Full Length Research Paper

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

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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.,

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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 QP2010 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
effect on the algal biomass concentration. These results reveal that a 4-fold increase in Mn or Cu concentration maximized the M. pusillum dry biomass. Our result was consistent with earlier reports showing that Mn had stronger impact on the growth of Ulothrix sp. (Rousch and Sommerfeld, 1999).

**Biomass yield and lipid productivity**

We harvested the algal cells after the 17 day incubation and examined lipid content, lipid productivity and biomass yield (Table 1). Depleting Zn, Mn, Co, or Cu from growth medium adversely affected algal biomass and lipid production. M. pusillum grown in BBM with 4X Cu or Mn produced more biomass (1.28 ± 0.04 or 1.25 ± 0.01 g/L) and lipid productivity (0.47 ± 0.05 or 0.45 ± 0.04 g/L) after 17 day of cultivation than the control (Table 1). Increasing the Mn or Cu concentration to 5X or higher had no further effect on the algal dry weight. This finding was consistent with the result of Wang et al. (2010b) who found that the increase of Mn concentrations stimulated the growth of blue green algae, while a further increase in Mn inhibited algal growth. The total lipid contents of M. pusillum in this study ranged from 31 ± 3.5% to 41 ± 1.5% of the dry biomass weight.

The highest lipid content (41 ± 1.5%) was presented by the algal strain grown in BBM containing 2X Mn. Cloez et al. (1987) found that lipid synthesis increased by three times after adding manganese, copper, and nickel at 2 mM. Hydrocarbon production was more sensitive to the change in Mn concentration. An increase in hydrocarbon production resulted from the increase of Mn concentrations (Song et al., 2012). Many microalgae species can be induced to accumulate substantial quantities of lipids (Sheehan et al., 1998), resulting in a high oil yield. Lipid contents of 20 to 50% of the dry biomass weight have been reported to be quite common (Spolaore et al., 2006; Li et al., 2008). It has also been reported that lipids accounting for more than 90% of the dry biomass of some microalgae have been reported in some culture conditions (Mata et al., 2010).

### Fatty acid composition

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

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**Table 1. Effect of trace metals concentration in the growth medium on biomass yield, lipid production, and lipid content of M. pusillum**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>0X</th>
<th>2X</th>
<th>3X</th>
<th>4X</th>
<th>5X</th>
<th>6X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass (g dw/L)</td>
<td>0.82±0.05</td>
<td>0.69±0.12</td>
<td>0.76±0.04</td>
<td>0.71±0.12</td>
<td>0.85±0.09</td>
<td>0.61±0.12</td>
<td>1±0.08</td>
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<tr>
<td>Lipid productivity (g/L)</td>
<td>0.33±0.02</td>
<td>0.26±0.01</td>
<td>0.30±0.02</td>
<td>0.24±0.02</td>
<td>0.28±0.01</td>
<td>0.23±0.01</td>
<td>0.41±0.02</td>
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<tr>
<td>Lipid content (%)</td>
<td>40±3.1</td>
<td>38±2.5</td>
<td>39±3.5</td>
<td>34±0.5</td>
<td>33±5.9</td>
<td>38±2.9</td>
<td>41±1.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>0X</th>
<th>2X</th>
<th>3X</th>
<th>4X</th>
<th>5X</th>
<th>6X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass (g dw/L)</td>
<td>0.82±0.05</td>
<td>0.60±0.04</td>
<td>1.04±0.16</td>
<td>1.21±0.12</td>
<td>1.28±0.04</td>
<td>1.19±0.09</td>
<td>0.99±0.01</td>
</tr>
<tr>
<td>Lipid productivity (g/L)</td>
<td>0.33±0.02</td>
<td>0.24±0.04</td>
<td>0.39±0.16</td>
<td>0.45±0.02</td>
<td>0.47±0.05</td>
<td>0.35±0.02</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>Lipid content (%)</td>
<td>40±3.1</td>
<td>40±1.9</td>
<td>38±0.5</td>
<td>37±3.7</td>
<td>38±1.5</td>
<td>32±1.1</td>
<td>31±3.5</td>
</tr>
</tbody>
</table>
Table 2. Effect of trace metals concentration in the growth medium on fatty acid composition (%) of M. pusillum

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>Zinc 0X</th>
<th>Zinc 2X</th>
<th>Cobalt 0X</th>
<th>Cobalt 2X</th>
<th>Manganese 0X</th>
<th>Manganese 2X</th>
<th>Manganese 3X</th>
<th>Manganese 4X</th>
<th>Manganese 5X</th>
<th>Manganese 6X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic (C16:0)</td>
<td>24.8±0.4</td>
<td>25.5±0.5</td>
<td>26.7±0.3</td>
<td>26.4±0.5</td>
<td>26.7±0.4</td>
<td>25.7±0.3</td>
<td>29.4±0.2</td>
<td>23.9±0.6</td>
<td>27.2±0.8</td>
<td>24.5±0.3</td>
<td>25.9±0.4</td>
</tr>
<tr>
<td>Oleic (C18:1n9c)</td>
<td>2.7±0.2</td>
<td>2.8±0.3</td>
<td>3.1±0.1</td>
<td>2.4±0.1</td>
<td>2.5±0.1</td>
<td>2.7±0.1</td>
<td>2.7±0.3</td>
<td>2.2±0.1</td>
<td>3.8±0.2</td>
<td>2.8±0.4</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>Linoleic (C18:2n6c)</td>
<td>51.9±0.8</td>
<td>53.6±0.6</td>
<td>53.1±0.9</td>
<td>52.3±0.7</td>
<td>52.5±0.9</td>
<td>52.2±0.7</td>
<td>52.2±1.1</td>
<td>54.0±0.9</td>
<td>49.8±0.5</td>
<td>53.9±0.9</td>
<td>50.4±0.8</td>
</tr>
<tr>
<td>Linolenic (C18:3n3)</td>
<td>20.6±0.4</td>
<td>18.1±0.2</td>
<td>18.4±0.2</td>
<td>18.9±0.3</td>
<td>19.4±0.4</td>
<td>15.7±0.2</td>
<td>19.9±0.3</td>
<td>19.2±0.3</td>
<td>18.8±0.2</td>
<td>21.1±0.1</td>
<td></td>
</tr>
</tbody>
</table>

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

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