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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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2000), silicon deficiency (Lynn et al., 2000), phosphate limitation (Reitan et al., 1994), high salinity (Rao et al., 2007), and some heavy metal stress (Guschina and Harwood, 2006).

The nutritional attributes of microalgae depend upon several intrinsic factors, such as their biochemical composition, average size, cell wall digestibility, and lipid accumulation. *Dunaliella tertiolecta* required inorganic phosphates and trace elements (that is Co, Mo, Fe, and Mn) to be grown optimally (Chen et al., 2011). Although several studies of individual nutrients such as carbon (Hosoglu et al., 2012), nitrogen (Shen et al., 2009), and iron (Liu et al., 2008) have been published, the effect of micronutrient concentration on algal growth and lipid production has not been reported.

Therefore, our study evaluated the effects of micronutrients (that is Zn, Mn, Cu, and Co) on biomass production and lipid content of *M. pusillum*. Furthermore, we investigated the fatty acid composition of this microalgal strain.

MATERIALS AND METHODS

Algal strain and medium preparation

M. pusillum GU732425 used in this study was isolated from the effluent of a municipal wastewater treatment plant in Wonju, South Korea (Abou-Shanab et al., 2011). The alga was cultivated in Bold Basal Medium (BBM) (Bischoff and Bold, 1963) containing KH_2PO_4 (175 mg/L), $CaCl_2{\cdot}2H_2O$ (25 mg/L), $MgSO_4{\cdot}7H_2O$ (75 mg/L), $NaNO_3$ (250 mg/L), K_2HPO_4 (75 mg/L), NaCl (25 g/L), H_3BO_3 (11.42 mg/L), trace metal solution (1 mL/L) consisting of $ZnSO_4{\cdot}7H_2O$ (8.82 g/L), $MnCl_2{\cdot}4H_2O$ (1.44 g/L), MoO_3 (0.71 g/L), $CuSO_4{\cdot}5H_2O$ (1.57 g/L), and $Co(NO_3)_2{\cdot}6H_2O$ (0.49 g/L), alkaline EDTA stock solution (1 mL/L) consisting of Na_2EDTA (50 g/L) and KOH (31 g/L), and acidified iron stock solution (1 mL/L) consisting of $FeSO_4$ (4.98 g/L) and H_2SO_4 (1 mL). Regular BBM was amended with different concentrations of zinc (8.82 and 17.64 g/L) as $ZnSO_4{\cdot}7H_2O$, manganese (1.44, 2.88, 4.32, 5.76, 7.20, and 8.64 g/L) as $MnCl_2{\cdot}4H_2O$, copper (1.57, 3.14, 4.71, 6.28, 7.58, and 9.42 g/L) as $CuSO_4{\cdot}5H_2O$, or cobalt (0.49 and 0.98 g/L) as $Co(NO_3)_2{\cdot}6H_2O$. The amended concentration of each nutrient was expressed as a fold change toward the concentration in regular BBM (control). Separate tests were conducted using micronutrient-depleted media, to evaluate the growth and biochemical properties of *M. pusillum* cultivated in growth media lacking a particular nutrient. The pH of the media used in this study was adjusted to 6.6 before autoclaving at 121°C for 15 min.

Microalgal cultivation and growth analysis

Liquid medium [100 mL at 10% (v/v)] was inoculated with the algal strain in 250 mL Erlenmeyer flasks. The flasks were incubated under continuous white fluorescent light at 40 µmol photon/m^2/s at 27 ± 2°C for 17 days, while shaking at 150 rpm on a rotary shaker (SH-804, Seyoung Scientific). Algal growth was measured by determining the optical density of the algal cell suspension at 680 nm using a DR/4000 v spectrophotometer (HACH, USA). The OD_{680} was then converted to dry weight concentration using a linear relationship between OD_{680} and dry cell weight (g/L) (American Public Health Association, 1998), which was obtained after an extensive data analysis in this study. Experiments were carried out 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

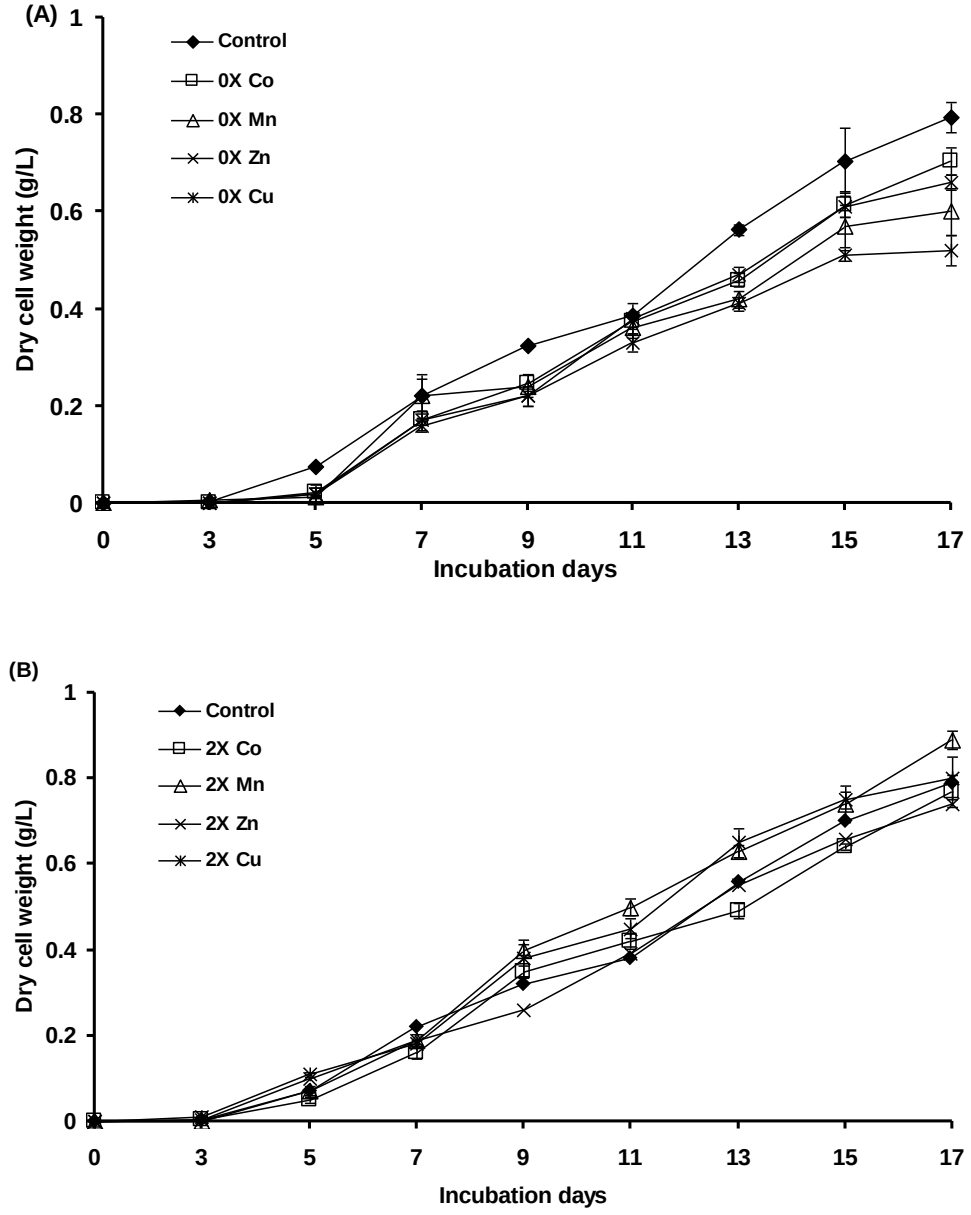

Figure 1. Dry cell weight of *M. pusillum* grown in (A) micronutrient-depleted media (0X) and (B) media containing a double each trace metal (2X).

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

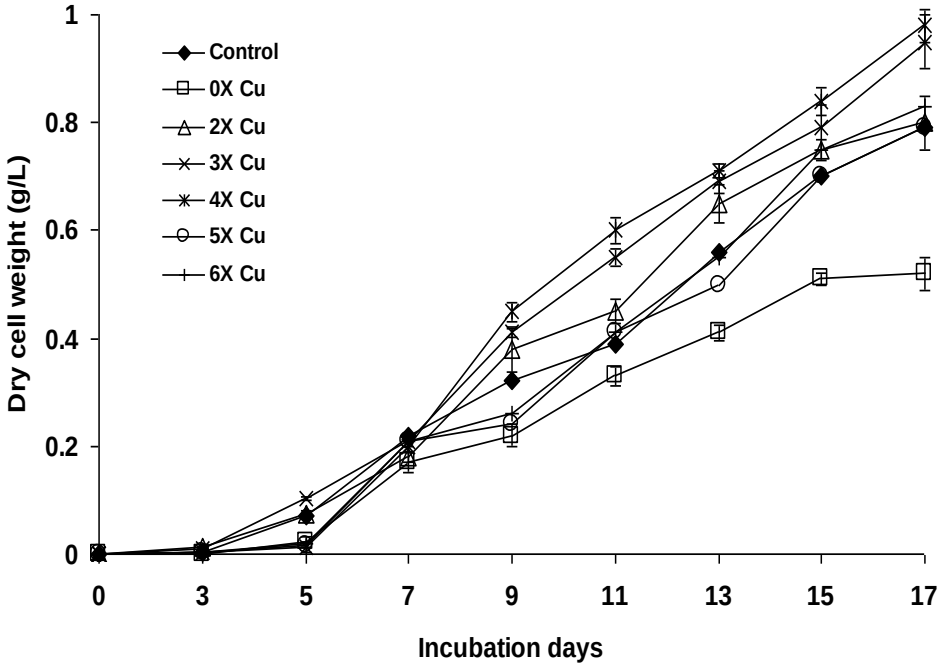

Figure 2. Dry cell weight of *M. pusillum* grown in BBM with different copper concentrations compared with control.

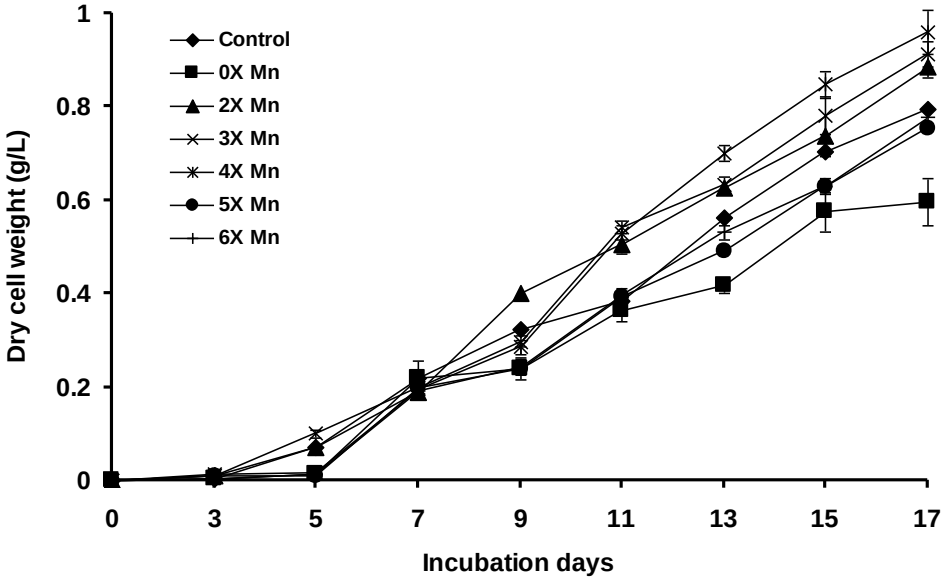

Figure 3. Dry cell weight of *M. pusillum* grown in BBM with different manganese concentrations compared with control.

± 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	Zinc		Cobalt		Manganese					
		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

Parameter	Control	Copper					
		0X	2X	3X	4X	5X	6X
Biomass (g dw/L)	0.82±0.05	0.60±0.04	1.04±0.16	1.21±0.12	1.28±0.04	1.19±0.09	0.99 ±0.01
Lipid productivity (g/L)	0.33±0.02	0.24±0.04	0.39±0.16	0.45±0.02	0.47±0.05	0.35±0.02	0.31 ±0.02
Lipid content (%)	40±3.1	40±1.9	38±0.5	37±3.7	38±1.5	32±1.1	31 ±3.5

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 alga 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. Cloe¨z 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

Table 2. Effect of trace metals concentration in the growth medium on fatty acid composition (%) of *M. pusillum*

Fatty acid	Control	Zinc		Cobalt		Manganese					
		0X	2X	0X	2X	0X	2X	3X	4X	5X	6X
Palmitic (C16:0)	24.8±0.4	25.5±0.5	26.7±0.3	26.4±0.5	26.7±0.4	25.7±0.3	29.4±0.2	23.9±0.6	27.2±0.8	24.5±0.3	25.9±0.4
Oleic (C18:1n9c)	2.7±0.2	2.8±0.1	1.8±0.1	2.4±0.2	1.9±0.1	2.7±0.1	2.7±0.3	2.2±0.1	3.8±0.2	2.8±0.4	2.6±0.1
Linoleic (C18:2n6c)	51.9±0.8	53.6±0.6	53.1±0.9	52.3±0.7	52.5±0.9	52.2±0.7	52.2±1.1	54.0±0.9	49.8±0.5	53.9±0.9	50.4±0.8
Linolenic (C18:3n3)	20.6±0.4	18.1±0.2	18.4±0.2	18.9±0.4	18.9±0.3	19.4±0.4	15.7±0.2	19.9±0.3	19.2±0.3	18.8±0.2	21.1±0.1

Fatty acid	Control	Copper					
		0X	2X	3X	4X	5X	6X
Palmitic (C16:0)	24.8±0.4	25.1±0.2	27.5±0.4	24.3±0.3	26.8±0.3	25.9±0.6	26.3±0.4
Oleic (C18:1n9c)	2.7±0.2	2.7±0.1	1.8±0.1	2.3±0.1	4.6±0.1	2.7±0.2	2.1±0.1
Linoleic (C18:2n6c)	51.9±0.8	52.6±0.6	52.6±0.7	54.1±1.1	49.2±0.8	53.9±0.9	50.0±0.7
Linolenic (C18:3n3)	20.6±0.4	19.6±0.2	18.1±0.2	19.3±0.4	19.4±0.5	17.5±0.2	21.6±0.5

biomass (Hu et al., 2008). Palmitic acid, oleic acid, and linoleic acid were found as the major fatty acids in both photoautotrophically and heterotrophically grown cultures of *Chlorella zofingiensis* (Liu et al., 2011). Of all the nutrients evaluated, nitrogen limitation is the single most critical nutrient affecting lipid metabolism in algae. A general trend towards accumulation of lipids, particularly triacylglycerols (TAG), in response to nitrogen deficiency has been observed in numerous species or strains of various algal taxa (Hu et al., 2008).

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 microalga 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.

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