

Comparative Studies of Cell Growth, Total Lipid and Methyl Palmitate of *Ankistrodesmus* sp. In Phototrophic, Mixotrophic and Heterotrophic Cultures for Biodiesel Production

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Abstract- Cultivation mode selection is considered to be an important aspect as this corresponded to the amount of lipid produced by microalgae and to be used for biodiesel production. In this study, phototrophic, mixotrophic and heterotrophic mode were used to cultivate *Ankistrodesmus* sp. in mon-culture for 17 days. It was found that, mixotrophic *Ankistrodesmus* sp. shown the highest cells number ($9.77 \pm 0.59 \times 10^6$ cell mL⁻¹), cell productivity ($1.23 \pm 0.08 \times 10^6$ cell mL⁻¹ day⁻¹), specific growth rate (0.43 ± 0.04 day⁻¹), biomass yield (1.61 ± 0.05 g L⁻¹), total lipid (10.55 ± 0.15 % wt), methyl palmitate yield (0.142 ± 0.005 µg mL⁻¹) and the shortest doubling time (1.63 ± 0.15 day). This was due to effect of glucose as an external carbon source in mixotrophic culture. However, without light *Ankistrodesmus* sp. grew poorly under heterotrophic mode affected by the lower pH of culture. It was also observed that the mix-cultures of *Ankistrodesmus* sp. and *Chlorella* sp. cultivated under mixotrophic mode shown better cell growth of *Ankistrodesmus* sp. than *Chlorella* sp. cells. This indicates that, *Ankistrodesmus* sp. has a higher tolerance towards the culture condition and has better chances of survival in the presence of other microalga species. Hence, the study shows that cultivating *Ankistrodesmus* sp. under mixotrophic mode will provide a better cell growth profile, enhance the lipid content and strong endurance.

Keywords Cultivation mode, *Ankistrodesmus* sp., cell growth, total lipid, methyl palmitate.

1. Introduction

The decreasing level of fossil fuel in line with the increasing of energy demands recently has made microalgae crops as one of the most potent and promising feedstock for renewable and sustainable energy resources. Microalgae capable of sequestering carbon dioxide (CO₂) efficiently

around 10 to 50 times higher [1] and has a rapid growth rate 40 times faster than terrestrial plants [2] as well as containing oils 7 to 31 times higher than soy oil and palm oil [3]. Because of this also, numerous studies had been conducted regards to microalgae, including pharmaceutical [4], nutraceutical [5], agricultures [6], biotechnology and bioengineering [7–10], waste water treatment [11, 12] and

aquacultures [13, 14]. By far, In Malaysia microalgae-based biodiesel is still under development and oil palm-based biodiesel is commonly used as the raw material. Besides that, the demand of biodiesel in Malaysia is still considered to be lower compared to other Asian countries such as Philippines, Thailand and Japan [15]. For that reason, this caused some biodiesel companies in Malaysia had switched biodiesel as their side product instead of their main product [16]. In fact, using oil palm as a biodiesel feedstock is not highly recommended due to the competition of domestic food industries and issues on food versus fuel [17, 18]. Because of this concern, microalgae crops can be used as an alternative raw material to substitute palm oil for biodiesel feedstock, especially in Malaysia.

Microalgae can be cultivated either phototrophically, heterotrophically, mixotrophically or photo-heterotrophically [19, 20]. Phototrophic microalgae utilised sunlight and CO₂ to grow. Even though, some phototrophic microalgae provide a lower cell density and cell productivity [21, 22], this cultivation mode is widely used especially in a large scale production due to its simplicity and low cost of cultivation [23]. Without light heterotrophic microalgae obtained energy from other organic compounds such as glucose, glycerol and acetate. Several literatures had reported that heterotrophic microalgae promote a better cell productivity with 2 times higher compared to phototrophic microalgae [24]. However, this cultivation mode is highly expensive. It must be cultivated in a dark condition and can be contaminated easily by other microorganisms [19]. Mixotrophic microalgae utilized sunlight, organic and inorganic compounds to grow. Similar to the heterotrophic mode, but with light provided, the photo-heterotrophic mode is rarely used due to the high cost of equipment and subtracts [25].

Having a rapid growth rate, able to grow in almost extreme conditions, a non-seasonal harvesting and less land requirement are the several advantages of microalgae crops [17, 26]. However, for a large scale production the selection of microalgae species is another criterion that should be highlighted especially if cultivated in an open system. The major drawback of using an open system for cultivation is the contamination issues [23]. Predators such as rotifers consumed microalgae in order to survive, thus this will affect the microalgae growth performance. Hence, microalga that has higher chances of survival and most dominant is preferred to be used.

Mata et al. [27] reported that marine and freshwater microalgae are capable of producing lipid up to 75 % wt (i.e. *Botryococcus braunii*) and lipid productivity up to 142.0 mg L⁻¹ day⁻¹ (i.e. *Nannochloropsis oculata*). Meanwhile, the cell productivity can increase up to 7.3 g L⁻¹ day⁻¹ (i.e. *Chlorella protothecoides*) [20]. In general, microalgae lipid enriches with fatty acid (FA) compositions, which can be classified into saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA). SFA such as palmitic acid (C16:0) is the major and common fatty acid existed in microalgae lipid (i.e. *Chlorella* sp., *Nannochloropsis* sp. and *Scenedesmus* sp.) [28, 29]. Such well-established microalgae are frequently used as the raw

materials either in research laboratories or in industrial sectors.

Other than these well-established microalgae, a fresh water microalga *Ankistrodesmus* sp. could be used as one of the potential candidates for biodiesel feedstock. As reported by Salim [30], under phototrophic mode and CO₂ induction, *Ankistrodesmus* sp. was capable of producing its cell number, specific growth rate and biomass as high as 7.73 x 10⁵ cell mL⁻¹, 1.59 day⁻¹, 2.4 g L⁻¹, respectively. Meanwhile, under heterotrophic mode of cultivation and using cassava starch hydrolysate (CSH) as the organic carbon source, the cell number, specific growth rate and biomass of *Ankistrodesmus* sp. can be up to 2.46 x 10⁶ cell mL⁻¹, 0.50 day⁻¹, 0.94 g L⁻¹, respectively [3]. Sukkrom and co-workers had investigated the amount of total lipid and lipid productivity of phototrophic *Ankistrodesmus* sp. by reusing the initial medium to culture the microalga [31]. They found the total lipid can be achieved up to 57.7 % wt from 1st reused medium and lipid productivity as high as 33.3 mg L⁻¹ day⁻¹ from 2nd reused medium. Under pilot scale (160 – 180 L) cultivation, the total lipid can be obtained around 28 – 39 % wt [32]. In addition, the FA compositions found in *Ankistrodesmus* sp. lipid were mainly consisted of C16 and C18 groups [31].

To the best of the authors' knowledge, by far there is no reported literature regards on the effect of cultivation modes towards the cell growth behaviour of *Ankistrodesmus* sp. in mono-culture or in mix-culture. Hence, it was expected that this study able to contribute a better understanding on the cell growth behaviour of this local microalga *Ankistrodesmus* sp. focussed on the effect of phototrophic, mixotrophic and heterotrophic mode cultivation. Apart from that, investigating the survival rate of *Ankistrodesmus* sp. with a well-established microalga such as *Chlorella* sp. in mix-culture was important to understand the level of competitiveness of the microalga.

2. Materials and Methods

2.1. Microalga Species

Local freshwater microalga *Ankistrodesmus* sp. and *Chlorella* sp. were selected as the raw materials. The microalgae were obtained from Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah.

2.2. Microalga Culture Condition

2.2.1. Culture Medium

Bold's Basal medium (BBM) was used to culture the microalgae [33]. The medium and apparatus used were autoclaved at 121°C and 103.4 kPa for 15 mins.

2.2.2. Phototrophic and Mixotrophic Culture Mode

A batch of culture in triplicates was prepared in a 500 mL Erlenmeyer flask. All cultures were exposed to a light and dark cycle a ratio of 12:12 hours. The cultivation was

performed under a fluorescent white cool lamp with a photon flux approximately $135 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Lux meter, LX-101, Lutron, India). The culture was aerated using an air bubble stone at 1.0 L min^{-1} (Flowmeter, Cole-Parmer, USA) [17]. The culture temperature was recorded and maintained at $32.0 \pm 2.0 \text{ }^\circ\text{C}$. Glucose 1.0 g L^{-1} was added into all mixotrophic mode samples (mono- and mix-culture). The growth study was employed for 17 days of cultivation period.

2.2.3. Heterotrophic Culture Mode

A batch of culture in triplicates was prepared in a 500 mL Erlenmeyer flask. The sample was sealed using a black paper and covered with aluminium foil. To achieve a homogeneous solution, the culture was placed on a magnetic stirrer at the speed level of 6 (HS0707V2, Favorit, Malaysia) [17]. The culture temperature was recorded and maintained at $32.0 \pm 2.0 \text{ }^\circ\text{C}$. Glucose 1.0 g L^{-1} was added into all samples and the cultivation was performed in a dark room condition. The growth study was employed for 17 days of cultivation period.

2.3. Microscopic Observation of Microalga

2.3.1. Microscopic View

Ankistrodesmus sp. and *Chlorella* sp. cells were viewed under a light microscope (Eclipse BO1, Nikon, USA) using the magnification power of 40.

2.3.2. Cell Morphology

Approximately 10 mL of *Ankistrodesmus* sp. was collected from the sample and dried in an oven at $70 \text{ }^\circ\text{C}$ for 2 hours. The surface morphologies of the *Ankistrodesmus* sp. cells were examined by scanning electron microscopy (SEM) (EVO MA 10, Carl Zeiss, Germany). The SEM sample preparation was performed using a chemical fixation method with 3% glutaraldehyde and was left for 12 hours at $4 \text{ }^\circ\text{C}$. Then, 0.1 M of phosphate buffer solution was added, gently mixed by swirling and left for 10 minutes. Then, dehydration processes using a series of ethanol with different concentrations (30, 50, 70, 90 and 100%) were performed. The sample was left for 30 mins before added with the new ethanol concentration. The sample was glued to a stub, sputter-coated with gold and then examined under SEM at 1000X magnification [34].

2.4. Determination of Growth Evaluation and Biomass Estimation

2.4.1. Cell Number and Cell Dry Weight

A sample of 10 mL from each culture was taken for every 48 hours to count the *Ankistrodesmus* sp. and *Chlorella* sp. cells number. The numbers of microalgae cells were determined using the Neubauer Improved Haemocytometer and a light microscope (Axiolab, Light Microscope) with the magnification power of 40. The

microalgae cell dry weights were determined via gravimetric method. Culture sample of 10 mL was harvested for every 48 hours and placed in a 25 mL clean container. The samples were dried at $70 \text{ }^\circ\text{C}$ overnight in an oven. Then was cooled to room temperature and measured using an analytical balance with a precision of 0.1 mg. The cell dry weight was expressed in grams per litre (g L^{-1}). All data presented in this work were expressed in average values \pm standard errors, unless was stated.

2.4.2. Cell Productivity, Specific Growth Rate and Doubling Time

The cell productivity in each culture was calculated according to Mansa et al. [35], using Eq. 1, whereas the specific growth rate (μ) and doubling time (T_2) were calculated according to Levasseur et al. [36] using Eq. (2) and (3):

$$\text{Productivity} = N_2 - N_1 / t_2 - t_1 \quad (1)$$

$$\text{Specific growth rate, } \mu = \ln(N_2/N_1) / (t_2 - t_1) \quad (2)$$

$$\text{Doubling time, } T_2 = 0.6931/\mu \quad (3)$$

Where;

N_1 and N_2 are the cells number (cell mL^{-1}) at the time (t_1) and time (t_2), respectively.

2.5. Determination of Glucose Content

A sample of 20 mL from each heterotrophic and mixotrophic culture was taken every 48 hours for glucose content determination. The sample was centrifuged (3-18K Sartorius, Sigma, UK) at 6000 rpm for 10 mins to separate the medium solution and microalga biomass. Benedict's reagent was used as the glucose indicator and the procedures can be referred from the previous study [17]. The glucose concentration was determined by using a UV-Vis spectrophotometer (Jasco, UV-Vis spectrophotometer, UK) at a wavelength of 550 nm.

2.6. Determination of Lipid and Esters

2.6.1. Total Lipid Estimation

The total lipid of microalga *Ankistrodesmus* sp. was quantified based on gravimetric method. At the last day of cultivation period, all culture samples were centrifuged at 8000 rpm for 10 mins and dried in an oven at $70 \text{ }^\circ\text{C}$ overnight. Total lipid was extracted from *Ankistrodesmus* sp. dried biomass according to Folch method (CHCl_3 -MeOH; 2:1; v/v) [37] and left for 15 hours without a continuous mixing. The biomass was filtered using two filter papers for each sample. The solvent was removed using a rotary evaporated at $62 \text{ }^\circ\text{C}$ (RV O5-ST, Kika®-WERKE, China) and was dried at $70 \text{ }^\circ\text{C}$ for 2 hours until a constant weight achieved [38]. The samples were left to cool at a room

temperature and the final weight of the extracted lipid was recorded. The total lipid content was calculated using Eq. (4).

$$\text{Total lipid (\% wt)} = \left[\frac{\text{g}_{\text{lipid}}}{\text{g}_{\text{dried microalga}}} \right] \times 100 \% \quad (4)$$

2.6.2. Fatty Acid Methyl Ester Conversion

The extracted lipid was converted to fatty acid methyl esters (FAME) using the method and procedures described by Zbinden et al. [9]. Approximately, 0.5 mL of a mixture of methanol and toluene (1:1; v/v) was added to dissolve the dried lipid sample. Then, 0.5 mL of 0.2 N methanol-potassium hydroxide solution was added. The sample was sealed in a 28 mL universal bottle and vortexed. Next, it was heated at 37 °C for 15 mins in a water bath to allow the transesterification reaction to occur and then was cooled at room temperature for a few minutes. About, 0.5 mL of 0.2 N acetic acid solution was added into the sample, followed by 2.0 mL of chloroform and 2.0 mL of deionized water. Mixing by swirling was performed for each addition of these reagents. The sample was then stirred using a magnetic stirrer at speed level of 7 (HS0707V2, Favorit) for 5 mins until two layers were formed. The bottom layer which was the chloroform phase (containing lipids) was extracted using a Pasteur pipette and was placed into a new and clean universal bottle. Finally, 1.0 mL of chloroform was added into the sample again for further purification and the sample was centrifuged once more, extracted, and placed into a new universal bottle. These steps were repeated twice. To ensure all water was removed a few grams of sodium sulphate anhydrous (Na₂SO₄) was added into the sample.

2.6.3. Fatty Acid Methyl Ester Compositions

The analysis of FAME was performed using GCMS (Agilent 19091S-433 GC with HP-5MS) and the parameters setting were followed according to Zbinden [39] but using zero holding time at maximum temperature. The flow rate of Helium gas (the carrier gas) was set at 1.4 mL min⁻¹ with an initial temperature of 120 °C held for 2 mins, then increased at 6 °C min⁻¹ to 180 °C, followed by 1.5 °C min⁻¹ to 198 °C, and finally at 5 °C min⁻¹ to 240 °C. The split ratio was set at 120:1. Methyl palmitate was used as the standard FAME. The standard was prepared with five different concentrations ranging from 0.36 mg mL⁻¹ to 3.63 mg mL⁻¹. The graph of peak area (cm²) against concentration (mg mL⁻¹) was plotted and a standard curve of $y = 18406792x$ with R² equivalent to 0.995 was obtained.

3. Results and Discussion

3.1. Cell Morphology and Sample Colour Observation of *Ankistrodesmus* sp.

The cells image for both microalga *Chlorella* sp. and *Ankistrodesmus* sp. under a light microscope were shown in Fig.1a and Fig.1b, respectively. Both are green freshwater microalgae unicellular. The *Chlorella* sp. has a spherical-shaped [2, 17], whereas *Ankistrodesmus* sp. has a needle-like shaped or crescent-shaped with slight curve at both ends [10]. Cell morphology of *Ankistrodesmus* sp. was shown in

Fig.2. It has a cell length from 14.7 to 38.1 μm (Fig.2a and Fig.2b). Generally, *Ankistrodesmus* sp. cell sizes were found to be approximately 5 times bigger than *Chlorella* sp. cell. Meanwhile, the cell morphologies of *Chlorella* sp. can be referred from the previous study [17].



Fig. 1. Cells image of microalga under a light microscope at magnification power of 40X (a) *Chlorella* sp. cells and (b) *Ankistrodesmus* sp. cells.

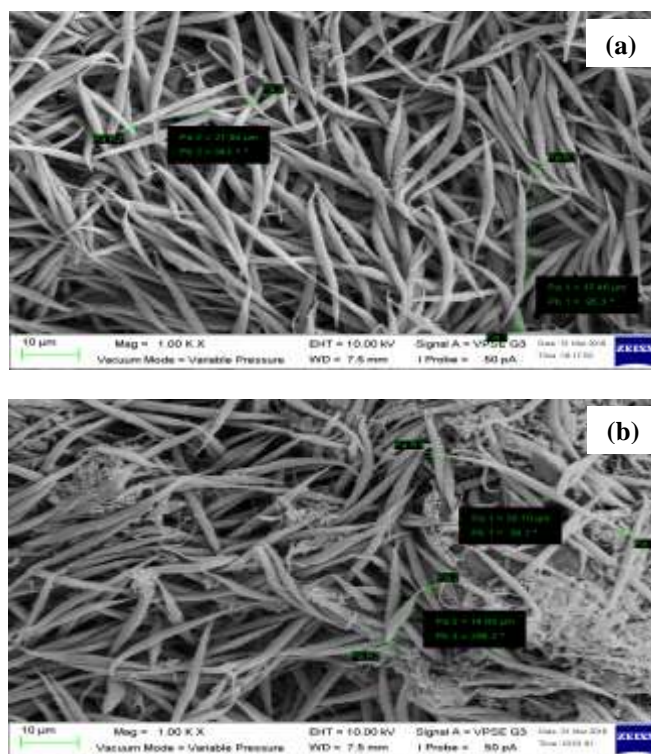


Fig. 2. SEM images of microalga *Ankistrodesmus* sp. under 1000X magnification power.

The sample colour of *Ankistrodesmus* sp. in all cultures on the final day of cultivation was shown in Fig.3. In comparison to the other modes, the sample colour in heterotrophic culture was observed to be green-yellowish and looks pale due to no light source was provided for this culture mode (see Fig.3c). The microalga was unable to undergo photosynthesis process, which was a similar observation gained in heterotrophic *Chlorella* sp. [17]. Hence, in sequence order from green to pale green colour, the pigment colour from each culture can be arranged to (d) > (b) > (a) > (c). Based on visual inspection, it can be concluded that, the greener the colour of the solution the higher the cells number of microalga in the culture sample.

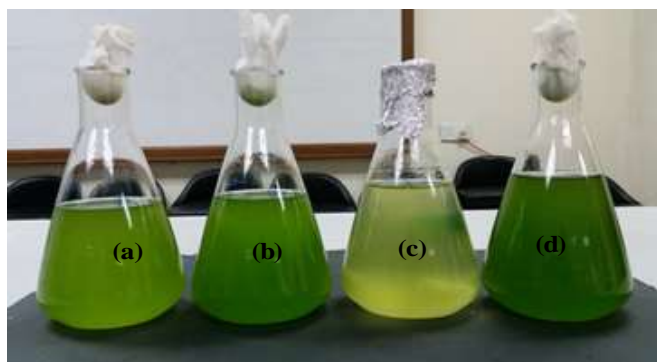


Fig.3. Comparison of solution colour among the cultures at day 7. Mono-culture samples cultivated under (a) phototrophic mode, (b) mixotrophic mode, (c) heterotrophic mode, and a mix-culture sample cultivated under (d) mixotrophic mode.

3.2. Effect of Glucose Concentration on *Ankistrodesmus* sp. Growth Profile

3.2.1. Preliminary Study on The Glucose Effect In Mixotrophic Culture

The effect of glucose concentration towards the *Ankistrodesmus* sp. growth was shown in Table 1. In this preliminary study, various glucose concentrations were prepared from 0.1 to 30.0 g L⁻¹ including no additional of glucose as the control sample. The cultivation was conducted under mixotrophic mode for 6 days of cultivation duration. Based on Table 1, the final biomass and specific growth rate for all samples that added with 0.1 and 1.0 g L⁻¹ glucose were higher compared to the control sample. From this result also, it was found that by adding a glucose concentration of 1.0 g L⁻¹ gave the highest biomass and specific growth rate of 0.96 ± 0.01 g L⁻¹ and 0.33 ± 0.01 day⁻¹, respectively.

Unfortunately, the cell productivity of *Ankistrodesmus* sp. had decreased as the level of glucose concentration increased from 5.0 to 30.0 g L⁻¹. From Table 1, the pH ranges recorded for glucose concentration 5.0 g L⁻¹ and above was in the pH range from 2.87 to 2.93, which was an acidic solution. Increasing the glucose concentration had contributed to a lower pH of the culture solution. This was most likely the reason that caused the cell productivity of *Ankistrodesmus* sp. to decrease. It was also observed that another microorganism existed in the sample. This indicates

that the pH was one of the important parameters that affect the microalgae cell productivity [30]. Based on Table 1, the solution pH range from 7.04 to 8.47 was compatible for *Ankistrodesmus* sp. to grow. In this study, 1.0 g L⁻¹ of glucose concentration was used for culturing the microalga in mixotrophic and heterotrophic cultures, since it was the optimum concentration.

Table 1. The cell productivity of *Ankistrodesmus* sp. cultivated in various glucose concentrations (Mean ± SE, n = 2)

Glucose (g L ⁻¹)	Final dried biomass (g L ⁻¹)	Specific growth rate, μ (d ⁻¹)	Glucose decreases (%)	Final pH
0.0	0.87 ± 0.01	0.12 ± 0.01	-	7.05 ± 0.01
0.1	0.91 ± 0.02	0.29 ± 0.01	100	7.17 ± 0.01
1.0	0.96 ± 0.01	0.33 ± 0.01	98	8.47 ± 0.01
5.0	0.14 ± 0.02	-0.05 ± 0.01	83	2.93 ± 0.01
10.0	0.13 ± 0.01	-0.07 ± 0.02	88	2.85 ± 0.01
15.0	0.12 ± 0.01	-0.08 ± 0.03	89	2.87 ± 0.01
20.0	0.11 ± 0.02	-0.08 ± 0.02	92	2.87 ± 0.01
30.0	0.10 ± 0.01	-0.08 ± 0.02	96	2.87 ± 0.01

3.2.2. Glucose Effect In Mono-Culture and Mix-Culture

The glucose concentration in mixotrophic and heterotrophic cultures was shown in Fig.4. As stated earlier, the initial glucose concentration used in this study was 1.0 g L⁻¹. The result had indicated that the glucose content was decreased from day 1 to day 9 in all cultures. Based on Fig.4, microalgae in the mix-culture cultivated under mixotrophic mode had showed the highest glucose reduction which was 0.0331 ± 0.0040 g L⁻¹ at day 3, 0.0116 ± 0.0015 g L⁻¹ at day 5 and was fully consumed at day 7. For microalga in mono-culture cultivated under mixotrophic mode, the glucose concentration was found to be 0.0361 ± 0.0017 g L⁻¹ at day 3, 0.0239 ± 0.0010 g L⁻¹ at day 5 and fully consumed at day 7. Meanwhile, microalga from heterotrophic culture had showed the lowest reduction of glucose, which was 0.3656 ± 0.0039 g L⁻¹, 0.0463 ± 0.0012 g L⁻¹, 0.0245 ± 0.0005 g L⁻¹ and 0.0013 ± 0.0001 g L⁻¹ at day 3, 5, 7 and 9, respectively.

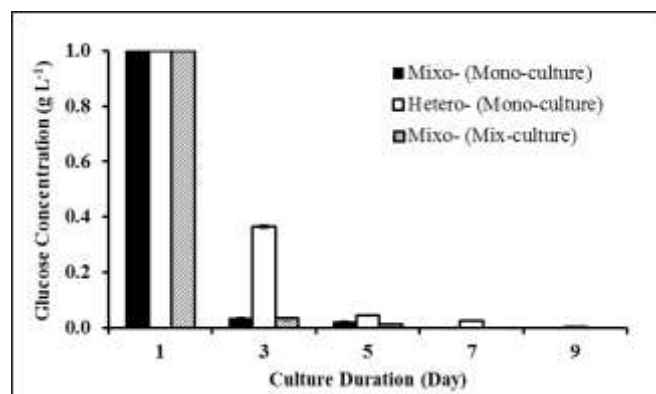


Fig. 4. Glucose concentration (g L⁻¹) in mixotrophic and heterotrophic culture.

In mixotrophic culture, the existence of two different microalgae species (*Ankistrodesmus* sp. and *Chlorella* sp.) and from mix-culture was the main reason for the higher glucose reduction (97 %) at day 3. Both species utilized the glucose and competing with each other to reproduce. Meanwhile, in heterotrophic culture, the pH reading at day 3 was 5.15 ± 0.02 , which was an acidic solution. *Ankistrodesmus* sp. was unable to reproduce efficiently under this pH level and likely the main reason for a lower glucose reduction (64 %) at day 3.

3.3. Growth Characteristic of Microalga

3.3.1. Survival Behaviour of *Ankistrodesmus* sp. In Mix-Culture

In an open pond system, microalgae are highly exposed to an open air and thus, contamination is always an issue. Because of this concern, it is important to select microalga that has the capability to survive in the presence of predators or other microalgae species. According to Costas et al. [40] some microalgae can be mutated into another strain to survive by changing their phycology. The survival rate can be determined based on the cell population (%) of the microalgae. In this work, microalga *Chlorella* sp. and *Ankistrodesmus* sp. were mixed and cultivated under a mixotrophic mode.

The comparison of *Ankistrodesmus* sp. cell growth in mono- and mix-cultures was shown in Table 2. The cell of *Ankistrodesmus* sp. increased, whereas *Chlorella* sp. cells number decreased with time. As shown in Table 2, at day 15 the cell population of *Ankistrodesmus* sp. was observed to be 100 %. Although the fresh media was added at day 10, the cell growth of *Chlorella* sp. kept on decreasing. One of the factors that caused *Chlorella* sp. cells to drop was the effect of culture temperature. According to Suali and Sarbatly [19] a high culture temperature (30 – 50 °C) may not be compatible for most microalgae to grow.

In In this work, the temperature in each culture mode was recorded between 30 °C to 34 °C, which was considered

to be higher, compared to the previous study, conducted by Joannes et al. and used *Chlorella* sp. as their sample [17]. Several of *Chlorella* species were suitable to be cultured under a culture temperature between 25 to 28 °C [19]. In addition, the decreasing of *Chlorella* sp. cells number was also attributed by the nutrient limitation due to microalgae competition to survive. On the contrary, *Ankistrodesmus* sp. was able to grow well in this culture temperature range. From the result, it shows that *Ankistrodesmus* sp. has a higher survival rate, tolerance and adaptation to culture conditions compared to *Chlorella* sp. cells.

The cells number of *Ankistrodesmus* sp. in mono-culture was higher compared to the cells number of *Ankistrodesmus* sp. in mix-culture from day 3 to 7. Lower cell productivity of *Ankistrodesmus* sp. in the mix-culture from day 3 to 7 was likely due to the microalgae competition occurred in the culture sample. At day 9 to 17, the cells number of *Ankistrodesmus* sp. in mix-culture ($10.87 \pm 0.57 \times 10^6$ cell mL⁻¹) had increased 1.1 times higher than that in mono-culture ($9.77 \pm 0.59 \times 10^6$ cell mL⁻¹). When microorganisms die, it decomposed and released its dissolved organic nutrients to the surroundings, and other living microorganisms will be able to consume these nutrients [40]. A similar case might be happening to the microalgae from mix-culture. The decreasing of *Chlorella* sp. cells number indicates the cells death. Since, the nutrient from *Chlorella* sp. dead cell had been utilized by *Ankistrodesmus* sp. this eventually contributes an increasing of cell productivity in the mix-culture.

3.3.2. Growth Profile of *Ankistrodesmus* sp. In Mono-Culture

Microalga *Ankistrodesmus* sp. was cultivated in mono-culture under three different modes to investigate its growth behaviour and was determined in term of its cells number per volume. The Fig.5 and Fig.6 displayed the cell growth and comparison of biomass of *Ankistrodesmus* sp. from phototrophic, mixotrophic and heterotrophic culture, respectively.

Table 2. Microalga cells number and population gained from mono- and mix-cultures cultivated under mixotrophic mode

Day	Mono-culture ^a	Mix-culture ^a	Individual species ^{a, b}		Population ^c (%)	
	<i>Ankistrodesmus</i> sp.	<i>Ankistrodesmus</i> sp.: <i>Chlorella</i> sp.	<i>Ankistrodesmus</i> sp.	<i>Chlorella</i> sp.	<i>Ankistrodesmus</i> sp.	<i>Chlorella</i> sp.
1	0.80 ± 0.20	1.77 ± 0.07	0.80 ± 0.20	0.97 ± 0.24	46 ± 13	54 ± 13
3	1.85 ± 0.36	1.78 ± 0.08	1.00 ± 0.23	0.78 ± 0.22	56 ± 13	44 ± 13
5	2.83 ± 0.36	2.38 ± 0.18	1.68 ± 0.34	0.70 ± 0.20	69 ± 11	31 ± 11
7	4.08 ± 0.44	4.67 ± 0.19	4.03 ± 0.39	0.63 ± 0.20	86 ± 5	14 ± 5
9	5.17 ± 0.52	5.77 ± 0.21	5.30 ± 0.43	0.47 ± 0.22	92 ± 4	8 ± 4
11	7.63 ± 0.63	8.28 ± 0.39	7.90 ± 0.60	0.38 ± 0.21	95 ± 3	5 ± 3
13	8.47 ± 0.66	9.12 ± 0.43	8.92 ± 0.56	0.20 ± 0.13	98 ± 2	2 ± 2
15	9.17 ± 0.55	10.37 ± 0.59	10.37 ± 0.59	0.00 ± 0.00	100 ± 0	0 ± 0
17	9.77 ± 0.59	10.87 ± 0.57	10.87 ± 0.57	0.00 ± 0.00	100 ± 0	0 ± 0

Notes: ^a Cell concentration in x10⁶ cell mL⁻¹, ^b Individual cell distribution in mix-culture, ^c Population cell distribution in the mix-culture.

The overall growth characteristics of the microalga were summarized in Table 3. Based on Fig.5 the cells number of *Ankistrodesmus* sp. in both phototrophic and mixotrophic modes were increased with time. Meanwhile, the cells number in heterotrophic mode was decreased from day 1 to day 9, then slightly increased from day 9 to 13, and decreased again from day 15 to 17. Details inspection in Fig.5, it was observed that the cells number of microalga in mixotrophic culture was always higher than the cells number in phototrophic culture along the cultivation period. This can be explained due to the presence of glucose in mixotrophic culture. In mixotrophic microalgae, aside from attaining the carbons directly from the CO₂, microalgae also consumed the carbons from glucose as a source of external energy. This excites the microalgae growth rate and thus enhanced its cell productivity [17, 42, 43].

Unlike Salim [3] and Lananan et al. [10] findings that obtained two growth peaks, here only one growth peak was observed. The different growth pattern of *Ankistrodesmus* sp. might due to the additional of nutrient. Maintaining the cell productivity higher as possible is crucial for biomass production. Hence, nutrient (20 mL of fresh BBM) was added into all cultures at day 10. This led to an increasing of cell growth at day 11 about 1.5 times higher than the previous growth in both phototrophic and mixotrophic cultures.

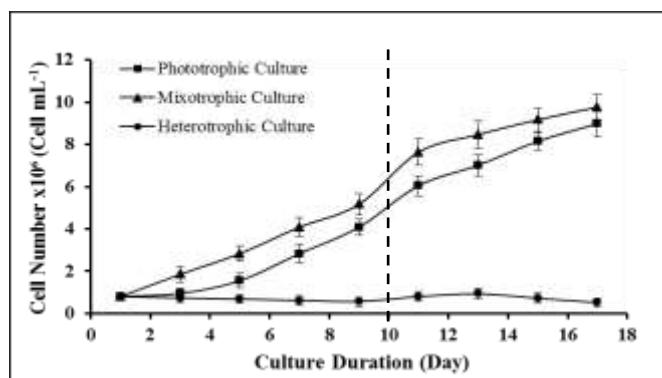


Fig. 5. Cell growth of *Ankistrodesmus* sp. in mono-culture under phototrophic, mixotrophic and heterotrophic mode.

Without the light source, heterotrophic microalga was fully dependent on the glucose as the main energy source. Glucose addition had caused the pH of the culture solution to decrease. The pH decreased from 6.74 ± 0.01 to 5.15 ± 0.02 at day 1 to day 3, respectively. The culture solution became more acidic and this had caused a poor cell growth. This shows that culture pH can affect the microalga cells growth [44]. The pH ranges from 7 to 9 were found to be the optimum value to obtain a higher cell growth [30, 44]. This support the results obtained in the study, where the cell growth has a positive response to an alkaline pH solution. Hence, this indicates that pH lower than 6 was unsuitable condition for *Ankistrodesmus* sp. to grow.

Although the culture solution pH had started to increase to 7.67 ± 0.06 at day 9, the cell still kept on decreasing. Based on microscopic inspection, besides *Ankistrodesmus* sp.

it was also observed that predators such as rotifers were existed in the culture. Unlike microalgae that can make its own food, rotifers consumed nutrient from a smaller microorganism in order to survive [45]. However, the additional of a fresh medium at day 10 enhanced the cells number 1.4 times higher than the previous growth and was recorded to be the highest cells number ($0.93 \pm 0.25 \times 10^6$ cell mL⁻¹) at day 13. But then, the cells number started to decrease again, even though the pH solution was above than 7. Hence, from the result, it indicates that heterotrophic mode was not the best mode to culture this microalga.

On the contrary, a different cell growth curve of heterotrophic *Ankistrodesmus* sp. was obtained by Salim [3]. The microalga cell growth had increased from day 1 to 8, and then decreased from day 8 to 12. The study also shows that, by employing 10 g L⁻¹ of CSH into the media, the cells number and specific growth rate were 2.6 times (2.46×10^6 cell mL⁻¹) and 2.2 times (0.50 day^{-1}) higher than the result obtained in this study. Using CSH as a carbon source in heterotrophic mode gave better results instead of using glucose [46]. Moreover, other factors such as the culture temperature and pH can affect the cell growth of heterotrophic microalga [20, 30]. Thus, this explains the results obtained were varied.

In Fig.6 the initial biomass in all cultures were $0.76 \pm 0.05 \text{ g L}^{-1}$. Culture mode from mixotrophic had achieved the highest biomass with 112.8% ($1.44 \pm 0.03 \text{ g L}^{-1}$) of increment, followed by phototrophic mode 89.5% ($1.61 \pm 0.05 \text{ g L}^{-1}$). On the other hand, the microalga biomass decreased about 85.5% ($0.11 \pm 0.02 \text{ g L}^{-1}$) in heterotrophic mode due to lower cell productivity. Additional of glucose enhanced the cell growth of microalga and therefore promotes a higher biomass production [3, 17].

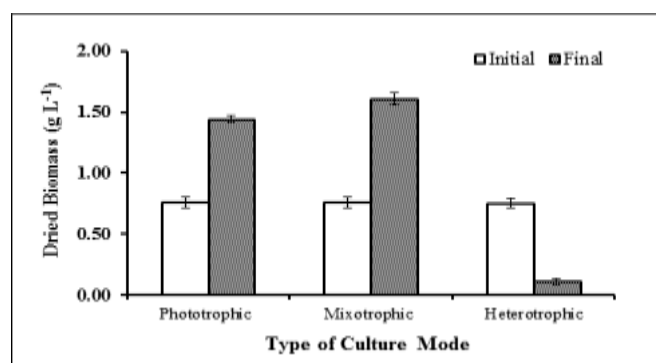


Fig. 6. Initial and final biomass of *Ankistrodesmus* sp. cultivated from three different modes.

From Table 3, mixotrophic *Ankistrodesmus* sp. in mono-culture achieved the highest cell number, specific growth rate, cell productivity and shorter doubling time. Followed by microalga culture in phototrophic mode and then in heterotrophic mode. The cells number in mixotrophic culture was 1.1 and 10.5 times higher than phototrophic and heterotrophic culture, respectively. Overall, mixotrophic microalga in mix-culture has shown better growth profiles result compared to mixotrophic microalga in mono-culture.

Table 3. Growth profile of *Ankistrodesmus* sp. in mono- and mix-culture under phototrophic, mixotrophic and heterotrophic mode

Microalga	Culture Condition	Max cell number, x_{max} ($\times 10^6$ cell mL ⁻¹)	Max cell productivity ($\times 10^6$ cell mL ⁻¹ day ⁻¹)	Max specific growth rate, μ_{max} (day ⁻¹)	Shortest doubling time, T_2 (day)	Final cell biomass (g L ⁻¹)
<i>Ankistrodesmus</i> sp. (mono-culture)	Phototrophic	8.98 ± 0.59 ¹⁷	0.98 ± 0.07 ¹¹	0.32 ± 0.04 ⁷	2.26 ± 0.27 ⁷	1.44 ± 0.03 ¹⁷
	Mixotrophic	9.77 ± 0.59 ¹⁷	1.23 ± 0.08 ¹¹	0.43 ± 0.04 ³	1.63 ± 0.15 ³	1.61 ± 0.05 ¹⁷
	Heterotrophic	0.93 ± 0.25 ¹³	0.12 ± 0.01 ¹¹	0.23 ± 0.09 ¹¹	3.85 ± 1.11 ¹¹	0.11 ± 0.02 ¹⁷
<i>Ankistrodesmus</i> sp. (mix-culture)	Mixotrophic	10.87 ± 0.57 ¹⁷	1.30 ± 0.10 ¹¹	0.46 ± 0.07 ⁷	1.58 ± 0.21 ⁷	1.77 ± 0.05 ¹⁷

Note: The subscript number referred to the cultivation days.

3.4. Total Lipid of *Ankistrodesmus* sp. In Mono-Culture and Mix-Culture

The total lipid of *Ankistrodesmus* sp. from mono- and mix-culture cultivated under different modes was presented in Table 4. The highest total lipid obtained was from the mix-culture sample (12.84 ± 0.35 % wt), followed by mono-culture sample cultivated under mixotrophic (10.55 ± 0.15 % wt), phototrophic (10.10 ± 0.20 % wt) and heterotrophic mode (1.67 ± 0.22 %). Additional of glucose in mixotrophic culture enhanced the total lipid [46]. Based on the result also, it can correlates that higher biomass in the culture will also promote a higher total lipid. Since this microalga grew poorly under the heterotrophic mode, lower total lipid was gained.

Table 4. Amount of total lipid of *Ankistrodesmus* sp. under different culture mode

Microalga Culture	Mode	Total Lipid (% wt)
<i>Ankistrodesmus</i> sp. (mono-culture)	Phototrophic	10.10 ± 0.20
	Mixotrophic	10.55 ± 0.15
	Heterotrophic	1.67 ± 0.22
<i>Ankistrodesmus</i> sp. (mix-culture)	Mixotrophic	12.84 ± 0.35

In mono-culture samples, mixotrophic microalga able to produce a total lipid of 1 times and 6 times higher than that in phototrophic and heterotrophic microalga, respectively. Although, mixotrophic mode had shown better lipid productivity, if this culture mode is applied to an industrial scale, the difference in term of cells number was not that much. In fact, an additional cost is required to buy the external carbon source [17, 23]. This could be the major limitation of using the mixotrophic mode, whereas phototrophic mode only required sunlight and carbon from CO₂ for cultivation.

3.5. FAME Composition of *Ankistrodesmus* sp. In Mono-Culture and Mix-Culture

The FAME components of *Ankistrodesmus* sp. were identified through Mass Spectrophotometer (MS) and predicted based on National Institute of Standards and Technology (NIST) library database. The GCMS chromatogram from each culture sample was shown in Fig.7 and the types of FAME found in each culture samples were presented in Table 5. Based on the GCMS chromatogram results, six significant peaks at the retention time of 16.09, 16.24, 16.95, 22.18, 22.41 and 22.81 were found in all culture modes as presented in Fig.7a to Fig.7d. However, only four of them were identified as FAME and two of them were alcohol groups. In Table 5, the four FAME components were 7, 10-hexadecadienoic acid methyl ester (C16:2), methyl palmitate (C16:0), methyl linoleate (C18:2) and methyl linolenate (C18:3) at retention time of 16.09, 16.95, 22.18 and 22.41, respectively. Meanwhile, the two alcohols were (Z, Z, Z)-9, 12, 15-Octadecatrien-1-ol and 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol at retention time of 16.24 and 22.81, respectively.

Table 5. Types of FAME detected in all culture samples

FAME	Common Name (Fatty Acid)	Formula	Molecular Weight	Retention Time (min)
7,10-Hexadecadienoic acid methyl ester	(C16:2)	C ₁₇ H ₃₀ O ₂	266	16.09
Hexadecadienoic acid methyl ester	Methyl palmitate (C16:0)	C ₁₇ H ₃₄ O ₂	270	16.95
9,12-Octadecadienoic acid (E,E) methyl ester	Methyl linoleate (C18:2)	C ₁₉ H ₃₄ O ₂	294	22.18
9,12,15-Octadecatrienoic acid, (Z,Z,Z) methyl ester	Methyl linolenate (C18:3)	C ₁₉ H ₃₂ O ₂	292	22.41

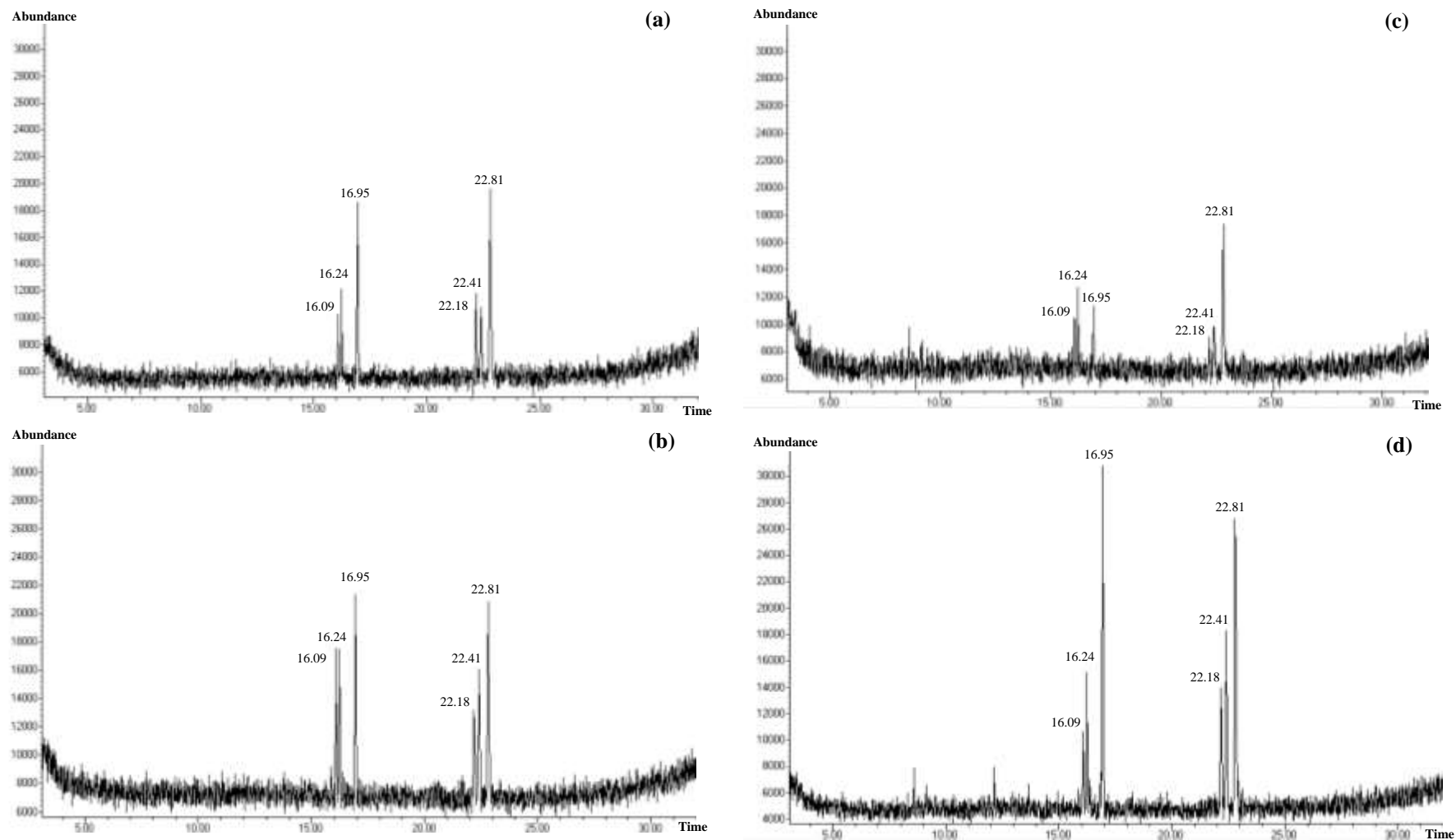


Fig. 7. GCMS chromatograms of *Ankiistrodesmus* sp. from mono-culture samples cultivated under (a) phototrophic mode, (b) mixotrophic mode, (c) heterotrophic mode, and a mix-culture sample cultivated under (d) mixotrophic mode.

In Fig.7a the methyl palmitate yield from mono-culture of phototrophic *Ankistrodesmus* sp. has shown the highest peak among the other three FAME, followed by methyl linoleate, methyl linolenate and 7, 10-hexadecadienoic acid methyl ester. The methyl palmitate yield from mono-culture of mixotrophic *Ankistrodesmus* sp. also has shown the highest peak among the FAME as presented in Fig.7b. However, from this chromatograph 7, 10-hexadecadienoic acid methyl ester was in the second highest followed by methyl linolenate and methyl linoleate. This indicates that fatty acids with C16:0 and C16:2 groups were mainly presented in mono-culture of mixotrophic *Ankistrodesmus* sp. lipids [31]. It was found that, in Fig.7c a similar trend of FAME peaks as in Fig.7b was obtained. The methyl palmitate in mono-culture of heterotrophic *Ankistrodesmus* sp. has shown the highest peak followed by 7, 10-hexadecadienoic acid methyl ester, methyl linolenate and methyl linoleate. As it can be seen, the level of peaks were low corresponded to the amount of total lipid extracted from the culture. From these GCMS chromatogram results, it were clearly elucidates that adding glucose into microalga culture affects *Ankistrodesmus* sp. FA profile. The FA groups such as C16:0 and C16:2 mainly found in mixotrophic and heterotrophic culture than C18:2 and C18:3 groups.

Based on Fig.7d, the methyl palmitate yield in mix-culture of mixotrophic *Ankistrodesmus* sp. detected was also the highest compared to the other chromatograms result. In second place was the methyl linolenate followed by methyl linoleate and 7, 10-hexadecadienoic acid methyl ester. This FAME peaks trend was the same as in Fig.7b. Higher peak level can be correlated to the amount of total lipid attained from the culture. From this results also, it shows that in mix-culture cultivated under mixotrophic mode can enhance the amount of FA especially the C16:0 groups. In overall, it can be observed that the most dominant composition of FAME presented from each culture sample was the methyl palmitate (a palmitic acid methyl ester) at retention time of 16.95. The C16:0 is a MSFA, whereas the C16:2, C18:2 and C18:3 are all PUFA. From engineering perspective view, the MSFA is preferred in biodiesel engine. This is because it provides a higher cetane number, which delivers a stable oxidation and promotes an excellent ignition of the engine [28]. As for PUFA, additional processes will be required to convert the PUFA to MSFA [47]. Thus, adding an extra cost for biodiesel conversion.

As previously mentioned, the methyl palmitate was used as the FAME standard. Hence, only the yield of methyl palmitate was determined and the results were presented in Table 6. For mono-culture, the highest yield of methyl palmitate was attained in mixotrophic culture with $0.142 \pm 0.005 \mu\text{g mL}^{-1}$ and followed by phototrophic culture with $0.066 \pm 0.009 \mu\text{g mL}^{-1}$ and heterotrophic culture with $0.009 \pm 0.001 \mu\text{g mL}^{-1}$. Whereas, in mix-culture the methyl palmitate yield obtained was up to $0.245 \pm 0.010 \mu\text{g mL}^{-1}$. This can be corresponded to higher total lipid was attained from the culture.

Table 6. Yield of methyl palmitate presented in each culture sample.

Microalga Culture	Mode	Methyl Palmitate ($\mu\text{g mL}^{-1}$)
<i>Ankistrodesmus</i> sp. (mono-culture)	Phototrophic	0.066 ± 0.009
	Mixotrophic	0.142 ± 0.005
	Heterotrophic	0.009 ± 0.001
<i>Ankistrodesmus</i> sp. (mix-culture)	Mixotrophic	0.245 ± 0.010

4. Conclusion

The effect of culture mode on the cells growth behaviour of *Ankistrodesmus* sp. was examined in this study. The study revealed that, using the mixotrophic mode cultivation with 17 days of cultivation period had shown to improve the *Ankistrodesmus* sp. cells number, cell productivity, specific growth rate, biomass yield, shortest doubling time, total lipid and methyl palmitate yield. The presence of glucose in mixotrophic culture has provided an addition of carbon source for the microalga cells to grow. Meanwhile, poor cell productivity was observed from heterotrophic culture attributed by the lower pH culture, which had turned it to an acidic solution. This led to another microorganism to grow under this pH value. In mix-culture, *Ankistrodesmus* sp. cells had showed an increasing of cell population compared to *Chlorella* sp. cells. This has clearly indicates that *Ankistrodesmus* sp. has strong competitiveness. For large scale production it impossible to culture single species of microalga using an open system. Therefore, microalga that has a strong endurance and competitive is favourable such as *Ankistrodesmus* sp. Aside from that, the lipids profile of *Ankistrodesmus* sp. was dominated by palmitic acid (C16:0). Biodiesel composition containing MSFA are preferable as compared to PUFA (i.e. C16:2, C18:2 and C18:3). Moreover, the glucose addition has contributed the increasing of C16 groups. Even so, the lipid content attained in this study was lower than other reported literatures the *Ankistrodesmus* sp. lipid can be increased via nitrogen starvation, which is an interesting future work. In conclusion, the selections of proper cultivation technique to attain a higher biomass as well as higher lipid content are crucial in the production of microalgae-based biodiesel. Besides that, selecting the most highly resistant and adaptable microalgae species such as *Ankistrodesmus* sp. should be emphasized in order to reduce or avoid contamination from unwanted microorganism.

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