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Technology of Wastewater Use For L-Lactic Acid Biosynthesis

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The increase in plastic wastes having a long term of natural decomposition has a serious impact on the deteriorating environmental situation and is an issue of global concern. One way to solve this problem is making more use of biodegradable polymers. However, their widespread availability is hampered by the high cost of raw materials, one of which is lactic acid. A promising source of stimulants and nitrogen, which reduces the cost of lactic acid production, is the filtrate of the culture fluid after the purification of municipal wastewater with microalgae *Chlorella vulgaris*. It has been experimentally established that using the culture fluid filtrate after municipal wastewater treatment with microalgae as a source of nitrogen and stimulating substances it is possible to increase the concentrations of: cells of lactobacilli bacteria of strains *Bacillus coagulans* and *Lactobacillus casei* 1.5-1.9 times on average; lactic acid in the culture fluid by 15-30 % (wt.) compared to the control samples. On the basis of the experimental data obtained using the equations of Verhulst and Monod-Yerusalimsky, a mathematical model of the process of culturing lactic acid bacteria has been identified, describing the kinetics of growth of lactic acid bacteria, the loss of the carbon-containing substrate and the accumulation of lactic acid.

1. Introduction

The rapid growth of human consumption is the main cause of increasing environmental pollution. The increase of plastic waste with a long term of natural decomposition seriously impacts the deteriorating ecological situation, especially in developing countries. At the same time, widespread use of biodegradable polymers is constrained by their high cost. Therefore, the search for solutions aimed at increasing the efficiency of technologies for the production of biodegradable polymers and reducing their cost is extremely urgent. One of the promising sources of raw materials for the production of biodegradable polymers is L-lactic acid, the demand for which grows annually.

Based on the analysis of studies in Table 1, it can be concluded that various sources of nitrogen and carbon provide for different rates of lactic acid accumulation: product output was in the range of 72 to 99 % (wt.) for the consumption rate of carbon-containing substrate of 1.1-3.3 g/(L·h), maximum concentration of lactic acid in the culture fluid (117 g/L) was obtained with the use of *Bacillus coagulans (B. coagulans)* LA-15-2 in the nutrient medium with white rice bran (carbon source) and yeast extract (nitrogen source). Thus, different strains of lactic acid bacteria vary in the efficiency of carbon and nitrogen consumption from nutrient media, and studies into the efficiency of nutrient media composition and selection of optimal sources of nitrogen and carbon for the cultivation of lactic acid bacteria are relevant. Moreover, in order to obtain nitrogen sources and stimulating substances, a time-consuming and energy-intensive extraction process is required. As such, the filtrate remaining after the cultivation of microalgae (Dvoretsky et al. 2017) on municipal wastewater, which contains ammonium salts, amino acids, B vitamins (Bogdanov 2007), cobalt, copper, manganese, molybdenum, iron, zinc, iodine, indole-3-acetic acid (Nazarenko et al. 1994), can be utilised .

The possibility of integrating the production of lactic acid with technologies for wastewater treatment and biofuel production will reduce the cost of lactic acid as a result of saving costs for water, energy and nutrient media components. The aim of the study was to determine the conditions for the accumulation of L-lactic acid,

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using nutrient media based on molasses and municipal wastewater purified by microalgae *Chlorella vulgaris (C. vulgaris)*. For this purpose the following tasks have been solved:
1) lactic acid biosynthesis using filtrate after the cultivation of microalgae in wastewater for the strains *B. coagulans* B-10468 and *Lactobacillus casei (L. casei)* B-3241 has been researched;
2) a comparative analysis of the biosynthesis of lactic acid on nutrient media based on microalgae-treated wastewater and wastewater after traditional biological treatment with activated sludge has been carried out;
3) a mathematical model describing the growth of the biomass of lactic acid bacteria, kinetics of lactic acid accumulation, and consumption of the substrate has been developed, and model parameters were experimentally determined.

Table 1: Literature review on lactic acid biosynthesis

Study	Strain	Nutrient medium composition		Max concentration of lactic acid, g/L	Substrate consumption rate, g/(L·h)	Average productivity, g/(L·h)	Product output, % (wt.)
		Carbon-containing source	Nitrogen containing source				
Van der Pol et al. (2016)	*Bacillus coagulans* DSM 2314	sugar cane lignocellulose	yeast extract	64	-	0.78	80
Zhou et al. (2016)	*Bacillus coagulans* CC17	glucose, sulphite pulp	yeast extract, NH_4Cl	110	1.6	0.79	72
Wang et al. (2015)	*Bacillus coagulans* LA-15-2	white rice bran	yeast extract	117	1.5	2.79	99
Wang et al. (2017)	*Bacillus coagulans* LA1507	sorghum	corn-steep	102	-	2.90	94
Xu and Xu (2014)	*Bacillus coagulans* H-1	molasses, glucose	corn-steep	57	1.5	1.40	97
Pleissner et al. (2016)	*Bacillus coagulans*	coffee bean hydrolyzate	yeast extract	32	1.1	3.57	95
Kunasundari et al. (2017)	*Bacillus coagulans* strain 191	palm tree juice	yeast extract, $[CO(NH_2)_2]$	75	1.1	2.64	92
Ma et al. (2016)	*Bacillus coagulans* NBRC 12714	cornstalk	yeast extract; $(NH_4)_2SO_4$	92	3.3	3.00	91
Zheng et al. (2014)	*Bacillus coagulans* NL-CC-17	xylose, powdered corn-steep liquor	yeast extract, tryptone	80	1.3	1.19	83

2. Materials and methods

2.1 Beetroot molasses was used as a carbon source. Its initial glucose content was ≈140 g/L. As a source of nitrogen and stimulating substances, the filtrate which formed after the purification of samples of municipal wastewater (with an initial content of 1.5 mg/L of ammonium cations and ≈ 3.5 mg/L of phosphate anions) by microalgae *C. vulgaris* IFR C-111 and *C. vulgaris Beijer* IPPAS C-2 was used.
2.2 Strains of *B. coagulans* B-10468 and *L. casei* B-3241 were used to produce lactic acid. To maintain the vital activity of the strains, a nutrient medium MRS was implemented: bacto-peptone - 10.0 g/L; meat extract - 10.0 g/L; yeast extract - 5.0 g/L; glucose - 20.0 g/L; Tween-80 - 1.0 g/L; ammonium citrate - 2.0 g/L; sodium acetate - 5.0 g/L; $MgSO_4 \cdot 7H_2O$ - 0.1 g/L; Na_2HPO_4 - 2.0 g/L; Agar - 20.0 g/L, pH 6.5.
2.3 Biosynthesis was carried out in an orbital shaker-incubator Biosen ES-20/60 (frequency 70 min^{-1}) under the following conditions: temperature T = 37 °C, initial pH 7.0, volume of culture medium 350 mL, seed culture quantity 10 % (vol.) from the culture medium, the seed culture titer was 700-900 million cells/mL, time 100 hours. The concentration of lactic acid and glucose was determined with the Biosen C-Line Clinic / Gp + bioanalyzer.

2.4 Microalgae biomass was separated from culture fluid in a centrifuge Sigma 2-16 PK/2-16P, rotation speed 3000 min^{-1} in 5 minutes. The amount of cells in the medium was determined by direct count in the Goryaev chamber.

2.5 *Experiment 1.* As a source of stimulants and nitrogen the following were used: malt extract – control sample; filtrates of the culture fluid after the cultivation of the microalgae *C. vulgaris* IFR C-111 and *C. vulgaris Beijer* IPPAS C-2 using municipal wastewater. The seed culture was a suspension of cells from a pure culture of strains of *B. coagulans* B-10468 and *L. casei* B-3241 obtained by flushing bacterial cells from a slanted agar medium MRS with sterile water. Table 2 presents variants of nutrient media that have been used for the biosynthesis of lactic acid. *Experiment 2*. The following have been used as sources of nitrogen and stimulating substances for the cultivation of *B. coagulans* and *L. casei* strains: municipal wastewater after biological treatment with activated sludge, and culture fluid filtrates after the cultivation of *C. vulgaris* IFR C-111 and *C. vulgaris Beijer* IPPAS C-2 in municipal wastewater.

2.6 Sanitary-bacteriological study of culture fluid. Wastewater samples of 0.1 mL volume were 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 $M = a \cdot 10^n / V$ (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).

Table 2: Composition of nutrient media for lactic acid biosynthesis

Strain	Source of nitrogen and stimulants	Glucose content in nutrient medium, g/L	pH
B. coagulans	malt extract, 25 % (vol.)	140	6.8
L. casei			7.0
B. coagulans	filtrate after the cultivation of *Chlorella vulgaris* IFR C-111, 100 % (vol.)		6.8
L. casei			6.9
B. coagulans	filtrate after the cultivation of *Beijer IPPAS C-2,* 100 % (vol.)		6.9
L. casei			6.9

2. Results and discussion

3.1 Experimental research into the process of lactic acid biosynthesis

Analysis of the results of the study (Figure 1) led to the conclusion that the highest concentrations of lactobacilla *B. coagulans* (1500 MCells/mL) and *L. casei* (900 MCells/mL) were achieved by 40 hours of cultivation in a nutrient medium using the culture fluid of *C. vulgaris* IFR C-111 as a stimulating substance and a source of nitrogen. The average concentration of cells was 1.5-1.9 times higher than in control samples in which malt extracts were used as sources of nitrogen and stimulants. After 40 hours of cultivation, the growth of cells of lactic acid bacteria slowed down, since by this time a significant lactic acid content of 10-58 g/L (depending on the strain) was observed in the culture fluid, which inhibited cell growth.

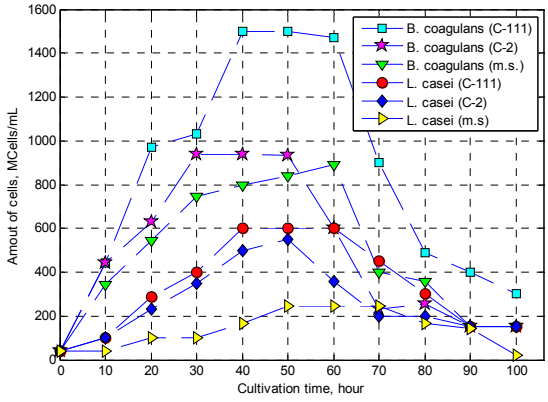

Figure 1: Kinetics of biomass accumulation.

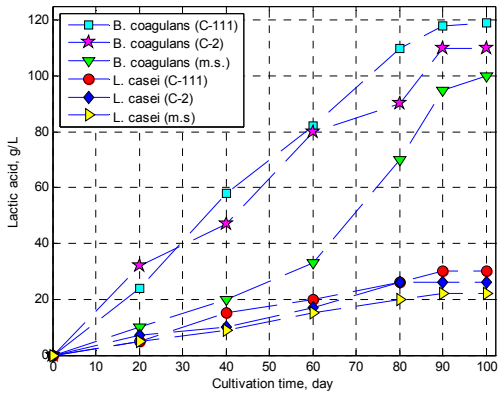

Figure 2: Kinetics of lactic acid accumulation.

The maximum concentration of the target product (Figure 2) was observed over 90-100 hours of cultivation with *C. vulgaris* IFR C-111 culture fluid filtrate: for the strain *B. coagulans* it constituted 118 g/L, and for the strain *L. casei* B-3241 it was 30 g/L. On average, when using algal culture fluid filtrate as a source of stimulants and nitrogen, the concentration of lactic acid in the culture fluid increases by 15-30 % (wt.), in comparison with the control sample (malt extract). This can be explained by the fact that B vitamins - pyridoxine, thiamin, riboflavin - are present in the filtrate of microalgae culture fluid (Bogdanov 2007). They intensify the reactions of protein synthesis, glycolysis and the formation of pyruvate, which allows the cells to actively divide and accumulate lactic acid. Analysis of the kinetics of glucose loss (Figure 3) allows to conclude that this carbon substrate is assimilated by cells at approximately the same rate during the entire cultivation time. This may be due to the fact that glucose is necessary both for the multiplication of cells, maintenance of their normal vital activity, and also for the synthesis of lactic acid. The maximum glucose uptake was observed when the *B. coagulans* strain was cultivated in the algal culture fluid filtrates, and was 93 % (wt.) when using the *C. vulgaris* IFR C-111 filtrate and 76 % when using the *C. vulgaris Beijer* IPPAS C - 2 filtrate, which is 1.3-1.4 times higher in comparison with control samples.

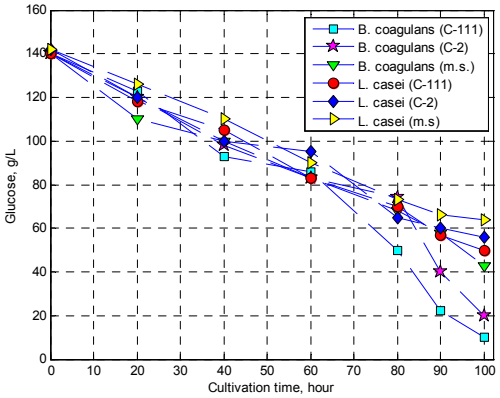

Figure 3: Carbon-containing substrate loss.

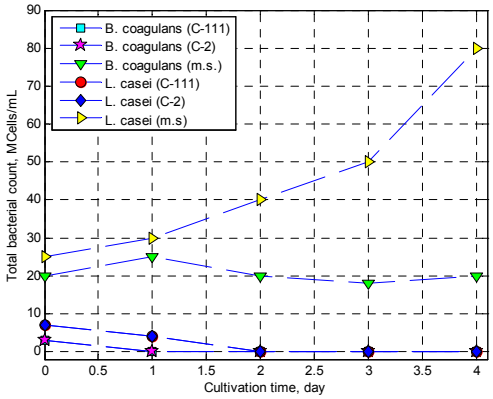

Figure 4: Changes in total bacterial count.

When cultivating *B. coagulans* and *L. casei* strains for 20 hours on nutrient media using municipal wastewater after activated sludge treatment, the death of lactic acid bacteria was observed. This can be explained by the fact that in municipal wastewater treated with activated sludge there are coliform and pathogenic bacteria (TBC = 20-25 cfu/mL) that suppress the vital activity of lactic acid bacteria. Also, lactic acid did not accumulate in the culture fluid, and the TBC of pathogenic bacteria increased up to 3 times.

Using filtrates of culture fluid after cultivation of *C. vulgaris* IFR C-111 and *C. vulgaris Beijer* IPPAS C-2 in municipal wastewater for the cultivation of *B. coagulans* and *L. casei* allows to sustain the vitality of strains, and to ensure their normal growth (Figure 1) and accumulation of lactic acid (Figure 2) up to the level of 119 g/L; this is 11.8 % higher than the maximum result (Wang et al. 2015) in Table 1. This is due to the fact that, during the cultivation in municipal wastewater, microalgae produce an extracellular metabolite (Plekhanov et al. 2013) that has an antibiotic effect and inhibits the activity of opportunistic pathogenic microflora and pathogens. Therefore, the TBC of the culture fluid filtrate after the cultivation of the microalgae *C. vulgaris* IFR C-111 and *C. vulgaris Beijer* IPPAS C-2 in municipal wastewater is 2.5-5 times lower than that of municipal wastewater after biological treatment with activated sludge and is 5 -10 cfu/mL (Figure 4).

3.2 Mathematical modelling of lactic acid biosynthesis process

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

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

where x is concentration of cell biomass, MCells/mL; μ is specific growth rate, day^{-1}; E_n is capacity of cell population, MCells/mL.

From the experimental data presented in Figures 1-3 it can be concluded that the dependence of the specific growth rate on the concentration of the carbon substrate S and the amount of accumulated lactic acid is described by the Monod-Yerusalimsky equation (Biryukov 2004):

$$\mu = \mu_{\max}\left(\frac{S}{K_s + S}\right)\cdot\left(\frac{1}{1+\frac{P}{K_P}}\right), \quad (2)$$

where S is concentration of carbon-containing substrate, g/L, μ_{max} is maximum specific growth rate, day^{-1}; K_S and K_P are constants of semi-saturation for the given substrates, g/L, P is the amount of lactic acid, g/L.
Glucose loss during the periodic cultivation of lactic acid bacteria is described as follows:

$$\frac{dS}{dt} = -\frac{1}{Y_{XS}}\cdot\frac{dx}{dt} + \frac{1}{Y_{PS}}\cdot\frac{dP}{dt} + m_s x, \quad (3)$$

where Y_{XS} is a coefficient referring to the amount of accumulated biomass Δx for the amount of substrate ΔS used over the time period Δt, calculated experimentally, MCells L/(mL·g); Y_{PS} is a coefficient of the amount of accumulated lactic acid ΔP for the amount of substrate ΔS used over the time period Δt, calculated experimentally; m_S is a coefficient of vitality sustainability.
The model of lactic acid accumulation process takes into account the relationship between the rate of its biosynthesis and the concentration of the carbon-containing substrate in the nutrient medium and the pH level that affects the vitality of the cells. Therefore, this process can be described by the following equation:

$$\frac{dP}{dt} = \mu_P \cdot \frac{dx}{dt}, \quad (4)$$

$$\mu_P = \mu_{p_{\max}}\left(\frac{S}{K_P + S}\right)\cdot\left(\frac{1}{1+\frac{P}{K_P}}\right). \quad (5)$$

Thus, the mathematical model of the process of lactic acid bacteria cultivation is a system of equations (1), (3) and (4) with initial conditions of the form of $x(t_0) = 50$ MCells/mL, $S\ (t_0) = 140$ g/L, $P(t_0) = 0$ g/L.

Having processed the experimental data, the following kinetic coefficients of the equations (Table 3) have been calculated for *B. coagulans* and *L. casei* strains.

Table 3: Kinetic coefficients of the model's equations

	B. coagulans		*L. casei*	
	C. vulgaris Beijer IPPAS C-2	*C. vulgaris IFR C-111*	*C. vulgaris Beijer IPPAS C-2*	*C. vulgaris IFR C-111*
$E_П$, MCells/mL	650	550	250	930
K_S, mg/L	0.5	0.1	0.1	3
K_P, mg/L	5.5	1.5	9	4.9
μ_{max}, day^{-1}	0.9	2	0.12	0.36
$\mu_{p_{\max}}$, day^{-1}	1.0	2.1	0.1	0.05
m_s	10^{-4}	10^{-4}	0.01	$3\cdot10^{-4}$
Y_{XS}, MCells L/(mL·g)	12	7	0.8	7.4
Y_{PS}, MCells L/(mL·g)	12	4	0.12	0.31

To assess the adequacy of the model, we used a function of the form: $\delta_i^j(t) = \max\left(\left|\xi^e(t) - \xi^m(t)\right| / \xi^e(t)\right)\cdot 100\%$, where $\xi^e = \left(x^e, S^e, P^e\right)$, $\xi^m = \left(x^m, S^m, P^m\right)$ are the vectors of the experimental and calculated values of the required model variables for the *i*-th microalgae strain and the *j*- th strain of bacteria. Checks for the model's adequacy yielded the following results: $\delta_{C-2}^{coag} = 12.5\,\%$, $\delta_{C-111}^{coag} = 29.5\,\%$, $\delta_{C-2}^{casei} = 11.5\,\%$, $\delta_{C-111}^{casei} = 16.3\,\%$.

3. Conclusions

It has been established through experimental studies that the use of the culture fluid filtrate after municipal wastewater treatment with microalgae *C. vulgaris* IFR C-111 and *C. vulgaris Beijer* IPPAS C-2 as a source of nitrogen and stimulants allows to increase the concentration of lactic acid bacteria *B. coagulans* and *L. casei* 1.5-1.9 times on average; and the concentration of lactic acid in the culture fluid by 15-30 % (wt.) compared to

the control samples. The use of nutrient media based on municipal wastewater treated with activated sludge for the cultivation of *B. coagulans* and *L. casei* strains results in the death of lactic acid bacteria due to the presence of coliform and pathogenic flora in water, which suppress the vital activity of lactic acid bacteria. On the basis of the experimental data obtained using the Verhulst and Monod-Yerusalimsky equations, a mathematical model of the process of cultivating lactic acid bacteria on the filtrates of the culture fluid after municipal wastewater treatment with microalgae *C. vulgaris* IFR C-111 and *C. vulgaris Beijer* IPPAS C-2. The model describes the kinetics of lactic acid bacteria growth, the loss of the carbon-containing substrate and the accumulation of lactic acid.

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