Full Length Research Paper

Manipulating nutrient composition of microalgal growth media to improve biomass yield and lipid content of *Micractinium pusillum*

Reda A.I. Abou-Shanab¹,2, Sapireddy V. Raghavulu¹, Nagah M.A. Hassanin², Seongheon Kim¹, Yong Je Kim³, Sang Un Oh⁴, You-Kwan Oh⁵, and Byong-Hun Jeon¹*

¹Department of Environmental Engineering, Yonsei University, Wonju, Gangwon-do 220-710, South Korea.
²City of Scientific Research and Technology Applications, New Borg El Arab City, Alexandria 21934, Egypt.
³Geologic Environment Division, KIGAM, Daejeon 305-350, South Korea.
⁴Department of Biological Environment, Kangwon National University, Chuncheonsi, Gangwon-do, South Korea.
⁵Korea Institute of Energy Research, Daejeon 305-343, South Korea.

Accepted 9 November, 2012

Biodiesel production from microalgae depends on the algal biomass and lipid content. Both biomass production and lipid accumulation are limited by several factors in which nutrients play a key role. We investigated the influences of micronutrients on biomass, and lipid content of *Micractinium pusillum* GU732425 cultivated in bold basal media (BBM). The average dry biomass of microalgal strain in control medium reached 0.34 ± 0.01 g/L, while doubling (2X) the levels of Mn and Cu concentration increased the dry biomass to 0.38 ± 0.01 and 0.37 ± 0.02 g/L, respectively. *M. pusillum* cultivated in control medium had a biomass of 0.82 ± 0.05 g/L and a lipid productivity of 0.33 ± 0.02 g/L after 17 day cultivation. The alga cultivated in BBM with 4X Mn or 4X Cu produced more biomass (1.25 ± 0.01 or 1.28 ± 0.04 g dw/L) and lipid productivity (0.45±0.04 or 0.47±0.05 g/L), respectively. *M. pusillum* cultivated in different growth media had fatty acid compositions mainly comprising linoleic (49-54%), palmitic (24-29%), linolenic (16-22%), and oleic acids (2-5%). These results can be used to maximize the production of microalgal biomass and lipids in optimally designed photobioreactors.

Key words: *Micractinium pusillum*, biomass, lipid production, media composition, fatty acids, trace metals.

INTRODUCTION

Both rapid growth and industrialization of nations have resulted in a steep increase in the production and consumption of fossil fuels. This increase has not only put severe stress on already depleting fossil fuels, but also resulted in an alarming increase in pollution across the globe. The current demand for biofuel as a gasoline substitute is extremely high due to the high cost of petroleum or the potential for a high cost. One such fuel showing great potential is biodiesel that has received much attention recently, as it is made from non-toxic, biodegradable, and renewable resources. Biodiesel also has environmental benefits, because they have fewer harmful emissions, such as carbon monoxide and hydrocarbons, and can decrease the greenhouse effect (Gouveia and Oliverira, 2009; Campbell et al., 2011).

Microalgae are emerging as one of the most promising resources of biodiesel with a projected yield of 58,700 to 136,900 L/ha/year (Chisti, 2007). Microalgae have a number of advantages as a potential feedstock to produce biodiesel, including higher photosynthetic efficiency, biomass production, and growth rates than other energy crops (Huang et al., 2010). Many microalgae have the ability to produce substantial amounts (1 to 70% dry cell weight) of triacylglycerols (TAG) as a storage lipid under photo-oxidative stress or other adverse environmental conditions (Richmond, 2004; Cheirsilp and Torpee, 2012). Lipid production from microalgae can be improved by manipulating growth conditions such as nitrogen deprivation (Illman et al., 2011).
in triplicate, and data are expressed as mean ± standard deviation.

Lipid extraction and fatty acid analyses

The total lipids were extracted from M. pusillum biomass (0.2 g/L) using a slightly modified method of Bligh and Dyer (1959). In brief, cells were harvested and lyophilized. Lipids were extracted with a mixture of chloroform and methanol (1:2, v/v), transferred into a glass tube, and indirectly sonicated for 30 min at a constant frequency of 40 kHz and at a power output of 700 W using a Powersonic 420 bath sonicator, South Korea. The tube was then incubated over night at 27°C with shaking at 100 rpm. An additional aliquot of chloroform (1.25 mL) was added to the tube and the content was sonicated again for 30 min. To separate the chloroform and aqueous methanol layers, 1.25 mL deionized water was added to the tube, which was then centrifuged at 4000 rpm for 10 min. The chloroform layer was collected from the bottom of the tube. A second extraction was performed by adding 2.5 mL chloroform and vortexing. The chloroform layer was gently collected from the bottom of the tube, washed with 5 mL of 5% NaCl solution, and evaporated in a dry oven at 50°C. The percent lipid of total dry biomass was calculated as weight of crude lipids that was used for fatty acid methyl ester analysis. Each experiment was carried out in triplicate and average values were reported.

Fatty acids were analyzed using a modification of the method proposed by Lepage and Roy (1984). The crude lipid (~10 mg) was dissolved in 2 mL of a freshly prepared chloroform and methanol mixture (2:1, v/v) and transferred to a 10 mL Pyrex tube with a Teflon-sealed screw-cap. 1 mL of chloroform containing an internal standard and transmethylation reagents was added to the tube and mixed for 5 min. The contents were transferred to a 10 mL Pyrex tube, incubated at 100°C for 10 min, cooled to room temperature, and separated into two phases by adding 1 mL deionized water. After 10 min of vigorous mixing and centrifugation at 4000 rpm for another 10 min, the chloroform layer was collected from the bottom of the tube using a hypodermic disposable polypropylene syringe and filtered through 0.2 μm syringe filters. Fatty acid methyl esters (FAMEs) in the extracted liquid were quantified by CP2010 Gas Chromatography–Mass Spectrometry (Shimadzu, Japan) with a flame ionization detector using a HP-5MS capillary column.

The oven temperature was set at 80°C, held for 5 min, raised to 290°C at 4°C/min, and held at 290°C for 5 min, and the temperature for injector and detector were set at 250 and 230°C, respectively. Helium was used as a carrier gas at a flow rate of 1.2 mL/min. The compounds were identified by comparing fragmentation patterns with those in the National Institute of Standards and Technology (NIST) library.

Statistical analysis

All data are represented as mean ± standard deviation of triplicate. Statistical analysis was performed using the SPSS package system version 11.

RESULTS AND DISCUSSION

Effect of media compositions on the growth rate of M. pusillum

Microalgae can grow profusely when supplied with sufficient nutrients under suitable conditions. Algal growth is directly affected by light and nutrient availability, pH and temperature stability, and the initial density of
inoculum (Wang et al., 2010a). A certain amount of trace metals (that is Mn, Cu, Zn, and Co) is capable to induce the growth of microalgae, while at the same time higher concentrations of these micronutrients can retard the growth of microalgae (Ilavarasi et al., 2011). Figure 1A shows that depleting individual micronutrients (that is Co, Mn, Zn, and Cu) from the culture media significantly decreased the M. pusillum growth rate compared with the control (paired t-test=3.42, P < 0.01). The average dry biomass concentration of M. pusillum grown in BBM (control) was 0.34 ± 0.01 g/L, while for micronutrient-depleted BBM, the dry biomass ranged from 0.24 ± 0.01 g/L (0X Cu) to 0.28 ± 0.01 g/L (0X Co) after 17 day of cultivation. Micronutrients (Co, Mn, Zn, and Cu) are essential for microalgal growth. These elements play vital roles in the active site of many algal enzymes and are involved in numerous metabolic processes, including photosynthesis and energy storage (Christensen, 1997; Liu et al., 2008; Chen et al., 2011). Thus, depleting micronutrients from the culture medium adversely affected M. pusillum growth.

The average dry biomass of M. pusillum increased with the increase of Mn or Cu concentrations (from 2X to 4X) in the growth medium (Figure 1B). The dry biomass concentration of microalgal strain in BBM supplemented with double concentration (2X) of Mn or Cu reached 0.38...
± 0.01 g/L or 0.37 ± 0.02 g/L, respectively, after 17 days of incubation, both of which were significantly higher (paired t-test -2.3, P < 0.05) than the control (0.34 ± 0.01). In contrast, increasing the Zn or Co concentration in the growth media had no noticeable effect on dry weight. Based on these results, further experiments evaluated M. pusillum growth as a function of Mn or Cu concentration in BBM. Increasing the Mn or Cu concentration to 4X, increased the M. pusillum biomass (0.39 ± 0.01 or 0.42 ± 0.01 g/L, respectively) compared to regular BBM (Figures 2 and 3). Interestingly, increasing the Mn or Cu concentration to 5X or higher had no further
Table 1. Effect of trace metals concentration in the growth medium on biomass yield, lipid production, and lipid content of *M. pusillum*

| Parameter                 | Control | 0X | 2X | 0X | 2X | 0X | 2X | 3X | 4X | 5X | 6X |
|---------------------------|---------|----|----|----|----|----|----|----|----|----|----|
| Biomass (g dw/L)          | 0.82±0.05 | 0.69±0.12 | 0.76±0.04 | 0.71±0.12 | 0.85±0.09 | 0.61±0.12 | 1±0.08 | 1.20±0.04 | 1.25±0.01 | 0.98±0.09 | 0.99±0.03 |
| Lipid productivity (g/L)  | 0.33±0.02 | 0.26±0.01 | 0.30±0.02 | 0.24±0.02 | 0.28±0.01 | 0.23±0.01 | 0.41±0.02 | 0.40±0.02 | 0.45±0.04 | 0.39±0.05 | 0.38±0.04 |
| Lipid content (%)         | 40±3.1 | 38±2.5 | 39±3.5 | 34±0.5 | 33±5.9 | 38±2.9 | 41±1.5 | 33±1.8 | 36±3.1 | 40±1.5 | 38±3.4 |

Table 2 shows the fatty acid composition in *M. pusillum* harvested from different culture media. Linoleic acid (C18:2n6c) ranged from 49 to 54% of all fatty acids, and was the dominant fraction for all experimental conditions. Linoleic acid was followed by palmitic acid (C16:0) and linolenic acid (C18:3n3) ranging from 24 to 29% and 16 to 22%, respectively. Oleic acid (C18:1n9c) accounted for <5% of all fatty acids. Biodiesel quality depends on the fatty acid composition. Petkov and Garcia (2007) found 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, and α-18:3 fatty acid components from green algae. A large number of double bonds in a fatty acid make it more susceptible to oxidation, thus results in economical loss (Chisti, 2007).

Nutrient composition of the growth medium, cultivation conditions, and growth phase can readily affect the fatty acid composition in algal
brazilian service waters. The present work investigated the effect of culture medium (BBM) supplemented with different concentrations of trace metals on the biomass yield, and lipid production of *M. pusillum*. The results demonstrate that trace metals play a major role in the algal biomass yield and lipid production. Increasing the Cu or Mn concentration in BBM increased the algal biomass and lipid productivity. BBM amended with 4X concentration of Cu or Mn resulted in 1.6 or 1.5-fold increase in biomass yield and 1.4 or 1.3-fold increase in lipid productivity when compared to control, respectively. The polyunsaturated fractions ranged from 68 to 73% of the total fatty acids (FA) in microalgae cultivated under all experimental variations. The lower percentage of polyunsaturated FA was obtained from alga grown in BBM amended with 4X Mn and 4X Cu. This study underlined the significance of medium development in achieving high-density cultures and lipid contents.

### Conclusion

The present work investigated the effect of culture medium (BBM) supplemented with different concentrations of trace metals on the biomass yield, and lipid production of *M. pusillum*. The results demonstrate that trace metals play a major role in the algal biomass yield and lipid production. Increasing the Cu or Mn concentration in BBM increased the algal biomass and lipid productivity. BBM amended with 4X concentration of Cu or Mn resulted in 1.6 or 1.5-fold increase in biomass yield and 1.4 or 1.3-fold increase in lipid productivity when compared to control, respectively. The polyunsaturated fractions ranged from 68 to 73% of the total fatty acids (FA) in microalgae cultivated under all experimental variations. The lower percentage of polyunsaturated FA was obtained from alga grown in BBM amended with 4X Mn and 4X Cu. This study underlined the significance of medium development in achieving high-density cultures and lipid contents.

### ACKNOWLEDGEMENTS

This work was supported by the Korea Institute of Energy Research, the Senior Researchers program (The National Research Foundation of Korea, 2010-0026904), the Eco-Innovation project (The Global-Top project) funded by the Korea Ministry of Environment, and the Brain Korea-21 (BK-21) and Brain Pool (KFSTS, Grant number: 11-150-152-1600-1658) programs administrated by the Ministry of Education, Science and Technology (MEST).

### REFERENCES

Abou-Shanab RAI, Hwang JH, Cho Y, Min B, Jeon BH (2011). Characterization of microalgal species isolated from fresh water bodies as a potential source for biodiesel production. Appl. Energy 88:3300-3306.
American Public Health Association (1998). Methods for biomass production. In: Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Baltimore, MD, USA.
Bischoff HW, Bold HC (1983). Phyiological studies IV. Some soil algae from Enchanted Rock and related algal species. University of Texas Publication 6318:1-95.
Bligh EG, Dyer WJ (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.
Campbell PK, Beer T, Batten D (2011). Life cycle assessment of biodiesel production from microalgae in ponds. Bioresour. Technol. 102:50-56.
Cheirsilp B, Torpee S (2012). Enhanced growth and lipid
production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation. Bioreour. Technol. 110:510-516.

Chen M, Tang H, Ma H, Holland TC, Ng KYS, Salley SO (2011). Effect of nutrients on growth and lipid accumulation in the green alage Dunaliella tertiolecta. Bioreour. Technol. 102:1649-1655.

Chisti Y (2007). Biodiesel from microalgae. Biotechnol. Adv. 25:294-306.

Christensen KK (1997). Differences in iron, manganese, and phosphorus binding in freshwater sediment vegetated with Littorella uniflora and benthic microalgae. Water Air Soil Pollut. 99:265-273.

Chloe” I, Dumont O, Picciotti M, Bourre JM (1987). Alterations of lipid synthesis in the normal and dysmyelinating trembler mouse scialic nerve by heavy metals (Hg, Pb, Mn, Cu, Ni). Toxicology 46:65-71.

Gousseia L, Oliverira CA (2009). Micro algae as a raw material for biofuel procoution. J. Ind. Microbiol. Biotechnol. 36:269-274.

Guschina IA, Harwood JL (2006). Lipids and lipid metabolism in eukaryotic algae. Prog. Lipid Res. 45:160-186.

Hosaglui M, Gultepe I, Elibol M (2012). Optimization of carbon and nitrogen sources for biomass and lipid production by Chlorella saccharophila under heterotrophic conditions and development of Nile red fluorescence based method for quantification of its neutral lipid content. Biochem. Eng. J. 61:11-19.

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008). Microalgal triacylglycerols as feedstocks for biofuels production: perspectives and advances. Plant J. 54:621-639.

Huang G H, Chen F, Wei D, Zhang XW, GuChen (2010). Optimization of various growth media to freshwater microalgae for biomass production. Biotechnology Biofuels 3:820.

Illman AM, Scragg AH, Shales SW (2000). Increase in nutrient availability on the biochemical and elemental stoichiometry in freshwater diatom Stephanodiscus minintus acilariophyceae. J. Phycol. 36:510-522.

Mata TM, Martins AA, Caetano NS (2010). Microalgae for biodiesel production and other applications: a review. Renew. Sustain. Energy Rev. 4:217-232.

Petkov G, Garcia G (2007). Which are fatty acids of the green alga Chlorella? Biochem. Syst. Ecol. 35:281-285.

Rao AR, Dayananda C, Sarada R, Shamala TR, Ravishankar GA (2007). Effect of salinity on growth of green alga Botryococcus braunii and its constituents. Bioreour. Technol. 98:560-564.

Reitan KL, Rainuzzo JR, Olsen Y (1994). Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. J. Phycol. 30:972-979.

Richtoand A (2004). Handbook of microalgae culture: biotechnology and applied phycology. Black Well Science Ltd., Oxford, UK.

Rousch JM, Sommerfeld MR (1999). Effect of manganese and nickel on growth of selected algae in pH buffered medium. Water Res. 33:2448-2454.

Sheehan J, Cambreco V, Duffield J, Garboski M, Shapouri H (1998). An overview of biodiesel and petroleum diesel life cycles. A report by US Department of Agriculture and Energy 98:1-35.

Shen Y, Pei Z, Yuan W, Mao E (2009). Effect of nitrogen and extraction method on algae lipid yield. Int. J. Agric. Biol. Eng. 2:51-57.

Song L, Qin JG, Su S, Xu J, Clarke S, Shan, Y (2012). Micronutrient requirements for growth and hydrocarbon production in the oil producing green alga Botryococcus braunii (Chlorophyta). PLoS ONE 7:41459. doi:10.1371/journal.pone.0041459.

Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006). Commercial applications of Microalgae. J. Biosci. Bioeng. 101:87-96.

Wang L, Yecong L, Chen P, Min M, Chen Y, Zhu J, Ruan R (2010a). Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae Chlorella sp. Bioreour. Technol. 101:2623-2628.

Wang Z, Chen S, Cao X (2010b). Micro-nutrients effects on algae colony: growth rate and biomass response to various micro-nutrients and competitive inhibitions among multi-microelements. Symp. 4th Int. Conf. Bioinformat. Biomed. Eng. pp. 1-8.