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Optimization of the Process of Cultivation of Microalgae *Chlorella Vulgaris* Biomass with High Lipid Content for Biofuel Production

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Experimental research into the conditions of cultivation of microalgae *Chlorella vulgaris* (*IFR №C-111 strain*) with high lipid content has been conducted. Nutrient media have been selected, and a concentration of potassium nitrate, which affects the formation of intracellular non-polar lipids, has been determined. Also, the influence of cultivation temperature on the biomass growth has been studied. As a result of research, kinetic coefficients of a mathematical model which describes the biomass growth of microalgae with high content of non-polar lipids were experimentally defined.

1. Introduction

Modern science is actively looking into new ecological types of raw materials and methods of their transformation into new products and environmentally friendly energy sources. Microalgae biomass is considered to be one of the most promising sources at the moment. Although *Chlorella vulgaris* strains have been studied over the last fifty years, this research is still topical.

Halim, Danquah and Webley (2012) demonstrate that microalgae will become a primary renewable source of industrial lipids for biofuel production. Held and Raymond (2011) studied the growth of *Chlorella vulgaris* (2714 strains) and two different strains of *Microcystis aeruginosa* (LB 2238 and LB 2061) in BG11, TAP and TP media. It was found that, out of these microalgae, *Chlorella vulgaris* 2714 showed the highest productivity, and the largest growth was achieved in TAP medium (1.6 times higher as compared to TP medium and 11.4 times higher than in BG11 medium). Cultivation of *Chlorella vulgaris Buitenzorg* for the production of biomass with high lipid content as a source of biofuel was researched in Wijanarko (2011). The strain was cultivated in Benneck medium, which – with the addition of dissolved nitrogen – was found to be an optimal nutrient medium for lipids production up to 0.42 g/g of biomass (potassium nitrate being the control source of nitrogen). With urea as a source of nitrogen present, a 30 % decrease in cell growth was observed; however, conditions for protein growth were created (up to 0.54 g/g of biomass). Additionally, it was determined that the use of ammonia-containing wastewaters increases the speed of *Chlorella* growth by 55÷60 % and the amount of intracellular lipids by 8.5 %. Concas et al. (2014) suggested a mathematical model which described the impact of iron on the biomass growth and lipid accumulation. It was concluded that the increase of iron concentration within a given range (up to 100 g/m3) allowed for the increase of growth rate and lipid content of *Chlorella vulgaris*.

The objective of the current research was determination of optimal conditions for the cultivation of microalgae *Chlorella vulgaris IFR № C-111* with high triglyceride content to use as a raw material for biofuel.

2. Experimental research into the conditions of cultivation of *Chlorella vulgaris IFR № C-111* biomass

Experiments were carried out under the following conditions: 1) seed material was 20 % of total suspension volume; 2) pH=6.2÷8.0; 3) in all experiments the bubbling of suspension was done by gas-and-air mixture

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(0.04 % of CO_2 content and 80 L/h discharge) for the purpose of intensive mixing of suspension layers; 4) the photoperiod was 24 h; 5) every 4 days a source of nitrogen was added to the suspension at the same rate as for the nutrient medium. The concentration of cells was determined by direct count in Goryaev chamber. The cells were cultivated in a laboratory unit shown in Figure 1.

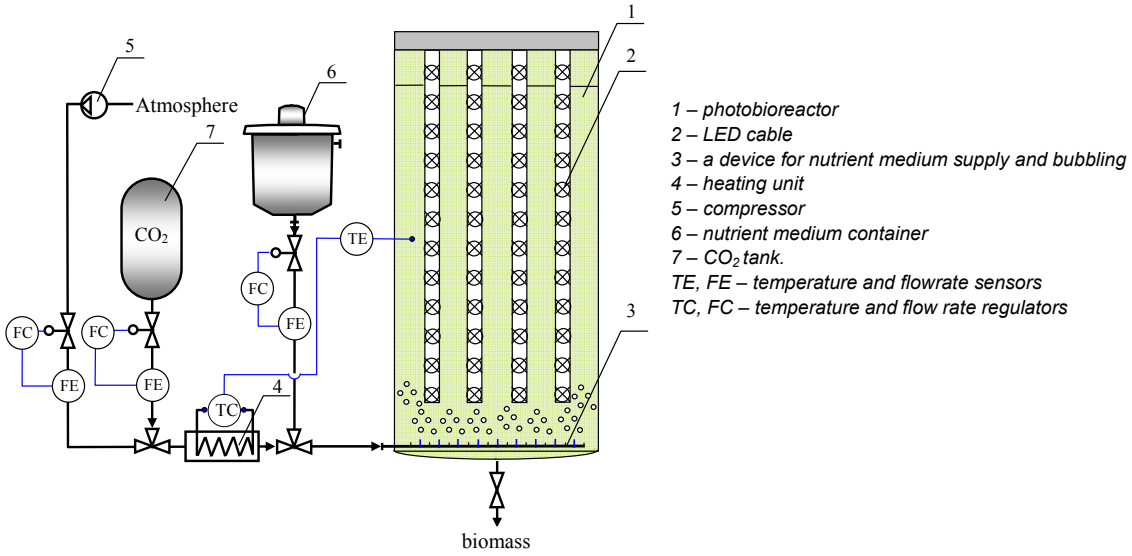


Figure 1: Basic diagram of a laboratory unit for the cultivation of microalgae biomass

The process of periodic cultivation of *Chlorella vulgaris* microalgae biomass in the technology of the 3rd generation biofuel production includes two stages: accumulation of the microalgae biomass up to the concentration of 50÷55 MCells/mL and higher; and creating stress conditions for the cells by changing a nutrient medium as to slow down the growth of microalgae and to stimulate the accumulation of lipids in cells.
In the first part of the experiment the efficiency of cultivation in Tamiya medium (Bogdanov, 2007), A5 medium (Upitis, 1983) and TAP medium (Held and Raymond, 2011) was assessed. On the 5th day of the experiment, the concentration of biomass in Tamiya medium was 14.1 MCells/mL, in TAP medium 2.1 MCells/mL, and in A5 medium 0.2 MCells/mL (see Figure 2). The graph shows that Tamiya medium is the most suitable one for the cultivation of this strain.

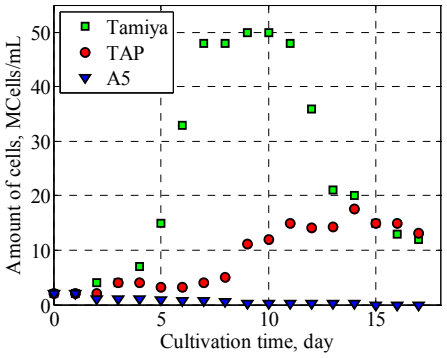


Figure 2: Dynamics of Chlorella vulgaris IFR №C-111 biomass growth in different media

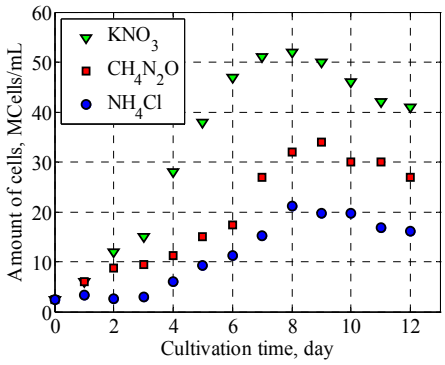


Figure 3: Dynamics of Chlorella vulgaris IFR №C-111 biomass growth in Tamiya medium

During the second stage of the experiment a source of nitrogen, an essential element of *Chlorella vulgaris* strain cultivation, was selected. Potassium nitrate (5.0 g/L), ammonium chloride (2.64 g/L), and urea (1.5 g/L) were implemented as nitrogen sources in nutrient media. Potassium nitrate yielded the best result (Figure 3): 52 MCells/mL on the 7th day of cultivation, whereas the use of urea in the nutrient medium allowed to receive 32 MCells/mL, and ammonium chloride – only 21 MCells/mL.
The third stage of the experiment involved the selection of suspension's illuminance level during the cultivation in Tamiya medium – 3 klux, 7 klux and 14 klux (the maximal level of illuminance for microalgae is 25÷30 klux).

Figure 4 shows that the level of illuminance of 14 klux is optimal as it allows to reach a steady state on the 7th day of cultivation.

The cultivation temperature for the maximal growth of the biomass was selected during the fourth stage. The microalgae were cultivated in Tamiya medium at temperatures of 27 °C, 30 °C, and 35 °C. The biomass has reached its maximal growth level (51 MCells/mL) on the 9th day of the experiment at the temperature of suspension 30 °C (Figure 5).

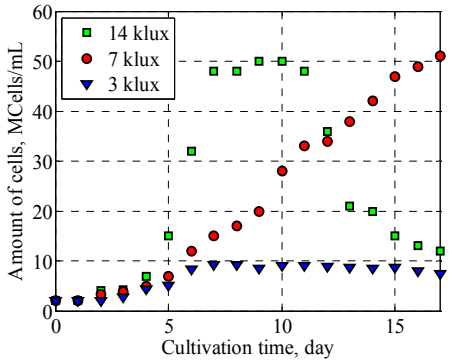


Figure 4: Dynamics of biomass growth in Tamiya medium at different levels of illuminance

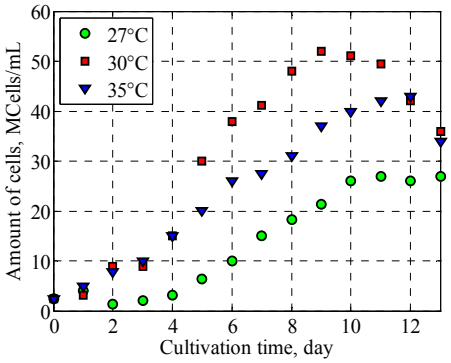


Figure 5: Dynamics of Chlorella vulgaris IFR № C-111 biomass growth at different temperatures

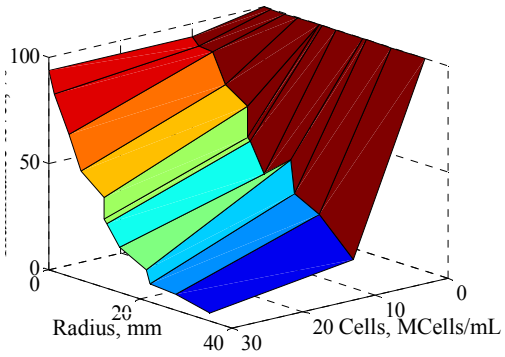


Figure 6: Dependency of illuminance level on the radius and the concentration of cells

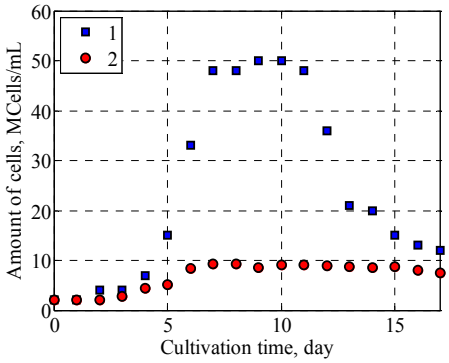


Figure 7: Chlorella vulgaris biomass increase in standard (1) and nitrogen-depleted Tamiya (2) media

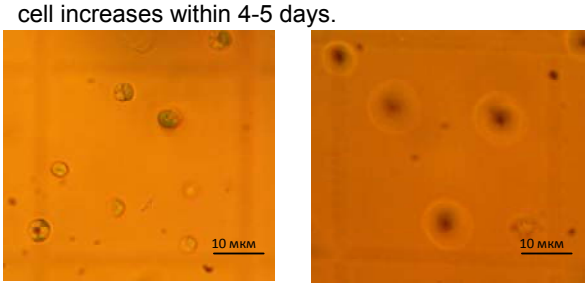
During the fifth stage of the experiment the distance between lighting elements was adjusted. The experiment showed that the level of illuminance of suspension at a depth of 30 mm constitutes only 7 % of that on the surface of the photobioreactor (Figure 6). For that reason, to optimize the regimes of exposure to light the suspension was intensively treated by bubbling, so that all cells would remain in highly illuminated areas for a sufficient period of time.

At stage six of the experiment, microalgae were grown in a standard and nitrogen-depleted Tamiya media (Figure 7). Nitrogen deficit creates stress conditions of cultivation, which stimulate accumulation of lipids in biomass cells. In the standard Tamiya medium the increment of growth was 48 MCells/mL (8 % lipid content) on the 8th day of cultivation, and in the nitrogen-depleted Tamiya medium the increment was 9 MCells/mL (32 % lipid content by Soxhlet extraction).

Additionally, a research into the influence of stress conditions of *Chlorella vulgaris* cultivation on the accumulation of intracellular neutral lipids was carried out. The following were used to create stress conditions: 1) sodium chloride (concentration 2 g/L) was added to the suspension; 2) the level of illuminance was increased to 32 klux (a level of photooxidation stress regime); 3) nitrogen content in the nutrient medium was kept at the deficit level of 50 mg/L of suspension.

All types of stress conditions slow down the growth of cells: on the seventh day of the experiment the concentration of cells decreased by 26 % as compared to the control suspension. Stress conditions also increase the size of cells (see Figure 8), protein biosynthesis is suspended, and cells begin to create nutrient storages in the form of triglycerides. At the fourth day of application of stress conditions the biomass cultivated in nitrogen-depleted medium showed the highest concentration of intracellular triglycerides (up to 36 % dry

basis of cells), which was seven times higher than in the control sample. Other types of stress influences led to only minor increases in the content of triglycerides as compared to the control sample.
Thus, the experimental results suggest that in order to reach high content of triglycerides in the biomass on the seventh or eighth day (after a steady biomass growth stage was reached) stress conditions must be created by reducing the content of nitrogen in the nutrient medium. The content of triglycerides in a microalgae cell increases within 4-5 days.



(a) (b)

Figure 8: Microscopy of Chlorella vulgaris: a – before stress, b – after stress

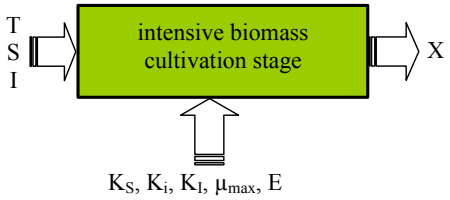


Figure 9: Flowchart of the biomass cultivation process

3. Mathematical modeling of the biomass cultivation process

The process of *Chlorella vulgaris* microalgae cultivation was considered as a system, which flowchart is presented in Figure 9.
When developing a mathematical model of the kinetics of *Chlorella vulgaris* biomass growth the following assumptions have been made: access of gas-and-air mixture bubbles to a cell is not inhibited; the concentration of oxygen is sufficient for energy metabolism; the concentration of mineral salts in all parts of the photobioreactor is the same; the level of illuminance of the biomass cells provides enough energy for photosynthesis; cells age and die-out rate were not considered in the model of lipid formation process; the cultivation process is carried out under pH=6.2÷8.0; the processes of feeding, photosynthesis, reproduction etc. are simultaneous.
The analysis of the observed dependence of the microalgae biomass accumulation (Figures 2, 4, 5, 7) proves that the curve corresponds to Verhulst's logical equation for population's limited growth (Kingsland,1995):

$$\frac{dx}{dt} = \mu \cdot x \cdot (1 - \frac{x}{E}), \quad (1)$$

x is biomass concentration, MCells/m^3, t is time, day, E is population capacity, MCells/m^3, μ is specific growth rate, day^{-1}.
The analytical solution of Verhulst's equation has the following form:

$$x(t) = \frac{x_0 \cdot E \cdot e^{\mu \cdot t}}{E - x_0 + x_0 \cdot e^{\mu \cdot t}}. \quad (2)$$

Nitrogen concentration in the nutrient medium is a limiting factor as it allows to create stress conditions for the accumulation of intracellular lipids. However, the experiments with the cultivation of *Chlorella vulgaris IFR № C-111* in the nutrient medium with high concentration of potassium nitrate have shown that the concentration of 25 g/L inhibits the biomass growth. Andrews (1968) suggested a model which takes into account the inhibition of growth by highly-concentrated substrates:

$$\mu(S) = \frac{\mu_{\max} \cdot S}{K_S \cdot S \cdot (1 + \frac{S}{K_i})}, \quad (3)$$

where $\mu(S)$ is a population's specific growth rate, day^{-1}, S is a concentration of a limiting substrate, kg/m^3, $\mu_{\max}$ is a maximal specific growth rate, day^{-1}, K_S is a saturation constant for a particular substrate, kg/m^3, and K_i is an inhibition constant, kg/m^3.

Besides, the specific growth rate of the biomass will depend on the temperature and the level of illuminance. To compute the specific growth rate in a multi-factor process a universal multiplicative dependence, each factor of which is autonomous, was used (Biryukov, 2004):

$$\mu = 0{,}5 \cdot (\mu(I) + \mu(S)), \tag{4}$$

where S is a concentration of substrate components in the bioreactor, kg/m^3 and *I* is the level of illuminance, lux.

The influence of the illuminance level on the dynamics of microalgae growth was described with the help of Michaelis-Menten equation:

$$\mu = \frac{\mu_{\max} \cdot I}{K_I + I}, \tag{5}$$

where K_I is a saturation constant of illuminance, lux.

Temperature mostly affects the maximal specific growth rate $\mu_{\max}$, whereas the values K_S and K_i are less influenced by it (Biryukov, 2004). Thus, the dependence of maximal specific growth rate on temperature can be formulated as:

$$\mu_{\max} = \mu_0 + \mu_1 \cdot T + \mu_2 \cdot T^2, \tag{6}$$

where $\mu_{\max}$ is a maximal specific growth rate, day^{-1}, $\mu_0, \mu_1, \mu_2, \mu_3$ are coefficients of the regression model obtained through experimental data processing (Figure 5).

The specific growth rate of *Chlorella vulgaris IFR № C-111* biomass can be found using the following formula:

$$\mu = (\mu_0 + \mu_1 \cdot T + \mu_2 \cdot T^2) \cdot 0.5 \cdot \left[\left(\frac{I}{K_I + I} \right) + \left(\frac{S_{KNO_3}}{K_S + S_{KNO_3} + S_{KNO_3}^2 / K_i} \right) \right]. \tag{7}$$

Analysis of the experimental data allowed to compute the following kinetic coefficients of the equation: μ_0 = -10.029, μ_1 =0.67, μ_2 = -0.01, $\mu_{\max}$ = 0.533 day^{-1}, K_S = 1.076 g/L, K_i = 30.3 g/L, K_I =2 klux, I =7 klux.

Thus, the equation which describes the biomass growth at illuminance level of 7 klux, *T*=30 °C, S_{KNO_3} =5 g/L is written as following:

$$\frac{dx}{dt} = 0.38 \cdot x \cdot (1 - \frac{x}{E}). \tag{8}$$

Table 1: Chlorella vulgaris IFR № C-111 biomass increase in standard and optimized Tamiya media

Tamiya	*Tamiya Optimum*
KNO_3 - 5.0 g/L, $MgSO_4 \cdot 7H_2O$ - 2.5 g/L, KH_2PO_4 - 1.25 g/L, $FeSO_4 \cdot 7H_2O$ - 0.003 g/L, EDTA - 0.044 g/L.	KNO_3 - 3.2 g/L, $MgSO_4 \cdot 7H_2O$ - 0.125 g/L, KH_2PO_4 - 0.25 g/L, $FeSO_4 \cdot 7H_2O$ - 0.003 g/L, EDTA - 0.044 g/L.
Arnon solution of microelements (1 mL per 1 L of nutrient medium):	*Solution of microelements (1 mL per 1 L of nutrient medium):*
H_3BO_3 - 2.86 g/L, $MnCl_2 \cdot 4H_2O$ - 1.81 g/L, $ZnSO_4 \cdot 7H_2O$ - 0.222 g/L, MoO_3 - 176.4 mg/10 L, NH_4VO_3 - 229.6 mg/ 10 L.	H_3BO_3 - 2.86 g/L, $MnCl_2 \cdot 4H_2O$ - 0.8 g/L, $CuSO_4 \cdot 7H_2O$ - 0.8 g/L, $ZnSO_4 \cdot 7H_2O$ - 0.1 g/L, MoO_3 - 176.4 mg/10 L, NH_4VO_3 - 229.6 mg/ 10 L.

The diagrams of biomass increase obtained as a result of modeling and of experimental research are presented in Figure 10. The maximal mismatch of data was 17 %. The developed mathematical model was used to determine the optimal nutrient medium for the cultivation of a maximal amount of biomass (Table 1). The data on the microalgae growth in the standard and the optimized Tamiya media are shown in Figure 11.

Having analyzed these data, it can be concluded that over 14 days the biomass increase in the optimized Tamiya medium amounted to 19 %.

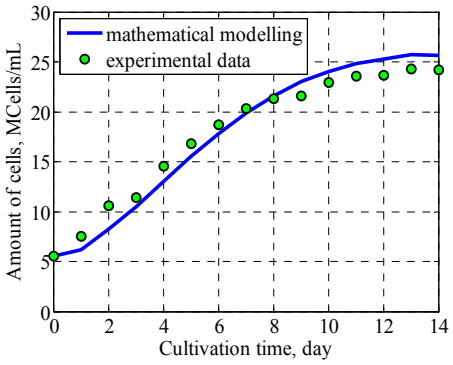


Figure 10: Biomass increase

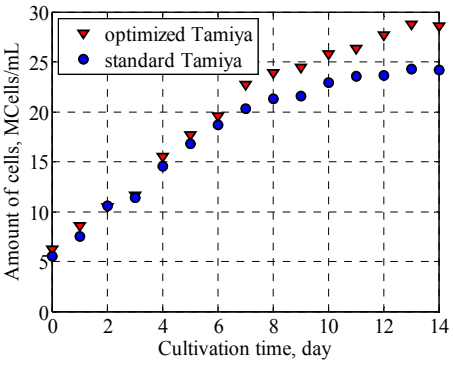


Figure 11: Chlorella vulgaris IFR № C-111 biomass increase in standard and optimized Tamiya media

4. Conclusions

The conducted research allowed to conclude that *Tamiya Optimum* medium is the most suitable one for the cultivation of *Chlorella vulgaris IFR № C-111* strain. The maximal increase in the biomass was observed when potassium nitrate was used as a source of nitrogen, the level of illuminance was 14 klux, and the temperature of suspension was 30 °C. Creation of stress conditions on the 7th-9th day of the experiment by decreasing the concentration of nitrogen in the nutrient medium resulted in the maximal accumulation of triglycerides in the biomass. A mathematical model of *Chlorella vulgaris IFR № C-111* biomass growth was developed.

Acknowledgements

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