

High-Density Photoautotrophic Algal Cultures: Design, Construction, and Operation of a Novel Photobioreactor System

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A photobioreactor system has been designed, constructed, and implemented to achieve high photosynthetic rates in high-density photoautotrophic algal cell suspensions. This unit is designed for efficient oxygen and biomass production rates, and it also can be used for the production of secreted products. A fiber-optic based optical transmission system that is coupled to an internal light distribution system illuminates the culture volume uniformly, at light intensities of 1.7 mW/cm² over a specific surface area of 3.2 cm²/cm³. Uniform light distribution is achieved throughout the reactor without interfering with the flow pattern required to keep the cells in suspension. An on-line ultrafiltration unit exchanges spent with fresh medium, and its use results in very high cell densities, up to 10⁹ cells/mL [3% (w/v)] for eukaryotic green alga *Chlorella vulgaris*. DNA histograms obtained from flow cytometric analysis reveal that on-line ultrafiltration influences the growth pattern. Prior to ultrafiltration the cells seem to have halted at a particular point in the cell cycle where they contain multiple chromosomal equivalents. Following ultrafiltration, these cells divide, and the new cells are committed to division so that cell growth resumes. The prototype photobioreactor system was operated both in batch and in continuous mode for over 2 months. The measured oxygen production rate of 4–6 mmol/L culture h under continuous operation is consistent with the predicted performance of the unit for the provided light intensity.

Key words: photobioreactor • ultrafiltration • photoautotrophic • DNA histograms • cell cycle • flow cytometry • *Chlorella vulgaris*

INTRODUCTION

Over the past decade we have seen the biotechnological use of genetically-engineered bacteria, yeast, and mammalian cells grow at a rapid rate and reach a relatively high degree of sophistication. However, despite their recognized biotechnological potential, the development of photoautotrophic cell cultures has been slower.⁶ The history of the commercial use of algal cultures spans about 50 years with application to wastewater treatment, mass production of different strains such as *Chlorella* and *Dunaliella*, and production of some specialty chemicals.^{1,12} Large-scale open algal cultures, such as lined and unlined ponds, typically reach low cell densities of 0.01–0.06% (w/v),^{2,13} resulting in expensive harvesting procedures and unfavorable economics.^{4,24} Even with

the development of closed tubular photobioreactors with controlled CO₂ supplementation, values of up to only 0.13% have been reported.⁷

Currently significant interest is developing in the use of high-density algal cultures to produce high-value products, such as pharmaceuticals and genetically-engineered products.⁵ These include antibacterial, antiviral, antitumor/anticancer, antihistamine, and many other biologically valuable products.^{9–11,20,23,25} Genetically engineered cyanobacteria have the potential to be used for a variety of purposes, such as biological control of insect larvae.²⁶ The genetic manipulation of cyanobacteria and microalgae, however, has lagged behind parallel developments with bacteria and yeast.

One of the impediments to the commercial use of high-density photoautotrophic algal cultures for production of high-value products is the availability of suitable photobioreactors. In this article we describe the design, construction, and implementation of a novel photobioreactor system for the cultivation of photoautotrophic cells. This photobioreactor system is capable of cultivating *Chlorella vulgaris* at densities of 10⁹ cells/mL [≈3% (w/v)].

ORDER-OF-MAGNITUDE CALCULATIONS

Before embarking on the detailed design of photobioreactor systems, it is useful to estimate their maximal achievable performance and identify the design limiting factors. Simple calculations based on known physicochemical and biological parameters enable us to set our design targets (Table I).

Volumetric Productivity

The volumetric oxygen production rate for growing cells can be used to specify reactor performance, and it can be calculated as follows:

$$\begin{aligned} \text{Volumetric oxygen production rate} &= \text{specific} \\ &\quad \text{oxygen yield} \quad (1) \\ &\quad \times \text{steady-state cell concentration} \quad (2) \\ &\quad \times \text{growth rate} \quad (3) \end{aligned}$$

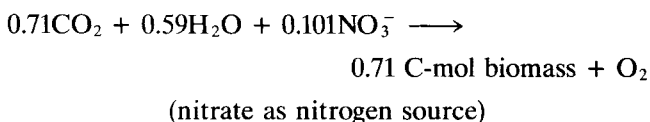
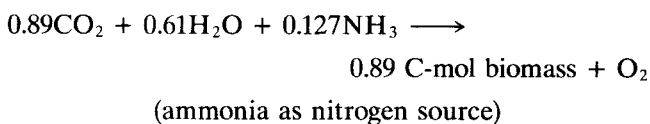
Numerical values for these three quantities are available.

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Table I. Design targets for an optimally performing photobioreactor.

Parameter	Target
Light intensity (I_0) (usable light)	1–2 mW/cm ²
Specific surface area (S/V)	5–10 cm ² /cm ³
Cell density	10 ⁹ cells/mL
Cell growth rate	0.3–0.4 g/L h
Volumetric productivity	0.02 mol O ₂ /L h

The specific oxygen yield (mol O₂/cell) can be estimated from the stoichiometry of photosynthesis. The stoichiometries presented here are based on those observed by Pirt et al.²²:



One C-mole of ash-free biomass (the amount that contains 1 mol carbon) is taken to be C_{1.0}H_{1.68}O_{0.365}N_{0.163}, which has the formula weight of 21.8 for nitrate as nitrogen source, and C_{1.0}H_{1.8}O_{0.432}N_{0.143}, with the formula weight of 22.7 for ammonia as nitrogen source.^{15,17,22} Based on photosynthetic stoichiometry, the specific oxygen yield is estimated to be in the range of 0.044–0.046 mol (≈1 L) of oxygen generated per gram dry weight generated biomass. This factor is an intrinsic property of algal biochemistry and for practical purposes must be considered as an inherent biological constraint.

The steady-state cell concentration is primarily a function of bioreactor design, with the illuminated surface-area-to-volume ratio and the rate of oxygen removal being the most important design variables. Assuming a cell volume on the order 30 fL/cell (representative of cell volume of *C. vulgaris* at high cell densities, >10⁸ cells/mL, in photoautotrophic cultures; the value was measured by a Coulter Channelyzer), the packing density of cells will be about 3 × 10¹⁰ cells/mL. Therefore, a density of about 10⁹ cells/mL, or 3% (w/v), may be considered as a reasonable design goal for the steady-state cell concentration. Above concentrations of 10% (w/v) the culture medium will turn to a creamy material⁵ and will become difficult to process.

The growth rate is assumed to be 0.06 h⁻¹, which corresponds to 12 h doubling time. The volumetric oxygen productivity can then be estimated to be 0.02 mol O₂/L h. An efficient photobioreactor must be able to grow the cells to high cell concentrations, sustain cells at these high concentrations, and maintain high growth and oxygen production rates.

Light Requirements

A major theoretical and practical limitation on microalgal productivity is the photosynthetic efficiency (biochemical energy stored in biomass per energy absorbed by the cells in the form of light). The minimum light requirement for production of 1 mol oxygen has been a source of controversy since the Z-scheme of photosynthesis mechanism was put forth in 1960.¹⁴ According to the Z-scheme, eight photons of light (quanta) are required to produce 1 mol oxygen. However, reviews on this topic^{15,21} have suggested that less than eight photons may be required for the formation of one oxygen molecule, and environmental conditions such as temperature have a strong influence on the efficiency of photosynthesis. The minimum photon requirement for *Chlorella* cells was estimated to be in the range of 5–6 $h\nu/\text{O}_2$.²¹ Based on this quantum requirement and assuming that the energy content (enthalpy) of *Chlorella* biomass is about 25 kJ/g dry wt,^{15,17,22} a maximum photosynthetic efficiency in the range of 32–54%, depending on assimilation quotient, is predicted. This efficiency is 10–20% higher than the predicted photosynthetic efficiency by the Z-scheme theory (23–30%).

We will use the Z-scheme stoichiometry of 8 $h\nu/\text{O}_2$ to specify the photobioreactor design requirement. This value corresponds to 160 $\mu\text{E}/\text{mL h}$, or in the range of 8–10 mW/mL (550–680 nm average light frequency) for production of 0.02 mol O₂/L h. Then, by assuming a photosynthetic efficiency of 23–30%, the target oxygen production rate will correspond to a biomass accumulation rate of 0.3–0.4 g dry wt/L h.

Specific Surface Area Requirements

Following the total light energy requirement, the next question that needs to be addressed is the light penetration distance at high cell densities. The light intensity may be described in Beer's law as an exponentially decaying function of distance into the culture volume with a characteristic length constant that is proportional to cell density.⁸ For *Chlorella pyrenoidosa*, the penetration distance at 680 nm has been measured to be about 1 cm at cell concentrations of about 10⁸ cells/mL.¹⁸ Thus at 10⁹ cells/mL the penetration distance is estimated to be about 1 mm.

The light penetration distance can also be calculated from the following empirical equation¹⁹:

$$d = \frac{6000}{C_c}$$

where C_c is the algal dry weight concentration in milligrams per liter and d is the light penetration distance in centimeters. This equation predicts a light penetration distance of about 2 mm at 30 g/L dry weight.

This information indicates that for a target cell concentration of 10⁹ cells/mL, the required specific area

for a reactor is about $5\text{--}10\text{ cm}^2/\text{cm}^3$, and the desired light intensity will range between 1 and 2 mW/cm^2 of usable light for the cells (in the red and blue regions of the spectrum). Table I summarizes the design targets for an optimal photobioreactor system.

PHOTOBIOREACTOR DESIGN

Our photobioreactor system consists of six major components: the light source, the optical transmission system, the photobioreactor, the gas-exchange unit, the ultrafiltration unit, and the on-line sensors for monitoring the condition of the culture (Fig. 1). The culture medium circulates in a closed loop between the reactor, the gas-exchange module, and the ultrafiltration unit. The circulation rate is calibrated so that the incoming stream to the photobioreactor is low in oxygen and the exiting stream is close to saturation. The gas supplied to the gas-exchange module is a mixture of nitrogen, oxygen, and carbon dioxide, whose compositions can be controlled. The effluent gases from the gas-exchange module flow through a condenser, trapping the water vapor prior to analysis of the gas composition. The pH, dissolved oxygen, and dissolved carbon dioxide concen-

trations in the inlet and outlet stream are measured continuously and fed to a computer which stores the data. The on-line data acquisition system provides a direct measurement of the carbon dioxide fixation and oxygen production rates. An on-line ultrafiltration unit is used to dialyze the culture medium at a relatively high flow rate. This unit allows us to selectively separate the waste and/or secreted products and exchange them with fresh medium. More detailed descriptions of the optical transmission system, the gas-exchange module, and the ultrafiltration unit are found in the Appendix.

As discussed above, one of the main challenges in the design and construction of the photobioreactor is to provide high illuminated surface-area-to-volume ratio. Figure 2 illustrates the geometric configuration of the prototype photobioreactor unit. The reactor has a culture volume of 0.6 L and the photobioreactor system has a total volume of 1 L. The light radiators inside the cylindrical bioreactor are arranged as concentric vertical cylinders to provide 3.2 cm^2 radiative surface per mL of algal suspension.

Light entering the reactor through the fiber-optic cables passes through $\frac{1}{4}$ -in.-thick acrylic cylinders. These cylinders form the center of light radiators. They consist

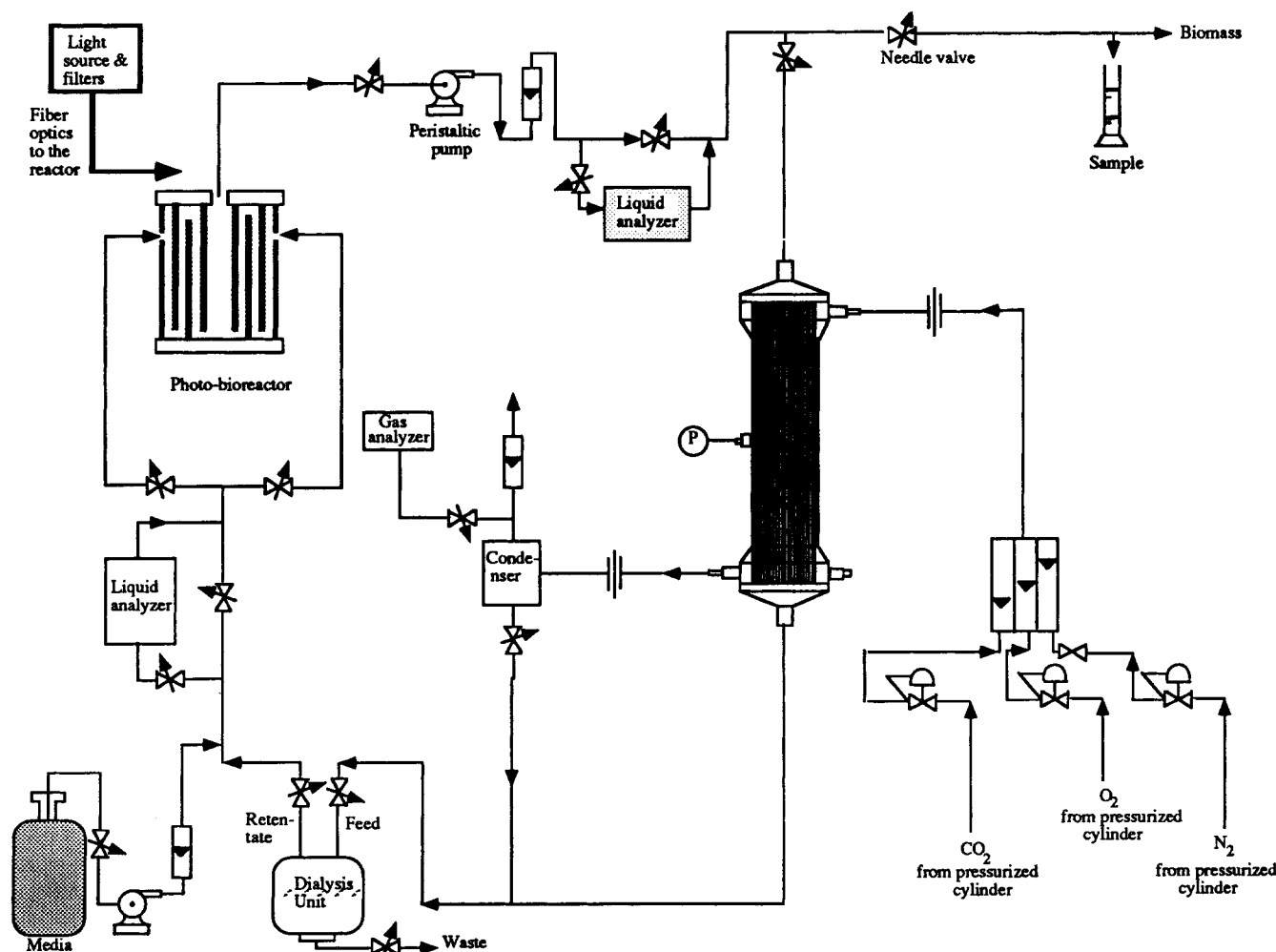
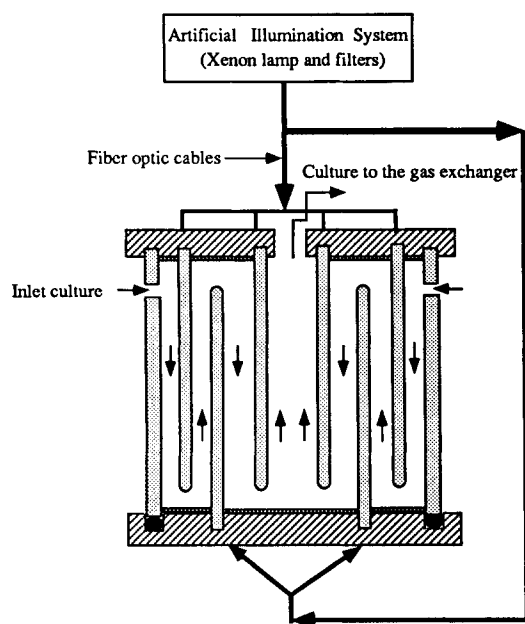


Figure 1. Flowsheet for photobioreactor system used in study.

SIDE VIEW



TOP VIEW

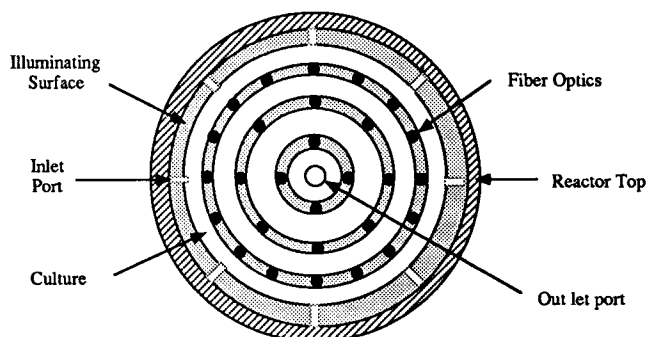


Figure 2. Details of prototype photobioreactor unit used in study.

of two $\frac{1}{8}$ -in.-thick annuli inserted into each other with wedge-shaped cuts of increasing depth in inner surface of the outer annulus, and outer surface of the inner annulus, allowing the light to escape evenly along the length of the annuli to illuminate the algal suspension. This method of construction provides the most efficient light radiation system, and since the rough surfaces are not in contact with cells, the clogging of light openings with microalgae will be prevented. The specifications of the prototype photobioreactor are summarized in Table II.

MATERIALS AND METHODS

Chlorella vulgaris, Emerson strain, from Carolina Biological Supply was cultured in the bioreactor system in N-8 medium at a pH of 5.6.²⁷ The medium consisted of (mg/L): $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 260; KH_2PO_4 , 740; CaCl_2 , 10; Fe EDTA, 10; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50; KNO_3 , 1000; and 1 mL of trace element stock with composition of (g/L): $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$, 3.58; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 12.98; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.83; and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.2.

Table II. Specifications of prototype photobioreactor unit.

Diameter	10 cm
Height	17 cm
Culture volume	600 mL
Illuminated surface/volume of culture	$3.2 \text{ cm}^2/\text{cm}^3$
Light radiator thickness	$\frac{1}{4}$ in.
Material	Acrylic
Irradiance (400–700 nm)	$1.7 \text{ mW}/\text{cm}^2$
Usable irradiance (red and blue part of spectrum)	$0.6 \text{ mW}/\text{cm}^2$
Number of fiber-optic bundles	100

Algal cell concentration was measured with a Coulter Counter Model ZM. This system also includes a Coulter Channelyzer which can measure particle size distributions. The culture was cultivated in the system with a reasonably constant temperature of $25 \pm 1^\circ\text{C}$ and a circulation flow rate of $\approx 2 \text{ L}/\text{min}$. The gas composition to the cartridge was controlled by multitube flowmeters. The input gas composition to the hollow fiber was kept at 20% O_2 , 5% CO_2 , and 75% N_2 with a total flow rate of 300 mL/min. The rate of ultrafiltration was about 8 mL/min, and the molecular cut-off of the membrane was 100 kD. The light intensity inside the reactor was measured by an LI-COR light meter model LI-185 from LAMBDA Instruments Corporation which indicated an intensity of $1.7 \text{ mW}/\text{cm}^2$ of light inside the reactor, and the total light energy provided to the reactor was about 3.2 W. The gas composition from hollow fiber cartridges was examined by a Hewlett-Packard gas chromatograph Model 5890 with autosampler for direct measurement of inlet and outlet gas to and from the cartridge.

The DNA content of the cells were quantified by using a flow cytometer. Samples containing a total number of 2×10^6 cells were centrifuged (280g, 10 min), suspended in a small volume ($<0.2 \text{ mL}$) of supernatant, and added to 1 mL ice-cold methanol. The samples were stored for 10 days in 4°C . For DNA staining the samples were centrifuged (280g, 7 min) and resuspended in methanol for 45 min to extract all the remaining chlorophyll. After this stage the samples were centrifuged (280g, 7 min), washed with phosphate buffer solution (PBS) once, then resuspended in 1 mg/mL RNase in PBS for 45 min at 37°C , after which the DNA was stained with propidium iodide (PI) in a concentration range between 30 and 40 $\mu\text{g}/\text{mL}$. Samples were stained and analyzed in groups of 30 samples. An internal standard such as chicken red blood cell was not used for DNA analysis of *Chlorella* cells due to the overlap of the standard and sample DNA histograms. Instead an external standard was used frequently as a staining control.

Stained samples were analyzed on a Coulter EPICS 751 flow cytometer (Coulter Corporation, Hialeah, FL) equipped with a 5-W argon laser. The laser was tuned to 488 nm, and the power output was set to 200 mW. The DNA histograms comprising 256 channels in a linear

scale were acquired to a total count of 30,000 events. The linear voltage was adjusted to allow the inclusion of all DNA peaks in a single linear histogram. The DNA histograms were bitmapped off two-parameter histograms to eliminate electronic noise and signals generated from cellular debris. The light energy emitted from the PI-DNA complex was directed to a photomultiplier tube by a 550-nm dichroic beam splitter followed by a 630-nm long-pass filter. The microscopic examination of the cells comprising peaks in the DNA histograms and sorted by flow cytometer revealed an insignificant number of clumps. Therefore, the DNA histograms are assumed to indicate the per-cell DNA content within a population.

RESULTS

The photobioreactor system can grow *C. vulgaris* into high cell densities. A typical growth curve for batch cultivation is shown in Figure 3. *C. vulgaris* inoculated at 10^7 cells/mL was grown up to 10^9 cells/mL over 16–20 days. The use of the ultrafiltration unit was critical (Fig. 3) in order to achieve cell concentrations above 10^8 cells/mL. As discussed below, the ultrafiltration unit serves to remove secreted autoinhibitory compounds enabling growth into high densities. A repeated ultrafiltration at densities of 10^9 cells/mL at day 26 did not lead to further growth.

Figure 4A shows the growth curve of an experiment of longer duration with the same initial conditions and more frequent use of the ultrafiltration unit. This experiment lasted about 2 months. The cells were cultivated in a batch mode until a concentration of 10^9 cells/mL was achieved. Again the key factor in achieving high cell concentration was the use of the on-line ultrafiltration unit. The pH and the specific oxygen production rate of the culture were monitored on-line through three periods of ultrafiltration (Figs. 4D, E). Following the onset of ultrafiltration the pH dropped rapidly to about 5.6, which is the pH of fresh medium, and then rose gradually after termination of ultrafiltration. The oxygen production rate increased following the period of ultrafiltration, and a concomitant expansion in the cell number took place. This result indicates that prior to on-line ultrafiltration, the culture is limited by either lack of nutrients or the accumulation of autoinhibitory compounds that are secreted by the cells.

To examine the effects of ultrafiltration on cell growth at high cell concentrations, flow cytometric DNA histograms were obtained prior to and following ultrafiltration (Fig. 5). Before ultrafiltration, the culture was not actively growing, and the DNA histogram had two distinct peaks showing the presence of two cell populations, one with a single set of chromosomes and another with multiple sets of chromosomes. This observation indicates that the growth is halted both at the commitment and at the division stages in the cell cycle. Fol-

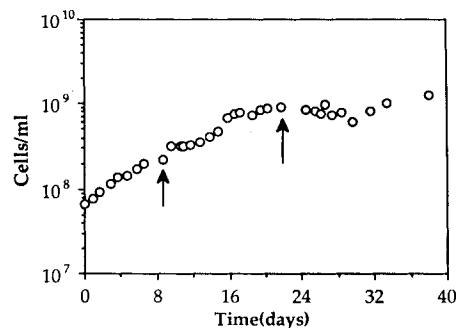


Figure 3. Growth curve for *C. vulgaris* grown in a batch mode in the photobioreactor system, shown in Fig. 1. Arrows indicate timing of on-line ultrafiltration. Each ultrafiltration was carried out for 8 h against fresh medium.

lowing ultrafiltration the multiple chromosome peak disappears, and more cells enter the cell cycle from the G_0/G_1 phase to the DNA synthesis phase (S phase), and the culture resumes its normal growth condition.

Based on this observation, it appears that at high cell densities the cells secrete autoinhibitory compounds that are responsible for the cell cycle alteration. To verify this phenomena, an experiment was conducted to investigate the effect of the autoinhibitory compounds on low-density cultures. Exponentially growing cells were inoculated at low density into fresh medium that was supplemented with spent medium from high density (10^9 cells/mL) culture. The DNA histograms from different cultures clearly show the effect of these compounds on the cell cycle of *C. vulgaris* (Fig. 6). Comparison between the control (0% conditioned medium) and 50% and 90% conditioned medium indicates that the broad exponential pattern of the DNA histograms resolves into separate peaks with two or three chromosomal equivalents. This observation verifies that the secreted products have the same effect on low-density cultures ($\approx 10^6$ cells/mL) as they do on high-density cultures. This observation also rules out nutrient limitation as the cause for the growth inhibition, since the number of the cells at low-density cultures is three orders of magnitude less than high-density cultures and in both cases the same composition of fresh medium was used.

The photobioreactor system was placed in continuous mode on day 52, with a dilution rate of 0.15 per day (150 mL/day). The oxygen production rate under these conditions was in the range between 4 and 6 mmol/L culture/h (Fig. 4F). The steady-state concentration at this dilution rate was measured to be 8×10^8 cells/mL (Fig. 4C). Assuming a cell volume of 30-femtol and dry-to-fresh-weight ratio of 0.25, 63 mg dry *Chlorella* was produced per liter of reactor per hour.

The total amount of light provided to the reactor from the xenon lamp was 3.2 W with 30–35% of the power in the usable frequency range for photosynthesis (≈ 1.2 W). Based on the usable provided light (0.6 mW/cm²), the oxygen production rate in this unit

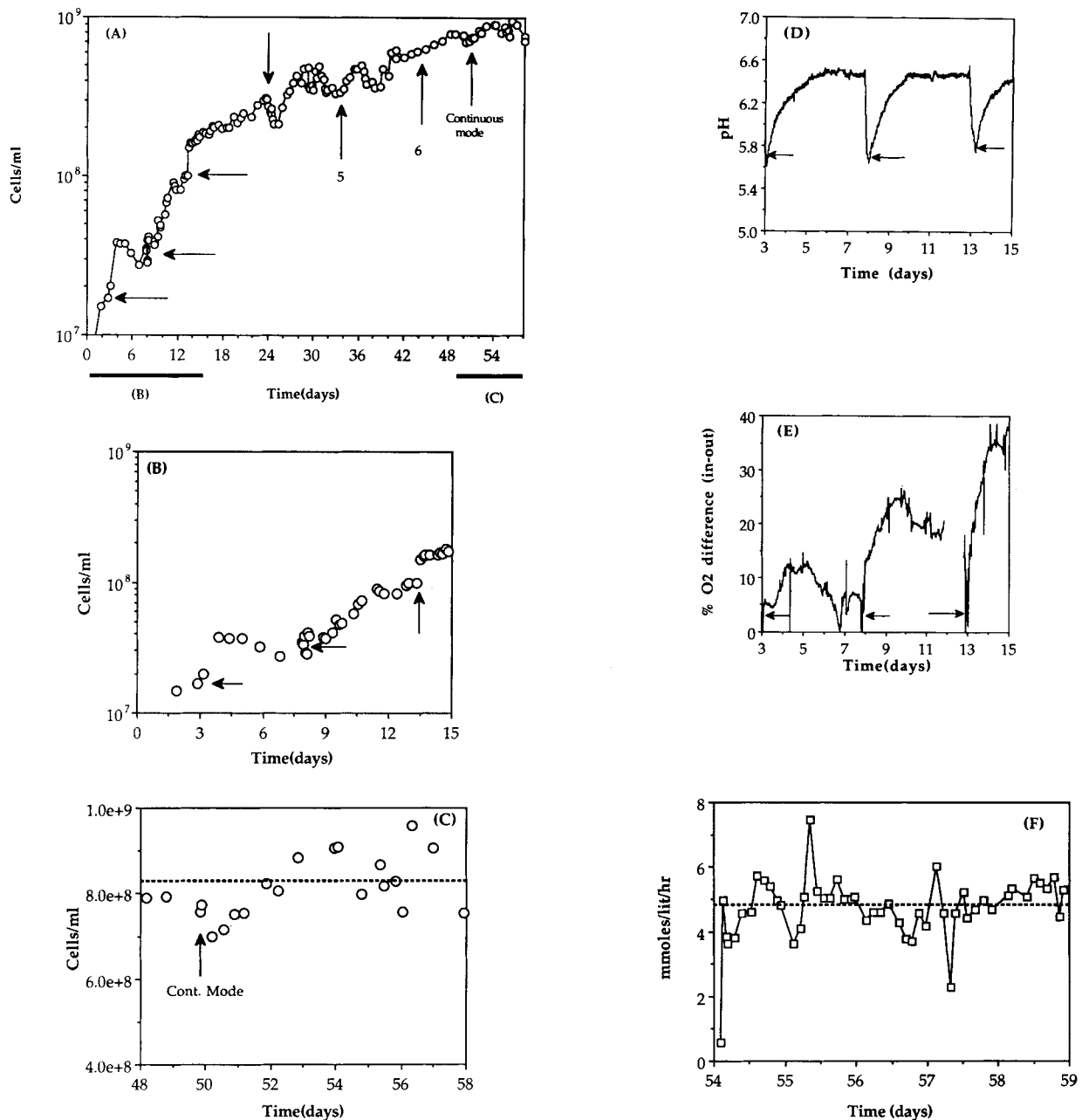


Figure 4. Operation of photobioreactor system over a 2-month period. (A) Growth curve of *C. vulgaris* in batch and continuous mode. Arrows indicate the timing of on-line ultrafiltration. Arrows 5 and 6 indicate ultrafiltration against medium with five-fold elevated potassium nitrate concentration (5 g/L) over normal medium (1 g/L). These particular ultrafiltrations were done to verify that the culture is not nutrient limited. The concentration of potassium nitrate was increased in the medium because this compound is the most likely nutrient to be depleted in the process of growth. As the growth curve shows, the additional availability of nitrogen source did not result in substantial stimulation of growth. After the reactor was operated over 8 days in continuous mode, it had to be stopped due to loss of light. (B) Growth curve during the first three ultrafiltrations. Arrows indicate time of ultrafiltration. (C) Growth curve during continuous mode. (D) On-line pH monitoring during ultrafiltration. Arrows indicate time of ultrafiltration. (E) On-line dissolved oxygen concentration monitoring during ultrafiltration. Arrows indicate time of ultrafiltration. (F) Oxygen production rate during continuous operation.

was very close to the predicted amount by the order-of-magnitude calculations. Assuming the Z-scheme for photosynthetic mechanism and 23% efficiency for converting light energy to biomass, it is expected to produce 60–80 mg dry biomass/L h, which agrees with the actual biomass production (63 mg/L h) rate. These results demonstrate that the reactor is performing as predicted.

DISCUSSION

A photobioreactor for mass cultivation of algal cells at high cell concentrations has been designed, constructed, and implemented. Our present unit meets all the specifications, based on physicochemical and biological constraints, except the surface light intensity. Based on the

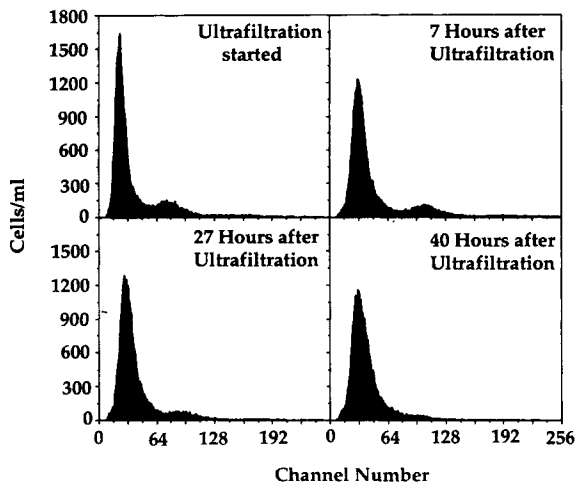


Figure 5. Time sequence of DNA histograms of *C. vulgaris* following ultrafiltration during second ultrafiltration period.

provided light and nutrients to the photobioreactor, the performance of the system meets the estimated achievable growth rate and oxygen production rate. This per-

formance could be improved further by increasing the light intensity at the illuminating surfaces inside the reactor to reach the estimated performance of 0.3–0.4 g/1 h biomass production and 0.02 mol/L h oxygen production.

Even with illumination that falls short of the desired design goal, we demonstrate that very high density algal cultures can be established and maintained. This technological achievement has led to the discovery of unusual cell cycle behavior at high cell densities. The autoinhibitory compounds alter the cell cycle kinetics of *Chlorella*. These factors may be analogous to the pheromonal mating factors known to be produced by other single cellular eukaryotes.¹⁶ These secreted compounds also may have bacteriocidal activity. There have been some reports about the bacteriocidal activity of spent medium from *Chlorella* cultures,²³ but this phenomenon has not been extensively studied.

The attainment of active high-density algal cultures will allow algal cultures to be economically feasible for the production of high-value pharmaceutical products and perhaps recombinant proteins.

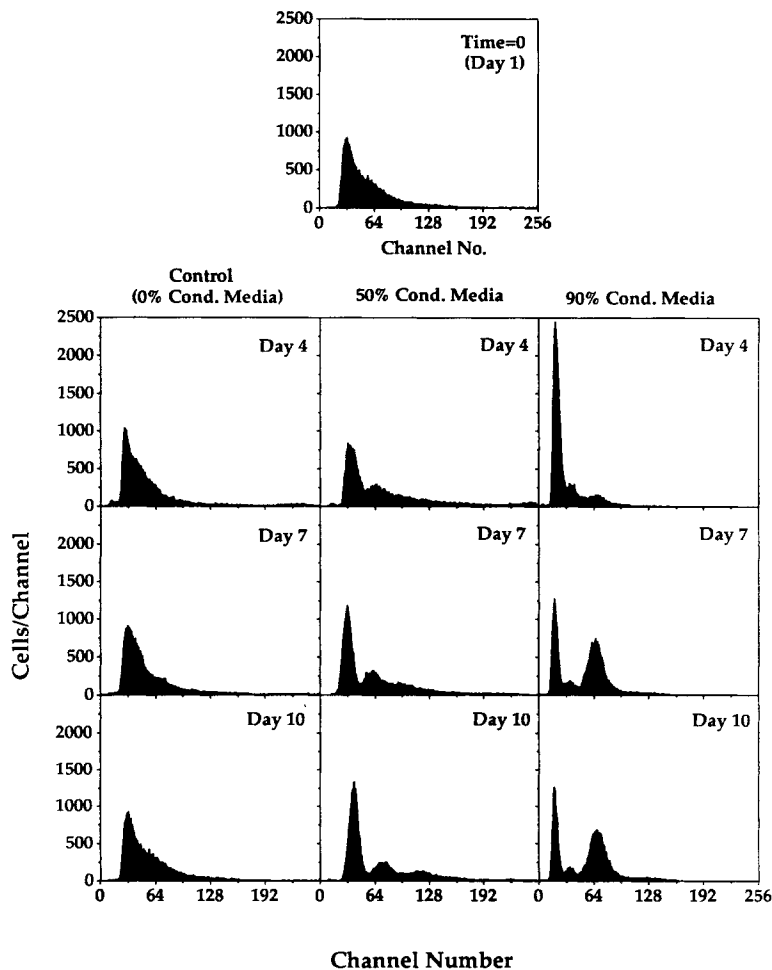


Figure 6. Time sequence of DNA histograms of *C. vulgaris* cultivated in conditioned medium.

APPENDIX

Optical Transmission System

The main goal in designing the optical transmission system was to provide uniform light with the required intensity at the illuminating surfaces of the reactor. This task was accomplished by conducting the required portion of the light spectrum via fiber-optic bundles to the illuminating surfaces of the reactor. In this fashion internal illumination is provided for the system, while the light source is kept out of the reactor, protecting the reactor from the lamp waste heat, and preventing unnecessary design and performance complications.

A xenon lamp is used as an artificial near-duplicate source of solar spectrum for controlled illumination. However, the efficiency of arc lamps is about 2–3%. The efficiency can be significantly improved by using other artificial light sources such as light emitting diodes (LEDs) if the use of artificial light proves to be necessary. These light sources can have efficiencies up to 80% in converting electrical energy to light at specific wavelengths, such as 660 nm (red light).³

Gas-Exchange Device

Hollow fiber cartridges with microporous hydrophobic polypropylene fibers are used as efficient devices to exchange CO₂ and O₂ in the suspension culture. The advantages of these units are their high efficiency at low flow rates (90%) and high mass transfer coefficients in the range of 0.05–0.3 mmol/h mm gas at liquid flow rates of 0.5–7.0 l/min. The gas-exchange membranes are external to the reactor to avoid unnecessary complications with respect to the light delivery to the culture. They can be used as a single unit or in serial arrangement. In serial arrangement oxygen can be stripped off the culture in one unit under low pressure, and carbon dioxide can be dissolved in the culture in another unit under high pressure to increase the solubility.

Ultrafiltration Unit

On-line ultrafiltration units allow the system to achieve high cell densities as well as the separation of autoinhibitory compounds without removing the cells from the system. The significance of this unit is demonstrated in the preliminary results. In this particular system, we have used Minisette acrylic cell from Filtron Technology Corporation, which is a high-performance tangential flow membrane unit and can be scaled up easily. The membrane is an open-channel polyethersulfone (PES) with a molecular cut-off of 100 kD. The surface area is 0.75 ft², the retentate flow rate is 2 L/min, and the filtrate flow rate is 8 mL/min, which can be increased to 30 mL/min.

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