J. Microbiol. Biotechnol. (2011), **21**(10), 1073–1080
doi: 10.4014/jmb.1103.03038
First published online 31 July 2011

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effects of Carbon and Nitrogen Sources on Fatty Acid Contents and Composition in the Green Microalga, *Chlorella* sp. 227

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Received: March 28, 2011 / Revised: July 7, 2011 / Accepted: July 8, 2011

In order to investigate and generalize the effects of carbon and nitrogen sources on the growth of and lipid production in *Chlorella* sp. 227, several nutritional combinations consisting of different carbon and nitrogen sources and concentrations were given to the media for cultivation of *Chlorella* sp. 227, respectively. The growth rate and lipid content were affected largely by concentration rather than by sources. The maximum specific growth was negatively affected by low concentrations of carbon and nitrogen. There is a maximum allowable inorganic carbon concentration (less than 500~1,000 mM bicarbonate) in autotrophic culture, but the maximum lipid content per gram dry cell weight (g DCW) was little affected by the concentration of inorganic carbon within the concentration. The lipid content per g DCW was increased when the microalga was cultured with the addition of glucose and bicarbonate (mixotrophic) at a fixed nitrogen concentration and with the lowest nitrogen concentration (0.2 mM), relatively. Considering that lipid contents per g DCW increased in those conditions, it suggests that a high ratio of carbon to nitrogen in culture media promotes lipid accumulation in the cells. Interestingly, a significant increase of the oleic acid amount to total fatty acids was observed in those conditions. These results showed the possibility to induce lipid production of high quality and content per g DCW by modifying the cultivation conditions.

Keywords: *Chlorella* sp., carbon, nitrogen, FAMEs, oleic acid

Biodiesel, long-chain alkyl esters, has been attracting attention as an alternative transportation fuel to petroleum-based fuels because it is renewable, biodegradable, and environment-friendly [4]. Microalgae can accumulate lipids whose chemical composition is similar to common vegetable oils used as a feedstock of biodiesel, and the production of biodiesel from microalgae is expected to be cost-effective because of its high productivity per unit area [4]. In addition, microalgae have further advantages because they can grow in various water sources such as wastewater, seawater, and river water [13, 23, 24, 43].

The photosynthetic mechanism of microalgae is capable of converting carbon dioxide into biomass, which can contribute to reducing carbon dioxide that causes global warming in the atmosphere. Single cellular microalgae have two dissolved inorganic carbon pumps capable of using not only gaseous CO_2 but also bicarbonate dissolved in liquid [28, 37]. Microalgae's CO_2 fixation ability is greater than that of other photosynthetic plants, and it is explained by the fact that microalgae do not have roots and stems [42]. Moreover, some microalgae are not strictly autotrophic, which means that mixotrophic or heterotrophic conditions can be applied to cultivating microalgae [44]. In addition, the fact that a high concentration of inorganic carbon is usually not utilized by microalgae acts as a limiting factor in photosynthesis, although inorganic carbon is an essential source in photosynthesis [5, 20].

In general, microalgae do not accumulate lipids in cells under normal conditions, and the maximum lipid capacity to accumulate in a cell varies greatly from strain to strain [15]. Several factors affecting lipid accumulation and fatty acid composition have been well studied to date, including nutritional factors (nitrogen deficiency, phosphorus limitation, iron) [18, 21, 22], physical environments (temperature, light intensity) [16, 28], and physiological factors (growth phase, physiological status) [33].

Nitrogen sources and concentration have been known as parameters to greatly affect yields of algal lipid. Various nitrogen sources, such as ammonia, nitrate, nitrite, and urea, can be used as the nitrogen sources for culturing

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microalgae. Since the nitrogen sources affect the heterotrophic growth of *Chlorella protothecoides*, urea is found to be a better nitrogen source than ammonium [35]. However, ammonium is preferred by other algal strains such as *Chlorella* sp. [33], and nitrate has been reported to be the most preferable nitrogen source in *Neochloris oleoabundans* [21].

Fatty acid profiles of bacteria or fungi can be used as an identification tool called Sherlock Microbial Identification [38]. However, the fatty acid composition varied in different researches even though the results were obtained from the same algal strain [30, 42]. A large quantity of lipid accumulation in microalgae was assumed to be an intrinsic ability of the species/strain-specific rather than genus-specific [15, 30]. Moreover, lipid contents in microalgae were assumed to have variation on growth periods.

Considering that one of the biggest huddles in commercialization of biodiesel is still a high cost for the production, it can be solved by maximization of lipid productivity with limited input energy. Therefore, it should be proposed which optimal nutritional condition on lipid production can bring maximum productivity and high-quality biodiesel. However, so far, the nutritional effects are reported with diversities, so it is necessary to generalize the nutritional effects on lipid production for high quantity and quality. With this viewpoint, we investigated the effects of different nutritional conditions on the contents and compositions of fatty acid produced in a microalgal species, *Chlorella* sp. 227.

Materials and Methods

Microalgal Strain and Culture Conditions

In our preliminary study, eight microalgae strains were used and *Chlorella* sp. 227 showed a higher growth rate and lipid content than the others (data not shown). Moreover, the results obtained from this study can be compared with results previously reported by others, because the species belongs to genera *Chlorella*, which is a universally well-known microalgae [9, 24, 25, 28]. Therefore, *Chlorella* sp. 227 was selected to investigate the effects of nutrients, carbon (bicarbonate/glucose) and nitrogen (ammonia/nitrate/urea) sources, and concentrations on the growth of and fatty acid methyl esters (FAMEs) production in the cells for this study.

Chlorella sp. 227, purchased from the National Institute for Environmental Studies in Japan, was cultured in a modified soil extract (SE) medium, which was composed of (per liter) 0.15 g $K_2HPO_4 \cdot 3H_2O$, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.35 g KH_2PO_4, 0.05 g NaCl, 2.86 mg H_3BO_3, 1.81 mg $MnCl_2 \cdot 4H_2O$, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.079 mg $CuSO_4 \cdot 5H_2O$, and 0.039 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and nitrogen and carbon sources were added according to the experimental purposes in the modified SE medium. All experiments were conducted by batch-fed types in 250 ml Erlenmeyer flasks having a working volume of 100 ml and cultured at the plant incubator (JEIO Tech GC-300, Korea) of 25°C with cool-white fluorescent lights of 27 μmol $m^{-2} s^{-1}$ on a 16:8 h light/dark cycle considering a natural condition.

Table 1. Sources and concentrations of carbon and nitrogen contained in the medium for this study.

Parameters	Carbon (mM)		Nitrogen (mM)		
	Inorganic bicarbonate	Organic glucose	Ammonia	Nitrate	Urea
Carbon (concentration)	0	-	5	5	-
	50	-	5	5	-
	100	-	5	5	-
	250	-	5	5	-
	500	-	5	5	-
	1,000	-	5	5	-
Carbon (concentration & source)	250	0	5	5	-
	250	0.5	5	5	-
	250	5	5	5	-
	250	50	5	5	-
Nitrogen (source)	250	-	10	-	-
	250	-	-	10	-
	250	-	-	-	10
Nitrogen (concentration)	250	-	0.2	-	-
	250	-	1	-	-
	250	-	2	-	-

Carbon Sources and Concentrations

The effect of carbon concentrations was investigated under several different concentrations (0, 50, 100, 250, 500, and 1,000 mM) of sodium bicarbonate in culturing *Chlorella* sp. 227 (Table 1). In addition, several different concentrations of glucose (0.5, 5, 50 mM) in media having 250 mM bicarbonate were applied additionally to investigate the growth and lipid productivity of *Chlorella* sp. 227, because the cultures showed a retarded growth when bicarbonate of more than 250 mM was given in the medium. Glucose was selected by the fact that most *Chlorella* spp. can utilize it [32], and the concentrations were established by a minimum range of concentrations showing some differences in growth or lipid production because it will demand some extra cost for supplying it. A fixed nitrogen concentration of 10 mM, which is regarded as enough not to cause depletion of nitrogen, as a mixture of 5 mM ammonia chloride and 5 mM sodium nitrate, was supplied for all these batch tests. Initial pH was adjusted to 7.1 (± 0.1) with 2 N HCl solution.

Nitrogen Sources and Concentrations

Ten mM of each ammonia-N, nitrate-N, and urea-N was used as different nitrogen sources to investigate the effect of nitrogen sources on the growth and FAMEs production of *Chlorella* sp. 227. The growth and lipid productivity under different nitrogen sources were estimated and compared. The effect on nitrogen concentrations was also investigated in the cultures having ammonia-N of 0.2 mM, 1.0 mM, and 2.0 mM (Table 1), concentrations of which can be supported by reuse of effluent released from municipal WWTPs, whereupon it would not demand the cost for supplying nutrients [6].

To minimize the effect of carbon concentration, 0.25 M bicarbonate, under which concentration of carbon the *Chlorella* sp. 227 showed the best growth rate, was supplied for these batch tests. The initial pH of all cultures was adjusted to 7.1±0.1 with 2 N HCl solution.

Dry Cell Weight Analysis

To measure total biomass changes in samples, the optical density (OD) of samples at 660 nm was measured routinely on growth time. The optical density was measured by a UV spectrophotometer (spectronic 20D+, Thermo Scientific, USA) and the OD values were converted to dry cell weight (DCW) by the predetermined conversion factor derived from the relationship between OD_{660} values and DCWs. The DCW of microalgal biomass was determined by the following procedure: 50 ml of sample was taken and filtered through preweighed GF/C filters (Whatman, England). After rinsing with distilled water, the filters were dried at 105°C for 2 h, and reweighed [1]. All assays were carried out in triplicate.

FAMEs Analysis

The content of fatty acids was analyzed using the modified transesterification method [19]. Microalgal cells were harvested by centrifugation (4,000 rpm, 10 min) and washed with distilled water (repeated twice). The cells were then dried in a freeze-dryer (FD5512, IlShin BioBase Co., Korea) for four days or longer. Cells were lyophilized in 10 ml Pyrex glass tubes (Teflon-sealed screw-capped) and the biomass in the tubes weighted. Lipid extraction reagent [chloroform/methanol, 2:1 (v/v)] of 2 ml was added to each tube. The tubes were vigorously mixed for 10 min at room temperature using a vortex mixer (Vortex Genius 3; Ika, Italy). Then 1 ml of chloroform that includes the internal standard (500 μg/l; Sigma Chemical Co., USA), 1 ml of methanol, and 300 μl of H_2SO_4 were sequentially added to the glass tube and mixed using a vortex mixer for 5 min. The tube was reacted in a 100°C water bath for 10 min. It was then cooled to room temperature, supplemented with 1 ml of water, and intensely mixed for 5 min. It was further centrifugally layer-separated at 4,000 rpm for 10 min. The lower layer (organic phase) was extracted using a disposable plastic syringe (Norm-ject, Henke Sass, Wolf GmBH, Germany) and filtered with a disposable 0.22 μm PVDF syringe filter (Millex-GV; Millipore, USA). Methyl esters of fatty acids were analyzed using a gas chromatograph equipped with a flame ionization detector and a 0.32 mm (ID)×30 m HP-INNOWax capillary column (Agilent Technologies, USA). Helium was used as a carrier gas at 2.2 ml/min. The temperatures of the injector and detector were set at 250°C and 275°C, respectively. Mix RM3, Mix RM5, GLC50, GLC70 (Supelco, USA) heptadecanoic acid, and g-linolenic acid (Sigma Chemical Co., USA), were used as standards. Other reagents used were of analytical grade.

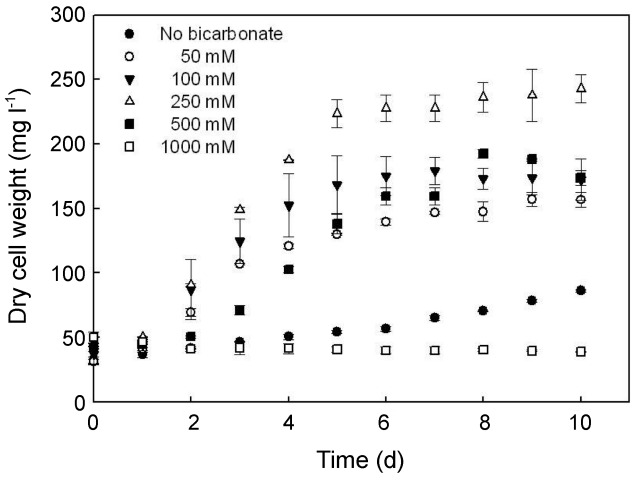

Fig. 1. Time course of *Chlorella* sp. 227 growth in media containing different bicarbonate concentrations.

Statistical Analysis

One-way ANOVA was used to determine the difference of averages on each DCW dataset. The DCWs for the analysis were obtained from after day 6 of the cultivation at each condition. The analysis was performed under the significance level of 0.05 [29]. SPSS ver. 18.0 was used for the analysis in this study.

Other Analysis

A sample pH was directly determined with a pH meter (B-212; Horiba, Japan). Determination of ammonia concentration in samples was achieved using a Humas kit (HS-NH_3(N)-L, HS-NH_3(N)-H; Humas, Korea).

RESULTS AND DISCUSSION

Carbon Concentrations

The inhibition of microalgal growth by high concentration of carbon has been already reported, even though inorganic carbon sources such as carbon dioxide are essential in photosynthesis [5, 19]. The increase of inorganic carbon concentration below 250 mM bicarbonate promoted biomass yields and growth rates in the cultures (Fig. 1). It suggests that the carbon dioxide concentration of 0.03% (v/v) in the atmosphere is not enough to get high productivity of biomass, so carbon can be potentially supplied for it with gases containing high concentrations of carbon dioxide such as power plant flue gases or landfill gases.

During the entire batch-cultivation period of 10 days, the highest biomass concentration was achieved from the culture given by 250 mM bicarbonate, and the delayed growth pattern was clearly observed in the culture given by 500 mM bicarbonate, and the maximum biomass in the culture given with 500 mM bicarbonate was less than it in the culture given with 250 mM bicarbonate (Fig. 1). Moreover, the culture with 1,000 mM bicarbonate, which concentration was the highest concentration established in this study, did not show any growth. This result demonstrates that the optimum concentration is lower than 500 mM bicarbonate, which corresponds to approximately 3.0% (v/v) as carbon dioxide dissolved.

Several studies have reported that this inhibition can be overcome by modified culture techniques or tolerable strains [8, 20, 42]. For example, this inhibition was partially overcome through application of a high carbon concentration after gradual adaptation with high-density inoculums and semicontinuous cultivation [20]. However, the growth and lipid content per g DCW obtained from the acclimated carbon concentration in the semicontinuous cultivation were lower than those obtained from the optimum carbon

Table 2. FAMEs contents produced by *Chlorella* sp. 227 according to different concentrations of inorganic carbon (bicarbonate) in media.

Day	Bicarbonate (mM)	Total FAMEs contents[1)]	% Ratio to total FAMEs					
			C14:0	C16:0	C16:1n	C18:1n	C18:2n	C18:3n
2	100	25.7		29.6			24.1	46.7
	250	32.2		29.5			23.9	46.6
	500	42.0		24.8		14.1	12.1	49.1
4	100	54.7	2.2	24.5		5.7	21.9	45.7
	250	44.8		22.8		8.3	17.2	52.0
	500	44.8		21.9		20.5	16.9	40.8
6	100	55.7	2.2	19.9	2.3	7.5	19.4	48.5
	250	54.0	1.7	20.2	1.7	7.4	18.9	50.4
	500	61.0		19.3		14.8	17.1	48.7

[1)]mg g^{-1} cell.

dioxide concentration among their other results. This suggests that excess carbon dioxide that resulted in inhibition of further growth will not be utilized by the biosynthesis system in photosynthetic organisms. Namely, there is a saturation limit in sequestration of CO_2 using photosynthesis, which links to the limit of lipid productivity.

The contents and compositions of FAMEs per g DCW in the cultures are indicated in Table 2. Certainly, excess carbon promotes lipid production from the early stage of growth in a cell, as the higher bicarbonate concentrations showed more FAMEs production at day 2. However, the differences of maximum FAMEs productions at day 6 were reduced in the cultures regardless of the given bicarbonate concentrations; the FAMEs productions on 100 mM and 500 mM of bicarbonate at day 2 were 25.7 and 42.0 mg per g DCW, while those at day 6 were 55.7 and 61.0 mg per g DCW. This assumed that there was a limit to the amount of carbon concentration that could be assimilated. The FAMEs were not measured from the samples of days 8 and 10 because within 6 days all cultures reached the late exponential growth period or stationary growth period [14], which are well known as the maximum lipid production period in the cells. Interestingly, we also found that the ratios of oleic acid to total FAMEs increased with the increase of carbon concentration, as shown in Table 2.

Carbon Sources

Some microalgae are known to utilize organic matters for their biosynthesis [41]. This would be desirable to allow the reuse of wastewater containing organic carbon for mass culture of microalgae. In addition, there is a maximum allowable limit to inorganic carbon dioxide concentration, and this limit can act as a restricting factor in growth and lipid production. To investigate the effect of additional organic carbon sources on growth and lipid production of *Chlorella* sp. 227, glucose, a representative organic compound, of 0.5 mM, 5 mM, and 50 mM, respectively, was supplied additionally in the media, which contained basically 250 mM bicarbonate as the inorganic carbon source. All experiments were done aseptically. The growth curve (Fig. 2) was obtained by measuring DCWs rather than by converting optical density to DCWs, because the color of the culture solution turned slightly yellowish from a classical green color after 2-day cultivation.

At first, the differences of maximum DCWs among conditions in which different concentrations of glucose were added were analyzed by one-way ANOVA. On this analysis, one molar of glucose was considered as one molar of sodium bicarbonate equally, and the DCW per one molar carbon source was used for the analysis. According to the analysis, there was no significant difference between each condition, with the p-values being larger than 0.05 in the growth. Shortly, additional glucose increased the lipid contents

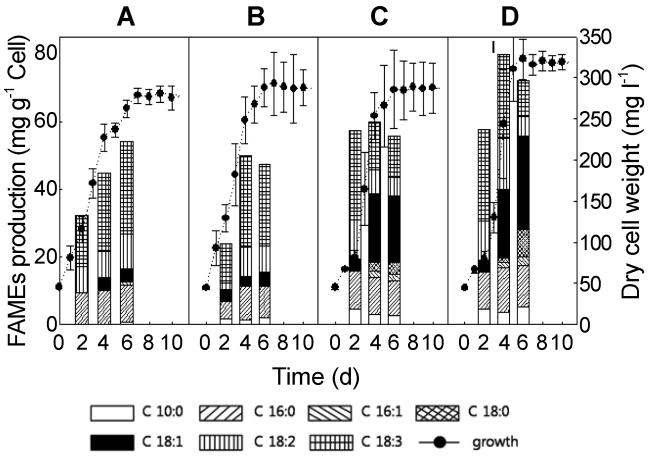

Fig. 2. Time course of *Chlorella* sp. 227 growth and FAMEs production per g DCW under mixotrophic condition; based on the same bicarbonate concentration (250 mM) and the addition of 0 mM (**A**), 0.5 mM (**B**), 5 mM (**C**), and 50 mM (**D**) of glucose in the medium, respectively.

per g DCW rather than the growth rate of *Chlorella* sp. 227. It was demonstrated by follows; the first was the lipid accumulation in cells that occurred from the early growth period with glucose of 5 mM and 50 mM. The second was that the highest lipid productivity per g DCW was shown in the culture having the highest concentration (50 mM) of glucose (Fig. 2). The third observation was at day 4 when the FAMEs production in the culture, with an additional 50 mM glucose (80.4 mg-FAMEs per g DCW), was approximately 2 times different compared with the culture with 0 mM glucose (44.8 mg-FAMEs per g DCW). The fact that lipid production increased under mixotrophic conditions is in accord with the results of Xiong *et al*. [40], but it is in contrast to the results of Heredia-Arroyo *et al*. [13], which reported the dramatic increase of biomass without the change of lipid content per cell *via* mixotrophic *Chlorella protothecoides*.

In addition, the lipid composition was also changed on the increase of glucose concentration. Specifically, the contents of oleic acid increased significantly as the glucose concentration increased (Fig. 2).

Nitrogen Sources

Unlike the results in which the maximum biomass production was obtained from nitrate, urea, and ammonia in order [21], the results obtained from the cultivation of *Chlorella* sp. 227 with the same concentration (10 mM) of different nitrogen sources showed a similar growth rate and FAMEs composition, regardless of nitrogen sources (Fig. 3), with no significant difference on the statistical analysis. The major FAMEs produced in *Chlorella* sp. 227 were linoleic acid (C18:2n6c) and α-linolenic acid (C18:3n3c), which occupied 70% or more. However, the different nitrogen sources slightly affected the FAMEs productivity per g DCW, and the maximum accumulation of FAMEs per g DCW occurred in the late-exponential growth phase with urea- and ammonia-N sources, but in the early stationary phase with nitrate-N. It might be connected to its assimilation process in which they reported that nitrate is usually utilized after *in vivo* transformation to nitrite or ammonia [27]. If so, various wastewaters having ammonia as a major nitrogen form can be used as a useful nitrogen resource for algal culture to reduce costs.

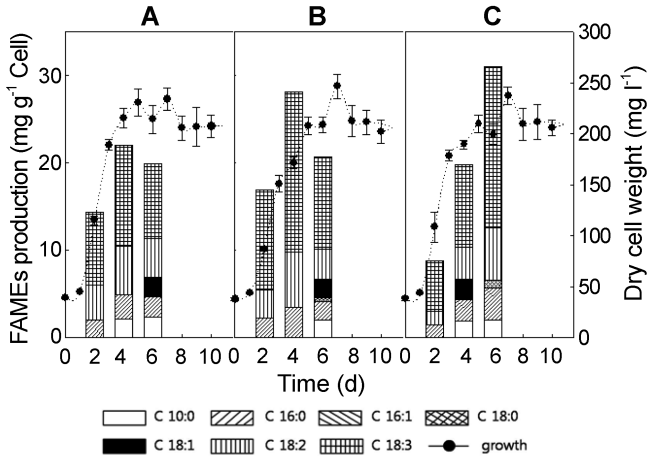

Fig. 3. Time course of *Chlorella* sp. 227 growth and FAMEs production per g DCW under different nitrogen sources of the same concentration; urea (**A**), ammonia (**B**), and nitrate (**C**).

The lipid content per g DCW in the cultures with ammonia and nitrate as nitrogen sources was higher than in the culture with urea, without the changes of FAMEs composition, respectively. The highest FAMEs content per g DCW was obtained from the nitrate-added culture, which was the same as Li *et al*'s study [20].

Nitrogen Concentrations

Based upon the experimental results above, ammonia was chosen as the nitrogen source to investigate the effect of nitrogen concentration on growth, and content and composition of FAMEs in *Chlorella* sp. 227 cultures. The different concentrations of 0.2 mM, 1.0 mM, and 2.0 mM were given to media, respectively. Considering that an average elemental composition for microalgae is given as $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$ [26], nitrogen is almost 10% (w/w) of total biomass and the amount is about 20% (w/w) of the carbon source. However, it is generally considered that the carbon source is taken for biomass (50%) as well as an energy source (50%). Then, the ratio of carbon to nitrogen is approximately 5~10. Therefore, the nitrogen concentration to not cause nitrogen depletion would be 2 mM of nitrogen, required because the DCW of approximately 250 mg was obtained as the maximum biomass in this study. Then, the concentration of 0.2 mM ammonia-N was assumed to cause a nitrogen depletion condition to enable lipid accumulation per g DCW.

The growth of *Chlorella* sp. 227 with the lowest ammonia concentration (0.2 mM) was retarded and stopped at the level of 0.69 times compared with that to the highest ammonia concentration (2 mM) with significant difference of p-value of 0.000 in the one-way ANOVA analysis. The maximum biomass obtained from the culture with 0.2 mM ammonia-N was 156.8 mg-DCW/l, while for those with 2 mM nitrogen it was 227.3 mg-DCW/l.

However, the maximum lipid production per g DCW with 0.2 mM ammonia-N (143.3 mg) was 2.3 times greater than that with 2 mM ammonia-N (63.0 mg) at day 6 (Fig. 4). This result followed the fact that nitrogen depletion promotes lipid production per cell. However, the growth rate of microalgae is also an important factor to determine lipid productivity. Therefore, considering the two factors, growth and maximum lipid production, the effect of lipid accumulation owing to nitrogen limitation (0.2 mM) calculated to 1.6 times; 0.2 mM nitrogen (22.5 mg-FAMEs/l) and 2 mM nitrogen (14.3 mg-FAMEs/l).

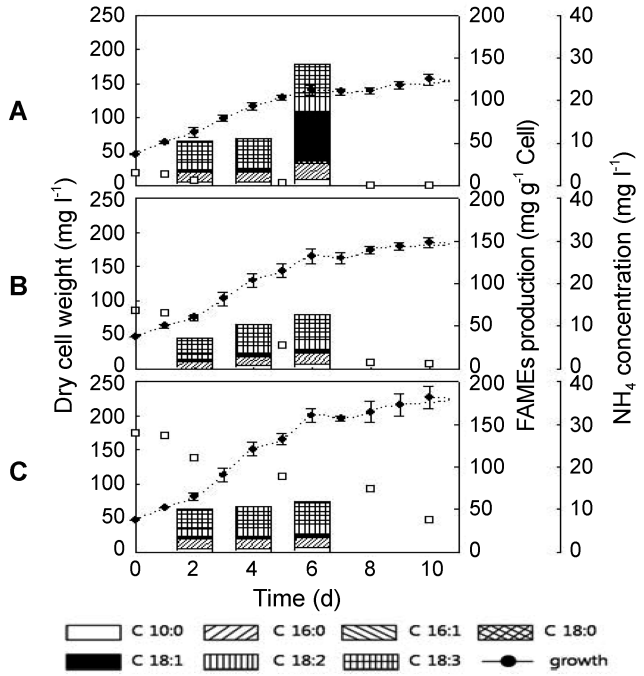

Fig. 4. Time course of *Chlorella* sp. 227 growth and FAMEs production per g DCW under different concentrations of ammonia-N; 0.2 mM ammonia-N (**A**), 1.0 mM ammonia-N (**B**), and 2.0 mM ammonia-N (**C**).

The increase of total lipid content under nitrogen limitation has been explained as the following; the enzymes involved in lipid synthesis are less susceptible to reduction in cellular soluble protein content than carbohydrate synthesis [2]. It demonstrated that the dramatic increase of FAME content per g DCW with 0.2 mM ammonia-N was observed soon after the nitrogen depletion in the medium at day 6 (Fig. 4). This corresponds with the previous results that lipid accumulation in microalgal cells was activated after consuming all nitrogen or when having lower nitrogen concentration than higher nitrogen concentration in the culture medium [7, 16, 36]. Even though nitrogen is an essential element in microalgal growth, it should be removed so as not to cause eutrophication before releasing water used for microalgae culture into natural water environments. Biodiesel production should be optimized with the lowest nitrogen concentration that minimizes growth inhibition.

With nitrogen depletion (0.2 mM ammonia-N), the oleic acid content was significantly increased to 39.3%, unlike the mere 7.9% under the nitrogen replete condition of 2 mM ammonia-N. This is a remarkable fact for high-quality biodiesel production. As a fuel, oleic acid is a recommendable FAME compound owing to its characteristics of liquid state at normal temperature, oxidative stability, and low-temperature behavior, relatively [15].

However, some studies did not show the improved lipid productivity under nitrogen-limited conditions, because limited nitrogen results in a decrease of biomass productivity [44]. In addition, the increase of lipid contents does not follow proportionally on the decrease of nitrogen concentration, while the biomass productivity was proportionally decreased on decrease of nitrogen concentration (Fig. 4).

Meanwhile, the nitrogen depletion condition occurred when we supplied 0.2 mM ammonia-N in the medium, and the maximum biomass obtained from the culture was 156.8 mg/l. However, nitrogen still remained in the culture where 2 mM ammonia-N was applied and the maximum biomass of 227.3 mg/l was obtained there (Fig. 4). Therefore, the 10 mM nitrogen given in the other experiments, in this study, might not induce a nitrogen depleted condition. Nevertheless, the highest lipid content per g DCW was achieved in the culture having the lowest nitrogen concentration (0.2 mM ammonia-N). The highest lipid productivity, in this study, was obtained in the culture having the most additional glucose (50 mM additional glucose), due to the low biomass productivity in the culture with 0.2 mM ammonia-N. On other hand, several strains did not follow the increase of lipid content in their cells as a response to only nitrogen limitation in their medium. According to another report, an increase of carbohydrate instead of lipid content was observed under low nitrogen environments [9, 33].

The maximum biomass production obtained in this study was 343 mg/l as the dry cell mass when the *Chlorella* sp. 227 was cultured mixotrophically with 250 mM bicarbonate and additional 50 mM glucose. In this study the relatively lower light intensity (27 μmol m^{-2} s^{-1}), temperature (25±0.5°C), and 16:8 h light/dark cycled illumination resulted in the low biomass productivity. This could be improved through optimizing the light intensity, pH, and illumination time in further study [13, 20, 40].

The growth rates of *Chlorella* sp. 227 were little affected by the sources of carbon and nitrogen, but low concentrations of carbon and nitrogen negatively affect the growth rates. The higher FAMEs productivities per g DCW were observed from the conditions having higher carbon and lower nitrogen concentrations, respectively. It indicates that a high ratio of carbon to nitrogen concentration (high C/N ratio) might promote lipid accumulation in the cells. However, the maximum lipid productivity should be considered with growth rates of *Chlorella* sp. 227 and lipid contents per g DCW at the same time.

Besides the increase of total lipid contents per g DCW under high C/N ratios, the significantly interesting fact found in this study is that the ratio of oleic acid content to total FAMEs produced was dramatically increased under nutrient conditions of high C/N ratios, such as nitrogen depletion conditions where carbon concentration was fixed and the conditions where an excess organic carbon source was supplied when nitrogen concentration was fixed. Therefore, it suggests that high C/N ratios in media

promote lipid accumulation as well as increase of oleic acid content in the total FAME profiles, relatively.

Several studies reviewed the effect of C/N ratios in alga-based biodiesel productivity [41, 42]. However, the increase of oleic acid content to total FAMEs produced by microalgae in media having high C/N ratios has never been reported before this study, even though it was referred to indirectly by Piorreck and Pohl [30]. On the other hand, the increase of oleic acid to total FAMEs in *Chlorella vulgaris* has already been reported as the response of temperature increase from 25°C to 38°C [7].

It has been previously reported that the fatty acid composition produced by microalgae varies with their physiological status and culture conditions, even with extraction methods for recovery of fatty acids [7, 15, 23, 30, 39]. In fact, the FAMEs composition of *Chlorella* sp. 227 is different according to nutrient composition, as shown in this study. There are some reports with the similar FAME composition of this study [12, 43] and some that are not [10].

In conclusion, the improvement of biomass and lipid productivity has become a major target of some studies for biodiesel production. This study shows the possibility that we can control other factors in producing high-quality biodiesel through modification of physiological characteristics besides strain selection. Therefore, this study suggests that the conditions to promote the lipid accumulation in microalgal cells extend to a high C/N ratio nutrient composition, including nitrogen limitation.

Acknowledgments

This work is supported by the Brain Korea 21 Program and by the Korea Institute of Energy Research.

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