Effect of Tris-(hydroxymethyl)-amino methane on microalgae biomass growth in a photobioreactor

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Abstract

One of the buffers namely Tris (Tris-(hydroxymethyl)-amino methane) was used to increase the growth of microalgae by stabilizing the pH value in microalgae cultures. The objective of this research is to determine the growth rate and biomass productivity of Chlorella sp. with and without Tris addition. Both conditions function at various N:P ratios cultured in photobioreactors (carbon dioxide of 5% (v/v), light intensity of 3.3 Klux). Daily variations in nutrient removal (nitrogen and phosphorus), cell concentration, DO, temperature and pH were measured for data analysis. The results show that the largest yield of biomass was achieved at the N:P ratio of 15:1 with and without Tris. After cultivation lasting 92 hours, the algae concentration at this ratio was 1250 mg L⁻¹ and 3568 mg L⁻¹ with and without Tris, respectively. This indicates that
adding Tris to the photobioreactor greatly reduces algae biomass due to bacterial competition.

Keywords: chlorella sp., tris (Tris-(hydroxymethyl)-amino methane, photobioreactor, N:P ratio, microalgae

1. Introduction

The rising global demand for energy is a serious issue with respect to fossil fuel depletion and carbon dioxide emissions linked to global greenhouse scenarios. Carbon dioxide is the most important anthropogenic greenhouse gas. Biofixation is the only economically feasible and environmentally sustainable technology in the long-term (Kumar et al., 2010). This biomass which is produced by converting carbon dioxide can be used to create products of high value, such as fatty acids, biodiesel, biogas, ethanol and organic fertilizers (Lopes et al., 2008; Giordano et al., 2014). It is worth noting that microalgae with 70% oil by weight can produce 23 times more oil compared to oil palm, the current major biofuel producer (Wang et al., 2009). The principle of biological fixation is that carbon dioxide is used to provide the carbon source for microalgae in the photobioreactor. Microalgae are known to contain large amounts of lipids within their cell structure, and so they are increasingly attracting interest as a biofuel feedstock. Microalgae photosynthesis will not produce any additional carbon dioxide while energy production and nutrient utilization for their growth can be achieved sustainably (Kumar et al., 2010; Pittman et al., 2011).
Chlorella sp., a genus of green unicellular microalgae which is highly efficient at removing the nutrient from wastewater (Shi et al., 2007; Imaizumi et al., 2014). It is spherical in shape, about 2-9 μm in diameter and without flagella. Although no useful microalgae have been found to be useful for carbon dioxide sequestration, Chlorella sp. has good commercial values and can grow under a high carbon dioxide concentration of 40% (Sakai et al., 1995). It is regarded as one of the most important energy microalgae due to its high protein accumulation, high lipid content and product variation (Das et al., 2011). The lipid content of Chlorella sp is about 32 ± 34% of the dry weight (Chiu et al., 2008). In addition, the carbon dioxide fixation rate is 0.68 mg L⁻¹ day⁻¹ (David and Prabakaran, 2012). They produce approximately half of the atmospheric oxygen and when used simultaneously the greenhouse gas carbon dioxide can grow photoautotrophically (Melanie, 2013). Indeed, Chlorella sp. is suitable microalgae for CO₂ mitigation and biodiesel production.

The process of microalgae cultivation is influenced by many factors. Nitrogen and phosphorous are two of the key factors in algae growth. The relative amounts of such essential nutrients required for growth and reproduce differ among algae species. Furthermore, the type of cultivation conditions for microalgae also need to be considered significantly. Phototrophic cultivation is the most commonly used cultivation condition for microalgae growth (Yoo et al., 2010). Namely photobioreactor is a closed system which is used to cultivate a single-species culture of microalgae for prolonged duration. It produces a large amount of microalgae biomass, which is about 13 times as concentrated as the biomass found in a raceway pond (Chisti, 2007). Bubble column reactors owe their many uses to their excellent mass and heat transfer characteristics. It is easy to construct and operate and has high surface area to volume
ratio (Ugwu et al., 2008), and requires only low maintenance costs. However, there may be significant phased back-mixing occurring. It is difficult to scale-up due to the complex interaction between the faces. The sole source of agitation is provided by the isothermal expansion of sparged gas (Chisti et al., 2006).

In previous studies, the cultivation of microalgae has incorporated the use of organic buffers to increase microalgae growth. Tris (Tris-(hydroxymethyl)-amino methane) is a buffer used to stabilize pH in microalgae cultures (Suzana et al., 2008). However, Tris is very controversial because the efficiency of pH stabilization has not been clearly demonstrated (Fabregas et al., 1993). In addition, the harmful effects of Tris have been observed in some phytoplankton species and freshwater algae (Harrison et al., 1980). Previous studies have indicated that Tris impacts on photosynthesis by inhibiting mechanisms such as the transportation of HCO$_3^-$ across the plasma membrane (Axelsson et al., 2000). This buffer also stimulates the growth of bacteria, leading to cultivation being severely curtailed (Fabregas et al., 1993). Nonetheless, it is questionable whether the beneficial effects of Tris are more or less than its effects on photosynthesis. Thus, this research aims to determine the growth rate and biomass productivity of *Chlorella* sp. with and without Tris. Both conditions are operated at various N:P ratios cultured in a photobioreactor.

2. Material and methods

2.1. Microalgae strain and culture medium

The microalgae strain used in this study was *Chlorella* sp. which was supplied by The Research Center of Aquaculture II. Ruan et al. (2011) cultivated *Chlorella* sp. in the
culture medium with the following solid ingredients: 100 mg L$^{-1}$ MgSO$_4$.7H$_2$O; 50 mg L$^{-1}$ CaCl$_2$.2H$_2$O. The liquid chemicals include: 1 mL L$^{-1}$ glacial acetic acid; 1 mL L$^{-1}$ trace elements solution consisted of 50 g L$^{-1}$ Na$_2$EDTA; 22 g L$^{-1}$ ZnSO$_4$.7H$_2$O; 0.05 g L$^{-1}$ CaCl$_2$.2H$_2$O; 11.4 g L$^{-1}$ H$_3$BO$_3$; 5.06 g L$^{-1}$ MnCl$_2$.4H$_2$O; 4.99 g L$^{-1}$ FeSO$_4$.7H$_2$O; 1.61 g L$^{-1}$ CoCl$_2$.6H$_2$O; 1.57 g L$^{-1}$ CuSO$_4$.5H$_2$O; 1.10 g L$^{-1}$ (NH$_4$)$_6$Mo$_7$O$_{24}$.4H$_2$O and 16 g L$^{-1}$ KOH.

2.2. Mass ratio adjustment

In this study involving an experiment to produce a *Chlorella* sp. biomass, the concentration of Tris and NH$_4$Cl was adjusted to suit the different N:P ratios (10:1, 15:1, 20:1, 25:1). The remaining chemical component remains unchanged. The concentration of Tris and NH$_4$Cl was altered but not the concentration of K$_2$HPO$_4$ and KH$_2$PO$_4$ in the culture medium. The final concentrations of Tris, NH$_4$Cl, K$_2$HPO$_4$, and KH$_2$PO$_4$ in the medium are presented in Table 1.

Table 1. Components of synthetic medium

2.3. Bubble column photobioreactor

A diagram of the experimental pilot used in this study is illustrated in Fig. 1. The photobioreactor was covered with a thick wood cover (5 mm) to retain a constant temperature and prevent outside light from affecting it, and to concentrate the light illuminated by three 18W lamps which were set up in the box. The microalgae were cultivated in two identical columns - scale photobioreactors with a diameter of 100 mm and a height of 600 mm. The working volume in the photobioreactor column was 4000...
mL. The aeration system for the reactor consisted of a 20 mm diameter air diffuser which was located at the bottom of the column. The system was operated under the following conditions: temperature of 29±2°C, 3 Klux of light intensity and 24:0 light-dark cycles (continuous illumination provided by three cool white lamps). Air mixture flow into the photobioreactor was provided via an air pump and a pure carbon dioxide tank through a 6 mm gas tube. With three rotameters which measured the air’s flow (from the air pump), the carbon dioxide gas and gas mixture, respectively, the carbon dioxide /air mixture at 2.0 L min⁻¹ flow rate was adjusted to achieve an air stream with 5% (v/v) of carbon dioxide. All experiments were carried out in batch mode.

Fig. 1 Bubble column photobioreactor system diagram

2.4. Relationship between cells density and dry mass

By using both methods together, cells density of *Chlorella* sp. was measured with a counting method and dry biomass was measured by filtering a known volume of culture medium through a 0.45 micrometer filter. It was then dried at 60°C for 24 h, and the standard curve of cell density and dry biomass were done. The formula of the standard curve equation: \( y = 29989x - 749565 \) (\( x \): dry biomass, mg L⁻¹; \( y \): cell density, cell mL⁻¹).

2.5. Biomass concentration analyses

Cell density was determined each day using a hemocytometer (Germany) under a microscope (Eclipse E50i; Nikon, Tokyo, Japan). Free-living algal growth was
determined daily (4 times per day). Cell density was measured by putting an algae
sample onto the mirrored surface of the Neubauer counting chamber. Then it was placed
under the microscope for cell counting, according to the Fuchs-Rosenthal and Burker
method. The formula to calculate the cell density after counting is $\alpha \times 0.25 \times 10^6$, with $\alpha$
being the average number of cells in 4 squares. When the cell density from the above
method was obtained, calculating the dry mass was done using the formula for the
standard curve equation.

3. Results and discussion

3.1. Growth of Chlorella sp. with and without Tris in photobioreactor

The growth curves of Chlorella sp. with and without Tris are shown in Fig. 2. It was
observed that the lag phase of culture conditions was lasted 20 h for the first cultivation,
reaching the logarithmic phase when the 20$^{th}$ hour of cultivation began. This was
followed by a stationary phase and then a death phase. Fig. 2 shows that a larger
maximum dry biomass without Tris was achieved than with Tris at the various N:P
ratios.

In the cultivation of N:P ratio of 10:1, the maximum dry biomass without Tris was 2.4
times larger than with Tris, achieving a concentration of 1404 mg L$^{-1}$ and 584 mg L$^{-1}$
after cultivation lasting 92 h and 80 h, respectively. Similar to the N:P ratio of 20:1 and
25:1, the maximum dry biomass without Tris was 1134 mg L$^{-1}$ and 1033 mg L$^{-1}$
whereas with Tris it was 800 mg L$^{-1}$ and 742 mg L$^{-1}$, respectively. Results of the
previous study conducted by Cabanelas et al. (2013) reported that the maximum dry
biomass of Chlorella sp. was approximately 1160 mg L$^{-1}$ and 1500 mg L$^{-1}$ at the N:P
ratio of 12:1; and 14:1, respectively. This indicates that optimal the N:P ratio for microalgae biomass production tends to the N:P ratio of 15:1.

In this study, with the cultivation at N:P ratio of 15:1, the dry biomass without Tris was also 2.9 times higher than with Tris, leading to the biomass concentrations of 3568 mg L\(^{-1}\) and 1250 mg L\(^{-1}\) under cultivating duration of 92 h and 96 h respectively. Similar results were obtained by Agwa et al. (2012) when cultivated *Chlorella* sp. in the same medium, dry biomass achieving at 3070 mg L\(^{-1}\). Cho et al. (2013) also revealed that *Chlorella* sp. cultivation could be achieved at the maximum dry biomass of approximately 3010 mg L\(^{-1}\) at the N:P ratio of 15:1, using mixed wastewater between from 10 % anaerobic digestion effluent and from 90% sludge dewatered supernatant. Reversely, the maximum dry biomass of *Chlorella* sp. obtained by the study of Chiu et al. (2008) was only approximately 899 mg L\(^{-1}\) with 5% of carbon dioxide and high density cell. However, the optimal N:P ratio of 15:1 for microalgae biomass production found from this study is contrast to typical N:P ratio of 8:1 conducted by USDA (1992).

Fig. 2 Dry biomass of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P ratios.

After a maximum of 5 days, the growth curves indicated characteristics of the stationary phase where the amount of newly formed cells are equal to that for dying cells. Similar results were obtained by Ruan et al. (2011) in the cultivation of *Chlorella* sp. Guerrero et al. (1999) reported that a lack of lag phase is due to high carbon and inorganic nutrient availability. However, the lag phase of this study occupies 20 h of the first
cultivation. This indicated that the concentration of carbon and inorganic nutrient was still low.

In the logarithmic phase, the growth was carried out continuously between 20 h and 72 h. The maximum dry biomass of 3568 mg L\(^{-1}\) (equivalent to cell density of \(1.05 \times 10^8\) cells mL\(^{-1}\)) obtained from the N:P ratio of 15:1 was found without Tris. Results of this study indicated that *Chlorella* sp. could be cultivated in the following conditions that enhanced biomass production: temperature of 29 ± 2°C, light intensity of 3 Klux, N:P ratio of 15:1 and without Tris.

Indeed, when cultivating the N:P ratio of 15:1, the maximum dry biomass was achieved both with and without Tris. This is closely similar to results reported by Cho et al. (2013) and Cabanelas et al. (2013). The above results indicated that a significant decrease in the dry biomass occurs with increasing applied nitrogen ratio (i.e., ratio of N:P greater than 15:1). For example, the study of Chiu et al. (2014) reported that the N:P ratios higher than 17:1 contributed to lower biomass production.

3.2. pH and DO curves of Chlorella sp. with and without Tris in photobioreactor

Fig. 3 illustrates the representative variation of pH with and without Tris as a function of residence time. Without Tris, during the 8 h lag phase, the pH value was stable in the range of 6.5 - 6.7 at the various N:P ratios. However, in the logarithmic phase, the pH value decreased slightly from 6.4 to 6.0. Especially at N:P ratio of 15:1, there was a significantly reduction in pH from the 56\(^{th}\) hour to the 96\(^{th}\) hour. Lazzaro et al. (2008) reported that the culture medium consisting of the predominant form of CO\(_3^{2-}\) will lead to an inefficient production of biomass. Thus, the initial pH adjusted in the range of 6.5
- 7.0 increased to form HCO$_3^-$ which easily uptaked by *Chlorella* sp. (Beardall et al., 1998). For this reason, in this study the consumption of HCO$_3^-$ started increasing during the logarithmic phase when pH have a downward trend by generating H$^+$. In addition, the pH value of the culture medium decreased according to the uptake of ammonia (Park et al., 1997). Tan et al. (2016) noted that the growth of *Chlorella* sp. was slower if the pH fell to 5.0 and this is the pH limitation value of *Chlorella* sp. With Tris, the pH value remained stable from 7.0 to 7.3 during the cultivation phase because Tris is one of the buffers often used to stabilize pH in microalgae cultures (Fabregas et al., 1993).

Fig. 3 pH curves of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P ratios

Fig. 4 shows the relationship of the concentration of dissolved oxygen with and without Tris over time. Dissolved oxygen concentration increases dramatically during the cultivation time for with and without Tris. It is generally agreed that photosynthetic oxygen is a product of photosynthesis. The daily DO peak increased gradually with the increase of cell mass when the algae were in the exponential growth phase. With the best N:P ratio cultured, the highest achievement of DO was 6.3 mg L$^{-1}$ and 8.6 mg L$^{-1}$ with and without Tris, respectively. The concentration of dissolved oxygen can be used to indicate good algal growth. However, residual dissolved oxygen may cause oxygen accumulation which can damage and reduce cell growth. Dissolved oxygen concentration should not reach a saturation level of 35 mg L$^{-1}$ (Carvalho et al., 2006). Therefore, it is important to have good mass transfer in the photobioreactor, which highlights the importance of photobioreactor design. Bubble column photobioreactor in
this study is not concerned with the accumulation of oxygen inside it (Das et al., 2011).

DO peak will continue to increase from the start of the logarithmic phase until the
stationary phase begins. All cultures confirmed such behavior and the dissolved oxygen
concentration declines after reaching the stationary phase. This finding is similar to the
results obtained by Chai et al. (2012). Dissolved oxygen level drops in the stationary
phase when the amount of dying cells is equal to that for newly formed cells.

Fig. 4 DO curves of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P ratios

3.3. Presence of protozoa (*Paramecium*) in during the cultivation of *Chlorella* sp.

Without Tris, there were no organic nutrients evident during the period of and no
*Paramecium* was present. However, with Tris, the presence of *Paramecium* was evident
at the various ratios of N:P. More specifically, they were observed by 100X
magnification and defined after cultivation lasting 44 h. Results obtained in this study
indicated that Tris is one of the most important factors determining the presence of
*Paramecium* in the cultivation of *Chlorella* sp. This conclusion is similar to that
reported by Fabregas et al. (1993). Furthermore, *Paramecium* disrupts the growth of
freshwater algae, and typically *Chlorella* sp. is the main food source for them

(Tillimann, 2004).

4. Conclusions
Some concluding remarks can be made regarding the impact of Tris on microalgal biomass growth in the photobioreactor as follows. Firstly, under operation the conditions of N:P =15:1 ratio, *Chlorella* sp. performed the best either with or without Tris, achieving a high biomass concentration after cultivation of 92 h and 96 h. Secondly, the dry biomass without Tris is 3 times larger than that with Tris. Thirdly, Tris is one of the factors that can determine the presence of *Paramecium* in the cultivation of *Chlorella* sp.

Acknowledgements

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References


**Figure captions**

Fig. 1. Bubble column photobioreactor system diagram

Fig. 2. Dry biomass of *Chlorella* sp. with and without Tris at various N:P ratios

Fig. 3. pH curves of *Chlorella* sp. with and without Tris at various N:P ratios

Fig. 4. DO curves of *Chlorella* sp. with and without Tris at various N:P ratios
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Fig. 2. Dry biomass of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P ratios.
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Fig. 4. DO curves of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P ratios
Table 1. Components of synthetic medium

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Graphical abstract
Highlights

- Effect of Tris on microalgae growth was investigated at different N:P ratios.
- *Chlorella* sp. performed well in both conditions with and without Tris at N/P of 15.
- Dry microalgae biomass without Tris was 3-fold higher than that with Tris.
- Tris can determine the presence of *Paramecium* in the cultivation of *Chlorella* sp.