Growth and Eicosapentaenoic Acid Production by *Phaeodactylum tricornutum* in Batch and Continuous Culture Systems

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Maximum specific growth rate (μ_{max}) of *Phaeodactylum tricornutum* increased with increasing culture reactor surface-to-volume ratio. Values for μ_{max} of 0.647, 0.377 and 0.339 day^{-1} were observed for the 75-mL tube, 5.6-L tank and the 16-L tank, respectively. Higher biomass was achieved in the 75-mL batch culture tube under continuous light as compared with light cycle conditions. Palmitic acid, palmitoleic acid and eicosapentaenoic acid (EPA) accounted for over 60% of total fatty acids in the batch tube culture, with EPA content increasing to a maximum after three days. In chemostat cultures, run at dilution rates of 0.15 day^{-1} (0.45 of μ_{max}) and 0.3 day^{-1} (0.9 of μ_{max}), cell concentration reached a steady state of 2.18 and 0.7 g/L, respectively, while contents of EPA per liter of culture at steady state were 100.9 and 82.5 mg/L, respectively. At both dilution rates, EPA content of total fatty acids was the same (35.0–35.2%). At a dilution rate of 0.3 day^{-1}, the continuous culture system manifested productivities of 0.51 g/L/d and 25.1 mg/L/d for biomass and EPA, respectively.

KEY WORDS: Algae, eicosapentaenoic acid, omega-3, *Phaeodactylum tricornutum*, polyunsaturated fatty acids.

Omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to have substantial beneficial effects on human health (1). Evidence suggests that these acids have potential for use in prevention or treatment of heart and circulatory diseases, inflammatory problems and cancer (2). The positive effects of omega-3 fatty acids in human physiology were first observed in populations dependent primarily on fish diets or human subjects fed on diets of fish oils. These polyunsaturated fatty acids are primarily produced by marine microorganisms upon which the fish feed (3). There is substantial interest in seeking alternative production sources of EPA and DHA from algae and fungi because of concerns regarding fish oil supply and the complex problems of purifying these omega-3 fatty acids from crude fish oils (4).

In an earlier investigation, we described the effects of nitrogen source, phosphate, sodium chloride, growth factors, precursors, CO_2, temperature, initial pH and inoculum size on biomass and EPA production by a freshwater algal strain of *Phaeodactylum tricornutum* in batch cultures (5). In this paper, the medium composition and optimum culture conditions observed have been used to investigate kinetics of growth of the diatom *P. tricornutum* in batch and continuous cultures.

MATERIALS AND METHODS

Organism. Phaeodactylum tricornutum UTEX 640 was obtained from the culture collection of Algae at the University of Texas at Austin. The culture was maintained and propagated as previously described (5).

Culture conditions. For both batch and continuous culture studies, composition of the medium and other conditions were based on earlier culture optimization studies (5) and consisted of 5.0 g/L NaCl, 0.7 g/L urea, 1.2 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L KCl, 0.3 g/L $CaCl_2$, 0.1 g/L K_2HPO_4, 0.55 g/L $NaHCO_3$, 30 mg/L Na_2 EDTA, 6 mg/L H_3BO_3, 2 mg/L $FeSO_4 \cdot 7H_2O$, 1.4 mg/L $MnCl_2$, 3.3 mg/L $ZnSO_4 \cdot 7H_2O$, 7.0 μg/L $Co(NO_3)_2 \cdot 6H_2O$ and 2.0 μg/L $CuSO_4 \cdot 5H_2O$. Initial pH was 7.6 and culture temperature was 20 ±1°C. Three types of culture units were used: the original 75-mL culture tube, a 5.6-L glass tank (dimensions 30 × 18 × 12 cm) and a 16-L glass tank (dimensions 40 × 25 × 20 cm). For tank cultures, temperature control was provided by a cooling coil and a heater with thermostat. Two submersible circulating pumps were used to ensure uniform temperature distribution and homogeneity of the culture. Two sets of double fluorescent lamps (GRO-LUX, Sylvania, F40712-GRO-WS) were provided laterally at both sides of the tanks to supply light intensity of *ca.* 4000 lux. Unless otherwise stated, a photoperiod of 16 h light to 8 h dark was used throughout the experiment. Air, supplemented with 1% carbon dioxide, was filtered through a Microfibre disposable filter unit (Grade AQ, Balston, MA) and then supplied to the culture at the outlet of the circulating pump which generated fine air bubbles to enhance gas exchange. Aeration rate was 1 vol of air per volume of culture per minute (VVM). For continuous cultures, a peristaltic pump with calibrated flow rate was employed to feed fresh nutrient medium into the culture system. Another pump attached to the level control apparatus was operated at higher flow rate than the feed pump to withdraw culture broth and to maintain constant volume.

Determination of growth parameters. For dry weight determinations, samples were taken at the middle of the light period. Culture broths (50 mL) were filtered through 0.8-μm membrane filters and washed twice with 50 mL of saline solution. Cells were dried at 60°C to constant weight. Specific growth rate and doubling time were calculated from the equations $\mu = 2.3(\log X_t - \log X_0)/t$; $T = 0.693/\mu$ (6), where μ = specific growth rate, d^{-1}; X = cell concentration at time t, cells/mL; X_0 = cell concentration at time 0, cells/mL; t = time, d; and T = doubling time, d.

In chemostat continuous cultivation, at steady state there is no change in cell concentration over time and sterile feed, so that $\mu = D$ (dilution rate, day^{-1}) and D = F/V (F is medium flow rate, mL/day; V is culture volume, mL).

Lipid analysis. Methods for extraction and analysis of lipids have been described previously (5).

RESULTS

The patterns of biomass production in batch cultures with the different size culture units are illustrated in Figure 1.

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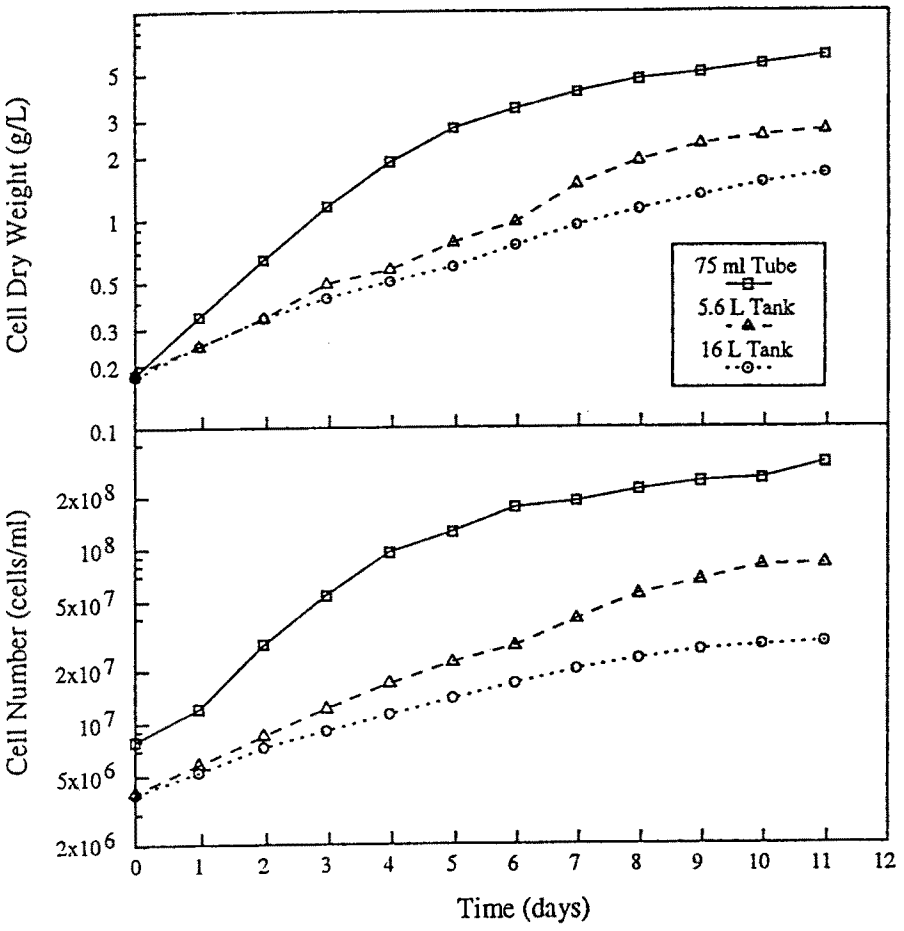

FIG. 1. Pattern of biomass production in batch cultures with different size culture units.

The smaller the culture unit, the higher the growth rate observed, and biomass dry weight values achieved after 11 d were 6.12 g/L, 2.67 g/L and 1.65 g/L for the 75-mL tube, the 5.6-L tank, and 16-L tank, respectively. In our study, the culture tube had the highest surface-to-volume ratio of approximately 1 cm^{-1}, while the corresponding ratios for the small and large tanks were 0.193 and 0.125 cm^{-1}, respectively. As far as growth behavior is concerned, there was no apparent lag phase in any of the three culture systems. In the 75-mL tube the culture exhibited a maximum specific growth rate (μ_{max}) of 0.647 day^{-1} (doubling time of 1.071 day). For the 5.6-L tank, μ_{max} of 0.377 day^{-1} (equivalent to a doubling time of 1.839 days) was observed on the third day of incubation. For the 16-L tank, μ_{max} was 0.339 day^{-1} (doubling time, 2.044 days) occurring on day 1. The changes in growth parameters, biomass, μ, and t, of *P. tricornutum* in the three culture units are presented in Figure 2. A semilogarithm plot of biomass *vs.* time revealed that *P. tricornutum* followed an exponential pattern of growth when light was saturated in the culture tube. Linear growth appeared to be prominent, particularly at high cell density at the later stages of the growth cycle.

When the organism was cultured in the 75-mL tube, under continuous light, a significant improvement in growth was observed, with cell biomass reaching 7.31 g/L compared to 5.26 g/L for light cycle conditions after 10 d (Fig. 3).

When the fatty acid composition of *P. tricornutum* was monitored throughout the growth cycle in 75-mL tube cultures, palmitic acid (16:0), palmitoleic acid (16:1) and EPA were the three predominant fatty acids formed, accounting for over 60% of total fatty acids (Table 1). Amounts of C16 acids were higher in the early growth phase and decreased gradually in the later stages of growth. In contrast, EPA content was lower during the lag phase and increased to a maximum value after three days. After four days of cultivation, proportions of the different fatty acids remained relatively constant (Fig. 4).

To study the kinetics of growth of *P. tricornutum* in continuous culture, the 5.6-L tank was used as a model system due to constraints in controlling feed rate and limitations on culture sampling for analysis encountered in the tube culture unit. Chemostat culture was the choice of operation because of its simplicity. Two levels of dilution rate (D), *i.e.* 0.15 day^{-1} (840 mL/d) and 0.3 day^{-1} (1680 mL/d), corresponding to 0.45 and 0.9 of μ_{max}, respectively, were chosen to avoid the possibility of wash-out at higher D. The time courses of biomass and EPA production in these continuous cultures are presented in Figures 5a and 5b. As the desired product, EPA, was directly related to biomass [Type 1 fermentation according to Luedeking (7)],

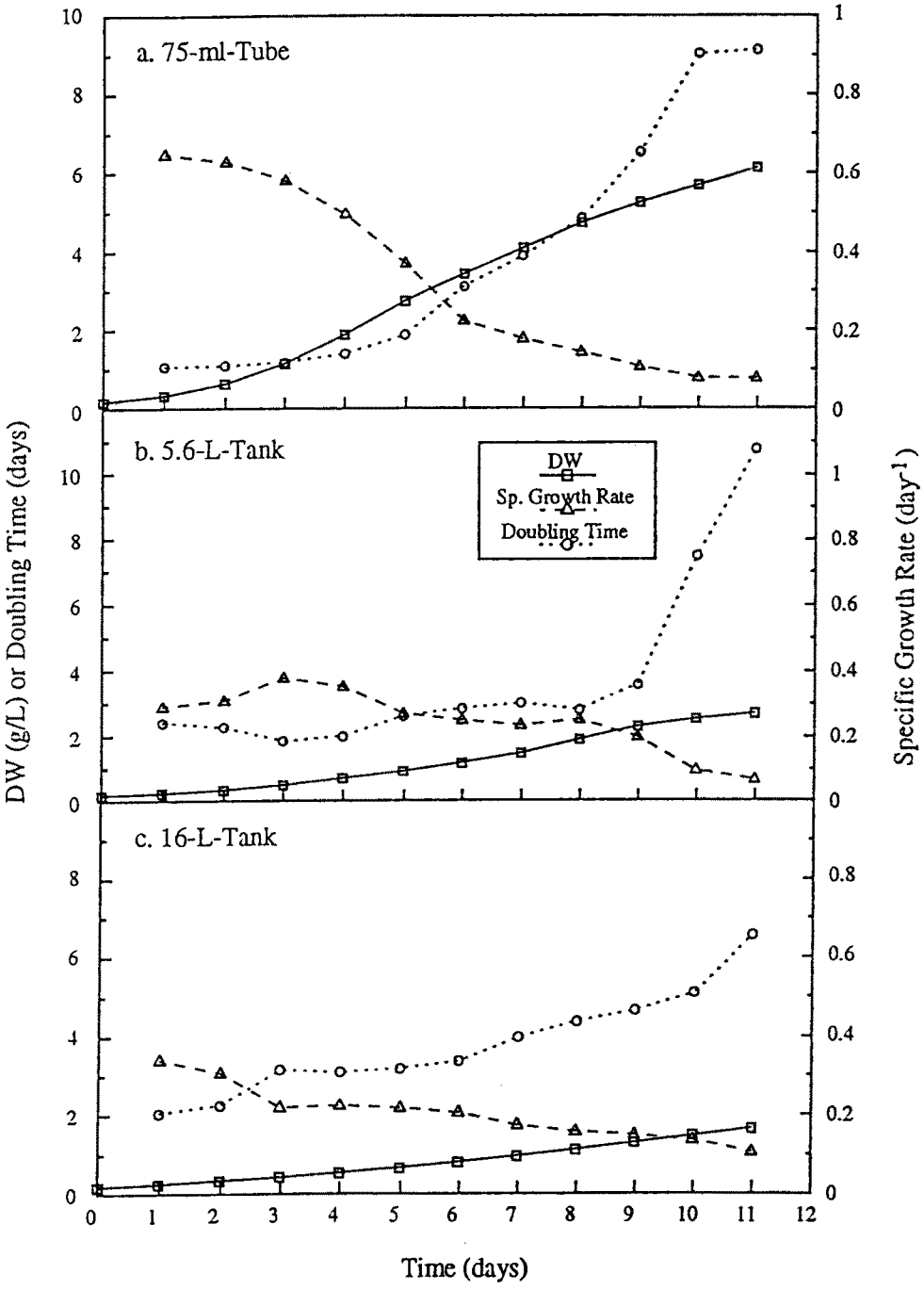

FIG. 2. Changes in growth parameters as a function of time in the three culture units.

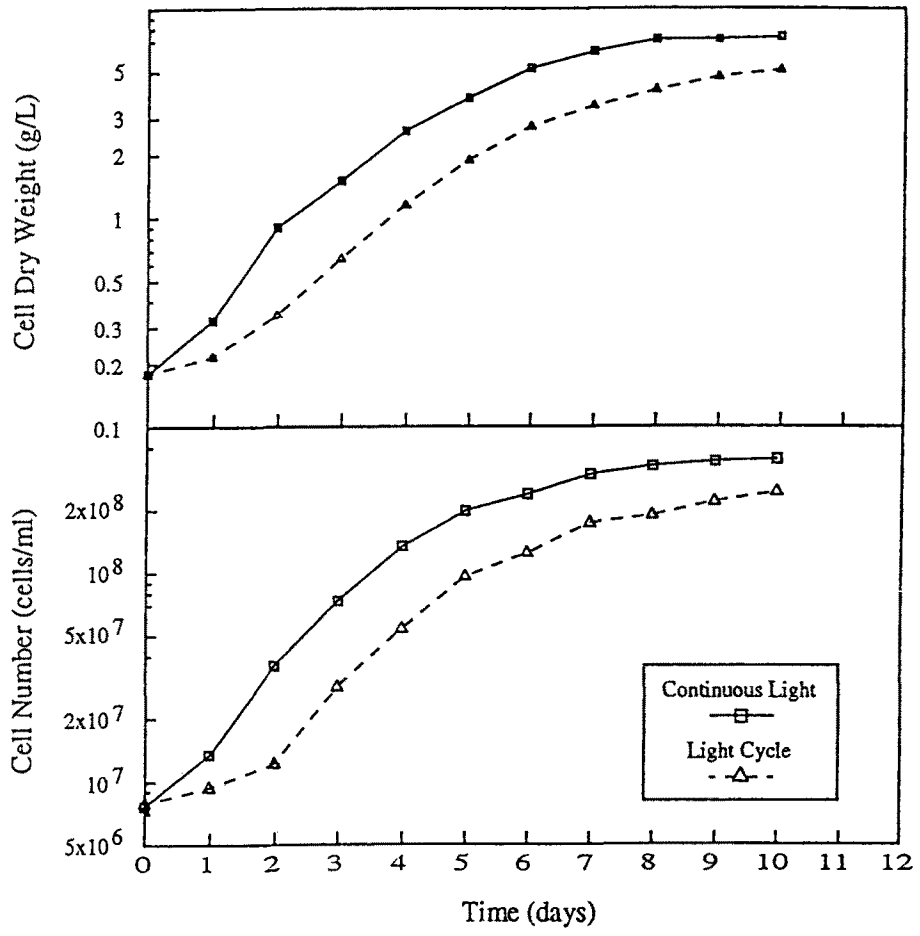

FIG. 3. Production of biomass with time under continuous light and light cycle conditions in 75-mL tube batch cultures.

TABLE 1

Changes of Fatty Acid Composition in *P. tricornutum* UTEX 640 During a Growth Cycle in 75-mL Tube Cultures

Fatty acid	Fatty acid content, % of total fatty acids					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
16:0	21.0	15.8	13.3	10.4	10.1	10.1
16:1	32.1	26.7	23.1	19.7	20.4	21.1
18:0	0.7	0.5	0.3	0.4	0.3	0.3
18:1	2.7	2.0	2.4	1.7	1.7	1.3
18:2	1.9	1.5	2.4	3.3	3.2	3.7
18:3	0.9	0.6	0.9	0.8	1.0	1.0
20:4	0.6	0.3	0.4	0.6	0.9	1.1
20:5	19.0	24.2	29.9	28.7	31.4	32.5
22:6	2.5	5.3	8.9	6.2	4.5	3.7
Others	18.7	23.0	18.4	28.2	26.7	25.2

fresh medium was fed to the culture tank at the tenth day where μ_{max} had been passed to ensure maximum initial cell concentration. Furthermore, at this period EPA accumulation in the cells had already attained highest value (approximately 35% w/w of total fatty acids). At D = 0.15 day^{-1}, cell concentration reached a steady state of 2.18 ± 0.03 g/L after the second day of fresh medium introduction. EPA content (% of total fatty acids) and EPA production (mg/L) were also maintained at constant levels of 35.0 ± 0.70% and 100.9 ± 2.48 mg/L, respectively. The same phenomenon was observed at the higher dilution rate (D = 0.3 day^{-1}) but with lower values of biomass concentration (1.7 ± 0.04 g/L), and EPA content of the culture (82.5 ± 2.35 mg/L) with a similar proportion of EPA in total fatty acids (35.2 ± 0.73%). A comparison of the production efficiency of the continuous culture systems indicated that, at a dilution rate of 0.3 day^{-1}, productivities for biomass and EPA were 0.51 g/L/d and 25.1 mg/L/d, respectively. Corresponding values for biomass and EPA at a dilution rate of 0.15 day^{-1} were 0.327 g/L/d and 15.14 mg/L/d, respectively.

DISCUSSION

The observation that continuous light exposure resulted in higher biomass production than light cycle conditions is consistent with cultivation studies with *Porphyridium* strains. High growth rates were observed when *Porphyridium* cultures were grown under continuous light, and no distinct requirements for a specific light-dark regime were observed (8,9). In contrast, Brand and Guillard (10) have

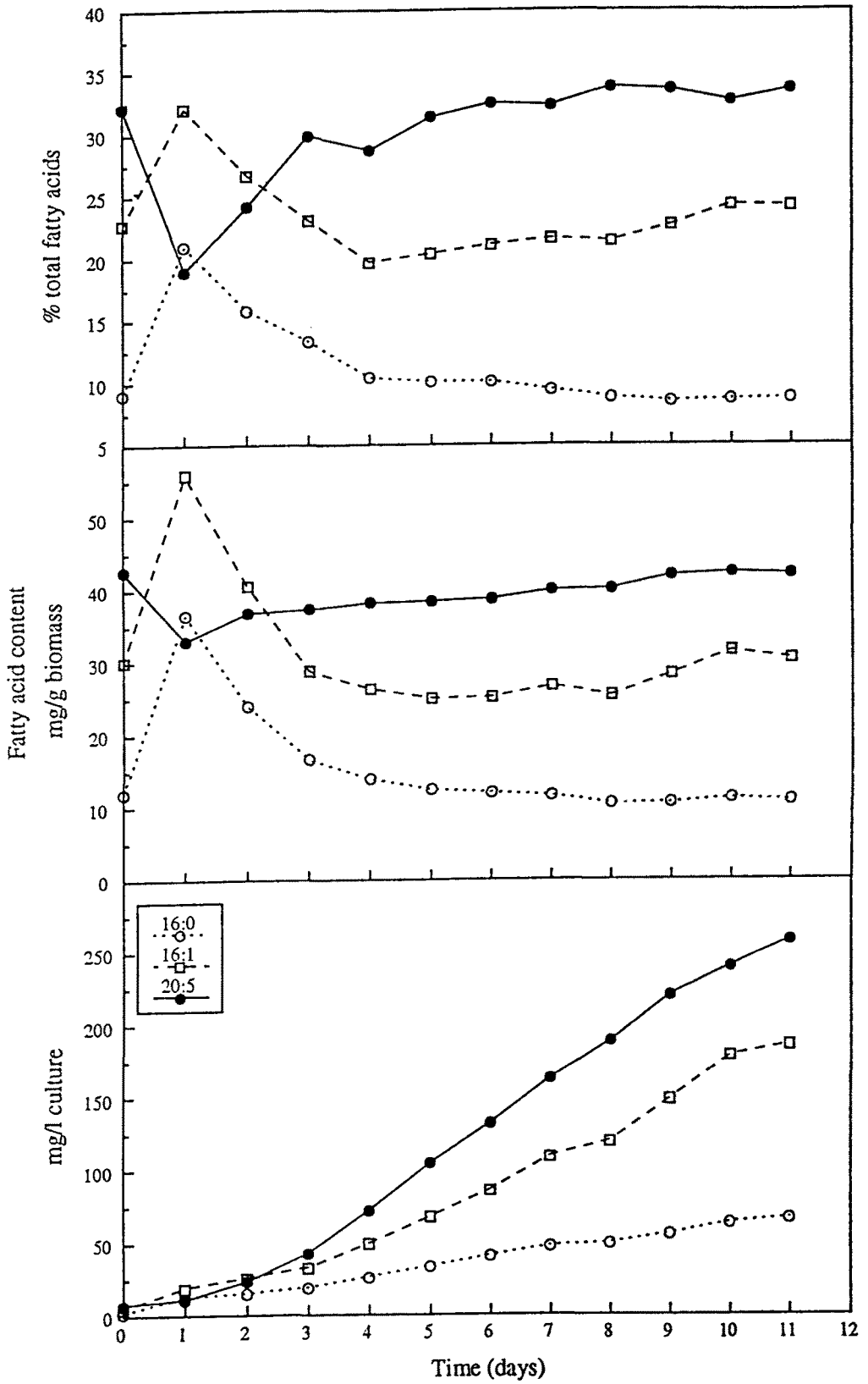

FIG. 4. Patterns of production of palmitic (16:0), palmitoleic (16:1) and eicosapentaenoic (20:5) acids by *P. tricornutum* in 75-mL tube batch cultures.

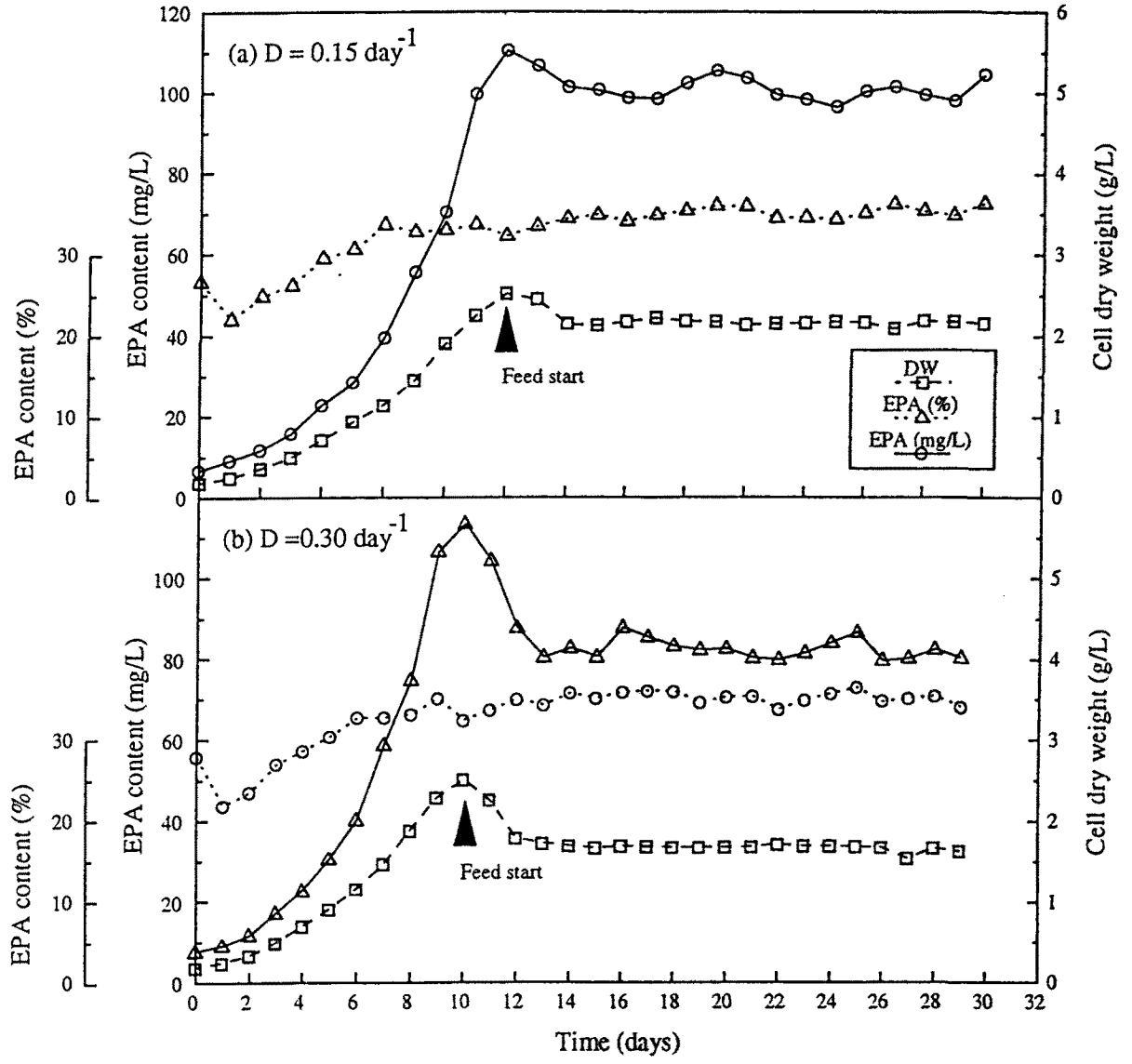

FIG. 5. Kinetics of growth and EPA production by *P. tricornutum* in chemostats at dilution rates of (a) D = 0.15 day^{-1} and (b) D = 0.30 day^{-1}.

shown that many micro-algae will not grow in continuous light and need a dark period. In most laboratories, light regimes of 12–14 h and dark periods of 10–12 h are typically used for algal cultivation. The finding that increasing algal growth rates were observed with increasing reactor surface-to-volume ratios has important implications for reactor design. Vertical glass tubular reactors were used to grow several species of algae under laboratory conditions by Miyamoto *et al.* (11), providing high surface-to-volume ratios similar to our tube culture. Outdoor mass cultivation of micro-algae was usually conducted in open raceway ponds where maximum light intensity may be obtained by limiting the culture depth (12). This type of reactor is being used successfully for the cultivation of *Chlorella, Spirulina, Porphyridium* and other algae in the United States, Israel and Thailand (13). Another type of reactor, consisting of an assembly of long (20 m), thin (6 cm diameter) polyethylene tubes in which the culture broth was circulated, has also been used in pilot-scale production of *Porphyridium cruentum* (9).

In growth studies with marine species of *P. tricornutum*, Siron *et al.* (14) noted that C16:0 and C16:1 fatty acids increased toward the end of the growth period with a loss of EPA, while Arao *et al.* (15) reported the opposite. Our observation that EPA content increases at the later stages of growth at the expense of C16 fatty acids is therefore consistent with the findings of Arao *et al.* (15). However, in two green algae, *Chlorella vulgaris* and *Scenedesmus obliquus*, larger amounts of polyunsaturated C16 and C18 fatty acids were observed during the initial growth stages while mainly saturated fatty acids were produced at the end of the growth stage (16).

Growth rate of our freshwater *P. tricornutum* species at 0.647 day^{-1} was comparable to that of marine species

reported at 0.14 to 0.87 day^{-1} in nutrient-deficient medium (17). When compared to *P. cruentum* (18), whose maximum specific growth rate reached 1.39 day^{-1}, our culture seemed to be inferior, but EPA content of *P. tricornutum* was higher (3.3% w/w of dry weight compared to 2.1% w/w).

Chlorella minutissima had EPA productivity of 3.01 mg/L/d (19) compared to 19.0 mg/L/d for our culture (5). The filamentous fungus *Mortierella alpina* produced 0.3 g/L of EPA (27 mg/g dry mycelia) equivalent to approximately 50 mg/L/d (20).

When compared with other algae of economic potential, *P. tricornutum* performed relatively well in terms of biomass production. For example, with *Chlorella pyrenoidosa* and *C. ellipsoidea* growth rate ranged from 0.16 to 0.50 day^{-1} depending upon carbon and nitrogen source (21). *Spirulina platensis* manifested a specific growth rate ranging from 0.11 to 0.30 day^{-1} depending on culture temperature (22). *Scenedesmus* sp., a unicellular green alga, had a specific growth rate as high as 0.65 day^{-1} under favorable growth conditions (13). The relative growth constant of *P. cruentum*, a unicellular red alga, was found to vary between 0.56 and 1.17 day^{-1} depending upon culture conditions, particularly light intensity and CO_2 supply (23).

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