

INFLUENCE OF INITIAL CELL CONCENTRATIONS ON THE GROWTH RATE AND BIOMASS PRODUCTIVITY OF MICROALGAE IN DOMESTIC WASTEWATER

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Abstract. The aim of this study was to compare the specific growth rate and biomass productivity of microalgae in domestic wastewater according to the initial cell concentration. The initial microalgae cell concentrations tested started from 10^3 cell/mL, 10^4 cell/mL, 10^5 cell/mL, 10^6 cell/mL, and 10^7 cell/mL under outdoor condition. The result revealed that the highest biomass productivity occurred at 10^6 cell/mL concentration with a value of 1.24×10^4 cell/mL/day, 0.26 day^{-1} of specific growth rate, and a doubling time of 2.63 days. Meanwhile, the lowest biomass productivity occurred at 10^3 cell/mL concentration with the lowest specific growth rate of 0.1 day^{-1} and the longest doubling time, which reached up to 7.14 day. As a result, the initial cell concentration of microalgae did influence the algal biomass productivity and growth rate differently. Thus, the maximum growth rate and biomass productivity were obtained at 10^6 cell/mL concentration which is recommended to be used in biotechnology industries and any wastewater treatment.

Keywords: *microalgae, growth rate, biomass productivity, wastewater, and cell concentration*

Introduction

Currently, growing microalgae in wastewater has become a topic of interest among researchers worldwide for its potential in phycoremediation, biofuel and hydrocarbon production, greenhouse gases mitigation and other factors (Gani, et al., 2015a; Gani, et al., 2015b; Onalo et al., 2014; Komolafe, et al., 2014; Rasoul-Amini et al., 2014; Lim and Vadivelu, 2014; Prakash et al., 2014; Shin et al., 2015; Abdelaziz et al., 2014; Mehrabadi et al., 2014; Slade and Bauen, 2013; Guo et al., 2013; Kothari et al., 2012; Park et al., 2011). The success of culturing mostly depends on the availability of nutrients in wastewater, culturing techniques, and environmental factors such as light, temperature, salinity, pH, and photoperiod (Komolafe, et al., 2014; Cai et al., 2013; Udom et al., 2013; Zhu et al., 2013; Sacristán de Alva et al., 2013; Kothari et al., 2013; Mata et al., 2012; Asulabh et al., 2012; Kirrolia et al., 2012; Qin and Li, 2006). The uniqueness of microalgae is that they like ordinary crops. In this case, microalgae absorb CO_2 in the atmosphere while producing O_2 by assimilating nutrients in wastewater (Muñoz and Guieysse, 2006; Mata et al., 2010; Rawat et al., 2011; Brennan

and Owende, 2010; Abdel-Raouf et al., 2012; Sivakumar and Rajendran, 2013; Slade and Bauen, 2013; Ramachandra et al., 2013; Gani, et al., 2015c). For example, nutrients taken up by microalgae in wastewater such as nitrogen, phosphorus, and carbon are able to reduce eutrophication in aquatic environments (Karthikeyan et al., 2012; Choul-gyun 2002; Chu et al., 2014; Aslan and Kapdan, 2006; Rasoul-Amini et al., 2014; Boonchai et al., 2012; Kim et al., 2013; Valley et al., 2012; Can et al., 2013).

As discussed by Oswald (1957) and De la Noüe et al. (1992), there are many species of algae in nature, some of which has already been grown by other researchers using wastewater as the alternative to synthetic media, such as *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp., and others. For example, Mata et al. (2012) cultivated *Scenedesmus obliquus* in synthetic brewery wastewater to analyse the potential of biomass production. They found that the maximum dry biomass was 0.9g per litre of culture. In the same study, *Scenedesmus obliquus* successfully reduced the value of COD and TN up to 57.5% and 20.8%, respectively. Other than that, Zhu et al. (2013) has successfully grown freshwater microalgae (*Chlorella zofingiensis*) in piggery wastewater to characterize the algal growth and nutrients removal. Their study used a column photobioreactor with varying nutrient concentration and found that biomass productivity was different subject to several concentrations. Also, Kothari et al. (2013) used dairy industry wastewater for the cultivation of *Chlamydomonas polypyrenoides* on biodiesel production. Results indicated that dairy wastewater was a great medium for algae growth when 75% concentration was applied. Meanwhile, culturing *Botryococcus braunii* in urban wastewater was studied by Can et al. (2013). The author found that the best growth using different loading concentrations also occurred in 75% urban wastewater, while without dilution (100%) the urban wastewater was better in terms of lipid production.

Although many researchers have investigated microalgae *Botryococcus* sp., a few of them focused on synthetic medium (Eroglu et al., 2011; Molnár et al., 2012; Wang et al., 2014; Dayananda et al., 2005; Cheng et al., 2013; Ashokkumar and Rengasamy, 2012; Suzuki et al., 2013) compared to domestic wastewater as the medium (Can et al., 2013; Órpez et al., 2009). So it is necessary to perform deeper research on *Botryococcus* sp. to be grown in domestic wastewater for the potential of biomass production. The aim of this paper is to compare the specific growth rate and biomass productivity of *Botryococcus* sp. in domestic wastewater according to the initial cell concentration, while the raw characteristics of domestic wastewater are also determined.

Materials and Methods

Preparation of microalgae

Microalgae used in this experiment were collected and isolated from a tropical rainforest in the Southern region of Peninsular Malaysia (between N 02° 30.711" E 103° 20.984" and N 02° 30.740" E 103° 20.996"), namely *Botryococcus* sp. Initial stock cultures of *Botryococcus* sp. were maintained in modified Bold's Basal medium (Bischoff and Bold, 1963) 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 14 days. Prior to inoculation, microalgae cultures were harvested using a centrifuge at low speed (3500rpm) for ten minutes and washed three

times with sterilized distilled water. This was followed by cell observation and cell concentration count using Neubauer haemocytometer.

Sampling and characterization of wastewater

The wastewater used in this study was effluent wastewater obtained from a domestic wastewater treatment plant located in Batu Pahat, Johor, Malaysia. The samples were collected in the morning at around 7:00am to 9:00am using acid washed sample bottles at the site and immediately transferred to the laboratory and preserved at temperatures below 4°C in a refrigerator. Then, wastewater quality parameters were immediately characterized once the samples reached the laboratory to avoid changes due to chemical and biological reactions. Chemical oxygen demand (COD), biochemical oxygen demand (BOD), total phosphorus (TP), dissolved oxygen (DO), and pH analysis were measured according to the standard methods (APHA, 2012). While total nitrogen (TN), total organic carbon (TOC), total carbon (TC), and inorganic carbon (IC) were obtained using TOC Analyzer (Brand: TOC-VCSH, Japan, Shimadzu). Before the inoculation process, the wastewater sample was filtered using a nylon membrane filter (Whatman) with a 0.45µm pore size to remove other microorganisms and suspended solids.

Experimental setup

A total of 15 Erlenmeyer flasks (500mL) were filled with 200 mL wastewater and were used in this experiment as the domestic wastewater. The wastewater experiment flasks (triplicate) were inoculated with microalgae starting with an initial cell concentration of 10³ cells/mL based on the standard methods (APHA, 2012) and increased up to 10⁷ cell/mL (10³ cell/mL, 10⁴ cell/mL, 10⁵ cell/mL, 10⁶ cell/mL, and 10⁷ cell/mL) (Kothari et al., 2013). The flasks were covered with sterile cotton plugs and kept under outdoor natural condition during the experimental period. All samples were shaken from time to time to ensure that the *Botryococcus* sp. was uniformly homogenized in the wastewater.

Determination of microalgae growth

The samples were taken daily from the culture for cell growth counting in wastewater started on day 3 using Haemocytometer (improved Neubauer chamber) according to Andersen's (2005) technique. The growth of *Botryococcus* sp. was determined according to the specific growth rate (µ/day), division per day (Dd), doubling time (td), and biomass productivity (cell/mL/day) using equations 1, 2, 3, and 4, respectively (Zhu et al., 2013; Komolafe et al., 2014; Asmare et al., 2013; Issarapayup et al., 2009; Wang et al., 2010; Andersen 2005). Nf and Ni were defined as the cell concentration (cell.mL⁻¹) at time Tf and Ti, respectively. The graph over time was required to plot the growth of batch culture to predict the exponential stage of the culture. At least three-time points were considered to satisfy or confirm the exponential stage (Andersen, 2005).

$$\text{Specific growth rate } (\mu/\text{day}) = \frac{\ln(N_f / N_i)}{T_f - T_i} \quad (\text{Eq. 1})$$

$$\text{Division per day (Dd)} = \frac{\mu/\text{day}}{\ln 2} \quad (\text{Eq. 2})$$

$$\text{Doubling time (td)} = \frac{1}{D_d} \quad (\text{Eq. 3})$$

$$\text{Biomass productivity} = \frac{N_f - N_i}{T_f - T_i} \quad (\text{Eq. 4})$$

Statistical analysis

All experiments were conducted in triplicates for each culture. Data analysis of average, mean differences, standard deviation, and the graph for each experiment were done using Microsoft Office Excel Professional Plus 2010.

Results and Discussion

Wastewater characterization

Wastewater characterization is compulsory and important for determining the nutrient supplements required for microalgae growth during the cultivation process. *Table 1* shows the physical and chemical parameters of domestic wastewater compared to the effluent standard which has been used in the formation of culture media in microalgae growth experiments. Concentrations of COD and BOD were 76.1 mg/L and 44 mg/L, respectively; this concentration was different from that used in other research papers. For example, Mostafa et al. (2012) used untreated domestic wastewater containing 50 mg/L COD and 15 mg/L of BOD to cultivate cyanobacteria and *Chlorella vulgaris*. Zhang et al. (2013) cultivated mixotrophic microalgae strains in domestic wastewater containing 142 mg/L of COD. However, the concentration of both COD and BOD in this study remains still under the limit of Standard B but slightly over the limit of Standard A. The wastewater used also contained 3.27 mg/L of TP, which was below both standard limits, while TN was 15.79 mg/L. Both parameters were compared to a study conducted by Zhang et al. (2013), who found that TP and TN were 1.59 mg/L and 27.7 mg/L, respectively. Other parameters were also determined such as TSS and TOC in which the values were 2250 mg/L and 21.06 mg/L, respectively. TSS concentration indicates that the wastewater was incomparable to the effluent standard limit, which was more than 100 mg/L of Standard B. Other than that, the pH value showed acceptable concentration compared to the effluent standard and suitable enough for microalgae cultivation (Creswell, 2010).

A few researchers (Zhang et al., 2013; Teles et al., 2013; Can et al., 2013; Ji et al., 2013) have shown the potential of microalgae in domestic wastewater treatment to biotransform pollutants into valuable biomass before the discharge of the cleaned water to the environment. As previously discussed in the introduction, the growth efficiency of microalgae in wastewater mostly depends on different variable factors such as the availability of nutrients and the influence of environmental factors. Thereby, this study may allow the use of domestic wastewater for the development of culture method in biomass production and for further purposes of phycoremediation study coupled with hydrocarbon production.

Table 1. Characteristics of the raw domestic wastewater used as the growth media

Physiochemical parameters	Concentration (^a mg/L)	Effluent standard, mg/L (Environmental Quality Act, 1974)	
		Standard A	Standard B
Chemical oxygen demand (COD)	76.10	50	100
Biochemical oxygen demand (BOD)	44.00	20	50
Total phosphorus (TP)	3.27	5	10
Total nitrogen (TN)	15.79	-	-
Total suspended solid (TSS)	2250	50	100
Total dissolved solid (TDS)	4900	-	-
Total carbon (TC)	21.06	-	-
Total organic carbon (TOC)	2.19	-	-
Inorganic carbon (IC)	18.86	-	-
Dissolved oxygen (DO)	14.76	-	-
pH	6.93	6.0 – 9.0	5.5 – 9.0

^aAll parameters unit in mg/L except for pH

*All experiments conducted in triplicate ($n=3$)

Growth of *Botryococcus sp.* and biomass productivity

In general, for most experiments, the growth curve which showed existing lag phase and the exponential phase was then followed by gradually increasing the biomass concentration over time except for 10^7 cell/mL concentration. There were no growth activities in this cell concentration due to the overpopulation. However, in other cell concentration experiments, a stationary and declining phases were also observed. All of the above explanations could be referred further in *Figure 1* below. Similar growth curve has been reported by Can et al. (2013); Cabanelas et al. (2013); Teles et al. (2013) and Chaput et al. (2012), who used domestic wastewater to grow microalgae but with different nutrients and pollutant load concentrations.

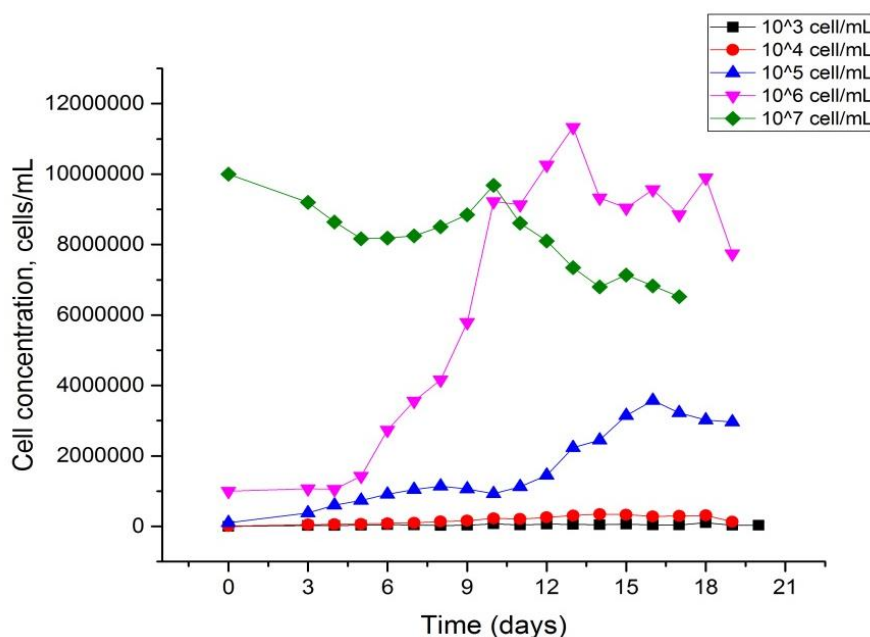


Figure 1. Growth of *Botryococcus sp.* in different cells concentration

Obviously, *Figure 1* shows that the growth in 10^6 cell/mL concentration was enhanced dramatically compared to other cell concentrations such as 10^3 cell/mL and 10^4 cell/mL while 10^5 cell/mL seemed to show us a better increment but still lower than 10^6 cell/mL. This means that the maximum peak growth for both cell concentration, 10^5 cell/mL and 10^6 cell/mL, occurred on day 16 at 3.57×10^6 cell/mL and day 13 at 1.1×10^7 cell/mL, respectively.

The exponential phase of *Botryococcus* sp. growth could be predicted by drawing a straight line in *Figure 1*, in which the points touched the straight line at least thrice according to Andersen's (2005) technique for each cell concentration except 10^7 cell/mL. Based on the 10^6 cell/mL concentration curve, the exponential phase occurred starting from day 5 to day 13 while for 10^5 cell/mL concentration the exponential phase started from day 11 to day 16. Also, according to the data in *Figure 1*, it was apparent that 10^3 cell/mL and 10^4 cell/mL concentration showed much less growth, with a maximum cell density achieved of only up to 1×10^5 cell/mL and 3.5×10^5 cell/mL, respectively. *Table 2* below thoroughly describes their biomass productivity.

Table 2. Computation of specific growth rate (μ /day), division per day (Dd), doubling time (td) and biomass productivity of *Botryococcus* sp. grown on domestic wastewater

Cell concentration (Cell/mL)	Specific growth rate (μ /day)	Division per day (Dd)	Doubling Time, td (day)	Biomass productivity, Cell/mL/day (10^4)
1×10^3	0.10	0.14	7.14	0.51
1×10^4	0.18	0.26	3.85	3.48
1×10^5	0.23	0.33	3.03	48.98
1×10^6	0.26	0.38	2.63	123.77
1×10^7	0	0	0	0

*All experiments conducted in triplicate ($n=3$)

After identifying the exponential phase, the specific growth rate (μ /day), doubling time (day), and biomass productivity (cell/mL/day) were determined scientifically based on the formula given in the methodology section in this paper. *Table 2* shows that the highest specific growth rate was at 10^6 cell/mL concentration with 0.26 day^{-1} compared to other concentrations. So, this result is quite similar to the results obtained by Teles et al., (2013) with specific growth rate value up to 0.23 day^{-1} when cultivating *Chlorella vulgaris* in domestic wastewater. Then, the lowest specific growth rate occurred at 10^3 cell/mL concentration with 0.1 day^{-1} as expected. Biomass productivity results are also provided in *Table 2* and have been plotted in a graph over cell concentration compared to specific growth rate in *Figure 2*.

From the data illustrated in *Figure 2*, obviously in various cell concentrations, biomass productivity, and the growth rate of *Botryococcus* sp. were very different. According to *Figure 2*, it occurred on 10^6 cell/mL (x-axis) concentration with the maximum biomass productivity and specific growth rate were 1.24×10^6 cell/mL/day and 0.26 day^{-1} , respectively; this finding is different from other previous research paper. For example, Shin et al. (2015) used *Scenedesmus bijuga* for growth on diluted food wastewater and was able to achieve biomass productivity between 39.4 mg/L/day to 50.75 mg/L/day in terms of dried weight. This difference is likely due to the strength of nutrients concentration available in the wastewater used. It may also be affected by environment factors, particularly weather conditions.

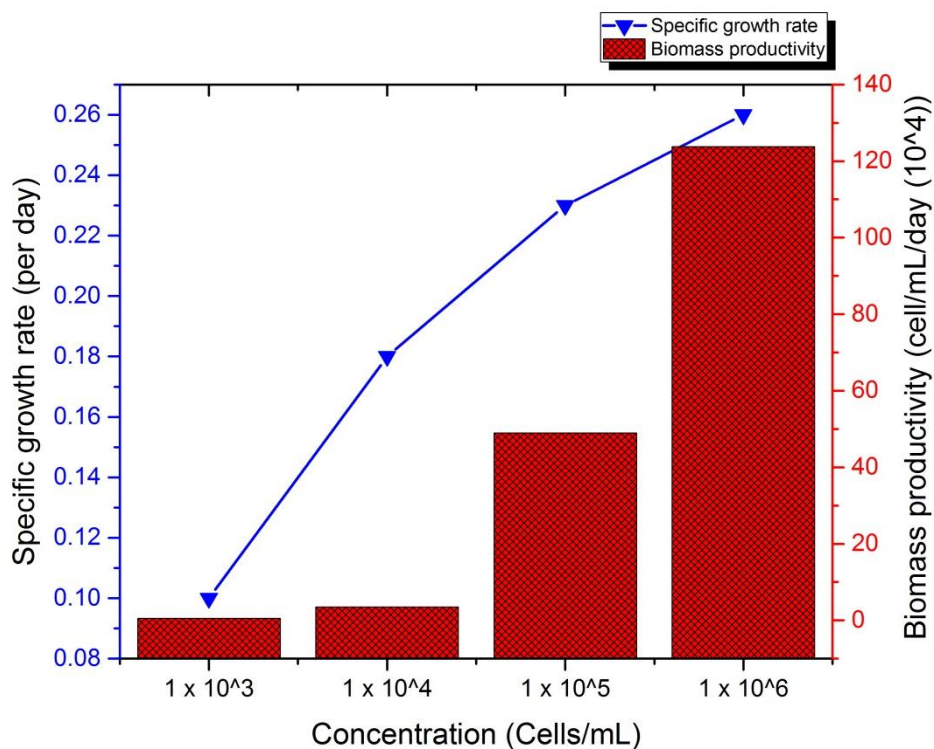


Figure 2. Specific growth rate (μ/day) and biomass productivity ($\text{cell}/\text{mL}/\text{day}$) of *Botryococcus* sp. in different cell concentration (cell/mL) on domestic wastewater

Conclusion

Recently, interest in cultivating microalgae as a source of biomass has grown. Growing microalgae requires a large quantity of water and nutrients. Amazingly, the use of domestic wastewater to cultivate microalgae is good from a sustainability perspective. However, the success of culturing microalgae in domestic wastewater is highly dependent on cell concentration at the initial stage of cultivation. This study has demonstrated how different cell concentration of microalgae makes it possible to produce microalgae biomass with high levels of productivity. Further research in this field regarding the role of microalgae *Botryococcus* sp. would be helpful for phycoremediation and hydrocarbon production.

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