COPPER STRESS ON CELLULAR CONTENTS AND FATTY ACID PROFILES IN *CHLORELLA* SPECIES

Sibi, G., T.S. Anuraag and Geeta Bafila

Department of Biotechnology, Indian Academy Degree College,
Centre for Research and Post Graduate Studies, Bangalore, India

ABSTRACT

Higher photosynthetic efficiency and biomass production with rapid growth makes microalgae as potential candidates over other energy crops in many applications. Heavy metals influence the production of secondary metabolites and lipd content of microalgae in particular. A study was conducted using six *Chlorella* species under heavy metal exposure to evaluate the copper stress on biomass, cellular and lipid contents. Preliminary growth studies indicated the growth tolerance levels of *Chlorella* in the presence of copper at 4.0 mg L^{-1} concentration. The total chlorophyll, protein and lipid content of the isolates were 1.7-3.45%, 0.43-0.70 mg g^{-1} and 0.02-0.11 mg g^{-1} respectively. Gas Chromatography-Mass Spectroscopy analysis revealed that the percent composition of fatty acids varied among the species studied and the major group of fatty acids were C16:0, C18:1 and C18:2. Highest percent of fatty acids were found in *C. vulgaris*, *C. protothecoides* and *C. pyrenoidosa*. Copper have an impact on *Chlorella* species where biomass content was directly proportional to the lipid productivity. The results reflects the fact that copper stress on *Chlorella* species as the evidence of lipid production in both qualitative and quantitative manner. In conclusion, *Chlorella* species can be used for the sustainable producion of renewable energy through copper stress and removal of copper from aqueous solutions.

Keywords: *Chlorella*, Lipid Productivity, Heavy Metal, Abiotic Stress, Copper

1. INTRODUCTION

Microalgae are used for many applications which include biofuel production, heavy metal removal, production of pharmaceuticals and nutraceuticals. Further, microalgae have advantages over other energy crops due to their higher photosynthetic efficiency, higher biomass production and faster growth (Huang *et al*., 2010; Chisti, 2007). The survival or proliferation of microalgae over a wide range of environmental conditions results in the production of many secondary metabolites (Anderson,

1996). Lipids are secondary metabolites produced by microalgae helps in maintaining specific membrane functions and cell signalling pathways while responding to the environment changes. The fatty acid content is influenced by the environmental and cultural conditions of microalgae (Petkov and Garcia, 2007).

Algal lipids have been recognized as suitable feedstock for biodiesel production (Griffiths and Harrison, 2009). Algae synthesize fatty acids principally for esterification into glycerol based polar lipids under favourable conditions. However, many algae alter their lipid biosynthetic pathways towards

Corresponding Author: Sibi, G., Department of Biotechnology, Indian Academy Degree College,
Centre for Research and Post Graduate Studies, Bangalore, India

the formation of neutral lipids in the form of triacylglycerol (Breuer *et al*., 2012; Li *et al*., 2011a) and can be readily converted to biodiesel (Hu *et al*., 2008). The lipid content can be altered by various factors (Hsieh and Wu, 2009; Rodolfi *et al*., 2009; Takagi and Yoshida, 2006; Liu *et al*., 2008) including heavy metals (Dragone *et al*., 2011; Rocchetta *et al*., 2006; Sunda *et al*., 2005; Vavilin *et al*., 1998). Copper has been used as pesticide to control algal blooms (Rai *et al*., 1981; Horne and Goldman, 1974) which disrupts photosynthesis, respiration, ATP production, pigment synthesis and inhibits cell division (Cid *et al*., 1996; Stauber and Florence, 1987). Copper is associated with large number of enzymes which catalyse oxidative reactions involving metabolic pathways (Marschner, 1995). However, little information is available on the effect of copper in modulating the fatty acid composition of *Chlorella* species. In this study, variations in lipid composition are compared with the concentrations of biomass, chlorophyll, protein and lipid content in order to elucidate the influence of copper stress on various *Chlorella* species.

2. MATERIALS AND METHODS

2.1. Sample Collection and Identification

Algal samples were collected from copper contaminated water habitats from Bangalore (13°04'N, 77°58'E) and washed several times with tap water and then with deionized water before analysed by using microscope. The family and genus were identified with reference to the biology of algae (Round, 1973).

2.2. Cultivation

Six *Chlorella* species were identified and were cultivated in Bold's basal medium (Andersen, 2005; Stanier *et al*., 1971) and http://web.biosci.utexas.edu/utex/default.aspx).

2.3. Copper Concentration

Stock copper solutions (CuCl$_2$. 2H$_2$O) were prepared at a concentration of 100 mg L^{-1} in deionized water. Solutions of varying concentrations were prepared by diluting with Bold's basal medium.

2.4. Heavy Metal Tolerance Studies

To determine algal tolerance to copper, exponential growth phase (10^4 cells m L^{-1}) algal cultures were used (OECD, 2002). Bioassays were carried out in 250 mL conical flasks containing 100 mL of Bold's basal media supplemented with Cu at initial concentrations ranging from 0.5, 1.0, 2.0, 4.0 and 8.0 mg L^{-1}. The flasks were kept under illumination at 2500 lux for 12 hr light-dark cycle at 24±2°C and agitation in an orbital shaker for 15 days. Metal-free and algae-free blanks were used as control groups. Separation of biomass from metal bearing solution after appropriate incubation time was achieved through centrifugation at 10,000 rpm for 10 min.

2.5. Cell Count and Biomass Determination

The cells were fixed with Lugol's iodine solution to measure the cell density using haemocytometer. The mean number of cells produced at each concentration after the incubation period was expressed as percentage growth reduction with respect to control. The algal biomass was determined by the spectrophotometric transmission of algal suspension (Schimadzu UV-2600) at 550 nm. The highest concentration of copper which produced maximum biomass content was used for further studies.

2.6. Chlorophyll Estimation

The chlorophyll content of the cells was estimated spectrophotometrically (Sartory and Grobbelaar, 1984). The cells were harvested by centrifugation at 3000 g and thoroughly ground in 96% ethanol. After incubation with ethanol for 2 h at 4°C, cell debris was cleared by centrifugation. The resulting supernatant was subjected to OD measurement at the wavelength of 645 nm and 663 nm, with 96% ethanol as blank. Total chlorophyll content (i.e., chl a and b) was estimated using a formula C (mg L^{-1}) = $20.2OD_{645}+8.05OD_{663}$ and was converted to % of cell dry weight.

2.7. Total Soluble Protein Estimation

Total soluble protein content was measured by the (Bradford, 1976) method using bovine serum albumin as standard. The total soluble protein was expressed as mg/g fresh weight.

2.8. Lipid Extraction

Algal cultures were centrifuged and the pellet was added with 10 mL of ice cold 0.2 N HClO$_4$. After 15 min at 4°C, the sample was centrifuged and 10 mL of chloroform-methanol (2:1 v/v) solution was added and allowed to stand for 5 min at 4°C. The sample was

centrifuged and to the supernatant, 0.2 volumes of distilled water were added. The solutions were shaken for 5 min and centrifuged for 15 min at 2000 rpm to separate the phases. The lower organic phase was collected and the chloroform-methanol solution was evaporated under a steam of nitrogen. Aliquots of lipid extracts were mixed with 2 mL of dichromate solution and the tubes were placed in boiling water bath for 45 min. The tubes were shaken for 2 or 3 times during the heating and were cooled, removed 1ml from each, diluted to 100 mL with water and the absorbance was read at 350 nm against water as blank. Palmitic acid was used a standard and the lipid content was determined from the standard curve (Folch *et al*., 1957).

2.9. Fatty Acid Methyl Ester (FAME) Analysis

Algae cultures (4 mL each) were centrifuged at 16,000 rpm for 3 min and the pellet was hydrolyzed and methyl-esterified by shaking (1,200 rpm) with 300 µL of a 2% H_2SO_4/methanol solution for 2 h at 80°C. This was followed by the addition of 50 µg of hexadecanoic acid as internal standard to the pellet prior to the reaction. A total of 300 µL of 0.9% (w/v) NaCl and 300 µL of hexane was then added and vortexed. Phase separation was performed by centrifugation at 16,000 rpm for 3 min. A total of 1 µL of the hexane layer was used for GC-MS analysis.

2.10. GC-MS Conditions

GC-MS analysis of lipid extract was performed using a Thermo Scientific TRACE GC Ultra comprising an Thermo DSQII auto-sampler equipped with Zebron ZB 5 ms column (30 mL ×0.25 mm ID ×0.25 µm). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1.0 mL min^{-1} and an injection volume of 1 µL was employed in a splitless mode. The initial temperature was 40°C with a hold time of 2 min and the ramp was 300°C at a rate of 10°C/min for a hold period of 5 min. Mass spectra were taken at a scan mass range of 30-600 m/z. The solvent delay was 0 to 2 min and the total GC/MS running time was 32 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Quadrupole mass analyzer along with Xcalibur and AMDIS software was adopted to handle mass spectra and chromatograms. Fatty Acid Methyl

Esters (FAMEs) were quantified by taking the ratio of the integral of each FAME's total ion current peak to that of the internal standard (50 µg).

3. RESULTS

Six species of *Chlorella* were identified namely *Chlorella vulgaris*, *C. pyrenoidosa*, *C. ellipsoidea*, *C. emersonii*, *C. sorokiniana* and *C. protothecoides*. Bioassay experiments involving Cu concentrations 0.5, 1.0, 2.0, 4.0 and 8.0 mg L^{-1} were carried during which cell count and biomass content were varied with Cu concentration. Highest cell count and biomass was obtained at 4.0 mg L^{-1} concentrations and was considered for determination of chlorophyll, total protein and lipid content.

The influence of copper stress among *Chlorella* species has been studied and a concentration dependent reduction in growth rate, content of chlorophyll, protein and lipid were compared and depicted in **Fig. 1-5**. Among the *Chlorella* species, *C. vulgaris* has produced maximum biomass (1.18 g L^{-1}) followed by *C. pyrenoidosa* and *C. protothecoides*. There were no significant differences in biomass content among the other species studied. The total chlorophyll content estimation revealed that amount of chlorophyll was in the range between 1.7-3.45%. Highest chlorophyll content was present in *C. vulgaris* (3.45%), *C. pyrenoidosa* (2.21%) and *C. protothecoides* (2.17%). The total protein estimation of isolated *Chlorella* species revealed the highest content in *C. protothecoides* (0.70 mg g^{-1}), *C. ellipsoidea* (0.69 mg g^{-1}) and *C. vulgaris* (0.61 mg g^{-1}). The least protein content was found in *C. emersonii* (0.43 mg g^{-1}). The total lipid estimation of the isolates revealed the lipid content between 0.02-0.11 mg g^{-1}, where *C. vulgaris* (0.21 mg g^{-1}) has recorded highest content followed by *C. protothecoides* (0.17 mg g^{-1}).

GC-MS analysis of the transesterified lipid extracts revealed the presence of various fatty acid groups (C15:0, C16:0, C16:1, C17:1, C18:0, C18:1, C18:2). The percent composition of fatty acids varied among the species studied and the major group of fatty acids were C16:0, C18:1 and C18:2 (**Table 1**).

In general, copper has influenced the fatty acid composition with various percent compositions. Highest percent of C16:0 (25.21%), C16:1 (2.67%), C18:0 (6.2%) and C18:2 (21.43%) were found in *C. vulgaris* whereas, C15:0, C17:1 and C18:1 were found in *C. protothecoides* at 1.72, 0.91 and 12.13% respectively.
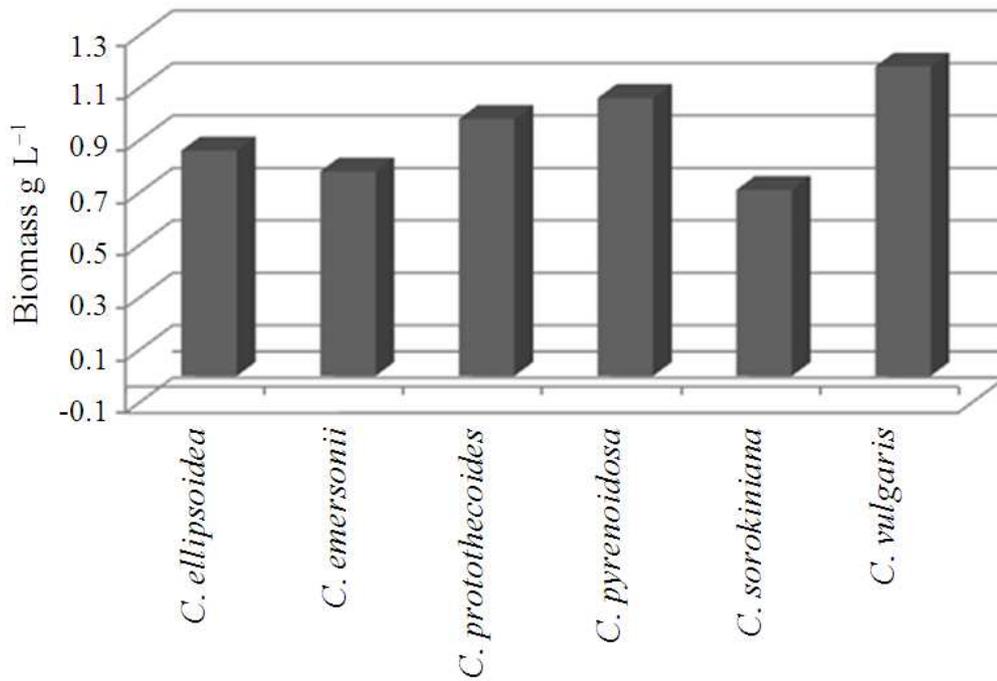
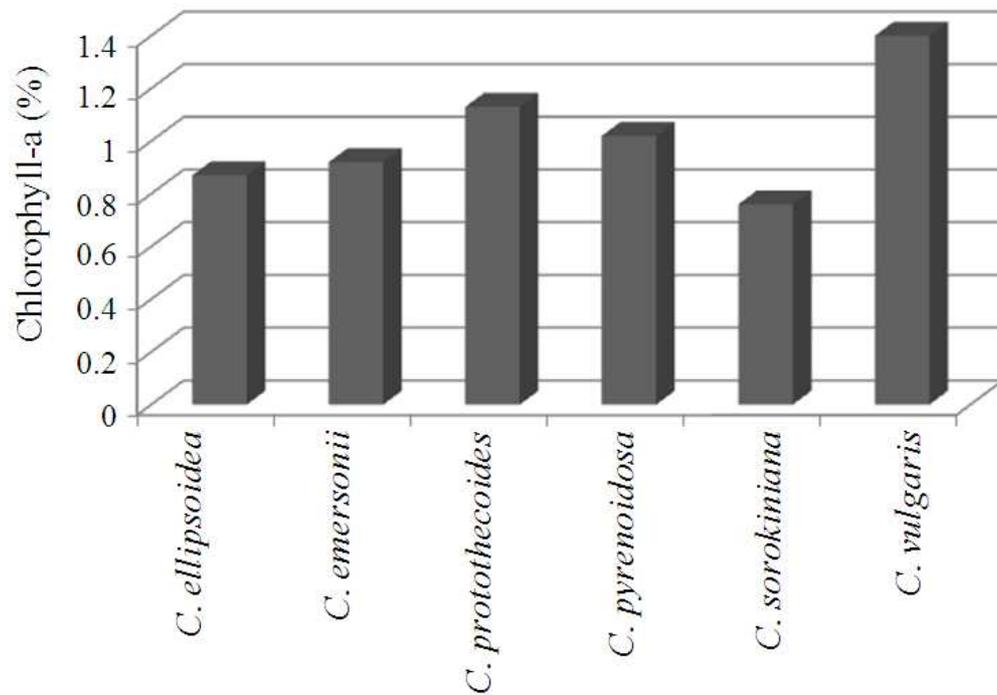**Fig. 1.** Biomass content of *Chlorella* species under copper stress

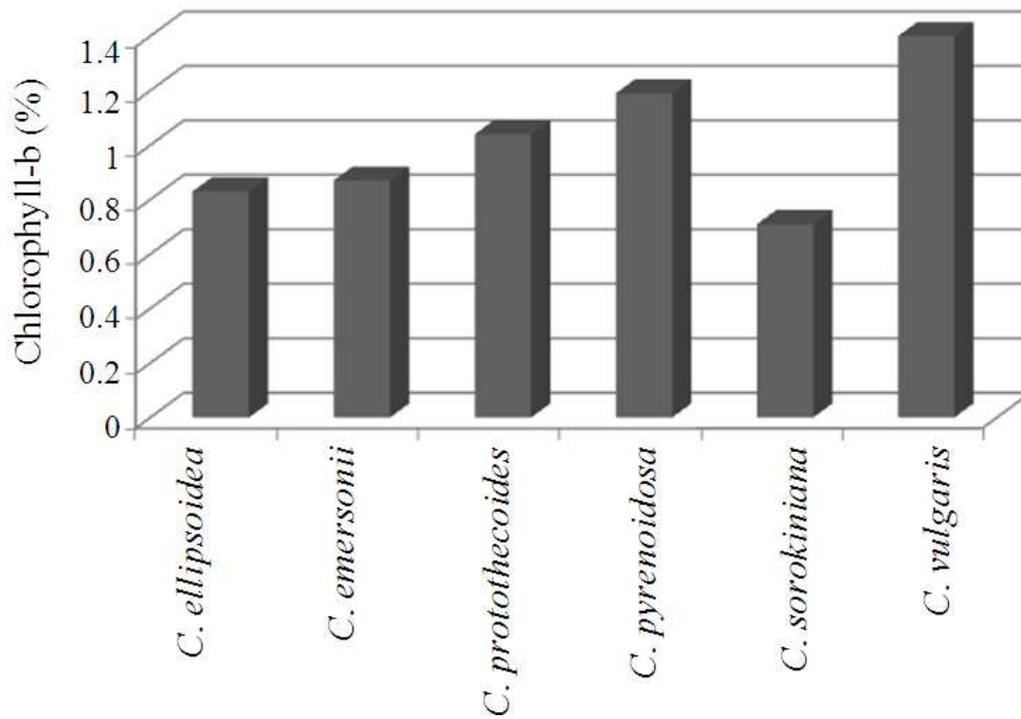**Fig. 2.** Chlorophyll-a content of *Chlorella* species under copper stress

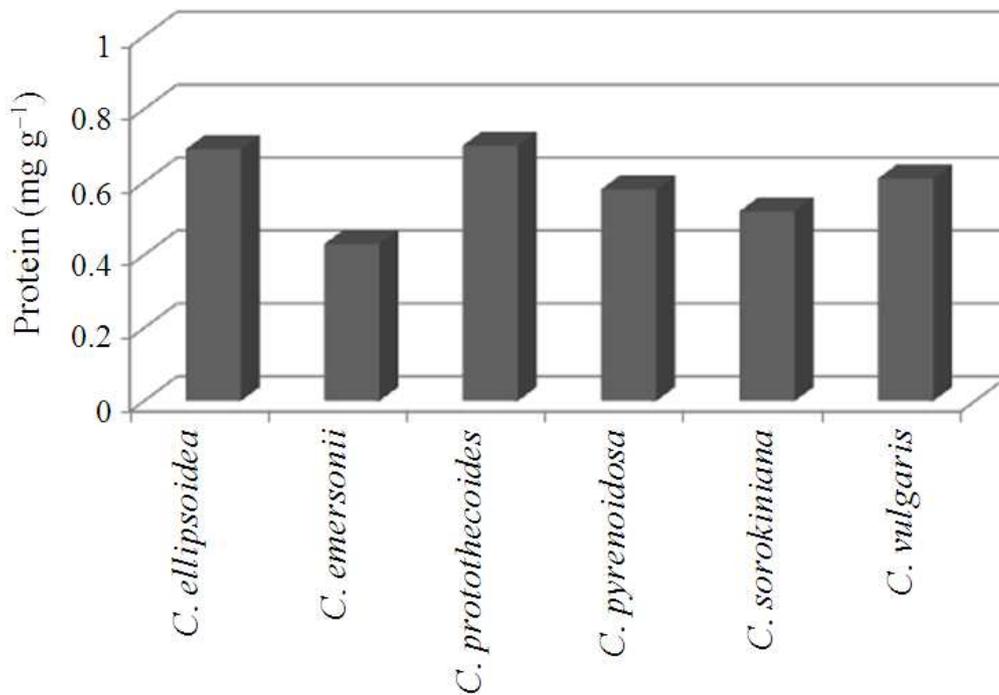**Fig. 3.** Chlorophyll-b content of *Chlorella* species under copper stress

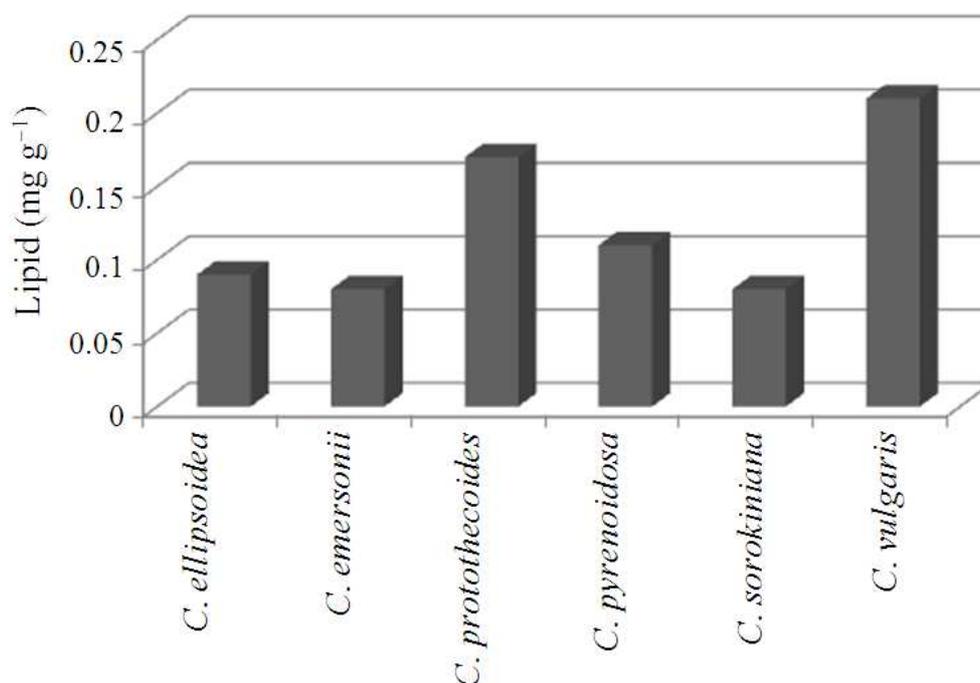**Fig. 4.** Protein content of *Chlorella* species under copper stress

Fig. 5. Lipid content of *Chlorella* species under copper stress

Table 1. Percent composition of fatty acids from *Chlorella species* under copper stress (4 mg L^{-1})

Species	C15:0	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2
C. ellipsoidea	1.67	9.03	1.61	ND	ND	7.04	11.13
C. emersonii	ND	11.25	ND	ND	0.81	7.73	12.93
C. protothecoides	1.72	18.42	1.85	0.91	ND	12.13	19.01
C. pyrenoidosa	ND	16.71	ND	0.25	2.64	7.92	12.24
C. sorokiniana	0.74	8.63	0.91	ND	0.71	3.05	6.79
C. vulgaris	1.53	25.21	2.67	ND	6.2	11.03	21.43

4. DISCUSSION

Most of the oleaginous algae belong to green algae and diatoms (Pulz and Gross, 2004). Green algae have more saturated and unsaturated fatty acids similar to that of vegetable oils. *Chlorella* species are particularly attractive due to their strong resistance characteristics to various unfavourable environmental conditions. Association of increased cellular lipid accumulation under different environmental stress conditions was observed in green microalgae (Li *et al*., 2011b). During biofuel production, high lipid production is crucial to achieve commercial feasibility. A suitable microalgae strain must have high lipid productivity, either by possessing high basal lipid content and/or be inducible to accumulate significant amounts of lipids (Rodolfi *et al*., 2009). Previous studies concerning the heavy metal tolerance of algal

species were reported (Bajguz, 2000; Takamura *et al*., 1989). Heavy metals can cause adverse effects on cell division, growth, photosynthesis and respiration (Wang and Chen, 2009). Variations in cell number, chlorophyll and protein content among the *Chlorella* species were observed in this study which could be due to copper exposure of *Chlorella*. Copper has been found to be one of more toxic metals to unicellular algae than other heavy metals (Bilgrami and Kumar, 1997; Ilangovan *et al*., 1992; Lam *et al*., 1999; Rachlin and Grosso, 1993). This study focused on effect of copper on growth rate, cellular and lipid composition of *Chlorella* species.

One of the goals of this study was to find a copper concentration that would not negatively affect the microalgae. It was found that increasing copper concentrations in the bioassay caused a significant decrease in algal growth rates among the *Chlorella*

species over 15 days. The growth rate was declined above 4.0 mg L^{-1} concentration and hence the further experiments were carried out at 4.0 mg L^{-1} concentration. Copper tolerance by *C. vulgaris* at 3.0 μg m L^{-1} concentration was reported in earlier studies (Mallick, 2004). The next goal was how copper would impact the growth, chlorophyll, protein content and lipid accumulation in *Chlorella* species. At 4.0 mg L^{-1}, significant cell numbers and biomass concentration were observed among the species. The determination of biomass, chlorophyll and lipid contents of microalgae would provide useful information concerning the growth status during cultivation. High lipid accumulation and biomass productivity are desired phenotypes in algae for biodiesel production. However, stress conditions reduce the biomass production and increase lipid content of algae (Li *et al.*, 2008). In this study, copper have an impact on *Chlorella* species where biomass content was directly proportional to the lipid productivity. Berglund *et al.* (2001) reported that both the quantity and quality of lipids produced vary with the identity of algal species. The same trend was observed in this study, where *C. vulgaris*, *C. protothecoides* and *C. pyrenoidosa* produced higher percent composition of fatty acids than other species.

5. CONCLUSION

In the case of value added products from microalgae, the isolates should adapt better to heavy metal stress and provide a more stable and productive culture in pilot scale. Most of the isolates were able to tolerate copper and produced diversified fatty acids at various concentrations irrespective of the species. Biochemical adaptations to abiotic stresses involve evolution of new metabolic pathways and synthesis of new proteins and the copper stress has influenced the lipid profile of microalgae in this study. This research presented here reflects the fact that copper stress on *Chlorella* species as the evidence of lipid production in both qualitative and quantitative manner. The presence of diversified fattyacids under copper stress is an evidence that *Chlorella* can be used for the sustainable production of renewable energy and other nutraceuticals. It also suggests the use of this species for the removal of copper from aqueous solutions. Further research on influence of other heavymetals in the cellular and lipid contents of *Chlorella* species need to be investigated for the efficeint use of the microalgae in the production of other value added products.

7. REFERENCES

Anderson, R.A., 1996. Algae. In: Maintaining Cultures for Biotechnology and Industry, Hunter-Cevera, J.C. and A. Belt (Eds.)., London Academic Press, Inc., ISBN-10: 978-0-12-361946-4, pp: 29-64.

Andersen, R.A., 2005. Algal Culturing Techniques. 1st Edn., Elsevier Academic Press. California, USA, ISBN-10: 0120884267, ppp: 578.

Bajguz, A., 2000. Blockade of heavy metals accumulation in *Chlorella vulgaris* cells by 24-epibrassinolide. Plant Physiol. Biochem., 38: 797-801. DOI: 10.1016/S0981-9428(00)01185-2

Berglund, O., P. Larsson, G. Ewald and L. Okla, 2001. The effect of lake trophy on lipid content and PCB concentrations in planktonic food webs. Ecology, 82: 1078-1088. DOI: 10.1890/0012-9658

Bilgrami, K.S. and S. Kumar, 1997. Effects of copper, lead and zinc on phytoplankton growth. Biol. Plantarum, 39: 315-317. DOI: 10.1023/A:1000600503710

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochem., 72: 248-254. DOI: 10.1016/0003-2697(76)90527-3

Breuer, C., K. Morohashi, A. Kawamura, N. Takahashi and T. Ishida *et al.*, 2012. Transcriptional repression of the APC/C activator CCS52A1 promotes active termination of cell growth. EMBO J., 31: 4488-4501. DOI: 10.1038/emboj.2012.294

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv., 25: 294-306. DOI: 10.1016/j.biotechadv.2007.02.001

Cid, A., P. Fidaglogo, C. Herrero and J. Abalde, 1996. Toxic action of copper on the membrane system of a marine diatom measured by flow cytometry. Cytometry, 25: 32-36. DOI: 10.1002/(SICI)1097-0320(19960901)25

Dragone, G., B.D. Fernandes, A.P. Abreu, A.A. Vicente and J.A. Teixeira, 2011. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. Applied. Energy, 88: 3331-3335. DOI: 10.1016/j.apenergy.2011.03.012

Folch, J., M. Lees and G.H. Sloane-Stanley, 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem., 226: 497-509. PMID: 13428781

Griffiths, M.J. and S.T.L. Harrison, 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. J. Applied Phycol., 21: 493-507. DOI: 10.1007/s10811-008-9392-7

Horne, A.J. and C.R. Goldman, 1974. Suppression of nitrogen fixation by blue-green algae in a eutrophic lake with trace additions of copper. Science, 183: 409-411. DOI: 10.1126/science.183.4123.409

Hsieh, C.H. and W.T. Wu, 2009. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresour. Technol., 100: 3921-3926. DOI: 10.1016/j.biortech.2009.03.019

Hu, Q., M. Sommerfeld, E. Jarvis, M. Ghirardi and M. Posewitz *et al.*, 2008. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. Plant J., 54: 621-639. DOI: 10.1111/j.1365-313X.2008.03492.x

Huang, G.H., F. Chen, D. Wei, X.W. Zhang and G. Chen, 2010. Biodiesel production by microalgal biotechnology. Applied Energy, 87: 38-46. DOI:10.1016/j.apenergy.2009.06.016

Ilangovan, K., M. Salazar, S. Dash, O. Monroy and A. Ramos, 1992. Interaction of cadmium, copper and zinc in *Chlorella pyrenoidosa* Chick. Environ. Technol., 13: 195-199. DOI: 10.1080/09593339209385144

Lam, P.K.S., P.F. Wut, A.C.W. Chan and R.S.S. Wu, 1999. Individual and combined effects of cadmium and copper on the growth response of *Chlorella vulgaris*. Environ. Toxicol., 14: 347-353. DOI: 10.1002/(SICI)1522-7278(199907)

Li, Y., M. Horsman, B. Wang, N. Wu and C.Q. Lan, 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Applied Microbiol. Biotechnol., 81: 629-636. DOI: 10.1007/s00253-008-1681-1

Li, X., H.Y. Hu and Y.P. Zhang, 2011a. Growth and lipid accumulation properties of a freshwater microalga *Scenedesmus* sp. under different cultivation temperature. Bioresour. Technol., 102: 3098-3102. DOI: 10.1016/j.biortech.2010.10.055

Li, Y., D. Han, M. Sommerfeld and Q. Hu, 2011b. Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp. (Chlorophyceae) under nitrogen-limited conditions. Bioresour. Technol., 102: 123-129. DOI: 10.1016/j.biortech.2010.06.036

Liu, Z.Y., G.C. Wang and B.C. Zhou, 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresour. Technol., 99: 4717-4722. DOI: 10.1016/j.biortech.2007.09.073

Mallick, N., 2004. Copper-induced oxidative stress in the chlorophycean microalga *Chlorella vulgaris*: Response of the antioxidant system. J. Plant Physiol., 161: 591-597. DOI: 10.1078/0176-1617-01230

Marschner, H., 1995. Mineral Nutrition of Higher Plants. 3rd Edn., Academic Press, London, ISBN-10: 0123849063, pp: 672.

OECD, 2002. Guidelines for the testing of chemicals. Fresh water Alga and Cyanobacteria, Growth Inhibition Test.

Petkov, G. and G. Garcia, 2007. Which are fatty acids of the green alga *Chlorella*? Biochem. Sys. Ecol., 35: 281-285. DOI: 10.1016/j.bse.2006.10.017

Pulz, O. and W. Gross, 2004. Valuable products from biotechnology of microalgae. Applied Microbiol. Biotechnol., 65: 635-648. DOI: 10.1007/s00253-004-1647-x

Rachlin, J.W. and A. Grosso, 1993. The growth response of the green alga *Chlorella vulgaris* to combined divalent cation exposure. Archives Environ. Contamination Toxicol., 24: 16-20. DOI: 10.1007/BF01061084

Rai, L.C., J.P. Gaur and H.D. Kumar, 1981. Phycology and heavy metal pollution. Biolog. Rev., 56: 99-151. DOI: 10.1111/j.1469-185X.1981.tb00345.x

Rocchetta, I., M. Mazzuca, V. Conforti, L. Ruiz and V. Balzaretti *et al.*, 2006. Effect of chromium on the fatty acid composition of two strains of *Euglena gracilis*. Environ. Pollut., 141: 353-358. DOI: 10.1016/j.envpol.2005.08.035

Rodolfi, L., G.C. Zittelli, N. Bassi, G. Padovani and N. Biondi *et al.*, 2009. Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng., 102: 100-112. DOI: 10.1002/bit.22033

Round, F.E., 1973. The biology of the algae. J. Marine Biolog. Associat. United Kingdom, 45: 799-799. DOI: 10.1017/S0025315400016672

Sartory, D.P. and J.U. Grobbelaar, 1984. Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia, 114: 177-187. DOI: 10.1007/BF00031869

Stanier, R.Y., R. Kunisawa, M. Mandel and G. Cohen-Bazire, 1971. Purification and properties of unicellular blue- green algae (order Chroococcales). Bacteriol. Rev., 35: 171-205. PMID: 378380

Stauber, J.L. and T.M. Florence, 1987. The mechanism of toxicity of ionic copper and copper complexes to algae. Marine Biol., 94: 511-519. DOI: 10.1007/BF00431397

Sunda, W.G., N.M. Price, and F.M.M. Morel, 2005. Trace Metal Ion Buffers and their Use in Culture Studies. In: Algal Culturing Techniques, R., Anderson, (Ed.,), Elsevier Academic Press, pp: 35-63.

Takagi, M.K. and T. Yoshida, 2006. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J. Biosci. Bioeng., 101: 223-226. DOI: 10.1263/jbb.101.223

Takamura, N., F. Kusai and M. Watanabe, 1989. Effects of Cu, Cd, Zn on photosynthesis of freshwater benthic algae. J. Applied Phycol., 1: 39-52. DOI: 10.1007/BF00003534

Vavilin, D.V., J.M. Ducruet, D.N. Matorin, P.S. Venediktov and A.B. Rubin, 1998. Membrane lipid peroxidation, cell viability and Photosystem II activity in the green alga *Chlorella pyrenoidosa* subjected to various stress conditions. J. Photochem. Photobiol. Biol., 42: 233-239. DOI: 10.1016/S1011-1344(98)00076-1

Wang, J. and C. Chen, 2009. Biosorbents for heavy metals removal and their future. Biotechnol. Adv., 27: 195-226. DOI: 10.1016/j.biotechadv.2008.11.002