CHEMOMETRICS APPROACH TO THE ANALYSIS OF A SYNERGISM OF TEMPERATURE, LIGHT INTENSITY AND CARBON DIOXIDE CONCENTRATION ON THE GROWTH OF *ANACYSTIS NIDULANS*

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The chemometrics approach has been applied to the analysis of the combined effects of temperature, light intensity and CO_2 concentration on the growth rate of the blue-green alga *Anacystis nidulans*. The strategy for the experiments is based on an extended factorial design. A statistically significant interaction is found between temperature and light intensity. Optimal conditions for maximal growth rate can be predicted by multiple regression analysis and its reliability has been confirmed by experiments. At the optimal growth condition the photosynthetic nature of *A. nidulans* was examined. The results suggest the importance of the utilization of these cells in the logarithmic growth phase for the purpose of CO_2 fixation.

Introduction

Public attention has been directed to the steadily increasing levels of CO_2 in the atmosphere. Among various proposals to solve this problem[1], CO_2 fixation by micro-algae appeared most promising to us in our preliminary survey of the literature. A search for algae species showing high CO_2 uptake activity and optimization of their culture environment are needed before actual use of this technique. A blue-green alga *Anacystis nidulans* which has long been known to have relatively high growth rate[13] is chosen for this investigation.

After the pioneering work of Kratz and Myers[13] on photosynthetic characteristics of *A. nidulans*, the physiology of the alga has been studied extensively. The works on the photosynthetic pigments of *A. nidulans* show that the ratio of phycocyanin to chlorophyll is affected by light intensity[9], light quality[16] and CO_2 concentration[5]. However, little attention has been given to the combined effect of such environmental factors on the photosynthetic activity of *A. nidulans*, since each factor has been treated individually in the previous works.

The objectives of this article are (1) to test the effects of combinations of temperature, light intensity and CO_2 concentration on the growth rates of *A. nidulans* using experimental planning by the chemometrics method[3,12], and (2) to investigate the kinetics of CO_2 fixation at the optimal growth condition.

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1. Materials and Methods

The blue-green alga *Anacystis nidulans* IAM M-6 was supplied from the Algal Culture Collection in the Institute of Applied Microbiology, University of Tokyo. The experimental apparatus is shown in **Fig. 1**. Growth experiments were performed in 500-ml cotton-stoppered oblong flat flasks (37 mm thickness) containing modified Detmer medium[20]. The flasks were immered in water baths thermostated to ± 0.1°C. The cultures were aerated by CO_2-enriched air or by plain air at 500 ml/min. One side of the flasks was illuminated continuously by parallel banks of fluorescent lamps mounted in the direction perpendicular to the flat surface of the flask. The light intensity on the medial surface of the vessel was measured and its intensity was regulated by varying the distance of the lamp from the vessel and the number of lamps. The cultures were inoculated with cells in a late logarithmic growth of the preculture which had been

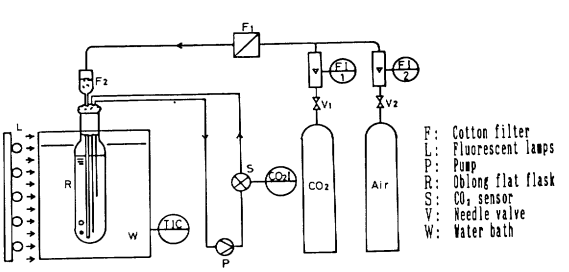

Fig. 1. Experimental apparatus

performed in a 50-ml flat flask at 30°C under a light intensity of 2 klux and the supply of plain air. Growth of a culture was followed by measuring optical density at 600 nm (1 unit of optical density at 600 nm = 0.31 g of dried cells per liter of culture).

The phycocyanin/chlorophyll ratio was determined from the absorption values of chlorophyll (680 nm) and phycocyanin (630 nm)[8]. The CO_2 concentration in the cultures was determined using a TOA Electronics IM-5S CO_2 electrode. By using this electrode, the k_La value for CO_2 in the cultures was determined at pH = 3.5. The details of the method are similar to those in the previous work[11].

2. Experimental Design

The strategy for the experimental planning is based on "factorial design"[3]. For the case of three experimental variables, two-level factorial design requires 2^3 experiments. Each variable is tested at only two values (high and low). To obtain more statistically meaningful information, the standard factorial design is expanded by adding three complemental experiments. Conditions for our experimental design are shown graphically in **Fig. 2**. Associated data are given in **Table 1**. The experiments numbered 1 to 8 are required for the basic two-level factorial design. They are performed under all possible combinations of minimum and maximum values of temperature (T = 30, 42°C), light intensity (I = 2, 8 klux) and mole fraction of CO_2 in the gas (y_{CO_2} = 0.5, 6.0%). Our preliminary experiment showed photo-bleaching of *A. nidulans* when the light intensity was more than about 10 klux. Run number 9 is the additional experiment, performed at the arithmetic means of minimum and maximum values of corresponding experimental variables. Runs number 10 and 11 are also additional experiments, located in the outer space of a cube. Four typical growth curves are shown in **Fig. 3**. Since no distinct difference is found in the final cell concentrations at different experimental conditions, we adopt the specific growth rate of the cell in logarithmic growth for evaluating the growth of *A. nidulans*. Experiments performed at each operating condition have been analyzed by multivariate data analysis to determine the individual and combined effects of the three factors on the growth rate of *A. nidulans*.

3. Results and Discussion

It is assumed that the specific growth rate μ (in terms of $\log_e$ units per day) is a function of experimental variables T, I, and y_{CO_2}:

$$\mu = f(T, I, y_{CO_2}) \tag{1}$$

Although the functional dependence of f is not clear in references, it is likely continuous with gradual

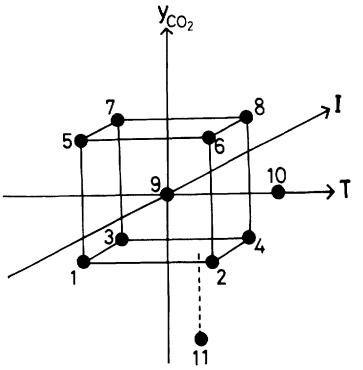

Fig. 2. Graphical presentation of the experimental design. The experimental conditions are shown in Table 1.

Table 1. Experimental conditions

Run no.	T [°C]	I [klux]	y_{CO_2} [%]
1	30	2.0	0.5
2	42	2.0	0.5
3	30	8.0	0.5
4	42	8.0	0.5
5	30	2.0	6.0
6	42	2.0	6.0
7	30	8.0	6.0
8	42	8.0	6.0
9	36	5.0	3.3
10	45	5.0	3.3
11	39	6.0	0.03

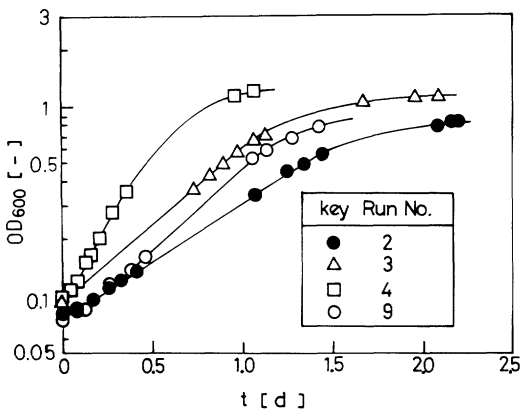

Fig. 3. Growth curves of *A. nidulans* at various experimental conditions. The conditions are shown in Table 1.

changes, provided that variations in the experimental variables are not too large. Considering these conditions, we analyze this function by a multiple regression technique according to the following equation:

$$\begin{aligned}\mu = {} & a_0 + a_1 T + a_2 I + a_3 y_{CO_2} + a_{11}(T)^2 \\ & + a_{22}(I)^2 + a_{33}(y_{CO_2})^2 + a_{12} TI \\ & + a_{13} T y_{CO_2} + a_{23} I y_{CO_2} + e\end{aligned} \tag{2}$$

The residual term, e, contains contributions from

higher-degree terms. The coefficients in the polynomial can be determined to fit a polynomial to available experimental results. All terms are not always significant and therefore their significance must be checked statistically. The equation relating the growth rate to light intensity presented by Tamiya *et al.*[19] and Steele[18] and that relating the growth rate to temperature presented by Koffler *et al.*[10] can be interpreted on the basis of Eq. (2). A comparison of the fitness of Eq. (2) to our experimental results by various combinations of the parameters are shown in **Table 2**. This analysis showed a fairly good fit for μ values with only three parameters, I, I^2 and $T*I$. We arrived at the following excellent correlation equation using seven descriptors:

$$\mu = 1.58T - 2.43I + 0.768y_{CO_2} - 0.0222T^2 + 0.190I^2 - 0.127y_{CO_2}^2 + 0.0230TI - 24.1 \quad (3)$$
$$(n=11,\ r=0.997,\ s=0.146,\ F=77.4)$$

Figure 4 shows the relationship between observed and calculated μ values using Eq. (3). The dependence of the μ curves as a function of light intensity upon temperature[15] appears in Eq. (3). Such synergism between temperature and light intensity on the growth rate is supported by the previous work by Bader on *A. nidulans*.[2] He suggested that the photosynthetic rate at low light intensity was expressible by a linear function of both the pigment content and light intensity. As shown elsewhere the pigment content is affected by temperature. Hence it turns out that the suboptimal temperature for growth changes with the increase of light intensity.

The ninth and tenth terms on the right-hand side of Eq. (2) were disregarded due to lack of statistical significance. Therefore, it appears that there is no significant interaction between temperature and CO_2 concentration (T, y_{CO_2}) and light intensity and CO_2 concentration (I, y_{CO_2}), within the range studied in these experiments. The relative importance of the terms in Eq. (3) was in the following order from F test: $I > I^2 > T^2 > T*I > T > y_{CO_2}^2 > y_{CO_2}$.

Contour projections at various levels of T, I and y_{CO_2} on various μ values, computed and plotted by computer, are shown in **Fig. 5**. Visual interpretation of the projections indicates that high growth rates are to be expected under the following conditions: increasing light intensity and temperature, and moderate CO_2 concentrations in the inlet gas. It should be noted that Eq. (3) is an experimental result based on the minimum number of tests to obtain information on the combined effect of environmental factors. Therefore, more extensive studies will be required for investigating the kinetics of the photosynthesis of *A. nidulans* in detail.

Table 2. Comparison of the correlation for Eq. (2) based on various combinations of descriptors

Descriptors	r	s
T, I, y_{CO_2}, T^2, I^2, $y_{CO_2}^2$, $T*I$, $T*y_{CO_2}$, $I*y_{CO_2}$	0.999	0.059
T, I, y_{CO_2}, T^2, I^2, $y_{CO_2}^2$, $T*I$, $T*y_{CO_2}$	0.999	0.044
T, I, y_{CO_2}, T^2, I^2, $y_{CO_2}^2$, $T*I$	0.997	0.146
T, I, T^2, I^2, $y_{CO_2}^2$, $T*I$	0.965	0.440
T, I, T^2, I^2, $T*I$	0.961	0.419
I, T^2, I^2, $T*I$	0.922	0.534
I, I^2, $T*I$	0.914	0.522

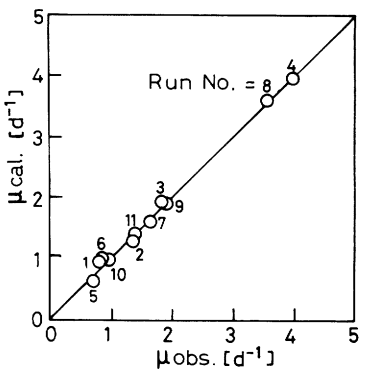

Fig. 4. Comparison of observed and calculated μ values according to Eq. (3)

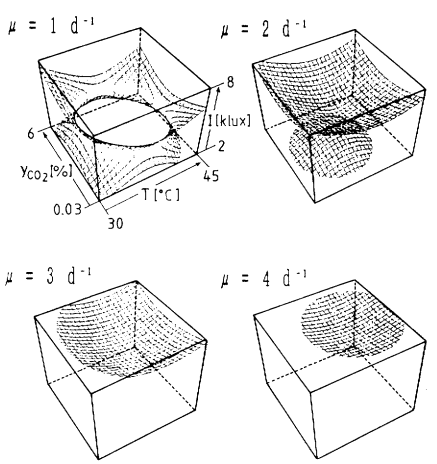

Fig. 5. Contour projections for various μ values obtained from Eq. (3)

Supplemental experiments were performed at two conditions, where (T, I, y_{CO_2}) were set at (32°C, 6.0 klux, 5.0%) and (42°C, 2.0 klux, 0.03%). All three variables were extremely different but show nearly equivalent μ values in Eq. (3). The results of these tests are shown in **Table 3**. Good agreement between experimental and calculated μ values was confirmed. In the range of our experimental conditions the optimum operating condition is found to be (40°C, 8.0 klux, 3.0%) from Eq. (3). A further growth experiment was performed at the optimum condition.

Table 3. Evaluation of Eq. (3) with additional experiments

Experimental conditions			μ [d^{-1}]	
T [°C]	I [klux]	y_{CO_2} [%]	Eq. (3)	Obsd.
32	6	5.0	1.11	1.24
42	2	0.03	1.01	0.97
40	8	3.0	4.87	4.68

The growth curve for this experiment yielded the highest μ value: 4.68 d^{-1}.

Although Kratz and Myers[13] observed a higher growth rate (μ=8.17 d^{-1}) of *A. nidulans* at 41°C and light-saturating illumination in 0.5 vol% CO_2-enriched air, their results should not be compared to ours because of their use of another carbon source, sodium citrate, in the medium in addition to CO_2. Research on the growth of *A. nidulans* in a medium containing no carbon source other than CO_2 is unexpectedly scarce in previous works. One such work by Pope shows the effects of various combinations of light intensity, oxygen concentration, and CO_2 concentration on growth of *A. nidulans* at 25°C using modified Detmer medium[17]. The data shown in his work range in μ value from 1.85 to 3.44 d^{-1}. Our μ value is higher than his value by a factor of 1.4, showing the possibility of increase in CO_2 fixation rate by proper choice of culture environments.

The photosynthetic activity of *A. nidulans* at the optimum growth condition is also examined. **Figure 6(A)** shows the growth curve and the variation of the phycocyanin/chlorophyll absorption ratio. The conversion efficiency of light energy into chemical energy is closely related to the pigment content.[7] The phycocyanin/chlorophyll absorption ratio was constant between 1.03 and 1.08 during the growth. Reported values by Goedheer and Kleinen Hammans[8] are between 1.30 and 1.76 in 5 vol% CO_2 enriched air and between 0.63 and 1.00 in plain air at 39°C and high light intensities. Our result is in the middle of their reported values.

Figure 6(B) shows the variation of pH and dissolved CO_2 in the medium. The pH value changed rapidly to 6.4–6.5, and after a while it went on increasing gradually. Since the medium used in this study contains K_2HPO_4, much CO_2 is dissolved in the medium not only as molecular CO_2 but also as HCO_3^-, more than predicted CO_2 from Henry's law constant. The concentrations of H_2CO_3 and CO_3^{2-} are negligibly small at this experimental condition. The amount of CO_2 dissolved is affected by the pH[6]. The dissolved free CO_2 during cultivation in **Fig. 6(B)** was determined from the total CO_2 ($CO_2+HCO_3^-$) concentration ($C^T_{CO_2}$) of the sample solution taken from the reactor. Before measurement of the total CO_2, the pH of the sample was adjusted to 3.5.

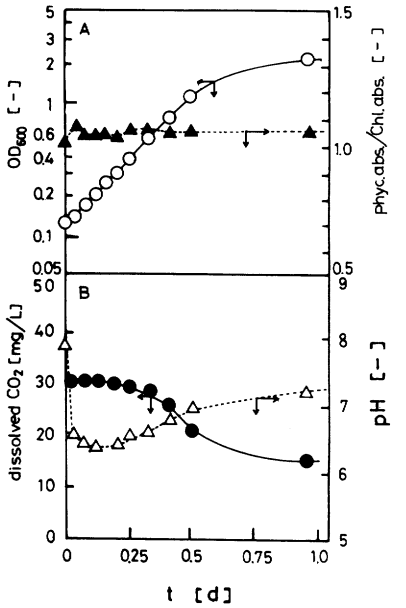

Fig. 6. Time courses of OD_{600} of *A. nidulans*, the phycocyanin/chlorophyll absorption ratio, and the variation of pH and dissolved CO_2 concentration. This test was performed at 40°C, aeration with 3 vol% CO_2-enriched air and light intensity of 8 klux.

When the volumetric carbon dioxide transfer coefficient k_La' is determined, the carbon dioxide balance can be expressed as Eqs. (6) and (7) by considering the following reactions:

$$CO_2+H_2O \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HCO_3^- + H^+ \tag{4}$$

$$CO_2+K_2HPO_4+H_2O \underset{k_4}{\overset{k_3}{\rightleftharpoons}} K_2H_2PO_4+K^++HCO_3^- \tag{5}$$

$$\frac{dC_{CO_2}}{dt} = k_La'(C^*_{CO_2}-C_{CO_2}) - k_1C_{CO_2}+k_2[HCO_3^-][H^+] + k_4[K_2H_2PO_4][K^+][HCO_3^-] - k_3C_{CO_2}[K_2HPO_4]-q_{CO_2}X \tag{6}$$

$$\frac{d[HCO_3^-]}{dt} = k_1C_{CO_2}-k_2[HCO_3^-][H^+] + k_3C_{CO_2}[K_2HPO_4] - k_4[K_2H_2PO_4][K^+][HCO_3^-] \tag{7}$$

where C_{CO_2} represents the mass of dissolved CO_2 in a unit volume of culture, $C^*_{CO_2}$ the mass of dissolved CO_2 at saturation, q_{CO_2} the specific fixation rate of CO_2, and X the cell mass concentration.

By combining Eqs. (6) and (7) we obtain the following equation:

$$\frac{dC^T_{CO_2}}{dt} = k_La'(C^*_{CO_2}-C_{CO_2})-q_{CO_2}X \tag{8}$$

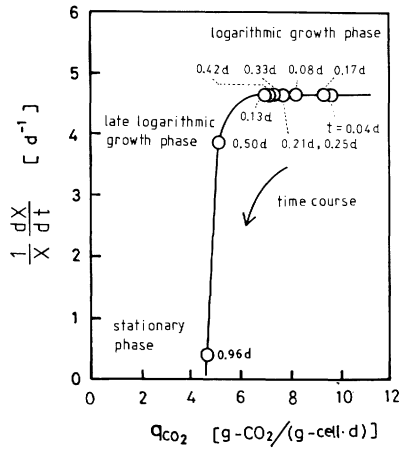

Fig. 7. Time course of the $(dX/dt)/X$ values against q_{CO_2} values in the run at optimal experimental condition

Our data showed that $dC^T_{CO_2}/dt \ll k_La'(C^*_{CO_2}-C_{CO_2})$, yielding

$$q_{CO_2} \fallingdotseq \frac{k_La'(C^*_{CO_2}-C_{CO_2})}{X} \quad (9)$$

Then the specific fixation rate of CO_2 is calculated based on Eq. (9). The experimentally observed $C^*_{CO_2}$ value was 32 mg/L. The k_La' value was measured for two solutions: fresh medium containing no cells and the final algal suspension. Since both values were nearly constant ($217\,d^{-1}$ for fresh medium and $194\,d^{-1}$ for the final algal suspension), we employed the mean value $206\,d^{-1}$ for Eq. (9).

Figure 7 shows a plot of $(dX/dt)/X$ against the q_{CO_2} value. The q_{CO_2} value was in the range 6.8 to 9.5 g-CO_2/(g-cell·d) in the logarithmic growth phase, whereas it decreased with time toward 4.5 g-CO_2/(g-cell·d) in the stationary phase.

If we assume that the chlorophyll content of *A. nidulans* is 2 wt% of the dry cell weight[2], the observed value of q_{CO_2} at 25°C by Lloyd *et al.*[14] for *A. nidulans* corresponds to 4.1 g-CO_2/(g-cell·d) and that at 28°C by Birmingham *et al.*[4] to 1.4 g-CO_2/(g-cell·d). Myers and Kratz[15] determined the specific rate of production of O_2 at 25 and 39°C. Their data correspond to the q_{CO_2} value in the range 4.5 to 14.5 g-CO_2/(g-cell·d). Hence the value of q_{CO_2} calculated here is comparable to the results of Myers and Kratz[15]. Our results reveal the importance of utilizing cells in the logarithmic growth phase for the purpose of CO_2 fixation.

Conclusion

Attempts were made to obtain basic knowledge about microbial CO_2 fixation using *A. nidulans* at a test organism. The results are as follows.

(1) The effects of various combinations of temperature, light intensity, and CO_2 concentration on photosynthetic growth in *A. nidulans* were studied, based on an extended factorial design statistical analysis. The results suggest that synergism between temperature and light intensity is significant. For the available experimental data the specific rate of logarithmic growth was successfully represented by a polynomial equation using operating variables.

(2) Optimal growth condition ($T=40$°C, $I=8.0$ klux, $y_{CO_2}=3.0$%) for maximal growth rate can be predicted based on this equation and it was experimentally confirmed (observed $\mu=4.68\,d^{-1}$).

(3) The photosynthetic characteristics of *A. nidulans* at the optimal growth condition showed that the specific fixation rate of CO_2 was relatively high in the logarithmic growth phase.

Nomenclature

C_{CO_2}	=	dissolved CO_2 concentration in the culture	[mg/L]
$C^*_{CO_2}$	=	dissolved CO_2 concentration at saturation	[mg/L]
$C^T_{CO_2}$	=	total CO_2 concentration in the culture	[mg/L]
F	=	F-value for multiple regression analysis	[—]
I	=	light intensity	[klux]
k_La'	=	coefficient of volumetric mass transfer of CO_2 into the culture	[d^{-1}]
n	=	number of data points in set	[—]
q_{CO_2}	=	specific fixation rate of CO_2	[g-CO_2/(g-cell·d)]
r	=	multiple correlation coefficient	[—]
s	=	standard deviation	[d^{-1}]
T	=	culture temperature	[°C]
t	=	time	[d]
X	=	cell mass concentration	[g/L]
y_{CO_2}	=	mole fraction of CO_2 in the gas	[%]
μ	=	specific growth rate	[d^{-1}]

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