The influence of photoperiod, light intensity, temperature and salinity on the growth rate and biomass productivity of *Botryococcus* sp.

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Abstract. Environmental factors such as photoperiod, light intensity, temperature, and salinity strongly influence the growth rate and biomass accumulation of microalgae in a culture. Therefore, the effects of photoperiod (24 h light: 0 h dark, 16 h light: 8 h dark, 12 h light: 12 h dark, 6 h light: 18 h dark and 0 h light: 24 h dark), light intensity (2.7, 48.6, 94.5, 176, 243 and 324 µmol m⁻² s⁻¹), temperature (18, 23, 28, 33 and 38 °C), and salinity (0, 0.15, 0.3, 0.45, 0.6 M of NaCl) factors on the growth rate and biomass productivity were studied for green microalgae, *Botryococcus* sp. *Botryococcus* sp. was found to experience optimum growth with a photoperiod of 24:0 hours, light intensity of 243 µmol m⁻² s⁻¹, temperature of 23 °C, and 0 M salinity. It was also observed that optimum biomass productivity of *Botryococcus* sp. was the same as that for growth rate optimum environmental factors. However, a temperature of 33 °C was shown to be optimum for biomass productivity. Freshwater green microalgae from genus *Botryococcus* sp. were examined for environmental factors in this study and show great potential for adoption in tropical climates such as Malaysia for the bio-based feedstock and biofuels industries.

1. Introduction

The assessment of different environmental factors regarding the reaction of microalgae in terms of growth rate and biomass productivity has received attraction among researchers due to the broad field of application of microalgae. Microalgae cultivation has been applied to many sustainable activities such as hydrocarbon production, biomass production, biofuel, CO₂ mitigation, the food industry, and phycoremediation of wastewater [1, 2, 3, 4, 5]. Microalgae technology has a number of advantages when compared to terrestrial plants because microalgae enjoy much higher biomass production. Growing algae does not require high-quality agricultural land [3]. However, the effectiveness of microalgae cultivation in terms of growth rate and biomass production not only depends on the availability of nutrient in the culture [6, 7], but is also significantly influenced by basic environmental factor such as photoperiod, light intensity, temperature, and salinity [8, 9, 10, 11].

Light is the most important factor influencing the growth of photosynthesis organisms such as microalgae. The effects of light on microalgae growth are usually categorized as photoperiod and light intensity, referring to the quality and quantity of light, respectively [12, 13, 14]. Photoperiod factor is necessary if the economic aspect is emphasized in microalgae cultivation, since the light is typically...
from artificial sources. Some researchers have reported on the effect of photoperiod or light regime on the growth rate and biomass productivity of *B. braunii*, *S. obliquus*, *N. conjuncta* and *N. Terrestris* [14], *Nannochloropsis* sp. [15], Chlorella vulgaris [13] and *Tetraselmis* chui [12]. Similarly, of the concentration of light that affects the spread of microalgae cells is of importance since light is an energy source during the photosynthesis process to convert carbon dioxide to organic compounds [10], [16]. Apart from that, direct sunlight provides the energy required to support metabolism, but if present in excess it may lead to photoinhibition [17]. Due to this, optimal light intensity for *Botryococcus braunii* as reported by [18] is 800 µmol m⁻²s⁻¹. Other species of microalgae such as *Scenedesmus obliquus*, *Spirulina platensis* and *Selenastrum minutum* have an optimum light intensity 20.3-33.8 µmol m⁻²s⁻¹ [6], 150 µmol m⁻²s⁻¹[17] and 420 µmol m⁻²s⁻¹[19], respectively.

Other parameters, such as temperature and salinity, also play an important role in microalgae growth and biomass productivity. This is because temperature influences algae cell size, biochemical composition, and also contributes to photoinhibition [16]. Temperatures are also reported to impact starch content in microalgae cells while an increase in temperature leads to degradation of starch production as well as affecting the formation of carotenoids [16]. Therefore, some researchers have reported that optimal temperatures for growth of cyanobacteria (*Chroococcusturgidus, Lyngbyaconervoides and Nostoc commune*), *Spirulina platensis*, and *Botryococcus braunii* are 20-30 °C, 25-30 °C and 25-35 °C, respectively. Meanwhile, salinity is also one of the most important factors to be considered affecting the growth of algae [20]. Optimal salinity concentrations have been found by previous researchers for the growth of *Thalassiosirarweissflogii, Nostoc oculata and Nostoc frustulum* at 25 ppt, 20-30 ppt, and 10-15 ppt, respectively [20, 21]. As reported by Juneja et. al. [16], higher salinity concentrations may reduce growth rates due to the inability of algae to adapt to salinity higher than their natural habitats.

However, to the best of the author’s knowledge, no report has been made so far investigating the local strain of green microalgae identified as *Botryococcus* sp. collected from freshwater body as influenced by environmental factors such as photoperiod, light intensity, temperature, and salinity. Realizing this gap, the objective of the present study is to investigate the effect of environmental factors on the growth rate and biomass productivity of *Botryococcus* sp. cultivated in a synthetic medium.

2. Materials and methods

2.1. Microalgae culture and medium

A local *Botryococcus* sp. (GenBank: JQ585723.1) was used in this study [22]. The initial stock cultures of *Botryococcus* sp. were maintained in modified Bold’s Basal medium containing the following chemicals: NaNO₃, CaCl₂.2H₂O, MgSO₄.7H₂O, K₂HPO₄, KH₂PO₄, NaCl, EDTA, KOH, FeSO₄.7H₂O, H₂SO₄ and micronutrients (ZnSO₄.7H₂O, MnCl₂.4H₂O, MoO₃, CuSO₄.5H₂O and Co(NO₃)2.6H₂O). The culture was inoculated in outdoor condition for 30 days as a stock media. Prior to inoculation for environmental factor examination, microalgae cultures were harvested by centrifuged at low speed (3500 rpm) for ten minutes and washed three times with sterilized distilled water. This is followed by observation and concentration of cell by using microscope counting chamber known as Neubauer haemocytometer.

2.2. Cultivation experimental design

There were four basic environmental factors tested in this study: photoperiod, light intensity, temperature, and salinity. The selection of these parameters was adopted from Qin and Li [23] with some modification of concentration parameters. According to a preliminary study of outdoor natural conditions, a photoperiod of 12:12 hours, light intensity of 94.5 µmole m⁻²s⁻¹, temperature of 28 °C, and salinity of 0 M were used as a control condition. Total five photoperiods (24 h light: 0 h dark, 16 h light: 8 h dark, 12 h light: 12 h dark, 6 h light: 18 h dark and 0 h light: 24 h dark) were used in this study in three replicates. Each flask contained 350 ml Bold’s Basal Medium inoculated with 1000 cell/ml of *Botryococcus* sp. and was cultured at 28 °C and 94.5 µmole m⁻²s⁻¹ irradiance. Meanwhile, five levels of temperature were used in these experiments: 18 °C, 23 °C, 28 °C, 33 °C and 38 °C, controlled with water.
bath equipment [24]. Three replicates were used in each examination. At all temperatures, the algae were cultured under the following conditions: 94.5 µmole m$^{-2}$s$^{-1}$, photoperiod of 12 h light: 12 h dark and 0 M salinity. A volume of 350 ml algal medium is bath watered in each 500 ml Erlenmeyer flask. Next, LED cool daylight lamps were used as light sources and were controlled in terms of intensity. Five light intensities (2.7, 48.6, 94.5, 176, 243 and 324 µmole m$^{-2}$s$^{-1}$) with three replicates were examined. Each flask contained 350 ml of algal culture inoculated with 1000 cell/ml of Botryococcus sp. and was exposed to a photoperiod 12 h light: 12 h dark. Temperature was constant at 28 ºC and the salinity was 0 M NaCl. A total of five different salinity levels were tested: 0 M, 0.15 M, 0.3 M, 0.45 M and 0.6 M of NaCl (0.1 M NaCl is equivalent to 5.85 %) with three replicates. Each flask contains growth media with the same photoperiod, temperature, and light intensity examination, with values of 28 ºC temperature, 94.5 µmole m$^{-2}$s$^{-1}$ of light intensity and 12:12 hour photoperiod. All samples were homogenized by manually shaking four times per day and the duration of examination was determined depending on the growth trends, up to 24 days.

2.3. Microalgae growth rate measurement
The kinetic growth of Botryococcus sp. was determined according to the maximum specific growth rate ($\mu_{\text{max}}$/day); meanwhile, division per day (Dd) and doubling time (td) were calculated according to equation (1) and (2), respectively [25, 26, 27, 28]. Maximum specific growth rate ($\mu_{\text{max}}$) was obtained from the slope of exponential stage of the growth curve [28,29]. Normally, this parameter is estimated by deciding subjectively, which part of the curve is approximately linear and then determining the slope of this curve section, eventually by linear regression. This method describes the number of cells (N) or the logarithm of the number of cells [Log(N)] as a function of time [28]. At least three time points were considered to satisfy or confirm the exponential stage [27, 30].

$$\text{Division per day (Dd)} = \frac{\mu_{\text{max}}}{\ln 2}$$

(1)

$$\text{Doubling time (td)} = \frac{1}{Dd}$$

(2)

2.4. Biomass productivity measurement
Biomass productivity measurement is a very important parameter to be evaluated for microalgae cultivation. In this study, biomass productivity was determined volumetrically based on growth kinetic parameter using equation (3), in which $\mu_{\text{max}}$, Xm and Xo were defined as maximum specific growth rate, maximum cell concentration in the culture, and initial cell concentration, respectively.

$$\text{Biomass productivity} = \frac{\mu_{\text{max}}}{\ln \frac{0.9X_m - 1.1X_0}{9X_m - 1.1X_0}}$$

(3)

2.5. Growth mathematical model
The Verhulst logistic model was used to predict Botryococcus sp. growth in the culture compared with an experimental curve [7, 31, 32]. Therefore, a logistic equation was selected for growth mathematical model per equation 4, where dx/dt is the microalgae growth rate and X is the cell concentration of microalgae in the medium. By integrating equation (4), we obtained equation (5). When t = 0, the Botryococcus sp. concentration may be derived via initial the cell concentration value (X = Xo).
\[
\frac{dX}{dt} = \mu_{\text{max}} \left(1 - \frac{X}{X_m}\right)X \\
X = \frac{X_0, X_m, e^{\mu_{\text{max}} t}}{X_m - X_0 + X_0, e^{\mu_{\text{max}} t}}
\]

3. Results and discussion

3.1. Photoperiod

The growth curves of various light photoperiods were analysed as shown in figure 1 (a). Comparisons were made with a calculated mathematical model curve of growth cell. Then, computation of the growth rate and biomass productivity was carried out as stated in table 1. According to figure 1 (a), *Botryococcus* sp. grew well when exposed to the light for more than 12 hours. However, the best photoperiod was observed on continuous light exposure (24:0 hours) with 40.55 \times 10^4 \text{cell/ml/day} of biomass productivity and 1.18 \mu_{\text{max}}/day of maximum specific growth rate. This was followed by the second best of biomass productivity 36.20 \times 10^4 \text{cell/ml/day}, which is 18:6 hours with a maximum specific growth rate of 0.99 \mu_{\text{max}}/day. These values are slightly higher than 12:12 hours (0.96 \mu_{\text{max}}/day). Even though there was not much difference in terms of maximum specific growth rate between 12:12 hours and 18:6 hours, results varied in terms of biomass production, as 12:12 hours was able to produce up to 25.84 \times 10^4 \text{cell/ml/day} compared to 18:6 hours (36.20 \times 10^4 \text{cell/ml/day}).

However, there is less significant difference in maximum specific growth rate among 6:18 hours, 12:12 hours and 18:6 hours’ photoperiod (table 1). These findings are also comparing the doubling or generation time of *Botryococcus* sp., which the highest maximum specific rate generates the lowest doubling time as, stated in table 1. According to Andersen [27], generation or doubling time refers to the length of time needed by the microalga to double of their cell numbers in the culture as calculated using equation (2). In this study, the lowest doubling time was 0.59 days, which is a 24:0 hour photoperiod as compared to Krzeminska et al. [14] which reached 0.78 days. This indicates that *Botryococcus* sp. is able to grow subjected to light for 6 hours or more but that it is quite miserable when cultivated without light. This indicates that algae are the type’s phototropic microalga which is they need light to growth. Light exposure also had an essential effect on specific growth rate and microalga biomass productivity.

The differences in the photoperiod also may change the biochemical composition such as protein, pigments, and fatty acid content in microalga biomass [14, 15]. In economic terms, a photoperiod is necessary if algal biomass is cultivated with a supply of artificial light sources, because continuous light definitely used more electrical energy and leads to higher cost. Harun et al. [10] and Andersen [27] reported that 12 to 15 hours illumination duration is generally considered as an economically optimal balance for algal growth between cost and productivity.

3.2. Light intensity

The photosynthetic utilization of light as an energy source is necessary for microalgae grown in water. Figure 1 (b) shows experimental and mathematical model data of cell growth of *Botryococcus* sp. cultured in batch condition with different light intensities. The best growth was when exposed to 243 \mu mole m^{-2}s^{-1} with biomass productivity and maximum specific growth rate are 81.52 \times 10^4 \text{cell/ml/day} and 1.03 \mu_{\text{max}}/day, respectively (table 1). Meanwhile, 94.5 \mu mol m^{-2}s^{-1}, 176 \mu mol m^{-2}s^{-1} and 324 \mu mol m^{-2}s^{-1} showed almost the same curve trend but different values for growth rate and biomass production. For example, on 94.5 \mu mole m^{-2}s^{-1}, 176 \mu mole m^{-2}s^{-1} and 324 \mu mole m^{-2}s^{-1} had 0.96\mu_{\text{max}}/day, 1.03\mu_{\text{max}}/day and 1.16\mu_{\text{max}}/day of maximum specific growth rate, respectively and biomass productivity were 35.12\times10^4 \text{cell/ml/day}, 28.62\times10^4 \text{cell/ml/day} and 70.35\times10^4 \text{cell/ml/day}, respectively (table 1). However, 2.7 \mu mole m^{-2}s^{-1} and 48.6 \mu mole m^{-2}s^{-1} intensity is only able to produce a biomass productivity 0.11\times10^4 \text{cell/ml/day} and 10.98\times10^4 \text{cell/ml/day}, respectively, and are considered not efficient enough to produce massive biomass due to the lower growth rates, which are 0.41\mu_{\text{max}}/day and 0.46\mu_{\text{max}}/day, respectively.
In terms of doubling time, 243 µmol m$^{-2}$s$^{-1}$ also still lead to the best generation time because their cells population could be double about 0.530 day which is within 12.72 hours. This value very differs from a study done by Wahidin et al. [15] in which they got 2.32 days (55.68 hours) when using 200 µmol m$^{-2}$s$^{-1}$ and 12:12 hours photoperiod for *Nannochloropsis* sp. Since light intensity plays an important role in microalgae photosynthesis, increasing light intensity levels would increase the biomass productivity and growth rate at an optimal level, but production will decrease if exposed to very high light intensity, as in the present study. Table 1 shows that this *Botryococcus* sp. was unable to accommodate the excess concentration of light intensity on 324 µmol m$^{-2}$s$^{-1}$ and causes a decrease in biomass productivity and maximum specific growth rate. This may be due to the higher light intensities can lead to photoinhibition [13, 15] and normally optimum light intensity would depend on the alga’s photosynthesis capability to fully capture photon energy [10].

**Figure 1.** Experimental and mathematical model growth curve of *Botryococcus* sp. (a) Photoperiod; (b) Light intensity; (c) Temperature; (d) Salinity. Symbols are experimental data and solid lines depict data calculated by Verhulst logistic model.

### 3.3. Temperature

The growth curve for both experimental and mathematical model of *Botryococcus* sp. in five different temperatures (18 °C, 23 °C, 28 °C, 33 °C, and 38 °C) were tested using constants light intensity (94.5 µmole m$^{-2}$s$^{-1}$), salinity (0 M) and photoperiod (12:12) as plotted in figure 1 (c). According to figure 1 (c), *Botryococcus* sp. was observed more tolerant at temperature between 23 °C to 33 °C. The highest biomass productivity is on 33 °C (43.91×10$^4$ cell/ml/day) follows by 23 °C (39.3×10$^4$ cell/ml/day) then 28 °C (35.1×10$^4$ cell/ml/day). This is slightly different from maximum specific growth rate, for which the best growth rate was 23 °C (1.1146 µ$\text{max}$/day). Meanwhile, the trend of maximum specific growth rate did not follow the biomass productivity trend (table 1). The maximum specific growth rate of 33 °C was 0.93 µ$\text{max}$/day which is lower than 23 °C and 28 °C (0.96 µ$\text{max}$/day). For significant results between 23 °C and 33 °C, if compared the maximum specific growth rate and biomass productivity, the *Botryococcus* sp. cell at 23 °C grew faster but produced lower biomass production than 33 °C. This is because *Botryococcus* sp. Cells under 33 °C conditions have a slow growth rate but are excellent in production compared to high growth rates. This situation may be due to the level of cell maturity much
faster in 23 °C causing the productivity was not as much as in 33 °C. These results reveal the Botryococcus sp. is able to adapt when subjected to sudden temperature changes with different specific growth rate and biomass production. This finding is almost in line with that reported for Botryococcus braunii, with a suitable growth temperature in a range of 25 °C to 35 °C and optimal temperature of growth at 30 °C [18]. Other species of microalgae such as Nannochloropsis oculata, Isochrysisaff galbana, Chaetocerosmuelleri and tetraselmischuiwere reported possess different optimal temperatures of 26 °C, 28 °C, 33 °C and 25 °C, respectively [9]. The optimal growth temperature served microalgae cell to undergo photosynthesis process without change or modify any biochemical and physiological parameters [33]. Obviously, temperature is an essential environmental factor that influences algal growth rate, cell size, biochemical composition and nutrient requirements. Moreover, temperature also plays an important role in photoinhibition which is well-known to have an impact on algal growth rate [10, 16].

From these findings, the optimal temperature of algal growth was revealed to be the same as the environment of the microalgae collected. The results of these investigations have indicated that Botryococcus sp. enjoys healthy living at temperature about 23 °C to 33 °C, the same as the natural outdoor condition climate in tropical rainforest located at in the Southern region of Peninsular Malaysia. Nevertheless, any batch bioreactor of microalgae operated indoors at a controlled temperature is also suitable for the optimal temperature provided in this study.

Table 1. Computation of kinetic growth parameter and biomass productivity of Botryococcus sp. with different environmental factors.

| Environmental factor | Kinetic growth parameters | | | Biomass productivity, cell/mL/day (10⁶) |
|----------------------|---------------------------|---|---|----------------------------------------|
|                      | Maximum specific growth rate (μmax/day) | Division rate (Dd) | Doubling time (td) | |
| Photoperiod(h)       | 0:24, 0.587±0.006, 0.847±0.006, 1.180±0.006, 0.183±0.009 | | | |
|                      | 6:18, 0.946±0.004, 1.365±0.004, 0.733±0.004, 2.192±0.009 | | | |
|                      | 12:12, 0.959±0.003, 1.383±0.003, 0.723±0.003, 25.838±0.038 | | | |
|                      | 18:6, 0.988±0.007, 1.426±0.007, 0.701±0.007, 36.203±0.121 | | | |
|                      | 24:0, 1.179±0.012, 1.701±0.012, 0.588±0.012, 40.533±0.435 | | | |
| Light Intensity      | 2.7, 0.412±0.000, 0.594±0.000, 1.682±0.000, 0.111±0.000 | | | |
| (µmol m⁻²s⁻¹)        | 48.6, 0.461±0.008, 0.665±0.008, 1.504±0.008, 10.970±0.175 | | | |
|                      | 94.5, 0.959±0.003, 1.383±0.003, 0.723±0.003, 35.079±0.384 | | | |
|                      | 176, 1.026±0.012, 1.480±0.012, 0.676±0.012, 28.602±0.343 | | | |
|                      | 243, 1.307±0.003, 1.886±0.003, 0.530±0.003, 81.520±0.215 | | | |
|                      | 324, 1.160±0.003, 1.673±0.003, 0.598±0.003, 70.338±0.194 | | | |
| Temperature (°C)     | 18, 0.572±0.009, 0.825±0.009, 1.211±0.009, 6.888±0.162 | | | |
|                      | 23, 1.115±0.002, 1.609±0.002, 0.622±0.002, 39.320±0.443 | | | |
|                      | 28, 0.959±0.003, 1.383±0.003, 0.723±0.003, 35.079±0.384 | | | |
|                      | 33, 0.933±0.006, 1.346±0.006, 0.743±0.006, 43.941±0.725 | | | |
|                      | 38, 0.804±0.007, 1.159±0.007, 0.862±0.007, 12.991±0.040 | | | |
| Salinity(M)           | 0, 0.959±0.006, 1.383±0.006, 0.723±0.006, 35.079±0.384 | | | |
|                      | 0.15, 0.607±0.007, 0.875±0.007, 1.142±0.007, 14.905±0.118 | | | |
|                      | 0.3, 0.428±0.008, 0.617±0.008, 1.621±0.008, 3.841±0.055 | | | |
|                      | 0.45, 0.476±0.015, 0.686±0.015, 1.457±0.015, 0.113±0.003 | | | |
|                      | 0.6, 0.554±0.011, 0.799±0.011, 1.251±0.011, 0.109±0.008 | | | |

Data are expressed as mean ± SE (n=3)

3.4. Salinity
The growth effects of different salinities concentration for both experimental and mathematical models (0 M, 0.15 M, 0.3 M, 0.45 M and 0.6 M) of NaCl are presented in figure 1 (d). The microalgae exhibited
low resistance to higher salinity, with a decrease in their growth when sodium chloride was added when compared to those with no sodium chloride. The most abundant growth occurred without addition of NaCl and decreased when mixed with 0.15 M and 0.3 M, while insignificantly increasing at 0.45 M and 0.6 M. This growth curve (figure 1 (d)) also shows that 0 M of NaCl was better for a maximum specific growth rate (0.96μmax/day) and biomass productivity (35.1×10^4 cell/ml/day), as shown in table 1. Apart from that, this microalgae remains tolerant to a range of salinities, i.e. 0.15 – 0.3 Mole (figure 1 (d)).

The study has gone some way towards enhancing understanding the optimal growth of Botryococcus sp. in media (BBM) without the addition of sodium chloride (NaCl). Table 1 shows the lowest doubling time at 0 M salinity (0.723 days) and the highest at 0.3 M salinity (1.621 days). However, this finding differs from other studies where the greatest growth of Botryococcus braunii is at 0.15 M salinity concentration [23]. This might due to the different strains and location adaptation used, as they obtained the Botryococcus sp. from Wuhan, China. The present study used a local strain collected from Malaysia’s tropical rainforest. According to Juneja et. al. [16], salinity is essential factor that changes the biochemical composition of microalga cells, including lipids, proteins, chlorophylls, and carbohydrates.

Exposing algal to lower or higher salinity levels than their natural habitat, however, can transform growth rate and change biochemical composition [8, 16, 34]. Similarly to the conditions of this study, the natural habitat of collected Botryococcus sp. is living in freshwater river. Therefore, volumetric biomass productivity (table 1) decreased with an increase in the concentration of NaCl. Other than that, different salinities also have a considerable effect on the morphology characteristic of microalgae [35] due to the inability of the alga to adapt to high salinity [16].

4. Conclusion

One of the more significant findings to emerge from this study is that differences in growth rate and biomass productivity of Botryococcus sp. were highly dependent on the environmental factors applied. The main findings may be summarized as follows: 1) Growth rate and biomass production increased when exposed much longer to light in terms of either duration exposure or light intensity; 2) the growth rate decreased when exposed to too much light intensity; 3) the growth rate tolerated temperatures between 23 °C and 33 °C and the samples grew well without any addition of salinity concentration. This research has led to more questions and a need for further investigation. Further work needs to be done to establish the phycoremediation process and sustainable biomass production for the future bio-based feedstock industry.

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