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Sequential heterotrophic/autotrophic cultivation – An efficient method of producing *Chlorella* biomass for health food and animal feed

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Abstract

Sequential heterotrophic/autotrophic cultivation method was investigated for production of high concentration of *Chlorella* biomass with high cellular protein and chlorophyll contents. By using autotrophic growth medium, which contains glucose as organic carbon source, for heterotrophic culture, the protein and chlorophyll contents of the cells could be increased by simply illuminating the culture broth and aerating with CO_2-enriched air at the end of the heterotrophic culture. A system was then constructed for continuous sequential heterotrophic/autotrophic production of algal biomass. The system was composed of the conventional mini-jar fermentor for the heterotrophic phase and a tubular photobioreactor for the autotrophic phase. The exhaust gas from the heterotrophic phase was used for aeration of the autotrophic phase in order to reduce the CO_2 emission into the atmosphere. With this system, it was possible to produce high *Chlorella* biomass concentration (14 g L^{-1}) containing 60.1% protein and 3.6% chlorophyll continuously for more than 640 h. During the steady state, about 27% of the CO_2 produced in the heterotrophic phase was re-utilized in the autotrophic phase. When the tubular photobioreactor was replaced with a 3.5-L internally illuminated photobioreactor, the productivity increased from 2 g L^{-1} d^{-1} to 4 g L^{-1} d^{-1}. However, the chlorophyll content of the cells was lower due to the lower light supply coefficient of the photobioreactor.

Introduction

The potential of microalgae as a food staple in the human diet has been investigated for many years in different countries (Vincent, 1969; Schwarz et al., 1995). Although research on the production of microalgae as protein supplement in foods is declining, the number of malnourished children are still on increase in many developing countries while in many developed countries such as Japan, algal biomass such as *Chlorella* and *Spirulina* are produced commercially, primarily for consumption as health food. The high production cost currently prevents wide usage of microalgae as animal feed but numerous nutritional experiments clearly demonstrate the high value of some species of microalgae as protein supplement for fish, cattle, hogs, and chickens (Soeder, 1986). There is thus a need for development of efficient systems for algae biomass production.

In commercial production of algal biomass, high cell density culture are desirable in order to reduce the cost for down-stream processing. Terry & Raymond (1985) made a detailed discussion on the historical background and various systems used for autotrophic production of microalgae. They classified them into ponds, channeled and shallow circulating systems and discussed the merits and demerits of each system. Because of variation in the climatic condition, growth characteristics of the microorganism used as well as methods of determining and reporting productivities, it is extremely difficult to compare the productivities of the various systems. However, the highest productivity reported was less than 30 g m^{-2} d^{-1}. This corresponds to a maximum productivity of 0.3 g L^{-1} d^{-1} assuming a uniform reactor depth of 10 cm. This is still very

low compared to more than 150 g L^{-1} d^{-1} productivity which can be obtained in heterotrophic cultures (Ogbonna & Märkl, 1993). Laws et al. (1986) gave a list of productivities achieved with various systems in various parts of the world but the highest productivity was still less than 35 g m^{-2} d^{-1}. They developed a system with a depth of 10.9 cm in which foils were inserted to create systematic vertical mixing. This resulted in two times increase in the productivity but even then, the maximum productivity was 35 g m^{-2} d^{-1} (0.32 g L^{-1} d^{-1}). Another problem with most of the systems reported is the low cell concentration which would result in an increase in the harvesting costs. As a solution to these problems, development of closed systems with better light supply and control of culture conditions has been investigated by many researchers. A lot of closed systems such as tubular (Richmond et al., 1993), helical (Watanabe et al., 1995), incline or horizontal thin panel (Tredici et al., 1991) and internally illuminated (Ogbonna et al., 1996a) photobioreactors have been developed. Although, with these closed systems, the productivities are higher than those of the open systems (as high as 1.5 g L^{-1} d^{-1} was reported by Richmond et al. 1993), the increased cost of construction and operation of the closed systems offset the increase in productivity. Thus most of the companies such as Cynotech Corporation and Dainippon Ink and Chemicals inc. still use the open cultivation ponds. There is therefore a need for development of alternative culture systems.

Some algae are capable of metabolizing organic carbon sources so that heterotrophic cultivation can be used to achieve high cell concentrations. However, under such conditions, the photosynthetically derived products are not accumulated and the main advantage of photosynthetic cell cultures is lost. Endo et al. (1974) reported that the chlorophyll content of heterotrophically grown *Chlorella regularis* is only 1.8% which is less than 50% of the value (3 8%) in autotrophically grown cells. Also the provitamin A, α-tocopherol and carotenoids contents of the heterotrophically grown cells were lower than those of the autotrophically grown ones. Experiments in our laboratory (unpublished) have also shown that in some other species such as *Euglena gracilis* strain IAM E-6 (strain Z), chlorophyll synthesis is almost completely inhibited under heterotrophic conditions while the α-tocopherol content is less than 25% of the values obtained in autotrophic cultures.

One possible method of producing high biomass concentration with high cellular concentration of photosynthetic products is the use of mixotrophic culture, where both organic carbon sources and light energy are simultaneously supplied to the reactor. However, in mixotrophic culture, the cellular concentrations of the photosynthetic products depend on the relative heterotrophic and autotrophic growth rates. At high cell concentrations, light becomes limiting and the autotrophic growth rate is very low in comparison with the heterotrophic growth rate. Under this condition, both the protein and chlorophyll contents of the cells are much lower than those of the autotrophic cultures. Ogawa et al. (1981) reported that for *Chlorella vulgaris* and *Scenedesmus acutus*, the chlorophyll contents of the cells (chlorophyll *a* + chlorophyll *b*) in mixotrophic cultures were 61.4% and 45.5%, respectively of their values in autotrophic cultures.

We have been working on development of systems for efficient cultivation of photosynthetic cells. We have shown that mixing in open ponds should be controlled in relation to the light intensity, cell concentration and depth of the reactor. On cloudy days, mixing should be minimal while at night, it is better to stop culture agitation (Ogbonna et al., 1995a). Also, night biomass losses were studied and we have reported that by adding very low organic carbon source during the night (a quantity that can be completely metabolized at night), continuous cell growth can be achieved under light/dark cycles without adverse effect on the composition of *Chlorella pyrenoidosa* (Ogbonna et al., 1996b). In order to develop a large scale stirred tank photobioreactor, the relative significance of the various growth phases during batch cultivation of photosynthetic cells was first investigated (Ogbonna et al., 1995b) and a light supply coefficient was proposed as an engineering parameter for design and quantitative evaluation of light condition inside photobioreactors (Ogbonna et al., 1995e). Using the above parameter, an internally illuminated stirred tank photobioreactor was developed and a method for its efficient scale up was proposed (Ogbonna et al., 1996a, Ogbonna & Tanaka, 1997). In this paper, sequential heterotrophic/autotrophic cultivation system, whereby high concentration of monoalgae biomass from a heterotrophic culture is passed through a photobioreactor for accumulation of photosynthetic products, was investigated as a means of producing high quality algal biomass for food and feed. Also utilization of the exhaust gas from the heterotrophic phase (high in CO_2) for aeration of the autotrophic phase was investigated as a means of reducing CO_2 emission into the atmosphere.

Materials and methods

Microorganism and medium composition

Chlorella pyrenoidosa C-212 was used in this study. It was obtained from the algal collection of the Institute of Applied Microbiology, University of Tokyo, Japan. The basal medium reported by Endo et al. (1974) was modified as follows (in g L^{-1}) Urea, 1.2, KH_2PO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.02; Sodium citrate, 0.05; Fe-solution, 0.16 mL; and A5 solution, 0.8 mL. This modified medium was used for both heterotrophic and autotrophic phases but in the case of heterotrophic phase, glucose was added as the carbon source. The Fe-solution was composed of 25 g $FeSO_4 \cdot 7H_2O$ and 33.5 g EDTA per liter of distilled water. The A_5 solution described in our previous paper (Ogbonna et al., 1995a) was used except that 2.5 g $MnSO_4 \cdot 7H_2O$ was replaced with 1.81 g $MnCl_2 \cdot 4H_2O$. The pH of the medium (excluding the urea) was adjusted to 6.5 before autoclaving at 121 °C for 15 min. Filter-sterilized urea solution was added to the autoclaved medium after cooling to room temperature.

Cultivation conditions

The pre-culture was done heterotrophically by inoculating two loops of the slant into a 500 mL Erlnmeyer flask containing 100 mL of the basal medium and 7 g L^{-1} of glucose as the carbon source. The flask was wrapped with aluminum foil (to shade off the light) and incubated on a rotary shaker at 200 rpm. The cultivation temperature was 30 °C.

Batch Sequential heterotrophic/autotrophic cultivation

Erlnmeyer flask containing 100 mL of a four times concentrated basal medium with 28 g L^{-1} of glucose as the carbon source, was inoculated with the pre-culture to give an initial cell concentration of 0.05 g L^{-1}. The cultivation conditions were as described for the pre-culture. When the glucose concentration was reduced to zero, the culture broth was transferred into a 100 mL Roux flask and cultivated autotrophically at 36 °C. Seven daylight fluorescent lamps for plant growth experiments (8FL-40-s-PG, National Electric Co. Ltd., Tokyo), arranged in parallel on a vertical plane, were used as the light source. The light intensity at the surface of the flask was 310 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.

Continuous sequential heterotrophic/autotrophic cultivation in a system using a tubular photobioreactor for the autotrophic phase

The experimental set-up used for the continuous sequential heterotrophic/autotrophic cultivation is shown in Figure 1. The heterotrophic phase is composed of a conventional 2.5 L aerated stirred mini-jar fermentor (Iwashiya Bioscience Co Ltd, Saitama, Japan) with a working volume of 2.0 L. It was inoculated with a pre-culture to give an initial cell concentration of 0.05 g L^{-1}. The cultivation temperature, aeration rate (with ordinary air), and agitation speed were 30 °C, 0.5 vvm and 300 rpm, respectively. During the cultivation, the pH was maintained at 6.5. When the glucose concentration was reduced to zero, continuous feeding of 5 times concentrated basal medium containing glucose (35 g L^{-1}) was started at a feed rate of 15 mL h^{-1}. The effluent from the heterotrophic culture was passed through the tubular photobioreactor whose working volume was 450 mL. The temperature of the autotrophic phase was maintained at 36 °C by immersing the photobioreactor in a water bath. It was illuminated using the system described for the batch culture. The light intensity at the surface of the photobioreactor was 310 μmol m^{-2} s^{-1}. The exhaust gas from the heterotrophic phase was used for aeration of the photobioreactor, through the glass ball filters, at 0.5 vvm. The average residence time of the broth in the photobioreactor was 30 h.

Use of an internally illuminated photobioreactor for the autotrophic phase

Continuous cultivation in which the tubular photobioreactor was replaced with an internally illuminated photobioreactor was also investigated. Easy of scale-up was the primary design criterion for this photobioreactor. It consists of units, with each unit composed of a light source in a glass tube surrounded by a tank-type reactor. The unit's size optimizes the light supply coefficient – an engineering parameter that reflects both the level of light energy supplied per unit volume and the distribution of the light energy within the photobioreactor (Ogbonna et al., 1995c). By increasing the number of units in three dimensions, a large photobioreactor with a light supply coefficient similar to that

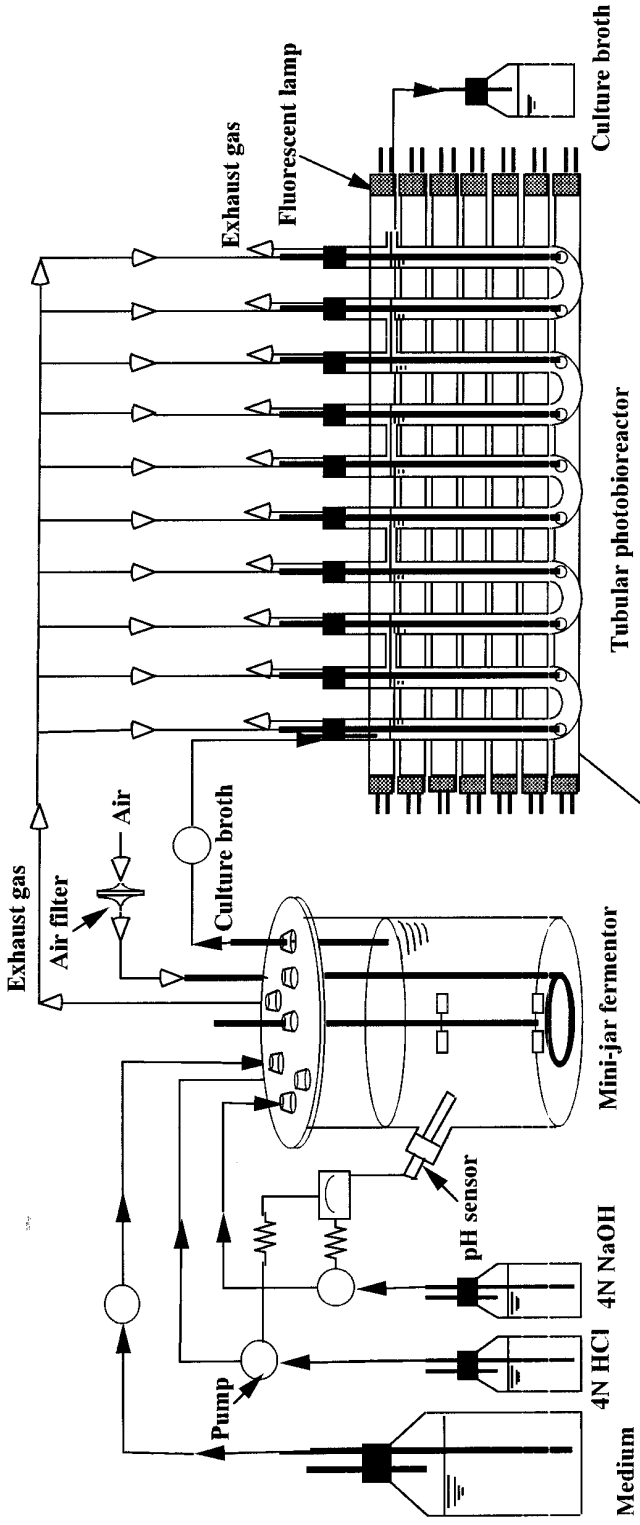

Figure 1. Schematic diagram of the system used for sequential heterotrophic/autotrophic cultivation.

of a single unit can be constructed. The photobioreactor was constructed for autotrophic cultivation of *Chlorella pyrenoidosa*, using a light supply coefficient with maximum cell yield from the supplied light energy. Detailed description of this photobioreactor was given in our previous paper (Ogbonna et al., 1996a).

The same mini-jar fermentor was used for the heterotrophic phase while the working volume of the photobioreactor was 3.0 L. Both bioreactors were filled with 28 g L^{-1} glucose in 4 times concentrated basal medium and inoculated with the pre-culture to give an initial cell concentration of 0.05 g L^{-1}. The photobioreactor was wrapped with aluminum foil without illumination and both bioreactors were cultivated heterotrophically. The agitation speed for the photobioreactor was 200 rpm while other cultivation conditions were as described above for the heterotrophic phase. In order to ensure homogeneity in both bioreactors, pump was used to circulate the culture broth between the two bioreactors. When the glucose concentration was reduced to zero, the medium circulation between the two bioreactors was stopped and the photobioreactor was illuminated by four 4-W fluorescent lamps. The light intensity at the surface of the glass tubes housing the lamps was 163 μmol m^{-2} s^{-1}, giving a light supply coefficient of 0.374 kJ kg m^{-6} s^{-1} (Ogbonna et al., 1995c, 1996a). Continuous sequential heterotrophic/autotrophic cultivation was started by continuously feeding the concentrated medium into the mini-jar fermentor and feeding the effluent from the mini-jar fermentor into the photobioreactor. The feed rate was 60 mL h^{-1} while other cultivation conditions were as described before.

Analytical methods

Cell dry 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 weighed (Ogbonna et al., 1996b). When the cell concentration was very low, it was estimated by measuring the optical density at 680 nm (Spectronic 20A. Shimadzu Scientific Instruments, 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 Pure Chemical Industries, 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 × 6.25 (Canizares-Villanueva et al., 1995). The chlorophyll content of the cells was measured according to the method described by Holden (1976). 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 carbon dioxide content of the exhaust gas was analyzed by gas chromatography (Model GC-8A, Shimadzu Co. Ltd, Japan).

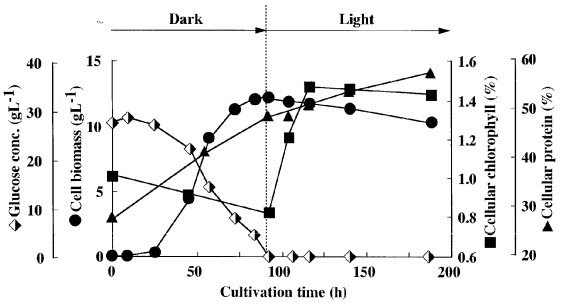

Figure 2. Changes in glucose and cell biomass concentrations in the broth as well as chlorophyll, and protein contents of the cells during batch sequential heterotrophic/autotrophic cultivation of *Chlorella pyrenoidosa*. After heterotrophic cultivation in Erlnmeyer flask, the cells were transferred into a Roux flask illuminated and aerated with 5% CO_2-enriched air.

Results

Batch process

The results of heterotrophic cultivation in an Erlnmeyer flask followed by autotrophic cultivation in a Roux flask is shown in Figure 2. When the heterotrophically grown cells were transferred into a Roux flask and cultivated autotrophically, the cell concentration decreased slightly but the chlorophyll content of the cells increased rapidly within the first 24 h. Longer autotrophic cultivation did not result in any further significant increase in the chlorophyll content of the cells. In the case of protein content, the increase was gradual but continued even after 96 h of autotrophic cultivation.

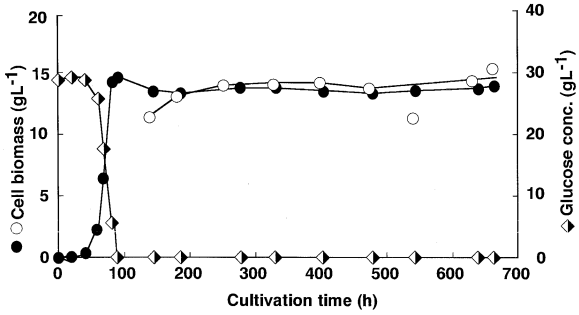

Figure 3. Time courses of glucose and cell biomass concentrations during continuous sequential heterotrophic/autotrophic cultivation of *Chlorella pyrenoidosa*, using the system shown in Figure 1. The closed and open symbols represent heterotrophic and autotrophic phases, respectively.

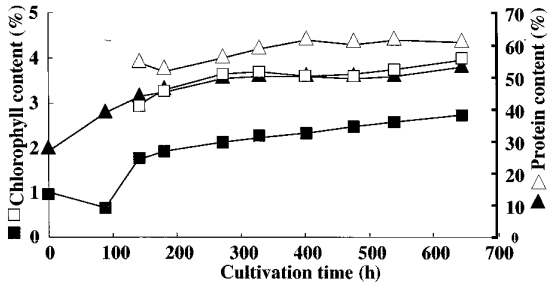

Figure 4. Changes in chlorophyll and protein contents of *Chlorella pyrenoidosa* cells during the continuous sequential heterotrophic/autotrophic cultivation using the system shown in Figure 1. The closed and open symbols represent heterotrophic and autotrophic phases, respectively.

Continuous process

As shown in Figure 3, after 91 h of batch heterotrophic cultivation, the glucose concentration was reduced to zero and the continuous process was started by continuously feeding the stock nutrient into the mini-jar fermentor and feeding the effluent from the mini-jar fermentor into the tubular photobioreactor. The glucose concentration in the effluent of the heterotrophic phase remained zero throughout the cultivation. During more than 640 h of cultivation, there was no significant differences in the cell concentrations between the effluents from the heterotrophic and autotrophic phases (Figure 3). The changes in the cellular protein and chlorophyll contents of the cells in the heterotrophic and autotrophic phases are shown in Figure 4. During the steady states, the protein and chlorophyll contents of the cells in the effluent of the autotrophic phase increased to very high values of 60.1 and 3.6%, respectively.

When the internally illuminated photobioreactor was used for the autotrophic phase, the productivity increased from 2 g L^{-1} d^{-1} to 4 g L^{-1} d^{-1}. The process was also stable for more than 600 h (data not shown) and during the steady state, the cellular protein and chlorophyll contents increased from 51 and 1.9 in the heterotrophic phase to 63.5 and 2.5% in the autotrophic phase, respectively.

Re-utilization of the CO_2

The CO_2 concentrations in the exhausts of the heterotrophic and autotrophic phases are shown in Figure 5. By using the exhaust gas from the heterotrophic phase for aeration of the autotrophic phase, the CO_2 content of the gas was reduced from 0.44% to 0.33% during the steady state.

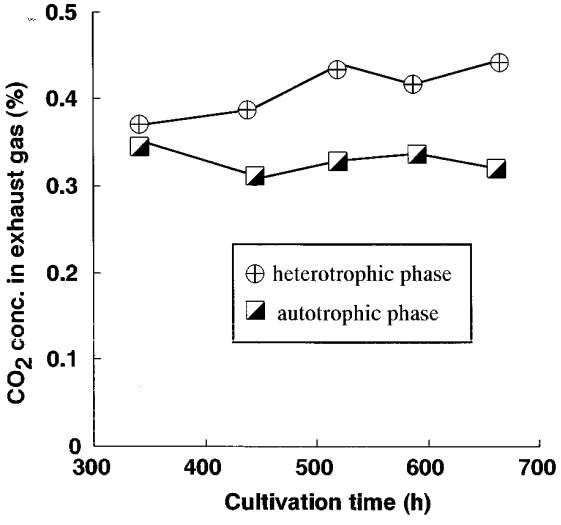

Figure 5. Carbon dioxide contents of the exhaust gases from the heterotrophic and autotrophic phases during the steady state of the continuous process. The exhaust gas from the heterotrophic phase was used for aeration of the autotrophic phase.

Discussion

The results of batch cultivation show that both the protein and chlorophyll contents of the heterotrophically grown cells can be increased by simply switching from heterotrophic to autotrophic conditions (illuminating the culture broth and aerating with CO_2-enriched air). The optimum duration of the autotrophic phase (in

the case of continuous process, the optimum residence time in the photobioreactor) would depend on the cell concentration and the light supply coefficient of the reactor. As shown in Figure 2, by considering the facts that the chlorophyll content of the cells reached a maximum value after 24 h of autotrophic culture and that by extending the period of autotrophic culture, the protein content increased only slightly while the cell concentration decreased, it was concluded that the optimum period of autotrophic culture under this experimental condition (12 g-cell L^{-1} in a 100 mL Roux flask illuminated from one surface at a light intensity of 310 μmol m^{-2} s^{-1}) is 24 h.

In this process, it is important that the glucose is completely consumed in the heterotrophic phase. If glucose is carried over to the autotrophic phase, the cells would grow mainly by the heterotrophic metabolism since at high cell concentration, the illuminated volume fraction is very low and the cells spend most of the time in the non-illuminated part of the reactor. This would result in cells with low protein and chlorophyll contents. Furthermore, although many efficient closed systems have been proposed, open ponds are still used for most commercial production of algal biomass because of the problems with scale-up as well as construction and operation costs of the closed systems. For economic reasons, it is desirable to use open ponds for the autotrophic phase. Passing over of glucose from the heterotrophic to the autotrophic phase could cause serious contamination problems in the open cultivation ponds.

The chlorophyll and protein contents of the cells obtained in the continuous process are close to the maximum values reported for purely autotrophically grown cells (Endo et al., 1974). This shows that by using the sequential heterotrophic/autotrophic cultivation system, high concentration of cells with high protein and chlorophyll contents can be produced continuously for long period of time. The cell concentration obtained in this study (14 g L^{-1}) is more than four times higher than the maximum values we have obtained for autotrophic cultivation of this strain using various types of photobioreactors (Ogbonna et al., 1995a, b, 1996 a, b) while the productivity achieved in this work (4 g L^{-1} d^{-1}) is more than twice higher than the value reported for most systems (Richmond et al., 1993, Laws et al., 1986). Higher cell concentration can easily be obtained by using higher glucose concentration in the heterotrophic phase, but it would be very difficult to efficiently supply light to such a dense culture. The chlorophyll content of the cells obtained with the internally illuminated photobioreactor was lower than that of the tubular photobioreactor because the light supply coefficient of the former (0.374 kJ kg m^{-6} s^{-1})was lower than that of the latter (1.9 kJ kg m^{-6} s^{-1}). Since the autotrophic phase is the limiting step in this process, the efficiency and productivity of this system can be improved by using a photobioreactor with high light supply coefficient.

The main advantages of this system over the mixotrophic culture is that the heterotrophic and autotrophic phases can be optimized independently. Efficient mixotrophic cultivation requires a sterilizable (to prevent contamination) reactor with high mass transfer (for efficient heterotrophic growth) and light supply (for efficient autotrophic growth) coefficients. Industrial scale stirred tank bioreactors are suitable for heterotrophic growth but are not good for autotrophic growth because of the difficulty of supplying light uniformly to the reactor.

On the other hand, most of the industrial scale photobioreactors (open ponds, tubular or tin panels) are not suitable for mixotrophic growth because of the relatively low mass transfer capacities and more especially because of the difficulties in maintaining sterility to avoid contamination in the presence of organic carbon sources. However, with the system proposed in this study, the conventional stirred tank bioreactors and photobioreactors can be used for the heterotrophic and autotrophic phases, respectively.

About 27% of the CO_2 produced in the heterotrophic phase was re-utilized in the autotrophic phase. The amount of CO_2 fixed in the autotrophic phase is comparable with the values reported for biological CO_2 fixation systems. Using a photobioreactor which is internally illuminated with optical fibers, the CO_2 concentrations in the exhaust gas were 0.37% and 0.935%, when the CO_2 concentration in the aeration gas were 0.55% and 1.10%, respectively (Takano et al., 1992). Longer residence time of the gas in the photobioreactor is required for further reduction in the CO_2 content of the exhaust gas. With this system, aerating only from one end and having the gas exit from the other end would result to high residence time of the gas in the photobioreactor but the gas bubbles would lead to poor flow of the culture broth. A compromise has to be made between the gas residence time and the culture flow characteristic when deciding the number and positions of the aeration and gas exit points.

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