

MICROORGANISMS - INHIBITORS OF ACTIVATED SLUDGE FILAMENT
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Abstract. A collection of microorganisms-inhibitors of filamentous swelling of activated sludge was created. The most promising for inhibiting the growth of filamentous prokaryotes are strains of *Pseudomonas sp. PT2* and *Bacillus sp. FL X-5*. It is shown that the introduction of microorganisms-inhibitors of filamentous prokaryotes has a positive effect on activated sludge at the initial stage of filamentous swelling: the oxidizing power of sludge increases by 10-40%, the efficiency of wastewater treatment increases by 1.8-2.2 times, the sedimentation properties of sludge improve, the number of representatives of the main indicator groups increases by 2 times, the filament index decreases from 3-4 to 1.

Keywords: filamentous bloating, activated sludge, filamentous prokaryotes, inhibitors, filament index, dehydrogenase activity of sludge.

Аннотация. Создана коллекция микроорганизмов-ингибиторов нитчатого вздувания активного ила. Наиболее перспективными для подавления роста нитчатых прокариот являются штаммы *Pseudomonas sp. ПТ2* и *Bacillus sp. FL X-5*. Показано, что внесение микроорганизмов-ингибиторов нитчатых прокариот положительно влияет на активный ил, находящийся в начальной стадии нитчатого вздувания: повышается окислительная мощность ила на 10-40%, увеличивается в 1,8-2,2 раз эффективность очистки сточных вод, улучшаются седиментационные свойства ила, увеличивается в 2 раза численность представителей основных индикаторных групп, снижается филаментный индекс с 3-4 до 1.

Ключевые слова: нитчатое вздувание, активный ил, нитчатые прокариоты, ингибиторы, филаментный индекс, дегидрогеназная активность ила

Annotatsiya. Faol il (loy) *ipsimon bo'rtmalarining ingibitor-mikroorganizmlari to'plami* yaratildi. *Ipsimon prokariotlarning o'sishini cheklovchi eng istiqbolli shtammlar bo'lib Pseudomonas sp. ПТ2 va Bacillus sp. FL X-5 hisoblandi. Ipsimon prokariotlar ingibitor-mikroorganizmlarining kiritilishi ipsimon bo'rtishning dastlabki bosqichida bo'lgan faol il (loy)ga ijobiy ta'sir etishi ko'rsatildi: il (loy)ning oksidlanish kuchi 10-40% ga oshadi, oqava suvlarni tozalash samaradorligi 1,8-2,2 baravar ortadi, il (loy)ning cho'kindi xususiyati yaxshilanadi, asosiy indikator guruhlar vakillari soni 2 barobar ortadi, filament indeksi 3-4 dan 1 gacha kamayadi.*

Kalit so'zlar: *ipsimon bo'rtish, faol il (loy), ipsimon prokariotlar, ingibitorlar, filament indeksi, il (loy)ning dегidrogenaza faolligi.*

Introduction. One of the problems arising in the operation of biological treatment plants is activated sludge bloating. This process leads to deterioration of wastewater treatment quality, as well as to the discharge of activated sludge into water bodies. The main reason is intensive development of filamentous bacteria, which are constantly present in normally functioning activated sludge in insignificant quantity, but under stress influences the number of flocculating bacteria is reduced to a minimum, while filamentous bacteria, due to their high resistance to

unfavorable factors, intensively develop in the aeration tank and displace other representatives of activated sludge biocenosis. Bacterial threads penetrate sludge flakes, make them loose and thus prevent their sedimentation. As a result, the swollen activated sludge is carried out of the secondary sedimentation tanks, thereby degrading the quality of treated water.

Despite the fact that filamentous sludge bloating is the most widespread problem of biological wastewater treatment, there are still no effective methods of its prevention and elimination. More and more attention is paid by researchers to the use of microorganisms with high destructive potential towards organic compounds of different chemical nature, as well as capable of producing surface-active compounds, biopolymers, which can have an inhibitory effect on filamentous prokaryotes. Application of such cultures will allow to develop new environmentally safe technologies for effective wastewater treatment from a number of pollutants, as well as methods of prevention and control of filamentous swelling of activated sludge.

Today, sludge bulking is the most common problem in biological wastewater treatment plants worldwide. However, until recently, there was no generally accepted system of measures for the prevention and suppression of sludge bulking. In the last decade, scientists have actively addressed this problem. The most common methods for controlling activated sludge filament swelling are physical-mechanical, chemical and biological. Various methods have been proposed both for prevention of activated sludge filamentous swelling and for its suppression and further restoration of biocenosis [1-3].

In our opinion, to solve this problem, a promising direction is the use of microorganisms with high destructive potential towards organic compounds of different chemical nature, as well as capable of producing various metabolites that may have an inhibitory effect on filamentous prokaryotes.

The aim of this work was the isolation and screening of microorganisms - inhibitors of filamentous prokaryotes development.

Materials and Methods.

Screening of microorganisms inhibiting the development of filamentous prokaryotes was carried out among museum cultures of the working collection of microorganisms destructors of xenobiotics from the Laboratory of Environmental Biotechnologies of the Institute of Microbiology of the National Academy of Sciences of Belarus, as well as among isolates isolated in the process of work with samples of municipal and industrial wastewater.

Preparation of microorganism suspension for the study of inhibitory effect: one or more isolated colonies of daily culture grown on dense flesh-and-butter agar were picked using a sterile loop and placed in a flask containing 50 ml of E-8 mineral medium with molasses of the following composition g/l: NaCl – 0,5, MgSO₄×7H₂O – 0,8; KH₂PO₄ – 0,7; (NH₄)H₂PO₄ – 1,3; and beet molasses – 5,0. Cultivation was carried out at a temperature of 28 °C, stirring intensity 180 rpm for 48 hours for bacteria of the genus *Bacillus* and 72-96 hours for bacteria of the genus *Rhodococcus*. The titer of the prepared microorganism culture fluids was at least 1×10⁹ CFU/mL.

To evaluate the effect of growth inhibition of test strains of filamentous bacteria, the well method was used. As test-cultures we used the most common for biological treatment facilities of the Republic of Belarus representatives of filamentous bacteria, isolated by us in pure culture: *Leucothrix* sp. NI-5, type 0411 NI-2, type 0803 NI-F. At the end of incubation, the inhibitory activity was evaluated by measuring the diameters of the growth suppression zones of the test strains (taking into account the well diameter). In the presence of an ellipse-shaped zone of growth

suppression, the maximum and minimum diameters were measured and then the average value was found.

Identification of isolated microorganisms-inhibitors of filamentous prokaryotes was carried out by analyzing a fragment of 16S rRNA gene.

The influence of microorganisms-inhibitors of filamentous prokaryotes on activated sludge biocenosis, its destructive activity and sedimentation properties was studied in 1000 ml Erlenmeyer flasks. A mixture of industrial wastewater with initial COD equal to 578 mgO₂/dm³ was used. The dose of activated sludge was 20 vol.%. Culture liquid of bacteria-inhibiting filamentous prokaryotes with a titer of (1,2-2,4)×10⁹ CFU/mL was added at the rate of 1 liter per 1 m³ of wastewater. The experiments were carried out on the 7th, 14th and 21st days. Sludge index, filamentous index (FI), chemical oxygen demand (COD), dehydrogenase activity, and hydrobiological analysis of activated sludge biocenosis were evaluated.

Establishment of key factors determining the ability of bacteria to inhibit filamentous prokaryotes was carried out in model conditions. Wastewater from the poultry farm of RUE «Belorusneft-Osobino» was used, the dose of activated sludge was 20%. The effect on activated sludge of culture liquids of bacteria *Bacillus* sp. FL X-5, *Pseudomonas* sp. PT2 and their supernatants. The dose of bacteria-inhibitor application was 0,1 vol. % (based on 1 L/m³ of effluent). The controls were filamentous index and sludge dose by volume. Drainage was carried out on the 7th, 14th and 21st days.

The sludge index was determined according to FR 1.31.2008.04398 «Methodology of measurement of sludge dose by volume and calculation of sludge index» [4].

The filament index (FI) was used as a quantitative indicator of the presence of filamentous microorganisms in the sludge. This index was determined by comparing the microscopic image of activated sludge with a series of reference photographs of different classes according to the Eikelboom methodology, where the gradation between the index classes (from 0 to 5) is about 10 times [5].

COD was determined photometrically using the analyzer «Expert-003-CDD» according to GOST 31859-2012 [6].

Sludge dehydrogenase activity (DAS) was determined according to the method described in the methodological guide for control of biological treatment of municipal wastewater [7].

Hydrobiological analysis of activated sludge was carried out using a Vizo-103 microimager (Russia). The «calibrated drop» method was used to study free-floating sludge [8]. Microscopy of the agitated sample was performed. The absolute number of organisms in the unit volume of the sludge mixture, as well as the total number of organisms belonging to the main indicator groups were taken into account during the study. Organisms in the studied samples were identified by morphological features according to the guidelines [8].

Statistical processing of the results was performed using Microsoft Excel, determining the arithmetic mean, standard deviation and confidence intervals for the 95% probability level.

Results and Discussion. Screening of microorganisms inhibiting the development of filamentous prokaryotes was carried out among museum cultures of the working collection of microorganisms destructors of xenobiotics of the Laboratory of Environmental Biotechnologies. Eighty-four strains were tested, of which 12 most active and capable of inhibiting the development of filamentous microorganisms were selected. The diameter of the growth inhibition zone of the test cultures varied from 8-46 mm depending on the type of filamentous bacteria.

Among the selected museum cultures, the maximum inhibitory effect against *Leucothrix* sp. NI-5 was shown by strains belonging to the genus *Bacillus*: *Bacillus* sp. 6/2-APF-1, *Bacillus coagulans* 1710, *Azotobacter* sp. FL-9MV, *Bacillus* sp. FL X-5. The diameter of the growth retardation zones of the test culture was 41-46 mm. For the other selected strains this figure was 11-35 mm.

In relation to filamentous bacteria types 0411 NI-2 and 0803 NI-F, the inhibitory effect was much lower. Only 7 museum cultures showed efficacy, and the diameter of growth retardation zones was only 8-24 mm. The most effective inhibited growth of filamentous prokaryotes were strains *Bacillus* sp. FL-9MV and *Bacillus* sp. FL X-5.

The isolation of microorganisms-inhibitors was carried out in the process of work with samples of municipal and domestic wastewater, effluents of organic synthesis enterprises, pulp and paper and meat and dairy industries. Seventy-three isolates were isolated, of which 32 showed inhibitory effect against test cultures of filamentous bacteria. These cultures were isolated: 8 – from wastewater of poultry farm RUE «Belorusneft-Osobino»; 5 – from wastewater of JSC «Paper Factory «Spartak»; 4 – from their municipal and domestic wastewater of Kobrinsky housing and communal services; 6 – from wastewater of Dobrush paper factory «Hero of Labor»; 2 – from municipal and domestic wastewater of Bereza; 4 – from municipal and domestic wastewater of Malorita.

The study of the ability to inhibit the growth of test cultures of filamentous bacteria allowed us to select isolates PT2 and SP5 as the most promising for further studies. These cultures showed the greatest inhibition effect against all three test cultures: *Leucothrix* sp. NI-5, types 0411 NI-2 and 0803 NI-F. The diameter of their growth retardation zones was 12-41 mm.

Based on nucleotide sequence analysis of the 16S rRNA gene fragment, the cultures were identified as *Pseudomonas* sp. PT2 and *Bacillus* sp. SP5.

Hydrobiological analysis of activated sludge is the main stage in studying the influence of microorganisms-inhibitors on its biocenosis. Organisms of biocenosis are sensitive to the slightest changes in the environment and immediately react to it by morphological, physiological, behavioral changes, easily noticeable by microscopy. By evaluating these changes it is possible to quickly identify violations of the purification process. Microscopy of the initial sample of activated sludge revealed the presence of filamentous bacteria types 0914, 1851, 0041, 021N, *Microthrix parvicella*, *Sphaerotilus* spp. The filamentous index was 3-4, which corresponds to the initial stage of bloat. At this index, the sedimentation capacity is markedly reduced, especially in the presence of strong filaments. The sludge flakes were dispersed, fine and friable with low density and blurred boundaries. The activated sludge used was numerically dominated by organisms of three indicator groups: small flagellates, gastropods and roundworm infusoria, indicating high organic matter loads on the sludge and low oxygen content.

During hydrobiological analysis during three weeks we noted a constant change in the number of organisms of indicator groups. On the seventh day of the study in the control sample numerically prevailed representatives of two groups: roundworms and free-swimming infusoria. A large number of them was also observed in all experimental samples. On the 14th day of the study in the control sample and in the sample with added culture of *Bacillus* sp. FL X-5 were dominated by small flagellates, while in all other samples circular infusoria prevailed numerically. The presence of attached barrel-shaped infusoria was found. Infusoria undergo this physiological

change when the load on activated sludge increases. The appearance of gastropod infusoria *Aspidisca costata* was noted in all samples, which indicates the beginning of flake formation.

The changes in the control and experimental samples on the 21st day of the study are the most indicative. In the control sample the numerical predominance remained for roundworm and free-floating infusoria, while in samples with added cultures of *Bacillus* sp. FL X-5, *Pseudomonas* sp. PT2 and *Bacillus* sp. SP5, there was an increase in diversity and even distribution among organisms of indicator groups. An increase in the number of gastropod infusoria *Aspidisca costata*, the appearance of benthic shell amoebae, colonial forms of attached roundworm infusoria of the Peritricha class with an elongated body shape, which complete the process of water purification and perform the functions of filtration agents by consuming non-flake-associated bacteria, which contributes to water clarification, was noted. It should also be noted the occurrence of rotifers in samples with cultures of *Bacillus* sp. FL X-5 and *Bacillus* sp. SP5.

One of the key points of application of microorganisms at biological treatment facilities is the study of their influence on the composition, structure and properties of activated sludge biocenosis. In this regard, an obligatory stage in the study of the possibility of using microorganisms-inhibitors of filamentous prokaryotes was the study of their influence on the destructive activity of activated sludge biocenosis and its sedimentation properties. At the same time, the studied indicators were sludge index, filament index, chemical oxygen consumption, and dehydrogenase activity.

It was found that already on the 7th day the filament index decreased in all studied samples to 2-3. On the 21st day in the samples with the addition of cultures of *Bacillus* sp. FL X-5 and *Pseudomonas* sp. PT2 the filamentous index was 1, while in the rest of the samples FI was at 2-3. In the control sample throughout the entire period of research FI remained unchanged and was 3-4.

The presence of filamentous swelling directly affects the sedimentation properties of activated sludge, which are characterized by the sludge index. Optimal values of sludge index are in the range of 90-120 cm³/g. Lower values are characteristic of sludge with high ash content caused by high mineralization of cellular matter or presence of heavy suspended solids. Increase of sludge index indicates deterioration of sedimentation ability of sludge. At values above 150, the sludge swells, which leads to a violation of separation of the sludge mixture in the secondary settling tanks and removal of suspended solids with constant water.

Initially, the sludge index of all studied samples was 157-172 cm³/g, which indicates the presence of filamentous swelling and is confirmed by filament index 3-4. Analysis of the change in silt index showed that a visible decrease was observed only on the 14th day of the study. A steady further decrease relative to the control was observed in samples with the addition of cultures of *Pseudomonas* sp. PT2 and *Bacillus* sp. SP5. On the 21st day the sludge index amounted to 148-152 cm³/g, respectively, for the control – 164-170 cm³/g.

As it is known, the efficiency of the biological wastewater treatment process is determined by the activity of the enzymatic system of activated sludge organisms. Often industrial wastewater with toxic contaminants is also supplied to urban wastewater. To control the toxicity of incoming wastewater, as well as the oxidizing capacity of activated sludge, it is possible to measure its DAS [7].

When bacteria-inhibiting bacteria of filamentous prokaryotes were applied and effluent was continuously supplied, the DAS on the 7th day was 76,2±2.2-81,9±1,4% in all samples; on

the 14th day – in the control – $61,9 \pm 1,9\%$, and in the other samples studied – $69,2 \pm 0,5$ - $78,0 \pm 1,7\%$. The change in DAS was most pronounced on the 21st day, when wastewater with COD equal to $1017 \text{ mgO}_2/\text{dm}^3$ was used for measurement. DAS in samples containing strains *Bacillus coagulans* 1710, *Bacillus* sp. 6/2-APF1, *Pseudomonas* sp. PT2 and *Bacillus* sp. SP5, was $63,4 \pm 1,8$ - $82,1 \pm 1,0\%$, which was 10-40% higher than the control sample. The introduction of these strains contributed to the improvement of activated sludge condition and its resistance to more polluted effluents, which is also confirmed by the analysis of COD changes in the studied samples. COD values on the 21st day were 15 - $18,3 \text{ mgO}_2/\text{dm}^3$, which is 1,8-2,2 times lower than in the control ($33 \text{ mgO}_2/\text{dm}^3$). It should be noted that the best destructive activity in relation to the analyzed wastewater showed strain *Pseudomonas* sp. PT2. On the 14th day the COD indicator was $180 \pm 9,9 \text{ mgO}_2/\text{dm}^3$, while in the control – $380 \pm 10,5 \text{ mgO}_2/\text{dm}^3$.

The key factors that determine the inhibitory ability of microorganisms against filamentous bacteria are either their high destructive activity or synthesis of metabolites that inhibit the growth and reproduction of filamentous prokaryotes [9].

In this regard, the effect on activated sludge of culture fluids of bacteria *Bacillus* sp. FL X-5, *Pseudomonas* sp. PT2 and their supernatants. The study was carried out in model conditions using wastewater from a poultry farm and 20% sludge.

It was found that on the 21st day of the study the best inhibition of filamentous bacteria occurred in samples with the addition of culture liquid of *Bacillus* sp. FL X-5 and its supernatant. The filamentous index was 1. In samples with *Pseudomonas* sp. PT2 the inhibition of filamentous bacteria was better when culture fluid was added than its supernatant.

Analysis of activated sludge sedimentation showed improved sedimentation properties in the sample with the addition of culture liquid of *Bacillus* sp. FL X-5 by 28% and its supernatant by 14%. In samples with *Pseudomonas* sp. PT2 no significant change in sedimentation properties was observed.

The above data allow us to conclude that the key factors responsible for the suppression of filamentous swelling are: for *Bacillus* sp. FL X-5 – inhibition of filamentous bacterial growth by the metabolites synthesized by it; for *Pseudomonas* sp. PT2 – its high destructive activity and consequently competition for substrate.

Conclusion. A collection of microbial inhibitors of filamentous prokaryotes has been established, among which the most active are *Pseudomonas* sp. PT2 and *Bacillus* sp. FL X-5. It was found that the introduction of microorganisms-inhibitors of filamentous prokaryotes has a positive effect on activated sludge at the initial stage of filamentous swelling: the oxidizing power of sludge increases, the number of representatives of the main indicator groups increases 2 times and the filamentous index decreases from 3-4 to 1. Application of microorganisms-inhibitors of growth of filamentous prokaryotes will allow to develop new environmentally safe technologies of effective wastewater treatment from a number of pollutants, as well as methods of prevention and control of filamentous swelling of activated sludge.

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