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Research into the Influence of Cultivation Conditions on the Fatty Acid Composition of Lipids of Chlorella Vulgaris Microalgae

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The effect of temperature and illumination intensity on the biomass growth of *Chlorella vulgaris Beijer* IPPAS C-2 microalgae cells, lipid biosynthesis and their fatty acid composition was studied experimentally. It has been established that temperature and photosynthetically active radiation (PAR) have a significant influence on the kinetics of microalgae biomass growth, biosynthesis of lipids and their fatty acid composition. Cultivation with increased levels of PAR and temperature leads to intensive cell division, which can be useful for seed preparation. At lower PAR levels and temperatures, the strain accumulates the maximum amount of biomass, lipids, saturated and unsaturated fatty acids. The analysis of fatty acid composition has shown that this strain may be promising in the technology of producing raw materials for biofuel production and food additives.

1. Introduction

Sustainable use of natural resources and production of minimal amount of environmentally harmful by-products has been on the agenda of many researchers worldwide. Technologies of complex use of microalgae for obtaining food ingredients, wastewater treatment, production of raw materials for biofuel, etc. have the potential for wide dissemination in the closed-loop economy. One of the key advantages of microalgae is their "flexibility" in terms of the composition and quality of the components they synthesize - the chemical composition of the biomass and the productivity of the microalgae strain can vary widely depending on the conditions of cultivation (Barghbani et al., 2012; Blair et al., 2014). Despite the plethora of studies (Ma et al., 2018; Chinnasamy et al., 2009) on microalgae cultivation regimes, their diversity and the peculiarities of the biosynthesis of external and internal metabolites existing in each strain, enables us to look for new approaches to their sustainable production. At the same time, one of the problems of rapid and reliable determination of optimal cultivation regimes is related to the fact that at the moment there is no clear understanding of the links between process conditions of microalgae cultivation and the intensity of biochemical and chemical reactions in the cell.

One of the promising microalgae species for industrial cultivation is *Chlorella vulgaris*, because it has a high growth rate, the ability to respond to different conditions of cultivation by changing the content of nutrients, and it is resistant to infection. *Chlorella vulgaris* microalgae cells contain up to 50 % of lipids - including saturated and unsaturated fatty acids used in the production of biofuels and nutritional supplements (ω-3 fatty acids), respectively (Araujo et al., 2013).

The analysis of the processes taking place in the *Chlorella vulgaris* microalgae cell (Figure 1) (Salati and Goodridge, 1996) shows that the main parameters in microalgae cultivation are the intensity of illumination and culturing temperature (Mayo, 1997; Gong et al., 2014). The temperature and illumination have a critical influence on the amount of the intermediate product, acetyl-CoA, which is the starting substance for the biosynthesis of lipids and fatty acids in the microalgae cell. In addition, the temperature influences membrane fluidity, cytoplasm viscosity and, as a consequence, the rate of reactions taking place in the cell. As a result of biochemical reactions from acetyl-CoA, saturated and unsaturated fatty acids are synthesized. The resulting

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acids form molecules of saturated and unsaturated triglycerides. At a higher temperature, there is a decrease in the proportion of unsaturated fatty acids as a result of their predominant oxidation and detachment. Under these conditions there is a decrease in the functional activity of photosystems and ATP-synthesizing complex, which leads to a slowdown in cell growth. At the same time, desaturase genes, which catalyze the formation of double bonds in fatty acids, are also expressed (Los, 2014).

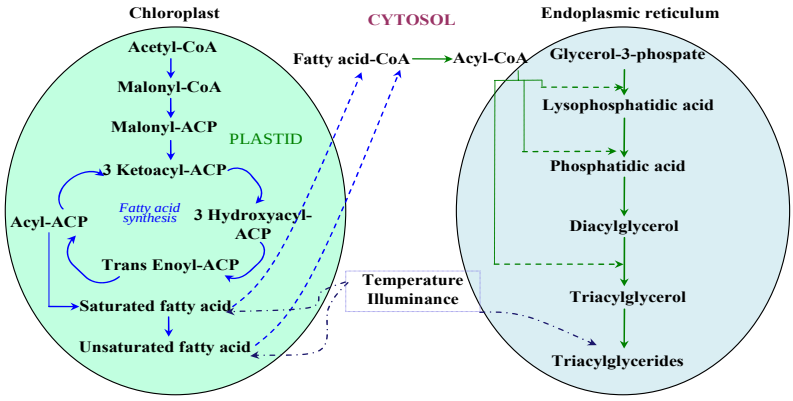

Figure 1: Biosynthesis of lipids in a Chlorella vulgaris cell

The increase in the level of illumination and temperature to some critical level has a positive effect on biomass accumulation (Takeshita et al., 2014). At low cultivation temperatures and illumination levels, metabolic pathways shift towards lipid accumulation (in particular, with unsaturated fatty acids). At the same time, the mass fraction of lipids in the cell is less affected by temperature than the illumination factor (Villarruel-López et al., 2017).

Converti et al. (2009) and Khoeyi et al. (2012) have found that the metabolism shifted to the synthesis of saturated fatty acids with a threefold increase in the intensity of illumination, while the amount of monounsaturated and polyunsaturated fatty acids decreased with an increase in illumination and the duration of the light phase. The maximum percentage of monounsaturated and polyunsaturated fatty acids (of the total amount of fatty acids): 15.93 - 27.40 % - was recorded with a 1.5 times decrease in the intensity of illumination.

The effect of light on the fatty acid content of *Chlorella vulgaris* biomass was studied in Seyfabadi et al. (2011), Dickinson et al. (2017), Kanokwan Chankhong et al. (2018). It was found that the total content of saturated fatty acids increases, while monounsaturated and polyunsaturated fatty acids decrease with increasing light intensity and duration of the light phase of the lighting period.

Changes in the composition of fatty acids are observed when the culturing temperature changes: as the temperature decreases, the amount of unsaturated fatty acids in lipids increases. Rohit and Venkata Rohan (2018) established that lipid content in microalgae depends on temperature. With a 5 °C increase in temperature, the lipid content in the *Chlorella vulgaris* biomass decreases by 40 %.

The analysis of the sources allows to conclude that the peculiarities of the microalgae lipid metabolism pathway of the *Chlorella vulgaris* species, as well as the influence of cultivation temperature and the level of illumination on the fatty acid composition of microalgae lipids, have not been fully studied.

Therefore, the aim of the study was to determine the regularities of the influence of *Chlorella vulgaris* cultivation conditions on the fatty acid composition of biomass.

2. Methods and materials

2.1 Biomass cultivation

For the experimental study, the strain *Chlorella vulgaris Beijer* IPPAS C-2 from the Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences was used.

Microalgae cultivation was carried out on the Tamiya nutrient medium (Tamiya, 1957). On the fourth day of cultivation, mineral salts were added to the microalgae suspension, which are part of the Tamiya nutrient medium (Dvoretsky et al., 2015). The process of cultivation was carried out in a cylindrical photobioreactor of 0.3 m height and 0.1 m diameter under the following conditions: 1) the amount of the introduced seed selected at the stationary growth stage was 10 % of the total suspension volume (cell titre - 13.5 million cells / mL) -

400 mL. The stock culture was grown at the temperature of 25 °C and the level of PAR of 105 μmol of photons /(m^2·s); 2) the pH level changed in the range of 6.2 - 8.0; 3) aeration of the suspension was carried out by a gas-air mixture (70 - 80 L/h) with the content of carbon dioxide 0.03 %. Microalgae were cultivated under the conditions presented in Table 1. The concentration of microalgae cells in the suspension was determined by direct count in the Goryayev chamber. Cells were counted in 25 large squares. The concentration was determined by the formula: $n = a / 100 \cdot 10^6$, where n is concentration of microalgae cells, million cells / mL; a is number of cells in 25 large squares, pcs.

Table 1: Process conditions of the experiment

Sample #	PAR, μmol of photons /(m^2·s)	Temperature, °C
1	105	20
2	315	20
3	105	30
4	315	30

2.2 Processing and analysis of microalgae cells

Sampling of microalgae biomass (500 mL) for lipid extraction was performed prior to the experiment, as well as on the 2nd, 4th, 6th, 8th and 10th day of culturing. Separation of fugate from microalgae biomass was carried out using a Sigma 2-16 RK/2-16P centrifuge at a rotation speed of 4000 r/min for 5 minutes. Microalgae cells were disintegrated in the form of paste with humidity of 98 - 99 % using an ultrasonic disintegrator Scientz IID at ultrasound frequency of 25 kHz and power of 100 W within 20 minutes. Microalgae cells were dried to determine the cell concentration in the suspension (g/L) in a dry-air oven "HS-121A" at 80 °C.

Extraction of lipids from microalgae biomass was carried out in the Soxhlet apparatus using petroleum ether as a solvent. Lipids were extracted within 6 hours. The fat content was calculated by the formula: $x = (m_1 - m_2) / m_3 \cdot 100$, where m_1 is the weight of the sachet with the attachment and anhydrous sodium sulphide before extraction, g; m_2 is the weight of the sachet with the attachment and anhydrous sodium sulphide after extraction, g; m_3 is the weight of the attachment after drying, g.

The solvent was distilled using a rotary evaporator IR-1 M3 at a temperature of distillation 85 °C and the speed of rotation of the flask 65 min^{-1}.

The lipid content (mg) of 1 litre of microalgae suspension was calculated by the formula: $m_1 = M \cdot x$, where m_1 is the mass of lipids in 1 L of microalgae suspension, mg/L; M is the mass of microalgae biomass, mg/L; x is the mass fraction of lipids in microalgae biomass.

Analysis of lipid fatty acids contained in 'microalgae extract' was performed using a gas chromatograph "Crystallux-4000M".

3. Results and discussion

3.1 Kinetics of biomass growth and lipid accumulation

Experimental studies have shown that the maximum growth rate of microalgae cells in all samples was observed at the exponential growth phase (Figure 2a). And for samples 1, 2, 4 it equaled ≈ 0.4 $days^{-1}$ on the 4th-6th day of cultivation (Table 2). The greatest number of cells was observed in sample 4, which was cultivated at high temperatures and PAR: 2.1 million cells / mL on the 8th day of cultivation with an average cell diameter of 7.4 μm. The highest mass concentration of cells (0.5 - 0.6 g/L) with the same number of cells (0.5 - 0.75 million cells / mL) was observed at a lower level of PAR (105 μmol of photons/(m^2·s) for samples 1, 3 on the 4th and 2nd day, respectively, see Figure 2b. At the same time, sample 1 reached the average cell diameter of 9 - 9.4 μm in 4 days, and sample 3 - in 2 days. Thus, in terms of biomass, the best performance was observed at lower levels of PAR.

The maximum amount of total lipids in microalgae cells was accumulated in samples 2, 4 and made up 45 % of cell dry matter on the 4th day (Figure 2c), which is due to the fact that stock culture was cultivated with low levels of PAR and samples were grown under stress conditions - with high levels of PAR (315 μmol of photons/(m^2·s)).

During the 4th to 6th day a decrease in the mass concentration of lipids in cells is observed, resulting from the addition of a source of nitrogen to the nutrient medium. The increase of mass concentration of lipids in samples 1 and 3 on 6 - 8 days is caused by nitrogen deficiency due to more intensive metabolism of the strain at low levels of PAR. During this period of time a slight decrease in mass concentration of lipids was

registered in samples 2 and 4, which is due to the fact that acetyl-CoA is used for biosynthesis of antioxidant substances necessary for the cell at higher levels of PAR (Salati and Goodridge, 1996). The greatest amount of lipids (0.19 g/L) was observed at cultivation of sample 1 for 4 days (Figure 2d), at cell diameter ≈ 9.4 μm and biomass concentration 0.58 g/L.

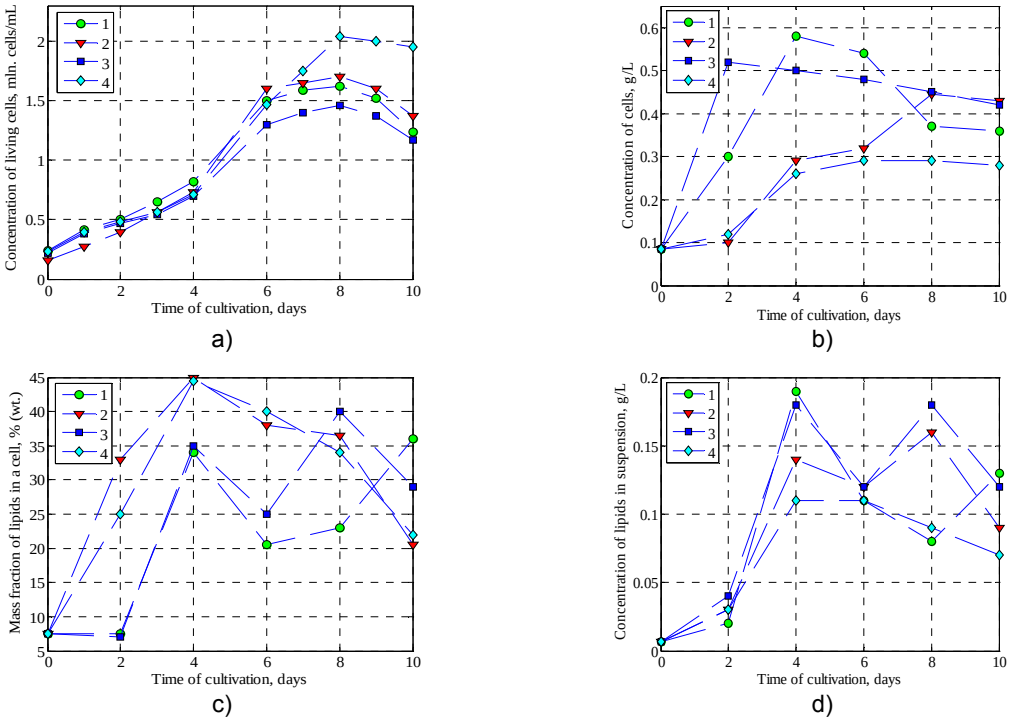

Figure 2: Kinetic characteristics of biomass with regard to cultivation conditions

3.2 Fatty acid composition

3.2.1 Saturated fatty acids

The maximum amount of saturated fatty acids in microalgae lipids (Figure 3a) was 181.5 mg/L on the 4th day of cultivation in sample 1; the same sample had the highest rate of saturated fatty acid accumulation of 81.85 mg/(L·days). This is explained by the fact that during the period of cultivation (2 - 4 days) there was an intensive growth of cells (Figure 2a), in which the active biosynthesis of fatty acids (primary product - saturated palmitic acid) was carried out. Cultivation conditions of stock culture and of samples 1 and 3 (in terms of illumination level) were identical, therefore these samples are assumed to have demonstrated the highest rates of metabolism and amounts of lipids (Table 2) containing saturated fatty acids - energy reserves necessary for cell growth and reproduction (Figure 3b). Chromatographic analysis of saturated fatty acids of lipids in sample 1 (on the 4th day) showed the presence of the following fatty acids: myristic acid (C14:0) - 0.6 % (mass), pentadecanic acid (C15:0) - 30.3 % (mass), palmitic acid (C16:0) - 41.2 % (mass), margaric acid (C17:0) - 18.4 % (mass), stearic acid (C18:0) - 2.4 % (mass). Thus, this cultivation regime can be considered as promising for obtaining raw materials for biofuel production.

3.2.2 Unsaturated fatty acids

Sample 1 also demonstrated the largest amount of unsaturated fatty acids (31.0 mg/L) and the rate of their accumulation (11.25 mg/(L·days)) on the 6th day of cultivation (Figure 3c). This is explained by the fact that in the process of intensive cell division a large number of unsaturated fatty acid molecules are required to form cytoplasmic membranes. Lower content of unsaturated fatty acids in samples 2 and 4 is associated with the process of photo-oxidative stress that may occur when microalgae cells are cultured at high levels of PAR. With temperature rise, the content of unsaturated fatty acids in cells (sample 3, Figure 3d) decreased.

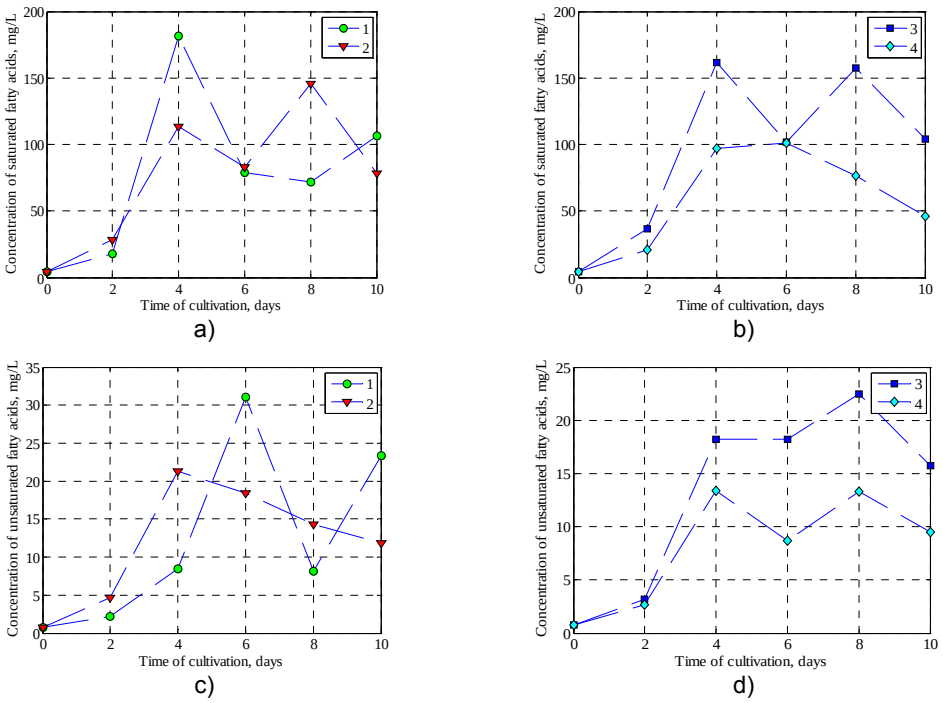

Figure 3: Kinetic characteristics of fatty acids in microalgae biomass

Table 2: Parameters of microalgae biomass during cultivation

	Parameter	Sample 1	Sample 2	Sample 3	Sample 4
Biomass	μ_{cells}, day^{-1}	0.38 (4 - 6 days)	0.41 (4 - 6 days)	0.31 (4 - 6 days)	0.38 (4 - 6 days)
	x_{max}, mln cells/mL	1.6 (8th day)	1.6 (8th day)	1.5 (8th day)	2.1 (8th day)
	$\mu_{biomass}$, g / L·day^{-1}	0.15 (2 - 4 days)	0.1 (2 - 4 days)	0.21 (0 - 2 days)	0.1 (8 - 10 days)
	x_{max}, g/ L	0.58 (4th day)	0.45 (8th day)	0.51 (2nd day)	0.29 (6th, 8th days)
Lipids	μ_{lipids}, g / L ·day^{-1}	0.085 (2 - 4 days)	0.05 (2 - 4 days)	0.07 (2 - 4 days)	0.04 (2 - 4 days)
	x_{max} , g / L	0.19 (4th day)	0.16 (8th day)	0.18 (4th, 8th days)	0.11 (4th, 6th days)
Saturated fatty acids	μ_{SFA}, mg / L·day^{-1}	81.85 (0 - 2 days)	42.7 (2 - 4 days)	62.51 (2 - 4 days)	37.97 (2 - 4 days)
	x_{max}, mg / L	181.5 (4th day)	145.76 (8th day)	161.82 (4th day)	101.31 (6th days)
Unsaturated fatty acids	μ_{UFA}, mg / L ·day^{-1}	11.25 (4 - 6 days)	8.3 (2 - 4 days)	7.47 (2 - 4 days)	5.35 (2 - 4 days)
	x_{max} , mg / L	31.0 (6th day)	22.12 (4th day)	23.58 (8th day)	13.42 (4th day)

(SFA - saturated fatty acids, UFA - unsaturated fatty acids, μ – maximum growth rate, x_{max} – maximum concentration)

The analysis of unsaturated fatty acids of lipids of sample 1 (on the 6th day) showed the presence of the following fatty acids: heptadecenoic (C17:1) - 21.5 % (mass), oleic (C18:1) - 3.9 % (mass), linoleic (C18:2) - 2.7 % (mass). That makes this cultivation regime a potentially attractive technology of obtaining raw materials for the production of food additives. The results obtained are consistent with the trends described in the works of Tsoglin and Pronina (2012) and Los (2014).

4. Conclusions

Experiments have shown that temperature and PAR have a significant influence on the kinetics of microalgae biomass growth, lipid biosynthesis and their fatty acid composition. Cultivation at elevated levels of PAR and temperature leads to intensive cell division, which may be useful for seed preparation. At lower PAR levels and temperatures, the strain *Chlorella vulgaris Beijer* IPPAS C-2 accumulates the maximum amount of

biomass (0.58 g/L (4th day)), lipids (0.19 g/L (4th day)), saturated (181.5 mg/L (4th day)) and unsaturated fatty acids (31.0 mg/L (6th day)). The analysis of fatty acid composition has shown that this strain may be promising in the technology of production of raw materials for biofuel and food additives.

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References

Araujo, G.S., Matos, L.J.B.L., Fernandes, J.O., Cartaxo, S.J.M., Goncalves, L.R.B., Fernandes, F.A.N., Farias, W.R.L., 2013, Extraction of lipids from microalgae by ultrasound application: prospection of the optimal extraction method. Ultrason.Sonochem. 20, 95–98.

Barghbani, R., Rezaei K., Javanshir A., 2012, Investigating the effects of several parameters on the growth of Chlorella vulgaris using Taguchi's experimental approach, International Journal of Biotechnology for Wellness Industries, 1, 128-133.

Blair M.F., Kokabian B., Gude V.G., 2014, Light and growth medium effect on Chlorella vulgaris biomass production, Journal of Environmental Chemical Engineering, 2 (1), 665-674.

Chinnasamy S, Ramakrishnan B., Bhatnagar A., Das K., 2009, Biomass production potential of a wastewater alga Chlorella vulgaris ARC 1 under elevated levels of CO_2 and temperature, International Journal of Molecular Sciences, 10, 518-532.

Converti A., Casazza A., Ortiz E., Perego P., Del Borghi M., 2009, Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production, Chem Eng Process; 48(6), 1146–51. DOI: 10.1016/j.cep.2009.03.006

Dickinson S, Ferreira S.P., Holz J.C.P., Lisboa C.R., Costa J.A.V., 2017, A review of biodiesel production from microalgae, Clean Technol Environ Policy, 19(3), 637–68.

Dvoretsky D.S., Dvoretsky S.I., Peshkova E.V., Temnov M.S., 2015, Optimization of the process of cultivation of microalgae Chlorella Vulgaris biomass with high lipid content, Chemical Engineering Transactions, 43, 361-366.

Gong Q., Feng Y., Kang L., Luo M., Yang J., 2014, Effects of light and pH on cell density of Chlorella vulgaris, International Conference on Applied Energy, 61, 2012-2015.

Kanokwan Chankhong, Wilaiwan Chotigeat, Jutarut Iewkittayakorn, 2018, Effects of culture medium on growth kinetics and fatty acid composition of Chlorella sp. T12, Songklanakarin J. Sci. Technol. 40 (5), 1098-1104.

Khoeyi Z.A., Seyfabadi J., Ramezanpour Z., 2012, Effect of light intensity and photoperiod on biomass and fatty acid composition of the microalgae Chlorella vulgaris, Aquaculture International, 20 (1), 41-49.

Los D.A., 2014, Fatty Acids Desaturases, Nauchny Mir, Moscow, Russia. (in Russian)

Ma X., Gao M., Gao Z., Wang J., Zhang M., Ma Y., Wang Q., 2018, Past, current, and future research on microalga-derived biodiesel: a critical review and bibliometric analysis, Environmental Science and Pollution Research, 25, 11, 10596-10610, DOI: 10.1007/s11356-018-1453-0

Mayo A.W., 1997, Effects of temperature and pH on the kinetic growth of unialga Chlorella vulgaris cultures containing bacteria, Water Environment Research, 69 (1), 64-72.

Rohit M.V., Venkata Mohan S., 2018, Quantum Yield and Fatty Acid Profile Variations With Nutritional Mode During Microalgae Cultivation, Front. Bioeng. Biotechnol., 25 September 2018. DOI: 10.3389/fbioe.2018.00111.

Salati L.M., Goodridge, A.G., 1996, Fatty acid synthesis in eukaryotes. Chapter in: D.E. Vance, J.E. Vance (Eds.) Biochemistry of Lipids, Lipoproteins and Membranes, New Comprehensive Biochemistry, Elsevier, Volume 31, 101-127. https://doi.org/10.1016/S0167-7306.

Seyfabadi J., Ramezanpour Z., Khoeyi Z.A., 2011, Protein, fatty acid, and pigment content of Chlorella vulgaris under different light regimes, Journal of Applied Phycology, 23 (4), 721- 726.

Takeshita T., Ota S., Yamazaki T., Hirata A., Zachleder V., Kawano S., 2014, Starch and lipid accumulation in eight strains of six Chlorella species under comparatively high light intensity and aeration culture conditions, Bioresour Technol.,158, 127–34.

Tamiya H., 1957, Mass culture of algae, Ann Rev Plant Physiol, 8, 309–334.

Tsoglin L.N., Pronina N.A., 2012, Biotechnology of Microalgae. Nauchny Mir, Moscow, Russia. (in Russian)

Villarruel-López A., Ascencio F., Nuño K., 2017, Microalgae, a potential natural functional food source – a review, Pol. J. Food Nutr. Sci., 67, 4, 251–263. DOI: 10.1515/pjfns-2017-0017.