Cytotoxicity of some preservatives for culture *Chlorella Vulgaris* GKO strain

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Abstract. One of the ways to preserve the culture of microalgae for a long time is conservation. All modern methods of preservation and long-term storage of microorganism cultures are based on the transfer of cells to a state of anabiosis, in which metabolic processes are either completely or partially stopped. One of the mechanisms that are realized during the transition to the anabiotic state is a change in cell permeability. This can be achieved by modifying the culture medium. Thus, the search for preservatives that contribute to the long-term preservation of biomass is very relevant. The primary condition for the use of any preservative or a new component of the culture medium should be the absence of a toxic effect on the cell culture. Goal of the work: to determine the cytotoxic effect of some preservatives for the cell culture of *Chlorella vulgaris* GKO strain. The cytotoxicity of ascorbic acid, lactic acid, citric acid, acetic acid, sodium chloride and urotropin was analyzed for the cell culture of *Chlorella vulgaris* strain GKO. The following parameters were determined: pH of *Chlorella vulgaris* suspension with preservative; Total number of cells, MM/ml; The ratio of dead cells to the total number of cells,%; Specific growth/death rate; The optical density of the suspension; The difference in the average optical density,%; Cell size (diameter), μm pH of suspension of *Chlorella vulgaris* with preservative; Total number of cells, MM/ml; The ratio of dead cells to the total number of cells,%; Specific growth/death rate; The optical density of the suspension; The difference in the average optical density,%; Cell size (diameter), μm pH of suspension of *Chlorella vulgaris* with preservative; Total number of cells, MM/ml; The ratio of dead cells to the total number of cells,%; Specific growth/death rate; The optical density of the suspension; The difference in the average optical density,%; Cell size (diameter), μm

1 Introduction

The specificity of the metabolism of a number of representatives of the algoflora, associated with the production of metabolites with valuable properties for humans, has made microalgae an important object of biotechnology [1]. The use of algae covers such areas as

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phycomediation, alternative energy, cosmetology, feeding of farm animals, biotesting, and other areas of biotechnology [2–4].

Among the microalgae cultivated on an industrial scale, the most famous are representatives of the following genera: *Chlorella*, *Chlorococcum*, *Spirogyra*, *Scenedesmus*, *Nostoc*, *Navicula*, *Nitzschia* [5-6]. One of the most promising genera of microalgae is *Chlorella*.

Chlorella **is** a genus of green algae (single-celled) that are spherical in shape with a diameter of 2 to 10 microns. Since the early 1960s, microalgae has been commercially produced in Japan as a dietary supplement using various types of *chlorella*. Subsequently, in the 1980s, the cultivation of microalgae spread throughout the world, including the United States, India, Israel, and Australia [7]. To date, Japan and China are the main producing countries of *chlorella*, with an annual production of more than 3500 tons of biomass [8]. Produced *chlorella* is sold mainly in the form of dried powder, tablets or capsules. It should be noted that *chlorella* is also used for feeding animals. The use of *chlorella* as a fodder crop for animals is a fairly young trend.

In recent years, due to the energy crisis and the growing interest in environmentally friendly fuels, *chlorella* has become a promising raw material for the production of biofuels. This species of microalgae is attracting more and more scientific and industrial attention due to its rapid growth, high fat content, and ease of cultivation [3].

Other interesting applications of *chlorella* are wastewater treatment and bioremediation (removal of heavy metals) [3]. In Russia, it was also proposed to use *chlorella* for the algolization of water bodies (the introduction of strains of *Chlorella vulgaris* into water bodies). However, in 2014, the Congress of the Hydrobiological Society under the Russian Academy of Sciences was held in Krasnoyarsk. An important result of the work of the Congress was the decision to consider the so-called method of "algolization" of water bodies as incorrect and causing significant damage to Russian hydrobiology [11].

The implementation of large-scale production based on *chlorella* is constrained by a number of difficulties, among which a serious problem ispreservation of biomass for a long time under various conditions. For example, biomass production in summer can be three to five times higher than biomass production in winter, therefore, this can lead to a biomass deficit in winter and crop surplus in factories in summer [12]. The use of refrigeration units is energy-intensive and requires large areas. Thus, the creation of special conditions for the conservation of biomass is required.

The stability of the biomass of algae during storage obeys the Mono-Jerusalem law, for example, an increase in the concentration of metabolic products in the medium or depletion of the substrate have a limiting effect on the culture [13]. The depletion of the environment and its pollution with metabolic products occurs with an increase in the rate of cell division. This can be especially acute for crops during transportation or storage of biomass at high temperatures. Thus, it is necessary to investigate the "shelf life" of biomass and look for potentially effective ways to preserve the culture of microalgae [14-16].

One of the ways to preserve culture for a long time is conservation. All modern methods of conservation and long-term storage of microorganism cultures are based on the transfer of cells to a state of suspended animation, in which metabolic processes either completely or partially stop [2]. One of the mechanisms that are realized during the transition to the anabiotic state is a change in cell permeability. This can be achieved by modifying the culture medium (adding additional components). Thus, the search for preservatives that contribute to the long-term preservation of *chlorella* biomass is very relevant. The primary condition for the use of any preservative or a new component of the culture medium should be the absence of a toxic effect on the cell culture.

Goal of the work: to determine the cytotoxic effect of some preservatives for the cell culture of *Chlorella vulgaris* strain GKO

2 Materials and Methods

2.1 Culture

In the work we used the culture of *Chlorella vulgaris* strain GKO (see Fig. 1, 2), produced in LLC "Algotek". The sample passed toxicological and microbiological tests at the Tverskaya Interregional Veterinary Laboratory (Test Protocol No. 1663-IL dated July 14, 2019). The manufacturer company offers various directions for using the strain, incl. for feeding fish. Long-term transportation of this strain to the place of application without refrigeration may contribute to the death of the culture.

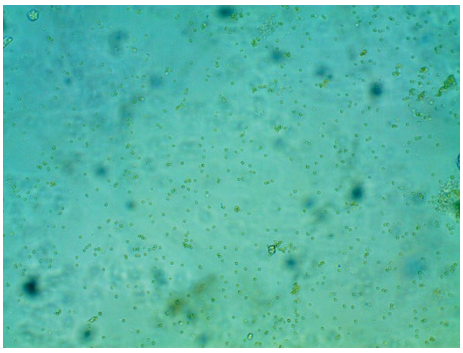

Fig. 1. Magnification x150

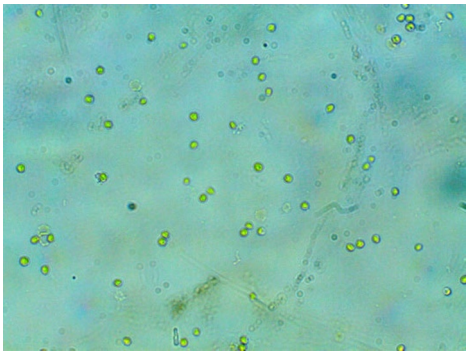

Fig. 2. Magnification x600

2.2 Culture conditions

The studies were performed on the basis of the Department of Biochemistry and Physiology of the St. Petersburg State University of Veterinary Medicine. In the laboratory, a strain of *chlorella* is cultivated on Tamiya medium. Tamiya nutrient medium consists of the following ingredients: macronutrients (g/l): KNO_3 - 5.0; $MgSO_4$ * $7H_2O$ - 2.50; KH_2PO_4 - 1.25; $FeSO_4$ * $7H_2O$ - 0.003 and trace elements (mg / l): H_3BO_3 - 286; $ZnSO_4$ * $7H_2O$ - 0.222; $MnCI_2$ * 7 H_2O - 1.81; MoO_3 - 17.64; NH_4VO_3 - 22.96; MnCl2 * $4H_2O$ - 1.81; pH index (7.8 -8.8) [17].

Conditions for cultivating mother culture: a glass bioreactor (dimensions 600*150*450, V=40.5) was used, at a temperature range of +27…+29°C (cylindrical heater AQUAEL AQN PLATINIUM HEATER 50W; temperature control was carried out with an aquarium thermometer), constant artificial illumination of 25–30 µmol of light quanta per m^2/s (Aquarium LED lamp, warm light 3200K, 280 mm.) and constant stirring. Before carrying out the experiments, the number of cells in the mother culture was measured. The characteristics of the uterine cell culture are presented in Table 1.

Table 1. Characteristics of the mother culture of *Chlorella vulgaris* GKO strain cells

Researched indicator	Meaning
pH of *Chlorella vulgaris* suspension with preservative	7
Total number of cells, MM/ml	12.2±0.25
The ratio of dead cells to the total number of cells, %	-
Suspension optical density	0.711±0.07
Cell size (diameter), µm	4.91±0.07

2.3 Preservatives used

In the work, the potential cytotoxicity of the following preservatives was determined: ascorbic acid, lactic acid, citric acid, acetic acid, sodium chloride, urotropine. All preservatives were purchased from Himmag-SPb, Russia. The working concentrations of preservatives are presented in Table 2. The choice of these preservatives is determined by the following reasons:

1. Available to a wide range of consumers;
2. Used as preservatives in the food industry;

Table 2. Working concentrations of preservatives

Preservative	Test concentrations				
	1	**2**	**3**	**4**	**5**
Ascorbic acid mg/ml	20	10	5	2.5	1
Lactic acid mg/ml	320	160	80	40	20
Citric acid mg/ml	40	20	10	5	2
Acetic acid mg/ml	40	20	10	5	2
Sodium chloride mg/ml	40	20	10	5	2
Urotropin mg/ml	40	20	10	5	2

2.4 Conditions for setting up experiments

Cultivation of the cell culture of *Chlorella vulgaris* strain GKO in combination with a preservative was carried out in 10 ml glass penicillin vials with a smooth neck for 24 hours.

5 ml of a stock culture of *Chlorella vulgaris* GKO strain cells was added to a penicillin vial, 5 ml of Tamiya medium was added, and a preservative was added at working concentrations. As a positive control (hereinafter referred to as PC), a complex of 5 ml of the uterine culture of *Chlorella vulgaris* GKO cells and 5 ml of Tamiya medium was used; as a negative control (hereinafter referred to as OK), 5 ml of the uterine culture of *Chlorella vulgaris* GKO strain cells was used. Three working samples were prepared for each preservative concentration. The experiment was carried out twice. The number of cells in the positive control was determined before placing in the incubator (6.0±0.23 MM/ml) and after daily exposure.

The samples were cultivated in a multi-cell algae cultivator (hereinafter KVM-05). Conditions for cultivation of algae in complex with a preservative in KVM-05 are presented in Table 3.

Table 3. Conditions for the cultivation of algae in combination with a preservative in KVM-05

Parameter characteristic	Meaning
Algae cultivation temperature, °C	28o
Light irradiation of algae culture (PAR area), W/m2.	60
Speed (adjustable) of rotation of the cassette with vials-reactors, rpm.	20
CO2 concentration in the gas phase, %.	0.03

2.5 Determination of the pH of a suspension of *chlorella*

The pH value of a suspension of *Chlorella vulgaris* strain GKO before and after the introduction of a preservative was measured by the potentiometric method on a pH-420 device (Aquilon-SZ, Russia).

2.6 Determination of the optical density of a suspension of *chlorella*

The growth of all cultures was controlled photometrically at a wavelength of 560 nm using a KFK-3-01 photoelectric concentration photocolorimeter (Analit-Lab, Russia), glass cuvettes (5.124 mm) were used. The relative (in %) difference in the average optical density for each working concentration was calculated compared to the positive control (see formula 1) [18].

$$l = ((D_к - D_о)/\text{DTo})*100 \quad (1)$$

where Dk and Do are the average values of optical density in the control and experimental groups, respectively.

The criterion for the toxicity of a sample is a decrease in the average value of optical density compared to the control variant by 20% or more in the case of suppression of the growth of the test culture or its increase by 30% or more in the case of stimulation of growth processes.

2.7 Quantitative analysis, determination of cell viability

The total number of *Chlorella vulgaris* cells was determined under an optical microscope (Mikmed-5, Russia) using a Goryaev counting chamber. Cells were counted at magnification (150X and 600X), and photofixation of the material was carried out in parallel (using the ToupTek FMA050 adapter). The unit of count was one cell. The suspension was stirred before counting.

The calculation was carried out depending on the density of cells: a) throughout the field - with a small number; b) in 25 large squares - with an average number; c) in 16 large squares diagonally - with a high number. The calculation was carried out according to the formula (see formula 2) [19]:

$$N = \frac{(1000 \times n)}{(S \times h)} \quad (2)$$

where N is the number of cells in 1 ml; 1000 - conversion factor mm³ to cm³; n is the number of calculated cells in a certain sector of the Goryaev chamber; h is the depth of the counting chamber 0.1 mm; S is the area of the sector (with the area of the "large" square 0.04 mm²).

Cell viability was determined using trypan blue azo dye (Himedia). The method is based on selective dye staining of dead cells in blue. 100 µl of the cell suspension was mixed with 50 µl of trypan blue in 96-well immunological plates (Khimmed, Russia), incubated for 3 minutes. Determination of the number of cells of dead cells was carried out in a Goryaev counting chamber; in parallel, albinization, lysis, and the appearance of malformations (signs of a dead cell) were taken into account [20]. The percentage of dead cells was determined by formula 4

$$dead\ cells\ (\%) = \frac{dead\ cells}{total\ number\ of\ cells} * 100 \quad (3)$$

2.8 Morphometric analysis

Morphometric studies of cells were performed using the ScreenMeter 1.0 software. A morphometric ruler (MS-1-4 Micrometers Stage) was used as a reference standard, at 1 DIV = 0.01 mm. The cell diameter was determined. During the work, single cells were taken into account.

2.9 Statistical analysis of data

Data are presented in the text as mean values and errors of the arithmetic mean. The comparison was carried out according to the following parameters: total number of cells, MM/ml; optical density of the suspension; Cell size (diameter), μm. Data were compared (comparison with positive control) using one-way analysis of variance (ANOVA) with 95% confidence. Differences between levels were identified using Tukey's test. Statistical significance was established at $p<0.05$. Statistical analysis was performed using STATISTICA Base software.

3 Results and discussion

The cytotoxicity of ascorbic acid, lactic acid, citric acid, acetic acid, sodium chloride and urotropin was analyzed for the cell culture of *Chlorella vulgaris* strain GKO. The following parameters were determined: pH of *Chlorella vulgaris* suspension with preservative; Total number of cells, MM/ml; The ratio of dead cells to the total number of cells, %; Specific growth/death rate; The optical density of the suspension; The difference in the average optical density, %; Cell size (diameter), μm

The results of the use of ascorbic acid (see table. 4) showed that its use at a concentration of 20 mg/ml reduces the pH to 6.0, when using a concentration of 10 mg/ml, the pH of the suspension was at the level of 6.5. The use of ascorbic acid at concentrations of 5 mg/ml, 2.5 mg/ml and 1 mg/ml maintained the pH at 7.0-7.5.

The results of assessing the effect of ascorbic acid on the total number of cells showed that its introduction leads to an increase in the number of cells at concentrations from 2.5 mg/ml to 20 mg/ml. The obtained data are consistent with the studies of other specialists. For example, the addition of ascorbic acid to Kuhl's medium with the addition of cadmium (Cd2+) significantly accelerated the growth and chlorophyll content of *Chlorella vulgaris* [21]. In addition, Desuki (1995), who worked with *Chlorella vulgaris*, showed that the addition of ascorbic acid 300 ppm resulted in stimulation of algae growth [22]. Thus, this compound cannot be considered as a potential preservative.

Determination of the ratio of dead cells in relation to living ones showed that at a concentration of 20 mg/ml, cell death reaches 12.5%, when using a concentration of 10 mg/ml and 5 mg/ml, cell death was 7%. A concentration of 2.5 mg/ml resulted in 2.5% cell death. Additional studies are required to determine the cause of the decrease in the total number of cells and the increase in the number of dead cells when using a concentration of ascorbic acid of 1 mg / ml.

The results of determining the optical density of the suspension showed an increase in the index in all experimental groups, with the exception of the experimental group with a concentration of 1 mg / ml. Optical density data are consistent with total cell counts.

The difference in the average value of optical density was calculated, an increase in the index was observed in all experimental groups, with the exception of the experimental group with a concentration of 1 mg/ml. In the experimental groups, the group with concentrations of 20 mg / ml and 10 mg / ml, the difference in optical density is over 30%

There were no significant changes in cell diameter when using ascorbic acid.

Table 4. The results of the study of the cytotoxicity of ascorbic acid for the cell culture of *Chlorella vulgaris* strain GKO

Index	Concentrations mg/ml					PC	OK
	20	10	5	2.5	1		
pH of *Chlorella vulgaris*	6.0	6.5	7.0	7.5	7.0	7.0	7.5

suspension with preservative							
Total number of cells, MM/ml	8±2.4	9.3±0.6*	8.4±0.25*	7.77±0.25	6.4±0.2*	7.3±0.07	13.77±0.33
The ratio of dead cells to the total number of cells, %	12.5	7.0	7.0	2.5	6.0	0	0
Suspension optical density	1.26±0.1*	1.26±0.2	1.107±0.2	0.92±0.07	0.85±0.11	0.955±0.04	1.035±0.08
Difference in the average optical density, %	- (32)	- (32)	- (15.9)	- (6,4)	+(12.3)	—	—
Cell size (diameter), µm	5.5±0.15*	5.08±0.14	4.86±0.13	5.06±0.18	4.97±0.1	4.98±0.15	5.2±0.1

Note: * - statistically significantly different compared to the indicators of the positive control group at p≤0.05;
-(..) – is used to denote an increase in crop growth;
+(..) - used to indicate the rate of culture suppression;

The results of using lactic acid (see Table 5) showed that its use at a concentration of 320 mg/ml lowered the pH to 2.0, when using a concentration of 160 mg/ml, the pH of the suspension was at the level of 3.0. The use of ascorbic acid at concentrations of 80 mg/ml, 40 mg/ml and 20 mg/ml maintained the pH at 5.0, 6.5 and 7.0, respectively.

The results of assessing the effect of lactic acid on the total number of cells showed that its introduction leads to a significant decrease in the number of cells at all concentrations studied; visual inspection noted a change in the color of *chlorella* biomass from green to brown. There are works on the study of the effect of inoculation of lactic acid bacteria on the safety of algae biomass [23,24]. Also, a number of studies have shown the preservative ability of *chlorella* in food products [25,26]. However, our studies have shown the detrimental effect of lactic acid on *chlorella* cells. The use of concentrations of 320 mg/ml, 160 mg/ml and 80 mg/ml resulted in the death of over 50% of cells in suspension (see Fig. 3.4).

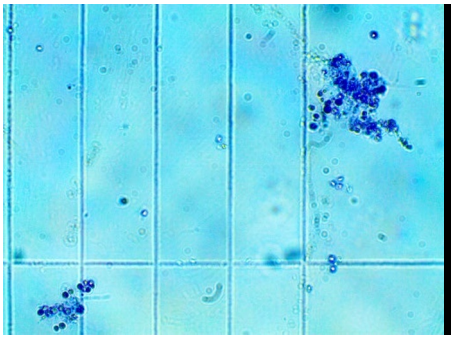

Fig. 3. Cell staining x600

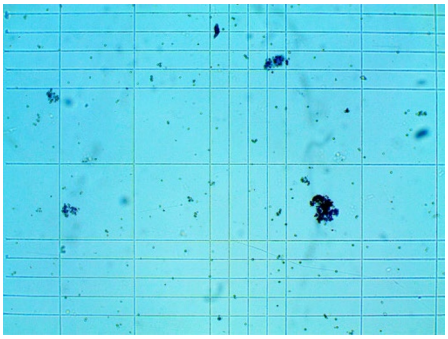

Fig. 4. Cell staining x150

The data obtained should not be considered unambiguously from the standpoint of the futility of using lactic acid as a preservative. Additional research and expansion of the line of tested working concentrations is required.

The results of determining the optical density of the suspension and the difference in the average value of the optical density showed a decrease in the studied parameters in all experimental groups. The results obtained show the toxicity of the studied concentrations of lactic acid for suspension

A decrease in cell diameter was recorded in the studied groups, the largest decrease was determined at concentrations of 40 mg/ml and 20 mg/ml up to 3.8±0.14 µm and 3.7±0.11, respectively.

Table 5. The results of the study of the cytotoxicity of lactic acid for the cell culture of *Chlorella vulgaris* strain GKO

Index	Concentrations mg/ml					PC	OK
	320	160	80	40	20		
pH of *Chlorella vulgaris* suspension with preservative	2.0	3.0	5.0	6.5	7.0	7.0	7.5
Total number of cells, MM/ml	3.37±0.1 *	3.4±0.25 *	3.53±0.04*	3.63±0.35*	3.63±0. 04*	7.3±0.0 7	13.77± 0.33
The ratio of dead cells to the total number of cells, %	57.6	58.8	63.7	38.3	20.6	0	0
Suspension optical density	0.205±0. 02*	0.208±0. 01*	0.31±0.07*	0.395±0.07 *	0.251± 0.04*	0.955± 0.04	1.035± 0.08
Difference in the average optical density, %	+(78.5)	+(78.2)	+(67.5)	+(58.6)	+(73.7)	—	—
Cell size (diameter), µm	4.45±0.0 9*	4.42±0.1 7*	4.02±0.19*	3.8±0.14*	3.7±0.1 1*	4.98±0. 15	5.2±0. 1

Note: * - statistically significantly different compared to the indicators of the positive control group at $p \leq 0.05$;
-(..) – is used to denote an increase in crop growth;
+(..) - used to indicate the rate of culture suppression;

The results of the use of citric acid (see table. 6) showed that its use at a concentration of 40 mg/ml reduces the pH to 1.0, when using a concentration of 20 mg/ml, the pH of the suspension was at the level of 2.0. The use of citric acid at concentrations of 10 mg/ml, 5 mg/ml and 2 mg/ml maintained the pH at 3.0, 4.0 and 5.0, respectively.

The results of assessing the effect of citric acid on the total number of cells showed no dose-dependent effect in changing the number of cells depending on the concentration of the solution. At concentrations of 40mg/mL, 20mg/mL, 10mg/mL, 5mg/mL, and 2mg/mL, significant cell death was observed 21.7%, 51.2%, 34.5%, 58.3 %, 43.4, respectively. A large number of destroyed cells were noted. The data obtained can be explained by the fact that the use of citric acid in working concentrations goes beyond the boundaries of the *chlorella* optimum zone. In other studies, it was shown that citric acid plays the role of a protector (at concentrations of 5, 10, 20 mg/l) during the action of graphene on the *Chlorella* pyrenoidesa cell culture [27]. The role of citric acid in the protection of higher plants during copper intoxication was also noted [28]. Thus, studies of the effect of citric acid on *Chlorella vulgaris* cell culture should be expanded, using lower concentrations. Given the data on the protective properties of citric acid, it can be proposed as an additional compound in a nutrient medium to prevent cell death from potentially toxic metabolites.

The results of determining the optical density of the suspension and the difference in the average value of the optical density showed a decrease in the studied parameters in all experimental groups. The results obtained show the toxicity of the studied concentrations of citric acid for suspension

It was recorded that when exposed to a medium with citric acid, an increase in cell diameter is observed in the studied groups. The highest values were determined at concentrations of 40 mg/ml, 20 mg/ml, 10 mg/ml.

Table 6. The results of the study of the cytotoxicity of citric acid for the cell culture of *Chlorella vulgaris* strain GKO

Index	Concentrations mg/ml					PC	OK
	40	20	10	5	2		
pH of *Chlorella vulgaris* suspension with preservative	1.5	2.0	3.0	4.0	5.0	7	7.5
Total number of cells, MM/ml	4.37±0.04*	4.47±0.11 *	4.7±0.07 *	3.43±0.2 2*	3.73±0.2 2*	7.3±0.07	13.77±0. 33
The ratio of dead cells to the total number of cells, %	21.7	51.2	34.5	58.3	43.4	0	0
Suspension optical density	0.254±0.01 *	0.27±0.01 2*	0.288±0. 08*	0.213±0. 01*	0.203±0. 05*	0.955±0. 04	1.035±0. 08
Difference in the average optical density, %	+(73.4)	+(71.7)	+(69.8)	+(77.6)	+(78.7)	—	—
Cell size (diameter), µm	5.99±0.14*	6.12±0.09 *	6.3±0.1*	5.21±0.1 2	5.55±0.2 *	4.98±0.1 5	5.2±0.1

Note: "*" - statistically significantly different from the indicators of the positive control group at $p \leq 0.05$;
-(..) – is used to denote an increase in crop growth;
+(..) - used to indicate the rate of culture suppression;

The results of the use of acetic acid (see table. 7) showed that its use at a concentration of 40 mg/ml reduces the pH to 3.0, when using a concentration of 20 mg/ml, the pH of the suspension was at the level of 4.0. The use of citric acid at concentrations of 10 mg/ml, 5 mg/ml and 2 mg/ml maintained the pH at 6.0, 7.0 and 7.0, respectively.

The results of evaluating the effect of acetic acid on the total number of cells allow us to see the presence of a dose-dependent effect in the change in the number of cells depending on the concentrations of the solution. At concentrations of 40mg/mL, 20mg/mL, 10mg/mL, 5mg/mL and 2mg/mL significant cell death was observed 47.4%, 34.1%, 40.5%, 35.9 %, 13% respectively. It should be noted that acetic acid is used in culture media for *chlorella* (TAP medium) [29]. It has also been shown that cultures using acetic acid as a carbon source (or addition of sodium acetate) under low light appear to increase total lipids, while addition of sodium acetate under normal light (20 µmol photons m-1 s-1) led to an increase in starch content [30]. The data obtained indicate that

The results of determining the optical density of the suspension and the difference in the average value of the optical density showed a decrease in the studied parameters in all experimental groups. The results obtained show the toxicity of the studied concentrations of acetic acid for suspension

The use of acetic acid resulted in significant changes in cell diameter at concentrations of 20 mg/mL and 10 mg/mL.

Table 7. The results of the study of the cytotoxicity of acetic acid for the cell culture of *Chlorella vulgaris* strain GKO

Index	Concentrations mg/ml					PC	OK
	40	20	10	5	2		
pH of *Chlorella vulgaris* suspension with preservative	3.0	4.0	6.0	7.0	7.0	7.0	7.5
Total number of cells, MM/ml	3.9±0.12 *	4.1±0.19 *	3.9±0.49 *	4.03±0.4 7*	4.6±0.07 *	7.3±0.07	13.77±0. 33

The ratio of dead cells to the total number of cells, %	47.4	34.1	40.5	35.9	13	—	—
Suspension optical density	0.23±0.09*	0.223±0.03*	0.216±0.1*	0.284±0.08*	0.3±0.06*	0.955±0.04	1.035±0.08
Difference in the average optical density, %	75.9	76.6	77.4	70.3	68.5	—	—
Cell size (diameter), μm	4.9±0.1	4.04±0.15*	4.11±0.11*	4.63±0.14	4.5±0.1*	4.98±0.15	5.2±0.1

Note: "*" - statistically significantly different from the indicators of the positive control group at p≤0.05
-(..) – is used to denote an increase in crop growth;
+(..) - used to indicate the rate of culture suppression;

The results of the application of sodium chloride (see table. 8) showed that at a concentration of 40 mg/ml reduces the pH to 6.0. The use of sodium chloride at concentrations of 20 mg/ml, 10 mg/ml, 5 mg/ml and 2 mg/ml maintained the pH at 7.0.

The results of evaluating the effect of sodium chloride on the total number of cells showed that its introduction maintained the number of cells at a level comparable to the positive control at the beginning of the experiment. When using 40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml and 2 mg/ml, the cell count was 5.67±0.04 MM/ml, 5.83±0.11 MM/ml, 5.9±0.24 MM/ml, 6.23±0.04 MM/ml, 5.3±0.07 MM/ml, respectively. The results of our work are similar to the data of other researchers who showed the suppression of the growth rate of *Chlorella* protothecoides CS-41 [31]. In another study, it was shown that the introduction of sodium chloride into the growth medium at a concentration of >0.4–0.5 M significantly reduced thermogenesis and inhibited the growth of the culture (up to complete cessation), but during long-term cultivation (up to 5 days) on a medium containing 0.05– 0.2 M NaCl, there was a slight increase in the growth of culture and an increase in its heat production [32]. It was also shown that sodium chloride inhibits the photosynthetic activity and respiration of *chlorella* already from the first minutes of exposure, without causing obvious changes in the dynamics of diffusion parameters of water transfer and cell ultrastructure [33,34]. Potentially, the safety of the cell culture can also be affected by the fact that sodium chloride reduces the rate of oxygen release during photosynthesis.

The results of determining the optical density of the suspension showed an increase in the index in all experimental groups. Optical density data are consistent with total cell counts. The difference in the average value of optical density was calculated, an increase in the index was observed in all experimental groups. Thus, these sodium chloride concentrations did not have a toxic effect on the *Chlorella vulgaris* cell culture after daily exposure.

Significant changes in cell diameter were noted when sodium chloride was used. An increase in cell size was also recorded by other specialists, for example, when using scanning electron microscopy, an increase in cell size by 33.52% (in *Chlorella* humicola) and 27.79% (in *Chlorella vulgaris*) was noted at 100 mm sodium chloride [35]. This may be due to the fact that sodium chloride leads to an increase in lipids, proteins, carbohydrates and thickening of the cell wall [31,36].

Table 8. The results of the study of the cytotoxicity of sodium chloride for the cell culture of *Chlorella vulgaris* strain GKO

Index	**Concentrations mg/ml**					**PC**	**OK**
	40	**20**	**10**	**5**	**2**		
pH of *Chlorella vulgaris* suspension with preservative	6.0	7.0	7.0	7.0	7.0	7.0	7.5

Total number of cells, MM/ml	5.67±0.04*	5.83±0.11*	5.9±0.24*	6.23±0.04*	5.3±0.07*	7.3±0.07	13.77±0.33
The ratio of dead cells to the total number of cells, %	0	0	0	0	0	—	—
Suspension optical density	0.823±0.01	0.841±0.08	0.896±0.09	0.91±0.04	0.812±0.1	0.955±0.04	1.035±0.08
Difference in the average optical density, %	+(13.2)	+(11.4)	+(6,17)	+(4,7)	+(14.9)	—	—
Cell size (diameter), µm	8.39±0.45*	5.3±0.2	6.06±0.14*	6.74±0.17*	6.27±0.12*	4.98±0.15	5.2±0.1

Note: "*" - statistically significantly different from the indicators of the positive control group at $p \leq 0.05$
-(..) – is used to denote an increase in crop growth;
+(..) - used to indicate the rate of culture suppression;

The results of the use of urotropin (see table. 8) showed that at a concentration of 40 mg / ml it increases the pH to 7.5. The use of sodium chloride at concentrations of 20 mg/ml, 10 mg/ml, 5 mg/ml and 2 mg/ml maintained the pH at 7.0.

The results of evaluating the effect of urotropin on the total number of cells showed that its introduction maintained the number of cells at concentrations of 20 mg/ml, 5 mg/ml and 2 mg/ml, at a level comparable to the positive control at the beginning of the experiment. It should be noted that the indicators in all experimental groups when using urotropin were lower in comparison with the positive control at the end of the experiment. When using 40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml and 2 mg/ml, the cell count was 6.77±0.04 MM/ml, 6.43±0.15 MM/ml, 6.6±0.07 MM/ml, 6.23±0.04 MM/ml, 6.27±0.15 MM/ml.

Data on the use of urotropin in the cultivation of *Chlorella vulgaris* in open literature are absent or limited to single reports. Possible prospects for the use of urotropin are based on the following:

1. Urotropin has antimicrobial activity, so adding it to the culture medium can help preserve biomass from microbial contamination [37];
2. There are reports that urotropine/glutathione can reduce the cytotoxic effect of sulfur mustard on cultured SVK-14 human keratinocytes in vitro [38]. Studies aimed at studying the ability of urotropin to reduce the cytotoxic properties of various compounds seem to be relevant.

The results of determining the optical density of the suspension showed no statistically significant changes in the experimental groups compared with the control. Optical density data are consistent with total cell counts. The difference in the average value of optical density was calculated, an increase in the index was observed in all experimental groups. Thus, these concentrations of urotropin did not have a toxic effect on the *Chlorella vulgaris* cell culture after daily exposure.

Significant changes in cell diameter were noted with the use of urotropin. Additional studies are required to determine the mechanisms of changes in cell morphology under the influence of urotropin.

Table 9. The results of the study of the cytotoxicity of urotropin for the cell culture of *Chlorella vulgaris* strain GKO

Index	**Concentrations mg/ml**					**PC**	**OK**
	40	**20**	**10**	**5**	**2**		
pH of *Chlorella vulgaris* suspension with preservative	7.5	7.0	7.0	7.0	7.0	7.0	7.5
Total number of cells, MM/ml	6.77±0.04*	6.43±0.15*	6.6±0.07*	6.23±0.04*	6.27±0.15*	7.3±0.07	13.77±0.33

The ratio of dead cells to the total number of cells, %	0.7	0	0	0	0	—	—
Suspension optical density	1.116±0.12	0.856±0.08	0.719±0.05	0.737±0.09	0.801±0.1	0.955±0.04	1.035±0.08
Difference in the average optical density, %	-(16.8)	+(10.4)	+(24.7)	+(22.8)	+(16.1)	—	—
Cell size (diameter), μm	6.12±0.11*	6.64±0.15*	6.46±0.12*	6.5±0.14*	6.47±0.12*	4.98±0.15	5.2±0.1

Note: "*" - statistically significantly different from the indicators of the positive control group at $p \leq 0.05$
-(..) – is used to denote an increase in crop growth;
+(..) - used to indicate the rate of culture suppression;

4 Conclusion

Thus, the cytotoxicity of ascorbic acid, lactic acid, citric acid, acetic acid, sodium chloride and urotropin was analyzed for the cell culture of *Chlorella vulgaris* GKO strain. The following parameters were determined: pH of *Chlorella vulgaris* suspension with preservative; Total number of cells, MM/ml; The ratio of dead cells to the total number of cells, %; Specific growth/death rate; The optical density of the suspension; The difference in the average optical density, %; Cell size (diameter), μm.

It is shown that the use of ascorbic acid as a preservative is meaningless. Although this compound does not lead to mass death of biomass, it stimulates the rate of cell division. The use of lactic acid at working concentrations led to a decrease in the number of cells in the culture; when stained with trypan blue, we detected over 50% of dead cells. The data obtained should not be considered unambiguously from the standpoint of the futility of using lactic acid as a preservative. Additional research and expansion of the line of tested working concentrations is required. Analysis of the action of citric acid showed that when using concentrations of 40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml and 2 mg/ml, a significant amount of dead cells was observed. The data obtained can be explained by that the use of citric acid in working concentrations goes beyond the boundaries of the optimum zone of *chlorella*. An analysis of the literature data showed that citric acid has protective properties in plants in case of intoxication with pollutants. Thus, studies of the effect of citric acid on *Chlorella vulgaris* cell culture should be expanded, using lower concentrations. The results of evaluating the effect of acetic acid on the total number of cells allow us to see the presence of a dose-dependent effect in the change in the number of cells depending on the concentrations of the solution. The data obtained suggest that acetic acid at these concentrations is not applicable as a cell culture preservative, but could potentially have a different value when used in combination with *chlorella*. The results of the application of sodium chloride showed that the studied concentrations did not have a toxic effect on the *Chlorella vulgaris* cell culture after daily exposure. It was noted that the introduction of sodium chloride maintained the number of cells at a level comparable to the positive control at the beginning of the experiment. Thus, sodium chloride should be studied further in the search for promising preservatives. The results of evaluating the effect of urotropin on the total number of cells showed that its introduction maintained the number of cells at concentrations of 20 mg/ml, 5 mg/ml and 2 mg/ml, at a level comparable to the positive control at the beginning of the experiment. Data on the use of urotropin in the cultivation of *Chlorella vulgaris* in open literature are absent or limited to single reports. Directions for further research on urotropin may be related to its antimicrobial activity and protection of cell cults from microbial contamination. In addition, the results of the use of urotropin after a daily exposure showed a slowdown in the rate of cell growth, therefore, studies of long-term exposure to *Chlorella vulgaris* in combination with urotropin are required.

Conflict of interest

All members of the team of authors declare no conflicts of interest

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