Article

Mixotrophic Cultivation of Marine Alga *Tetraselmis* sp. Using Glycerol and Its Effects on the Characteristics of Produced Biodiesel

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Abstract

As a possible feedstock for biodiesel, the marine green alga *Tetraselmis* sp. was cultivated under different conditions of phototrophic, mixotrophic and heterotrophic cultures. Glycerol, a byproduct from biodiesel production process, was used as the carbon source of mixotrophic and heterotrophic culture. The effects of glycerol supply and nitrate-repletion were compared for different trophic conditions. Mixotrophic cultivation exhibited higher biomass productivity than that of phototrophic and heterotrophic cultivation. Maximum lipid productivity of 55.5 mg L^{-1} d^{-1} was obtained in the mixotrophic culture with 5 g L^{-1} of glycerol and 8.8 mM of nitrate due to the enhancement of both biomass and lipid accumulation. The major fatty acid methyl esters (FAME) in the produced biodiesel were palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). The degree of unsaturation was affected by different culture conditions. The biodiesel properties predicted by correlation equations based on the FAME profiles mostly complied with the specifications from the US, Europe and Korea, with the exception of the cold-filter plugging point (CFPP) criterion of Korea.

Keywords: Biodiesel, Microalgae, Glycerol, Mixotrophic, Fuel quality

1. Introduction

It is essential to enhance lipid productivity of microalgae during cultivation to produce microalgal biodiesel on a commercial scale. The lipid productivity of microalgae can be enhanced through a variety of cultivation strategies including the application of selected environmental stress factors during cultivation to increase cellular lipid synthesis and intracellular accumulation of produced lipids[1]. Mixotrophic or heterotrophic cultivation also enables us to enhance lipid productivity by increasing either cell growth rate or cellular lipid content compared to photoautotrophic cultivation[2,3]. Although both mixotrophic and heterotrophic culture can achieve increased cell density, mixotrophic cultivation is usually more advantageous than heterotrophic or photoautotrophic one. Mixotrophic microalgae normally performs photosynthesis under light condition using CO_2 and also able to utilize organic carbon sources under dark condition, minimizing biomass loss during dark respiration compared to phototrophic cultivation[4,5]. Under mixotrophic cultivation, the CO_2 generated from respiration can be reused for photosynthesis, whereas it is supposed to be wasted or released to the atmosphere under heterotrophic cultivation. Heterotrophic cultivation requires a substantial amount of organic substrates and oxygen supply - which incurs increased expenses, while mixotrophic culture can reduce the organic substrate consumption compared to heterotrophic cultures[6].

Previous studies have shown that the mixotrophic cultivation with supplementation of glucose, acetate or other organic substrates enhanced lipid productivity of some microalgae species such as *Botryococcus braunii*, *Chlorella* and *Chlorococcum* species[7-9]. However, glucose or acetate is not an appropriate carbon source on a large-scale cultivation, particularly for producing commodity products like biofuel which are not higher value-added products than the consumed carbon sources. Thus, in order to secure the economic feasibility of microalgal biodiesel on a commercial scale, it is necessary to use alternative organic carbon sources which are relatively inexpensive[10].

Glycerol is a byproduct of the biodiesel production process, and a substantial amount of glycerol is generated from the biodiesel production facilities, in the conversion step from triacylglycerol (TAG), or neutral lipids, to biodiesel in the form of fatty acid alkyl esters[11]. It has been known that some microalgae are able to utilize glycerol as a carbon source for cell growth and lipid synthesis[12,13]. A versatile species including *Chlorella*, *Haematococcus*, *Nannochloris* and *Scenedesmus* were reported to increase their cell growth rate and TAG content simultaneously under mixotrophic conditions with glycerol[14,15].

The aim of this study was to increase lipid productivity of the marine microalga *Tetraselmis* sp. utilizing glycerol as an organic carbon source in mixotrophic cultivation. *Tetraselmis* sp. is a green algal genus among the order Chlorodendrales which are found in both marine

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and freshwater. The *Tetraselmis* strain used in this study has been employed in floating photobioreactor systems on natural seawater to produce biodiesel because it has a wide salt tolerance, grows well in relatively cold seawater, and exhibits high lipid content[16,17]. The effects of the changes in glycerol and nitrate concentrations were investigated under different trophy conditions. The produced fatty acid methyl ester profile was analyzed and the resulting biodiesel properties were evaluated.

2. Materials and methods

2.1. Microalgal strain and culture medium

Green marine alga *Tetraselmis* sp. Ganghwa (KCTC 12236BP, Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology) was provided by the Marine Bioenergy Research Center in Korea[16]. The algal cells were cultured in sterilized artificial seawater enriched with F/2 nutrients. The artificial seawater had the following composition (g L^{-1}): NaCl, 24.72; KCl, 0.67; $CaCl_2 \cdot 2H_2O$, 1.36; $MgCl_2 \cdot 6H_2O$, 4.66; $MgSO_4 \cdot 7H_2O$, 6.29; $NaHCO_3$, 0.18; Tris, 0.606 (adjusted to pH 8.2). F/2 nutrients had the following composition (g L^{-1}): $NaNO_3$, 0.075 (0.882 mM); $NaH_2PO_4 \cdot H_2O$, 0.005; trace metals and vitamins including (mg L^{-1}) $FeCl_3 \cdot 6H_2O$, 3.15; $Na_2EDTA \cdot 2H_2O$, 4.36; $CuSO_4 \cdot 5H_2O$, 0.0098; $Na_2MoO_4 \cdot 2H_2O$, 0.0063; $ZnSO_4 \cdot 7H_2O$, 0.022; $CoCl_2 \cdot 6H_2O$, 0.01; $MnCl_2 \cdot 4H_2O$, 0.18; thiamine HCl, 0.2; biotin, 0.001; cyanocobalamin, 0.001.

2.2. Culture conditions

A two-stage culture process was applied: photoautotrophic culture was performed as the first stage for 5 days followed by the second stage under photoautotrophic, mixotrophic or heterotrophic conditions for 7 days. For the photoautotrophic and mixotrophic cultures, *Tetraselmis* cells were inoculated in aerated photobioreactors (PBRs) with 500 mL of working volume, and the light was supplied continuously at an intensity of 110~120 µmol m^{-2} s^{-1} using white fluorescent lamps (FL20SD/19, Kumho, Korea). Filtered ambient air was supplied through the bottom of the PBRs at a constant aeration rate of 0.2 vvm (volume to volume per minute) using a gas flow meter (Rate-Master, Dwyer, USA). Heterotrophic cultivation was conducted in shaking flasks with a constant agitation rate of 150 rpm, in which light was blocked by covering the flasks with aluminum foil. Refined glycerol (99.0%, Samchun, Korea) was added to the mixotrophic and heterotrophic cultures, at the concentration range of 1~15 g L^{-1}. The temperature was controlled within the range of 20~25 °C, and the experiments were repeated thrice.

2.3. Cell growth measurement

Cell growth was monitored by measuring the optical density at a wavelength of 680 nm (OD_{680}) using a UV/Vis spectrophotometer (DR-4000U, HACH, USA). The dry cell weight (DCW) was measured by filtering the cells using glass microfiber filters (GF/F 0.7 µm pore size, 47 mm diameter, Whatman, UK). Filters were dried at 80 °C for 12 h before and after filtering the cells. Biomass productivity was cal-

culated by dividing the difference between DCW at the start and end of the cultivation by the elapsed time. Nitrate concentrations remaining in the culture medium were monitored by the ultraviolet spectrophotometric screening methods (Standard Methods 4500-NO_3^- B, APHA, 1995).

2.4. Lipid analysis

The harvested cells were dried in a vacuum oven at 40 °C overnight for the analyses of lipid content and fatty acid compositions. The moisture-free cells at a weight of 50.0 mg were used for lipid extraction[18] after cell disruption using the ultrasonicator (VCS 130, Sonics & Materials Inc., CT, USA). Lipid content (wt %) was calculated by dividing the obtained lipids (mg) by the cell weight (50.0 mg). Lipid concentration per reactor volume (mg L^{-1}) was calculated by multiplying the DCW (g L^{-1}) and lipid content (g g^{-1}) for each cultivation period. The lipid productivity (mg L^{-1} d^{-1}) was calculated by dividing the lipid concentration (mg L^{-1}) by the elapsed time (d).

2.5. Transesterification and FAME analysis

The harvested cells were converted to fatty acid methyl ester biodiesel through the acid-catalyzed transesterification process[19] with some modifications. A 1.0 mL mixture of chloroform/methanol (2:1, v/v) was added to the moisture-free biomass cells, and 1.5 mL of 0.6 M HCl in methanol was also added. The sample mixtures were heated at 85 °C for 1 h and cooled for 15 min at room temperature. After adding 5.0 mL of *n*-hexane, samples were left undisturbed at room temperature for 1 h to allow phase separation to occur. The hexane layer was transfered into a new vial to analyze the FAME composition.

Fatty acids composition was analyzed using GC-FID (YL6500 GC, Younglin Instrument, Korea) equipped with an HP-INNOWAX capillary column (Agilent 19091N-213) at the following conditions: sample injection volume was 1 µL at 10:1 split ratio, inlet temperature was 260 °C, carrier gas was helium with a constant flow of 1.2 mL/min, the oven temperature was initially held at 140 °C for 5 min, increased at a rate of 4 °C min^{-1} up to 240 °C and held for 10 min. The detector temperature was 260 °C, detector gases were hydrogen, 35 mL min^{-1}; air zero, 300 mL min^{-1}; and helium, 20 mL min^{-1}. Each FAME was identified by the retention time of the FAME standard mixture (FAME mix C4-C24, Sigma Aldrich #18918, USA). GC analysis for each sample was conducted in triplicate, and the data are expressed as mean ± SD (standard deviation).

2.6. Estimation of biodiesel properties based on FAME profiles

The average degree of unsaturation (ADU) value of the produced FAME biodiesel was calculated as Eqn. (1) using the FAME profiles data in Table 1. The properties of the produced biodiesel such as including kinematic viscosity (ν), specific gravity (ρ), cloud point (CP), cetane number (CN) and iodine value (IV) were determined based upon the ADU value by empirical equations (2) through (6) where D is the number of double bonds and N_i is the percentage of each FAME component[20].

Table 1. FAME Composition under Phototrophic, Mixotrophic and Heterotrophic Conditions. For Mixotrophic and Heterotrophic Cultures, Glycerol Concentration was 5 g L^{-1} and Nitrate Concentration was 8.8 mM

		Fatty acid methyl esters (wt %)		
		Phototrophic	Mixotrophic	Heterotrophic
C16:0	Palmitic	27.26 ± 0.01	28.84 ± 0.10	26.00 ± 0.71
C16:1	Palmitoleic	2.16 ± 0.04	2.26 ± 0.06	6.32 ± 0.90
C17:0	Heptadecanoic	5.82 ± 0.16	6.18 ± 0.10	2.08 ± 0.02
C16:4	Hexadecatetraenoic	6.32 ± 0.05	6.15 ± 0.05	5.84 ± 0.17
C18:0	Stearic	2.17 ± 0.01	n.d.	3.64 ± 0.04
C18:1	Oleic	25.00 ± 0.10	25.09 ± 0.10	36.30 ± 0.47
C18:2	Linoleic	13.13 ± 0.03	13.16 ± 0.07	4.60 ± 0.08
C18:3	linolenic	11.97 ± 0.06	11.76 ± 0.03	9.10 ± 0.14
C18:4	Stearidonic	1.76 ± 0.04	2.02 ± 0.13	1.49 ± 0.08
C22:0	Behenic	4.41 ± 0.01	4.54 ± 0.15	4.63 ± 0.19
SFA		39.7	39.6	36.3
UFA		60.3	60.4	63.7
MUFA		27.2	27.4	42.6
PUFA		33.2	33.1	21.0

1) Data expressed as mean ± SD (n = 3)
2) n.d. = Not detected

$$ADU = \Sigma \; D \cdot N_i \tag{1}$$
$$\upsilon = -0.6316 \cdot ADU + 5.2065 \tag{2}$$
$$\rho = -0.0055 \cdot ADU + 0.8726 \tag{3}$$
$$CP = -13.356 \cdot ADU + 19.994 \tag{4}$$
$$CN = -6.6687 \cdot ADU + 62.876 \tag{5}$$
$$IV = 74.373 \cdot ADU + 12.71 \tag{6}$$

The oxidation stability/instability can be measured by induction period (IP). The induction period represents the time which is elapsed before increasing carboxylate level rapidly as a result of oxidation[21]. The cold filter plugging point and the induction period of the produced FAME biodiesel were predicted by Eqns. (7) and (8), respectively, where CP is the estimated cloud point and L is the sum (wt %) of linoleic and linolenic acid fractions among the produced FAME[22].

$$CFPP = 1.0191 \cdot CP - 2.9 \tag{7}$$
$$IP = 117.9295/L + 2.5905 \; (0 < L < 100) \tag{8}$$

3. Results and discussion

3.1. Effects of glycerol addition

Figure 1 shows the effects of glycerol supply on cell concentration and lipid content under different trophy conditions. The initial cell concentration was photoautotrphically grown 1.20 g L^{-1}. and the cultivations were carried out for three days in mixotrophic and heterotrophic conditions with different glycerol concentrations. One photoautotrophic cultivation was also maintained as a control. Figure 1(a) shows that mixotrophic cultures with glycerol of 5, 10 and 15 g L^{-1} resulted in cell concentrations of 1.70, 1.72 and 1.73 g L^{-1} on the final

day of cultivation, which corresponded to 0.167, 0.173 and 0.177 g L^{-1} d^{-1} as growth rate, respectively. The photoautotrophic culture achieved the final cell concentration of 1.61 g L^{-1} at day 3 (as 0.137 g L^{-1} d^{-1} as growth rate), while heterotrophic cultures did not show any increase in the cell concentrations. The growth rate under mixotrophic culture with 5 and 15 g L^{-1} glycerol was increased more than 21.9 and 29.2%, respectively, compared to autotrophic culture. However, the effect of the difference in glycerol concentrations in the range of 5 to 15 g L^{-1} on cell growth was not significant. When the glycerol concentration was below 5 g L^{-1} under mixotrophic conditions, cell concentrations showed no significant difference from those of autotrophic culture (data not shown). Therefore, about 5 g L^{-1} dose was considered as a threshold concentration of glycerol to stimulate the cell growth of *Tetraselmis* sp. in a mixotrophic condition.

Lipid content at the start of the glycerol addition was 22.5 %, and the changes in the lipid content due to the different concentrations of glycerol were not distinct as seen in Figure 1(b). The results indicated that although the supply of glycerol in mixotrophic cultures could improve the cell biomass productivity over that of autotrophic or heterotrophic cultures, the contribution of glycerol to lipid synthesis was not significant. Similar results were reported by Babuskin et al. [23] that a marine microalga *Isochrysis galbana* showed an increase in cell growth rate with glycerol supplementation in mixotrophic cultures compared to the growth in phototrophic cultures, while the lipid content was not increased concurrently by glycerol. In the present study, the average lipid contents in heterotrophic cultures were higher than those in phototrophic or mixotrophic cultures. However, the lipid profinal cell density were lowest. Therefore, heterotrophic cultivation is not recommended to enhance lipid productivity in microalgal biodiesel production with current *Tetraselmis* strain. It is important to consider increasing both biomass productivity and cellular lipid content to en-
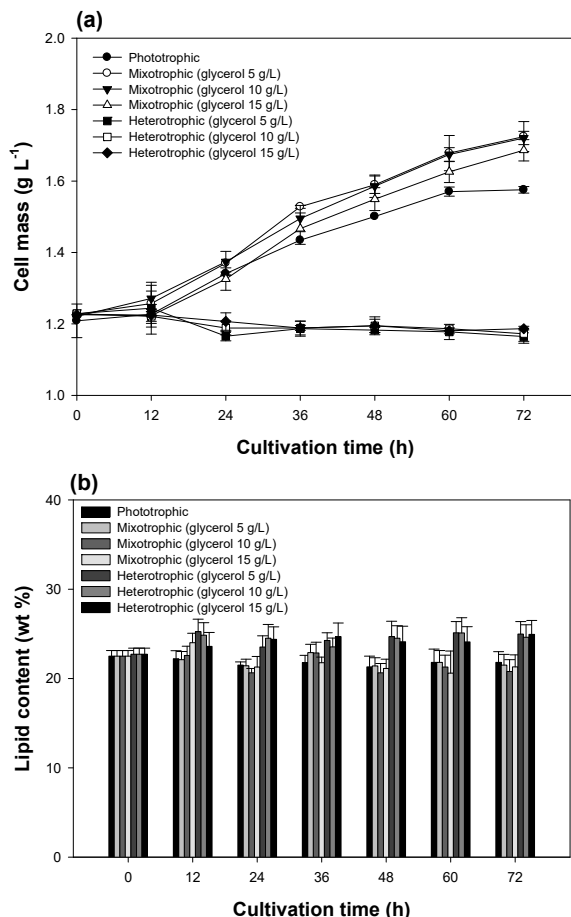
(The productivity of heterotrophic cultures was much lower than those of phototrophic or mixotrophic ones because biomass productivity and the)

Figure 1. Effect of different glycerol concentrations on (a) cell growth and (b) lipid content under mixotrophic and heterotrophic conditions. Photoautotrophic data are given as a reference.

Figure 2. Combined effects of nitrate-repletion (8.82 mM) and glycerol supplementation (5 g L^{-1}) on (a) cell growth and (b) lipid content in mixotrophic and heterotrophic conditions (In non-repleted controls, nitrate level = 0.88 mM).

hance lipid productivity.

3.2. Effect of nitrate repletion

The probable reason for not increasing lipid productivity in accordance with the glycerol supply was considered that there other major nutrients such as nitrogen might not be sufficient although carbon source was sufficient, since the *Tetraselmis* sp. was previously reported that lipid accumulation increased in nitrate-replete conditions[24]. Mixotrophic and heterotrophic cultivations were conducted in the presence of glycerol of 5 g L^{-1} and nitrogen repleted to 8.8 mM which was 10-times higher concentration than nitrogen content in usual F/2 medium (i.e. 0.88 mM, non-repleted control). The contribution of nitrogen repletion is shown in Figure 2. In Figure 2(a), the cell concentration reached up to 2.55 g L^{-1} for 7 days of cultivation under the mixotrophic culture with nitrate-repletion, while it was 2.08 g L^{-1} in the mixotrophic culture with non-repleted control. Autotrophic cultures also showed a little enhancement of cell growth with nitrogen repletion, achieving a cell concentration of 1.88 g L^{-1} at 7th day, compared to non-repleted autotrophic culture. Meanwhile the cells in heterotrophic condition showed no growth at all regardless of nitrogen repletion.

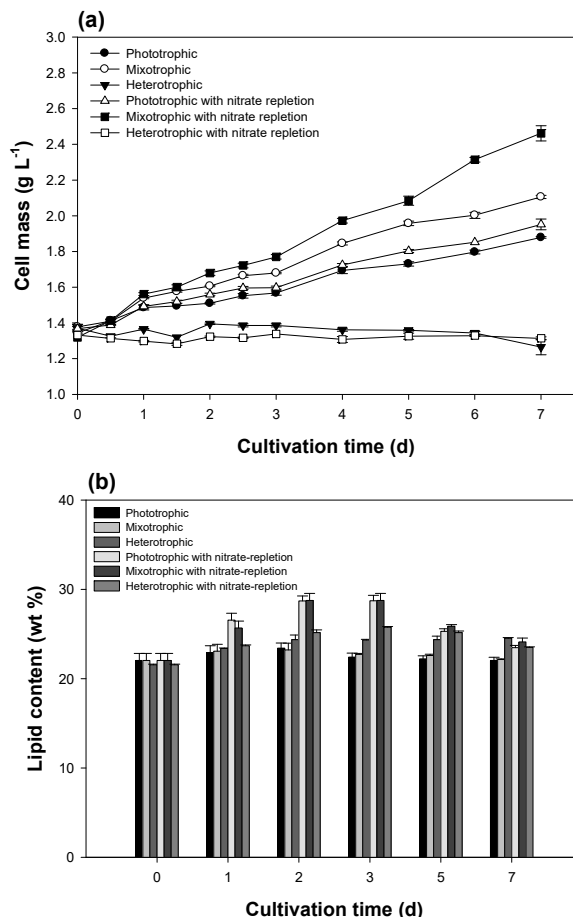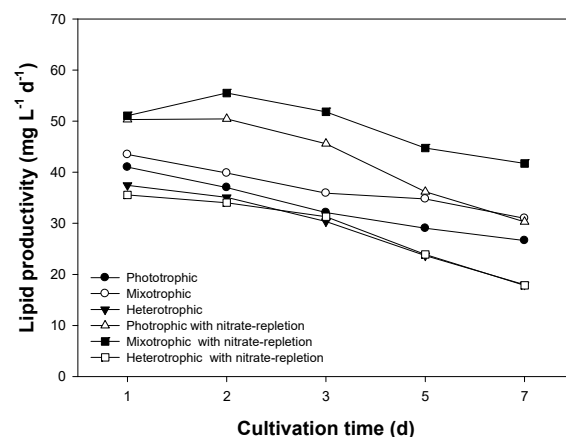As seen in Figure 2(b), the maximum lipid content of 28.7 % was

Figure 3. Lipid productivity under phototrophic, mixotrophic and heterotrophic cultures with glycerol and different nitrate levels.

observed in both phototrophic and mixotrophic cultures with nitrate-repletion within 2 days of cultivation. These results indicated that lipid accumulation was induced by the nitrate-repletion, not by the glycerol supplementation. Figure 3 shows the change of lipid productivity

Table 2. Fuel Properties of the Biodiesel Derived from the *Tetrselmis* Biomass Cultures under Different Trophy Conditions and Their Comparison with the Standards of US, Europe and Korea

	Phototrophic	Mixotrophic	Heterotrophic	US (ASTM D6751-08)	Europe (EN 14214)	Korea (KS M 2619)
Cetane number (CN)	54.8	54.8	55.6	47 (min)	51 (min)	45 (min, BD20)
Kinematic viscosity 40 °C (mm^2 s^{-1})	4.4	4.4	4.5	1.9-6.0	3.5-5.0	1.9-5.0
Specific gravity (kg L^{-1})	0.879	0.879	0.879	-	0.86-0.90 (density)	0.86-0.90 (density)
Cloud point (°C)	3.75	3.75	5.51	Report	-	-
Cold filter plugging point (°C)	0.9	0.9	2.7	-	Country-specific	0 (max)
Oxidation induction time, 110 °C (h)	7.3	7.3	11.2	3 (min)	8 (min)	6 (min)
Iodine value (g I$_2$ 100 g^{-1})	103.2	103.2	93.4	-	120 (max)	-
Average degree of unsaturation (ADU)	1.22	1.22	1.08	-	-	-

through the time course. Owing to the increase in both cell growth and lipid content under mixotrophic cultures with nitrate-repletion, the highest lipid productivity of 55.5 mg L^{-1} d^{-1} was achieved at day 2. Autotrophic culture with nitrate-repletion also showed an increased lipid productivity due to the increased lipid content. Regardless of the nitrate level, heterotrophic cultures showed the lowest lipid productivity. The current *Tetraselmis* strain is not capable of utilizing heterotrophic condition with glycerol for cell growth and lipid synthesis.

According to the experimental results, it can be concluded that the supplementation of glycerol as an organic carbon to the mixotrophic culture is responsible for the enhancement of cell growth, but does not contribute significantly to lipid synthesis, compared to the conventional photoautotrophic cultivation. However, nitrogen repletion using nitrate contributed greatly to increasing lipid synthesis and further cell growth.

3.3. FAME profiles and fuel properties

Neutral lipids were extracted from the cells grown in phototrophic, mixotrophic and heterotrophic cultures with nitrate-repletion, and then were converted to fatty acid methyl ester biodiesel. The fatty acid composition of the obtained FAMEs were analyzed and the obtained profiles are given in Table 1. The major fatty acids of FAME were palmitic (C16:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids, accounting for over 75 %. There was no significant change in FAME compositions between phototrophically and mixotrophically grown cells, as they showed a similar ratio between saturated and unsaturated fatty acids. Heterotrophically grown cells had higher content of monounsaturated fatty acids (MUFA) than the phototrophic or mixotrophic grown cells. Conversely, the polyunsaturated fatty acid (PUFA) content was smaller than that in phototrophic or heterotrophic cells.

The length of the carbon chain and the number of double bonds in FAME directly influence biodiesel properties such as ignition quality, oxidation stability, low-temperature flow properties, viscosity and density. Particularly, the fraction of PUFA greatly affects the oxidation stability of biodiesel[25,26]. As shown in Table 2, the predicted fuel properties based on the FAME profiles mostly complied with the standards for biodiesel (BD100) fuel in the US, Europe and Korea. The cetane numbers (CNs) were in the range of 54.8~55.6, which were higher than the minimum limits of the standards. The CN value is re-

lated to the ignition delay and combustion quality. The higher the CN, the shorter the ignition delay and also the lower the amount of NO$_x$ emission[27]. The kinematic viscosity and the specific gravity were also in the range of the standard limits, which are important physical properties related to the behavior of fuel injection and engine performance[25,28]. The predicted kinematic viscosity was approximately 4.5, and it was reported that most biodiesel fuels obtained from vegetable oils exhibit viscosity values in the range of 4~5 mm^2 s^{-1} which is relatively higher than those of petrodiesel fuels, 2~3 mm^2 s^{-1}.

The low-temperature flow property is indicated by the cloud point, pour point and cold filter plugging point. The CP is the temperature at which the fuel starts to form wax crystals, and the PP is the temperature at which the fuel ceases to flow. The CFPP is the temperature at which the fuel causes a filter to plug due to crystallization, and is limited by the Korean Standard (KS) to less than 0 °C (-16 °C for BD20). The predicted CFPPs did not satisfy the KS requirement showing values above 0 °C, probably due to the high content of SFA with long carbon chains in FAME. The problem of low-temperature flow is common in biodiesel fuels, but can be improved by employing cold flow additives/improvers, blending with conventional diesel oil, or winterization which reduces SFA fraction[28,29].

Oxidation stability, another major issue of biodiesel use, is also related to the degree of unsaturation. The oxidation stability/instability can be measured by induction period. The induction period represents the time which is elapsed before increasing carboxylate level rapidly as a result of oxidation[21]. Particularly, biodiesel fuels with high PUFA content, such as C18:2 and C18:3, are vulnerable to oxidation. It was reported that biodiesel derived from *Dunaliella tertiolecta* biomass exhibits poor oxidation stability due to the high fraction of C16:4 and C18:3[30]. Although the *Tetraselmis* sp. in the present study also had not so small fractions of C18:2, C18:3 and C16:4, the induction period for oxidation instability was predicted in the range of 7~11 h, meeting the minimum requirement of the Korean Standard and US standard. Iodine value which is proportional to the degree of unsaturation[25] was also within the maximum limit of European Standard.

4. Conclusions

Simultaneous enhancement in the cell growth and lipid content of *Tetraselmis* sp. was achieved through mixotrophic cultivation using glycerol with nitrate-replete conditions. Autotrophically grown cells were divided into three different trophy-conditioned cultivations. The mixotrophic culture showed the highest growth rate compared to heterotrophic and autotrophic cultivation. Although the supply of glycerol in mixotrophic culture could improve the cell biomass productivity, its contribution to lipid synthesis was not significant. Nitrate repletion greatly induced lipid synthesis instead of glycerol supplementation. Owing to the increase in both cell growth and lipid content under mixotrophic cultures with and 5 g/L glycerol and 8.8 mM nitrate-repletion, the highest lipid productivity of 55.5 mg L^{-1} d^{-1} was achieved. There was no significant change in FAME compositions between phototrophically and mixotrophically grown cells, while heterotrophically grown cells had higher content of monounsaturated fatty acids than the phototrophic or mixotrophic grown cells. The fuel properties such as cetane number, viscosity and oxidation stability meet biodiesel quality specifications of three different standards, except that CFPP value was slightly higher than the minimum limit of Korean Standard.

Acknowledgment

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