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Effects of CO_2 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 CO_2 enriched gas and controlled amounts of bicarbonate were added. An empirical model correlating the equilibrium pH to bicarbonate and CO_2 stream concentrations was established experimentally. Finally, the isolated impact of either pH or CO_2 concentration on *Dunaliella salina* growth was studied.

Keywords: Isolated impact; pH; CO_2; *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 CO_2 on microalgal growth. At saturating light intensities, the rate of CO_2 supply is crucial for algal photosynthesis as CO_2 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 CO_2 on microalgal growth have been well studied by many researchers [9-14], however, neither pH nor dissolved CO_2 were solely controlled during their experiments due to the interactions between pH and dissolved CO_2. It seems to be infeasible to keep pH constant while varying the dissolved CO_2, or vary the pH while keeping dissolved CO_2 constant. Therefore it will be interesting to find out the isolated effect of pH or CO_2 on algal growth. In this study, a method was proposed to achieve a constant pH and variable dissolved CO_2, or a constant CO_2 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 CO_2 concentrations were tested.

Methodology

Under a constant bubbling condition, the equilibrium concentration of dissolved CO_2 ($[CO_2]^*$), according to Henry's law and Two-film theory, should only depend on the CO_2 partial pressure in the gas phase under constant gas/liquid properties and temperature. Therefore, for a fixed CO_2 percentage in the bubbling gas, the $[CO_2]^*$ will not be altered when varying the concentrations of $NaHCO_3$ in the medium (assuming the changes in liquid physical properties by adding $NaHCO_3$ into the water are negligible, as long as the concentrations of $NaHCO_3$ are low). On the other hand, the dissolved CO_2 concentration is correlated to pH by Equation 1 [15,16]. Since the concentration of Na^+ varies for different concentration of $NaHCO_3$, while the $[CO_2]^*$ does not change, it is therefore reasonable that the equilibrium pH (pH*) changes for the medium with different $NaHCO_3$ concentration.

$$[CO_2] = \frac{(10^{-pH} - 10^{(pH-14)} + \Delta[Na^+])10^{(-2pH)}}{10^{(-6.381-pH)} + 2\times 10^{(-16.758)}} \quad (mol/L) \qquad (1)$$

In Ying et al. [16], the effects of $NaHCO_3$ concentration on equilibrium concentration of dissolved CO_2 and CO_2 mass transfer rate in water were studied. The results proved the above hypothesis, indicating the feasibility of using $NaHCO_3$ to control the equilibrium pH of the medium without affecting the $[CO_2]^*$ and CO_2 mass transfer rate. However, only one concentration of CO_2 (5%) in the bubbling gas was tested, the relationship between pH* and $NaHCO_3$ established was only suitable for 5% CO_2 dosing. Therefore, in this study, experiment a) was designed to find a comprehensive model correlating pH*, $NaHCO_3$ and CO_2%, which would facilitate the experimental designs on b) pH impact and c) CO_2 effect on algal growth.

Experiment a): Relationship between pH*, $NaHCO_3$ and CO_2%

To study the interaction between pH*, $NaHCO_3$ and CO_2%, a gas mixture containing a certain percentage of CO_2 balanced with N_2 is injected to the airlift bioreactor containing 1.5 L of distilled water and a certain concentration of $NaHCO_3$. 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 $NaHCO_3$ and 5 CO_2 stream concentrations tested. The equilibrium concentration of CO_2 ($[CO_2]^*$) 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 $NaHCO_3$ 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. CO_2 gas mixture was constantly dosed into each reactor with a fixed stream

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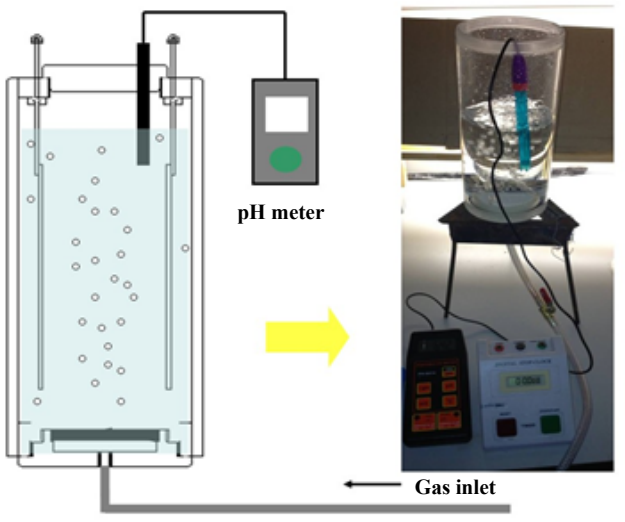

Figure 1: The setup for equilibrium pH measurement.

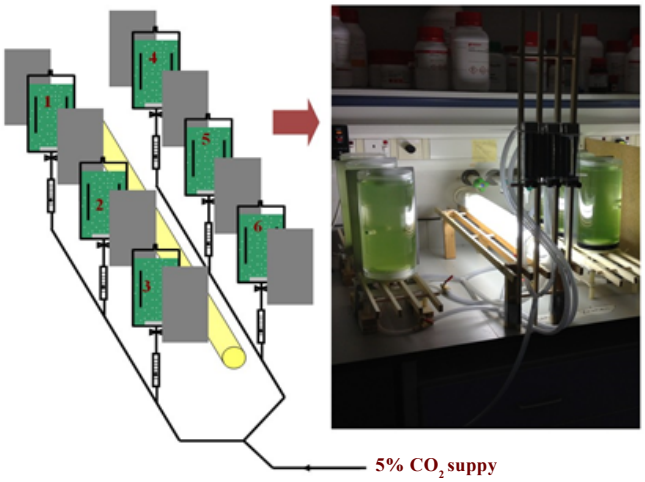

Figure 2: The experimental setup for studying the impact of pH on *D. salina* growth.

concentration (5% CO_2 balanced with N_2) under 0.3 L/min. Although the algal growth may consume some dissolved CO_2, a new equilibrium would be achieved immediately after that due to the constant CO_2 dosing (CO_2 mass transfer >> CO_2 consumption, the consumed CO_2 would be balanced with the CO_2 transferred into the medium). In other words, the dissolved CO_2 is maintained constant at its equilibrium concentration. However, the equilibrium pH for each reactor differs, due to the different $NaHCO_3$ concentrations in the medium. As regard to the specific $NaHCO_3$ concentration for each culture, it was determined by the empirical model established based on the results from experiment (a). The whole set of cultures were illuminated by a fluorescent cool white lamp providing continuous light of 90 μmol quanta m^{-2} s^{-1}. The light intensity was measured by a Hansatech Quantum Sensor. 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.

Experiment c): 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 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 activity of each culture was measured at day 5 and day 16. The experimental setup and culture conditions are shown in Figure 3 and Table 2, respectively.

Results and Discussion

The correlations between pH*, $NaHCO_3$ and CO_2%

Figure 4 summarized the relations between $[CO_2]$*, $NaHCO_3$ and

Reactor	Culture conditions			
	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.

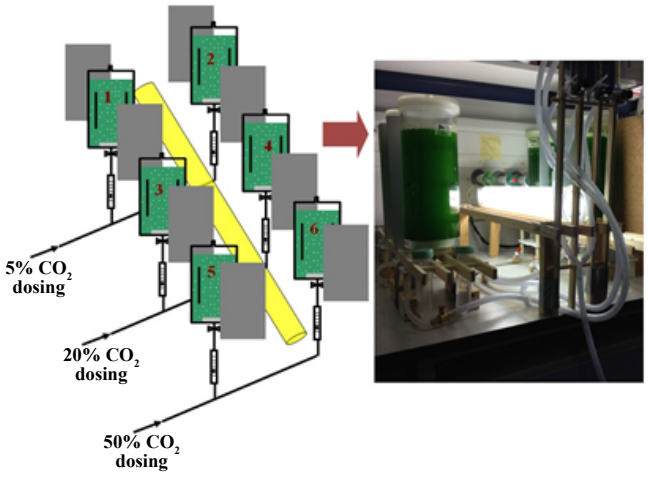

Figure 3: The experimental setup for studying the effect of CO_2 on *D. salina* growth.

CO_2%. The equilibrium concentration of dissolved CO_2 ($[CO_2]^*$) is found to be only dependent on the CO_2 stream concentration (CO_2%). $[CO_2]^*$ was enhanced with the higher CO_2% supply. The variation of $NaHCO_3$ concentration did not affect $[CO_2]^*$ when CO_2% 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 $NaHCO_3$ concentration and CO_2 stream concentration (CO_2%) were plotted in Figure 5a. As can see, for a fixed CO_2% in the gas supply, pH* was altered by varying the $NaHCO_3$ concentration. Higher $NaHCO_3$ 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 $NaHCO_3$ and CO_2% 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 $NaHCO_3$ and CO_2% 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 CO_2 mass transfer rate needs to be controlled sufficiently to balance the CO_2 consumption by algal growth. Otherwise, pH* would not stay constant but increase.

$$pH^* = 7.6543 + 0.4063\ln(CO_2\%) - 0.4551\ln([NaHCO_3]_{mol/L}) \quad (2)$$

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 CO_2 concentration for each culture was maintained the same (about 0.002 mol.L^{-1}) through the constant dosing of 5% CO_2. 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*-$NaHCO_3$-CO_2% 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			
	Dosing condition	Concentration of $NaHCO_3$ (mol/L)	Expected pH*	Expected $[CO_2]^*$ (mol/L)
No. 1	5% CO_2 dosing	6.97×10^{-3}	7	0.002
No. 2	5% CO_2 dosing	6.97×10^{-3}	7	0.002
No. 3	20% CO_2 dosing	3.29×10^{-2}	7	0.008
No. 4	20% CO_2 dosing	3.29×10^{-2}	7	0.008
No. 5	50% CO_2 dosing	9.19×10^{-2}	7	0.020
No. 6	50% CO_2 dosing	9.19×10^{-2}	7	0.020

Table 2: The culture condition of each reactor in the study of CO_2 impact on *D. salina* growth.

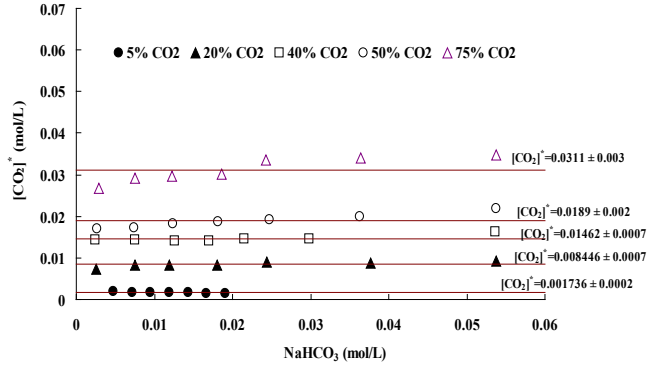

Figure 4: Plots of $[CO_2]^*$ versus $NaHCO_3$ concentration for different CO_2 stream concentrations.

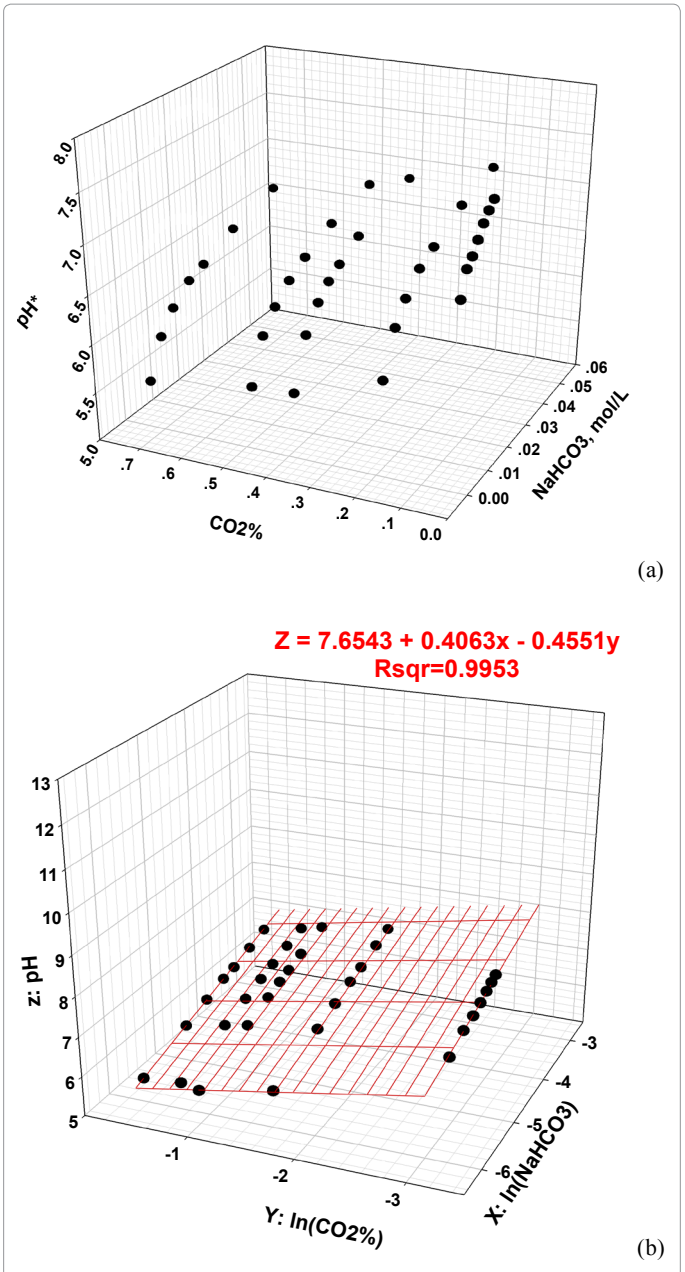

Figure 5: 3D-plot of the relationship between pH*, $NaHCO_3$ and CO_2%. (a) Plot of pH* versus $NaHCO_3$ and CO_2%; (b) plot of pH* versus ln($NaHCO_3$) and ln(CO_2%).

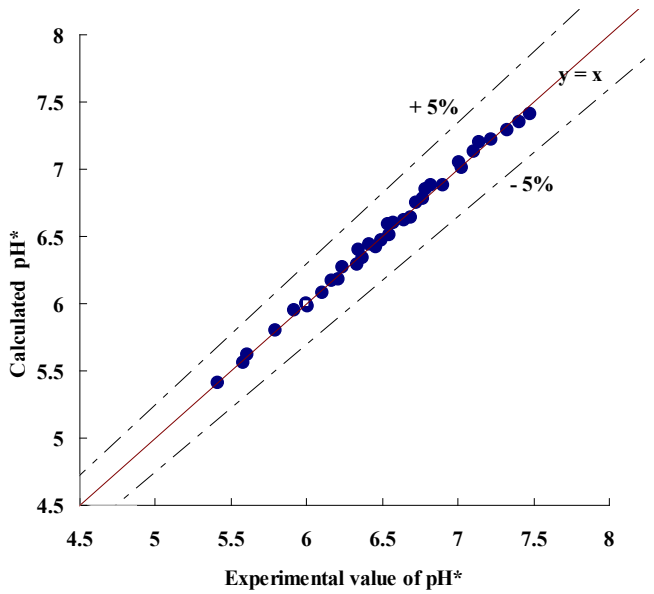

Figure 6: Comparison between experimental pH* value with the one calculated based on Equation 2. This figure consists of 35 points, covering the pH* values under 7 $NaHCO_3$ and 5 CO_2 stream concentrations.

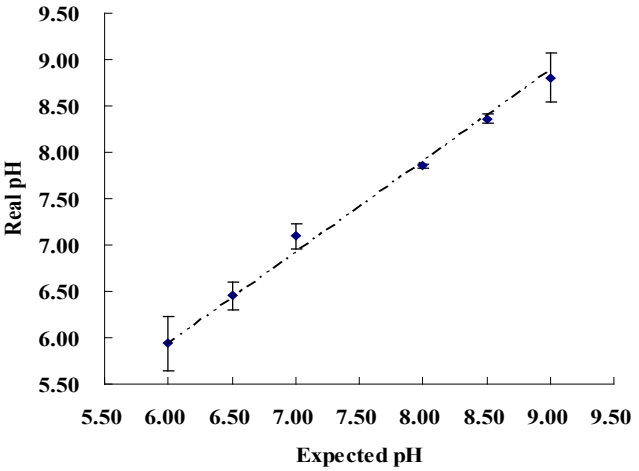

Figure 7: Plot of the real pH versus the expected pH for the experiment 'pH effect on *D. salina* growth'. For each culture, the real pH value presented in this figure was calculated as the average value of the daily recorded pHs of which the standard deviations are shown as error bars.

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 O_2 yield rate. An example of the typical photosynthetic O_2 concentration versus time was plotted in Figure 10, from which the photosynthetic O_2 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 CO_2 is maintained the same for each culture, the intracellular CO_2 concentration was speculated

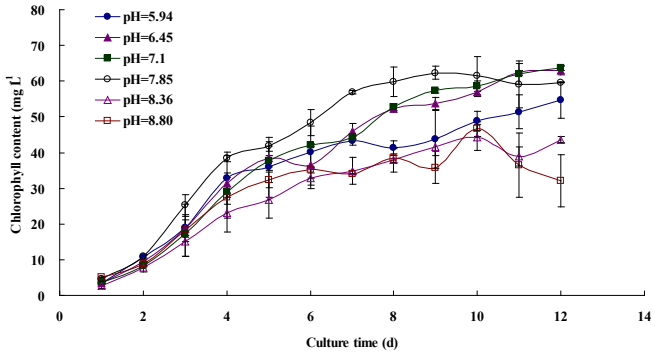

Figure 8: The plot of daily chlorophyll content against culture time for different pH levels. According to the diagram, from day 2 to day 5, the increase in chlorophyll was obviously quicker than the increase between day 5 and day 10. Therefore, for each culture condition, two specific growth rates were calculated on day 2 - day 5 and day 5 - day 10, separately. The method for estimating the specific growth rate is shown in Appendix 2.

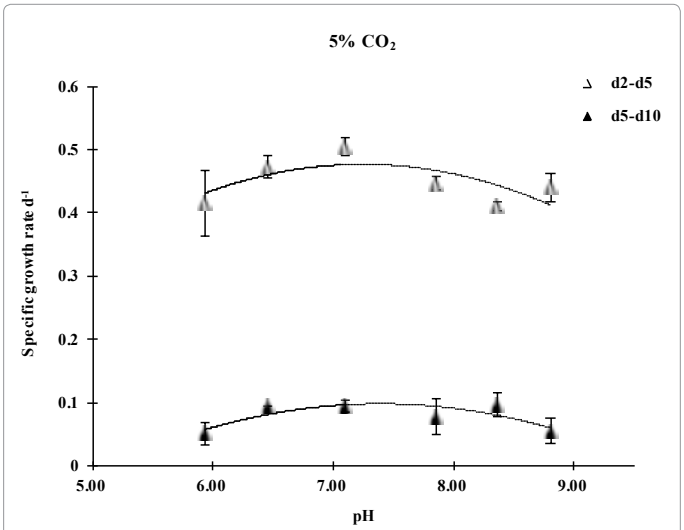

Figure 9: The specific growth rate of *D. salina* culture under each pH condition. The hollow triangles represent the specific growth rates of the first 5 days (light-unlimited) while the solid triangles stand for the specific growth rates between day 5 and day 12 (light-limited).

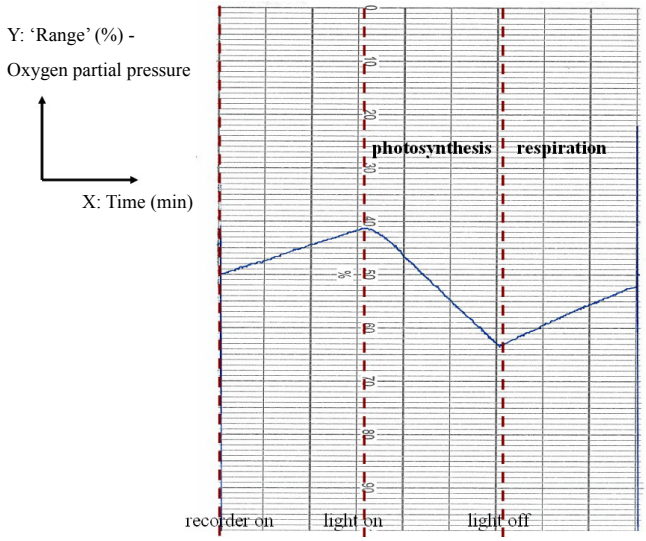

Figure 10: Typical graph of photosynthetic O_2 concentration (partial pressure) versus time. Each unit in X-axis was set to be 1 min. After several minutes when the recorder system was on, the light was turned on to trigger the algal photosynthetic activity, and the oxygen concentration started to increase. After several minutes, the light was turned off to observe the oxygen consumption (net respiration). The total photosynthetic oxygen generation rate was then calculated assuming that the rate of respiration in the light was the same as the respiration measured in the dark (see Appendix 1).

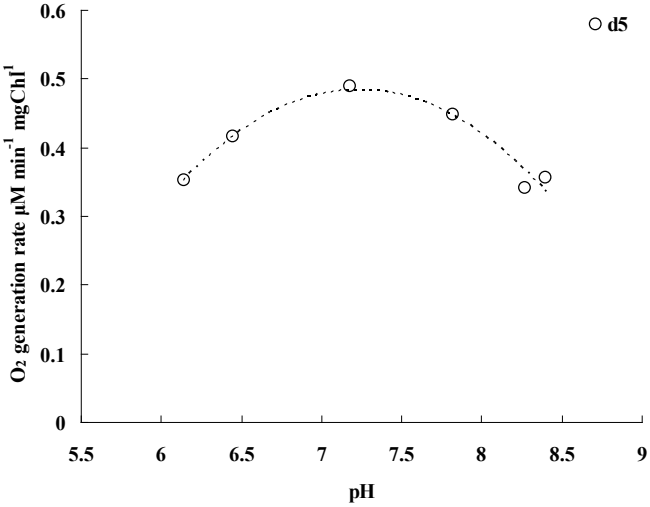

Figure 11: The photosynthetic O_2 yield rates under different pH conditions. The O_2 yield rates were measured on the samples taken at the 5th day of the culture (light-unlimited).

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 *D. salina* culture.

Effect of dissolved CO_2 concentration ($[CO_2]^*$) on *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 *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 $[CO_2]^*$), while a slight growth inhibition was observed when increasing the CO_2 dosing concentration up to 20% (0.008 mol L^{-1} of $[CO_2]^*$), 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 $[CO_2]^*$) strongly inhibited *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 *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

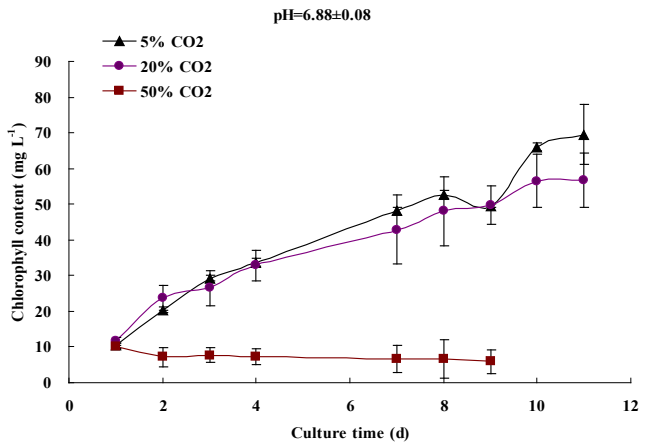

Figure 12: The plot of daily chlorophyll content against culture time for different CO_2 stream concentrations.

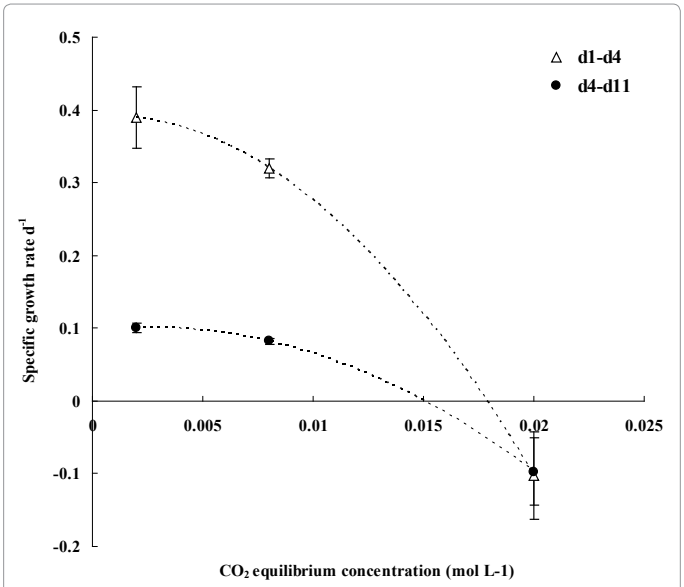

Figure 13: The specific growth rate of *D. salina* culture for different CO_2 stream concentrations. Under the 0.02 mol L^{-1} of dissolved CO_2 concentration, the specific growth rate was shown as negative, representing the decrease in algae concentration.

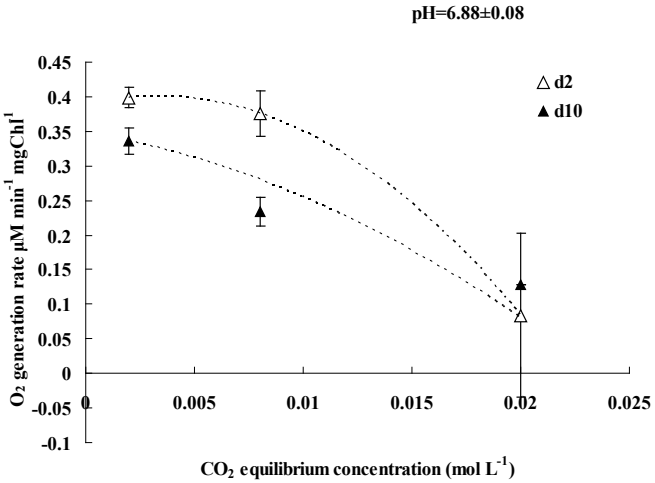

Figure 14: The photosynthetic O_2 yield rates under different dissolved CO_2 concentrations. The O_2 yield rates were measured on the samples taken at the 2nd day (light-unlimited) and at the 10th day (light-limited) of the culture.

d^{-1} by increasing the $[CO_2]^*$ from 0.002 mol L^{-1} to 0.008 mol L^{-1}, whilst the photosynthetic O_2 yield dropped from approximately 0.40 μmol min^{-1} $mgChl^{-1}$ to 0.38 μmol min^{-1} $mgChl^{-1}$. Under 0.02 mol L^{-1} $[CO_2]^*$, although the decrease in chlorophyll content and the negative value of specific growth rate indicated a strong inhibition in photosynthesis, a photosynthetic activity was still detected, showing the photosynthetic O_2 rate to be 0.08 μmol min^{-1} $mgChl^{-1}$. Due to the significant weakening of photosynthesis at this high CO_2 concentration, the photosynthetic activity (assimilation) is highly inhibited and exceeded by the respiration activity (dissimilation), negative growth is therefore observed. When the light become limiting (d4 – d11), the effect of different dissolved CO_2 concentrations on *D. salina* growth remains the same when increasing $[CO_2]^*$ from 0.002 mol L^{-1} to 0.008 mol L^{-1}. For 0.02 mol L^{-1} of $[CO_2]^*$, neither specific growth rate nor O_2 yield showed any obvious changes, because the growth is inhibited at the beginning of the culture, which did not lead to a light-limited situation.

To sum up, under the same extracellular pH, an increase in dissolved CO_2 concentration (i.e. the CO_2% in a constant dosing condition) resulted in an inhibition of photosynthesis for *D. salina* culture at 50% CO_2 in the dosing stream (or 0.02 mol L^{-1} of $[CO_2]^*$ in the culture), this level of CO_2 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 CO_2 equilibrium concentration, whilst higher extracellular equilibrium CO_2 concentration leads to a lower intracellular pH which may damage or inhibit the enzymes involved in photosynthesis.

Conclusions and Future work

A methodology was proposed to achieve a constant pH and variable dissolved CO_2, or a constant CO_2 level and variable pH for microalgal culture. An empirical equation correlating pH* to $NaHCO_3$ and CO_2% 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 CO_2 concentration on *Dunaliella salina* growth was then studied by using 'pH*-$NaHCO_3$-CO_2% system'. According to either specific growth rate or photosynthetic O_2 generation rate, pH around 6-9 was found to support *D. salina* culture. Both specific growth rate and photosynthetic O_2 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 CO_2 concentration on *D. salina* growth, both specific growth rate and photosynthetic O_2 generation rate decreased when the CO_2 concentration increased. Under 0.02 mol L^{-1} CO_2 concentration, a strong growth inhibition was observed. More than 0.02 mol L^{-1} of dissolved CO_2 (i.e. constant dosing of 50% CO_2) was fatal to *D. salina* growth.

Due to the lab limitations, only 3 different CO_2 stream concentrations were studied, an optimal dissolved CO_2 concentration was not determined for *D. salina* culture. More CO_2 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 CO_2 concentrations, and to find out the relationship between intracellular pH and extracellular pH.

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