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Carbon Dioxide Fixation and Polyunsaturated Fatty Acid Production by the Red Alga *Porphyridium Cruentum*

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ABSTRACT

Focusing on CO_2 fixation, photoautotrophic cultivation of the red alga *Porphyridium cruentum* was investigated by means of a batch culture under a 5% CO_2-enriched atmosphere. The algal growth kinetics was successfully described with a logistic model, and simulation of a continuous culture under the optimum growth conditions (30°C, 12 klux and 1.18 g-cells/L) showed that the algal CO_2-fixation activity could reach 0.66 g-CO_2/(L × d). Under the same growth conditions, eicosapentaenoic acid (20:5 n-3, EPA) and arachidonic acid (20:4 n-6, ARA) yields were similarly calculated to be 3.6 mg-EPA/(L × d) and 6.5 mg-ARA/(L × d), respectively.

Index Entries: Carbon dioxide; *Porphyridium cruentum;* environment; eicosapentaenoic acid; arachidonic acid; red alga; fatty acid.

INTRODUCTION

Global warming is a serious problem. It is caused by the massive release of so-called greenhouse gases such as CO_2. Microalgae contain protein, lipid, and fine chemicals such as vitamins *(1)*, and have thus been used for food *(2)*, animal feeds *(3)*, and a source of chemicals *(4)*. Large-

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scale cultivation of microalgae is simple and inexpensive because they only require sunlight, water, CO_2, and minerals for their growth. Therefore, large-scale cultivation of microalgae is a promising method for CO_2 fixation. Recently, we have investigated the CO_2-fixation activities of the hot-spring alga *Cyanidium caldarium* and the green alga *Chlamydomonas reinhardtii (5,6).* In these works, the maximum CO_2-fixation activities were calculated in the continuous cultures by assuming the availability of artificial lighting and temperature control.

The marine red alga *Porphyridium cruentum* has been investigated as a potential source of eicosapentaenoic acid (20:5 n-3, EPA) and arachidonic acid (20:4 n-6, ARA) *(7–12),* which are rare fatty acids of pharmaceutical value. ARA is a precursor of prostaglandins and leukotrienes *(7),* and EPA has been shown to be effective for preventing and curing thrombosis and arteriosclerosis *(13,14)* and inhibiting the growth of a human lung carcinoma *(15).* The feasibility of mass cultivation of *P. cruentum* outdoors has already been studied *(8).*

In the present work, we determined the optimal growth conditions for *P. cruentum.* Then we calculated the CO_2-fixation activity of this alga and EPA and ARA yields under the optimal growth conditions and compared them with those of other microalgae.

MATERIALS AND METHODS

Reagents

All reagents and solvents used were of guaranteed grade or of reagent grade. Deionized water was used in all experiments.

Strain

P. cruentum IAM R-3 was donated by the Microbial and Microalgal Research Center, Institute of Applied Microbiology, the University of Tokyo.

Culture System

An artificial seawater culture medium (pH 7.6) based on that described by Jones et al. *(16)* was used. The medium was sterilized by autoclaving at 120°C for 30 min. Stock cultures were maintained in cotton-plugged glass bottles under the illumination with cool-white fluorescent lamps at 1.5 klux with a light-dark rhythm of 12 and 12 h at 23°C. They were transferred to the fresh medium every two months. *P. cruentum* was

always precultured for 3 d at 25°C and 4 klux in a 500-mL cotton-plugged oblong flat flask (37 mm thickness, 300 mL of culture medium) with aeration by 5 vol% CO_2-enriched air (100 mL/min). The preculture in an exponential growth stage was then inoculated at the fixed concentration of OD_{760} (optical density at 760 nm) of 0.10. Growth experiments were then carried out axenically for 7 d at 15 ~ 32°C in a 1-L shaking flask (500 mL of culture medium) with reciprocal shaking at 110 strokes/min. The flask was aerated with 5 vol% CO_2-enriched air (100 mL/min) under sterile conditions and continuously illuminated by cool-white fluorescent lamps at 90 ~ 480 w/m^2. Light intensity was measured at the upper surface of shaking flask by use of a lux meter. Growth was monitored turbidimetrically at 760 nm (OD_{760}) *(16)* with a Shimadzu UV-120-type spectrophotometer (Shimadzu, Kyoto, Japan). A thermostatic shaking water bath "Thomastat T-22S" (Thomas Kagaku Kikai, Tokyo, Japan) was used in the growth experiments. The decrease in pH of the culture was <0.5 during the course of the growth experiments.

The cells were harvested by centrifugation at 2000*g* for 25 min at 4°C and were lyophilized after being washed twice with a physiological saline solution. Preliminary experiments showed that reproducibility of the growth experiments was satisfactorily good. Therefore, each growth experiment was usually carried out one time. In the experiments, we first optimized temperature and then light intensity separately because their synergistic effect on the growth had been suggested to be not important, especially at the optimal temperatures *(17)*. The yield of algal cells is always presented as the mass obtained per 1 L of culture broth on a dry basis and one unit of OD_{760} corresponded to 0.536~0.747 g-cells/L according to the cultivation conditions employed.

Fatty Acid Analysis

Fatty acid methyl esters were prepared from 100 mg of dry cells directly by transmethylation with 5 mL of 6 wt% methanolic HCl for 3 h at 95°C, extracted with *n*-hexane, and analyzed by gas chromatography (GC) using a 5 wt% Advance DS/Chromosorb W glass column (3 mm diam. × 2 m) at 150°C (C_{14} ~ C_{18} esters) and at 165°C(C_{18} ~ C_{21} esters), respectively. Methyl heneicosanoate was employed as the internal standard for GC. Fatty acid methyl esters used as the standard for GC were purchased from Sigma, St. Louis, MO. Carbon content of dry cells was determined by means of microelemental analysis with a Yanaco MT-type CHN corder (Yanagimoto, Tokyo, Japan). The plotting software package "Delta Graph Pro.3.5" (Delta Point, Monterey, CA) was used to calculate growth constants from growth data.

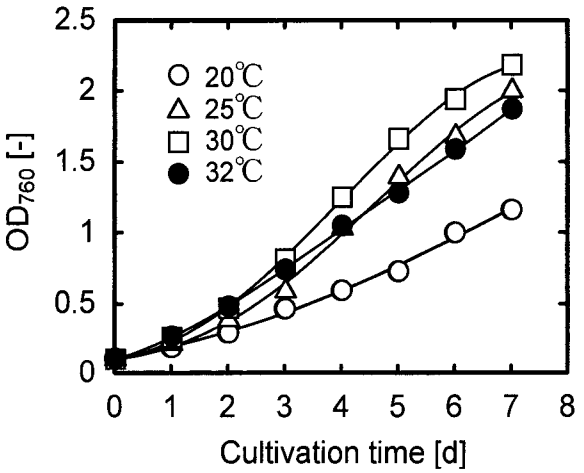

Fig. 1. Growth curves for *P. cruentum* at different temperatures and 6 klux.

RESULTS

Growth of *P. cruentum*

We first investigated the effect of temperature on the growth rate of *P. cruentum* and its fatty acid content at a fixed light intensity of 6 klux (Fig. 1, Tables 1 and 2). At 20°C, OD_{760} of the culture reached 1.16 after 7 days of the cultivation. At higher cultivation temperatures, *P. cruentum* grew more rapidly and at 30°C, the OD_{760} was 2.18 after 7 d. At a cultivation temperature of 32°C, however, *P. cruentum* grew slower and the final OD_{760} was only 1.87 (Fig. 1). Yield of algal cells after 7 d of the cultivation was a maximum 1.26 g-cells/L at 30°C as well (Table 1). Thus, the growth rate of *P. cruentum* was a maximum at 30°C and a fixed light intensity of 6 klux.

Cell growth was successfully described by the logistic law *(18)*.

$$dX/dt = \mu X \qquad \mu = \mu_m(1-X/X_{oo}) \tag{1}$$

in which dX/dt is the rate of cell growth [g-cells/(L × d)], and X and μ are the algal cell concentration [g-cells/L] at time t [d] and growth rate constant[d^{-1}] at algal concentration X, respectively. μ_m and X_{oo} are the growth rate constant at X = 0 and the upper limiting value of algal cell concentration, respectively, under the cultivation conditions employed. Experimentally obtained values of μ_m and X_{oo} for the cultivation at 20 ~ 32°C and 6 klux are given in Table 1. At higher cultivation temperatures, μ_m increased and reached a maximum value of 0.76d^{-1} at 30°C. However, it then decreased at 32°C. In contrast, X_{oo} was maximum 1.39 g-cells/L at 25°C (Table 1). The percent values of cell yield/X_{oo} were 73%(20°C) ~ 98%(32°C).

Table 1
Cell Yields and Growth Constants for *P. cruentum* Cultivated at Different Temperatures[a]

Cultivation temperature (°C)	Cell yield (g-cells/L)	Growth constants[b]	
		μ_m (d^{-1})	X_{oo} (g-cells/L)
20	0.87	0.50	1.19
25	1.21	0.68	1.39
30	1.26	0.76	1.29
32	1.15	0.61	1.25

[a] Cultivated at 6 klux for seven d.
[b] Average deviation between experimental and calculated algal cell concentrations was 2.3 ~ 3.6%.

Table 2
Fatty Acid Contents for *P. cruentum* Grown at Different Temperatures[a]

	Cultivation temperature (°C)			
	20	25	30	32
Fatty acid content (mg/g-cells)				
16:0	10.8	11.3	12.2	13.3
18:2	3.3	3.1	4.1	4.2
20:4 n-6(ARA)	14.0	15.3	16.9	17.1
20:5 n-3(EPA)	13.5	8.0	3.9	3.1
others[b]	2.2	1.7	1.8	1.6
total	43.8	39.4	38.9	39.3
DBI[c]	2.85	2.58	2.34	2.21

[a] Cultivated at 6 klux for seven d.
[b] 14:0 + 16:1 + 18:0 + 18:1 + 18:3.
[c] Double bond index of cellular fatty acids.

The effect of light intensity on the growth rate of *P. cruentum* and its cellular fatty acid content was investigated at 30°C, at which the maximum growth rate was observed (Figs. 2 and 3, Table 3). At higher light intensities, *P. cruentum* grew rapidly and the final OD_{760} of the culture increased markedly. For example, the final OD_{760} tripled from 1.07 to 3.41 when the light intensity was increased from 4 to 12 klux (Fig. 2). Algal cell yield reached the maximum 2.25 g-cells/L at 12 klux as well (Fig. 3).

Growth constants μ_m and X_{oo} were similarly determined based on Eq. 1 (Fig. 3). At higher light intensities, X_{oo} increased markedly and reached 2.36g-cells/L at 12 klux. In contrast, μ_m increased gradually and then

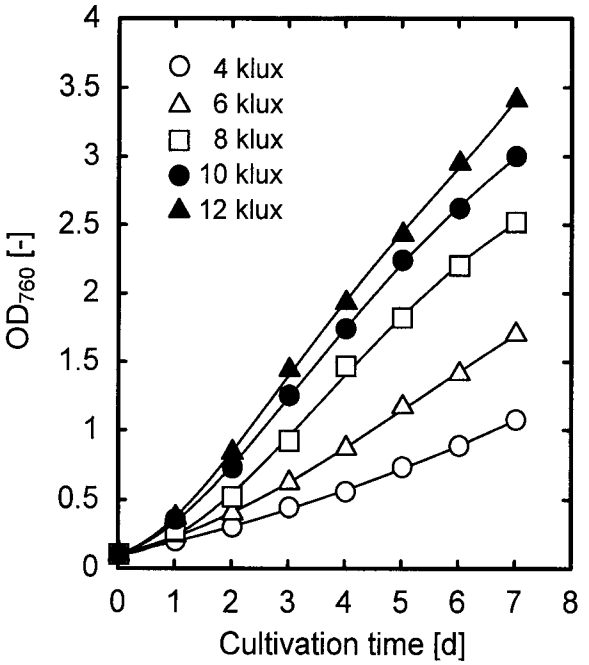

Fig. 2. Growth curves for *P. cruentum* at 30°C under different light intensities.

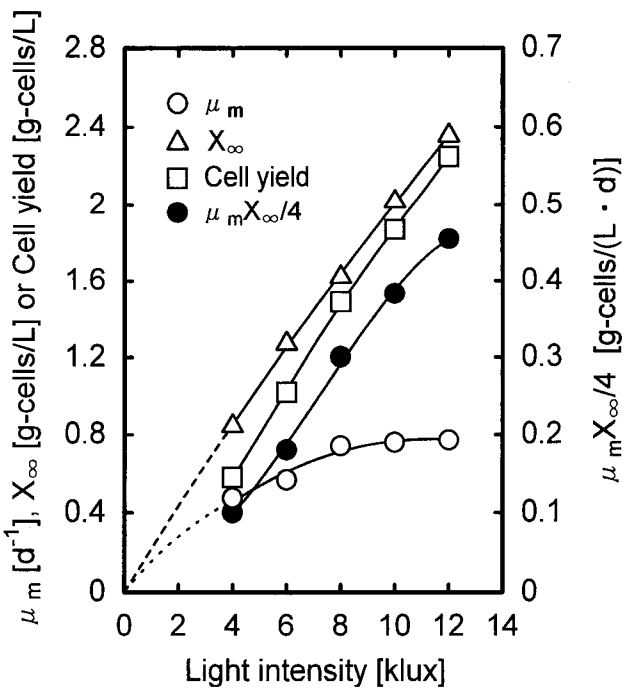

Fig. 3. Cell yields, growth constants, and maximum growth rates for *P. cruentum* at 30°C under different light intensities. *P. cruentum* was cultivated for 7 d. Average deviation between experimental and calculated algal cell concentrations was 1.7~5.6%.

Table 3
Fatty Acid Contents for *P. cruentum* Grown under Different Light Intensities[a]

	Light intensity (klux)					
	4	6	8	10	12	12[b]
Fatty acid content (mg/g-cells)						
16:0	13.3	11.8	10.6	10.4	9.8	13.5
18:2	3.3	3.9	3.4	3.3	3.4	3.7
20:4 n-6(ARA)	17.7	16.8	15.6	15.3	13.8	14.2
20:5 n-3(EPA)	4.8	4.0	3.8	3.8	3.7	7.9
others[c]	1.4	1.4	1.1	1.2	0.9	1.4
total	40.4	37.9	34.3	34.0	31.6	40.7
DBI	2.35	2.36	2.41	2.42	2.39	2.39

[a] Cultivated at 30°C for 7 d.
[b] Cultivated at 30°C for 4 d (final cell concentration = 1.20 g-cells/L).
[c] 14:0 + 16:1 + 18:0 + 18:1 + 18:3.

nearly leveled off at 0.77 d^{-1} at higher light intensities (Fig. 3). This variation of μ_m could be described by the well-known Monod equation.

$$\mu_m = \mu_{m0} I/(K_1 + I) \quad (2)$$

in which I is the light intensity [klux], and μ_{m0} (= 1.19 d^{-1}) and K_I(= 4.97 klux) are the maximum growth rate constant and the saturation constant, respectively. The average deviation between experimental and calculated values of μ_m was 3.8%.

At 4 klux, the percent value of cell yield/X_{oo} was 68%, but it was as high as 80 ~95% at 6 ~ 12 klux.

Fatty Acid Content

Tables 2 and 3 summarize the cellular fatty acid contents for *P. cruentum* grown under these different cultivation conditions. The major fatty acids are 16:0 (palmitic acid), 18:2 (linoleic acid) (20:4 n-6, ARA) and (20:5 n-3, EPA). The cellular content of ARA was always higher than that of EPA (13.8 ~ 17.7 mg/g-cells ↔ 3.1 ~ 13.5 mg/g-cells). When the cultivation temperature was raised from 20 to 32°C, cellular ARA content increased (14.0 → 17.1 mg/g-cells) whereas cellular EPA content decreased drastically (13.5 → 3.1 mg/g-cells). At higher temperatures, the double bond index (DBI) of cellular fatty acids decreased (i.e., 2.85 at 20°C → 2.21 at 32°C, Table 2).

At higher light intensities and 30°C, cellular ARA and EPA contents decreased gradually and the DBI of cellular fatty acids remained nearly

constant (Table 3). When algae cultivated at 30°C and 12 klux were harvested after 4 d cellular EPA content was higher than for similar cultures grown for 7 d. Cellular EPA content doubled (3.7 → 7.9 mg/g-cells) and cellular ARA content remained nearly equal (Table 3).

Calculation of the Algal Activity for CO_2 Fixation

The algal activity for CO_2 fixation per unit volume of the culture in photobioreacter can be calculated by the following equation.

$$CO_2\text{-fixation rate } [g\text{-}CO_2/L \times d)] = 44/12) \times Cc/100) \times (dX/dt) \quad (3)$$

in which Cc is carbon content of dry cells (39.4wt%) and dX/dt is the algal growth rate described by Eq. 1. Equation 3 shows that the maximum CO_2-fixation activity is obtainable when dX/dt is maximum. This growth rate becomes the maximum value of $\mu_m X_{oo}/4$, when the algal cell concentration X is $X_{oo}/2$. The value of $\mu_m X_{oo}/4$ for *P. cruentum* at 30°C increased markedly at higher light intensities and reached 0.46g-cells/(L × d) at 12 klux (Fig. 3). Thus, the algal growth rate was the maximum 0.46 g-cells/(L × d) at 30°C, 12 klux and X = 1.18 g-cells/L. We then calculated the maximum CO_2-fixation activity of *P. cruentum* by assuming a continuous culture under the optimal growth conditions (30°C, 12 klux and 1.18 g-cells/L). Thus, the activity was 0.66 g-CO_2/(L × d). Under these growth conditions, cellular EPA and ARA contents were 7.9 and 14.2 mg-fatty acid/g-cells, respectively (Table 3). Thus, EPA and ARA yields were calculated to be 0.46 × 7.9 = 3.6 mg-EPA/(L × d) and 0.46 × 14.2 = 6.5 mg-ARA/(L × d), respectively.

DISCUSSION

The effect of environmental conditions on the fatty acid content of *P. cruentum* has been investigated by many workers *(9–12)*. Cohen et al. *(11)* found a clear correlation between growth rate and cellular fatty acid content. That is, EPA became the main polyunsaturated fatty acid under growth conditions conducive to fast growth rate. In contrast, under suboptimal growth conditions of light and temperature, cellular EPA content decreased sharply, whereas cellular ARA content increased *(11)*. The variations of algal EPA and ARA contents under the different growth conditions, which were observed in our experiments (Tables 2 and 3), do not agree with the view found by Cohen et al. *(11)*. Our cultivation was carried out by means of a batch culture and it was discontinued at the stages near to stationary phase (cell yield/X_{oo} = 80 ~ 98%, *see* Results). In as the fatty acid content of the algal cells was thus measured in stationary phase, the content appears to be independent of the growth rate. However, if algal

samples had instead been taken during exponential growth, the fatty acid contents measured might have been expected to be a variable of the growth rate as reported by Cohen et al. *(11)*. At any rate, the decrease in DBI of cellular fatty acids at higher temperatures (Table 2) can be understood by using the concept of membrane fluidity *(5,19–21)*.

The optimal growth temperature for *P. cruentum* (30°C) found in the present work (Fig. 1, Table 1) is different from one (25°C) already reported *(8–12)*, possibly due to the difference in strains. However, a maximum value of X_{oo} was found at 25°C (Table 1). At any rate, the rise in X_{oo} at higher light intensities (Fig. 3) indicates that the algal cell concentration at stationary phase is substantially determined by the density of light flux passing through the culture system. The successful description of μ_m by the Monod equation (Eq. 2) suggests that light is a limiting substrate for the algal growth.

Although the availability of artificial lighting and temperature control was assumed, the maximum CO_2-fixation activity of *P. cruentum* was calculated to be 0.66 g-CO_2/(L × d) in the continuous culture at 30°C, 12 klux and 1.18 g-cells/L (*see* Results). This CO_2-fixation activity is comparable to that of the hot-spring alga *Cyanidium caldarium* (0.72 g-CO_2 [L × d]) *(5)*. But, the activity is smaller than those of *Spirulina platensis* (1.13 g-CO_2/[L × d]) *(22)*, *Anacystis nidulans* (0.96 g-CO_2/(L × d) *(17)* and of *Chlamydomonas reinhardtii* (1.01 g-CO_2/[L × d]) *(6)*. Both *C. caldarium* and *C. reinhardtii* contain α-linolenic acid(18:3n-3), whereas *S. platensis* does γ. linolenic acid (18:3n-6)*(23–25)*. In contrast, *A. nidulans* contains no such valuable fatty acids *(26)*. Figure 3 suggests that the CO_2-fixation activity of *P. cruentum* could be raised further under illumination above 12 klux.

Our *P. cruentum* strain also revealed that under the optimal growth conditions EPA and ARA yields could reach 3.6 and 6.5 mg-fatty acid/(L × d), respectively (*see* Results). This ARA yield is nearly half the value already reported for *P. cruentum* by Ahern et al. (0.46 mg-ARA/[L × h]) or 11.0 mg-ARA/[L × d]) *(7)*.

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