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Nitrogen Limitation Increases Lipid Content of *Chlorella vulgaris* at Photobioreactor System

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Abstract: This study aimed to produce *C. vulgaris* which is easy to culture with limited cost and whose outside culture proved practical by increasing its lipid content. *Chlorella vulgaris* was cultured at photo bioreactor in non-nitrogen nutritive medium with different flow rates (0.3, 0.5 and 0.6 m sec^{-1}) and inoculataion densities of 20 and 50%. The highest lipid content in the study was obtained from the culture with a biomass rate of 38.16±0.8% and 0.409±0.01 g L^{-1}, inoculation density of 50% and a flow rate of 0.6 m sec^{-1}. The lowest lipid content was found as 21.34±0.5% in the culture with an inoculation density of 50% and a flow rate of 0.3 m sec^{-1}. The highest protein content was found as 21.73±0.1% in the culture with inoculation density of 50% and a flow rate of 0.5 m sec^{-1}. The lowest biomass content found as 0.035±0.007 g L^{-1} in the culture with an inoculation density of 20% and a flow rate of 0.3 m sec^{-1}. The lipid content in that culture was found as 29.69±1% and the protein content as 14±0.5%. These results suggest that N limitation increases *C. vulgaris* lipid content.

Key words: N limitation, *C. vulgaris*, lipid, biomass, photobioreactor system, Turkey

INTRODUCTION

The microalgae are valuable natural products used as animal and human food sources and in pharmaceuticals and medicines. In some countries, microalgal biomass is added into food products such as noodles in Japan and Chile to improve the nutrient profile (Hu, 2004; Fuentes *et al.*, 2000; Ginzberg *et al*., 2000). Microalgae have significant advantages over higher plants in the production of future biofuels (Day *et al*., 2012) and in main aplications of microalgae for aquaculture (Hemaiswarya *et al*., 2011).

The chemical composition of microalgae is influenced by environmental conditions including temperature and growth phase (Richmond, 1986; Renaud *et al*., 1995; Fidalgo *et al*., 1998). Especially, nitrogen sources and their concentrations effected of the growth and biochemical structures of microalgae (Fidalgo *et al*., 1995).

Biodiesel is commonly produced from vegetable oils or animal fats. Alternatively, biodiesel could be obtained using the lipid of microalgae. Some types of microalgae are known to have a high lipid content that is desirable for biodiesel production. For >50 years, the use of microalgae and production technology is known. Microalgae can be used alone for biodiesel as a new source (Chisti, 2007).

Hundreds of microalgal strains capable of producing high content of lipid have been screened and their lipid production metabolisms have been determined and reported (Sheehan *et al*., 1998). Several studies have shown that the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions (temperature and light intensity) or nutrient media characteristics (concentrations of nitrogen, phosphates and iron) (Illman *et al*., 2000; Xin *et al*., 2010).

The aim of the study was to increase lipid content of *C. vulgaris* in non-nitrogen medium at photobioreactor system with different flow rates and inoculataion densities. The lipid, protein and biomass rates were determined at the end of the experiment.

MATERIALS AND METHODS

Algae material and culture conditions: Chlorella species are eukaryotic, unicellular and non-motile freshwater green algae that belong to the Chlorophyta division (Kay, 1991). Chlorella cells have hemicellulotic cell walls and are spherical with a diameter ranging from 2-10 μm (Kay, 1991; Becker, 2007). Microalgae *C. vulgaris* cultures were kept at a constant room temperature of 18±2°C and illuminated with fluorescent lamps (Philips TLM 40W/54RS) at an irradiance level of 80 μmol

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photon/m^2/sec. The irradiance was measured by a Radiation Sensor LI-COR (LI-250, Inc. USA). The microalgae stock culture was grown in 8 L glass jar in a batch culture system and the culture was continuously stirred by air. For inoculation to the photobioreactor, 140 L of algae were cultured in laboratory.

The control culture group, at which the original Jaworski's medium was used, was also formed. The culture was grown in Jaworski's medium and the content of the medium consists of the following composition (g/200 mL): 4 $Ca(NO_3)_2.4H_2O$, 2.48 KH_2PO_4, 10 $MgSO_4.7H_2O$, 3.18 $NaHCO_3$, 0.45 EDTA FeNa, 0.45 EDTA Na_2, 0.496 H_3BO_3, 0.278 $MnCl_2.4H_2O$, 0.2 $(NH_4)6Mo_7O_{24}.4H_2O$, 16 $NaNO_3$ and 7.2 $Na_2HPO_4.12H_2O$.

Tubular photobioreactor system volume is 280 L. The diameter of the transparent acrylic tubes was 2.6 cm and tubes were set up horizontally. The collectiontank of working volume was 300 L. The CO_2 gas inlet was provided with flowmeter. The pH was arranged with pH controller as 7 and light intensity was measured 3 times a day.

In this study, *C. vulgaris* was cultured at photobioreactor in non-nitrogen nutritive medium with different flow rates (0.3, 0.5 and 0.6 m sec^{-1}) and with inoculataion densities of 20 and 50%.

Analytical methods: *C. vulgaris* cell concentration was determined daily by optical density measurements at 500 nm (Liu *et al*., 2008) by a UV-visible spectrophotometer (Shimadzu, UV mini, 1240 model, Japan). Biomass was determined by 10 mL of algal culture through glass fibre filter (Whatman GF/C, 1.2 μm, UK). Algae biomass was dried at 105°C for 2 h and weighed (Boussiba *et al*., 1992). For pigment analyses, 10 mL samples were centrifuged at 3500 rpm for 10 min and the pellet extracted with 5 mL acetone. The extracts were centrifuged again and chlorophyll a and total carotenoids were determined spectrophotometrically, recording the absorption at 661.6, 644.8 and 470 nm and using the equations of Lichtenthaler (1987). All measurements were made with three replications.

For lipid and protein analyses, samples of microalgae were collected in the stationary growth phase. *C. vulgaris* cells were separated from the medium by centrifugation at 3500 rpm for 10 min, using the centrifuge model of Hereaus Supragufe 22. However, biomass was dried at 55°C for 2 h, pulverized in a mortar and stored at -20°C for later analysis. Dry extraction procedure according to Zhu *et al*. (2002), as a modification of the wet extraction method by Bligh and Dyer (1959) was used to extract the lipid in microalgal cells. Typically, cells were harvested by centrifugation at 3500 rpm for 10 min. After drying, the samples were pulverized, overnight in a mortar and extracted using a mixture of chloroform: Methanol (2:1, v/v). About 120 mL of solvents were used for every gram of dried sample in each extraction step. The solid phase was separated carefully using filter paper (Advantec filter paper, No. 1, Japan) in which two pieces of filter papers were applied twice to provide complete separation. The solvent phase was evaporated in a rotary evaporator under vacuum at 60°C and the procedure was repeated three times until the entire lipid was extracted. The effects of solvents having different polarities for extracting the lipid as well as the effect of drying temperature and ultrasonication time were investigated in this study. As a result, the total crude protein content (Nx6.25) was determined by Kjeldahl Method (AOAC, 1995).

Statistical analysis: Two-way Analysis of Variance (ANOVA) was used to test the effects of nitrogen deficiencies and flow rates of culture on lipid, protein, optical density, chlorophyll a and biomass. When differences were found in the two-way ANOVA, Duncan multiple comparison test (HSD) of the one-way ANOVA was used to compare the mean differences (Zar, 1999) by the Statistical Package for the Social Sciences (SPSS) (Version 12.0, SPSS, Chicago, IL). As such, the differences were considered to be significant at $p \leq 0.05$.

RESULTS

The maximum optical density was reached to use the *C. vulgaris* continuous culture in photobioreactor system in Jaworski's nutrient medium. The experiment was created as a control group. Culture reached the maximum optical density of 8 days. The initial optical density was 0.3336±0.001, last day of optical density was 1.1341±0.006. The biomass was increased from 0.074±0.002 to 0.304±0.04 g L^{-1}, respectively. At the end of the trial, total lipid and protein ratios were determined as 12.34±0.4 and 50.79±0.8%, respectively.

Trial 1: The first stage of the experiment *C. vulgaris* was cultured in non-nitrogen Jaworski's medium and 20% inoculation density. In the experiment flow rate of culture, 0.3 m sec^{-1} was to be fixed. The experiment was completed in 9 days.

The optical density and biomass were started with 0.321±0.001, 0.046 g L^{-1} at the experiment, respectively. However, optical density and biomass values were declined at the end of the experiment and the values of optical density and biomass were 0.189±0.001 and 0.035±0.007 g L^{-1}, respectively.

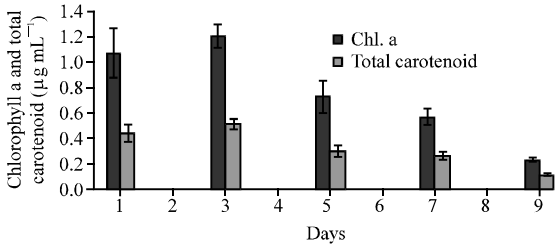

Fig. 1: The chlorophyll a and total carotene values of *C. vulgaris* culture non-nitrogen Jaworski's medium and 20% fertilization density and 0.3 m sec^{-1} flow rate at photobioreactor system; each value indicates the Mean±SD of duplicates

The initial chlorophyll a and total carotene were 1.0789±0.1 and 0.4431±0.06 µg mL^{-1}, respectively. At the end of the experiment, chlorophyll a and total carotenoid amounts were determined as 0.2314±0.01 and 0.1201±0.009 µg mL^{-1}, respectively (Fig. 1).

The temperature of 7-23°C and light intensity of 51-784 µmol photon/m^2/sec were recorded in culture at photobioreactor system. On the last day of the experiment, total lipid ratio of 29.69±1% was recorded when the last day protein content of 14±0.5% was identified.

Trial 2: The second stage of the experiment *C. vulgaris* was cultured in non-nitrogen Jaworski's medium and 50% inoculation density. In the experiment, flow rate of culture 0.3 m sec^{-1} was to be fixed. The experiment was completed in 8 days.

The initial optical density was 0.4847±0.003; at the last day this value increased with 0.5582±0.001. The amount of the first day of 0.133±0008 g L^{-1} biomass while the last day, this value was 0.117±0.01 g L^{-1}.

The initial chlorophyll a and total carotene were 1.3997±0.04 µg mL and 0.5654±0.01 µg mL^{-1}, respectively. The end of experiment chlorophyll a and total carotenoid were declined as 0.2782±0.04 and 0.3952±0.02 µg mL^{-1}, respectively (Fig. 2).

The temperature of 25-31°C and light intensity of 742-1084 µmol photon/m^2/sec were recorded in culture at photobioreactor system. On the last day of the experiment, total lipid ratio of 21.34±0.5% was recorded and protein content of 15.51±0.4% was identified.

Trial 3: *C. vulgaris* was cultured in non-nitrogen Jaworski's medium and 50% inoculation density. In the experiment, flow rate of culture 0.5 m sec^{-1} was to be fixed. The experiment was completed in 7 days.

The initial optical density and biomass were 0.9044±0.005 and 0.244±0.02 g L^{-1}, respectively. At the

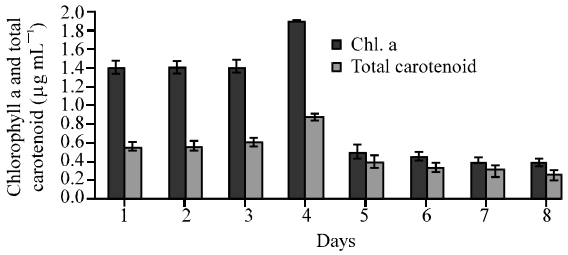

Fig. 2: The chlorophyll a and total carotene values of *C. vulgaris* culture non-nitrogen Jaworski's medium and 50% fertilization density and 0.3 m sec^{-1} flow rate at photobioreactor system; each value indicates the Mean±SD of duplicates

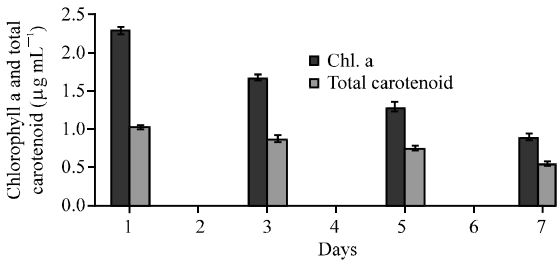

Fig. 3: The chlorophyll a and total carotene values of *C. vulgaris* culture non-nitrogen Jaworski's medium, 50% fertilization density and 0.5 m sec^{-1} flow rate at photobioreactor system; each value indicates the mean±SD of duplicates

end of the experiment, the values of 0.9715±0.003 optical density and biomass of 0.24±0.01 g L^{-1} were determined. The optical density increased but biomass was not changed.

The initial chlorophyll a and total carotene were 2.2768±0.05 and 1.0213±0.02 g mL^{-1}, respectively. At the end of the experiment, chlorophyll a and total carotenoid amounts were determined as 0.8915±0.03 and 0.5473±0.01 µg mL^{-1}, respectively (Fig. 3).

The temperature of 29-31°C and light intensity of 997-1216 µmol photon/m^2/sec were recorded in culture at photobioreactor system. At the last day of the experiment, total lipid ratio of 29.66±0.3% was recorded while the last day protein content of 21.73±0.1% was identified.

Trial 4: The last stage of the experiment *C. vulgaris* was cultured in non-nitrogen Jaworski's medium and 50% inoculation density. In the experiment, flow rate of culture 0.6 m sec^{-1} was to be fixed. The experiment was completed in 14 days.

The initial optical density was 0.5560±0.001 but at the last day this value was increased (1.6356±0.005). The

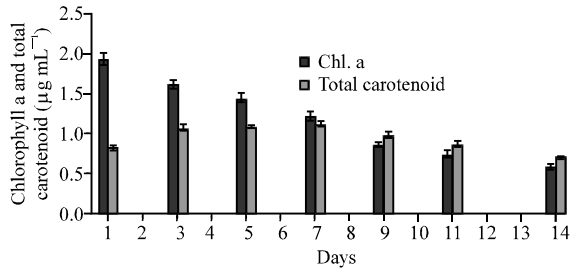

Fig. 4: The chlorophyll a and total carotene values of *C. vulgaris* culture non-nitrogen Jaworski's medium, 50% fertilization density and 0.6 m sec^{-1} flow rate at photobioreactor system; each value indicates the mean±SD of duplicates

Table 1: Main parameters of biomass, lipid and protein content of *C. vulgaris* at different groups

Groups	Biomass (g L^{-1})	Total lipid (%)	Total protein (%)
Control	0.304±0.04^{b}	12.34±0.4^{d}	50.79±0.8^{a}
20% fertilization on 0.3 m sec^{-1} flow rate	0.035±0.07^{e}	29.69±1^{b}	14.00±0.5^{d}
50% fertilization on 0.3 m sec^{-1} flow rate	0.117±0.01^{d}	21.34±0.5^{c}	15.51±0.4^{c}
50% fertilization on 0.5 m sec^{-1} flow rate	0.240±0.01^{c}	29.66±0.3^{b}	21.73±0.1^{b}
50% fertilization on 0.6 m sec^{-1} flow rate	0.409±0.01^{a}	38.16±0.8^{a}	12.60±0.8^{a}

Mean values, n = 3; Different letters between the lines indicate significant difference at 5% by Duncan multiple range test

amount of biomass at the first and last day were 0.122±0.009 and 0.409±0.01 g L^{-1}, respectively. The initial chlorophyll a and total carotene were determined 1.9337±0.07 and 0.8305±0.03 µg mL^{-1}, respectively but at the end of the experiment, chlorophyll a and total carotenoid amounts were declined as 0.5874±0.03 and 0.7214±0.02 µg mL^{-1}, respectively (Fig. 4).

The temperature of 29-31°C and light intensity of 1052-1368 µmol photon/m^2/sec were recorded in culture at photobioreactor system. On the last day of the experiment, total lipid ratio of 38.16±0.8% was recorded when the last day protein content of 12.60±0.8% was identified.

The effects of nitrogen limitation, flow rates and inoculation densities on *C. vulgaris* growth, lipid and protein content are summarized in Table 1.

DISCUSSION

There are various types of difficulties in microalgae species cultures even though the developments of algal biotechnology. The main purpose of the production of phototrophic organisms in a continuous culture in general is to provide an optimal optical density. When an algal culture was carried out outdoor, the environment factors were changed in both daily and seasonally. Growth factors like environmental factors, nutrient medium, temperature, salinity, pH, light can affect the biochemical composition of the biomass (Sukenik, 1991; Cohen *et al*., 1988; Brown *et al*., 1989; Roessler, 1990; Lourenco *et al*., 2002; Hu, 2004).

In this study, *C. vulgaris* was cultured in nitrogen-free media in a tubular photobioreactor, outdoor at the pilot plant of Algal Biotechnology Fisheries Faculty, Cukurova University with the aim of optimizing the yield and lipid content of the organism.

It is known that different N sources and concentrations effect on the growth and biochemical structure of microalgae (Fidalgo *et al*., 1995). The amount of biomass decreased, lipid content of biomass increased when the nitrogen deficiency applied in the microalgae culture (Xin *et al*., 2010). *C. vulgaris* were cultured in the medium N deficiency conditions (Mutlu *et al*., 2011), the biomass amount was decreased. In similar studies, it was reported that the nitrogen limitation effected to decrease of optical density and biomass (Kilham *et al*., 1997; Pruvost *et al*., 2009). In this study, N limitation caused the optical density and biomass to decrease.

Production of protein is favoured during periods of nitrogen sufficiency with limited carbohydrate synthesis; carbohydrates accumulate and protein production drops (Lourenco *et al*., 1998) whereas lipids usually increase (Shifrin and Chisholm, 1981). The imposition of nitrogen limitation increased the amount of lipid, such as *I. affinis galbana*, *Nannochloropsis* sp., *P. tricornutum*, *C. vulgaris*, *S. platensis* (Sukenik and Wahnon, 1991; Reitan *et al*., 1994; Fidalgo *et al*., 1995; Mutlu *et al*., 2011; Uslu *et al*., 2011). In this study, nitrogen limitation cause of decrease the growth rate of *C. vulgaris* but increased the cellular lipid.

In another study at which the effects of nitrogen limitation on the metabolites were studied, optical density and chlorophyll a quantity were decreased while organic carbon compounds as lipid was increased. However, yellowing of colors was observed due to increasing carotene quantity in the microalgae cultures (Shifrin and Chisholm, 1981; Sukenik *et al*., 1989). In this study, chlorophyll a amount were decreased and the color of the biomass were changed from green to yellow.

In this study, the inoculation rate was adjusted to 20 and 50%. The high dry matter in inoculation groups was 50%. This is because half of the total volume of culture is that of *C. vulgaris*. The highest amount of the lipid and the best growth rates were obtained at 50% inoculation and 0.6 m sec^{-1} flow rate in culture. The low flow rates were caused to low growth and lowlipid content in the biomass. Scragg *et al*. (2002) describes that

0.63 m sec^{-1} tubular photobioreactor system operating at their study, low nitrogen concentration, *C. vulgaris* growth was higher than the control group and reported that the rate of lipid was 58%.

As a result from these experiments, groups treated with the same amount of lipid, 20% of the amount of inoculation with a very low biomass present in culture, the amount of this inoculate is not suitable germination. In this study, the main observation was increased the amount of both lipid and biomass. The highest yield was obtained from the experimental group (50% inoculation rate and of 0.6 m sec^{-1} flow rate) when it was considered biomass and lipid. It was observed that the flow rates of 0.3 and 0.5 m sec^{-1} did not increase the biomass and lipid amount at the same inoculation ratios.

CONCLUSION

Finally, *C. vulgaris* could not grow well and circulate in reactor system at the flow rates of 0.3 and 0.5 m sec^{-1}. The optimum culture flow rate was 0.6 m sec^{-1} for *C. vulgaris* in photobioreactor culture experiments.

High temperature changes prevented the growth of algae and caused to lower biomass. At the end of the study, it was determined that the optimum temperatures are 23°C (Spring) and 31°C (Summer) for *Chlorella vulgaris* culture.

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REFERENCES

AOAC, 1995. Official Methods of Analysis. 16th Edn., Association of Official Analytical Chemists, Washington, DC., USA.

Becker, E.W., 2007. Micro-algae as a source of protein. Biotechnol. Adv., 25: 207-210.

Bligh, E.C. and W.J. Dyer, 1959. A rapid method for total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911-917.

Boussiba, S., L. Fan and A. Vonshak, 1992. Enhancement and determination of astaxanthin accumulation in green alga *Haematococcus pluvialis*. Methods Enzymol., 213: 386-391.

Brown, M.R., S.W. Jeffry and C.D. Garland, 1989. Nutritional aspects of microalgae used in mariculture: A literature review. CSIRO Marine Laboratories Report No. 205, pp: 1-44.

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

Cohen, Z., A. Vonshak and A. Richmond, 1988. Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum*: Correlation to growth rate. J. Phycol., 24: 328-332.

Day, J.G., N.J. Thomas, U.E.M. Achilles-Day and R.J.G. Leakey, 2012. Early detection of protozoan grazers in algal biofuel cultures. Bioresour. Technol., 114: 715-719.

Fidalgo, J.P., A. Cid, J. Abalde and C. Herrero, 1995. Culture of the marine diatom *Phaeodactylum tricornutum* with different nitrogen sources: Growth, nutrient conversion and biochemical composition. Cahiers de Biologie Marine, 36: 165-173.

Fidalgo, J.P., A. Cid, E. Torres, A. Sukenik and C. Herrero, 1998. Effects of nitrogen source and growth phase on proximate biochemical composition, lipid classes and fatty acid profile of the marine microalga *Isochrysis galbana*. Aquaculture, 166: 105-116.

Fuentes, M.M., G.G. Fernandez, J.A. Perez and J.J. Guerrero, 2000. Biomass nutrient profiles of the microalga *Porphyridium cruentum*. Food Chem., 70: 345-353.

Ginzberg, A., M. Cohen, U.A. Sod-Moriah, S. Shany, A. Rosenshtrauch and S.M. Arad, 2000. Chickens fed with biomass of the red microalga *Porphyridium* sp. have reduced blood cholesterol level and modified fatty acid composition in egg yolk. J. Applied Phycol., 12: 325-330.

Hemaiswarya, S., R. Raja, R. Ravi Kumar, V. Ganesan and C. Anbazhagan, 2011. Microalgae: A sustainable feed source for aquaculture. World J. Microbiol. Biotechnol., 27: 1737-1746.

Hu, Q., 2004. Environmental Effects on Cell Composition. In: Handbook of Microalgal Culture: Biotechnology and Applied Phycology, Richmond, A. (Eds.). Blackwell Publishing Company, England, pp: 83-93.

Illman, A.M., A.H. Scragg and S.W. Shales, 2000. Increase in chlorella strains calorific values when grown in low nitrogen medium. Enzyme Microb. Technol., 27: 631-635.

Kay, A.R., 1991. Microaglae as food and supplement. Crit. Rev. Food Sci. Nut., 30: 555-573.

Kilham, S.S., D.A. Kreeger, C. Goulden and S. Lynn, 1997. Effects of nutrient limitation on biochemical constituents of *Ankistrodesmus falcatus*. Freshwater Biol., 38: 591-596.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol., 148: 350-382.

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.

Lourenco, S.O., E. Barbarino, U.M.L. Marquez and E. Aidar, 1998. Distribution of intracellular nitrogen in marine microalgae: Basis for the calculation of specific nitrogen-to-protein conversion factors. J. Phycol., 34: 798-811.

Lourenco, S.O., E. Barbarino, J.C. De-Paula, L.O.S. Pereira and U.M.L. Marquez, 2002. Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. Phycol. Res., 50: 233-241.

Mutlu, Y.B., O. Isik, L. Uslu, K. Koc and Y. Durmaz, 2011. The Effects of Nitrogen and Phosphorus Deficiencies and Nitrite Addition on the Lipid Content of *Chlorella vulgaris* (Chlorophyceae). Afr. J. Biotechnol., 10: 453-456.

Pruvost, J., G. Van Vooren, G. Cogne and J. Legrand, 2009. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. Bioresour. Technol., 100: 5988-5995.

Reitan, K.I., J. R. Rainuzzo and Y. Olsen, 1994. Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. J. Phycol., 30: 972-979.

Renaud, S.M., H.C. Zhou, D.L. Parry, L.V. Thinh and K.C. Woo, 1995. Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae *Isochrysis* sp., Nitzschia closterium, Nitzschia paleacea and commercial species *Isochrysis* sp. (clone T. ISO). J. Applied Phycol., 7: 595-602.

Richmond, A., 1986. Cell Response to Environmental Factors. In: Handbook of Microalgal Mass Culture, Richmond, A. (Ed.). CRC Press, Boca Raton, Florida, pp: 69-99.

Roessler, P.G., 1990. Environmental control of glycerolipid metabolism in microalgae: Commercial implications and future research directions. J. Phycol., 26: 393-399.

Scragg, A.H., A.M. Illman, A. Carden and S.W. Shaless, 2002. Growth of microalgae with increased calorific values in a tubular bioreactor. Biomass Bioenergy, 23: 67-73.

Sheehan, J., T. Dunahay, J. Benemann and P. Roessler, 1998. A look back at the US department of energy's aquatic species program: Biodiesel from Algae, Close out report TP-580-24190. Golden, CO: National Renewable Energy Laboratory, US Department of Energy's Office of Fuels Development, USA.

Shifrin, N.S. and S.W. Chisholm, 1981. Phytoplankton lipids: Interspecific differences and effects of nitrate, silicate and light-dark cycles. J. Phycol., 17: 374-384.

Sukenik, A. and R. Wahnon, 1991. Biochemical quality of marine unicellular algae with special emphasis on lipid composition. I. *Isochrysis galbana*. Aquaculture, 97: 61-72.

Sukenik, A., 1991. Ecophysiological considerations in the optimization of eicosapentaenoic acid production by *Nannochloropsis* sp. (Eustigmatophyceae). Bioresour. Technol., 35: 263-269.

Sukenik, A., Y. Carmeli and T. Berner, 1989. Regulation of fatty acid composition by irradiance level in the eustigmatophyte *Nannochloropsis* sp. J. Phycol., 25: 686-692.

Uslu, L., O. Isik, K. Koc and T. Goksan, 2011. The Effects of Nitrogen Deficiencies on the Lipid and Protein Contents of *Spirulina platensis*. Afr. J. Biotechnol., 10: 386-389.

Xin, L., H. Hong-ying, G. Ke and S. Ying-Xue, 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresour. Technol., 101: 5494-5500.

Zar, J.H., 1999. Biostatistical Analysis. 4th Edn., Prentice Hall International Inc., New Jersey, USA., pp: 231-272.

Zhu, M., P.P. Zhou and L.J. Yu, 2002. Extraction of lipids from *Mortierella alpina* and enrichment of arachidonic acid from the fungal lipids. Bioresour. Technol., 84: 93-95.