Effect of different concentrations of sodium nitrate, sodium chloride, and ferrous sulphate on the growth and lipid content of *Chlorella vulgaris*

Battah, M.G.[1*], El-Ayoty, Y.M.[2], Esmael, A.E.[1] and Abd El-Ghany, S.E.[2]

[1]Botany Department, Faculty of Science, Benha University, [2]Botany Department, Faculty of Science, Zagazig University

Chlorella vulgaris is one of the better sources of energy. Nitrogen deficiency of *Chlorella vulgaris* to the level 0.1mM $NaNO_3$ as shown in results gave the minimum mean growth rate and highest lipid content (54.88 % oil) as compared with control after 12 days old cultures (29.7% oil), which represents 84% more than its corresponding control. High salinity to the level of 0.45 mM also gave the minimum mean growth rate and highest lipid content (43.2 %). In a similar response to salinity 35.25 µM of ferrous sulphate gave the highest lipid content (34.7 % oil) as compared with control after 12 days old cultures. Thirteen fatty acids were identified. Total saturated fatty acids percentages ranged from 19.98-50.34% and total unsaturated fatty acids ranged from 41.88-80.00%, where the most of the applied treatments caused an obvious increase in unsaturated fatty acid contents comparing with control, and the major constituents of the lipid fraction of *Chlorella vulgaris* was found to be C14, C16, C18, C18:1, C18:2 and C18:3 fatty acids. Also, it was found that all of the treatments in the present work resulted in formation of C24 fatty acid which was not recorded in the lipid profile of untreated alga.

Keywords: Algal oil – Biodiesel fuel - *Chlorella vulgaris*, Lipid – Fatty acids – Salinity.

Introduction

Nowadays modern world is currently facing a dangerous energy problem due to the increase of world consumption of energy (Vasudevan and Briggs, 2008). As a result new alternatives and sustainable energy sources are vital (Bruce, 2008; Sims *et al*., 2010). Of these alternatives biodiesel exhibit particular promising (Miao and Wu, 2006; Behzadi and Farid, 2007). Biodiesel is an alternative liquid fuel produced by chemical reaction between a plant oils or an animal fats in presence of a catalyst which is eco-friendly and renewable (Meher *et al*., 2006; Marchetti *et al*., 2007) but, the use of edible vegetable oils for fuel production will compete significantly with food uses, and this would

---

[*] Corresponding author: Battah, M.G.; e-mail: maamay57@yahoo.com

result in undesirable increase in food and biodiesel costs (Demirbas, 2008). Algal oils were found to be a good alternative for the production of biodiesel rather than vegetable oils (Schenk *et al*., 2008). Microalgae are sunlight-driven cell factories that convert carbon dioxide to potential biofuels (Singh *et al*., 2005; Spolaore *et al*., 2006). Unlike traditional oil seed crops, microalgae can grow in places away from the farmlands and forests such as ponds, fermentation units and even wastewater (De-Bashan *et al*., 2004), thus minimizing the damages caused to the eco- and food-chain systems (Chisti, 2007).

The biochemical composition in algae can be modulated with altering environmental conditions (Miller, 1962; Fabregas *et al*., 1995). In algae lipids are widely distributed, especially in several resistance stages (Miller, 1962). A nutrient limitation is an efficient environmental pressure to increase lipid accumulation (Goldberg and Cohen, 2006; Rodolfi *et al*., 2009). Of these nitrogen limitation (Parrish and Wangersky, 1990), high salinity (Walsby, 1982) and iron (Liu *et al*., 2008) could modify and improve algal lipid content.

The aim of the study was to obtain highly quantitative and qualitative biodiesel from a micro alga *Chlorella vulgaris* through the technology of transesterification. So the present study focused on determination the effect of nitrogen deficiency, high salinity and stress of iron on the growth and lipid content of *Chlorella vulgaris*. The fatty acid methyl esters of lipid were analyzed for each effect.

Materials and methods

The organism and culture medium: The organism used in this study known as *Chlorella vulgaris* which is a unicellular green micro alga belonging to Division Chlorophyta.. The isolate was obtained from lab of algae at Faculty of Science, Zagazig University. It was selected due to its growth potentially and lipid contents. The algal *Chlorella vulgaris* was aseptically grown on Bold Basal medium (BBM) and prepared according to Nichols *et al*. (1973). The control of treatments used equal to it's concentration in BBM medium.

Preparation of inocula and growth conditions: 250 ml Erlenmeyer flasks each containing 100 ml of BBM were autoclaved. After cooling; the flasks were inoculated under aseptic condition with 5 ml of *Chlorella vulgaris* inocula, then incubated under photoperiod of light: dark regime (16-8) and at temperature 25 ± 2^oC. The optimum light intensity 5000 lux was supplied with cool white fluorescent tubes for 12 days. The growth curve of the alga was spectrophotometrically estimated after 2 days intervals through the incubation periods12 days old.

Effect of different concentrations of sodium nitrate (NaNO₃) on Chlorella vulgaris: To 250 ml Erlenmeyer flasks 100 ml BBM were added. Then, in triplicates the flasks were supplemented with different concentrations of sodium nitrate (0.10 mM, 1.00 mM, 2.00 mM, 3.00 mM and 5.00 mM). Then all the flasks were autoclaved. After cooling, the flasks were inoculated with 5 ml inoculum (O.D = 0.45). Then the growth and determinations were carried out at conditions as mentioned above.

Effect of different concentrations of sodium chloride (NaCl) on Chlorella vulgaris: To 250 ml Erlenmeyer flasks 100 ml BBM were added. Then, in triplicates the flasks were supplemented with different concentrations of NaCl (0.30 mM, 0.40 mM, 0.42 mM and 0.45 mM). Then the growth and determinations were carried out at conditions as mentioned above.

Effect of different concentrations of ferrous sulphate (FeSO4.7H2O) on Chlorella vulgaris: To 250 ml Erlenmeyer flasks 100 ml BBM were added. Then, in triplicates the flasks were supplemented with different concentrations of ferrous sulphate (17.62 µM, 26.43 µM, 35.25 µM and 44.06 µM). Then the growth and determinations were carried out at conditions as mentioned above.

Growth assessment

Optical density: Optical density of *Chlorella vulgaris* culture was measured according to Hsieh and Wu (2009) using spectrophotometer (spectronic 20D).

Dry weight: The biomass was evaluated by the dry weight (g L^{-1}), and its relationship with the optical density (OD) at 450 nm (Fan *et al*., 2008) was referred as following: Dry wt $= 0.49 \times OD_{450}$

Mean growth rate: The mean growth rate ($R^{\backslash}$) was calculated according to Robert (1979).

Determination of lipid content

Spectrofluorometric determination of lipid using Nile red stain

Preparation of Nile Red was prepared from Nile blue according to (Thorpe, 1907) and (Davis and Hetzer, 1966). The algal cells were directly stained with 0.1µg/ml Nile red for 10 min. Then, the fluorescence was measured on a fluorometer with excitation and emission wavelengths of 470 and 570 nm respectively.

Determination of lipid content by gravimetric method: Oil content of *Chlorella vulgaris* was determined according to (Sadasivam and Manickam,

1996) by Soxhlet apparatus using n-hexane as the extraction solvent for 6 hours under reflux (Frenz *et al.*, 1989).

Extraction and determination of fatty acids

Separation of fatty acids

The lipid samples were saponified over-night with ethanoic KOH (20%) at room temperature. The fatty acids were freed from their potassium salts by acidification with hydrochloric acid (5N), followed by extraction with ether or (petroleum .ether 40-60°C). The ether extract was washed three times with distilled water then dried over anhydrous sodium sulfate, and filtered off (Vogel, 1975).

Gas liquid chromatography (GLC): One μl of fatty acid methyl ester was injected into a 6 feet × 1/8 inch internal diameter column packed with 20% diethylene glycol succinate (DEGS) on chromsorb 60-80 mesh by using Hewellet-Packard (model:HP-GC-MS) according to the standard conditions of determination .

Identification of fatty acids: A set of standard fatty acids of 10:0, 11:0, 12:0, 13:0, 14:0, 15:0, 16:0, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1 and 22:0 with purity of 99% were used. The purity of each fatty acid methyl ester was checked by GLC and gave one peak.

Quantification of fatty acids: The response of each fatty acid separated on the chromatograph was determined as peak area per unit weight of sample, as recommended by Radwan (1978).

Results and discussions

The results in Fig. (1) revealed that the growth of *Chlorella vulgaris* is proportionally directed with increasing concentration of sodium nitrate more than its concentration in BBM (3mM), especially following the application of higher concentration (5mM). Where as at 5mM of nitrate the mean growth rate of *Chlorella vulgaris* was maximally recorded at the elapse of 8 days (R$^\backslash$ = 0.32 d^{-1}). On the opposite hand, the rate of growth was subsequently shifted to the lowest one as nitrate deprives to the level 0.10 mM which need more time for regeneration. Inspection of the biosynthesis and accumulation of lipids of *Chlorella vulgaris* under different concentrations of nitrate was manifested in Fig.1. It was revealed that the biosynthesis and accumulation of lipids of *Chlorella vulgaris* was maximally recorded 54.88% as nitrate deprives to the level 0.10 mM after 12 days of incubation. This value was two folds more as it compared with the corresponding control (29.74%). But on the other hand, after

12 days of culturing *Chlorella vulgaris* with high nitrate concentration (5mM NaNO$_3$) the lipid content inhibited with 67.2% from the corresponding. The previous results indicate that nitrogen deprivation is a feasible tool for the over production of lipid contents and the relation between lipids and growth was found to be inversely. These results are in agreement with those obtained by Illman *et al*. (2000), who observed that the lipid content (40%) of *Chlorella vulgaris* in low-N was about two times more than that (18%) in the control. Moreover the growth of *Chlorella vulgaris* at this condition of nitrogen deficiency did not parallel to the obvious increases of lipids .Such results were coincident with, Rodolfi *et al*. (2009) who found that N- deficiency could stimulate the lipid accumulation, but the biomass productivity was reduced. The obvious increase in the lipid content under nitrogen limitation could activate diacylglycerol acyltransferase, which converts acyl-CoA to triglyceride or may be attributed to the inhibition of cell division under the environmental stresses (Sukenik and Livne, 1991). Ahlgren and Hyenstrand (2003) reported that under nitrogen-deficient conditions, algal cells often accumulate carbon metabolites as lipids. It was also reported that microalgae respond to the nitrogen starvation condition by degrading nitrogen containing macromolecules and accumulating carbon reserve compounds, as fats (Banerjee *et al*., 2002; Dayananda *et al*., 2005).

The mean growth rates and cell productivity of *Chlorella vulgaris* grown at different salinities (0.3, 0.4, 0.42 and 0.45 mM NaCl) are shown in Fig. (2). The resulted data indicated that the mean growth rates for *Chlorella vulgaris* was subsequently decreased with increasing the level of salinity. The maximum percentage of reduction was recorded (26.7%) following seeding of culture media with 0.45 mM NaCl less than the corresponding control at 6 days old culture. Mean while these parameters were maximally attained at 6 days old culture in untreated control (0.4mM NaCl) where, the mean growth rate was recorded 0.34 d^{-1}. Inspection of the biosynthesis and accumulation of lipids of *Chlorella vulgaris* under the stress of salinity was recorded in Fig. (2), where the over production of lipids was directly increased with increasing the level of salinity. The maximum percent of yield was recorded (43.15%) following the treatment of *Chlorella vulgaris* with 0.45mM NaCl at 12 days old culture but, in control samples the percent of accumulation of lipids was (29.47%). The previous results indicate that salinity is a feasible tool for the over production of lipid contents and the relation between lipids and growth was found to be inversely. Rodriguez *et al.* (2006) showed that, salt-exposed plants exhibited a reduction in the growth and biomass as compared with control. Our results are in agreement with Sinah and Hader (1996) who found that NaCl concentration > 5mM inhibited the growth of *Anabaena sp*. Ben-Amotz *et al*. (1985) and Hu

(2004) reported that an increase in salinity may result in a slightly increase in total lipid content of algae. Ben-Amotz *et al*. (1985) also, found that the lipid content of *Botryococcus braunii* in salt concentration was higher than that without salt. Walsby, (1982) suggested that the increases in lipid content under hypertonic conditions makes the plasma membrane more viscous and fluid to increase the turgor pressure of the cell and thus prevent the out flux of water from the cells as a mechanism of adaptation. Inhibition of the growth of *Chlorella vulgaris* in response to salinity may be due to: Partial inhibition of cell division (Hagemann *et al*., 1989).

Shifting the metabolites to the synthesis of osmoregulant compounds rather than synthesis of cellular constituents (Mohammed and Shafea, 1990).

In this regard the ability of algae to survive and grow in saline environment involves in maintenance of a constant turgid with the osmotic potential in their cells higher than the osmotic potential of the surrounding fluid (Kauss, 1967). Integration of intracellular adaptive mechanisms was noted (Reuveni *et al*., 1990). Production of endogenous compatible solutes and substances do not inhibit the metabolism even at very high concentrations (Brown and Edgley, 1980).

Results in Fig. 3 revealed that there was a variation in the growth of *Chlorella vulgaris* under the effect of different concentrations of ferrous sulphate (μM). Where after 6 days of culturing *Chlorella vulgaris* with 26.43μM and 35.25 μM of ferrous sulphate, the mean growth rate increases with 10.34% and 6.89% respectively from their corresponding control. On the other hand, after 6 days of culturing the alga with 44.06 μM of ferrous sulphate the mean growth rate of *Chlorella vulgaris* inhibited with 51.72% from its corresponding control. The data also revealed that after 6 days of culturing the alga with 26.43μM of Ferrous sulphate which the mean growth rate reached its maximum value (0.32 d^{-1}). Inspection of the biosynthesis and accumulation of lipids of *Chlorella vulgaris* under the stress of ferrous sulphate was recorded in Fig. 3, where the over production of lipids was subsequently increased slightly with increasing the level of ferrous sulphate up to 35.25 μM, but any further excess more than 35.25 μM resulted in inhibition in the lipid accumulation as the lipid content decreased with 28.94% from the corresponding control after 12 days of culturing *Chlorella vulgaris* with 44.06 μM of ferrous sulphate. The maximum lipid accumulated was recorded (34.6%) after 12 days old culture treated with 35.25 μM of ferrous sulphate. The results are in agreement with Liu *et al*. (2008) who also found that the total lipid content of *Chlorella vulgaris* in cultures supplemented with higher iron concentration was increased as compared with those supplemented with lower iron concentrations. In this regard Liu *et al*. (2008) concluded that the relatively high ferrous sulphate

concentration can induce the considerable lipid accumulation, but the superfluous iron in the media might restrict the metabolic pathways related to the lipid synthesis. The previous results are explained on the basis that Iron is one of the most important elements required by most microalgae because ferric ion is involved in fundamental enzymatic reactions, photochemistry in photosystem II and nitrogen consumption and chlorophyll synthesis in the algal cells (Naito *et al*., 2005). Deficient or excessive iron can generally reduce the photosynthetic efficiency of microalgae (Oijen *et al*., 2004) so; the previous deficiency in the photosynthesis may reduce algal growth.

Data in Table 1 revealed that, under the maximum stress factors there were a great variation in the lipid contents of *Chlorella vulgaris*. The obtained data also, manifested that among the used stress factors 0.10 mM $NaNO_3$ considered a feasible tool for the over production of lipid where, it maximally recorded 52.3% with increasing of 83.5% from the corresponding control. The data also, showed that the lipid content determined gravimetrically don't differ so much from the previous amount of lipid determined Spectrofluorometrically using Nile red stain.

The relative percentages of fatty acids extracted from *Chlorella vulgaris* are presented in Table 2. Thirteen fatty acids were identified from Capric to Lignoceric fatty acids. It was found that treating *Chlorella vulgaris* with 0.1mM of NaNO3 and 0.45mM of NaCl resulting in an obvious increase in total saturated fatty acids (SFA) with percent 14% and 3.3% respectively from their corresponding control. On the other hand, treating the alga with 35.25µM ferrous sulphate resulted in obvious decreases in total SFA with percentages 3.3% from the corresponding control. So, the result decreased in total SFA may be shifted towards the biosynthesis of UFA and vice versa. Where as total SFA percentages ranged from 50.13%-59.07 % and the maximum yield of saturated fatty acid was found in the lipid extract of cultures treated with 0.1 mM of $NaNO_3$. Data also showed that the major constituents of the lipid fraction of *Chlorella vulgaris* were found to be C16, C18, and C18:1, C18:2 and C18:3 fatty acids. Also, it was found that all of the treatments in the present work resulted in formation of C24 fatty acid which wasn't recorded in the lipid profile of untreated alga.

Treating the alga with 0.1 mM of $NaNO_3$ resulted in an obvious increase in the relative percentages of saturated fatty acid (SFA) from its corresponding control. The increase in SFA is due to increases in the content of stearic acid (C18). On the other hand, there was a decrease in unsaturated fatty acid (UFA) contents from the corresponding control (especially in Palmitolec (C16:1), Linoleic (C18:2) and α-Linoleic C18:3. It was found also that, in response to sodium nitrate stress each of C10, C12, C16:1, C17, C20 and C22 fatty acids

were not recorded in the resulted lipid profile compared with the control lipid profile. Not only 0.1 mM of $NaNO_3$ but also, treating the alga with 0.45mM NaCl resulted in an obvious increase in SFA contents from their corresponding control. This increase is due to biosynthesis of Lignoceric acid (C24:0= 24% of total content) which wasn't recorded in the corresponding control. On the other hand, there were a slightly decreases in UFA (especially in C16:1 and C18:2) from the corresponding control. On the other hand, treating *Chlorella vulgaris* with 35.25µM ferrous sulphate resulted in a slightly decreases in the total contents of SFA with a slightly increases in total UFA as compared with control. The previous decreases in SFA (especially in the contents of C14:0 and C16:0) may be shifted in the synthesis of long chain fatty acid (C24:0=12.72% of total content) which wasn't recorded in untreated culture.
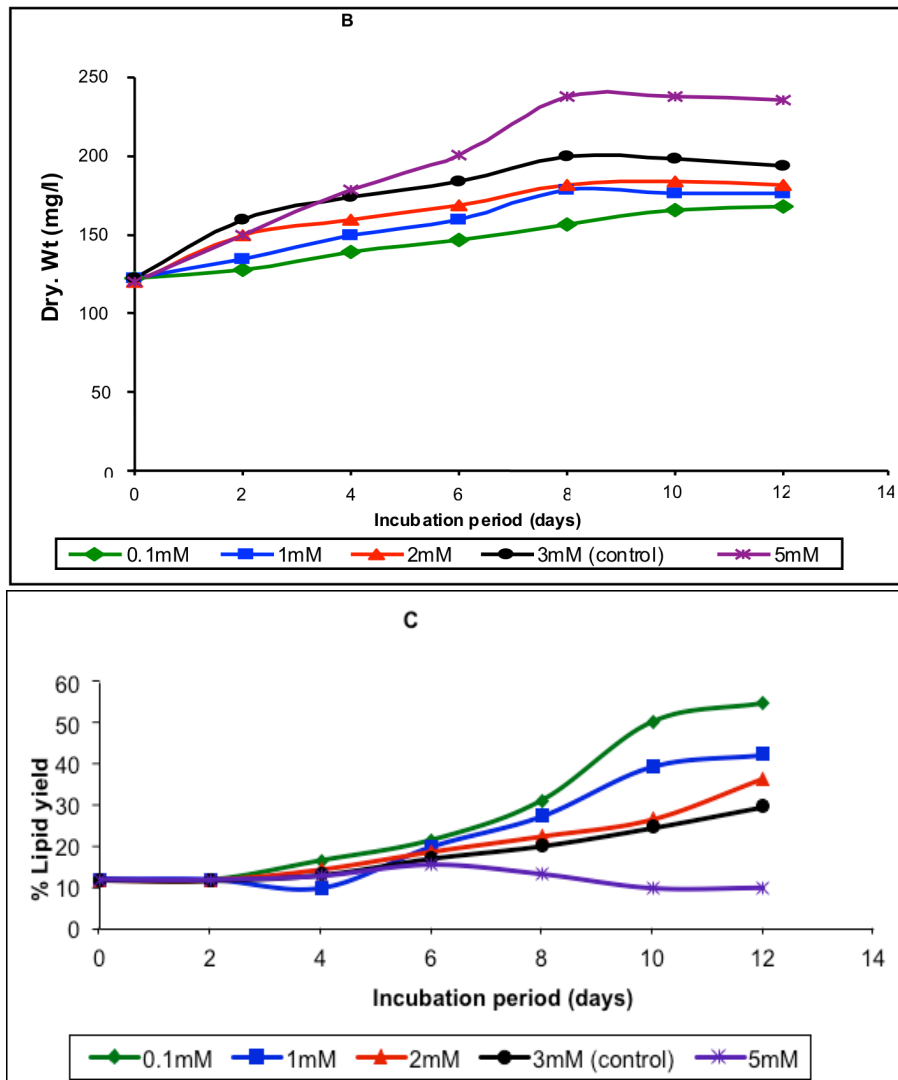

346
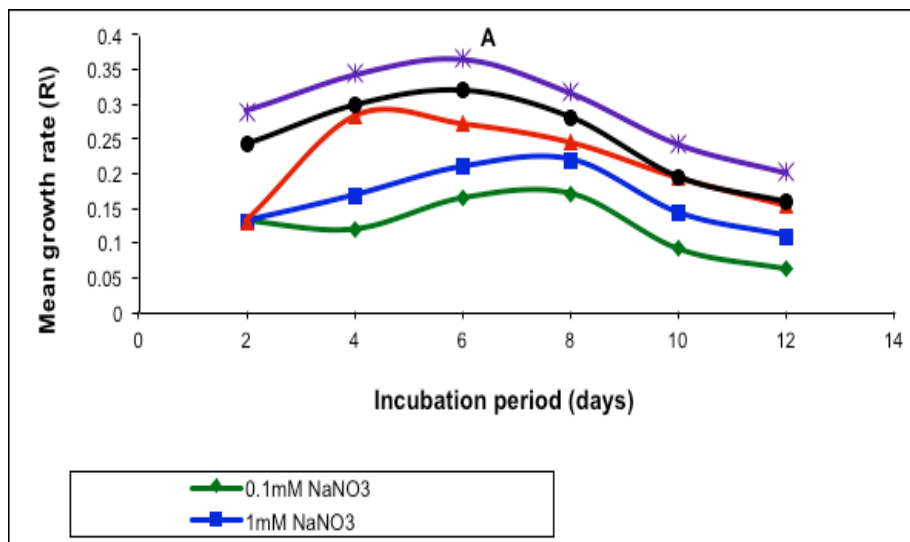
Fig. 1. Effect of variable concentrations of NaNO₃ (mM) on *Chlorella vulgaris* measured as:
(a) Mean growth rate (R\)   (b) Dry wt. (mg/l)   (c) Percentage of Lipid yield (%)
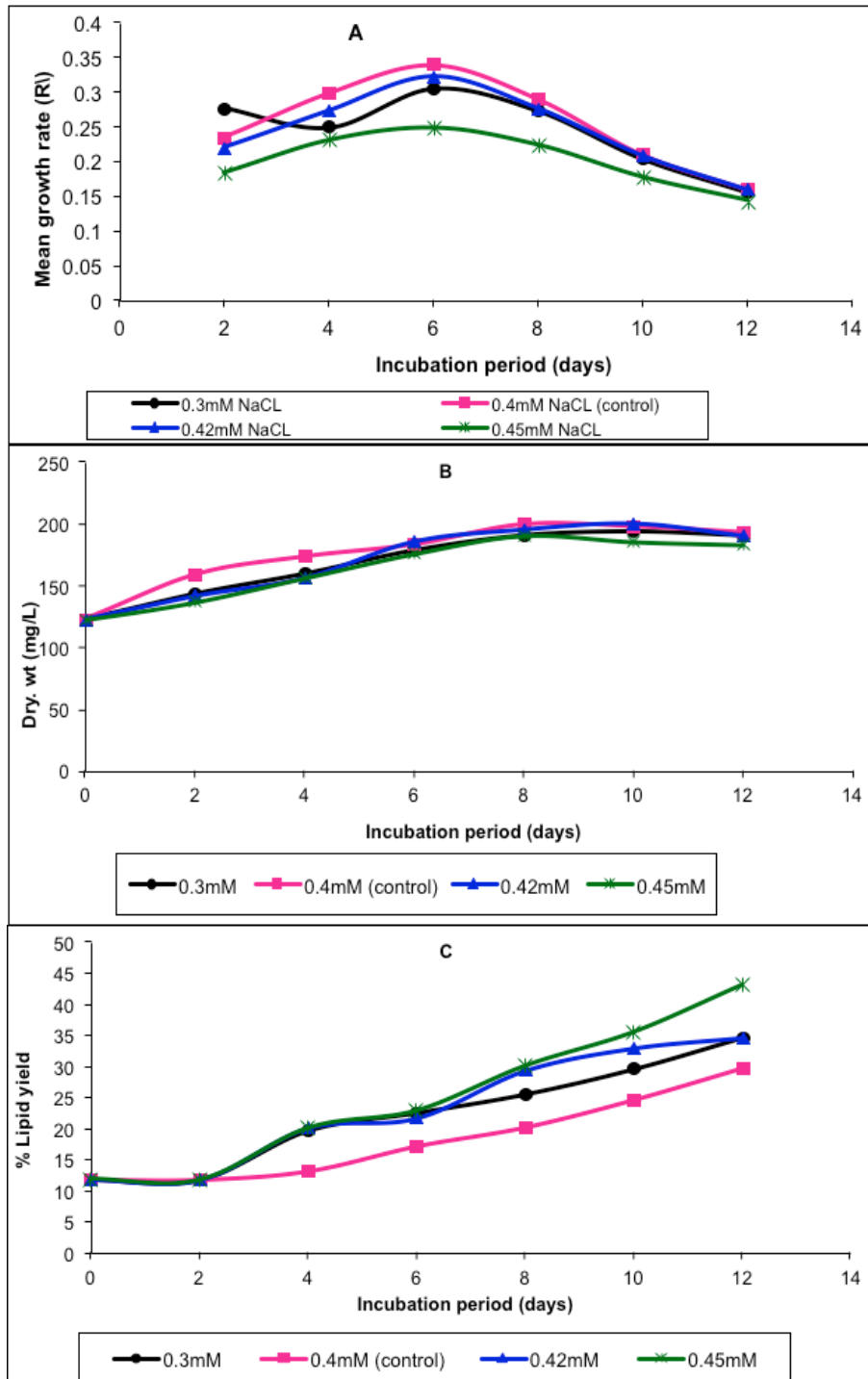
Fig. 2. Effect of variable concentrations of NaCl (mM) on *Chlorella vulgaris* measured as:
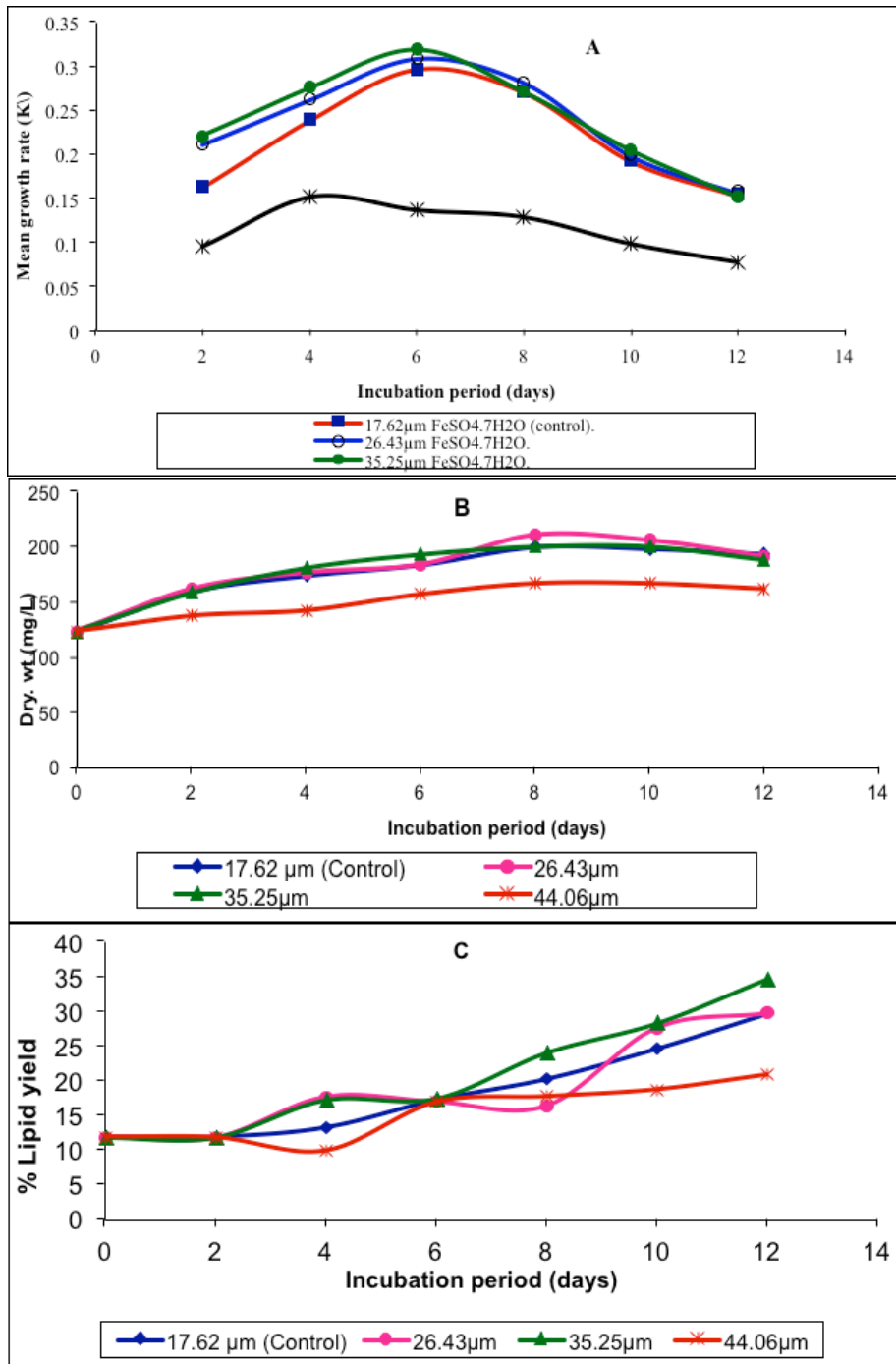(a) Mean growth rate (R\)   (b) Dry wt. (mg/l)   (c) Percentage of Lipid yield (%)

348

Fig. 3. Effect of variable concentrations of FeSO$_4$.7H$_2$O (mM) on *Chlorella vulgaris* measured as:(a) Mean growth rate (R\)   (b) Dry wt. (mg/l)   (c) Percentage of Lipid yield (%)

Table 1. Effect of nitrogen deficiency, high salinity and iron concentration on the lipid content of *Chlorella vulgaris* (%)

Treatments	Lipid %
Control	28.50
0.1mM NaNO$_3$	52.30
0.45mM NaCl	44.00
35.25μM FeSO$_4$.7H$_2$O	32.70

Table 2. Gas Chromatography analysis of n-hexane lipid extract of *Chlorella vulgaris* treated with the best nutrient concentrations for lipid content

Fatty acids	C-Number of fatty acids	Control	0.1mM NaNO$_3$	0.45mM NaCl	35.25μM Ferrous sulphate
Capric	C10:0	1.38	-	2.5	2.36
Lauric	C12:0	4.67	-	2.13	2.51
Myristic	C14:0	14.57	1.45	7.02	6.46
Palmitic	C16:0	19.83	11.17	12.93	14.24
Palmitoleic	C16:1	7.56	-	3.7	4.47
Margaric	C17:0	2.79	-	0.42	2.75
Stearic	C18:0	5.54	44.11	2.33	5.52
Oleic	C18:1	10.69	38.50	11.48	14.66
Linoleic	C18:2	17.64	1.64	16.49	17.50
α-Linoleic	C18:3	12.24	0.77	14.27	13.22
Arachidic	C20:0	1.57	-	0.66	2.27
Behenic	C22:0	1.49	-	1.57	1.30
Lignoceric	C24:0	-	2.34	24.00	12.72
Saturated fatty acid (SFA)		51.84	59.07	53.56	50.13
Unsaturated fatty acid (UFA)		48.13	40.90	45.94	49.85
Ratio of SFA / UFA		1.08	1.44	1.17	1.01
Total fatty acid		99.97	99.97	99.50	99.98

References

Ahlgren, G. and Hyenstrand, P. (2003). Nitrogen limitation effects of different nitrogen sources on the nutritional quality of two freshwater organisms, *Scenedesmus quadricauda* (Chlorophyceae) and *Synechococcus sp*. (Cyanophyceae). J. Phycol., 39:906–917.

Banerjee, A., Sharma, R., Chisti, Y. and Benerjee, U.C. (2002). *Botryococcus braunii*: A renewable source of hydrocarbons and other chemicals. Crit. Rev. Biotechnol., 22(3):245–279.

Behzadi, S. and Farid, M.M. (2007). Review: examining the use of different feedstock for the production of biodiesel. Asia Pacific J. Chem. Eng. 2:480–486.

Ben-Amotz, A.; Tomabene, T.G. and Thomas, W.H. (1985). Chemical profile of selected species of microalgae with emphasis on lipids. J. Phycol., 21:72–81.

Brown, A.D. and Edgley, M. (1980). Osmoregulation in yeast. In: Genetic Engineering of Osmoregulation (Ed. By D.W. Rains. R. C. Valentine and A. Hollander). plenum publishing Co., New York. pp. 75-89

Bruce, E.R. (2008). Opportunities for renewable bioenergy using microorganisms. Biotechnol. Bioeng., 100(2):203–212.

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

Davis, M.M. and Hetzer, H.B. (1966). Titrimetric and equilibrium studies using indicators related to Nile blue. A. Anal. Chem., 38:451-461.

Dayananda, C., Sarada, R., Bhattacharya, S. and Ravishankar, G.A. (2005). Effect of media and culture conditions on growth and hydrocarbon production from *Botryococcus braunii*. Process Biochem., 40:3125–3131.

De-Bashan, L.E., Hernandez, J.P., Morey, T. and Bashan, Y. (2004). Microalgae growth-promoting bacteria as ''helpers'' for microalgae: a novel approach for removing ammonium and phosphorus from municipal wastewater. Water Res., 38:466–474.

Demirbas, A. (2008). Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. Energy Convers Manage., 49:2106–2116.

Fabregas, J., Patino, M., Arredondo-Vega, B.O., Tobar, J.L. and Otero, A. (1995). Renewal rate and nutrient concentration as tools to modify productivity and biochemical composition of cyclostat cultures of the marine microalga *Dunaliella tertiolecta.* Appl. Microbiol. Biotechnol., 44:287-292.

Fan, L.H., Zhang, Y.T., Zhang, L. and Chen, H.L. (2008). Evaluation of a membrane-sparged helical tubular photobioreactor for carbon dioxide biofixation by *Chlorella vulgaris*. J. of Membrane Sci.; 325:336–345.

Frenz, J., Largeau, C. and Casadevall, E. (1989). Hydrocarbon recovery by extraction with a biochemical solvent from free and immobilized cultures of Botrycoccus braunii. Enzy. Microb. Technol., 11: pp. 717.

Goldberg, I.K. and Cohen, Z. (2006). The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water eustigmatophyte *Monodus subterraneus*. Phytochemistry, 67:696–701.

Hagemann, M., Erdmann, N. and Schiewer, U. (1989). Salt adaptation of the cyanobacteria *Microcystis firma* and *Synechocystis aquatilis* in turbidostat cultures. I. Steady state values. Arch. Hydrobiol. Suppl. 82, 4. Algological Studies (57):425-435.

Hsieh, C. and Wu, W. (2009). Cultivation of microalgae for oil production with a cultivation strategy of area limitation. Bioresour. Technol., 100:3921-3926.

Hu, Q. (2004). Environmental effects on cell composition. In: Richmond, A. (Ed.), Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwell Science, Victoria, pp. 83–93.

Illman, A.M., Scragg, A.H. and Shales, S.W. (2000). Increase in *Chlorella* strains calorific values when grown in low nitrogen medium. Enzy. and Microb.Technol., 27:631–635.

Kauss, H. (1967). Isofloridosid und Osmoregulation bei *Ochromonas malhamensis*. Z. Pflanzen Physiol., (56):553-565.

Kolber, Z.S., Barber, R.T., Coale, K.H., Fitzwater, S.E., Greene, R.M., Johnson, K.S., Lindley, S. and Falkowski, P.G. (1994). Iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. Nature; 371:145–149.

Liu, Z.Y., Wang, G.C. and Zhou, B.C. (2008). Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresour. Technol.; 99:17-22.

Marchetti, J.M., Miguel, V.U. and Errazu, A.F. (2007). Possible methods for biodiesel production. Renew Sust Energy Rev; 11:1300–1311.

Meher, L.C., Vidya Sagar, D. and Naik, S.N. (2006). Technical aspects of biodiesel production by transesterification –a review. Renew Sust. Energy Rev, 10:248–268.

Miao, X.L. and Wu, Q.Y. (2006). Biodiesel production from heterotrophic microalgal oil. Bioresour Technol; 97:841–846.

Miller, J.D. A. (1962). Fats and Steroids. *In*: R. A. Lewin (1962), Physiology and Biochemistry of Algae. Academic Press, NY, pp. 929.

Mohammed, A.A. and Shafea, A.A (1990). Growth and some metabolic activities of various Stalinized Scendesmus obliqus. Bull. Fac. Sci. Assiut Univ., 19:73-91.

Naito, K., Matsui, M. and Imai, I. (2005). Ability of marine eukaryotic red tide microalgae to utilize insoluble iron. Harmful Algae, 4:1021–1032.

Nichols, H.W. (1973). Growth media – freshwater. In Stein, J. (Ed.) Handbook of Phycological Methods, Culture Methods and Growth Measurements, Camb. Univ. Press, pp. 7-24.

Oijen, T.V., Van Leeuwe, M., Gieskes, W.W.C. and De Baar, H.J.W. (2004). Effects of iron limitation on photosynthesis and carbohydrate metabolism in the Antarctic diatom *Chartoceros brevis* (Bacillariophyceae). Eur. J. Phycol., 39:161–171.

Parrish, C.C. and Wangersky, P.J. (1990). Particulate and dissolved lipid classes in cultures of *Phaeodactylum tricornutum* in cage culture turbidostats with a range of nitrogen supply rates. Mar. Ecol. Prog. Ser., 35:119-128.

Radwan, S.S. (1978). Coupling of two dimension thin layer chromatographywith gas chromatography for the quantitative analysis of lipids classes and their constituent fatty acids. J. Chromatog. Sci., 16:538-542.

Reuveni, M., Bennett, A.B., Bressan, R.A. and Haswgawa, P.M. (1990). Enhanced H^+ transport capacity and ATP hydrolysis activity of the tonoplast H^+ ATP-ase after NaCl adaptation. Plant Physiol., 94:524-530.

Robert, R.L.G. (1979). Growth measurements. Division rate. In R. J. Stein (Ed) physiological methods. Culture methods and growth measurements. Cambridge Univ. press, Cambridge, pp. 29-311.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N. and Bonini, G. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering., 102(1):100-112.

Rodriguez, A.A., Stella, A.M., Storni, M.M., Zulpa, G. and Zaccaro, M.C. (2006). Effects of cyanobacterial extracellular products and gibberellic acid on salinity tolerance in *Oryza sativa* L. Saline System. J., 2:1-7.

Sadasivam, S. and Manickam, A. (1996). Biochemical methods. 2nd ed. New age International (P) limited,Tmail Nadu Agricultural University.

Schenk, P.M., Thomas-Hall, S.R., Stephens, E., Marx, U.C., Mussgnug, J.H. and Posten, C. (2008). High efficiency microalgae for biodiesel production. Bio-Energy Res., 1:20-43.

Sims, R.E.H., Mabee, W., Saddler, J.N. and Taylor, M. (2010). Anoverview of second generation biofuel technologies. Bioresour. Technol., 101:1570–1580.

Sinah, R.P. and Hader, D.P. (1996). Response of a rice field cyanobacterium *Anabaena* sp to physiological stressors. Germany Environmental and Experimental Botany, 36(2):147-155.

Singh, S., Kate, B.N. and Banerjee, U.C. (2005). Bioactive compounds from cyanobacteria and microalgae: an overview. Crit. Rev. Biotechnol., 25:73–95.

Spolaore, P., Joannis-Cassn, C., Duran, E. and Isambert, A. (2006). Commercial application of microalgae. J. Biosci. and Bioeng., 101(2):87-96.

Sukenik, A. and Livne, A. (1991). Variations in lipid and fatty acid content in relation to acetyl CoA carboxylase in the marine Prymnesiophyte *Isochrysis galbana*. Plant Cell Physiology, 32:371–378.

Thorpe, J.F. (1907). A reaction of certain coloring matters of the oxazine series. J. Chem. SOC., 91:324-336.

Vasudevan, P.T. and Briggs, M. (2008). Biodiesel production-current state of the art and challenges. J. Ind. Microbiol. Biotechnol., 35:421–430.

Vogel, A.J. (1975). A text book of practical organic chemistry .3rd ed. English Language Book Society and Longman Group Ltd. London, pp. 969-971.

Walsby, A.E. (1982). Cell-water and cell-solute relations. In: Carr, N. G., Whitton, B.A. (eds,). The Biology of Cyanobacteria, Blakwell, Oxford, pp. 237-262.