

# Technology of Using Municipal Wastewater for Obtaining *Chlorella Vulgaris* Biomass with High Lipid Content for Biofuel Production

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An approach based on the integration of technologies that transform human activity wastes into feedstock and energy sources may help address the issues of reducing the burden on the environment and improving the economic efficiency of renewable energy production. The possibility of using municipal wastewater for cultivation of *Chlorella vulgaris* microalgae was investigated. It has been established that for 7-10 days of cultivation the concentration of ammonium cations and phosphate anions is reduced to the maximum permissible level, and the maximum concentration of intracellular lipids of microalgae was  $18 \pm 2$  % (wt.). With the use of the Verhulst and Monod equations, a mathematical model of the process of microalgae cultivation in wastewater has been developed, which describes the kinetics of accumulation of microalgae cells, the loss of nitrogen and phosphorus-containing substrates, with an error not exceeding 15 %.

## 1. Introduction

The need to protect the environment urges humanity to make effective use of all material resources at their disposal. One of the approaches to reducing the burden on the environment and improving the economic efficiency of renewable energy production is based on the integration of technologies that enable the processing of increasing volumes of human waste, and transforming them either into useful feedstock or energy sources. A promising area for obtaining renewable energy sources is the production of third generation biofuel using municipal wastewater for the cultivation of microalgae. The use of sewage as a nutrient medium will reduce the cost of biofuels, increase the profitability of production, while ensuring a reduction in the level of chemical pollution and pathogenic microflora in wastewater.

Integration of the infrastructure of treatment plants with the production of third generation biofuels will also allow to optimize the costs of facilities construction, communications, heating, electricity, etc. However, despite the attractiveness of the integration of wastewater treatment technologies and biofuel production, it is not implemented yet in existing plants. This, apparently, is due to insufficient economic efficiency of individual stages of biofuel production.

Based on the analysis of studies in Table 1, it can be concluded that different strains of microalgae treat wastewater at different speed and with various degrees of efficiency: the time of cultivation of microalgae, depending on the type of strain, ranged from 7 to 22 days at a nitrogen consumption rate of 0.5-4.8 mg/day, phosphorus 0.2-1.2 mg/day, the temperature range was 18-30 °C, and the illumination level was 55-174  $\mu\text{mol photons}/(\text{m}^2 \cdot \text{s})$ . The concentration of microalgae accumulated in the lipid cells varied from 7.7 to 32.2 % (wt.). Thus, different strains of microalgae with different degrees of efficiency purify municipal wastewater from cities located at different geographical latitudes, and therefore, studies to assess the effectiveness of strains for wastewater treatment and their ability to accumulate lipids are relevant.

The purpose of this work was to study the conditions of cultivation of microalgae *Chlorella vulgaris* (*C. vulgaris*) in municipal wastewater, which allow obtaining biomass with high lipid content for biofuel production.

To achieve this goal, the following tasks were set and solved:

- 1) experimental studies on the cultivation of strains *C. vulgaris* IFR C-111 and *C. vulgaris* Beijer IPPAS C-2 in municipal wastewater were conducted, allowing to reduce the concentrations of ammonium and phosphate anions and pathogenic microflora in wastewater to values below the threshold limit (as per Russian Sanitary Regulations and Norms Document 2.1.5.980-00);
- 2) lipids extracted from the microalgae cells were analysed quantitatively and qualitatively;
- 3) a mathematical model for the cultivation of microalgae in wastewater was developed, which included equations of the kinetics of microalgae cells accumulation and nitrogen- and phosphorus-containing substrates loss.

Table 1: Studies on the treatment of wastewater with microalgae

Author	Strain	Time, days	Temperature, °C	Changes of nitrogen concentration, mg/L			Changes of phosphorus concentration, mg/L			Lipids, % (wt.)
				initial	final	Change rate, mg/(L · day)	initial	final	Change rate, mg/(L · day)	
Rinna et al. (2017)	<i>Botryococcus braunii</i> UTEX-USA	10	25 ± 1	24.9	9.2	-1.6	10.2 (PO <sub>4</sub> <sup>3-</sup> )	≈0	-1.0	20.0
	<i>Botryococcus braunii</i> IBL-Brazil			24.9	9.7	-1.5	10.2 (PO <sub>4</sub> <sup>3-</sup> )	≈0	-1.0	14.0
Komolafe et al. (2014)	<i>Oscillatoria Arthrospira</i>	11			13	-1.5		24.8	-1.0	26.3
	<i>Desmodesmus</i> sp.	16			5.8	-1.1		21.7	-0.9	13.0
	<i>Desmodesmus</i> sp., <i>Navicula</i> , <i>Chlorella</i> , <i>Oscillatoria</i> , <i>Chlamydomonas</i>	22	-	29.1 (NH <sub>4</sub> <sup>+</sup> )	4.7	-1.1	35.4 (TP)	14.2	-1.0	14.3
Gupta et al. (2016)	<i>Chlorella vulgaris</i> (FC-16)	12	25 ± 2	40.0 (TN)	33.7	-0.5	9.2 (TP)	7.1	-0.2	7.7
Samorì et al. (2014)	<i>Desmodesmus communis</i>	7	18-25	32.4 (NH <sub>4</sub> <sup>+</sup> )	0.3 - 17.1	-4.6 - (-2.2)	2.4 (PO <sub>4</sub> <sup>3-</sup> )	≈0	-0.3	11.7 - 21.3
Nayak et al. (2016)	<i>Scenedesmus</i> sp. (+ CO <sub>2</sub> )	7	25 ± 2	38.6 (NH <sub>4</sub> <sup>+</sup> )	5.3	-4.8	9.2 (PO <sub>4</sub> <sup>3-</sup> )	1.0	-1.2	23.1
Zhang et al. (2014)	<i>Scenedesmus</i> sp. ZTY1	21	25	41.0 (TN)	2.9	-1.8	8.4 (TP)	0.1	-0.4	32.2
Sacristán de Alva et al. (2013)	<i>Scenedesmus acutus</i>	21	27 ± 3	49.4 (OrN+ +NH <sub>4</sub> <sup>+</sup> )	3.2	-2.2	9.5 (PO <sub>4</sub> <sup>3-</sup> )	3.2	-0.3	28.3

## 2. Materials and methods

**2.1** Samples of municipal wastewater taken from an urban wastewater treatment plant with a capacity of ≈80,000 m<sup>3</sup>/day were used. Initial content of ammonium cations was 35-50 mg/L and phosphate anions - 15-38 mg/L. For the purification of wastewater, the following strains of microalgae were used: *C. vulgaris* IFR C-111 and *C. vulgaris* Beijer IPPAS C-2, which are mesophiles and are capable of floating in the water.

**2.2** Determination of nitrogen was carried out using Russian Federal Standard GOST 33045-2014 "Water. Methods for determination of nitrogen-containing substances". The amount of phosphorus was determined according to Federal Environmental Regulations 14.1;4.248-07 "Quantitative chemical analysis of waters. Technique for the measurement of mass concentrations of orthophosphates, polyphosphates and phosphorus".

**2.3** Sanitary-bacteriological study of wastewater. Wastewater samples of 0.1 mL volume are plated to Petri dishes containing the MPA and Endo culture medium for the detection of coliform bacteria. The cultures were placed in a thermostat and kept at 37 °C for 24 hours. The number of grown colonies (total bacterial count -

TBC) was calculated by the formula  $M = a \times 10^n / V$  ( $M$  is the number of cells in 1 mL,  $a$  is the average number of colonies,  $V$  is the volume of the suspension, in mL, and  $10^n$  is the dilution).

**2.4** The treatment of municipal wastewater was carried out in a photobioreactor with a volume of 2 L for the sample - "autumn" (temperature 22 °C, illumination 30  $\mu$ moles of photons / ( $m^2$  s)), for the sample - "summer" (temperature 32 °C, illumination 350  $\mu$ moles of photons / ( $m^2$  s)); The time of accumulation cultivation was 3-4 days, stress cultivation in the samples (with the concentration of ammonium cations less than 22.5 mg/L) began on the 3rd-4th day.

**2.5** Separation of microalgae biomass from wastewater was carried out using a centrifuge with a rotation speed of 3000  $min^{-1}$  for 5 minutes. The destruction of the cell walls was carried out by successive treatment with a mixture of enzymes (Cellulux A-Protosubtilin g3x taken at a ratio of 12 mg/mL to 4 mg/mL, exposure for 10 minutes at 55 °C) and microwave radiation (280-400 W, 2450 MHz, processing time 30-40 s).

**2.6** Lipids were extracted from the biomass of microalgae with disrupted cell walls with a mixture of solvents of ethanol and petroleum ether taken in the ratio 1:2 (vol.), the ratio of the microalgae biomass (g) to the volume of the solvent mixture (mL) was 1:20, extraction temperature of 50-55 °C. Estimation of lipids extracted from biomass was done by Zoellner and Kirsch method of determination of total lipids (Zoellner and Kirsch 1962).

**2.7** The qualitative composition of the lipid fraction was determined by thin-layer chromatography. Analysis of the fatty acid composition of non-polar lipids extracted from the microalgae cells was carried out using a gas chromatograph "Crystallux-4000M".

### 3. Results and discussion

#### 3.1 Experimental studies into the process of cultivating microalgae in municipal wastewater

A comparative analysis of the kinetics of biomass accumulation shows that the maximum growth of microalgae cells for both strains (Figure 1) occurred in the "summer" period and amounted to 100 % over for days of cultivation. In the "autumn" period, the maximum cell growth was 250 % over eight days of cultivation. Thus, the temperature of cultivation and the initial concentration of microalgae are optimizable variables when solving the problem of optimization of the biomass accumulation process.

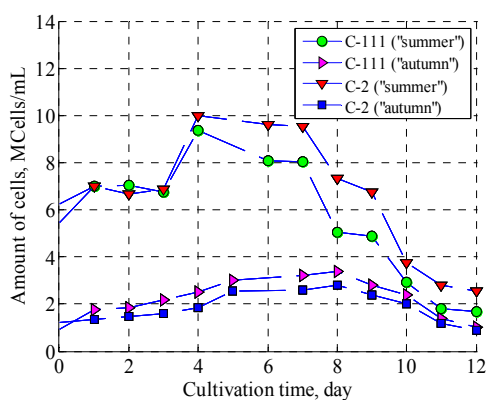


Figure 1: Kinetics of microalgae biomass accumulation.

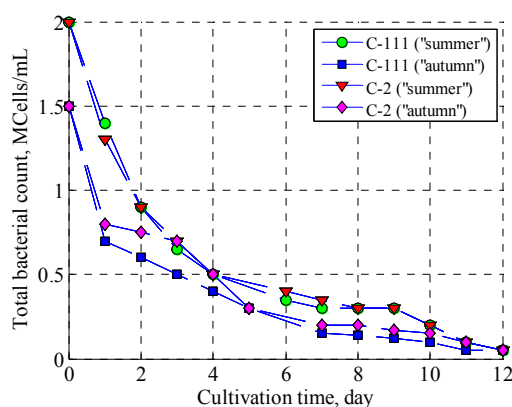


Figure 2: Kinetics of decrease in extraneous microflora in wastewater.

The capacity of the population of strains compared to the process of microalgae cultivation in model media (Dvoretzky et al. 2015) decreased by 80-85 % on average, which can be explained by the presence of coliform bacteria and pathogenic microflora in municipal wastewaters. At the same time, the concentration of the accompanying microflora (Figure 2) was constantly decreasing during the cultivation and by the 11<sup>th</sup>-12<sup>th</sup> day reached the values of  $\approx 0.15$ - $0.35$  MCells/mL during the "summer" period and 0.1-0.2 MCells/mL in the "autumn" season. This is most likely due to the fact that *C. vulgaris* microalgae secreted substances with an antibiotic effect into the culture fluid (Amaro et al. 2011), which inhibit bacterial activity and allow to reduce the total bacterial count to 10 cfu/mL during the "summer" period and up to 5 cfu/mL in the "autumn" period, which is 75 % and 88 % lower compared to the wastewater treated with activated sludge, respectively.

For 10 days of cultivation in municipal wastewater (Figure 3) during the "summer" period, the concentration of ammonium cations decreased by 92 % (wt.) and 93.5 % (wt.) for *C. vulgaris* Beijer IPPAS C-2 and *C. vulgaris* IFR C-111. In the "autumn" period, during 10 days of cultivation, the concentration of ammonium ions decreased by 88 % (wt.) and 89.5 % (wt.) for *C. vulgaris* Beijer IPPAS C-2 and *C. vulgaris* IFR C-111 strains.

The concentration of ammonium cations during cultivation in the "autumn" period (Figure 3) increases significantly during the first day of cultivation, which is explained by the presence in the water of bacteria decomposing organic substances to ammonium cations. And in the "autumn" period due to a lower temperature (22 °C) this process is slower, and this explains the surge in the concentration of ammonium cations. In the "summer" period (at 32 °C), the process goes much faster, so the organic decomposes much faster and this surge is not observed. The concentration of phosphate anions (Figure 4) is reduced by 75 % and by 88 % when cultivated for 10 days in wastewater during the "summer" period and by 96.8 % and by 96.1 % during the "fall" period for *C. vulgaris Beijer* IPPAS C-2 and *C. vulgaris* IFR C-111, respectively.

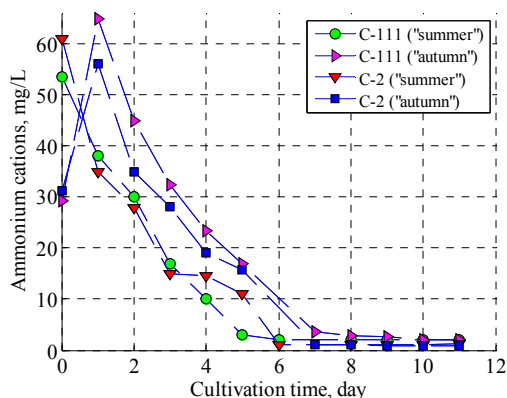


Figure 3: Kinetics of ammonium cations loss.

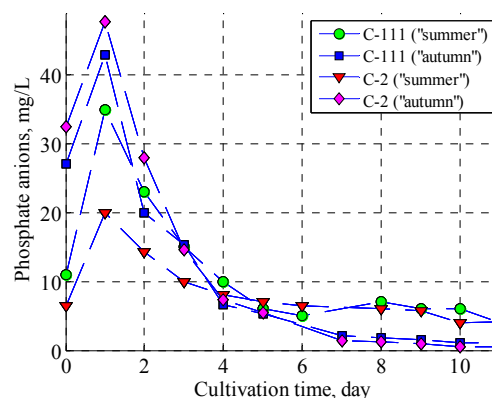


Figure 4: Kinetics of phosphate anions loss.

Comparative characteristics of the conducted experiments are presented in Table 2. Due to a more intensive flow of biochemical processes at a higher temperature, the rate of nitrogen loss in summer time for both strains is 1.9 - 2.1 times higher than in "autumn" and is 4-5 mg/day. The rate of phosphate anion loss is higher in "autumn" time for both strains and is 2.1-2.6 mg/L, and in "summer" time it is 3.5-8.7 times lower, which agrees with the data (Upitis 1983). The time of accumulative cultivation for "summer" samples was 3 days, for "autumn" samples - 4 days. Stress cultivation (i.e. with the content of ammonium cations in the effluent below 22.5 mg/L) continued until reaching a level below the threshold limit (for ammonium cations - 1.5 mg/L, for phosphate anions - 3.5 mg/L) and was 3 days ("autumn" samples) and 7 days ("summer" samples) for *C. vulgaris Beijer* IPPAS C-2, and 6 days ("autumn" samples) and 7 days ("summer" samples) for *C. vulgaris* IFR C-111.

Table 2: Results of the studies on microbial wastewater treatment

Strain		NH <sub>4</sub> <sup>+</sup>				PO <sub>4</sub> <sup>3-</sup>				Lipids, % (wt.)
		Concentration, mg/L		Consumption rate, mg/day	Time till threshold limit (1.5 mg/L), days	Concentration, mg/L		Consumption rate, mg/day	Time till threshold limit (3.5 mg/L), days	
		Initial	Final			Initial	Final			
C-111	"autumn"	29	2	-2.3	10	27	2	-2.1	6	12± 3
	"summer"	53	1.5	-4.3	10	11	4	-0.6	10	14 ± 2
C-2	"autumn"	31	1.5	-2.4	7	32	1	-2.6	6	17 ± 3
	"summer"	61	1	-5.0	7	7	4	-0.3	10	18 ± 2

The maximum concentration of intracellular lipids was reached in the "summer" period for the *C. vulgaris Beijer* IPPAS C-2 strain and was 18±2 % (wt.), and for *C. vulgaris* IFR C-111 - 14±2 % (wt.) after 7 days of stress cultivation. During the "autumn" period, the concentration of intracellular lipids decreased by 6 % (wt.) for *C. vulgaris Beijer* IPPAS C-2 and by 15 % (wt.) for *C. vulgaris* IFR C-111.

Analysis of the composition of the extracted lipid fractions of both strains showed that they contain sterols, coenzyme Q, triacylglycerides, vitamin K, methyl esters of fatty acids, and squalene. The fatty acid composition of triacylglycerides extracted from biomass include the following acids: palmitic (C16: 0) - 11.8 % (wt.), stearic (C18: 0) -17.9 % (wt.), oleic (C18: 1) - 12.5 % (wt.), linoleic (C18: 2) - 5.8 % (wt.), behenic (C22: 0) - 5.3 % (wt.), and erucic (C 22: 1) - 4.2 % (wt.).

### 3.2 Mathematical modelling of the process of cultivating microalgae in wastewater

Analysis of the experimental dependence (Figure 1) of microalgae biomass accumulation showed that the curve character corresponds to the Verhulst logistic equation for a limited growth of population (Kingsland 1995):

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

where  $E_n$  – population capacity, MCells/mL; and  $\mu$  is specific growth rate, days<sup>-1</sup>.

It was found that the concentration of nitrogen and phosphorus in the nutrient medium are limiting factors, since these biogenic elements have a significant effect on the growth of microalgae cells. In addition, varying the concentrations of these elements allows to create the stressful conditions necessary for the accumulation of intracellular lipids.

Based on the analysis of the experimental curves, it can be concluded that the dependence of the specific growth rate of microalgae on the concentration of nitrogen- and phosphorus-containing substrate is described by the Monod equation (Biryukov 2004):

$$\mu(S) = \frac{\mu_{\max} \cdot S}{K_S + S}, \quad (2)$$

where  $\mu(S)$  – population's specific growth rate, days<sup>-1</sup>,  $S$  – concentration of a limiting substrate, mg/L,  $\mu_{\max}$  – maximum specific growth rate, days<sup>-1</sup>,  $K_S$  – a constant of semi-saturation for a given substrate, g/L.

Since the specific growth rate depends on temperature, concentration of ammonium cations and phosphate anions in wastewater, a universal multiplicative dependence (Biryukov 2004) was used to calculate the specific growth rate in a multifactor process, in which each factor is autonomous:

$$\mu = 0,5 \cdot (\mu(S_N) + \mu(S_P)), \quad (3)$$

where  $S_N$  is concentration of nitrogen-containing component in wastewater, mg/L,  $S_P$  is concentration of phosphorus-containing component in wastewater, mg/L.

From equations (1) - (3), the specific growth rate of the microalgae biomass in wastewater can be determined by the following formula:

$$\frac{dx}{dt} = \mu_{\max} \cdot \left( \frac{\frac{S_N}{K_{SN} + S_N} + \frac{S_P}{K_{SP} + S_P}}{2} \right) \cdot x \cdot \left(1 - \frac{x}{E_n}\right). \quad (4)$$

The processes of nitrogen- and phosphorus-containing substrates' loss during the periodic cultivation of microalgae are described by equations:

$$\frac{dS_N}{dt} = -\frac{1}{Y_N(t)} \cdot \frac{dx}{dt}, \quad \frac{dS_P}{dt} = -\frac{1}{Y_P(t)} \cdot \frac{dx}{dt}, \quad (5)$$

where  $Y$ , MCells-L/(mL·g) is a coefficient, referring to the amount of accumulated microalgae biomass  $\Delta x$  for the substrate  $\Delta S$  used over the time period  $\Delta t$ , calculated experimentally.

The processing of the experimental data made it possible to calculate the kinetic coefficients of the equation for strains of *C. vulgaris* Beijer IPPAS C-2 and *C. vulgaris* IFR C-111, which are presented in Table 4.

Table 4: Kinetic coefficients of the model's equations

	<i>C. vulgaris</i> Beijer IPPAS C-2		<i>C. vulgaris</i> IFR C-111	
	"summer"	"autumn"	"summer"	"autumn"
$E_n$ , MCells/mL	9.9	9	3.4	3.5
$K_{SN}$ , mg/L	3.5	12.5	2.5	2.5
$K_{SP}$ , mg/L	2	0.5	0.1	0.1
$\mu_{\max}$ , days <sup>-1</sup>	0.45	0.35	0.6	0.3
$Y_N(t)$ , MCells-L/(mL·g)	$0.1 \cdot t^2 - 1.3 \cdot t + 6.2$	0.13	0.1	0.13
$Y_P(t)$ , MCells-L/(mL·g)	$0.1 \cdot t^2 - 0.5 \cdot t + 1.8$	0.8	0.1	$0.1 \cdot t^2 - 1.3 \cdot t + 3.6$

To assess the adequacy of the model, a function  $\delta_i^j(t) = \max\left(\left|\frac{\xi^e(t) - \xi^m(t)}{\xi^e(t)}\right|\right) \cdot 100\%$  was used, where  $\xi^e = (x^e, S_N^e, S_P^e)$ ,  $\xi^m = (x^m, S_N^m, S_P^m)$  are the vectors of the experimental and calculated values of the unknown variables of the model for the  $i$ -th microalgae strain and the  $j$ -th time of the year. Checks for the model's adequacy yielded the following results:  $\delta_{C-2}^{summer} = 14.4\%$ ,  $\delta_{C-111}^{summer} = 13.9\%$ ,  $\delta_{C-2}^{autumn} = 14.5\%$ ,  $\delta_{C-111}^{autumn} = 15.3\%$ .

#### 4. Conclusions

Based on the results of the experimental studies, it can be concluded that municipal wastewater can be used as a nutrient medium for the cultivation of microalgae of *C. vulgaris* IFR C-111 and *C. vulgaris* Beijer IPPAS C-2 strains without preliminary treatment. During 7-10 days of cultivation the concentration of ammonium cations and phosphate anions decreases to the threshold limit level. The maximum concentration of intracellular lipids was achieved for the *C. vulgaris* Beijer IPPAS C-2 strain and was  $18 \pm 2\%$  (wt.) with a cell population of  $\approx 10$  MCells/mL. The lowest decrease in the concentration of intracellular lipids (by 6% (wt.)) was observed for *C. vulgaris* Beijer IPPAS C-2 in the "autumn" period. Based on the experimental data obtained using the Verhulst and Monod equations, a mathematical model of the process of microalgae cultivation in wastewater is developed, which describes the kinetics of microalgae cells accumulation, and the loss of nitrogen- and phosphorus-containing substrates.

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