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Effect of Light Irradiance on the Production of Sulfolipids from *Anabaena 7120* in a Fed-Batch Photobioreactor

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ABSTRACT

Sulfolipids have recently emerged as promising antiHIV and antitumor therapeutics. These lipids have been found in association with the photosynthetic apparatus in most photoautotrophic organisms. To date there have been no quantitative studies on the effect of environmental factors on the production of sulfolipid. In this study, we present results on the effect of light irradiance on the production of sulfolipids using the cyanobacterium *Anabaena 7120*. The cyanobacteria are grown in a 2 L fed-batch photobioreactor at various external-light intensities. Total lipids are extracted using the Folsch procedure and sulfolipids are quantified using thin-layer chromatography and scanning densitometry. We have achieved a maximum of 14 mg sulfolipid/g dry weight of cell. Our results indicate that there are two stages in the specific rate of production of sulfolipids, one in the declining exponential-growth phase of the cells and the other in the light-limited stage of growth.

Index Entries: Sulfolipid; light intensity; fed-batch culture; cyanobacteria; photobioreactor.

INTRODUCTION

The plant sulfolipid, sulfoquinovosyl diacylglycerol (SQDG), has been found in all photosynthetic plants, algae, and bacteria investigated to date (*1–3*). They represent a very interesting class of high-value products.

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Gustafson et al. *(4)* demonstrated potential applications of sulfolipids as antitumor/antiAIDS human therapeutics and, as a result, the National Institute of Cancer has selected the sulfolipid as a high priority for further preclinical evaluations.

Since the discovery and structural elucidation of SQDG *(5,6)*, relatively little progress has been made toward the details of the biosynthetic pathways and the function of the sulfolipid. This is somewhat surprising because the occurrence of substantial amounts of the sulfolipid in all photosynthetic organisms examined makes it one of the most abundant sulfur-containing organic compounds *(7)*. Because of the presence of sulfolipids in the photosynthetic apparatus, it has been proposed that the sulfolipid plays some role in photosynthesis. In *Euglena gracilis*, it was noted that there was a direct correlation between the appearance of chlorophyll and the appearance of galactoglycerides and sulfolipid *(8)*. Several other experimental observations have implicated diacylsulfoquinovosyl glycerol in photosynthetic functions. Shibuya and Hase *(3)* studied the accumulation of sulfolipid (by introducing S^{35}-labeled magnesium sulfate into the medium) and chlorophyll in *Chlorella protothecoides* under growth conditions which resulted in either greening or bleaching of the cell mass. They found that the sulfolipid level increased or decreased almost in parallel with the process of greening or bleaching, respectively. Therefore, it would be expected that light would play an important part in controlling lipid production. The production of sulfolipids is thought to be linked to the amount of thylakoid membrane present. It has long been observed that photosynthetic microorganisms and plants increase their complement of photosynthetic pigments with decreasing intensities of ambient light, in a process called shade adaptation *(9)*. The consequences of shade adaptation include increasing the total amount of photosynthetic membranes, the amount of light-harvesting pigments per reaction center and in some cases, and the ratio of photosystem II to the photosystem I reaction center *(9)*.

This paper presents results delineating the effect of light irradiance on growth and sulfolipid-production kinetics in fed-batch photobioreactors using the cyanobacterium *Anabaena 7120*. Characteristic features of the growth and sulfolipid production kinetics are demonstrated and a theoretical model for the transient internal light distribution is used to explain experimental observations. Chlorophyll levels within the cultures were also monitored as a function of time to determine correlations between pigments and sulfolipid as a function of light intensity.

MATERIALS AND METHODS

Organism and Culture Conditions

Anabaena 7120 was cultivated in a fed-batch photobioreactor. All experiments were conducted using BG-11 medium with ammonium chlo-

ride as the nitrogen source *(13)*. A 2 L New Brunswick MultiGen bioreactor equipped with temperature and pH controllers was used. The temperature was maintained at 27 ± 1°C and the pH at 7.0 ± 0.2 by addition of 0.2 *M* sodium carbonate as required. The photobioreactor was operated at a working volume of 1.5 L and agitated at 200 rpm with a single-turbine impeller located near the bottom. The photobioreactor was inoculated with pure cultures grown in shake flasks. The initial biomass concentration after inoculation was 1.5×10^{-6} g/mL for experiments with an external-light intensity of 170 μE m^{-2} s^{-1}; 5×10^{-6}g/mL for experiments with an external-light intensity of 120 μE m^{-2} s^{-1}; 5.5×10^{-6}g/mL for experiments with an external-light intensity of 88 μE m^{-2} s^{-1} and 3.5×10^{-5} g/mL for experiments with an external-light intensity of 66 μE m^{-2} s^{-1}. Although initial biomass concentrations were not precisely controlled in these studies, the cultures were very dilute following inoculation. With the possible exception of the run at 66 μE m^{-2} s^{-1}, the initial biomass concentrations were so dilute they had negligible impact on the initial light-intensity distribution. The photobioreactor was sparged with humidified, filter-sterilized air containing 1% carbon dioxide at a rate of 0.4 L/min. To assure that the growth rate was limited by light, not nutrient availability, concentrated nutrients were added to the photobioreactor on a daily basis. Light was provided by concentric 32-W circular fluorescent bulbs. Light levels were measured prior to charging the photobioreactor and found to be relatively uniform throughout the reactor. The experimental arrangement is shown in Fig. 1.

Four experiments were conducted at four different external-light levels: 66, 88, 120, and 170 μE m^{-2} s^{-1}, as measured at the wall of the photobioreactor. All experiments were run for at least 21 d, during which the ammonium concentration was maintained at 2.5 m*M* or higher by the addition of ammonium chloride and the phosphate concentration was maintained at 32 mg L^{-1} or higher by addition of monobasic potassium phosphate. The pH was measured by an in-dwelling pH electrode (Ingold, type 465-35-90-K9) and displayed on a digital pH meter (Cole Parmer, model 5656-10). When the pH level dropped below 7.0, a pump was activated to add sodium carbonate in order to adjust the pH to approx 7.4.

Measurement of Light Irradiation Intensity

Irradiance was provided by two or three 32 W circular cool-white fluorescence lamps (Philips FC12T9/cw) around the photobioreactor. Light intensity was varied by shading the vessel with a white cloth. The light intensities on the surface of the photobioreactor were measured by a light meter (Lambda Instruments, type L1-170). The light distribution was measured at 10 equally spaced points around the circumference of the empty vessel. Six values of light intensity, measured inside the photobioreactor along the vertical coordinate, were averaged to get the average light intensity at the surface of the vessel.

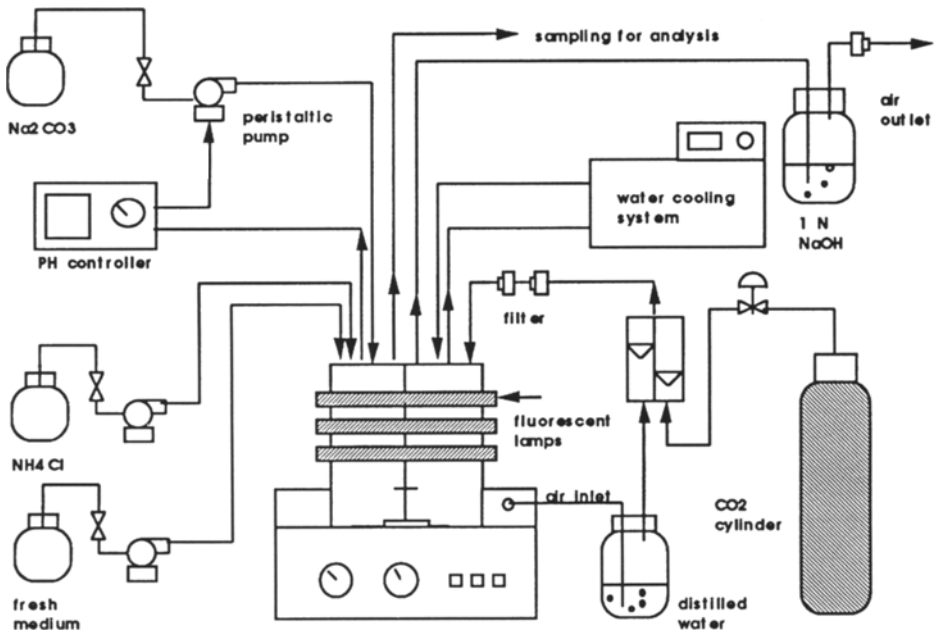

Fig. 1. Experimental setup. The base of the photobioreactor contains an air pump that provides the pressure head for sparging. The air is bubbled into a distilled-water flask to prehumidify the air. The prehumidified air is mixed with CO_2 gas from a gas cylinder. The sparging gas is filtered through two 0.22 μm filters before entering the photobioreactor. The effluent gas is bubbled through a 1 *N* NaOH solution and a 0.22 μm filter to minimize contamination.

Analytical Methods

A 15-mL sample was taken from the photobioreactor every 24 h and analyzed for biomass, phosphate, and ammonium concentration in the media. Chlorophyll and sulfolipids were extracted from the cyanobacteria and quantified to obtain intrinsic levels of these compounds in the biomass. The biomass concentration was determined by measuring the optical density of the sample at 680 nm. To minimize the effect of algal-chain length on the optical density, the sample was homogenized for 15 s with a Tissue Tearer at low speed (Biospec Products, model 985-370) to achieve a fairly uniform chain length. The biomass concentration (grams dry weight per liter) was calculated from a measurement of the optical density by means of a calibration equation. The calibration curve was determined by taking samples from three different *Anabaena 7120* cultures, homogenizing as described above and serially diluting each of the three samples. Optical density of each of the diluted samples was measured at 680 nm. A known volume of each sample was filtered onto a preweighed, predried filter (0.2 μm, Whatman) and the filters were dried at 70°C until the weight

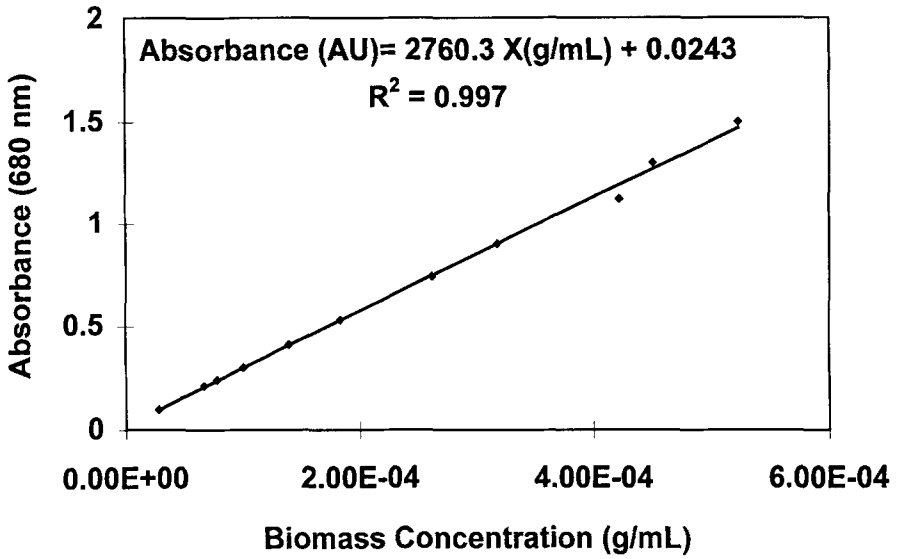

Fig. 2. Calibration curve for biomass dry-weight concentration vs absorbance.

remained constant. Figure 2 shows the resulting calibration curve relating optical density to biomass dry-weight concentration. The calibration was linear up to an absorbance of 1.5; samples with high biomass concentrations were diluted to obtain measurements in the linear region. The error associated with the biomass concentration measurement is estimated to be within 17% at low-cell densities (<0.0004 g/mL) and closer to 5–8% at the higher densities >0.0008g/mL). The concentrations of NH_4^+ and PO_4^{3-} were measured by an ammonia probe (Orion model 95-12) and a phosphate colorimetric kit (Industrial Municipal Equipment). The chlorophyll content was determined by the method of Becker *(11)*.

Sulfolipid Analysis

To date there has been no simple way to quantify sulfolipids. Methods such as column chromatography, high-performance liquid chromatography (HPLC) and sulfur analysis have had problems such as poor separation and unsuitability for routine analysis *(12–14)*. We have used a simple method for quantifying sulfolipids involving thin-layer chromatography (TLC) and photodensitometric scanning. Extraction of lipids from cyanobacterial cells was carried out by traditional organic-solvent methods *(15)*. Fresh cells were extracted by homogenization with chloroform-methanol (2:1 v/v). In order to remove any water-soluble impurities, potassium chloride was added to the extract. Two phases resulted; the upper-aqueous phase was removed and the organic phase, containing lipids, was evaporated under nitrogen.

Because the samples used in the kinetic studies of sulfolipid production are generally quite small, it was necessary to use a quantitative method

that had the advantages of sensitivity, simplicity, and speed. Thin-layer chromatography followed by photodensitometric scanning is an efficient method for quantifying sulfolipids. The crude lipid extract obtained from a 15-mL photobioreactor sample was separated with TLC using commercial precoated silica gel plates (EM Separations, HPTLC silica gel 60) with chloroform:methanol:acetic acid:water (85:15:10:3) as the solvent. To detect the lipids, a copper sulfate solution was sprayed on the plate and the plate was heated at 180°C for 20 min to char the lipids. The charred TLC plate was scanned by a Microtex ScanMaker 600 7S, using the software program Adobe Photoshop. The scanned image was analyzed by NIH *Image*, an image processing and analysis program for the Macintosh. The image-analysis program provides a density-profile plot where the areas of the peaks on the plot are proportional to the amount of lipid originally present *(16)*. A vertical slice in the center of the sulfolipid bands was analyzed and the mean density was correlated with a calibration curve. The calibration curve was obtained by scanning and analyzing TLC plates with known sulfolipid amounts in the range 0.5–4 μg. The reproducibility of the photodensitometric TLC-quantitative method using sulfolipid standards was within 12%. The concentration of sulfolipid in the sample was obtained and then divided by the concentration of cells in the sample to provide an intrinsic sulfolipid concentration (mg/g dry weight cells).

RESULTS AND DISCUSSION

Anabaena 7120 was grown in a fed-batch photobioreactor under four different light intensities: 66, 88, 120, and 170 $\mu E\ m^{-2}\ s^{-1}$. The growth curves are shown in Figs. 3–6. The biomass data are plotted as dimensionless biomass concentration, i.e., biomass concentrations are divided by the initial biomass concentration. In all experiments, the data show the characteristic stages of growth: the lag phase, the exponential-growth phase, and a phase of declining relative growth. It should be noted that there is an absence of a true stationary phase in these fed-batch cultures. When biomass has increased sufficiently such that absorbance of light by the dense culture causes a deviation from exponential growth, light-limited growth kinetics are observed.

Sulfolipid production as a function of light intensity is shown also in Figs. 3–6. Sulfolipid production under all light intensities show similar profiles, a sharp increase just after the exponential-growth phase followed by a gradual increase during the culture's severely light limited-growth phase.

The final dry weight of the cells increased with increasing external-light intensity. After 20 d, cultures grown under external-light intensities of 170, 120, 88, 66 $\mu Em^{-2}\ s^{-1}$ achieved concentrations of 2.5, 2, 1.53, and 1.24 g/L respectively. The sulfolipid concentrations after 20 d were 11.1, 11.5,

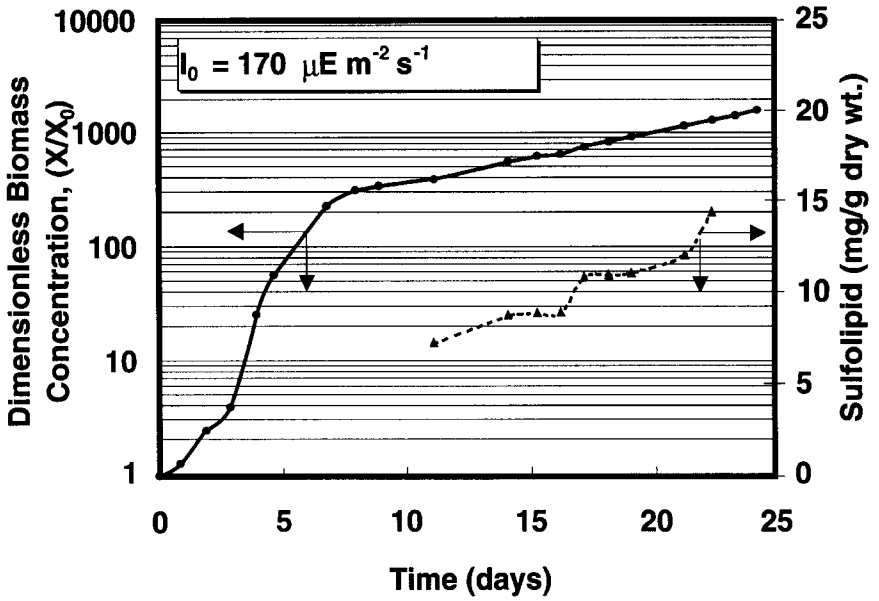

Fig. 3. Dimensionless biomass concentration and sulfolipid concentration vs time at a light intensity of 170 μE m^{-2} s^{-1} [X—biomass concentration (mg/L), X_O—initial biomass concentration (mg/L), I_O—external-light intensity (μE m^{-2} s^{-1})].

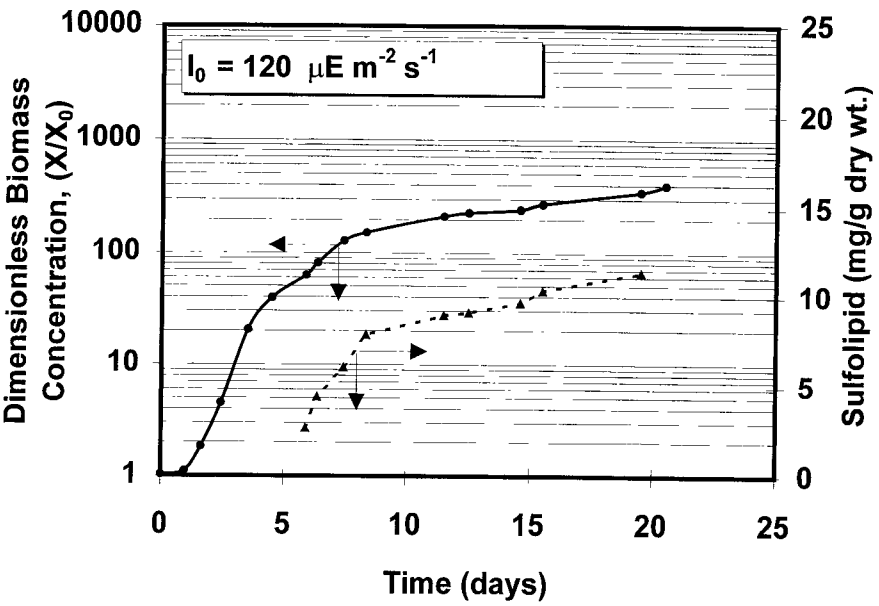

Fig. 4. Dimensionless biomass concentration and sulfolipid concentration vs time at a light intensity of 120 μE m^{-2} s^{-1} [X—biomass concentration (mg/L), X_O—initial biomass concentration (mg/L), I_O—external-light intensity (μE m^{-2} s^{-1})].

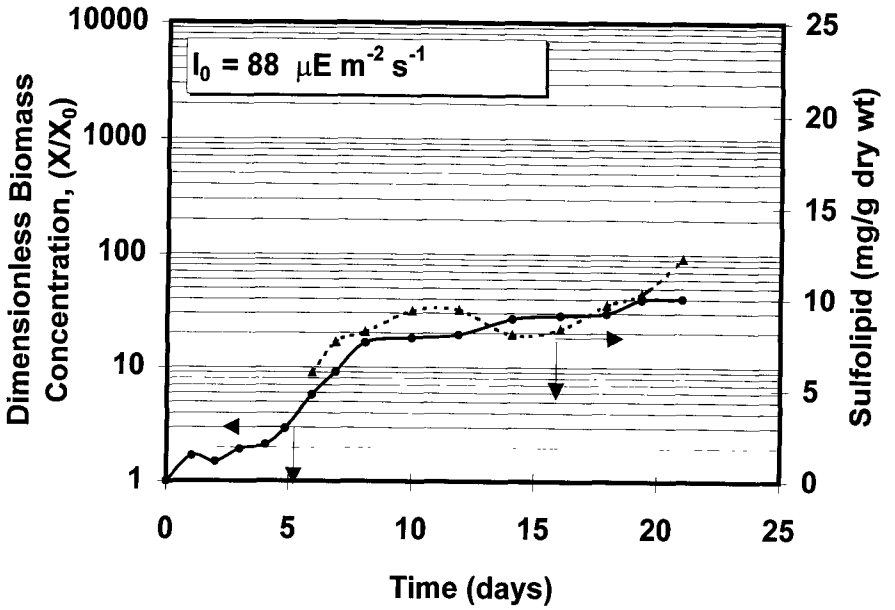

Fig. 5. Dimensionless biomass concentration and sulfolipid concentration vs time at a light intensity of 88 μE m^{-2} s^{-1} [X—biomass concentration (mg/L), X_O—initial biomass concentration (mg/L), I_O—external-light intensity (μE m^{-2} s^{-1})].

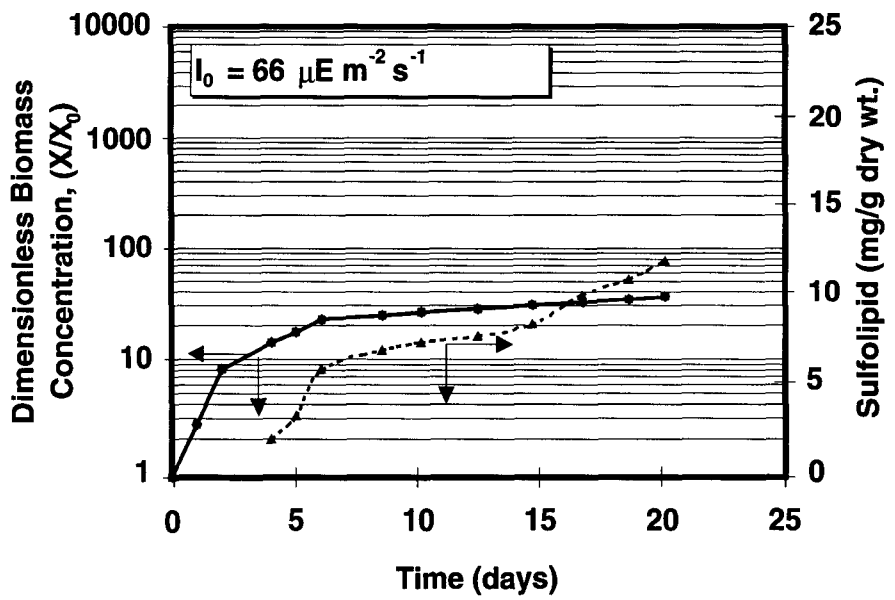

Fig. 6. Dimensionless biomass concentration and sulfolipid concentration vs time at a light intensity of 66 μE m^{-2} s^{-1} [X—biomass concentration (mg/L), X_O—initial biomass concentration (mg/L), I_O—external-light intensity (μE m^{-2} s^{-1})].

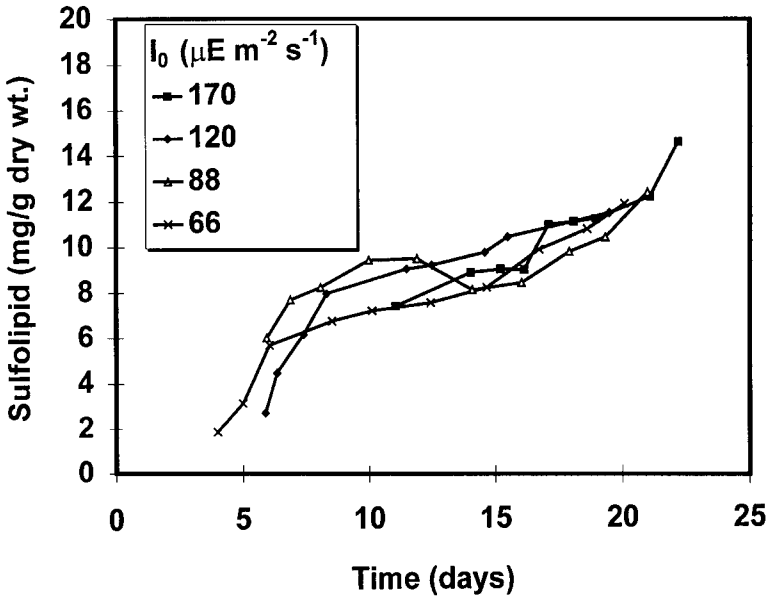

Fig. 7. Intrinsic sulfolipid concentration as a function of time for various external light intensities.

11.4, 10.6 mg/g dry weight cell at light levels of 170, 120, 88, 66 μEm^{-2} s^{-1} respectively. Benson and O'Brien *(10)* reported sulfolipid levels of 6.4 mg/g dry weight for *Chlorella pyrenoidosa* and 4.4 mg/g dry weight for alfalfa leaves. With our experimental arrangement, we have been able to achieve a sulfolipid production rate of 0.06 mgL^{-1} h^{-1} at a light intensity of 170 μEm^{-2} s^{-1} (Production rate = (dry cell weight/reactor volume) × (mass of sulfolipid/gram dry weight)/(length of time for batch culture)).

Our data show sulfolipid production is associated with specific stages in the growth of the cyanobacteria. The initial increase in sulfolipids can be considered to be associated with the declining exponential growth of the cells. The second stage, which occurs during extreme light-limited growth, can be considered non-growth associated.

It is shown in Fig. 7 that after approximately 2 wk, there is is no discernible effect of external-light intensity on intrinsic sulfolipid levels. This would lead one to believe that the actual light intensity within the photobioreactor after 2 wk is almost identical, irrespective of the different external-light intensities. This is most likely caused by reduction of light intensity by self-shading. At a high external-light intensity, the culture becomes dense quickly, resulting in a decrease in available light inside the photobioreactor. At the lower-light intensities, the combination of cell density and lower external-light intensity gives an average internal-light intensity that is similar to the average light intensity obtained with a higher external-light intensity. This phenomena can be explained by considering a simple model of the transient light-intensity distribution in the photobioreactor.

Light Model

Because light-irradiance affects the cyanobacterial growth and, presumably, sulfolipid production, the light distribution in the culture vessel is an important parameter in the design and analysis of photobioreactors for cyanobacterial cultures. Currently, there are no commercially available autoclavable light sensors for on-line monitoring of light intensity in laboratory-scale bioreactors. Even if such sensors were available, measurement of the transient-light distribution throughout the photobioreactor for a filamentous cyanobacteria culture would be very challenging. However, theoretical models can be developed that approximately solve the coupled growth and transient light-intensity distribution problem. In this model, the incoming irradiation is distributed by both scattering and absorbance. Thus, it is not uniform inside the culture and the light-intensity distribution changes with time as a result of changes in the culture density and optical properties of the culture medium. The concentration of organisms, the geometry of the light source, and culture vessel determine the distribution. Matsuura and Smith *(17)* proposed a modified Lambert-Beer relationship for a cylindrical vessel with cylindrical illumination. By using Lambert-Beer's law for this cylindrical system, the radiant-energy equation is

$$\frac{1}{r}\frac{\delta(rI)}{\partial r} = \pm\sigma\bar{n}I \tag{1}$$

where I is the intensity of radiation at radial position r, $\bar{n}$ is the volume averaged concentration of microorganisms (which is time-dependent)

$$\bar{n} = \frac{1}{\pi R^2}\int_0^R n(r,t)2\pi r dr \tag{2}$$

and σ is the absorption coefficient. The absorption coefficient was determined by measuring the absorbance at 680 nm of *Anabaena 7120* cultures at different concentrations; for our cultures $\sigma = 2819 cm^2/g$ dry weight.

The growth rate of microorganisms at any point in the photobioreactor is considered to depend on the light intensity at that point. Using the Post et al. *(18)* Monod-type model for light-limited kinetics we have

$$\frac{dn(r,t)}{dt} = \mu\bar{n}(t) = \left(\frac{I(r,t)}{K_I + I(r,t)}\right)\mu_{max}\bar{n}(t) \tag{3}$$

where $dn(r,t)/dt$ is the rate of growth at a point in a vessel, μ_{max} is the maximum specific growth rate, K_I is the half saturation coefficient, and t is the culturing time. This model assumes that light is the only rate-limiting factor and

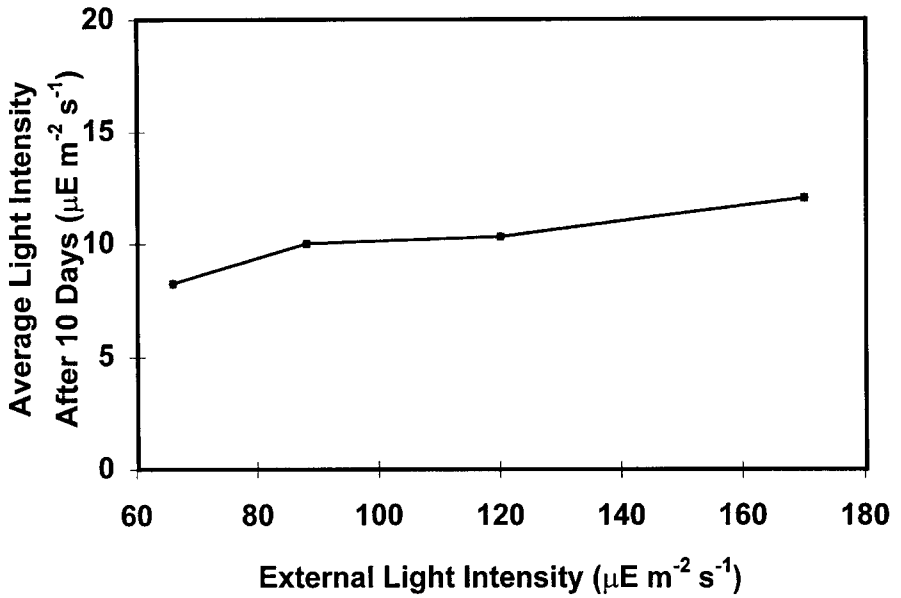

Fig. 8. Effect of external-light intensity on average-light intensity within the photobioreactor after 10 d.

the photobioreactor content is optically homogenous. When low-light intensities are applied ($K_I \gg$ I), the Monod equation approximates a first-order equation. Under those restrictions, the Monod equation can be presented as

$$\frac{dn(r,t)}{dt} = (\frac{I}{K_I})\mu_{max}\overline{n}(t) = \beta I(r)\overline{n}(t) \tag{4}$$

where $\beta = \mu_{max}/K_I$ is the first-order rate constant associated with all environmental factors (e.g., T, pH, medium composition). Because the environmental conditions are kept constant, it is expected that β should be constant as well.

Equation 4 is volume averaged to obtain the average rate of growth. This equation, coupled with Eq. 1 can be solved simultaneously to provide the light distribution within the photobioreactor as a function of time and radial position, with the boundary condition at the surface of the vessel, I = external-light intensity. The light profile can be averaged over the volume to give a volumetric average-light intensity.

$$I_{avg} = \frac{1}{\pi R^2}\int_0^R I(r,t)2\pi r dr \tag{5}$$

Figure 8 predicts that after 10 d the volume averaged-light intensity within the photobioreactor is very similar, regardless of the external-light

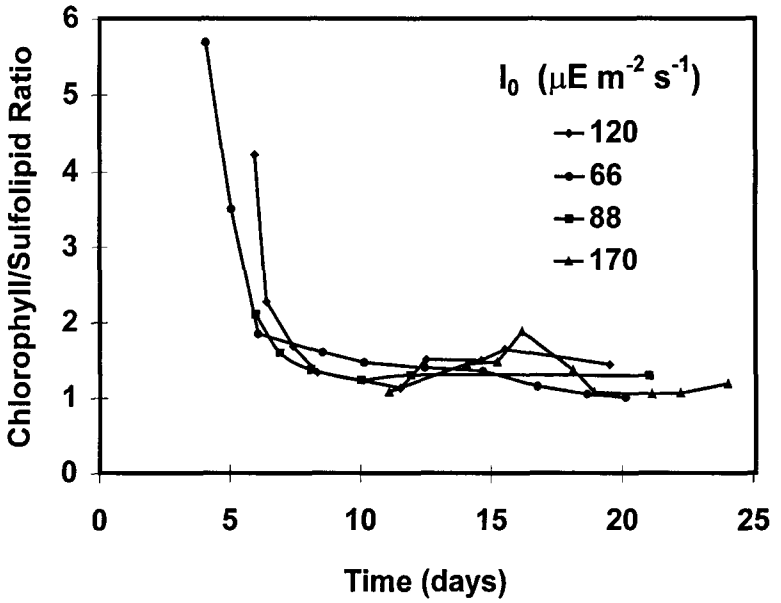

Fig. 9. Chlorophyll/sulfolipid ratio vs time for various external-light intensities.

intensity. The effect of the variations in the initial biomass concentration are included in the light-distribution analysis. This leads us to believe the similar profiles and similar sulfolipid levels after 10 d are caused by a comparable average-light distribution within the vessel.

Qualitative studies have been done which provide evidence for the accumulation of sulfolipids under low-light and low-growth conditions. It has been shown by Sallal et al. *(19)*, that freshwater cyanobacteria tend to increase their complement of sulfolipids when grown in the dark, after being grown in the light for 10 d. Miyachi et al. *(20)*, found that the S^{35} level increased in lipid samples when cultures initially grown for 15 d in the light were placed in the dark with CO_2-free air. This compares favorably with what we have found. In the latter stages of growth—around 10–15 d—the culture becomes severely light-limited. During this period, we see a gradual but significant increase in the specific sulfolipid concentration in the cells. A simple experiment was performed where *Anabaena 7120* cultures approximately 12 d after inoculation were grown in the dark for 10 d after having been grown at a light intensity of 15 μE $m^{-2}s^{-1}$. They were grown in 50-mL test tubes with air sparged through Pasteur pipets. The test tubes were covered with foil and no pH or temperature control was used. The cell concentration decreased from 0.00022 g/mL to 0.00007 g/mL in 10 d. However, the sulfolipid level increased from 9.1 mg/g dry wt to 25 mg/g dry wt. These results support the findings of Sallal et al. *(19)*.

The ratio of intrinsic chlorophyll concentration to intrinsic sulfolipid concentration was also determined, as shown in Fig. 9. After 10 d, the ratio

appears to be constant. This can be interpreted to mean that under severe light limitation, chlorophyll *a* and sulfolipid levels follow similar trends. This evidence supports the suspected role of sulfolipids in photosynthesis, because chlorophyll and sulfolipids follow similar trends. Several researchers have noted a correlation between the appearance of chlorophyll and the appearance of sulfolipids. Helmy et al. *(8)*, using radioactive S^{35}, exposed, dark-grown *Euglena gracilis* to light and found a direct correlation between the appearance of chlorophyll and sulfolipids. Weier and Benson *(21)* have speculated on the arrangement of lipids, including the sulfolipid, within the chloroplast or thylakoid membrane. They suggested that the sulfolipid may help orient the chlorophyll molecules; evidence of this was suggested by the interactions between the two species in vitro. By using space-filling models of SQDG, they were able to show very good potential interactions (both ionic and hydrophobic) with chlorophyll models. Also, it has been shown that antibodies raised against sulfolipids, can inhibit photosynthetic electron transport *(22)*. The inhibition was observed for a photosystem I mediated reaction between the electron donor 2, 6-dichlorophenol indophenol and the electron-acceptor methylviologen. Harwood *(23)* has presented various functions for sulfolipids in photosynthetic organisms. It is likely that sulfolipids have more than one function in the cell, such as thylakoid/chloroplast structure and photosynthesis.

CONCLUSIONS

Quantitative kinetic data are presented for growth and sulfolipid production during fed-batch photoautrophic culture of the filamentous cyanobacterium *Anabaena 7120*. This work shows there are two stages in sulfolipid production, an initial increase during the declining-exponential phase of growth followed by a gradual increase during the severely light-limited phase. Under various external-light irradiance conditions, sulfolipid production profiles were found to be quite similar. A simple light-distribution model coupled with a biomass-growth equation predicted the average light intensity in the photobioreactor after 10 d. The various external-light intensities studied resulted in very similar average internal-light intensities after 10 d owing to differences in the biomass concentrations. This explains the similarity of the final sulfolipid levels from photobioreactors with very different levels of external illumination. The correlation of chlorophyll *a* and sulfolipid levels gives supporting evidence for the role of sulfolipids in photosynthesis.

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