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Антибактериальный и фунгицидный потенциал хитозана

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Аннотация. Введение. *Изучение антибактериального и фунгицидного потенциала хитозана является перспективным направлением исследований и имеет достаточно высокое прикладное значение в области применения хитозана, как технологического вспомогательного материала в технологиях вин, пива и напитков брожения с целью элиминации потенциальных мутеобразующих компонентов биологической этиологии.* ***Материалы и методы.*** *Научная задача исследования – оценить антибактериальную и фунгицидную активность хитозана. Определение бактерицидной активности хитозана в отношении E. coli осуществляли методом поверхностного посева на питательную среду Эндо, селективную в отношении БГКП.* ***Результаты и обсуждение.*** *Определение фунгицидной активности в отношении Saccharomyces cereviviae осуществляли методом поверхностного посева на питательную среду сабуро с хлорамфениколом, селективную в отношении дрожжевых грибов.* ***Заключение.*** *Хитозан характеризуется антибактериальной и фунгицидной активностью, выявлено, что добавление хитозана даже в минимальном количестве обеспечивает фунгицидный эффект в отношении тест-культуры дрожжевых грибов S. cereviviae и позволяет значительно усилить бактерицидный эффект в отношении тест-культуры бактерий E.coli.*

Ключевые слова: мутеобразующие компоненты биологической природы, антибактериальная активность хитозана, фунгицидная активность хитозана.

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Research article

Antibacterial and fungicidal potential of chitosan

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Abstract. Introduction. *The study of the antibacterial and fungicidal potential of chitosan is a promising area of research and has a fairly high applied value in the field of using chitosan as a technological auxiliary material in wine, beer and fermented beverage technologies to eliminate potential components of biological mutagenesis.* ***Materials and methods.*** *The scientific objective of the study is to evaluate the antibacterial and fungicidal activity of chitosan. Determination of the bactericidal activity of chitosan against E. coli was carried out by surface inoculation on Endo nutrient medium selective for coliforms.* ***Results and discussion.*** *Determination of fungicidal activity against Saccharomyces cereviviae was carried out by surface inoculation on Sabouraud nutrient medium with chloramphenicol selective for yeast fungi.* ***Conclusion.*** *Chitosan has antibacterial and fungicidal activity. It has been established that the addition of chitosan even in minimal quantities provides a fungicidal effect in relation to the test culture of yeast fungi S. cereviviae and allows to significantly enhance the bactericidal effect in relation to the test culture of bacteria E. coli.*

Key words: turbidity-forming components of biological nature, antibacterial activity of chitosan, fungicidal activity of chitosan.

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Introduction. The positive charge of chitosan imparts numerous and unique physiological and biological properties to this biopolymer in a wide range of industries, such as pharmacology, medicine, ecology, agriculture, and the food industry. Chitosan and its modifications can be used as an antibacterial and fungicidal agent against various types of fungi and bacteria, which undoubtedly has significant practical significance [1, 7, and 9]. Numerous studies have demonstrated the influence of the molecular weight and concentration of chitosan on antibacterial and antifungal activity. Thus, it has been established that chitosan, depending on the type and concentration, inhibits mycelial growth in *Penicillium digitatum* and *Penicillium italicum,* it was revealed that the best fungicidal activity on mycelium is observed in media supplemented with low-molecular-weight chitosan. Some studies show a higher fungicidal effect due to increased chitosan concentrations (0.5–2.0%). Chitosan and its derivatives are considered universal biopolymers for use in agriculture as antimicrobial compounds. [2, 7].

The antioxidant and antimicrobial activity of chitosan has led to its use as a preservative in some food products, particularly those prone to lipid oxidation or spoilage, against common foodborne pathogens including *Staphylococcus aureus and Escherichia coli* in milk, apple juice, etc. [1, 7]. Scientists have successfully synthesized a group of new water-soluble ammonium salts of chitosan with halogens, including chitosan-bromoacetate, chitosan-chloroacetate, chitosan-dichloroacetate, chitosan-trichloroacetate, and chitosan-trifluoroacetate, and investigated their antifungal activity on three types of phytopathogens by measuring hyphae in vitro. Fungicidal evaluation showed that the synthesized chitosan derivatives possessed higher antifungal activity than chitosan, and the order of antifungal activity corresponded to the electronegativity of various halogen-substituted groups. Substituted groups with stronger electronegativity could increase the positive charge density of cationic amino groups by withdrawing more electrons from the cationic amino groups of chitosan ammonium salts, demonstrating that protonation of amino groups is important for the antifungal activity of chitosan derivatives [3, 8].

The effect of chitosan products obtained from shrimp shells with medium-viscosity molecular weights (22 to 387 kDa) on their antimicrobial activity against various phytopathogenic bacteria and fungi was studied in vitro and compared with standard high-molecular-weight chitosan (846 kDa). Antibacterial activity was assessed against *Agrobacterium tumefaciens, Erwinia carotovora, Corynebacterium fascians, and Pseudomonas solanacearum.* Antifungal activity against *Alternaria alternata, Fusarium graminearum, F. oxysporum, F. solani, Phytophthora infestans and Rhizoctonia solani,* as the effective concentration of

chitosans causing 50% inhibition of mycelial growth ranged from 480 to 3037 mg/l depending on the tested fungus and the molecular weight of chitosan products [4].

Chitosan nanoparticles were obtained by adding anionic proteins isolated from *Penicillium oxalicum cultures* to chitosan solutions, which were characterized by high antifungal activity. The chitosan nanoparticles were evaluated for their ability to inhibit the growth of phytopathogens, namely *Pyricleria grisea, Alternaria solani, and Fusarium oxysporum.* Chickpea seeds treated with chitosan nanoparticles showed positive morphological effects, such as increased germination percentage, seed viability index, and seedling vegetative biomass. The authors' results indicate that chitosan nanoparticles can be used in the field to protect various crops from destructive fungal pathogens, as well as as growth promoters. [5].

In gram-positive bacteria, chitosan non-covalently binds to teichoic acids embedded in the peptide glycan layer. Teichoic acids on the surface play an important role in cell division and other fundamental aspects of gram-positive bacterial physiology. The functions of teichoic acids can be divided into three main groups: protection against environmental stress, control of enzyme activity, and regulation of cation concentration in the cell membrane. The electrostatic interaction of chitosan with teichoic acids likely disrupts the functioning of the acids themselves, which in turn leads to disruption of the cell's functioning. Therefore, it can be concluded that the primary mode of action is associated with electrostatic interactions between chitosan and teichoic acids, which can disrupt various functions, leading to cell death [6].

Chitosan acts by two mechanisms in Gram-negative bacteria: chelating various cations, which disrupts the integrity of the cell wall and impairs the absorption of essential nutrients; and electrostatic interactions between chitosan and the anionic moieties of lipopolysaccharides on the outer membrane. Research shows that chitosan disrupts the inner membrane, leading to leakage of intracellular material. Furthermore, chitosan has been shown to penetrate bacterial cell membranes, suggesting that chitosan may interfere with DNA/RNA synthesis and trigger intracellular reactions. Electrostatic interactions between cell surface anions and chitosan are important factors determining chitosan's antimicrobial activity against fungi and bacteria. Although electrostatic interactions are undoubtedly fundamental, chitosan is also able to non-covalently bind to cholesterol, indicating that there may be other non-covalent interactions that also play a role in antimicrobial activity alongside electrostatic ones [6].

Unlike gram-positive and gram-negative bacteria, the differences between chitosan-sensitive and chitosan-resistant fungi are smaller. First, chitosan affects the cell membrane through electrostatic interactions with negatively charged phospholipids. When the cell membrane is disrupted, chitosan is able to penetrate the cell, which can lead to the inhibition of DNA/RNA synthesis and disruption of protein synthesis. However, for chitosan-resistant fungi, the biopolymer is unable to penetrate the cell membrane and remains on its outer surface. This contrasts with chitosan-sensitive fungi, which experience membrane disruption along with leakage of the intracellular matrix due to chitosan penetration. The reason why chitosan is unable to destroy the cell membrane of chitosan-resistant fungi is due to the difference in the fluidity of the cell membrane; a study of the phospholipid-fatty acid composition of the cell membrane of chitosan-sensitive and chitosan-resistant fungi showed that an increase in the activity of chitosan is associated with a higher amount of unsaturated fatty acids in the cell membrane; this indicates that membrane fluidity affects the activity of chitosan and its mode of action largely depends on the type of fungi [6].

The aim of the study was to evaluate the antibacterial and fungicidal activity of chitosan samples obtained by acid-base hydrolysis.

Materials and methods. The bactericidal activity of chitosan with a deacetylation degree of 92-97% (solution in 0.1% acetic acid) against *E. coli* was determined by surface inoculation on Endo nutrient medium selective for coliform bacteria. A solution of chitosan in 0.1% acetic acid was pre-mixed with a 1-day bacterial culture in the following ratios: 1:1; 0.1:1; 0.25:1; 0.50:1; 0.75:1. 0.1 cm^3 of each mixture was added to the surface of the medium, previously

poured into Petri dishes (Figure 1), and triturated with a Drigalski spatula. The dishes were turned upside down and thermostatted at 37 ^{0} C for 24–72 hours.

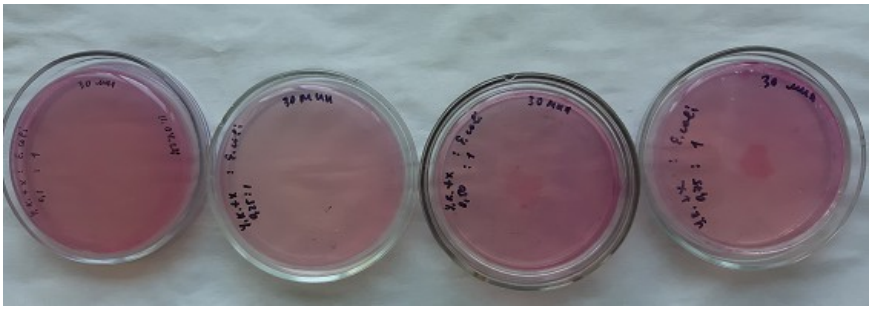
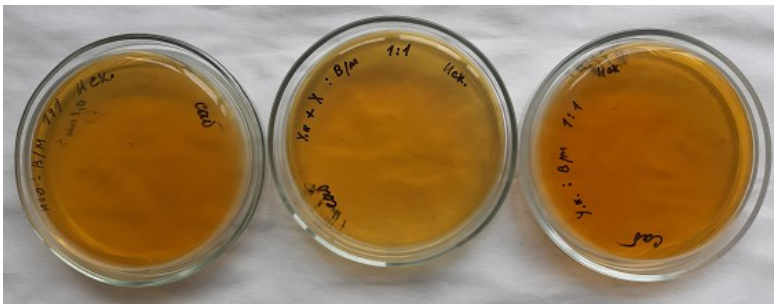


Figure 1. Dishes with Endo medium

E. coli suspensions with sterile water and *E. coli suspensions with 0.1% acetic acid* were used as control samples. After completion of cultivation, dark crimson colonies with a metallic sheen were counted.

Determination of the fungicidal activity of chitosan (solution in 0.1% acetic acid) against *Saccharomyces cereviviae* was grown by surface inoculation on a Sabouraud nutrient medium containing chloramphenicol, which is selective for yeast fungi. A solution of chitosan in 0.1% acetic acid was pre-mixed with a suspension of yeast fungi in 10% apple wine material in the following ratios: 1:1; 0.1:1; 0.25:1; 0.50:1; 0.75:1. 0.1 cm^3 of each mixture was added to the surface of the medium, previously poured into Petri dishes (Figure 2), and ground with a Drigalski spatula. The dishes were turned upside down and thermostatted at 25 ^{0} C for 5 days.



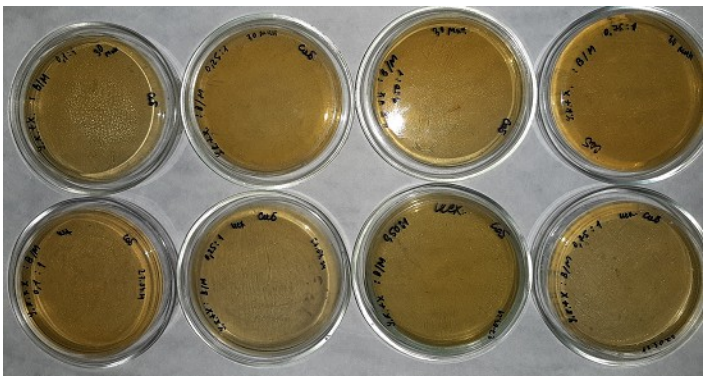


Figure 2. Petri dishes with Saburo medium

S. cereviviae suspension with sterile water and *S. cereviviae suspension* with 0.1% acetic acid were used as control samples. After completion of cultivation, smooth cream- *colored colonies were counted.*

Study results. In a preliminary inoculation with a 1:1 ratio of components, colony growth was observed in all Petri dishes inoculated with the initial suspensions, but after a 30-minute exposure, *E. coli colonies* were detected only in the suspension with H2O (Figure 3).

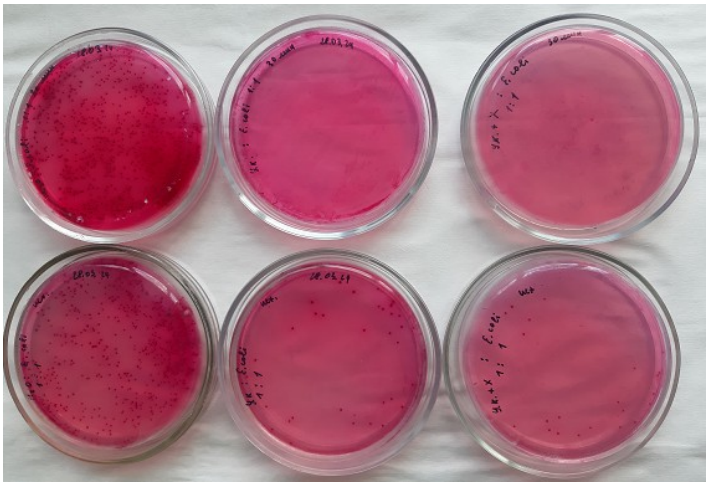


Figure 3. Macroscopic picture of the initial mixtures sown on Endo medium.

In the initial acetic acid suspension, the bacterial count was an order of magnitude lower than in the aqueous suspension (Table 1). Adding chitosan to the acetic acid further halved the number of viable *E. coli cells* in the first 2-3 minutes after mixing.

Table 1 – Dynamics of the number of bacteria in various *E. coli suspensions* in a 1:1 ratio

Variants of *E. coli* suspension components	Observation options	
	Original	In 30 minutes
In H_2O	7.77×10^{3} CFU/cm^{3}	9.27×10^{3} CFU/cm^{3}
In 0.1% acetic acid	4.50×10^{2} CFU/cm^{3}	0 CFU/ ^{cm3}
In 0.1% acetic acid with chitosan	2.20×10^{2} CFU/cm^{3}	0 CFU/ ^{cm3}

In 30-minute mixtures of *E. coli suspensions* with chitosan and acetic acid, cell death occurred (Figure 3, Table 1), while in the control mixture with H2O, the bacterial count increased by 19% compared to the initial mixture due to bacterial proliferation. After 72 hours, the culture results remained unchanged, confirming the bactericidal effect (Figure 4).

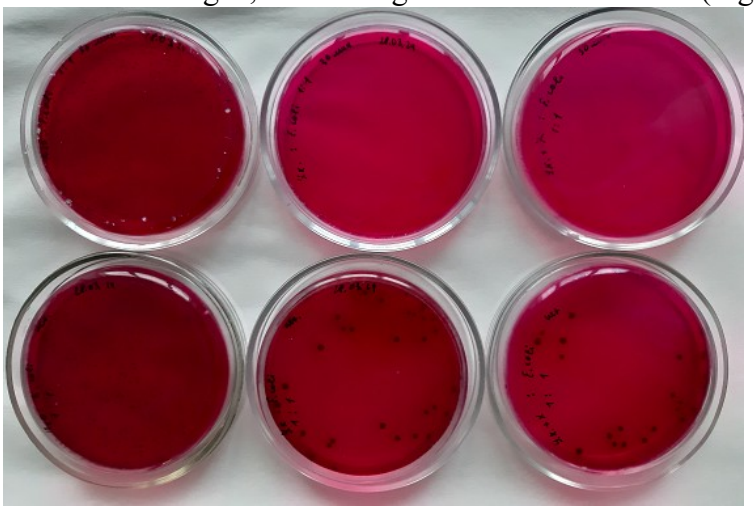


Figure 4. Macroscopic picture of seeding on Endo medium of 30-minute mixtures

In the second stage of the study, a series of mixtures were created in which the amount of chitosan solution increased from 0.1 parts to 0.75 parts relative to the volume of *E. coli culture.*

It was found that cell death occurred within the first 2-3 minutes after the mixtures were created when 0.1 and 0.25 parts were added to the *E. coli culture* chitosan solution (Figure 5).

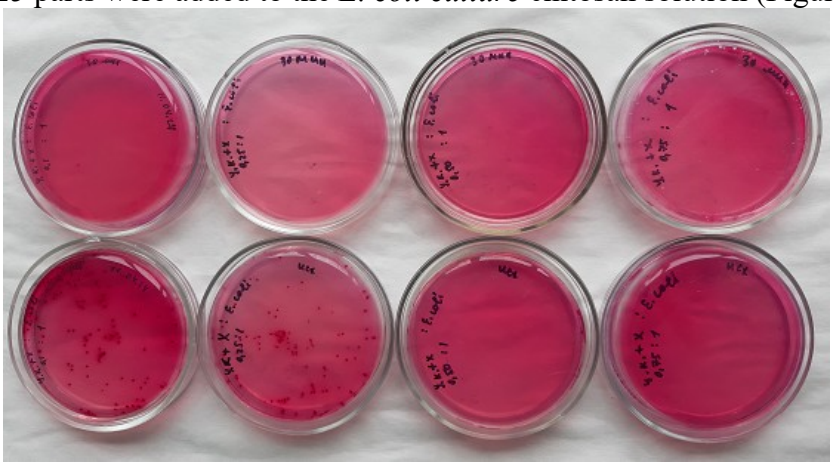


Figure 5. Growth pattern of *E. coli colonies* on Endo medium in suspensions treated with chitosan

After 30 minutes, in a mixture with 0.25 parts of chitosan solution, complete death of *E. coli cells also occurred*, and in a mixture with 0.1 parts of chitosan solution, their number decreased by 54 times (Table 2).

Table 2 – Dynamics of the number of bacteria in E. coli suspensions in *0.1* % acetic acid with chitosan

Ratio of suspension components ((0.1% acetic acid + chitosan) *: E. coli)*	Observation options	
	Original	In 30 minutes
0.1:1	1.04×10^{3} CFU/cm^{3}	20 CFU/ cm^{3}
0.25:1	9.80×10^{2} CFU/cm^{3}	0 CFU/ cm^{3}
0.50:1	0 CFU/ cm^{3}	0 CFU/ cm^{3}
0.75:1	0 CFU/ cm^{3}	0 CFU/ cm^{3}

In control mixtures with H2O, colony growth was observed on all Petri dishes (Figure 6).

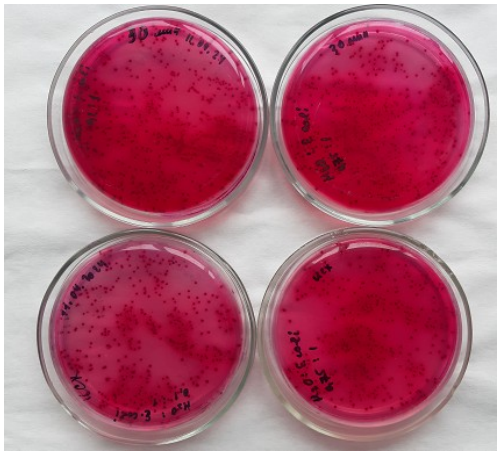


Figure 6. Growth pattern of *E. coli colonies on* Endo medium in seeding from mixtures with H2O

The number of colonies after 30-minute exposure became 18–21% higher than in the culture from the initial mixtures (Table 3).

Table 3 – Dynamics of bacterial numbers in *E. coli suspensions* in $_{H2O}$

suspension components (H_2O : *E. coli*)	Observation options	
	Original	In 30 minutes
0.1:1	5.41×10^3 CFU/cm^3	6.55×10^3 CFU/cm^3
0.75:1	4.85×10^3 CFU/cm^3	5.73×10^3 CFU/cm^3

In control mixtures with acetic acid, colony growth was observed in all sowing variants immediately after mixing and in three variants after 30-minute exposure (Figure 7).

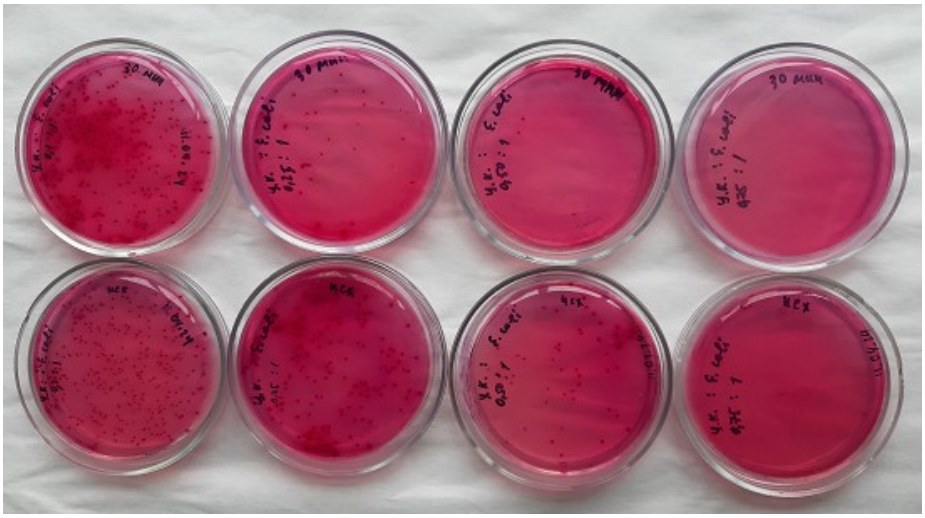


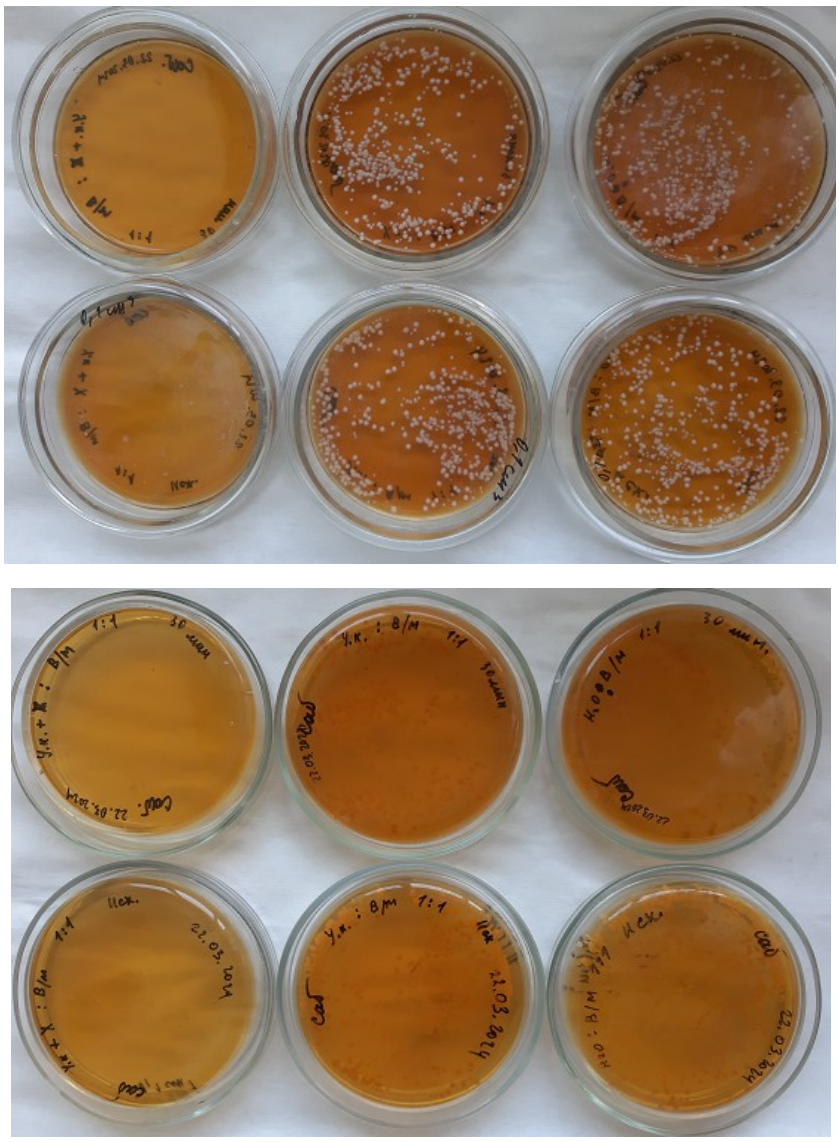
Figure 7. Growth pattern of E. coli colonies on *Endo* medium in seeding from mixtures with acetic acid

It was found that a solution of 0.1% acetic acid added to a suspension of *E. coli* in the amount of from 0.1 to 0.75 parts allows the number of bacteria in mixtures to be reduced by 1.4–40 times after 30-minute exposure (Table 4).

Table 4 – Dynamics of bacterial numbers in *E. coli suspensions* in 0.1% acetic acid

Ratio of suspension components (0.1% acetic acid: E. coli)	Observation options	
	Original	In 30 minutes
0.1:1	4.53×10^3 CFU/cm^3	3.15×10^3 CFU/cm^3
0.25:1	1.52×10^3 CFU/cm^3	3.40×10^2 CFU/cm^3
0.50:1	5.70×10^2 CFU/cm^3	30 CFU/ ^{cm3}
0.75:1	40 CFU/ ^{cm3}	0 CFU/ ^{cm3}

In preliminary sowing to determine the fungicidal activity of chitosan (solution in 0.1% acetic acid) against *Saccharomyces cereviviae cultures* with a 1:1 component ratio revealed that, in both the initial mixtures and the 30-minute exposure mixtures, yeast colony growth was observed only on the control plates containing H2O and acetic acid. Yeast colonies were absent from both cultures from mixtures containing a chitosan solution in acetic acid (Figure 8).



Macroscopic picture of the initial mixtures sown on Saburo medium

Sseries of mixtures were compiled in which the amount of chitosan solution increased from 0.1 parts to 0.75 parts relative to the volume of the *S. cereviviae suspension.*

It was established that immediately after the mixtures were prepared, cell death occurred when 0.1 to 0.75 parts of yeast were added to the suspension. Chitosan solution (Figure 9).

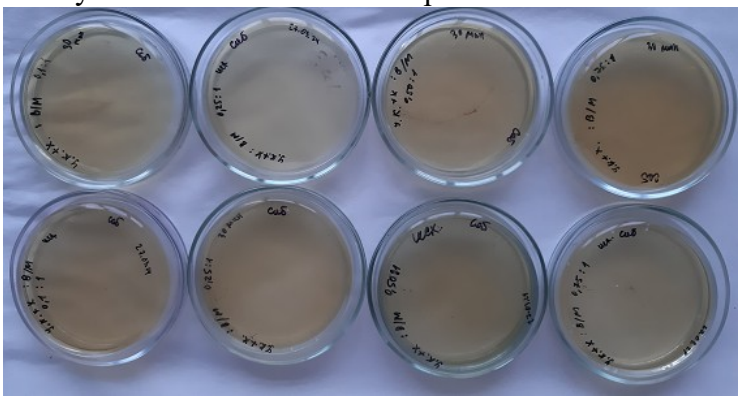


Figure 9. Petri dishes on the 5th day after sowing yeast cell suspensions treated with chitosan.

After 30 minutes of exposure of the mixtures, a similar picture was observed: in mixtures with a chitosan solution, complete cell death also occurred (Figure 9).

In control mixtures with 0.1% acetic acid solution and with H2O (0.1 h: 1 h yeast), colony growth was observed on all Petri dishes (Figure 10).

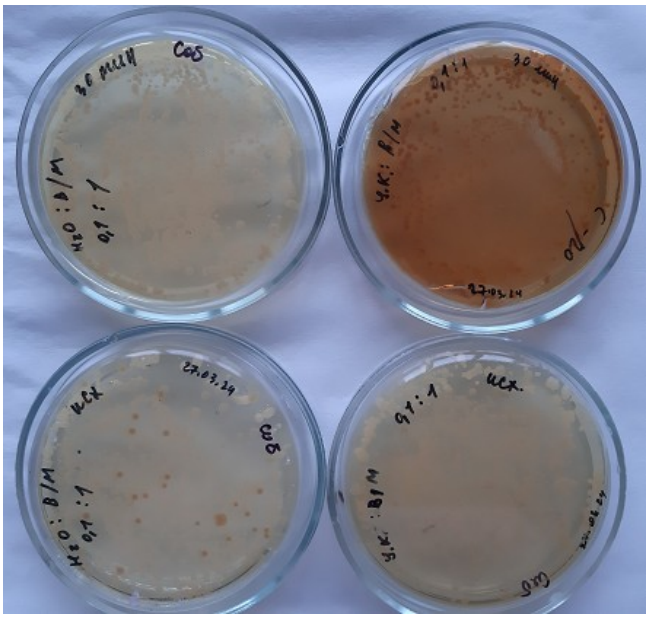


Figure 10. Growth pattern of yeast colonies on Saburo medium in seeding from mixtures with 0.1% acetic acid solution and H2O

The number of colony-forming units in the control mixtures after 30-minute exposure did not change significantly compared to the initial mixtures (Table 5).

Table 5 – Number of yeast fungi in mixtures with H2O $_{and}$ 0.1% acetic acid solution on the 5th day after sowing

Components of mixtures	Observation options	
	Original	In 30 minutes
H2O: S. cereviviae 0.1:1	7.34×10^{3} CFU/cm ^{3}	7.14×10^{3} CFU/cm ^{3}
0.1% acetic acid solution: S. cereviviae 0.1:1	7.35×10^{3} CFU/cm ^{3}	7.04×10^{3} CFU/cm ^{3}

Conclusion. Chitosan is characterized by antibacterial and fungicidal activity. It was found that the addition of chitosan, even in minimal quantities, provides a fungicidal effect against the test culture of yeast fungi *S. c. ereviviae* and significantly enhances the bactericidal effect against the test culture of bacteria *E. coli*.

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