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Production of pure photosynthetic cell biomass for environmental biosensors

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Abstract

The feasibility of producing pure photosynthetic cell biomass heterotrophically and activating their photosynthetic apparatus through light illumination was investigated using *Chlorella sorokiniana* and *Euglena gracilis*. Although the chlorophyll contents of *Chlorella* and especially *Euglena* cells decreased during heterotrophic cultivation, photoactivation of the cells resulted in sharp increases in their chlorophyll and some other metabolites contents. Furthermore, the cells swiftly changed from heterotrophic to photoautotrophic metabolism and vice versa, when the culture conditions were cyclically changed from heterotrophic to photoautotrophic. Under this condition, the cells grew continuously and, depending on the light condition during the photoautotrophic phase, there was a stable fluctuation in the intracellular metabolites concentrations. In the case of *Chlorella*, for example, the protein content of the cells decreased during the heterotrophic phase and then increased during the photoautotrophic phase. The results implied that the cells retained their photosynthetic apparatus during the heterotrophic cultivation and can be easily activated by illuminating the culture.

An internally illuminated photobioreactor, which can be used for both heterotrophic cultivation and photoactivation of the cells was therefore constructed. Using this reactor, the cells are first cultivated heterotrophically to a high cell concentration and when the organic carbon source is completely exhausted, the reactor is illuminated for photoactivation of the cells. The photobioreactor has high mass transfer capacity (for efficient heterotrophic culture) and high light supply capacity (for efficient photoactivation). It can be illuminated by both artificial and solar light, can be maintained under strict sterile condition and easy to be scaled up.

Keywords: Biosensors; Photosynthetic cells; Photoautotrophic metabolism; Heterotrophic metabolism; Photoactivation; Chlorophyll de-differentiation

1. Introduction

There are a lot of metabolic and structural diversity among photosynthetic microorganisms. Some are very simple and specific in their nutritional requirement while others are very versatile. Although many members of the Cyanophyta can grow only photoautotrophically, many photosynthetic bacteria and green algae can grow photoautotrophically, mixotrophically and heterotrophically. Under heterotrophic condition, some are specific in their organic carbon metabolism while others can metabolize a wide range of carbohydrates and organic acids. Furthermore, some of them can grow under both aerobic and anaerobic conditions. Diversity of the photosynthetic microorganisms, their structural simplicity and nutritional versatility make them suitable for construction of different types of biosensors for environmental monitoring. For example, *Euglena* can grow under various conditions utilizing a wide range of organic compounds such as sugars, amino acids, fatty acids, fatty alcohols as well as protein [1]. Yet they are very sensitive to heavy metals and their rate of photosynthesis can be used as a sensitive indicator of heavy metal toxicity [2]. Thus, the heterotrophic and photoautotrophic metabolism in photosynthetic cells can be exploited for environmental monitoring under wide range of conditions. For biosensors based on the photosynthetic system (e.g. biosensors for herbicides), the cells must be photosynthetically active. Currently, most of the commercial cultivation of photosynthetic cells is done photoautotrophically in open cultivation ponds. Aside from the low productivities of such systems, the cells are usually contaminated by a variety of microorganisms [3]. In fact, successful production systems have been established only for those strains, which grow under extreme environments such as high pH and salinity.

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Heterotrophic cultures can be used for large-scale production of pure photosynthetic cell biomass. However, the problems of chloroplast de-differentiation under heterotrophic culture must be addressed if heterotrophically grown cells are to be used for construction of biosensors based on photosynthetic pathway. In other words, it is necessary that the cells retain their photosynthetic apparatus during heterotrophic cultivation. In this work, photoreactivation of chloroplast formation and metabolites syntheses in cells grown under heterotrophic condition was investigated, using *Chlorella sorokiniana* and *Euglena gracilis* as examples of photosynthetic cells. A photobioreactor was then constructed for efficient heterotrophic production of pure photosynthetic cell biomass and photoactivation of chlorophyll and metabolites syntheses.

2. Experimental

2.1. Microorganisms and media composition

C. sorokiniana and *E. gracilis* IAM E-6 (strain Z) were used in this study. They were obtained from the algal collection of the Institute of Applied Microbiology, University of Tokyo, Japan. The medium used for photoautotrophic cultivation of *Chlorella* was composed of (in g/l) KNO_3, 1.25; KH_2PO_4, 1.25; $MgSO_4 \cdot 7H_2O$, 1.25; $CaCl_2$, 0.04; $FeSO_4 \cdot 7H_2O$, 0.002; and A_5 solution, 1.0 ml l^{-1} (pH = 6.8). The composition of the A_5 solution was given in our previous paper [4]. *Euglena* was cultivated photoautotrophically in a modified Hutner medium, which was modified as follows (in g/l): $(NH_4)_2SO_4$, 2.25; KH_2PO_4, 0.44; $(NH_4)_2HPO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCO_3$, 0.2; H_3BO_3, 0.0144; vitamin B_1, 0.0025; vitamin B_{12}, 20 μg/l; trace element solution, 1.0 ml l^{-1} and Fe-solution, 1.0 ml l^{-1} (pH = 3.5). The composition of the trace element solution was $ZnSO_4 \cdot 7H_2O$, 4.4 g; $MnSO_4 \cdot H_2O$, 1.16 g; $Na_2MoO_4 \cdot 2H_2O$, 0.3 g; $CuSO_4 \cdot 5H_2O$, 0.32 g; $CoSO_4 \cdot 5H_2O$, 0.38 g and distilled water 100 ml while the Fe-solution was composed of 1.14 g $(NH_4)_2SO_4Fe(SO_4)_2 \cdot 6H_2O$ and 1.0 g EDTA per 100 ml of distilled water. In the case of heterotrophic cultivation of *Chlorella* cells, 15 g/l of glucose was added to the photoautotrophic medium as the organic carbon source. Also for heterotrophic cultivation of *Euglena*, the photoautotrophic medium was modified by replacing the ammonium sulfate with 5 g/l of sodium glutamate and adding 15 g/l of glucose as the organic carbon source. All the media were sterilized by autoclaving at 121°C for 15 min.

2.2. Pre-culture

The pre-culture for *Chlorella* was done by inoculating a loop of a slant culture into 80 ml of the medium in a 100-ml Roux flask and cultivating them under continuous illumination at 37°C for 24 h. Seven daylight fluorescent lamps for plant growth experiments (8FL-40-s-PG, National Electric, Tokyo), arranged in parallel on a vertical plane, were used as the light source. The light intensity at the surface of the flask was 200 μmol m^{-2} s^{-1}. Aeration and mixing were achieved by sparging air enriched with 5% CO_2 through a glass-ball filter, which was inserted to the bottom of the Roux flask, at 0.3 vvm. The pre-culture of *Euglena* cells was done as described for *Chlorella* cells except that 8 ml of the stock culture was used for inoculation while the light intensity at the surface of the Roux flask was 100 μmol m^{-2} s^{-1}.

2.3. Batch heterotrophic, mixotrophic and photoautotrophic cultivation of Chlorella

For the heterotrophic culture, the pre-culture was used to inoculate 500-ml Erlenmeyer flask containing 200 ml of the heterotrophic medium. The flask was wrapped with aluminum foil and incubated on a rotary shaker at 200 rpm. The cultivation temperature was 30°C. The conditions for the batch photoautotrophic and mixotrophic cultures were the same as described for the pre-culture. The preculture was used for the inoculation and the heterotrophic medium was also used for the mixotrophic cultivation.

2.4. Fed-batch heterotrophic cultivation of Euglena

The cultivation was done in a jar fermentor using the heterotrophic medium and 50 ml of 60% glucose solution was added each time glucose concentration in the medium was reduced to less than 1 g/l. Furthermore, when a decrease in the glucose uptake rate was observed, 25 ml of five times concentrated medium (without glucose) was added. The aeration rate (with a mixture of air and pure oxygen gas), the agitation speed and dissolved oxygen concentration in the broth were controlled at 0.5 vvm, 150 rpm and 0.8 ppm, respectively [5].

2.5. Photoactivation of heterotrophically grown Chlorella and Euglena cells

In the case of *Euglena* cells, an internally illuminated photobioreactor [6,7] containing 3.0 l of the heterotrophic medium was inoculated with the pre-culture broth to give an initial cell concentration of 0.05 g/l. It was cultivated heterotrophically, using glucose as the organic carbon source, until the glucose was completely consumed. During this heterotrophic phase, a mixture of ordinary air and nitrogen gas was used for aeration (0.5 vvm) through the ring sparger. The agitation speed and dissolved oxygen concentration were controlled at their optimum values of 100 rpm and 0.81 ppm, respectively. The dissolved oxygen concentration was controlled by varying the oxygen partial pressure in the aeration gas. The photobioreactor was not illuminated and aluminum foil was used to wrap the glass vessel. The cultivation was done at 25°C while the pH was

controlled at 3.5. When the glucose concentration was reduced to zero, 0.5% corn steep liquor was added and the reactor was illuminated by inserting four 4-W fluorescent lamps into the four housing glass tubes. The light intensity at the surface of the glass tubes was 163 μ mol m^{-2} s^{-1}, which is equivalent to a light supply coefficient of 0.374 kJ kg m^{-6} s^{-1} [8]. Air containing 5% CO_2 was used for aeration (0.5 vvm) and the temperature was increased to 30°C. The heterotrophic cultivation conditions for *Chlorella* were the same as described for *Euglena* except that only ordinary air was used for aeration and the dissolved oxygen concentration was not controlled, the cultivation temperature was 30°C and increased to 36°C during the photoactivation phase. The pH was controlled at 6.8 while corn steep liquor was not added to the culture during the photoactivation phase.

2.6. Cyclic autotrophic–heterotrophic metabolism in the cells

The cultivation was done in 2.0-l Roux flasks with a 1.5-l working volume under an alternating light/dark cycle of 10-h light illumination followed by 14-h dark incubation. They were inoculated with 200 ml of the pre-culture broth. Aeration and mixing were done as described for pre-culture except that two glass ball filters were used to ensure good mixing within the flask. Aeration during the dark period was provided by ordinary air. The light intensity at the surface of the flask during the light period was either 250 or 150 μmol m^{-2} s^{-1} for *Chlorella* or *Euglena* cells, respectively. During the dark period of each cycle, the Roux flasks were wrapped with aluminum foil and an organic carbon source was added to the culture. The amounts of organic acids added during the night were determined from their specific rates of uptake. However, in order to ensure that all the added organic carbon source was consumed during the night, the rates of addition were kept slightly lower than the rates estimated from the uptake rates. In the case of *Chlorella* cells, the rate of addition of glucose was 0.11 g g-cell^{-1} h^{-1} while in the *Euglena* cultures, ethanol was added at a rate of 0.04 ml g-cell^{-1} h^{-1}.

2.7. Analytical methods

Dry cell weight determinations were made using duplicate samples of the culture. The cells were washed with 0.5 M HCl to remove the precipitated salts and other non-organic substances, rinsed with distilled water, dried at 105°C for 24 h, cooled over silica gel in a desiccator, and weighted [9]. When the cell concentration was very low, it was estimated by measuring the optical density at 680 nm (Spectronic 20A, Shimadzu Scientific Instruments, Tokyo, Japan). In the latter case, the O.D. readings were converted to dry cell concentrations using predetermined calibration curves. The glucose concentration was determined with a glucose test kit (Wako, Osaka, Japan). The light intensities were measured by an analogue photometer (LI-185B, Licor, Nebraska, USA). Elemental analysis of the biomass (CHN) was performed on duplicate samples with a CHN elemental analyzer (Perkin-Elmer 2400, Norwalk, Conn., USA). The protein content was calculated as $N \times 6.25$ [10]. The chlorophyll content of the cells was measured according to the method described by Holden [11]. The absorbance of the methanol extract was measured at 650, 665 and 750 nm and the total chlorophyll concentration (μg ml^{-1}) was calculated as $25.5(A_{650}-A_{750}) + 4.0(A_{665}-A_{750})$. The ethanol concentrations were determined by liquid chromatography (Nihon Bunko Model 860-CO, Tokyo, Japan). The α-tocopherol content of the cells was determined from chloroform-methanol (1:2 v/v) extract as described by Shigeoka et al. [12], using a vitamin E homologues kit for biochemistry and analysis (Eisai, Tokyo, Japan).

3. Results and discussion

3.1. Growth curves of C. sorokiniana under photoautotrophic, heterotrophic and mixotrophic cultures

Fig. 1 shows the growth characteristics of *Chlorella* under various culture conditions. Although the maximum cell concentration under the photoautotrophic culture was only about 4 g/l, the final cell concentrations for the heterotrophic and mixotrophic cultures were 12 and 15 g/l, respectively, when the initial glucose concentration was 15 g/l. The above results were obtained under relatively good light condition. Thus, in large scale systems, the cell growth rate and thus the final cell concentration in the photoautotrophic culture are expected to be much lower while by increasing the organic carbon source con-

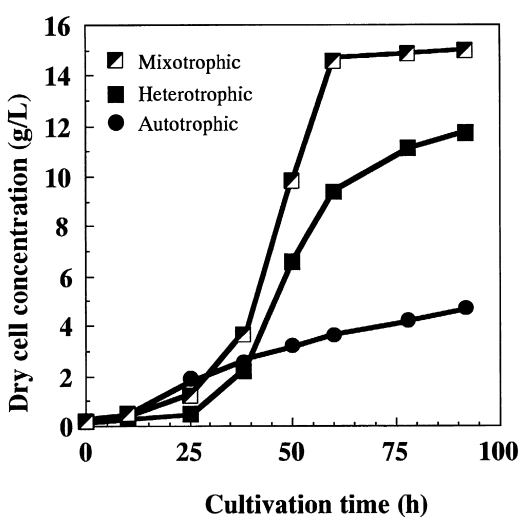

Fig. 1. Growth curves of *C. sorokiniana* under mixotrophic, heterotrophic and photoautotrophic conditions.

centration, much higher cell concentration can be obtained in the heterotrophic and mixotrophic cultures. In this work, the final cell concentration was higher in the mixotrophic than in the heterotrophic culture. This is consistent with some reports, which indicate that in mixotrophic culture, both the heterotrophic and photoautotrophic metabolisms proceed simultaneously and independently, resulting in the specific growth rate, and the final cell concentration in the mixotrophic culture being the sum of those in the photoautotrophic and heterotrophic cultures [13–17]. However, in general, the relationship between the photoautotrophic and heterotrophic metabolism under mixotrophic condition depends on the type of cell, the cell concentration, the light condition inside the reactor as well as the organic carbon concentration. For example, under light saturating condition, the mixotrophic growth rate of *Chlorella* was reported to be almost the same as the photoautotrophic growth rate [18,19] while under very high light intensity, cell growth is inhibited in the mixotrophic culture so that the mixotrophic growth rate is lower than that of the photoautotrophic culture [14].

3.2. Fed-batch heterotrophic cultivation of Euglena gracilis

An example of how heterotrophic cultivation can be used to produce high concentration of cell biomass is shown in Fig. 2. The cell growth rate and thus, the final cell biomass of *Euglena* are low in photoautotrophic culture when compared to the values usually obtained for *Chlorella*. Yet, by using a fed-batch cultivation method, almost 60 g/l of cell biomass is obtained in 1 week. We have already reported the use of fed-batch heterotrophic cultivation for efficient production of tocopherol and showed that although the growth rate was relatively lower with ethanol, high cell concentration was also obtained. In comparison with glucose, the tocopherol content was higher when ethanol was used as the organic carbon source [5]. The above results have shown that heterotrophic culture can be used to produce high concentration of pure photosynthetic cell biomass. However, several reports indicate that metabolites composition and contents in heterotrophically grown cells are different from those cultivated photoautotrophically [20,21]. Biosensors are based on the metabolic activity of the cells. Depending on the type of biosensor, heterotrophically cultivated cells may be suitable but when reactions in the photosynthetic pathway are involved, heterotrophic culture can be used only if the cells maintain their photosynthetic apparatus during the heterotrophic cultivation.

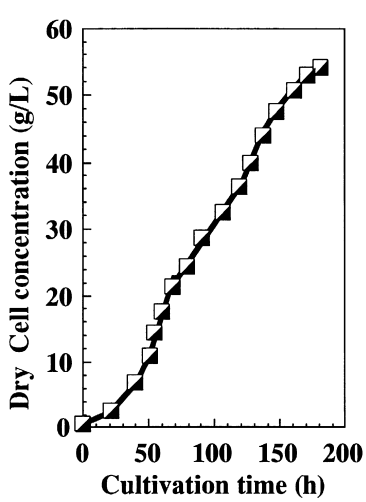

Fig. 2. Fed-batch heterotrophic cultivation of *E. gracilis* using glucose as the organic carbon source.

3.3. Activation of the heterotrophically grown cells

The chlorophyll and protein contents of heterotrophically cultivated *Chlorella* cells were 0.8 mg/g-cell and 47%, respectively. As shown in Fig. 3A, when the cells were illuminated, the chlorophyll content increased by almost 100% within 48 h. Also the protein content of the cells increased linearly with the illumination time, reaching 58% in 48 h. Fig. 3B shows that by illuminating heterotrophically cultivated *Euglena* cells, there were linear increases in both the chlorophyll and tocopherol contents. In comparison with *Chlorella*, the chlorophyll content of heterotrophically cultivated *Euglena* cells was very low and even after illumination, the chlorophyll content was much lower than the values usually obtained in photoautotrophic culture. The rate and extent of chlorophyll recovery during the photoactivation was dependent on the cell concentration and the light intensity. High light intensity was inhibitory to chlorophyll but stimulatory to tocopherol synthesis. Depending on the cell concentration, the higher the light intensity, the lower was the chlorophyll but the higher was the tocopherol content of the cell (data not shown).

The above results have shown that the chlorophyll and other metabolites contents of heterotrophically cultivated cells can be increased by simply illuminating the cells and aerating with carbon dioxide fortified air. Furthermore, the extent of chlorophyll degeneration during the heterotrophic culture and its recovery during illumination depends on the type of cell. In the case of *Chlorella*, by optimizing the illumination condition, both the chlorophyll and protein contents of the cells increased to the same levels as the cells cultivated photoautotrophically. Thus, pure photosynthetic cell biomass can be produced heterotrophically and then subjected to photoautotrophic condition to activate their photosynthetic system and bring them to the desired metabolic status before using them for biosensor construction.

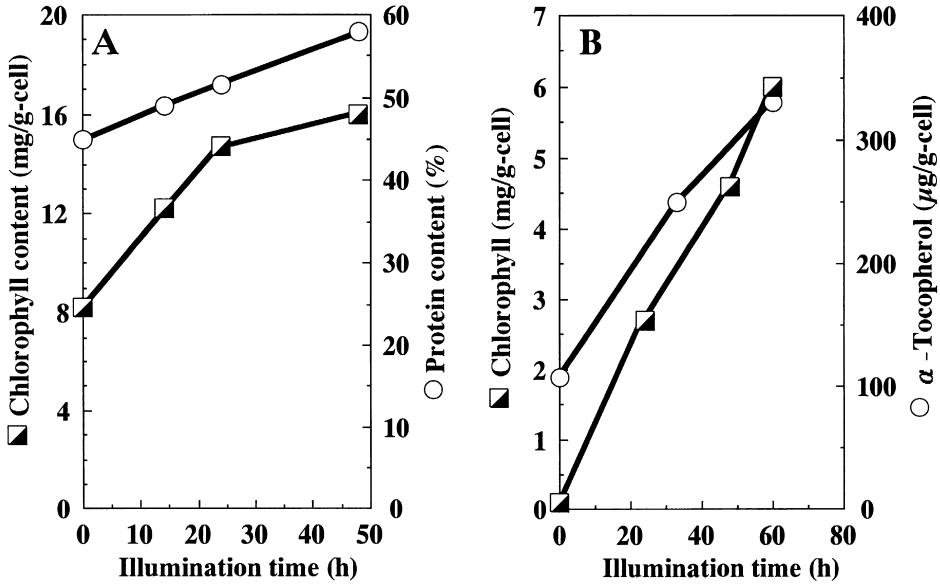

Fig. 3. Changes in chlorophyll and protein or tocopherol contents of *Chlorella* (A) or *Euglena* (B) during photoactivation of heterotrophically cultivated cells.

3.4. Cyclic heterotrophic–photoautotrophic metabolism in Euglena and Chlorella cells

In order to know if photosynthetic cells can easily adapt when transferred from heterotrophic to photoautotrophic conditions and vice versa, the cells were subjected to cyclic photoautotrophic–heterotrophic conditions by cultivating them photoautotrophically during the day and adding an amount of organic carbon source, which can be completely metabolized during the night [22]. As shown in Fig. 4, *Chlorella* cells showed continuous growth both in the day (photoautotrophic) and at night (heterotrophic). This shows that the cells are capable of swiftly switching from photoautotrophic metabolisms to heterotrophic metabolism and vice versa. Under this condition, the protein content of the cells increased during the day and then decreased at night, thus a stable fluctuation in the protein content of the cells was observed. However, at high cell concentration, light became limiting so that the protein content continued to decrease even during the day.

Fig. 5 shows that *Euglena* cells are also capable of changing from photoautotrophic to heterotrophic metabolism and vice versa. As in the case of *Chlorella*, there was continuous cell growth under cyclic photoautotrophic–heterotrophic conditions. However, the fluctuation in the metabolite (tocopherol) concentration was not very apparent. This is because ethanol was used as the organic carbon source. Tocopherol synthesis by *Euglena* under

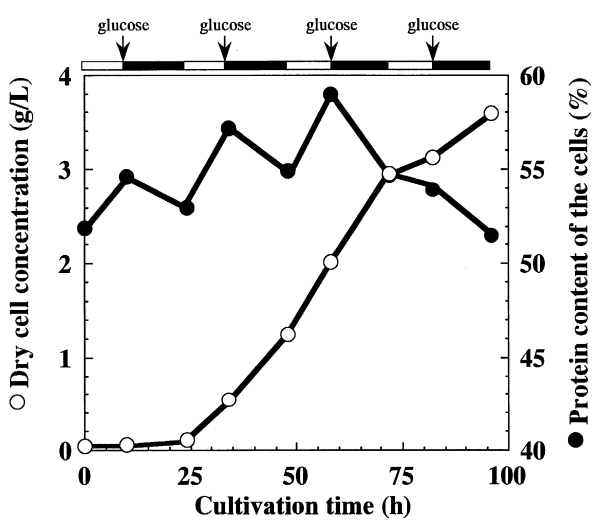

Fig. 4. *Chlorella* cell growth and changes in their cellular protein content under cyclic photoautotrophic–heterotrophic conditions.

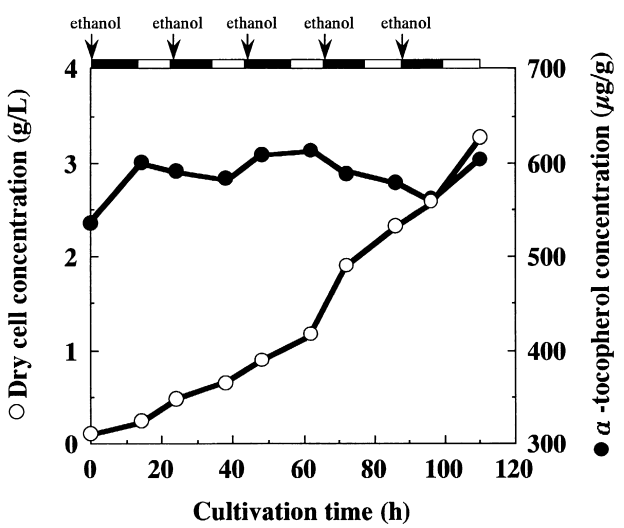

Fig. 5. Growth and tocopherol production by *E. gracilis* under cyclic photoautotrophic–heterotrophic conditions.

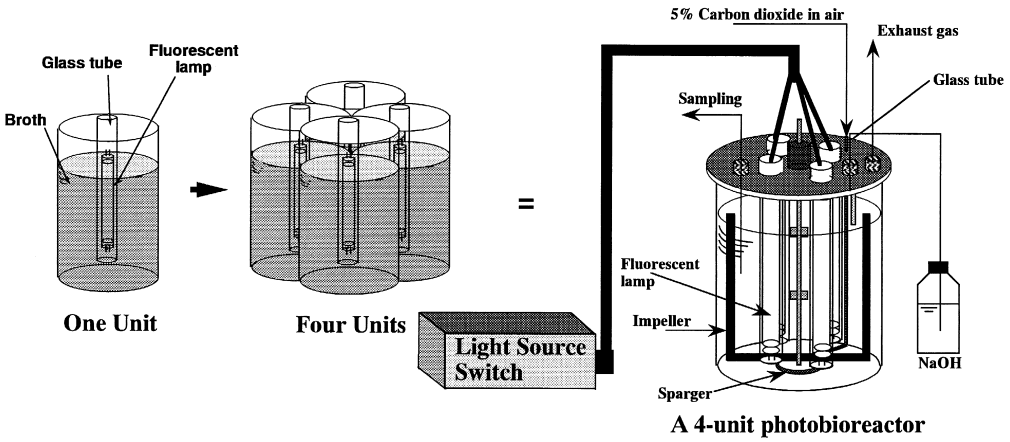

Fig. 6. Schematic diagram of an internally illuminated photobioreactor which can be used for efficient heterotrophic cultivation and photoactivation of photosynthetic cells.

heterotrophic condition is relatively high when ethanol is used as the organic carbon source [5]. The above results have also demonstrated that heterotrophic culture could be used for efficient production of pure photosynthetic cell biomass for biosensors even if the sensing is based on photosynthetic activity.

3.5. Photobioreactor for efficient heterotrophic cultivation and photoactivation of photosynthetic cells

A photobioreactor, which can be used for efficient heterotrophic production of pure photosynthetic cell biomass and photoactivation of the cells has been developed (Fig. 6). Efficient heterotrophic cultivation of photosynthetic cells requires a reactor with good mass transfer characteristics for efficient oxygen supply but many photosynthetic cells are very sensitive to hydrodynamic stress. Thus, a modified paddle impeller, which generate very low hydrodynamic stress and at the same time has very high mass transfer capacity [23,24] was installed for mixing. Aeration was done through a ring sparger (5.5 cm in diameter) with four holes (each 1 mm in diameter) and the glass housing units served as baffle plates to break up the gas bubbles, thus increasing the mass transfer capacity.

On the other hand, light activation of the heterotrophically grown cells requires a photobioreactor with high light supply coefficient. This is especially true for high cell density cultivation. Thus, internal illumination was used to achieve uniform light distribution within the reactor. In order to construct such a photobioreactor, the photobioreactor was considered as consisting of a number of units. One unit consists of a reactor volume (space) with a single lamp. The optimum volume of the reactor, which can be efficiently illuminated by the centrally placed lamp, is first determined experimentally. A larger reactor with the same light supply capacity as the single unit can then be constructed by increasing the number of these units in three dimensions [6].

The reactor can also be sterilized just as other bioreactors. Maintaining sterile condition is especially important during the heterotrophic phase. The risk of contamination by fast growing heterotrophs is very high during the heterotrophic cultivation since it contains organic carbon source.

The lamps were not fixed to the reactor so that illumination is achieved simply by inserting the lamps into the

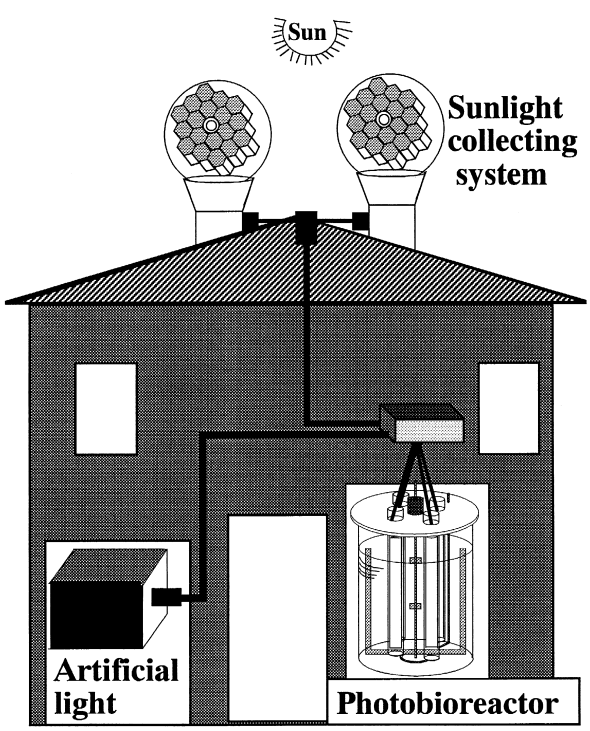

Fig. 7. Schematic diagram of the integrated solar and artificial light system for internal illumination of stirred tank photobioreactor.

glass tube housings. The lights are removed, sterilized and used for heterotrophic cultivation. At the end of the heterotrophic phase, the reactor is illuminated by inserting the lamps into the housing glass tubes.

3.6. Integrated solar and artificial illumination system

As shown in Fig. 7, the reactor could be illuminated by both artificial and solar light energy. The solar light was collected by a solar light collector, which is equipped with light tracking sensor so that the lenses rotate with the position of the sun. Thus, efficient light collection can be achieved irrespective of the position of the sun. The solar light collected by the lens was transmitted through optical fibers, which were connected to light radiators inside the reactor. The light radiators were cylindrically shaped and made of vertically etched transparent glass or quartz. The light intensity is almost uniform throughout the illumination surface of the light radiators.

In order to overcome the problems of diurnal variation in solar light intensity and prolonged periods of bad weather, an illumination system with integrated solar and artificial light sources was developed [25]. Metal halide lamp was used as the artificial light source. Parabolic mirrors were used to produce parallel rays from the lamps, which were then filtered and transmitted through the optical fibers to the light radiators inside the photobioreactor as described for the solar light. The same light radiators were used for both the solar light and metal halide lamp. A light intensity sensor monitors the solar light intensity and the artificial light is automatically switched on or off, depending on the solar light intensity. In this way, continuous light supply to the reactor is achieved by using solar light during sunny period, and artificial light at night and on cloudy days.

By using the photobioreactor described here, high cell concentration of pure photosynthetic cell biomass can be produced for environmental monitoring. For biosensors utilizing reaction systems that are not affected by heterotrophic cultivation, the biomass can be used directly. However, if photosynthetic or other enzymatic systems that are induced by light are required, the cell should be photoactivated before using them for biosensor construction.

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