

Effect of Agitation on Growth and Fatty Acid Composition of Green Microalgae *Acutodesmus obliquus* Q2-12E

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Different environmental factors including agitation can affect the growth of microalgae and fatty acid composition. It is necessary to agitate the culture to ensure that each cell received enough amount of nutrition, air, and light exposure. This study aimed to analyze the growth and fatty acid composition of *Acutodesmus obliquus* Q2-12E cultivated with molecular diffusion (no agitation) and shaking and compare those with a previous study employing the aeration method. The results showed that in comparison to molecular diffusion, agitation by shaking and aeration on the culture significantly increased the specific growth rate. Culture under aeration was revealed to exhibit the fastest growth rate (0.1444 day⁻¹) compared to shaking (0.0951 day⁻¹) and molecular diffusion (0.0614 day⁻¹). The lipid obtained using modified Folch's method was esterified and analyzed using gas chromatography with a flame-ionization detector (GC-FID) with heptadecanoic acid (C17:0) as the internal standard. The analyzed result shows that the major fatty acids produced both by molecular diffusion and shaking method were monounsaturated fatty acids which were 39.5% and 46%, respectively. While the major fatty acid produced by the aeration method was polyunsaturated fatty acid (38.6%). Molecular diffusion and shaking could not produce eicosatetraenoic acid (C20:4) which was detected with the aeration method. This study demonstrated that the same microalga strain exhibited a different growth and fatty acid composition depending on the agitation methods.

1. Introduction

Microalgae have received a lot of interest recently because of their potential advantages as a bioresource that might be utilized for a variety of goods, including fish feed, human food, pharmaceuticals, and different sources of feedstock for the upcoming generation of biofuels (Kamyab et al., 2019). It has been considered an essential resource to fulfill the global demand for sustainable energy and green technologies. *Acutodesmus obliquus* (also known as *Scenedesmus obliquus* or *Tetradesmus obliquus*) (Oliveira et al., 2021) is one of the most prevalent species of green microalgae in freshwater ecosystems. It is one of the oleaginous microalgae which has been identified as an excellent freshwater oleaginous microalgal species for research because of its rapid growth in large quantities, ease of cultivation, strong resistance to bacterial infections, and its ability to adapt to a variety of environmental conditions (Othman et al., 2019). Many environmental factors, including temperature, pH, oxygen content, salinity, and light intensity, can have an impact on the growth of microalgae and the biochemical or physiological characteristics of microalgal cells (Ajala & Alexander, 2022). Because culture conditions differ from species to species, all of these growth variables must be specified for successful microalgae cultivation for a particular application (Chowdury et al., 2020). Another factor that may affect some microalgae's growth rates and fatty acid profile is agitation. The frequency with which cells are exposed to light and dark volumes as well as the amount of mass transferred between the cell and the nutrients will all be improved by effective mixing. The culture grown in flasks can be mixed in a variety of ways. For instance, it can be manually mixed by handshaking and bubbling utilizing aeration. Another type of mixing is orbital shaking which is caused by the orbital displacement of a vessel and provides a rotating path with a set angular velocity as well as a permanent

direction for an inertial force. It is advised to use orbital shaking to maintain a low-shear stress environment and avoid cellular damage in active fluid suspension (Samadi et al., 2022). The high rates of aeration flows can cause hydrodynamic stress that impacts growth, cell damage, and finally cell death while maintaining a high level of mixing and incurring higher energy and carbon dioxide costs. Agitation's impact on microalgal growth and biochemical parameters is influenced by both the algal strain and the culture conditions such as the medium's ionic strength, salinity, temperature, and nutrition. Besides, microalgae growth and biofiltration are not dependent on aeration per se in cases where nutrient concentrations are high enough (Mansouri et al., 2022). In this present study, the effect of agitation on microalgae growth and fatty acid composition in *A. obliquus* Q2-12E was analyzed and compared with a previous study employing aeration with carbon dioxide supplied.

2. Materials and methods

2.1 Microalgae strain and culture conditions

A. obliquus (Q2-12E, MJIIT-UTM, Malaysia), a green microalga was used in this study. It was initially studied by researchers from Algal Biomass Research Lab, Malaysian-Japan International Institute Technology (MJIIT), Universiti Teknologi Malaysia (UTM) for the production of omega-3 fatty acids. Before conducting the experiment, stock culture was prepared with inoculum size in the culture is 10% (v/v) and the cultivation for the main experiment was performed using the batch of culture in the exponential growth phase. Cells were cultivated in no agitation and agitation methods which were molecular molecular diffusion and shaking. 10 % (v/v) of stock culture was cultivated in Erlenmeyer flasks with 100 mL of the modified AF6 medium under sterile conditions. The pH of the medium was adjusted to pH 6.6. Microalgal cells were grown for a 12h photoperiod at 25°C under a light intensity of approximately 80 $\mu\text{mol photons/m}^2/\text{s}$. For molecular diffusion mode, the Erlenmeyer flasks were placed standstill without agitation. In the shaking mode, cells were agitated at 140 rpm using TAITEC double shaker NR-30. The microalgae were cultivated for 14 days in the growth chamber until early stationary growth phase. Meanwhile, from previous study of aeration mode, 2% of CO₂ was supplied.

2.2 Growth of microalgae

The growth of the microalgae was studied by plotting the growth curve and monitored until the death phase. Microalga culture was incubated at an optimum temperature under light sources for 14 days. Microalgae growth was monitored at 750 nm of optical density (OD_{750nm}) with an ultraviolet-visible (UV-Vis) spectrophotometer (UV-1900, Shimadzu) on an alternate day. The specific growth rate (μ , day⁻¹) was to evaluate the rate of increasing cell number per time during the exponential growth phase by using Eq (1).

$$\text{Specific growth rate } (\mu, \text{day}^{-1}) = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (1)$$

Where X_1 , X_2 , t_1 , and t_2 are OD_{750nm} value at the early exponential growth phase, OD_{750nm} value at the late exponential growth phase, and day corresponding to X_1 , and the day corresponding to X_2 .

The doubling time of the culture is calculated to determine the number of cell divisions per day based on the value of the specific growth rate using Eq (2).

$$\text{Doubling time, } t_d(\text{day}) = \frac{\ln 2}{\mu} \quad (2)$$

2.3 Fatty acid methyl esters (FAME) extraction

Following the extraction of the lipid content, the lipid undergoes a transesterification process to produce the FAMES of this three-agitation method. Centrifugation was used to harvest 100 mL of the microalgae culture for 10 min at 25°C at 12,000 x g. using High Speed Refrigerated Centrifuge (SRX 201, TOMY). Prior freeze-drying for 3 days using a freeze dryer (FDU-1100, EYELA), the microalgae pellet was frozen at -80°C for one day. The total lipid was extracted using chloroform-methanol solvent-based Folch's method (Folch et al., 1957) with some modifications. The dry lipid obtained were weighed using a weighing scale and expressed in percentage of dry cell weight. Dry lipid samples were treated with 4 mL of 0.1N methanolic: hydrochloric acid (MeOH: HCl) and left in a water bath at 100°C for 1 h. 4 mL of hexane was added to the cooled mixture. Then, the mixture was shaken vigorously to form a double layer. The upper layer of the mixture was transferred to a new extraction tube. 2 mL of distilled water and 2 mL of hexane are added to the remaining layer. Again, the upper layer was transferred to the first upper layer. The mixture was dried using a rotary evaporator for 30 min using a centrifugal concentrator (CC-105, TOMY). The dry lipid samples were dissolved in hexane and transferred to a glass vial for gas chromatography analysis.

2.4 Gas chromatography analysis

After reaching the stationary phase, FAs were collected and analyzed by Gas chromatography with a flame-ionization detector (GC-FID) to determine the FA composition. GC-FID: GC-2014, Shimadzu, Japan and Agilent CP-Sil 5 CB column (50 m x 0.32 mm x 0.12 μ m) was used to measure and classify the FAMES profile. Helium was used as carrier gas with a constant flow rate in splitless mode. The initial temperature was set to 60 °C for 1.5 min then increased to 130 °C at 20 °C/min. Then, the temperature increased to 250 °C at 4 °C/min. 1 μ L of the sample was injected into GC. By comparing the retention times of the peaks of known FAMES in the standard solution (FIM-FAME-7 Mixture, C4:0-C22:6, Matreya) to the retention times of the FAMES in the microalgal lipid sample, the FAMES in the sample were identified. By comparing the total FAME chromatogram's peak area to the peak area of the internal standard, the amount of FAMES was determined (C17:0) using Eq (3):

$$FAME \text{ content, \%} = \left(\frac{A_{FAME}}{\sum A - A_{C17}} \right) \times 100\% \quad (3)$$

Where A_{FAME} , $\sum A$, A_{C17} are the peak area of FAME, the total peak area of the FAMES chromatogram, and the internal standard peak area (C17:0).

3. Result and discussion

3.1 Effect of agitation on cell growth

The growth curve of *A. obliquus* Q2-12E with different agitation modes was constructed by monitoring the OD_{750nm} every alternate day within 14 days period. In the microalgal growth curve, there are four stages. The first stage is the lag phase, where the microalgae undergo physical adjustments to adapt to the nutrition in the new medium. The second stage will be the exponential phase, in which microalgae rapidly consume the nutrients in the culture medium and the amount of biomass is expected to increase significantly. The next stage is the stationary phase, in which there will be a nutrient limitation and the growth and death of microalgae at the same rate. Finally, the death phase will be the last stage of the growth curve where the microalgae are unable to grow due to nutrient depletion and pH disturbance (Thong et al., 2022). Based on Figure 1, the growth profile of *A. obliquus* Q2-12E cultivated under aeration + CO₂ was found to be higher compared to molecular diffusion and shaking mode. Algal growth was limited by CO₂ lower than 5% because the cell was unable to access the carbon requirement needed for full cell growth. Besides, cells that were cultivated under the shaking and aeration method entered the exponential phase on day 2 of cultivation, while the molecular diffusion method entered the exponential phase on day 4. The quantity of nutrients and light made available to cells by their spatial displacement and homogeneity is the reason why the exponential phase of agitation cultures begins more quickly compared to molecular diffusion (Samadi et al., 2022). At the stationary phase, molecular diffusion entered the phase later than the culture under shaking and aeration which were on days 12, 8, and 6. Compared to the stationary cultures, efficient mixing within the batches permitted the cell to effectively absorb nutrients from uncharted areas and be exposed to light.

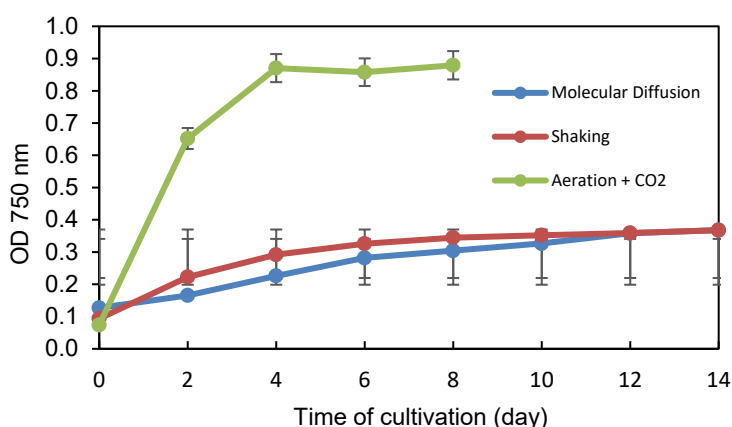


Figure 1: Growth curve of *A. obliquus* Q2-12E for molecular diffusion (blue circles), shaking (red circles), and aeration+CO₂ (green circles). Data for aeration+CO₂ was obtained in the previous study

Figures 2 show the specific growth rate of *A. obliquus* Q2-12E under three different agitation modes during the exponential phase. The specific growth rate assessed an microorganism's ability to grow by counting how many

times it could reproduce over time. The result demonstrates that a specific growth rate of *A. obliquus* Q2-12E differed significantly under all agitation modes. There are various ways that agitation promotes the growth of microalgae. It prevents the cell from self-shading, breaches the boundary layer in the culture system, and makes it easier for cells to absorb CO₂, nutrients, and light (Samadi et al., 2022). Figure 2 shows that the specific growth rate of cultures cultivated by shaking and aeration modes was higher, which were 0.1444 day⁻¹ and 0.0951 day⁻¹ compared to cultures grown in molecular diffusion (0.0614 day⁻¹) method. Nevertheless, the specific growth rate for aeration was slightly higher than shaking which indicates that the aeration+CO₂ was much more effective to agitate the culture. CO₂ concentration plays various important roles for microalgae, one of which is photosynthesis. On supplementation of CO₂, it facilitates the specific growth rate of cells in aeration mode by improving the photosynthetic activity and different photosynthetic pigment patterns. Besides, the result shows that due to nitrogen and phosphorus limitation and no supplementation of CO₂ give an extreme impact on algal growth for both modes, with no agitation and agitation (Kumari et al., 2021). In addition, according to these findings, agitation has an important impact on the interaction with other microalga growth conditions.

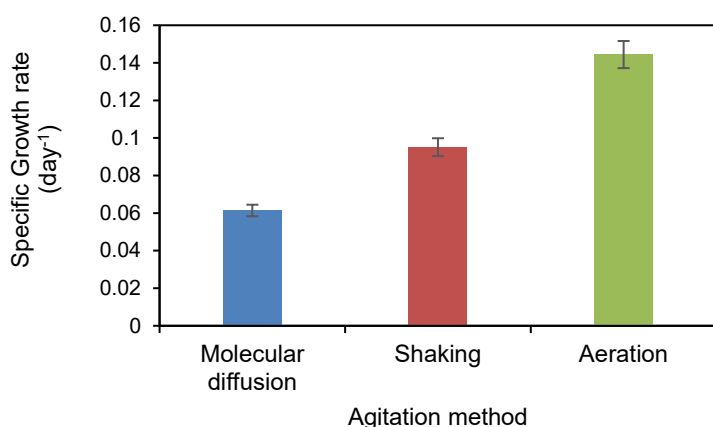


Figure 2: Specific growth rate of *A. obliquus* Q2-12E for three different agitations. Data for aeration+CO₂ was obtained in the previous study

3.2 Effect of agitation on fatty acid composition

Microalgal cultivation has a noticeable effect on fatty acids (FAs) composition, which is also useful as a biochemical marker of cultivation differences. In this study, the FAMES profile of the culture under three agitation modes was examined and compared to determine the type of FAs produced by *A. obliquus* Q2-12E with a different agitation mode. Table 1 depicts the major FA composition detected in different agitation modes. These findings revealed that the molecular diffusion mode has a higher content of saturated fatty acids (SFA) compared to the other two agitation modes. Meanwhile, monounsaturated fatty acid (MUFA) was the highest under shaking mode which was at 46 %. It can be deduced that under aeration+CO₂ have the highest concentrations of polyunsaturated fatty acid (PUFA), which was 38.6 %. Meanwhile, the PUFA percentage under molecular diffusion and shaking has not much different which were 24.1 % and 20.5 %. According to the result, the production of PUFA by *A. obliquus* Q2-12E was more favorable under aeration with supplementation of CO₂. CO₂ concentration facilitates the accumulation of total lipids and PUFAs in microalgae. The greater PUFA content in cells with CO₂ supply may have resulted from the inhibition of FA synthesis, which encourages the desaturation of existing FAs (Liang et al., 2020).

Table 1: The major fatty acid composition (%) of *A. obliquus* Q2-12E under three different agitation modes

Types of fatty acids	Percentage to total fatty acids (%)		
	Molecular diffusion	Shaking	Aeration + CO ₂
SFA	36.5	33.5	26.0
MUFA	39.5	46.0	35.4
PUFA	24.1	20.5	38.6

Note: Data of aeration+CO₂ was obtained in the previous study

Table 2 shows the fatty acid profile of *A. obliquus* Q2-12E varied under different agitation modes. In this study, a total of 9 and 11 FAs were detected by *A. obliquus* Q2-12E under molecular diffusion and shaking, respectively. While, under aeration+CO₂, five FAs were found. The shares of FA C16:1 and C20:3 in total fatty acid (%) were found low (<1 %) under molecular diffusion mode. While, under shaking mode, the shares of FA C14:0, C14:1, and C20:3 was relatively low (<1 %). Stearic acid (C18:0), which occurs most frequently in living things, was the predominant SFA in all agitation modes. Oleic acid (C18:1 cis-9) was the most MUFA detected in this study for molecular diffusion and shaking method which was 36.20 % and 41.28 %. However, no C18:1 cis-9 was detected for aeration+CO₂. The untreated microalgae with CO₂ profile are excellent for biodiesel synthesis since the fatty acid profiles high in SFAs and MUFAs are the most suitable for those applications. *A. obliquus* Q2-12E under those cultivation conditions has the potential to be one of the microalgae that are important for biofuel feedstock for the future. The primary components of the oil produced by microalgae are typically unsaturated fatty acids, mainly palmitoleic (16:1), oleic (18:1), linoleic (18:2), and linolenic acid (18:3), with only a minor amount of palmitic (16:0), SFAs percentage derived from saturated stearic acids (C18:0) (Rahman et al., 2019). *Acutodesmus* sp. was known as α -Linolenic acid (C18:3) producing microalgae (Othman et al., 2019), however, surprisingly under aeration+CO₂ there was no C18:3 detected. Under molecular diffusion and shaking mode, the amount of C18:3 detected was relatively similar which was 8.98 % and 8.10 %, respectively. Interestingly, only aeration+CO₂ can generate eicosatetraenoic acid (C20:4). The addition of CO₂ into the culture has proven to elevate the omega-3 FAs content or synthesis in the microalgae (Barati et al., 2022). In addition, the approach of investigating novel microalgal strains as a potential source of ETA has generated scientific interest. This is due to persistent concerns about the increased demand for high-purity omega-3 fatty acids, specifically ETA, from the growing global population (Mohamad et al., 2021). An increase in ETA consumption can lead to enhanced overall health and well-being, hence effective strategy to boost the amount of ETA in this strain should be conducted.

Table 2: The fatty acid (FA) profile of *A. obliquus* Q2-12E with different agitation modes

Type	Fatty acids	Common name	Percentage to total acids (%)		
			Molecular diffusion	Shaking	Aeration+CO ₂
Saturated	C14:0	Myristic acid	ND	0.05	22.11
	C16:0	Palmitic acid	35.07	32.14	ND
	C18:0	Stearic acid	1.57	1.49	26.04
Monounsaturated	C14:1	Myristoleic acid	ND	0.18	ND
	C16:1	Palmitoleic acid	0.63	2.83	ND
	C18:1 trans-9	Elaidic acid	2.82	1.95	ND
	C18:1 cis-9	Oleic acid	36.20	41.28	ND
	C20:1	Eicosenoic acid	ND	ND	13.39
Polyunsaturated	C16:4	Hexadecatetraenoic acid	ND	ND	22.09
	C18:2 cis-9	Linoleic acid	11.58	9.93	ND
	C18:3	α -Linolenic acid	8.98	8.10	ND
	C18:2 trans-9	Linoelaidic acid	3.22	2.26	ND
	C20:3	Eicosatrienoic acid	0.42	0.29	ND
	C20:4	Eicosatetraenoic acid	ND	ND	16.65

Note: Data of aeration+CO₂ was obtained in the previous study

4. Conclusions

The effect of the agitation method of *A. obliquus* Q2-12E on growth and fatty acid composition was determined and compared with the previous study by aeration with 2% CO₂. The result shows that agitation of culture can enhance the specific growth rate of the microalga compared to the motionless method. Agitation by aeration with supplementation of CO₂ depicts to be more efficient on the cell growth followed by shaking mode and molecular diffusion. This difference can be attributable to the two mixing types' different distributions of wall shear stress and fluid motion. The culture's nutritional uptake, as well as the uniformity of the light and temperature distribution, are all improved by agitation. Besides, supplementation of CO₂ had a notable impact on the FAs profiles which, the PUFA content was significantly increased and rare ETA was able to be produced. Nevertheless, without supplementation of CO₂, the cells are able to produce an interesting amount of MUFAs and SFAs for biofuel production. These findings demonstrated that agitation interacts with other culture medium variables in significant ways, and that optimization is necessary for microalgal cultivation systems to scale up effectively.

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