

Experimental Research into the Antibiotic Properties of *Chlorella Vulgaris* Algal Exometabolites

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An experimental research into the antibiotic properties of *Chlorella vulgaris* Beijer IPPAS C-2 microalgae exometabolites under different cultivation conditions was carried out. It has been established that the inhibitory effect of triglycerides, O-dialkyl monoglycerides, fatty acids, O-dialkyl glycerol esters and long-chain alcohols on the microflora of wastewater is highly dependent on the intensity and time of illumination. The antibiotic effect of non-polar substances - exometabolites of microalgae of lipid nature varies depending on the intensity and time of illumination.

1. Introduction

The search for ways to reduce the cost of microalgae biomass and to solve the problem of creating sustainable industrial production of microalgae biomass in the last decade has been the focus of attention of researchers around the world (Ma et al., 2018). However, Richardson et al. (2014) have shown that, to date, no cultivation system is economically viable. The use of wastewater for the cultivation of microalgae, despite the difficulties caused by the negative effects of the microflora present in it, is a promising direction for reducing the cost of microalgae biomass. According to a report published by the United Nations in 2017, only 20 per cent of wastewater is treated, while the remaining 80 per cent is discharged into the environment without pre-treatment, thus causing serious harm (WWAP, 2017). Creating technologies for the integrated use of microalgae to provide humans with valuable renewable feedstock, as well as to purify wastewater could be a potentially attractive approach to address these problems.

One of the problems in the industrial-scale cultivation of microalgae is the degeneration of strains from contaminants in photobioreactors (Roux et al., 2017). One of the useful properties of some microalgae species is their ability to produce substances exhibiting antibiotic effect (Amaro et al., 2011).

Pratt et al. (1944) noticed the ability of microalgae to release antibiotic agents in the course of their life activity. It was found that microalgae *Chlorella vulgaris* and *Chlorella pyrenoidosa* exhibit antibiotic properties against Gram-positive and Gram-negative organisms such as *Staphylococcus aureus*, *Streptococcus pyogenes* (scarlet fever agents), *Bacillus subtilis* (hay bacterium), *Bacterium coli* and *Pseudomonas pyocyanea* (*Ps. aeruginosa*, or blue pus bacillus). It was also established that the appearance of antibacterial properties depended on illuminance.

Bacteriostatic effect of culture fluid after cultivation of algae *Scenedesmus obliquus*, *Scenedesmus quadricauda* and *Chlorella vulgaris* on the growth of opportunistic pathogenic microflora *Staphylococcus aureus*, *Citrobacter sp.*, *Pseudomonas sp.*, *Klebsiella sp.* was documented in Maksimova and Sidorova (1986), Zenova et al. (1995), Goldin and Goldina (1999).

Ghasemi et al. (2007) studied the antibacterial activity of *Chlorella vulgaris* microalgae extracted from soil samples taken from rice fields in Iran. Analysis of the results of the study showed that supernatant and methanolic extract from biomass of *Chlorella vulgaris* showed high activity against Gram-positive bacteria, but antibiotic activity against Gram-negative bacteria was insignificant, which could be explained by the fact that the cell wall of these bacteria has a more complex multi-layered structure, which makes it difficult for antibiotic substances to penetrate into the cell.

In their review, Amaro et al. (2011) noted that the substances that cause antimicrobial activity of microalgae pertain to such classes of compounds as indoles, terpenes, acetogenins, phenols, fatty acids, and volatile halogen hydrocarbons.

Ward and Singh (2005) found that microalgae exometabolites inhibit the development of Gram-positive pathogenic bacteria and this action is associated with the presence of polyunsaturated fatty acids (eicosapentaenoic and hexadecatrienic acids) in the culture fluid. The exact mechanisms of action of fatty acids remain unknown. Desbois et al. (2009) suggest that fatty acids are directly related to the peroxidative process, causing the mitochondria to age and disrupting the respiratory chain. The degree of antibacterial properties depends on the length of the carbon chain and the level of their unsaturation.

It can be concluded that the influence of microalgae cultivation conditions on the chemical composition of the culture fluid and its effect on the life activity of symbiotic associations of opportunistic and pathogenic microorganisms has not been sufficiently studied. Microalgae of the genus *Chlorella vulgaris* were chosen for the study, as they have one of the best indicators of adaptation to adverse conditions of cultivation and rate of biomass accumulation.

The aim of this work was to study the antibiotic properties of exometabolites of *Chlorella vulgaris* microalgae, as well as to determine the modes of microalgae cultivation, in which they have the maximum bactericidal effect on the microflora of wastewater.

2. Methods and materials

This research used the strain *Chlorella vulgaris* Beijer IPPAS C-2, obtained at the Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences. All measurements were made at three times repetition.

2.1 Experiment 1: Identification of *Chlorella vulgaris* microalgae exometabolites with antibiotic effect.

Microalgae cultivation was performed on Tamiya OPTIMUM medium (Dvoretzky et al., 2015). Nitrogen was added to the nutrient medium on the fifth day of cultivation. The process was carried out under the following conditions: 1) the seed material was 10% of the total suspension volume (cell titre - 180000 cells / mL); 2) the pH value was set within the range of 6.2...8.0; 3) in all experiments the suspension was bubbled with a gas-air mixture with a carbon dioxide content of 0.03 % and a flow rate of 80 L / h. Sampling of culture fluid was carried out on the eighth day of cultivation (stationary phase).

Centrate was separated from microalgae biomass using a Sigma 2-16 RK/2-16P centrifuge at a rotation speed of 4000 rotations / min for 5 minutes. Extraction was performed with the use of petroleum ether as a solvent. The solvent was distilled using a rotary evaporator IR-1 M3 at a temperature of distillation 85 °C and the speed of rotation of the flask 65 min⁻¹. Qualitative determination of substances in the extracts of culture fluid was carried out using the method of thin-layer chromatography with the help of densitometer (Kates, 1986). "Petroleum ether-diethyl ether-acetic acid" mixture in the ratio 90:10:1 (vol.) was used as a system of solvents. Sulphuric acid was a carrier substance.

The analysis of fatty acids contained in the culture fluid of microalgae was carried out using a gas chromatograph "Crystallux-4000M". The total bacterial number (TBC) of wastewater was determined by the Koch method.

Meat and peptone agar (MPA) nutrient media were used. The volume of the sample taken for measurement was 0.1 mL. The plates were incubated at 37 °C for 24 hours. The grown colonies were counted using the STEGLER SCM-2 microbial colony counter.

To determine the sensitivity of wastewater microorganisms to the action of extracellular microalgae metabolites, the disc method was used (CLSI, 2012). Municipal wastewater containing microorganisms (0.07 million cells / mL) is seeded on the surface of a Petri dish with MPA medium. After the drying of the suspension of microorganisms on the surface of the agar, it is placed on a disk with a substance that exhibits antibiotic properties. In the experiment, paper disks with the corresponding spots of substances were analysed. The presence or absence of antibiotic properties of the substance to the culture of microorganisms of wastewater was estimated by the phenomenon of growth delay around the disk after incubation in the thermostat. A petroleum ether-treated disc was used as control sample.

Identification of lipids in microalgae cells was performed by staining lipids (Vyas and Chhabra, 2017) with the fluorescent Red Nile dye, the samples were studied with the help of the microscope Micromed 3 - Lum.

Incubation was performed under the following conditions (illuminance level / photoperiod (light / darkness) / temperature): sample 1 - 7 kLx, 24/0 h for 24 h, 37 °C, sample 2 - 7 kLx, 12/12 h for 24 h, 37 °C; sample 3 (control) - incubation in darkness for 24 h at 37 °C.

2.2 Experiment 2. Determination of the influence of cultivation conditions on the antibiotic properties of microalgae exometabolites in different growth phases.

Cultivation conditions similar to experiment 1 were realized. Microalgae cultivation modes are given in Table 1.

Table 1: Experimental conditions

Mode	Illuminance, kLx	Temperature, °C	Light/darkness, h
1	7	20	24/0
2	7	20	16/8
3	7	30	24/0
4	7	30	16/8
5	21	20	24/0
6	21	20	16/8
7	21	30	24/0
8	21	30	16/8

Sampling of culture fluid was carried out during the exponential growth phase, stationary phase and dying out phase in order to identify antibiotic substances. Calculation of microalgae biomass concentration in the process of cultivation was carried out by the method of direct cell count in the Goryayev chamber.

3. Results and discussion

It has been established that the culture fluid of microalgae contains substances of lipid nature: triglycerides (1), O-dialkyl monoglycerides (2), fatty acids (3), long-chain alcohols (4), O-dialkyl esters of glycerol (5). The following fatty acids were identified in the culture fluid: 1) saturated - myristin (C14:0), pentadecan (C15:0), palmitic (C16:0), margarine (C17:0), stearic (C18:0); 2) unsaturated - margarinoic (C17:1), oleic (C18:1), erucic (C22:1).

The largest zone of inhibition of wastewater microflora growth (Figure 1) was observed for O-dialkyl monoglycerides (substance 2), while the change in the photoperiod from 24/0 h to 12/12 h led to a 2.0-fold decrease in antibiotic effect. In the absence of illumination O-dialkyl monoglycerides display low antibiotic activity, and their effect diminishes by 5.7 times in comparison with the sample illuminated for 24 h. In the 24/0 h photoperiod, triglycerides and fatty acids had an inhibitory effect on bacterial activity (substances 1, 3 in Figure 1), in the 12/12 h photoperiod, the antibiotic effect of triglycerides and fatty acids decreased by 12.1 and 2.7 times respectively. In the absence of illumination the antibiotic effect of triglycerides and fatty acids was insignificant, respectively 21.25 and 4.64 times lower.

Long-chain alcohols and O-dialkyl glycerol esters (substances 4, 5) showed the least antibiotic effect. At decrease in illuminance level O-dialkyl esters of glycerol did not produce death of bacteria. The inhibitory effect of long-chain alcohols depended on illumination and was six times lower in the absence of illumination.

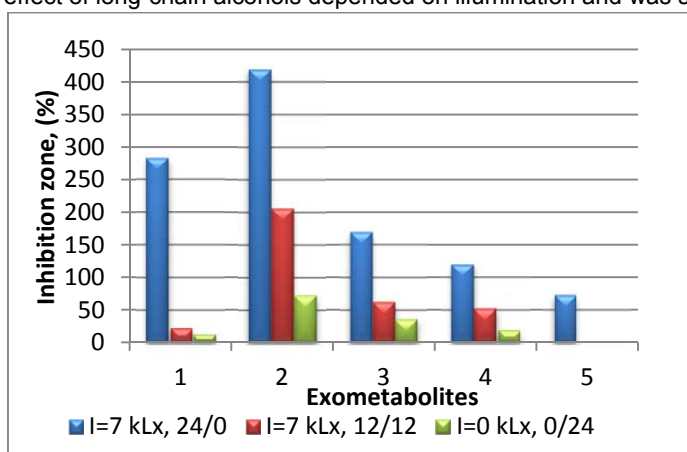
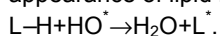


Figure 1: Antibiotic effect in different "light/darkness" photoperiods

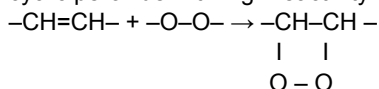
Analyzing the chemical structure of microalgae exometabolites, we can conclude that the antibiotic effect of triglycerides, O-dialkyl monoglycerides, fatty acids, O-dialkyl glycerol esters and long-chain alcohols (which are likely to have double bonds) depends on the level of illumination. It can be assumed that the flow of

photons initiates the formation of reactive oxygen species (photooxidative stress) that interact with lipidic substances, which leads to the appearance of lipid radicals:

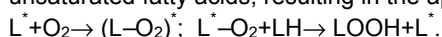
photon flux initiates the formation of reactive oxygen species, which interact with substances of lipid nature: the interaction of conjugated fatty acids with HO^* и HO_2^* (active oxygen species) occurs, which leads to the appearance of lipid radicals.



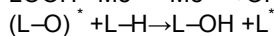
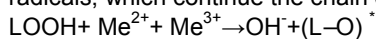
Long-chain alcohols (most likely unsaturated) will be oxidized at the site of double bonds with formation of cyclic peroxide with high reactivity:



Lipid radicals react with oxygen molecules to form peroxy radicals that interact with new molecules containing unsaturated fatty acids, resulting in the appearance of lipid peroxides:



Lipid hydroperoxides in interaction with transition metals in the culture fluid or inside cells also turn into active radicals, which continue the chain of lipid oxidation:



The high speed of these reactions suppresses the antioxidant system of bacteria, so large quantities of the formed lipid radicals violate the structure of molecules of proteins and nucleic acids, which leads to a metabolism disorder and cell death.

Thus, microalgae exometabolites oxidize under the influence of light and initiate oxidation of cell membrane lipids, and the resulting active radicals also disrupt the metabolism of bacteria that make up the microflora of wastewater. This process is described by the theory of lipid peroxidation of Bach-Engler and branching chain reactions of Semenov (Rubin, 2017).

According to the results of experiment 1, it can be concluded that the main components of the extract of microalgae culture fluid suppress the activity of symbiotic associations of opportunistic and pathogenic microorganisms at the illuminance level of 7 kLx, and it is important to note that the quantitative and qualitative chemical composition of the culture fluid will vary greatly depending on the phase of cultivation of microalgae cells. Therefore, it can be assumed that the magnitude of the antibiotic effect will depend significantly on the phase of microalgae cultivation.

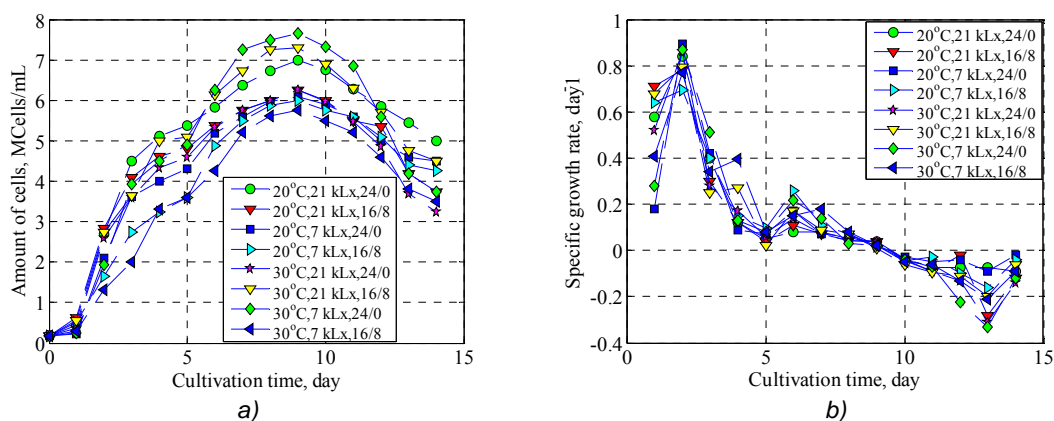


Figure 2: Kinetics of microalgae cultivation (experiment 2)

In the course of experiment 2 the character of kinetic curves of microalgae growth at periodic cultivation has been established: the phase of exponential growth is observed from 1st to 7th day inclusive; the phase of stationary growth lasts from 7th to 9th day, the phase of dying out from 9th to 14th day (Figure 2a, 2b). The maximum concentration of microalgae cells was achieved under the following conditions of cultivation: 30 °C, 7 kLx, 24/0 h, and amounted to 7.7 million cells/mL (Figure 2a); under the same conditions, the maximum specific cell growth rate of 0.895 days⁻¹ was observed (Figure 2b). The chemical composition of microalgae exometabolites changed during cultivation: the greatest inhibitory effect on the microflora of wastewater was observed at the minimum specific growth rate (Figure 2a, 2b) at the stationary growth phase (7th day of cultivation (Figure 2.a)). This can be explained by the fact that when the stationary phase is reached, the culture fluid contains the minimum amount of nitrogen and phosphorus, which in turn leads to the creation of

stressful conditions for cultivation (Soru et al., 2019), accompanied by the cessation of cell division, chlorophyll decomposition, and slowdown of the protein biosynthesis process. Cellular metabolism shifts towards the formation of lipid substances, the concentration of which increases in cells and culture fluid, which can be observed in Figure 3.

The analysis of Table 2 shows that the greatest antibiotic effect of exometabolites was observed during cell cultivation at low illuminance level (7 kLx), temperature 20 °C and photoperiod 24/0 h. This can be explained by the fact that at low levels of temperature and illuminance a greater number of unsaturated compounds of lipid nature is formed in the culture fluid, while at high temperature and illuminance level the biosynthesis of lipid compounds containing saturated fatty acids is observed.

Table 2: Antibiotic effect of microalgae culture fluid

Sampling phase	Cultivation modes, effect on wastewater microflora*							
	1	2	3	4	5	6	7	8
Exponential	+	+	-	-	+	+	-	-
Stationary	+++	+++	+	++	++	+++	+	+
Dying out	++	++	+	+	+	++	+	+

* +++ strong antibiotic effect, ++ medium antibiotic effect, + low antibiotic effect, - no antibiotic effect

This is consistent with Khoeyi et al. (2012), in which it was determined that the maximum percentage of monounsaturated and polyunsaturated fatty acids from the total amount of fatty acids was observed at low illuminance level and illumination period of 8 hours a day; and with experiments of Lynch and Thompson (1982), which established that a decrease in the cultivation temperature leads to an increase in the amount of unsaturated fatty acids.

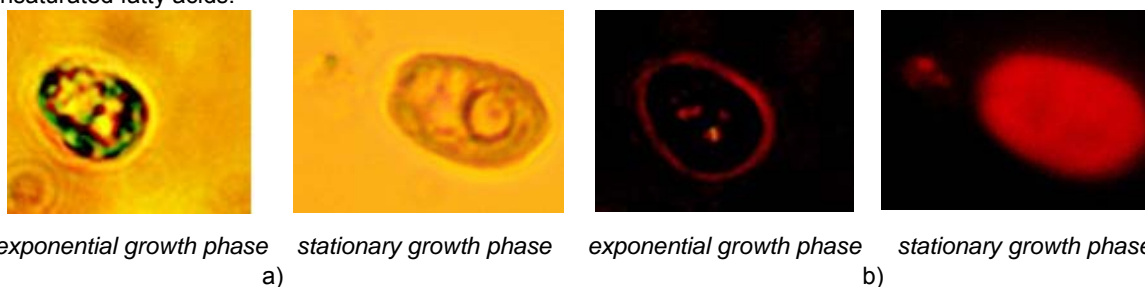


Figure 3: Microscopy of *Chlorella vulgaris* microalgae cell at the: (a) in visible light; (b) lipid fluorescence at 500-550 nm

The high content of saturated fatty acids in microalgae culture fluid compounds reduces the antibiotic effect due to the fact that saturated fatty acids are not oxidized, unlike unsaturated fatty acids. This results in the formation of fewer lipid radicals and, consequently, has less impact on the microflora of wastewater.

The low antibiotic effect on the exponential growth phase (4th day of cultivation (Figure 2a, 2b)) can be explained by the fact that during this period there is an active growth of cells, so the number of lipid compounds in the culture fluid is smaller, since cell metabolism is focused on protein biosynthesis and reproduction processes. Decrease of antibiotic effect at the phase of cell dying out is connected with the fact that the number of viable cells and their metabolism decreases, that is why the rate of biosynthesis of lipid-type exometabolites and their release into the culture fluid becomes low.

4. Conclusions

The inhibitory effect of monoglycerides, O-dialkyl diglycerides, fatty acids, O-dialkyl glycerol esters and long-chain alcohols on the microflora of wastewater depends to a large extent on the intensity and time of illumination. At the illuminance level of 7 kLx and photoperiod 24/0 h, the antibiotic effect of these substances increases in average by 2-5 times in comparison with the sample incubated in the dark. The greatest antibiotic effect of *Chlorella vulgaris* microalgae exometabolites on the microflora of wastewater is manifested at a cultivation temperature of 20 °C, the illuminance level of 7 kLx photoperiod 24/0 h at the stationary growth phase.

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