Effects of CO₂ and pH on Growth of the Microalga *Dunaliella salina*

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Abstract

A potentially cost-effective and scalable method to stabilize pH in microalgal batch cultures is proposed in this study. The cultures were supplied with different concentrations of CO₂ enriched gas and controlled amounts of bicarbonate were added. An empirical model correlating the equilibrium pH to bicarbonate and CO₂ stream concentrations was established experimentally. Finally, the isolated impact of either pH or CO₂ concentration on *Dunaliella salina* growth was studied.

Keywords: Isolated impact; pH; CO₂; *Dunaliella salina*

Introduction

The cultivation of microalgae has been studied and developed for more than 60 years [1]. Some parameters affecting algal growth have been well studied, e.g. light illumination [2-8], while some are still worthwhile to be studied, for instance the effects of pH and CO₂ on microalgal growth. At saturating light intensities, the rate of CO₂ supply is crucial for algal photosynthesis as CO₂ is major source for the carboxylation of RuBP. pH is also one of the important factors for algal growth as it can affect the activity of different enzymes. In general, different algal species have various ranges of tolerance to pH.

The effects of pH and CO₂ on microalgal growth have been well studied by many researchers [9-14], however, neither pH nor dissolved CO₂ were solely controlled during their experiments due to the interactions between pH and dissolved CO₂. It seems to be infeasible to keep pH constant while varying the dissolved CO₂, or vary the pH while keeping dissolved CO₂ constant. Therefore it will be interesting to find out the isolated effect of pH or CO₂ on algal growth. In this study, a method was proposed to achieve a constant pH and variable dissolved CO₂, or a constant CO₂ level and variable pH. Their effects on microalgal growth were studied based on the culture of the unicellular green alga *Dunaliella salina*. Six different pH levels and three different dissolved CO₂ concentrations were tested.

Methodology

Under a constant bubbling condition, the equilibrium concentration of dissolved CO₂ ([CO₂]⁺) according to Henry’s law and Two-film theory, should only depend on the CO₂ partial pressure in the gas phase under constant gas/liquid properties and temperature. Therefore, for a fixed CO₂ percentage in the bubbling gas, the [CO₂]⁺ will not be altered when varying the concentrations of NaHCO₃ in the medium (assuming the changes in liquid physical properties by adding NaHCO₃ into the water are negligible, as long as the concentrations of NaHCO₃ are low). On the other hand, the dissolved CO₂ concentration is correlated to pH by Equation 1 [15,16]. Since the concentration of Na⁺ varies for different concentration of NaHCO₃, while the [CO₂]⁺ does not change, it is therefore reasonable that the equilibrium pH (pH*) changes for the medium with different NaHCO₃ concentration.

\[
[\text{CO}_2] = \frac{10^{-pH} \cdot 10^{-0.631 \cdot pH^{-1.84} + 2 \times 10^{-16.758}}}{10^{-8.311 \cdot pH^{-1.84} + 2 \times 10^{-16.758}}} \quad (\text{mol} / \text{L})
\]

In Ying et al. [16], the effects of NaHCO₃ concentration on equilibrium concentration of dissolved CO₂ and CO₂ mass transfer rate in water were studied. The results proved the above hypothesis, indicating the feasibility of using NaHCO₃ to control the equilibrium pH of the medium without affecting the [CO₂]⁺ and CO₂ mass transfer rate. However, only one concentration of CO₂ (5%) in the bubbling gas was tested, the relationship between pH* and NaHCO₃ established was only suitable for 5% CO₂ dosing. Therefore, in this study, experiment a) was designed to find a comprehensive model correlating pH*, NaHCO₃ and CO₂%, which would facilitate the experimental designs on b) pH impact and c) CO₂ effect on algal growth.

Experiment a): Relationship between pH*, NaHCO₃ and CO₂%

To study the interaction between pH*, NaHCO₃, and CO₂%, a gas mixture containing a certain percentage of CO₂ balanced with N₂ was injected to the airlift bioreactor containing 1.5 L of distilled water and a certain concentration of NaHCO₃. The initial temperature is adjusted to 22°C. pH was measured by a SevenGo Duo pro (pH/DO/Ion) meter. When the pH reading stops changing for 10 minutes, this value is recorded and considered as the equilibrium pH. The experimental procedure was repeated 35 times using 7 concentrations of NaHCO₃ and 5 CO₂ stream concentrations tested. The equilibrium concentration of CO₂ ([CO₂]⁺) was calculated by Equation 1. The experimental set up is shown in Figure 1.

Experiment b): The effect of pH on algal growth

Six 1.5 L-airlift bioreactors containing artificial sea water medium designed for Dunaliella species [17,18] but with different NaHCO₃ concentrations were run simultaneously for *Dunaliella salina* (strain 19/30, Culture Centre of Algae and Protozoa, Oban, UK) culture. (Figure 2) At the beginning, 50 ml of healthy pre-cultured *D. salina* was added to 1.5 L of fresh culture medium for each culture. CO₂ gas mixture was constantly dosed into each reactor with a fixed stream rate.
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Experiment (a): The effect of dissolved CO$_2$ on algal growth

To study the impact of dissolved CO$_2$ on D. salina growth dissolved CO$_2$ concentration needs to be varied while the pH for each culture should be maintained constant. To achieve this, three different CO$_2$ stream concentrations (5%, 20% and 50%) were applied to provide three corresponding CO$_2$ equilibrium concentrations. The equilibrium pH for each reactor was expected to be 7 by adding the proper amount of NaHCO$_3$. The concentration of NaHCO$_3$, required for each culture is estimated by the empirical equation found from experiment (a). The whole set of cultures was illuminated by a fluorescent lamp providing continuous light of 90 μmol quanta m$^{-2}$ s$^{-1}$. Non-transparent baffles were placed between every two reactors to ensure even illumination for each culture. The temperature for each culture was maintained around 23°C, due to the empirical heat transfer from the fluorescent lamp. pH, OD and chlorophyll content for each culture were measured daily. The photosynthetic oxygen generation rate of each culture was measured at day 5. The method for photosynthetic oxygen generation rate measurement is described in Appendix 1 and 2. The detailed culture condition for each reactor is listed in Table 1. The whole set of experiments were repeated once for error analysis.

Results and Discussion

The correlations between pH*, NaHCO$_3$ and CO$_2$%

Figure 4 summarized the relations between [CO$_2$]*, NaHCO$_3$ and

| Reactor | Dosing condition | Concentration of NaHCO$_3$ (mol/L) | Expected pH* | Expected [CO$_2$]* (mol/L) |
|---------|------------------|------------------------------------|--------------|--------------------------|
| No. 1   | 5% CO$_2$ constant dosing with fine-bubbles (d32: 719 μm) | 5.95×10$^{-4}$ | 6 | 0.002 |
| No. 2   | 2.03×10$^{-3}$ | 6.5 | 0.002 |
| No. 3   | 6.97×10$^{-3}$ | 7 | 0.002 |
| No. 4   | 8.17×10$^{-2}$ | 8 | 0.002 |
| No. 5   | 0.280           | 8.5 | 0.002 |
| No. 6   | 0.957           | 9 | 0.002 |

Table 1: The culture condition of each reactor in the study of pH impact on D. salina growth.
CO₂%. The equilibrium concentration of dissolved CO₂ ([CO₂]*) is found to be only dependent on the CO₂ stream concentration (CO₂%). [CO₂]* was enhanced with the higher CO₂% supply. The variation of NaHCO₃ concentration did not affect [CO₂]* when CO₂% was fixed. This phenomenon can be supported by Henry’s law that the equilibrium concentration of a gas is in direct proportion to the partial pressure of that gas over the solution.

In terms of equilibrium pH (pH*), its changes along with the NaHCO₃ concentration and CO₂ stream concentration (CO₂%) were plotted in Figure 5a. As can see, for a fixed CO₂% in the gas supply, pH* was altered by varying the NaHCO₃ concentration. Higher NaHCO₃ concentration resulted in a higher pH*. Such a trend is also consistent with findings from Ying et al. [16]. An empirical equation correlating pH* to NaHCO₃ and CO₂% was created in the logarithmic plot (Figure 5b), shown in Equation 2. The accuracy of Equation 2 was examined by comparing the experimental pH* values with the calculated values, shown in Figure 6. The results showed a less than 5% deviation between the real and the estimated pH* values by using Equation 2. Therefore, under a constant gas bubbling condition, pH can be controlled at a specific level for microalgae culture by choosing the right concentration of NaHCO₃ and CO₂% in the gas supply, without applying additional ‘auto-pH regulating systems’ or expensive buffers. For the gas dosing, microbubbles or fine bubbles (e.g. less than 800 - 1000 μm in diameter) are recommended as the CO₂ mass transfer rate needs to be controlled sufficiently to balance the CO₂ consumption by algal growth. Otherwise, pH* would not stay constant but increase.

\[ pH^* = 7.6543 + 0.4063 \ln(\text{CO2}_\%\text{)} - 0.4551 \ln([\text{NaHCO}_3]) \]  

Effect of pH on *D. salina* growth

To study the pH effect on *D. salina* growth, six different pH levels were tested (expected pH= 6, 6.5, 7, 8, 8.5 and 9). The dissolved CO₂ concentration for each culture was maintained the same (about 0.002 mol.L⁻¹) through the constant dosing of 5% CO₂. The real pH value for each culture versus the expected value was plotted in Figure 7. The results showed that the pH for each culture was controlled at the expected level, which again proved the feasibility of using pH*-NaHCO₃-CO₂% model (Equation 2) for pH control in the real algal culture. The daily algal growth under each pH level was shown in Figure 8. First of all, two different growth phases were observed for each culture. The growth was logarithmic in the first 5 days while 5 days after it became linear-like. The same scenario was discussed by Richmond [8]. For a certain high light intensity, assuming all the photons of a flux density can be captured by the algal culture; cell density will keep

| Reactor | Culture conditions | Concentration of NaHCO₃ (mol/L) | Expected pH* | Expected [CO₂]* (mol/L) |
|---------|-------------------|---------------------------------|--------------|-------------------------|
| No. 1   | 5% CO₂ dosing     | 6.97×10⁻²                      | 7            | 0.002                   |
| No. 2   | 5% CO₂ dosing     | 6.97×10⁻²                      | 7            | 0.002                   |
| No. 3   | 20% CO₂ dosing    | 3.29×10⁻²                      | 7            | 0.008                   |
| No. 4   | 20% CO₂ dosing    | 3.29×10⁻²                      | 7            | 0.008                   |
| No. 5   | 50% CO₂ dosing    | 9.19×10⁻²                      | 7            | 0.020                   |
| No. 6   | 50% CO₂ dosing    | 9.19×10⁻²                      | 7            | 0.020                   |

Table 2: The culture condition of each reactor in the study of CO₂ impact on *D. salina* growth.
increasing exponentially until all photosynthetically available photons are absorbed. Then, cell density increases linearly until light per cell becomes limiting which leads to growth inhibition. Therefore the cell concentration at 5th day of the culture can be considered as the ‘threshold’ between light-unlimited growth and light-limited growth, which was about 30-40 mg/L in chlorophyll content. Secondly, no pH level between 6 and 9 was found to completely inhibit to D. salina growth, however, the differences in the growth for different pH conditions were also observed. The specific growth rate for each pH level was compared by plotting Figure 9.

In Figure 9, the differences between the specific growth rates of light-unlimited growth phase and light-limited growth phase were obvious; the former were about 4 times higher than the latter. Therefore, a better geometry design of ALB to extend the light-unlimited growth phase is important and should be mainly considered in future work, for example enhancing the Light/Dark ratio [8]. In terms of the pH effect on D. salina growth, the plot of specific growth rate against each pH condition presented a ‘parabola trend’ with an optimal value achieved at around pH 7 for either light-unlimited or light-limited growth phase. Besides, D. salina had a wide range of tolerance to pH, and pH between 6 and 9 was found not to completely inhibit growth.

The pH effect on growth was also studied in terms of photosynthetic O2 yield rate. An example of the typical photosynthetic O2 concentration versus time was plotted in Figure 10, from which the photosynthetic O2 generation rate was calculated. An identical ‘parabola trend’ as in Figure 9 was obtained in Figure 11, again indicating the optimal pH level of around 7.

Since the concentration of dissolved CO2 is maintained the same for each culture, the intracellular CO2 concentration was speculated...
to be the same according to the two-film theory, which would suggest that the intracellular equilibrium pH for each culture is identical. In general, the results (Figures 9 and 11) indicated that even for the same intracellular pH, the changes in extracellular pH could still affect the algal growth via an as yet unknown mechanism, possibly related to the pH gradient across the cell membrane. pH around 7 was found to be the optimal pH for \textit{D. salina} culture.

**Effect of dissolved CO\(_2\) concentration ([CO\(_2\)]\textsuperscript{+}) on \textit{D. salina} growth**

In this experiment, the pH level for each culture was designed to be 7 by using ‘pH*-NaHCO\(_3\)-CO\(_2\)% model’ (Equation 7.1), while the practical pH value was actually controlled at 6.88 ± 0.08. The daily chlorophyll content change of \textit{D. salina} under different CO\(_2\) equilibrium concentrations was plotted in Figure 12. The chlorophyll content increased from 10 mg L\(^{-1}\) to 70 mg L\(^{-1}\) within 11 days under constant 5% CO\(_2\) dosing (0.002 mol L\(^{-1}\) of [CO\(_2\)]\textsuperscript{+}), while a slight growth inhibition was observed when increasing the CO\(_2\) dosing concentration up to 20% (0.008 mol L\(^{-1}\) of [CO\(_2\)]\textsuperscript{+}). In this case, the chlorophyll content increased to less than 60 mg L\(^{-1}\) in 11 days. The 50% CO\(_2\) dosing (0.02 mol L\(^{-1}\) of [CO\(_2\)]\textsuperscript{+}) strongly inhibited \textit{D. salina} growth as the chlorophyll content started decreasing from day 2 onwards. Figures 13 and 14 clearly show the effect of dissolved CO\(_2\) concentration on \textit{D. salina} growth in terms of specific growth rate and the photosynthetic O\(_2\) generation rate, respectively. In the first 4 days, the light was still sufficient for growth due to the low concentration of algae in the culture; the specific growth rate decreased from about 0.39 d\(^{-1}\) to 0.32
The photosynthetic O₂ yield dropped from approximately 0.40 μmol dissolved CO₂, or a constant CO₂ level and variable pH for microalgal culture, this level of CO₂ was fatal to D. salina culture. An empirical equation correlating pH* to NaHCO₃ and CO₂% equilibrium concentration, whilst higher extracellular equilibrium CO₂ concentration leads to a lower intracellular pH which may damage or weaken of photosynthesis at this high CO₂ concentration, the explanation behind the situation is that despite the same extracellular condition) resulted in an inhibition of photosynthesis for D. salina. Under 0.02 mol L⁻¹ CO₂ concentration, a strong growth inhibition was observed. More than 0.02 mol L⁻¹ of dissolved CO₂ (i.e. constant dosing of 50% CO₂) was fatal to D. salina growth. Due to the lab limitations, only 3 different CO₂ stream concentrations were studied, an optimal dissolved CO₂ concentration was not determined for D. salina culture. More CO₂ stream concentrations (especially between 5%-20%) are expected to be tested in the future. It will be interesting to measure the intracellular pH under different dissolved CO₂ concentrations, and to find out the relationship between intracellular pH and extracellular pH.

**Conclusions and Future work**

A methodology was proposed to achieve a constant pH and variable dissolved CO₂, or a constant CO₂ level and variable pH for microalgal culture. An empirical equation correlating pH* to NaHCO₃, and CO₂% is obtained. The accuracy of this empirical equation was examined by comparing the experimental pH* values with the calculated values. The results showed a less than 5% deviation between the practical and the estimated pH* values.

The isolated impact of either pH or CO₂ concentration on Dunaliella salina growth was then studied by using pH*-NaHCO₃, CO₂% system. According to either specific growth rate or photosynthetic O₂ generation rate, pH around 6-9 was found to support D. salina culture. Both specific growth rate and photosynthetic O₂ generation rate versus different pH levels presented a ‘parabola trend’ with an optimal value achieved at around pH 7 for either light-unlimited or light-limited growth phase. With regard to the isolated effect of CO₂ concentration on D. salina growth, both specific growth rate and photosynthetic O₂ generation rate decreased when the CO₂ concentration increased. Under 0.02 mol L⁻¹ CO₂ concentration, a strong growth inhibition was observed.

To sum up, under the same extracellular pH, an increase in dissolved CO₂ concentration (i.e. the CO₂% in a constant dosing condition) resulted in an inhibition of photosynthesis for D. salina culture at 50% CO₂ in the dosing stream (or 0.02 mol L⁻¹ of [CO₂]* in the culture), this level of CO₂ was fatal to D. salina growth. The possible explanation behind the situation is that despite the same extracellular pH, the intracellular pH can be affected by the extracellular CO₂ equilibrium concentration, whilst higher extracellular equilibrium CO₂ concentration leads to a lower intracellular pH which may damage or inhibit the enzymes involved in photosynthesis.

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