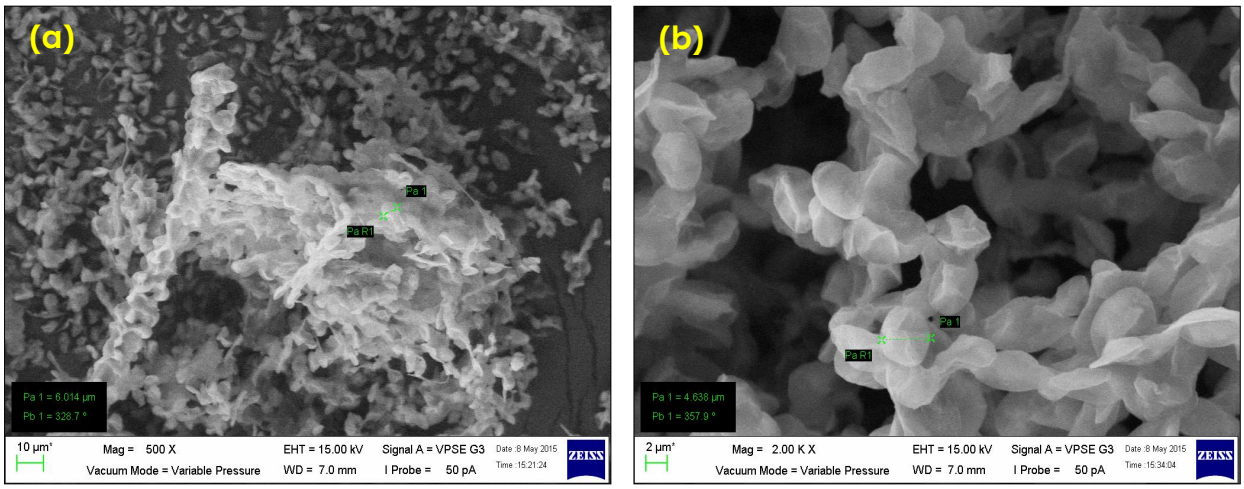


Figure 5 SEM images of surface morphology of the lyophilized *Chlorella* sp. cells viewed **(a)** under 500x magnification power and **(b)** under 2000x magnification power

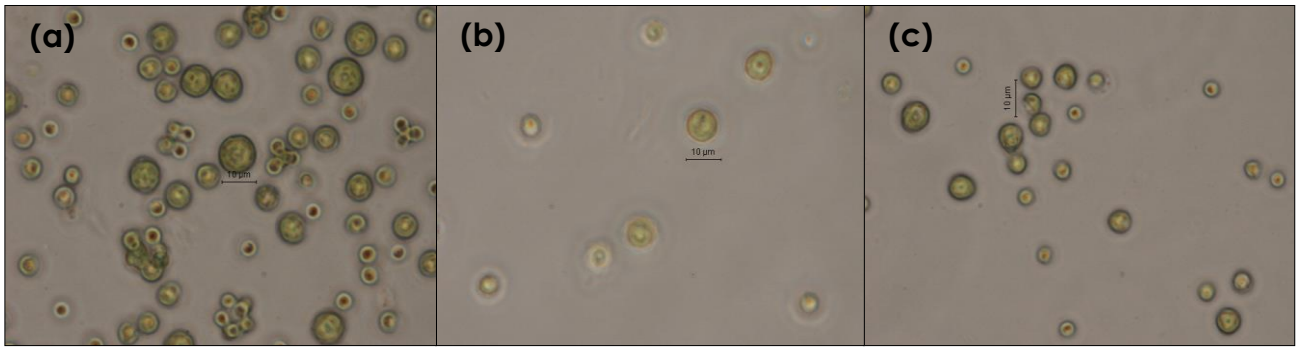


Figure 6 Image of *Chlorella* sp. cells viewed under a light microscope (40x magnification power) on the 23rd day **(a)** Photoautotrophic mode, **(b)** Heterotrophic mode and **(c)** Mixotrophic mode

4.0 CONCLUSION

The growth evaluation of a local freshwater microalga *Chlorella* sp. was conducted in this study. The results indicated that *Chlorella* sp. has the highest specific growth rate in heterotrophic cultivation, but achieved a highest cell concentration in photoautotrophic for 23 days of cultivation period. In the photoautotrophic mode, light is required for the microalga to grow. The addition of glucose in heterotrophic and mixotrophic cultures enhanced the cell concentration at the initial stage. Afterward, the growth curve decreased when the glucose content was fully consumed by microalga in the heterotrophic mode. Nevertheless, with the exposure of light in mixotrophic mode, the growth curve continues to increase with time. In conclusion, although *Chlorella* sp. cultivated under the heterotrophic mode has a higher specific growth rate, the cultivation mode dependable with an external energy source such as glucose to grow. Unfortunately, in a large-scale production point of view, higher biomass production will increase the glucose consumption and this directly contributes to a higher production cost. For this concern, aside from using the photoautotrophic mode as the culturing technique, the mixotrophic mode also could be an alternative technique for microalgae cultivation specifically for biodiesel production. However, the addition of the carbon source must be enough and continuously supplied to the microalga cell for maintaining its cell productivity as high as possible. Hence, the flexibility of microalgae to grow in various conditions has solidified its potential to replace other conventional biomass feedstock, such as oil palm in Malaysia.

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