Enhancement of the Growth of Marine Microalga *Chlorella* sp. from Mixotrophic Perfusion Cultivation for Biodiesel Production

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For biodiesel production, marine alga *Chlorella* sp. was grown by continuously perfusing 35–40 °C warm waste seawater in a 14 L photobioreactor. The temperature of the culture system was maintained at minimum of 25 °C at 1.0 L h^{-1} of perfusion rate with no other heating systems. Under mixotrophic conditions with only 1 g L^{-1} of glucose, a maximum cell concentration of 7.1 ($g_{dry\ mass}$ L^{-1}) was observed for over 95 days cultivation while 3.3 ($g_{dry\ mass}$ L^{-1}) of maximum cell concentration was maintained for autotrophic growth. The highest total lipid production was also obtained as w = 21.6 % with w = 50 % of palmitic and oleic acids, which would be a proper composition for biodiesel. The specific lipid production rate remained relatively constant until the end of the perfusion cultivation while it decreased in latter periods of batch cultivation. In general, the perfusion process shows a better performance because of the continuous supply of fresh medium and high accumulation of biomass. These results prove that this unique recycling system can be employed where geographically the temperature is a more critical factor for outdoor mass cultivation.

Key words:
Chlorella sp., mixotrophic, lipid production, biodiesel, photobioreactor

Introduction

There has been a great demand to increase various renewable resources for the production of biodiesel. These natural resources mainly include agricultural products and their by-products, such as energy crops, trees, food, and feed, etc.[1] However, for biodiesel, the use of agriculture-based raw materials poses a serious problem in disrupting supply and demand. In addition, the yield of biomass from agricultural biomass is low; for example, the oil yields from sunflower and soybean were in the ranges of 18.0 % to 25.5 % from 1,500 kg ha^{-1} ~ 2,700 kg ha^{-1}).[1,2] Recently, marine microalgae have been extensively investigated as a potential natural resource to produce biomass for biodiesel, because they has great ability to absorb CO_2, a major cause for global warming, and under suitable culture conditions produce a higher yield per unit of arable area than agricultural biomass. In particular, the light source and the composition of the culture medium play a major role in increasing the biomass yield (i.e., the amounts of biomass per unit area) from marine microalgae. Such higher yields are critically important for raw materials to be produced on a commercial scale.[3]

It has been considered important to maintain the maximum cell concentration of microalgae under scaled-up outdoor conditions to meet the economic feasibility of using microalgae for the production of biodiesel on a commercial scale.[3–5] However, many studies have been done using the batch or fed-batch process on relatively small scales in indoor systems, and this process showed significant limitations in terms of maximizing the cell concentration under scaled-up outdoor conditions.[6,7] The maximum cell concentration of *Porphyridium cruentum* after 20 days of cultivation was determined to be 0.7 ($g_{dry\ mass}$ L^{-1}) from chemostat cultivation and 4.0 ($g_{dry\ mass}$ L^{-1}) from batch cultivation.[8] The amounts of lipid extracted from *Chlorella protothecoides* cultured under autotrophic and heterotrophic conditions were compared, and 55 % of the lipid could be obtained from 15.5 ($g_{dry\ mass}$ L^{-1}) of maximum cell density in a 5 L bioreactor[10] under heterotrophic cultures, which was 4 times higher than that extracted under autotrophic conditions.[9]

However, in most batch cultivation processes, a limit in cell concentration is unavoidable because the nutrients could be used up easily and toxic by-product could be accumulated during the batch cultivation. Compared to the batch process, the fed-batch cultivation could overcome such limita-

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tions. In fed-batch cultivations, the cell concentration could be increased by continuously providing nutrients during cell growth. *Chlorella protothecoides* showed a biomass concentration of 51.2 ($g_{dry\ mass}\ L^{-1}$) and a lipid level of w = 50.3 % in the fed-batch cultivation,[11] but it showed a relatively low growth rate at the end of the cultivation process due to the accumulation of by-products and the unbalanced amount of nutrients in the medium; thus, this method still has limitations for use in scaled-up outdoor cultivation.

For maintaining the optimum culture condition of microalgae, many factors should be controlled, and the culture conditions also depend on the kinds of microalgae. The optimum culture conditions of *Porphyridium cruentum* have been studied by varying several growth parameters, such as pH, light intensity, inoculation ratio, and liquid volume, having 0.34 ($g_{dry\ mass}\ L^{-1}$) to 3.27 ($g_{dry\ mass}\ L^{-1}$) of maximum cell concentration.[12] According to Theodoridou *et al.,*[13] *Chlorella sorokiniana* produced maximum cell concentration of 7 ($g_{dry\ mass}\ L^{-1}$) at the optimum temperature of 35 °C. In addition, the optimal temperature for *Chlorella protothecoides* was shown to be 30 °C.[9] It has been found that temperature and light intensity in the batch or the fed-batch processes are the most important factors in controlling cell concentration for large scale indoor or outdoor cultivation. Especially for outdoor cultivation, the temperature should affect cell growth the most, since the temperature during outdoor cultivation cannot be readily controlled compared to indoor photo-bioreactor systems. Usually the temperature would be seriously fluctuated for several months during outdoor cultivation, which causes the cell concentration to decrease and limits the production of biodiesel.

Therefore, it is necessary to develop a system to maintain the proper temperature during outdoor cultivation to achieve mass amounts of biomass production for industrial scale outdoor cultivation. In this study, a novel type of perfusion cultivation process was introduced to solve the most serious problems caused by fluctuating temperature. This new method was designed to maintain the temperature in the photo-bioreactor by continuously feeding warm fresh medium heated by waste heat from the power plants and recycling the cells into the reactor. This system could overcome the geographical limitations of outdoor cultivation especially in countries with colder climates. For the perfusion process, cell growth was correlated with temperature and lipid production by mixotrophic growth of *Chlorella* sp. that could grow rapidly and contain a higher portion of polyunsaturated fatty acids, which can produce large amounts of lipid.[14,15]

Material and methods

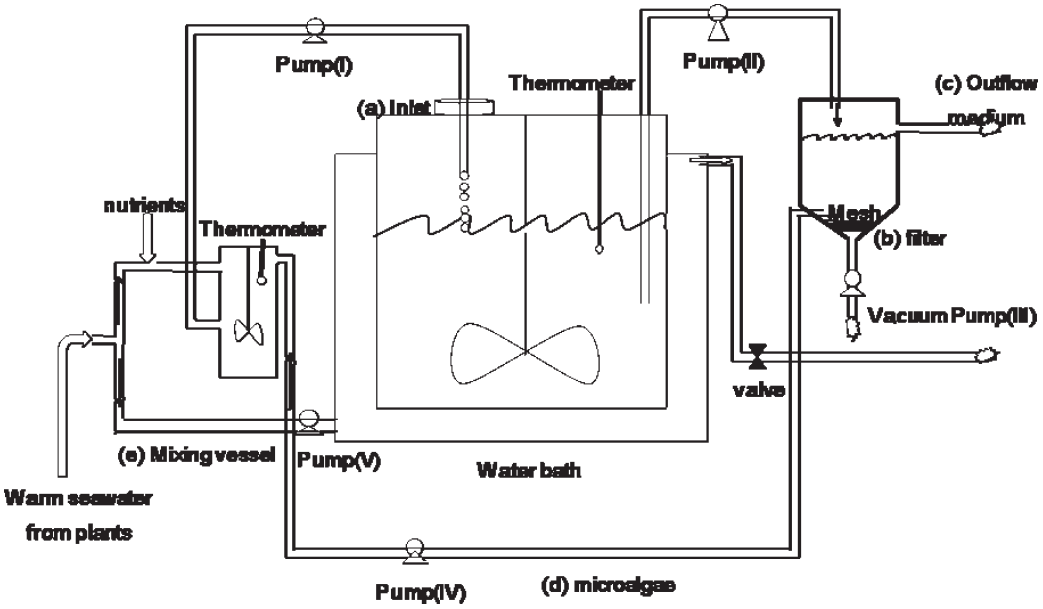
Materials

The green alga, *Chlorella* sp. (C-156) was kindly purchased from Korea Marine Microalgae Culture Center (Pukyoung National University, Pusan, Korea). *Chlorella* sp. was cultured in F/2 medium $NaNO_3$, $NaH_2PO_4 \cdot 9H_2O$, Ferric EDTA, $MnCl_2$, $CoCl_2$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $Na_2SiO_3 \cdot 9H_2O$, Vitamin B12, Biotin, Thiamine-HCl and filtered seawater. Before being mixed with the culture medium, the F/2 medium was sterilized for 15 minutes at 121 °C in an autoclave and the seawater was filtered with 0.45 μm filter paper. For mixotrophic cultivation, 1 g L^{-1} of glucose was added into the culture medium.

Cultivation methods

To understand the effects of temperature on cell growth under mixotrophic conditions, 2.5 g L^{-1} of the cells was inoculated into a 1 L Erlenmeyer flask (working volume 500 mL) for batch cultivation at various temperature: 25 °C, 30 °C, 35 °C and 40 °C with illuminating light intensity of 30 μE m^{-2} s^{-1} by using four 40 W fluorescent lamps, whose intensity was about similar to the the light intensity in outdoor cultivations by shading the screens on the top of the photoreactor light conditions: light:dark = 12 (h):12 (h). CO_2 was constantly supplied at a rate of 250 (mL min^{-1}) at an agitation speed of 150 rpm.

A photobioreactor was designed to continuously perfuse the warm medium and to recycle only the cells into the reactor by separating through a 0.45 μm membrane filter (Fig. 1b) with less than 0.01 Pa of pressure drop due to thin and non-clogging wire membrane and slow flow rate of max. 16 mL min. The dimension of a photobioreactor was 25 cm in diameter and 45 cm in height (14 L working volume) for minimizing the heat loss (Fig. 1). For perfusion cultivation, after 1 L of medium with cells was filled, the waste seawater from power plants near the sea was collected and warmed to 35 °C, same as the outlet temperature from the power plant. The waste seawater was mixed with the F/2 medium, and added into the reactor through the inlet (a) by a pump (I). The temperature inside the reactor was only controlled by perfusing the waste warm seawater at 35 °C to the water jacket and inside the reactor. The whole culture system (14 L reactor with accessories in Fig. 1) was put in a walk-out cold chamber to maintain the outside temperature of the photo bioreactor at 1 °C, which was close to the average temperature of winter seasons in Korea. The internal temperature change in the reactor was measured by a ther-



F i g . 1 – *A schematic diagram of perfusion type photobioreactor with recycling the cells: (a) Input flow for the warm waste seawater (35 °C) with nutrient; (b) Membrane filter for the separation of microalgae; (c) Outflow for the medium from the filter; (d) Inflow of the microalga; (e) Mixing vessel for the mixture of microalga and the fresh medium*

mometer inside the system. The cold chamber was completely covered with black curtains to block the light from the outside, and the system was continuously illuminated with the light intensity of 30 μE/(m^{-2} s^{-1}). The main purpose of this experiment was to maintain the proper temperature of the reactor and to determine whether the perfusion system could allow the cells to grow in a cold environment. A volume of 2 L of a reverse conical shape filter (Fig. 1b) was used to settle down the cells naturally to the bottom of the filter through not only gravitation force but also by a vacuum pump (III) in Fig. 1. This was used to efficiently separate the cells from the outflow medium through 3 μm pore size membrane filter s (5 cm inner diameter) connected to the recycling pumps (IV) in figure 1. The applied vacuum was not high enough to clog the membrane filter and only helped the cells to settle down easily to the bottom of the filter. The filter was cleaned by flushing the outflow medium back every day to prevent the filter from being clogged by cell debris. After the filter, the cells were separated from the outflow medium (Fig. 1c), and then returned to the reactor. The recycled cells and new warm medium were mixed in a 3 L mixing vessel (Fig. 1e) after passing through the pumps. pH was also monitored using pH meters (SevenEasy Ph, Mettler-Toledo Inc, Columbus, USA) installed inside the reactor. This system has the advantage of being easily scaled-up for outdoor cultivation since the temperature inside the photo bioreactor was only maintained by perfusing warm medium and required no extra heating.

For batch cultivation, the photo bioreactors were heated by continuous inflow and outflow of waste seawater at 35 °C in a water jacket surrounding the reactor. The data of batch cultivation from this system were only used to compare perfusion cultivation using the same reactor. Both the batch and perfusion cultivation were operated for about 95 days. For autotrophic growth during perfusion and batch cultivation in 14 L reactor, light and CO_2 were supplied under the same conditions used for the 1 L flask cultures.

Measurement of cell concentration and lipid contents

To measure the cell concentrations, 40 mL of the medium was collected from the reactor every 5 days. The medium was filtered by a 0.45 μm membrane filter and the filtered cells were dried at 105 °C for 12 hours.[11,16,17] Based on the dry mass of the cells, the specific growth rate was calculated by the following equation.[18]

$$\mu = 1/t \ln(X_m/X_0) \quad (1)$$

where X_m and X_0 are the concentrations of biomass at the end and the beginning of a batch run, respectively, and t is the duration of the run.

To estimate fatty acid composition in the lipid, the dried cells were extracted by the Folch method, and acid-catalyzed esterification was applied to measure the total lipid content.[15,16,18–21] The fatty acid composition was analyzed with a gas chromatograph (GC) (5890 SERIES II, GMI Inc, MN, USA).[16–21] The GC contained an HP-88 column

(100 m × 0.23mm × 0.2 μm) equipped with flame ionization detector column, and the oven temperature was programmed to increase from 100 °C to 240 °C at a rate of 4 °C min^{-1}. The injection temperature and the detector temperature were 250 °C and 280 °C, respectively. The column flow and split ratio were 1.0 (mL min^{-1}) and 30:1, respectively, and each were injected by 1 μL.[23]

Statistical analysis

The data are expressed as mean ± SD (standard deviation) and the mean is the average of three test results per experiment. The experiments were repeated at least three times to confirm the results.

Results

The effect of temperature on cell growth was examined in a 1 L flask under autotrophic conditions for 20 days of batch cultivation (Fig. 2). The cell growth seemed to be greatly affected by temperature, maintaining 1.18 ($g_{dry\ mass}$ L^{-1}) of the highest cell concentration at 30 °C and 1.0 ($g_{dry\ mass}$ L^{-1}) at 25 °C. However, a relatively low maximum cell concentration of 0.55 ($g_{dry\ mass}$ L^{-1}) was observed at 40 °C even though cell growth rapidly decreased at the highest temperature after 15 days cultivation. These results clearly demonstrate that *Chlorella* sp. could not grow well at high temperatures compared to low temperatures. This also indicates that controlling the temperature inside the reactor will be essential to the cell growth in outdoor cultivations. The optimum temperature ranges of other marine algae were also reported as 25 °C – 35 °C for *C. vulgaris* and *C. sorokiniana,*[13,24] which was similar to the optimal temperature for *Chlorella* sp. Also, the effect of temperature on the growth of *Chlorella* sp. growth was analyzed by response surface methodology, and showed 0.128 h^{-1} of maximum specific growth rate at 35 °C.[25] Based on these results, the optimum temperature for *Chlorella* sp. could be determined as 25 – 30 °C, which implies that this range of the culture temperature has to be maintained during outdoor cultivation. Other studies also showed the importance of temperature for both cell growth and lipid production from *Nannochloropsis oculata* and *Chlorella vulgaris.*[17]

It was found that the perfusion rate was a critical factor to maintain proper temperature for the growth of *Chlorella* sp. because only warm waste seawater at 35 °C was supplied into the culture system as shown in Fig. 1. At 0.1 (L h^{-1}) of perfusion rate, the medium temperature increased to only 8 °C for two days, which was not enough to maintain proper temperature. The medium temperature inside the reactor quickly increased faster than 0.3 (L h^{-1}), then reached a temperature of about 28 °C when the perfusion rate was 1.0 (L h^{-1}) after 5 days of operation. The medium temperature increased up to 31 °C when the perfusion rate was 1.2 (L h^{-1}) after 9 days of operation, which was the highest temperature obtained for this system. However, the inside temperature remained relatively constant once the temperature reached about 28 °C even though the warm medium was continuously supplied. This occurred because the perfusion rates were relatively high enough to replace the entire 14 L volume of the vessel in one day with seawater that has higher specific heat than pure water. This result indicates that the medium temperature could be maintained at minimum 25 °C for the growth of *Chlorella* sp. only by perfusing a warm medium at 35 °C into the reactor. Therefore, the following experiments were conducted using this perfusion system to cultivate the cells at 4 °C without providing extra heat under simulated outdoor environmental conditions for cold seasons.

The cell growth and total lipid production from both batch and perfusion cultivations should be compared under autotrophic and mixotrophic con-

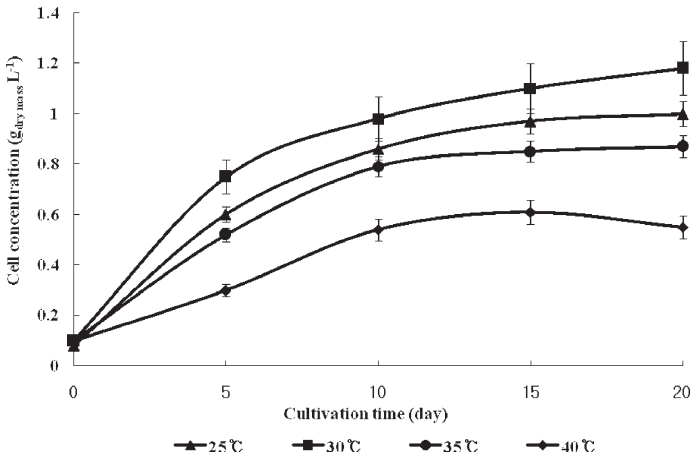


Fig. 2 – *Effect of temperature on the growth of Chlorella sp. under autotrophic conditions in a 1 L flask*

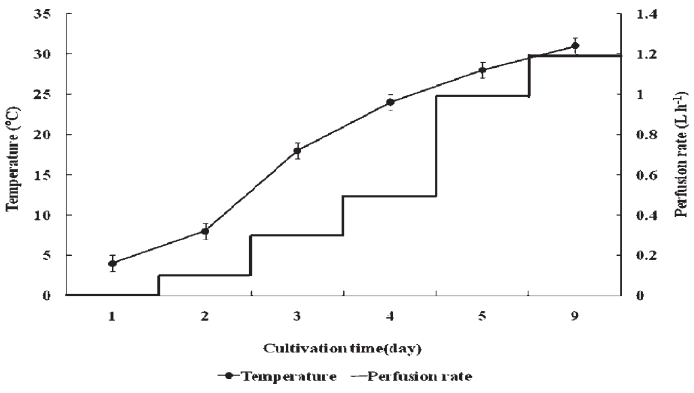
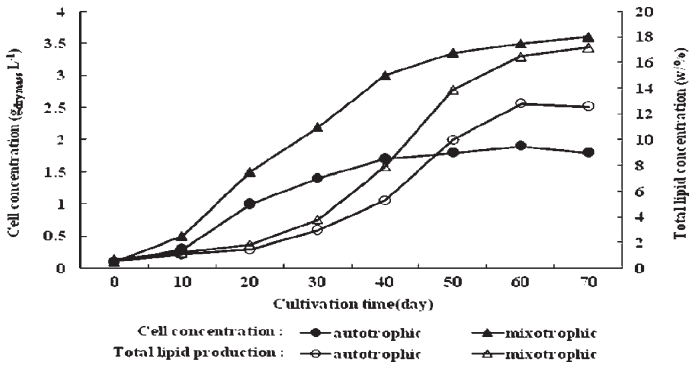


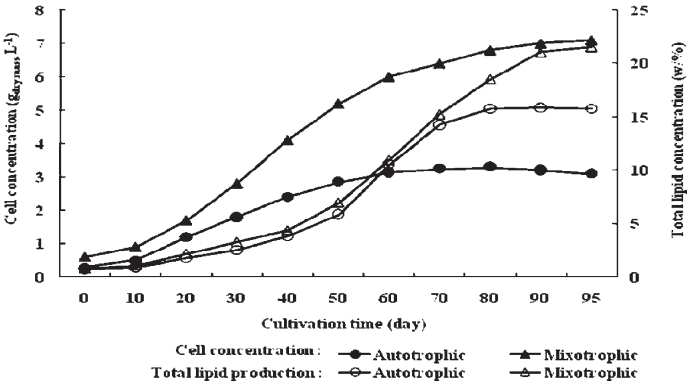
Fig. 3 – *Temperature changes in a 14 L photo-bioreactor as function of perfusion rates*

ditions in a 14 L photobioreactor since the growth and lipid production patterns might be different from small scale cultures due to the light path and agitation speed, etc. (Figs. 4 and 5). For batch cultivation, the reactor was heated by a water jacket continuously supplying 35 °C warm seawater to hold approximately 30 °C during the cultivation since the warm medium was not fed into the system. About 1.9 ($g_{dry\ mass}\ L^{-1}$) of maximum cell concentration was obtained on the 60th day of batch cultivation under autotrophic conditions as shown in Fig. 4. However, under mixotrophic conditions the cells continuously grew until the 70th day, maintaining a higher maximum cell concentration of 3.6 ($g_{dry\ mass}\ L^{-1}$) (Fig. 4). Generally, under mixotrophic conditions, cell growth was significantly higher due to the provided glucose as an organic carbon source with carbon dioxide and continuous illumination of light.[6] The total lipid concentration also showed a similar pattern as the cell growth. Under autotrophic conditions, w = 12.8 % of maximum lipid accumulation was observed on the 55–60th days, even after that the total lipid production remained relatively constant until the end of the cultivation, which was different from the cell growth. Under the mixotrophic conditions, the highest lipid production was estimated as w = 17.2 % at the later periods of the 70 days of cultivation. Interestingly enough, continuous lipid production was also observed at the later periods of cultivation when the cell growth ceased for mixotrophic cultivation. This result indicates that the lipid accumulation within the cells could partially be a cell-growth related process but not closely related to the cell growth. Similar patterns have been reported elsewhere for lipid production and other chemicals can affect lipid production pathways by assimilating organic and inorganic carbon sources.[26,27]

There were significant differences in cell growth and lipid production between autotrophic and mixotrophic conditions in perfusing the warm



F i g . 4 – *The cell growth and total lipid production from Chlorella sp. under autotrophic and mixotrophic conditions in batch cultivation*



F i g . 5 – *Comparison of cell growth and total lipid production from Chlorella sp. under autotrophic and mixotrophic conditions in a 14 L photo bioreactor at perfusion rate of 1.0 L h^{-1}*

medium at a rate of 1.0 (L h^{-1}) under (Fig. 5). At this perfusion rate, temperatures remained relatively constant between 27 and 30 °C during cultivation without supplying extra heat, and the pH ranged from 7.1–8.0 (data not shown here). It was found that the overall cultivation time for the perfusion process was also longer than the batch process (70 days vs. 95 days) based on cell growth because fresh medium was continuously supplied into the reactor. As a result of the continuous feeding of fresh medium, the maximum cell concentration and total lipid concentration were much higher than those from batch cultivations under both autotrophic and mixotrophic conditions: Under mixotrophic conditions, the cell concentration was 7.1 ($g_{dry\ mass}\ L^{-1}$) vs. 3.3 ($g_{dry\ mass}\ L^{-1}$) and the lipid production w = 21.6 % vs. w = 15.9 %; under autotrophic conditions, the cell concentration was 3.3 ($g_{dry\ mass}\ L^{-1}$) vs. 1.9 ($g_{dry\ mass}\ L^{-1}$) and the lipid production w = 15.9 % vs. w = 12.8 % . Interestingly, the lipid production did not improve much during both mixtotrophic and autotrophic growth by the perfusion process, compared to the significant increases in cell growth. However, correlation patterns between cell growth and lipid production were the same as those for batch cultivations, where the lipid production seemed to be partially related to the growth process.

Several key parameters to indicate the overall performance of the culture systems such as specific growth rate, the maximum cell growth and total lipid production should be estimated under autotrophic and mixotrophic conditions according to cultivation modes based on the data in Figs. 4 and 5 (Table 1). From the perfusion process, maximum specific growth rates for autotrophic and mixotrophic growth were estimated as 0.035 (d^{-1}) and 0.076 (d^{-1}), respectively, which can be compared to 0.021 (d^{-1}) and 0.061 (d^{-1}) from the batch cultivation. This means that under both autotrophic and mixotrophic conditions, the specific growth

Table 1 – *Estimation of maximum cell density, specific growth rate, and total lipid production from Chlorella sp. under various culture conditions*

Culture conditions	Batch			Perfusion		
	maximum cell density, X ($g_{dry\ mass}\ L^{-1}$)	specific growth rate, μ (d^{-1})	total lipid contents (w/%)	maximum cell density, X ($g_{dry\ mass}\ L^{-1}$)	specific growth rate, μ (d^{-1})	total lipid contents (w/%)
Autotrophic	1.9	0.021	12.8	3.3	0.035	15.9
Mixotrophic	3.6	0.061	17.2	7.1	0.076	21.6

rates for the perfusion cultivation were faster than those for the batch cultivation. This result indicates that cell growth in perfusion cultivation maintained higher exponential phase than the growth during batch cultivation because of recycling the cells into the reactor as well as continuously feeding fresh nutrients. These results strongly suggest that the mixotrophic condition with the perfusion cultivation is more efficient in increasing both cell growth and lipid production, which is highly important for biodiesel production.

The 14 L photobioreactor was designed less high than typical photo bioreactors to prevent temperature loss during cultivation. However, dense cell concentration in a photobioreactor should have a deep path of light into the center of the reactor, which is a very critical factor that affects cell growth and lipid production in large-scale outdoor culture systems. That is why the light intensities were measured at three points – two points on the front surfaces and one point at the center of the reactor, by shining light from both sides. The light intensity profiles inside the reactor must be monitored to let the light pass through the center of the reactor in maintaining the maximum cell density of 3.3 ($g_{dry\ mass}\ L^{-1}$) and 7.1 ($g_{dry\ mass}\ L^{-1}$) from autotrophic and mixotrophic cultivations, respectively under perfusion cultivation conditions (Fig. 6).

Under autotrophic and mixotrophic conditions, about 10–12 % increase in light intensity was observed at the surface of the reactor since the light was illuminated from both sides, which would not inhibit the cell growth. For the case of 3.3 ($g_{dry\ mass}\ L^{-1}$) of maximum cell concentration, the light intensity at the center was measured to be 28.5 μE $m^{-2}\ s^{-1}$, which was only 5 % less than the input light intensity. For 7.1 ($g_{dry\ mass}\ L^{-1}$), the light intensity at the center was 25.5 μE $m^{-2}\ s^{-1}$, about 15 % less than the input light intensity. When about 15 % of total input light intensity was reduced at the highest cell concentration, light-limited conditions at the center of the reactor might not be harmful because it could be presumed that the slight light limitation would occur only for a short period. It could even accelerate lipid synthesis within the cells. This was probably why the highest lipid production was obtained under mixotrophic conditions. Similar results were also previously reported, where better lipid production was observed under light and/or substrate-limited conditions.[11,28]

During the perfusion process, specific lipid production rate from the growth of *Chlorella* sp. was observed according to cultivation time (Fig. 7). Under the mixotrophic conditions, the specific lipid production rate decreased until 40 days of cultivation, then maintained relatively constant at 1.12 (% lipid $g^{-1}_{dry\ mass}\ d^{-1}$). This suggests that, as

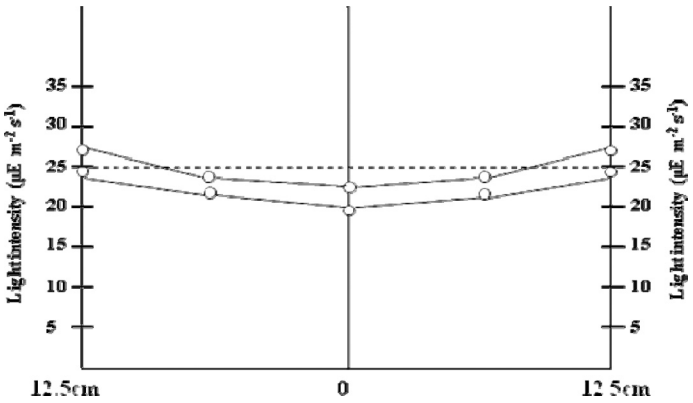


Fig. 6 – *Light intensity profiles inside the bioreactor in maintaining two different maximum cell concentrations for perfusion cultivation*

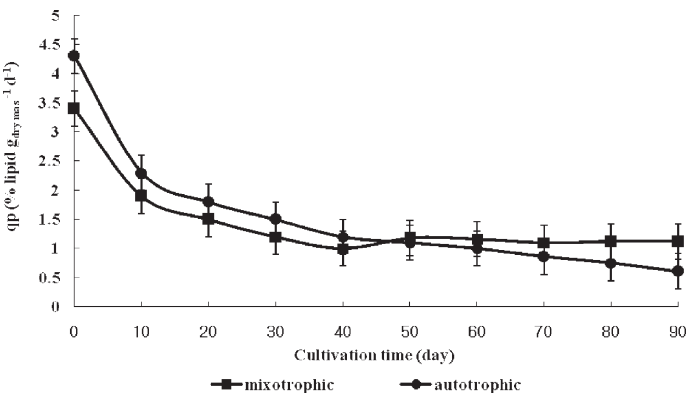


Fig. 7 – *Comparison of specific lipid production rates from Chlorella sp. under autotrophic and mixotrophic conditions in a 14 L photo bioreactor for 90 days of perfusion cultivation*

long as the constant cell concentration is maintained in the perfusion system, relatively constant lipid production could also be maintained during indoor and outdoor cultivations. Under autotrophic conditions, the specific lipid production rate gradually decreased during the 90 days of cultivation, having 0.61 (% lipid $g^{-1}_{dry\ mass}$ d^{-1}) of specific lipid production rate at the later period of cultivation. Under mixotrophic conditions, to understand the lipid production yield from both carbon sources of glucose and carbon dioxide, residual concentrations of glucose and carbon dioxide in the medium were measured (data not shown). In general, a relatively high ratio of glucose consumption was observed from 1.0 (g L^{-1}) of initial concentration down to 0.1–0.2 (g L^{-1}) of residual concentrations after 40 days cultivation, the later period cultivation when the lipid production rate remained relatively constant since the fresh medium containing 1.0 (g L^{-1}) of glucose was continuously fed into the system. While about 3.0 mbar of carbon dioxide was consumed from supplying 7.2 mbar of gaseous CO_2 into the medium, which was a smaller amount of carbon consumption, compared to the case of glucose at the same cultivation times. These results imply that under mixotrophic conditions, there was no substrate-limited condition and most of input organic carbon source was converted into lipid production and some inorganic carbon was used for lipid production and most for cell growth. Similar carbon substrate consumption patterns were also reported elsewhere.[40,41] It also explains the results of the relatively high maximum cell density obtained during perfusion mixotrophic cultivations.

For biodiesel applications, it is also very important to find the fatty acid profiles of the lipids obtained from perfusion cultivation under mixotrophic (Fig. 8b) and autotrophic (Fig. 8c) conditions, which have to be compared with standard fatty acids (Fig. 8a) (Fig. 8). The lipids from the perfusion process contain seven major C_{16}-C_{18} saturated fatty acids and some unsaturated fatty acids. There was also a difference between the two culture conditions, where relatively larger amounts of saturated and unsaturated fatty acid lipids were obtained under mixotrophic conditions, such as higher amounts of palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$), compared to those obtained under autotrophic conditions. The lipids obtained under autotrophic conditions contained relatively larger amounts of longer chain fatty acids. Based on these results, it could be said that a higher amount of lipid production could be obtained from mixotrophic cultivation than from autotrophic cultivation, which is the similar pattern of the results in Fig. 5. The concentrations of key fatty acids for biodiesel production were measured from the data in Fig. 8, according to two different culture conditions (Table 2).

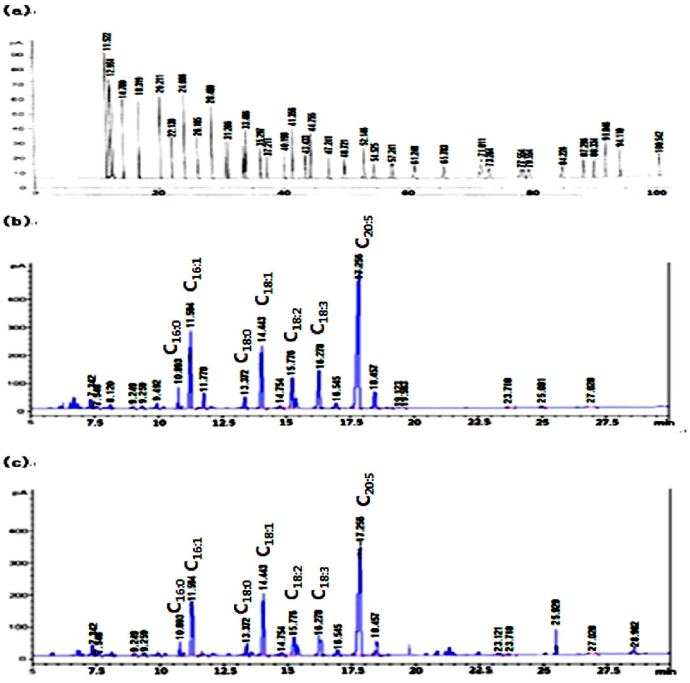


Fig. 8 – *Fatty acid profiles in the lipids of Chlorella sp. from autotrophic and mixotrophic perfusion cultivations: (a) standard fatty acids; (b) mixotrophic conditions; (c) autotrophic conditions. Notations: C16:0 (RT: 10.893 min); C16:1 (RT: 11.594 min); C18:0 (RT: 13.372 min); C18:1 (RT: 14.443 min); C18:2 (RT: 15.776 min); C18:3 (RT: 16.278 min); C20:5 (RT: 17.256 min).*

Table 2 – *Comparison of fatty acid compositions from Chlorella sp. under various culture conditions*

Culture conditions		Fatty acid composition				
		$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Perfusion	Autotrophic	11 %	5 %	13 %	6 %	14 %
	Mixotrophic	14 %	6 %	17 %	9 %	15 %

The mixotrophic growth had higher amounts of fatty acid composition in all the contents, and specifically showed higher amounts of C_{18} unsaturated fatty acids than C_{16} saturated fatty acids. However, there were no significant differences in the fatty acid compositions between the two cultivation conditions for batch cultivation although a somewhat higher amount of longer unsaturated fatty acids were observed in the mixotrophic condition (Fig. 8). The mixotrophic conditions produced more unsaturated fatty acids, which would be suitable for biodiesel applications, compared with autotrophic conditions. This proves that the perfusion process could produce higher amounts of good quality fatty acids for biodiesel than those from other cultivation methods.

Discussion

Even though there have been many studies about increasing cell concentrations for large outdoor cultivations[11,29,30,34–36] most current cultivation processes have several limitations in maintaining mass amounts of biomass, especially due to the depletion of nutrients and the economic feasibility of maintaining the temperature for outdoor systems. Therefore, in this work, a perfusion system was introduced to maintain the proper temperature for the growth of *Chlorella* sp. for outdoor cultivations. This system was proved to maintain an optimal temperature of 28 °C by continuously providing both a heating source and a fresh nutrient from warm waste seawater (35–40 °C), which helps scale-up for an outdoor culture system where extra heating methods would be unavailable. The proper perfusion rate was estimated higher than 1.0 ($L\ h^{-1}$), which also corresponded well to the heat flux estimation from warm (28 °C) to cold (1 °C) temperature based on the heat capacity of seawater and the reactor design. The pH was also shown to be relatively stable in the range of 7.1–8.0. However, for the case of *Spirulina platensis*, the pH increased as the cell concentration increased in batch cultivation[32] because the CO_2 level in the system could be lowered during cultivation due to photosynthesis. From standpoint of the kinetic analysis of this perfusion system, overall cell growth would follow the batch growth pattern. However, in considering the operation of the system, the inflow and outflow of the medium was continuous, which is similar to continuous cultivation and not turbidostat cultivation, since the cell concentration gradually increased.

For open pond type cultivations, especially in cold regions such as Korea and Russia, etc., the effect of temperature on the algal cell growth and to the method of supplying a cheap and efficient heating source would be the key factors, since in general the optimum temperature for the growth of *Chlorella* sp. was about 25–30 °C. From other works, the optimum temperature for cell growth and lutein production from *Chlorella protothecoides* was also shown to be 28 °C and 35 °C, respectively 31, indicating that temperature plays a major role in controlling the growth of microalgae for outdoor cultivations. The results from this perfusion system proved to maintain the temperature inside the reactor within the range 27–30 °C by only feeding warm waste seawater at 35–40 °C from power plants near the sea with no additional heat supply, which is the most critical factor for reducing production costs.[31] Moreover, this seawater is directly drawn from the sea to cool down the reactors in the plants without coming into contact with anything in the systems and it is then returned to the sea, which can act as a perfect heating source as well as good source of nutrients for algal growth.

In batch cultivation, the cell growth decreased after 50 days of cultivation under autotrophic conditions but continuously increased under mixotrophic conditions when both glucose and light were provided. This mixotrophic culture conditions resulted in a maximal cell concentration of 7.1 ($g_{dry\ mass}\ L^{-1}$) in adding only 1.0 ($g\ L^{-1}$) of glucose, compared to the results that about 5–10 ($g\ L^{-1}$) of glucose or other organic carbon sources have been added to maintain at least 5–10 ($g_{dry\ mass}\ L^{-1}$) of biomass for most mixotrophic cultivations.[7,9,37,38] However, the addition of this amount of organic carbon source would be less economically feasible for bioenergy production. That was why only 1.0 ($g\ L^{-1}$) of glucose was added in this work, and it was found that the cultures did not reach the carbon limitation stage due to feeding them continuously into the system. This result proves that a perfusion system would be appropriate to supply least amounts of organic carbon sources under mixotrophic conditions. The specific lipid production under mixotrophic conditions was also higher than under autotrophic conditions by showing a relatively constant specific lipid production rate under mixotrophic conditions as 1.12 (% lipid $g^{-1}_{dry\ mass}\ d^{-1}$). This result also indicated that cell metabolism to produce lipids was relatively higher at high cell concentration obtained under mixotrophic conditions.

Generally, for batch cultivation, the cell metabolism to produce lipids is relatively lower under mixotrophic conditions because the light intensity, which activates lipid metabolism in cells, is lower due to the high cell concentration in the photo bioreactor. However, the perfusion process could maintain the cells in a condition that is not completely balanced such as chemostat cultivation, but is more favorable for both cell growth and lipid production by recycling the cells and feeding fresh nutrients into the reactor, which has not been reported elsewhere for microalgal growth including *Chlorella* sp. In addition, even the light intensity profiles within the reactor also remained relatively consistent at a high cell concentration of 7.1 ($g_{dry\ mass}\ L^{-1}$). This maximum cell concentration from mixotrophic growth doubled when the cultivation system was changed from the batch system to the perfusion system, whereas the cell concentration under autotrophic conditions increased ca. 1.5 times. When the autotrophic conditions were changed to mixotrophic conditions, the maximum cell concentration increased 1.9 times in the batch system, whereas in the perfusion system it increased 2.2 times. This suggests that the perfusion cultivation system would be more effective in

maintaining high cell concentrations under mixotrophic conditions. In terms of lipid production, long chain fatty acids, which are important for biodiesel applications,[33,39] were found in the cells cultivated under the mixotrophic conditions as shown in Table 2. The total lipid production in the perfusion system was also 1.4 times higher in mixotrophic conditions relative to autrophic conditions, and 1.3 times higher in the batch system. In the case of mixotrophic conditions, the total lipid production increased 1.3 times when the cultivation system was changed from batch to perfusion system. In the case of autotrophic conditions, the increase was only 1.2 times. The lipid production from the growth of *Chlorella* sp. was found to most likely be a partially growth-related production process. This novel approach holds great promise for the use of outdoor cultivations, since both temperature and light intensity directly affects cell concentration during the cultivations. In addition, the fatty acid composition shown in Table 2 indicates that the cells grown under mixotrophic conditions in the perfusion system contain higher amounts of better quality fatty acids.

Conclusions

Mass production of algal biomass has been one of the major bottlenecks in meeting the economic feasibility of industrial-scale biodiesel production. Many techniques of cultivating mass amounts of biomass have been developed for outdoor or indoor cultivation systems. However, so far, open-pond type outdoor processes seem to be economical once the proper temperature is maintained during cultivation, but there are geological limitations to this system. Therefore, in this work, a unique perfusion cultivation system has been introduced to keep an optimal temperature in a closed type photo-bioreactor by continuously feeding warm waste seawater from the plants near the sea. This process has proved to be able to maintain at least 25 °C in the vessel at a perfusion rate of 1.0 L h^{-1} with no extra heating, which resulted in 7.1 ($g_{dry\ mass}$ L^{-1}) of maximum cell density under mixotrophic conditions. It was interesting that the perfusion rate affected the cell growth more than the lipid accumulation in the cell. This maximum cell concentration seemed to be similar or even higher than other data of *Chlorella* sp. grown from mixotrpphic conditions because the biomass was continuously accumulated into the reactor by recycling the cells and only the waste medium was pumped out. This perfusion system also showed high lipid metabolism by accumulating high amounts of total lipids in the cell. For this system, mixotrophic cultivation could maintain higher biomass concentration and better lipid profiles for biodiesel production than the autotrophic process. In general, this interesting process has been proved efficient and could be easily scaled-up without geological limitations and contamination problems for outdoor cultivations. However, there might be a need for further studies of developing cheaper organic carbon sources for this alga rather than the relatively expensive glucose, as well as a more reliable and easy scaling-up system for large-scale cultivations.

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Symbols and abbreviations

w – mass fraction, %

μ – the specific growth rate, d^{-1}

X_m – the concentrations of biomass at the end of a batch run, $g_{dry\ mass}$ L^{-1}

X_0 – the concentrations of biomass at the beginning of a batch run, $g_{dry\ mass}$ L^{-1}

t – the duration of the run, d.

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