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High Cell Density Culture of *Anabaena variabilis* with Controlled Light Intensity and Nutrient Supply

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Controlling the light energy and major nutrients is important for high cell density culture of cyanobacterial cells. The growth phase of *Anabaena variabilis* can be divided into an exponential growth phase and a deceleration phase. In this study, the cell growth in the deceleration phase showed a linear growth pattern. Both the period of the exponential growth phase and the average cell growth rate in the deceleration phase increased by controlling the light intensity. To control the light intensity, the specific irradiation rate was maintained above 10 μmol/s/g dry cell by increasing the incident light intensity stepwise. The final cell density increased by controlling the nutrient supply. For the control of the nutrient supply, nitrate, phosphate, and sulfate were intermittently added based on the growth yield, along with the combined control of light intensity and nutrient concentration. Under these control conditions, both final cell concentration and cell productivity increased, to 8.2 g/l and 1.9 g/l/day, respectively.

Keywords: Cyanobacteria, high cell density culture, specific irradiation rate, nutrient supply, growth yield

Cyanobacteria are O_2-evolving photosynthesizing prokaryotes that have an extensive history of use as a human food source and as a fertilizer in rice fields [18, 26]. Cyanobacteria have great potential as a health food [1, 16], a natural colorant for food and beverages [28], a biofertilizer [11], a source of a fluorescent tag [7], a source of exopolysaccharides [27], and a source of renewable energy [25, 32, 35, 36].

To produce such products efficiently, the development of a photobioreactor and a mass cultivation technique was investigated [17, 19, 20, 30]. Both light energy and nutrients are important factors in culturing cells at high densities [13, 34]. When cells grow at high density, the distance of light penetration is reduced. Therefore, a high incident light intensity is necessary. However, the exposure of cells to excessive light or a surplus of nutrients often leads to a decline in cell growth. Therefore, controlled supplies of light energy and nutrients are necessary. The suggested parameters for the control of light are as follows: photosynthesis rate [6], average cell growth rate [10], specific light uptake rate [3, 24], and average light intensity [31]. Whereas the photosynthesis rate, the average cell growth rate, and the average light intensity are good parameters at low cell density, the specific light uptake rate is more appropriate for high cell density cultures.

In addition to the light energy, the nutrient supply is an important factor [14]. For this purpose, nitrogen or carbon sources have been added intermittently or continuously [2, 5, 29], or culture medium was exchanged with fresh medium [13]. However, few investigations have reported on the provision of major nutrients [4] and the control of their concentrations in the medium. In this study, in addition to the control of light energy mentioned above, major nutrients (nitrogen, sulfur, and phosphorus) were supplied to the culture in an intermittent manner based on growth yield in order to control their concentrations. The objectives of this study were to investigate the combined effect of controlled light and nutrient supply on the high cell density culture of *Anabaena variabilis* and also to obtain the optimum combined condition of light and nutrients for high cell density culture.

MATERIALS AND METHODS

Microorganism and Maintenance

Anabaena variabilis (ATCC 29413) in an Erlenmeyer flask (500 cm^3) containing 100 cm^3 of BG11 medium was cultured in a shaking incubator at 30°C and 190 rpm [37].

Cultivation Equipment and Conditions

Batch and fed-batch cultures were carried out in a photobioreactor. The photobioreactor consisted of five parts: a bubble column, fluorescent lamps, a temperature-controlled water bath, a gas recycle

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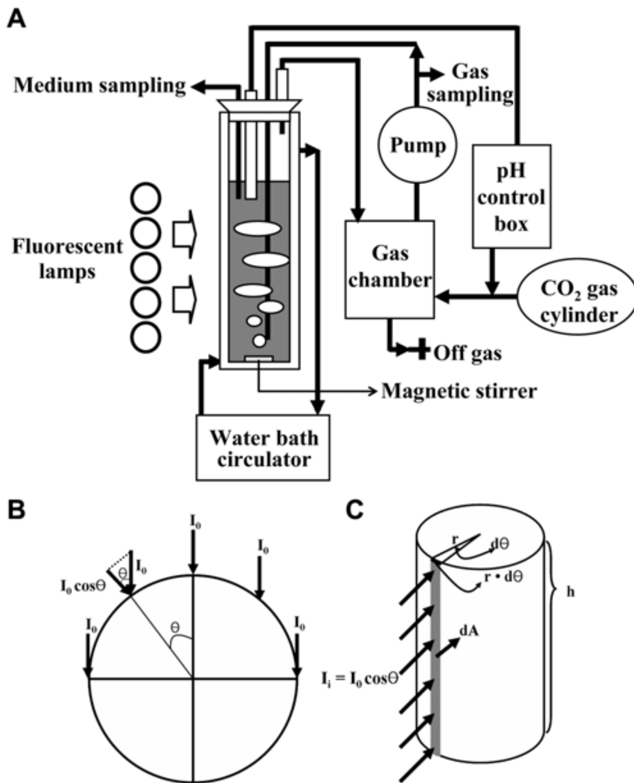

Fig. 1. Schematic diagram of the photobioreactor.
A. Photobioreactor system. **B**. Incident light on the surface of the bubble column (top view). **C**. Illuminated area on the surface of the bubble column (side view).

unit, and a pH controller (Fig. 1A). The bubble column (working volume 260 cm^3) was composed of Pyrex glass. Its inner diameter was 2.6 cm and its height was 63 cm. The temperature was maintained at 30°C using the temperature-controlled water bath (VS-1205CW; Vision Corp., Republic of Korea), which circulated thermostated water through a water-jacket that surrounded the bubble column.

Ten fluorescent lamps were placed on one side of the photobioreactor, and the supplied illumination was in the range of 100−110 to 1,530−1,700 μmol/m^2/s. The light intensity was adjusted to various levels by changing either the distance between the fluorescent lamps and the bubble column or the number of fluorescent lamps used. The other three sides were surrounded by mirrors.

The gas recycle unit consisted of a peristaltic pump, a gas chamber, and Tygon food-grade tubing (Cole-Parmer Instrument Company, U.S.A.). The gas chamber (1,200 cm^3) was used to mix air and CO_2. The cultures were aerated through a sparger consisting of a straight stainless-steel tube (3 mm diameter) that was positioned above the bottom of the photobioreactor, and they were stirred by a magnetic stirrer on the bottom of the photobioreactor. Bubbles in this sparger formed slug flow. The superficial gas velocity was 2.0 cm/s.

The pH was controlled at 7.0 with CO_2 using the pH controller (KF-L; KoBioTech Corp., Republic of Korea). When CO_2 was injected into the gas chamber, it was sterilized by a bacterial gas filter with a pore size of 0.2 μm (Minisart SRP-25, Sartorius, U.S.A.). The culture medium was BG11 medium with 100 mg/l K_2HPO_4.

Determination of Specific Irradiation Rate

When photobioreactors are illuminated in one direction, such as toward sunlight, it is difficult to calculate the specific light uptake rate because of the outgoing light energy from the outer surface column. A modified parameter for the controlled incident light, a specific irradiation rate (q_i), was defined as follows [3, 23]:

$$q_i = \frac{I_i \times A}{C \times V} \quad (1)$$

where I_i=intensity of the light illuminated at a right angle on the surface area, μmol/s/m^2; A=illuminated surface area of the bubble column, m^2; C=cell concentration, g/l; and V=volume of medium, m^3.

Light intensity illuminated at a right angle on the surface area, I_i, is

$$I_i = I_0 \cos\theta \quad (2)$$

where I_0 is the incident light intensity on the surface of the bubble column (μmol/s/m^2) and θ is the angle of incidence (radian) (Fig. 1B). The illuminated surface area of the bubble column, dA, is

$$dA = h \bullet r \bullet d\theta \quad (3)$$

where h is the height of the bubble column (m), r is the radius of the bubble column (m), and dθ is the angle of arc of dA (radian) (Fig. 1C). Therefore, combining Eq. (2) and Eq. (3) with Eq. (1) gives

$$q_i = \frac{2\int_0^{\pi/2} I_i \times dA}{C \times V} = \frac{2\int_0^{\pi/2}(I_0 \cdot \cos\theta)(h \cdot r \cdot d\theta)}{C \cdot V}$$
$$= \frac{2I_0 \cdot h \cdot r \cdot \int_0^{\pi/2}\cos\theta\, d\theta}{C \cdot V} = \frac{2I_0 \cdot h \cdot r}{C \cdot V} \quad (4)$$

Determination of Cell Concentration

The cell concentration was measured by optical density at 680 nm, and was plotted versus dry weight (g/l) on a standard curve [36].

Measurement of Light Intensity

The light intensity was measured using a quantum sensor (LI-COR, Model LI-190SA; Lambda Instrument Corp., U.S.A.) and a light meter (LI-COR, Model LI-250; Lambda Instrument Corp., U.S.A.).

Analysis of Nitrate, Sulfate, and Phosphate Concentrations

Nitrate, sulfate, and phosphate were analyzed using a Dionex ion-chromatography system (Dionex, DX-120) equipped with a conductivity detector, an AS40 automated sampler, a 0.5 cm^3 injection loop, and an IonPac AS14A anionic column. The detector, the automated sampler, the injection loop, and the anionic column were supplied by Dionex Coporation (U.S.A.).

RESULTS AND DISCUSSION

Control of Light and Nutrients

Control of the light energy and major nutrients is necessary for high cell density cultures. The cell growth curve drawn in logarithmic scale (Fig. 2B) shows that the growth pattern consists of an exponential growth phase for 2 days and a subsequent deceleration phase. Since nitrate, phosphate, and sulfate were not depleted during the first 2 days in the

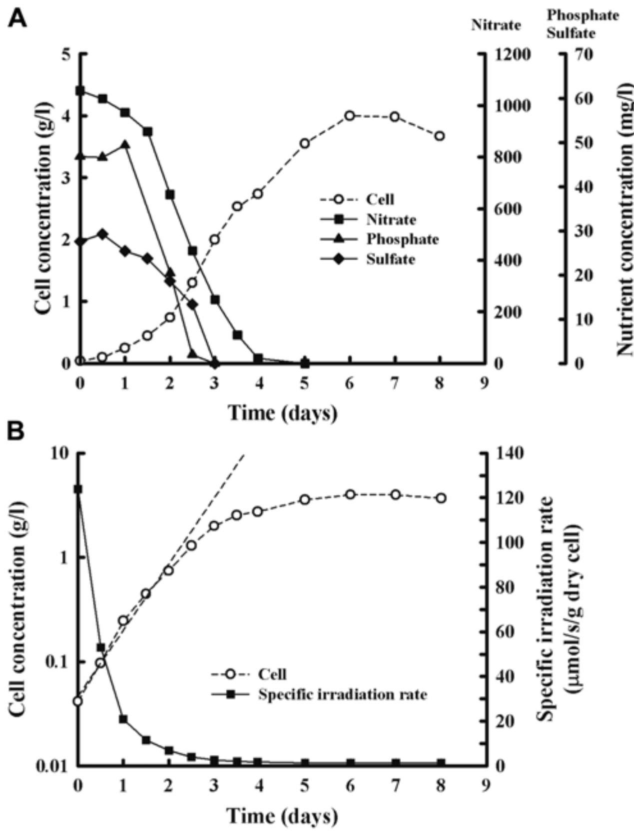

Fig. 2. **A**. Profiles of N, S, and P sources in the batch culture. **B**. Profile of the specific irradiation rate. The dashed line is the regression line between cell concentration and time in the exponential growth phase.

exponential growth phase (Fig. 2A), we assumed that the light limited the cell growth. The light intensity was increased at 1.5 days before the end of the exponential growth phase. Since the specific irradiation rate (q_i) was 11.5 μmol/s/g dry cell at 1.5 days (Fig. 2B), we attempted to maintain the specific irradiation rate above that value by increasing the incident light intensity in a stepwise manner (Figs. 3B and 3C). In Fig. 3, "L" denotes the number of stepwise increases in light intensity, and "M" denotes the number of additions of nutrient to the medium. For example, L2M1 means that the light intensity was increased twice and the nutrient was added once. By increasing the light intensity from L0M0 to L2M0, the period of the exponential growth phase increased from 2 days to 2.5 days, and the average cell growth rate (a) in the deceleration phase also increased from 0.81 g/l/day to 1.48 g/l/day as shown in Fig. 3A.

However, the final cell concentration of L2M0 was slightly lower than that of L0M0. To obtain a high cell concentration, the nutrient was added intermittently to the medium. Since phosphate is depleted more rapidly than nitrate or sulfate (Fig. 2A), nutrients should be added to the

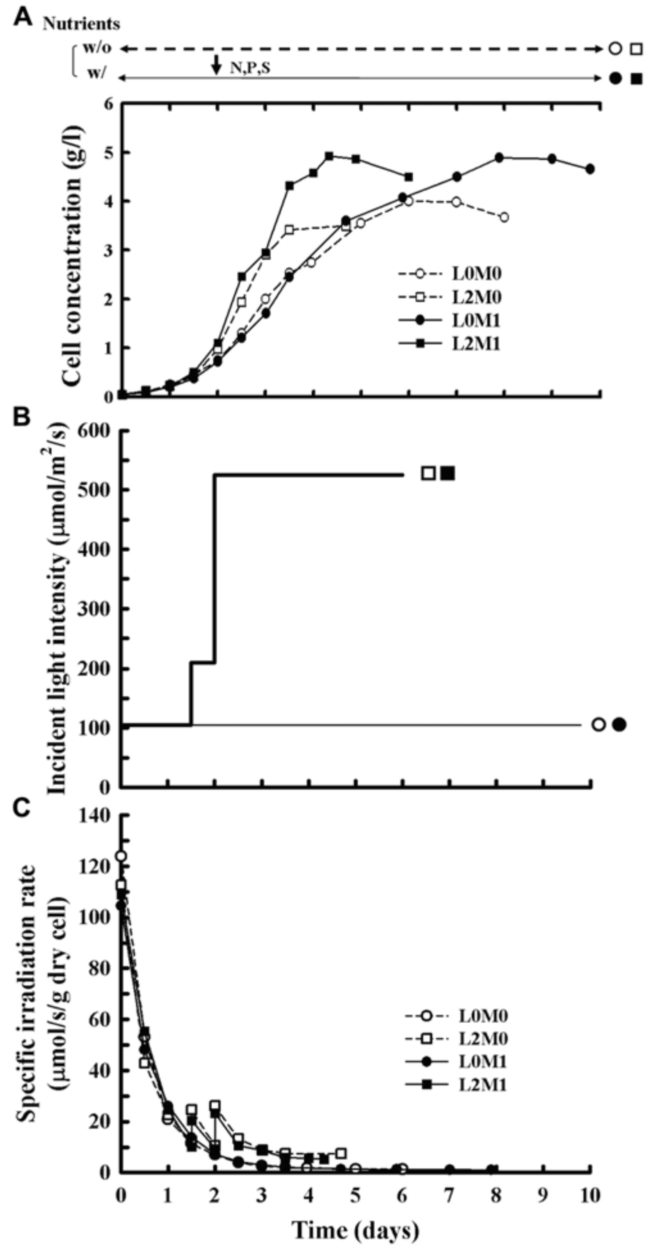

Fig. 3. Effect of controlled light intensity and nutrient supply on cell growth. In L0M0, L0M1, L2M0, and L2M1, "L" denotes the number of light intensity increases and "M" denotes the number of nutrient additions to the medium. For example, L2M1 means that light intensity was increased twice and nutrient was added once.
A. Cell growth profile. The downward arrow in the diagram indicates the timing of nutrient addition. **B**. Profile of incident light. **C**. Profile of specific irradiation rate.

medium before the phosphate deficiency occurs. A nutrient solution containing nitrate, phosphate, and sulfate was added at 2 days (Fig. 3A). The amounts of nutrients in the solution (2 cm^3) were 124.7 mg $NaNO_3$, 11.92 mg K_2HPO_4, and 7.334 mg $MgSO_4 \cdot 7H_2O$. The quantity of phosphate

added was 30% smaller than the amount consumed because a high concentration of phosphate was found to inhibit cell growth. Compared with the case without the addition of nutrient (L0M0 in Fig. 3A), the final cell concentration increased with the addition of nutrient from 4.0 g/l to 4.9 g/l (L0M1 in Fig. 3A).

When the specific irradiation rate (q_i) was controlled and three nutrients were also added simultaneously (L2M1), the specific growth rate in the exponential growth phase increased from 1.47 day^{-1} to 1.60 day^{-1}, compared with L2M0. The average cell growth rate (α) in the deceleration phase was comparable to that seen for L2M0, and the cell concentration increased from 3.5 g/l in 4.7 days to 4.9 g/l in 4.3 days (L2M0 and L2M1 in Fig. 3A). These results show that the high light intensity increases the growth rate, whereas the addition of nutrients increases the final cell concentration.

High Cell Density Culture in Controlled Light and Nutrient Supply Conditions

To achieve a higher cell concentration, more nutrients were added intermittently, as shown in Fig. 4A. The amounts of the nutrients added were the same as those consumed during cell growth, and were calculated using the growth yield ($Y_{x/s}$) of each nutrient. When the specific irradiation rate (q_i) was controlled above 10 μmol/s/g dry cell by increasing the incident light intensity in a stepwise manner during cell growth (Figs. 4B and 4C), the growth profiles in the exponential growth phase were found to be similar, although cells grew more rapidly in the deceleration phase (Fig. 4A). As a result, the final cell density reached a level higher than 8 g/l. The final cell concentration and cell productivity were similar in L6M4 and L6M5, which indicated that four additions of nutrients are sufficient. With the four nutrient additions and the specific irradiation rate (q_i) controlled above 10 μmol/s/g dry cell (L6M4), the final cell concentration and the cell productivity increased from 4.9 g/l to 8.2 g/l and from 1.1 g/l/day to 1.9 g/l/day, respectively, compared with L2M1. Even in the high cell density culture such as L5M3, L6M4, and L6M5, there was no decrease in cell quality in the growth phase and at least until the early stationary phase.

The distance of light penetration is short in high cell density cultures, and light intensity is not homogenous along the radial direction in the bubble column. For this reason, light is the most important and limiting factor that affects cell growth at high cell concentrations. The exponential growth phase ended when cell density reached approximately 2 g/l at 2.5 days (Fig. 5A). Since the concentrations of nitrate, phosphate, and sulfate were not limited until this time (Figs. 5B, 5C, and 5D), it can be assumed that cell growth is limited by available light. Further investigation is required to increase the period of exponential growth. We need to understand the relationship between high cell

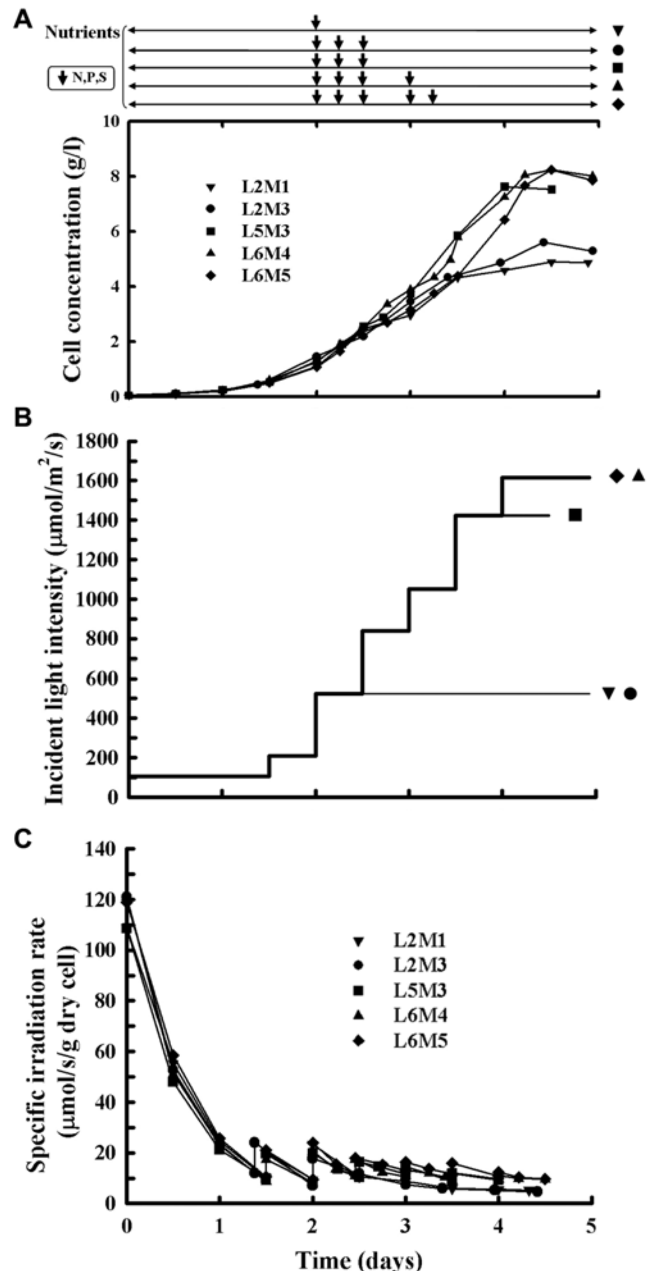

Fig. 4. Combined effect of controlled light intensity and nutrient supply on cell growth.
A. Cell growth profile. Downward arrows in the diagram indicate nutrient addition. **B**. Profile of incident light. **C**. Profile of the specific irradiation rate.

density and the mutual shading effect. It will be useful to develop a mutant strain that can grow exponentially, even in the condition of excessive light.

Fig. 5 shows that the concentration of each nutrient was maintained above a certain level between 2 days and 3 days owing to the addition of nutrients based on the growth yield. Although a phosphate deficiency began at 3.25 days, cells continued to grow until 4.25 days. This result is in

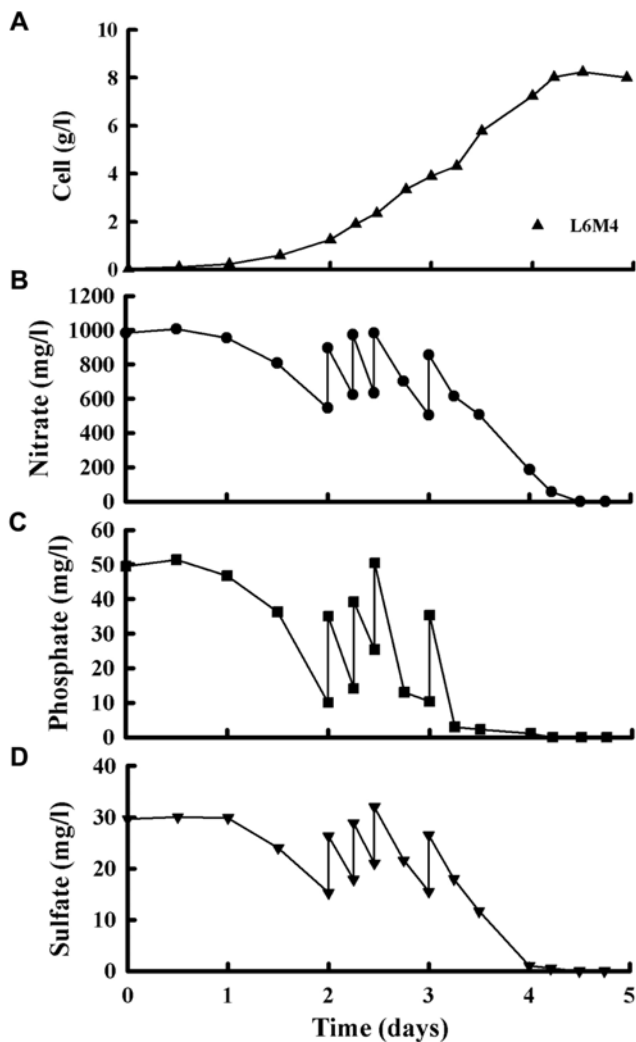

Fig. 5. Profiles of N, S, and P sources of L6M4.
Cell growth profile (**A**), and profiles of nitrate (**B**), phosphate (**C**), and sulfate (**D**).

agreement with the previous study, which showed that, prior to phosphate deficiency, cells accumulated sufficient polyphosphate to support several generations in the absence of external phosphorus [9, 15]. However, we needed to confirm whether the phosphate deficiency after 3.25 days affected cell growth during this period. Therefore, we examined the effect of a controlled phosphate supply on cell growth after 3.25 days.

Effect of Controlled Phosphate Supply in the Deceleration Phase

To confirm whether the phosphate deficiency affected cell growth in the deceleration phase, phosphate was supplied to the culture by intermittent additions based on growth yield (Fig. 6C). The specific irradiation rate (q_i) was maintained above 10 µmol/s/g dry cell during cell growth by increasing the incident light intensity in a stepwise manner as done in L6M4. The nitrate, phosphate, and sulfate concentrations were individually controlled by intermittent additions based on growth yield (Figs. 6B, 6C, and 6D). Compared with L6M4, more additions of phosphate considerably inhibited (L6M8) or delayed cell growth (L6M14), as shown in Fig. 6A. The average cell growth rates (α) in the deceleration phases of L6M8 and L6M14 were even lower than that of L6M4. This result suggests that a phosphate deficiency in the deceleration phase is important for high cell density cultures.

When plants and algal cells were exposed to irradiation at levels higher than they experienced during their normal growth, photoinhibition occurred in photosystem II [33]. The growth of green algae was also inhibited by the photoinhibition of photosystem II, even at low light intensity in phosphate-deficient conditions [8, 12, 21]. At high light intensity, however, increased photosystem II activity of phosphate-deficient cells was demonstrated in experiments

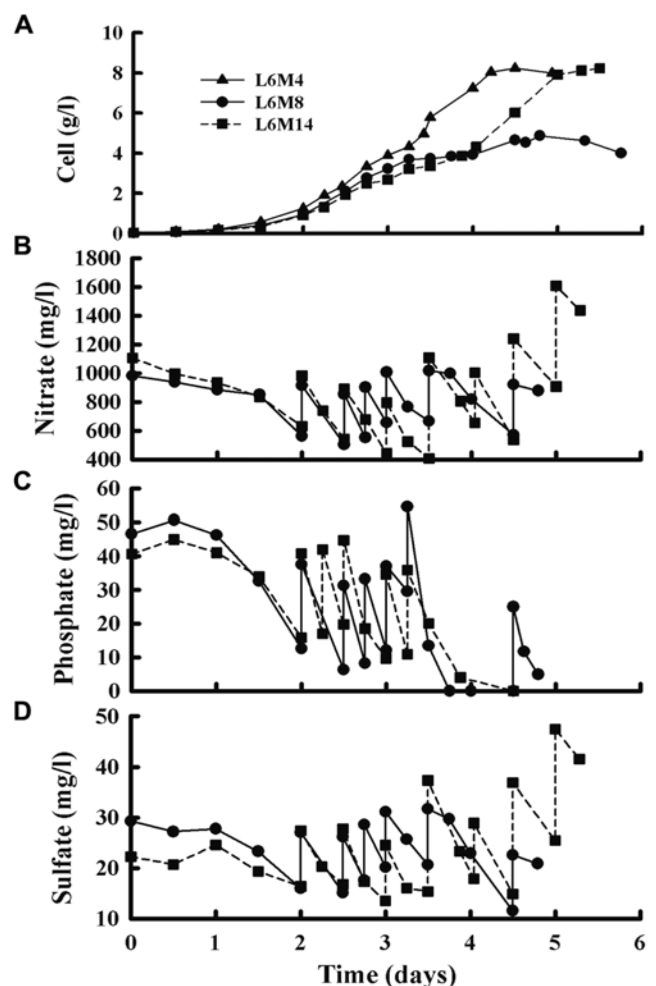

Fig. 6. Effect of controlled phosphate supply on cell growth in the nonexponential growth phase.
Cell growth profile (**A**), and profiles of nitrate (**B**), phosphate (**C**), and sulfate (**D**).

Table 1. Comparison of maximum cell density and cell productivity using different light control parameters.

Parameter for light control	Microorganism	Initial cell density (g/l)	Maximum cell density (g/l)	Cell productivity (g/l/day)	Ref.
Average cell growth rate	*Marchantia paleacea*	0.2	5.1	0.38	[10]
Specific light uptake rate	*Haematococcus pluvialis*	0.13[a,b]	8[a]	0.75	[3, 24]
Average light intensity	*Synechococcus* PCC 6301	0.2[b]	2.92	0.17	[31]
Specific irradiation rate	*Anabaena variabilis*	0.04	8.2	1.91	This study

[a]In this datum, cell density unit is "g fresh cell/l".
[b]Data calculated from the graph [3, 24, 31].

with green algae, sunflowers, and maize leaves [12, 22]. These results showed that photoinhibition of phosphate-deficient cells could be relieved by supplying a high light intensity. Whereas previous experiments have shown that the growth of phosphate-deficient cells was inhibited at low light intensity, our investigation showed that, at high light intensity (above 1,000 μmol/s/m^2), phosphate-deficient cells had a higher growth rate than phosphate-sufficient cells in the deceleration phase.

Table 1 shows that our strategy of controlling light and nutrients improved the maximum cell density and cell productivity, compared with other results reported in the literatures. Using our strategy, although the initial cell density was 3–5 times lower than that in other studies, the maximum cell density was achieved in a very short cultivation period, which was 2–3.6 times shorter than that in other studies. Table 2 compares the performances at various light intensities and nutrient supplies. The final cell concentration and cell productivity increased with the controlled provision of light and nutrients. The maximum final cell concentration and cell productivity were obtained when the nutrients were added four times and the specific irradiation rate (q_i) was maintained above 10 μmol/s/g dry cell.

Rapid growth and high cell concentration were obtained under controlled light intensity and nutrient supply conditions. For the control of light intensity, the specific irradiation rate (q_i) was maintained above 10 μmol/s/g dry cell by increasing the incident light intensity in a stepwise manner. For the control of the nutrient supply, nitrate, phosphate, and sulfate were intermittently added based on the growth yield. The controlled light supply increased the period of the exponential growth phase and the average cell growth rate (α) in the deceleration phase, while the controlled nutrient supply increased the final cell density. More than four additions of phosphate inhibited cell growth, and the phosphate deficiency in the deceleration phase was quite beneficial in terms of the high cell growth rate. When the nutrients were added four times and the specific irradiation rate (q_i) was maintained above 10 μmol/s/g dry cell during cell growth, the final cell concentration and the cell productivity were 8.2 g/l and 1.9 g/l/day, respectively.

Acknowledgment

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Table 2. Combined effects of controlled light and nutrient supply on cell growth.

Number of increases of light intensity	Number of nutrient additions			μ^b (day^{-1})	α^c (g/l/day)	Final cell concentration (g/l)	Cell productivity (g/l/day)
	N, P, S[a]	N, S[a]	P[a]				
0	0	0	0	1.46	0.81	4.0	0.67
0	1	0	0	1.32	0.71	4.9	0.62
2	0	0	0	1.47	1.48	3.5	0.98
2	1	0	0	1.60	1.42	4.9	1.14
2	3	0	0	1.63	1.70	5.6	1.27
5	3	0	0	1.61	3.52	7.6	1.91
6	4	0	0	1.66	3.22	8.2	1.91
6	5	0	0	1.64	3.26	8.2	1.83
6	5	1	2	1.60	1.07	4.9	1.02
6	3	7	4	1.56	2.21	8.2	1.58

[a]Abbreviations: N, nitrate; P, phosphate; S, sulfate.
[b]Specific growth rate in the exponential growth phase.
[c]Average cell growth rate in the deceleration phase.

NOMENCLATURE

A : illuminated surface area of the bubble column (m^2)
C : cell concentration (g/l)
dA : surface area at the point of incidence (m^2)
h : height of the bubble column (m)
I_0 : incident light intensity on the surface of the bubble column (μmol/s/m^2)
I_i : light intensity illuminated at a right angle on the surface area (μmol/s/m^2)
q_i : specific irradiation rate (μmol/s/g dry cell)
r : radius of the bubble column (m)
V : volume of the culture medium (m^3)
$Y_{x/s}$: growth yield based on nutrients (g/g)
α : average cell growth rate in the deceleration phase (g/l/day)
θ : angle of incidence (radian)
$d\theta$: angle of arc of dA (radian)
μ : specific growth rate in the exponential growth phase (1/day)

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