

POSSIBILITY OF OBTAINING HYALURONIC ACID FROM CYANOBACTERIA

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Abstract. The results of studies on the possible producing valuable substances from cyanobacterial biomass are presented. It is shown that the main source of hyaluronic acid is the cyanobacteria from surface waters known as *Microcystis aeruginosa* and cyanobacterial associations of actinomycetes. The possibility of hyaluronic acid extraction has been experimentally proven. The dynamics of its quantitative characteristics were determined.

Keywords: hyaluronic acid, cyanobacteria, biomass, biotechnology.

1. Introduction

The problem of natural and technogenic-chemical pollution of the surface of natural waters by specific organic substances produced by aquatic flora, fauna and humans is quite urgent today.¹ Therefore, much attention is paid to the studies focused on the prevention or limitation of the massive expansion of cyanobacteria (CB) in both artificial and natural water reservoirs. Cyanobacteria “blooming” raises the level of toxicity of surface waters, deteriorates the regime of water reservoirs, and inhibits the vital activities of aquatic organisms and inhabitants of adjacent ecosystems. The annual seasonal process of “blooming” and further mortality of aquatic organisms require a thorough analysis of the impact of environmental conditions on water production,²⁻⁵ mathematical modeling of eutrophication process,⁶ development of technological solutions to the environmental problem,⁷

and assessment of the risk of air pollution by products of uncontrolled biodegradation of cyanobacterial biomass.^{8,9}

It has been determined that about 40 representatives of different species of toxic cyanobacteria, including *Microcystis*, *Anabaena*, *Nodularia*, *Nostoc*, *Cylindrospermopsis*, are sources of eutrophication. However, the main accumulator of organic substances during the “blooming” period of the Dnieper is a representative of photosynthetic cyanobacteria – *Microcystis aeruginosa*.⁵ It contributes up to 90% of the biomass in blooming spots, the places with the biggest concentration of cyanobacteria in the reservoir. In addition, cyanobacterial associations of actinomycetes and the actinomycetes *Streptomyces pluricolorescens* and *S. cyaneofuscatus* are sources of blooming in natural surface waters.

The solution of the problem of CB extraction and processing will enable the targeted use of natural biomass sources and their components containing important food, feed, medical, pharmaceutical, perfumery, agricultural and forestry substances.^{2,5} Nowadays, the most widespread and well-developed approach in this area is the use of cultivated biomass as a component of reclamation mixtures.¹⁰⁻¹² However, in this case, the innovation potential of the area is not optimal.^{13,14}

Due to the development of biotechnology since the end of the 20th century, there has been a clear tendency to implement various bioprocesses in industry and replace traditional methods of producing a large number of substances for medical, cosmetic, food and feed purposes with biotechnological production methods. At the same time, the study of the properties and mechanisms of biological action of a number of biopolymers contributes to the production of new products and drugs based on them.

One of these biopolymers of animal and plant origin is hyaluronic acid (HA). This glycosaminoglycan is used in various medical, cosmetic and veterinary products. HA has been used in surgery as a substitute for synovial fluid in joints and as a lubricant and chondroprotective component; in dermatology as a remodeling agent in the correction of age-related deformations of the face, especially of the skin around the eyes, and gynecology.

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The field of application of HA is constantly expanding, which means that the need for this type of biopolymer is growing, and, consequently, the interest in alternative sources of its production is also increasing.

Regarding the above, this study is devoted to the possibilities of extracting hyaluronic acid from cyanobacterial biomass and further development of biotechnology for the production of hyaluronic acid using different associations of actinomycetes, including *Streptomyces* actinomycetes, which are present in the biomass of CB.

2. Experimental

2.1. Materials

Hyaluronic acid is one of the main components of the intercellular matrix of the vertebral connective tissue. It makes a significant contribution to cell proliferation and migration, and may also prevent the formation of tumors. A 70 kg person's body contains on average 15 g of hyaluronic acid, a third of which is renewed daily.¹⁵

Hyaluronic acid – $(C_{14}H_{21}NO_{11})_n$ – an organic compound that belongs to the group of non-sulfated glucose aminoglycans (Fig. 1). The presence of numerous sulfated groups in related glucose aminoglycans causes numerous isomerisms. This phenomenon is not observed in hyaluronic acid, which is always chemically identical, regardless of the methods and sources of production.¹⁶

The HA molecule is characterized by the formation of internal intermolecular hydrogen bonds both within the molecule and between adjacent carbon residues that are at a considerable distance from each other, and in aqueous solution even between adjacent molecules through carboxyl and acetamide groups. HA solutions have $pH < 7$ because of the carboxyl group. The acidic properties of hyaluronate enable the production of water-soluble salts with alkali metals. HA is an anionic linear polysaccharide with a different molecular weight. The molecular weight depends on the method of production; the obtained HA is always chemically identical to natural HA due to the absence of isomerism.

HA is an amorphous polymer. The purified product is a white fine powder. HA macromolecules have a linear

structure and are characterized by a high degree of asymmetry. The molecular weight of HA depends on the method of production and can range from 50-8000 kDa. It is estimated that natural hyaluronic acid in its native state has a molecular weight of 1000-20000 kDa. For example, the average molecular weight of the polysaccharide found in human synovial fluid is 3000-3500 kDa.¹⁷

HA is a hydrophilic polymer and is characterized by a high sorption capacity for water molecules. In the presence of water, hyaluronic acid forms elastic, resilient (soft) gels, absorbing 10 000 times its volume of water. Due to its hydrophilic properties, HA regulates the water balance of the tissue. High hydrophilicity determines the widespread use of the polysaccharide in cosmetics.¹⁸ The main materials from which hyaluronic acid is produced are animal raw materials and biomass. It should be noted that the composition of the substrate of animal raw materials (Table 1) and the biomass of the CB have much in common. The extraction of HA from animal raw materials is often complicated by the fact that in the tissues and organs of mammals and birds (e.g., in chicken combs), the biopolymer exists in a complex with proteins, proteoglycans, and, in addition, related glycosaminoglycans are often present in animal raw materials.¹⁵

Therefore, CB biomass can be used to produce hyaluronic acid.

2.2. Methods

Traditional methods of producing HA are based on the extraction of the biopolymer from various mammalian and bird organs, such as the vitreous of the cattle eye, chicken combs or newborn umbilical cords. However, the raw material base for the industrial production of this polysaccharide is limited and cannot fully satisfy the ever-growing demand for HA. In addition, the availability of these raw materials may be seasonal or irregular. Furthermore, there is always a risk of being infected with nonspecific viruses and other infectious pathogens. The extraction of HA from animal raw materials is often complicated by the fact that the biopolymer exists in a complex with proteins.

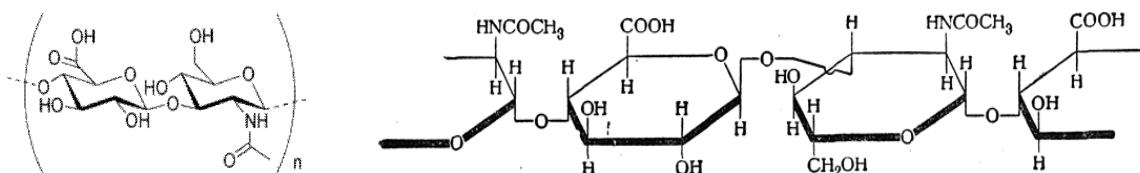


Fig. 1. The chemical formula of hyaluronic acid¹⁶

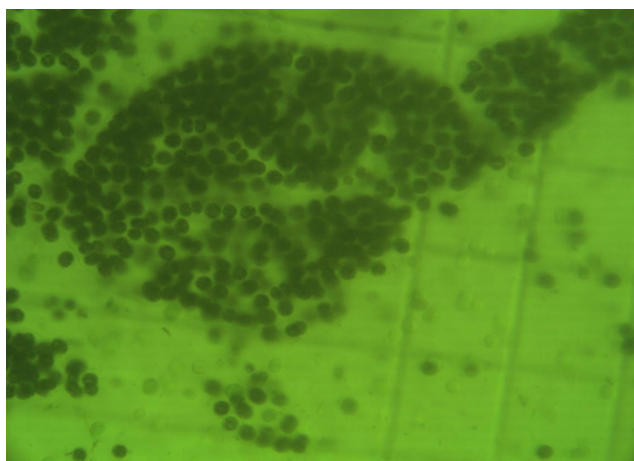
Table 1. The chemical content of chicken combs and cyanobacteria biomass^{16,18-20}

Chicken combs		Cyanobacteria biomass	
Macronutrients		humid	dry
Mg	46.8 mg/kg	130 mg/dm ³	105 mg/dm ³
K	393.7 mg/kg	115 mg/dm ³	120 mg/dm ³
Ca	102.3 mg/kg	70 mg/dm ³	30 mg/dm ³
Na	2500.9 mg/kg	undefined	undefined
P	undefined	0.4 %	6.6 %
Micronutrients			
Fe	46.3 mg/kg	1.49 %	1.69 %
Zn	1.90 mg/kg	–	–
Ck	5.60 mg/kg	–	–
Mn	0.19 mg/kg	1.14 %	1.46 %
Co	0.1 mg/kg	–	–
Content of amino acids, vitamins and fatty acids			
Vitamins (thiamine, ascorbic and pantothenic acids)	9.1–11.3 %	95–263 ‰	15–72 ‰
Essential Amino Acids: Valine, Threonine, Tyrosine	7.9–9.1 %	undefined	undefined
Total Saturated Fatty Acids	13.76 %	380 mg/dm ³	43 mg/dm ³
Humidity	87.6 %	100 %	12–15 %
pH	6.4	–	–
Gluconic acid racemate	–	98	91
Lactose	–	21	<20
Galacturonic acid	–	25	<20
Xylose	–	<20	<20
N-acetyl-D-glucosamine	–	30	<20
Glycerin	–	36	<20

The biotechnological method of HA production, based on the cultivation of appropriate microorganisms-producers, is free of these disadvantages. The ability to synthesize HA is provided by cultures of various microorganisms (*Streptococcus*, *Pasteurella* and *Streptomyces*) and some species of microalgae of the genus *Chlorella*. Hyaluronic acid (HA) is a biotechnological product obtained by biological fermentation.¹⁴ There are known methods of HA production, which are divided into two groups: the physico-chemical method, which involves the extraction of hyaluronic acid from animal tissues, and the microbiological method of HA production based on bacterial producers.

The microbiological method of HA production, based on the *Streptomyces* producers, enables its relatively easy purification and fractionation by molecular weight in the absence of HA-related proteins. Therefore, biotechnological HA products have higher quality indicators. As can be seen from Table 1, the chemical composition of the cyanobacterial biomass of the species *Microcystis aeruginosa* (Fig. 2) is more suitable for the production of hyaluronic acid compared to animal raw materials. CB biomass contains carbon as the main component for construction of the HA structure, glucose, and has a high content of proteins, including enzymes (23.0-82.6%), carbohydrates (6.6-70.0%), and lipids (2.0-

12.0%), which include polyunsaturated fatty acids, as well as vitamins: B1 (thiamine), B2 (riboflavin), B3 (vitamin PP - nicotinamide), B6 (pyridoxal, pyridoxine), B7 (biotin), B9 (folic acid), vitamins C, A, E, K, minerals (iron, magnesium, calcium, boron iodine, zinc, copper).¹⁵ All these components are catalysts for the release of HA.

**Fig. 2.** *Microcystis aeruginosa*

Our attention was focused on comparing the technology of producing hyaluronic acid from animal raw

materials and the possibility of using the waste water (digestate) after separation of cyanobacterial biomass as a fermentation material. The main material for fermentation is various mammalian and bird organs, the vitreous of the cattle eye, chicken combs, newborn umbilical cord, and actinomycetes microorganisms. The supporting material is water after the removal of excess cyanobacteria biomass for biogas production using *Actinomyces* bacteria based on *Streptomyces* producers.

Hyaluronic acid is soluble in water and in an aqueous solution of sodium chloride, insoluble in organic solvents, which was taken as the basis for the extraction of HA from the digestate of CB biomass.

Experimental studies were carried out in two directions:

- a) extraction of hyaluronic acid directly from CB biomass;
- b) production of hyaluronic acid from CB digestate.

In both cases, the experiments were conducted under the same conditions: samples of 500 cm³ were taken. In the first case, the samples were settled for 120 hours to ensure complete biomass settling. The samples were filtered, acetate buffer, to maintain the acidity of the medium, and 2 dm³ of sodium chloride solution of the appropriate concentration were added (Table 2).

In the second case, acetate buffer and 2 dm³ of sodium chloride solution of the appropriate concentration were also added directly to the digestate. After 5-6 hours, we observed the start of turbidity of the solution, which increased when the samples were kept at low temperatures (from 0 to -60°C). Gradually, a slightly yellowish gel-like substance precipitated at the bottom (Figs. 5, 6).

3. Results and Discussion

Experimental studies of the digestate of CB biomass for the presence of bacteria of the genus *Actinomyces*, which are a source of hyaluronic acid, were carried out in the laboratory. These bacteria were detected in the water remaining after the separation of CB biomass. They are absent after biomethanogenesis, because under methanogenic fermentation, the main mass of *Actinomyces*, including *Streptomyces*, dies. They are aerobic he-

terotrophs, requiring nutrients and molecular oxygen. Under anaerobic conditions of methanogenesis, all components of HA synthesis are converted into methane, carbon dioxide and hydrogen.²

Moreover, the possibility of obtaining hyaluronic acid after lipid extraction from CB biomass²¹ and after its cavitation treatment was studied.²² As it was expected, we failed to extract hyaluronic acid. There are several reasons for this. Firstly, hyaluronic acid forms complexes with lipids, and after their extraction by cavitation or laser treatment, not only the complexes but also the chemical bonds of biopolymers are destroyed with the formation of mono- and dimolecules of glucose aminoglycan. During direct cavitation treatment of CB biomass, not only are organic biopolymers destroyed, but also molecules of saturated organic fatty acids involved in the stabilization of "hyaluronic acid-lipid" complexes. In the process of electrochemical treatment of CB biomass,²³ further studies also did not identify the presence of hyaluronic acid.

It should be noted that cyanobacterial actinomycete associations and actinomycetes, which are present in small quantities in natural waters, play an important role in the synthesis of HA. They get into the water from the places of initial soil formation through underground aquifers of sedimentary carbonate rocks, where actinomycetes participate in stabilizing the bacterial block of the system and enhance the photosynthetic activity of algae. Obviously, actinomycetes as associative symbionts have a positive impact on the ecosystem as a whole, due to their stimulating effect on the nitrogen-fixing capacity of cyanobacteria and increased protection of the entire system from pathogens through the release of antibiotics in the form of waste products.

HA and its derivatives are macromolecular polysaccharide biopolymers consisting of repeating single units - disaccharides. Acetylglucosamine and glucuronic acid alternate in the complex hyaluronic acid molecules, which are the structural components of the polymer. HA is synthesised by a type of built-in membrane proteins, *i.e.*, enzymes called hyaluronate synthetases. The hydrolytic degradation of HA with complete acid hydrolytic cleavage of hyaluronic acid produces β -D-glucuronic acid, β -N-glucosamine, and acetic acid (Fig. 3).²⁴

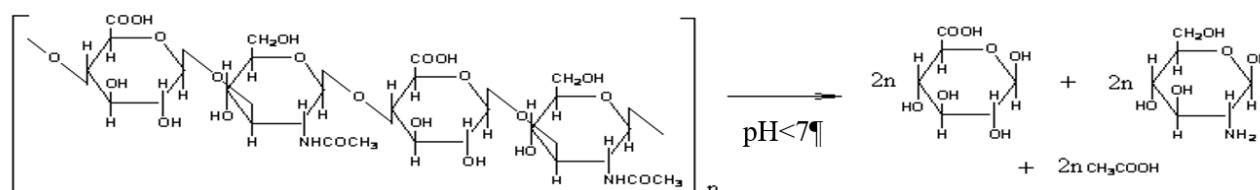


Fig. 3. Hydrolytic destruction of hyaluronic acid¹⁶

Enzymes within the hyaluronidase class cause the biodegradation of HA. They are divided into two main types: testicular and bacterial hyaluronidases. The products of enzymatic degradation of HA under the action of heat-resistant testicular hyaluronidase are tetra-oligosaccharides (consisting of two elementary links of the hyaluronic acid macromolecule connected by a β -(1-

4)-glycosidic bond), and under the action of bacterial hyaluronidase - disaccharides (β -N-glucosamine, β -D-glucuronates) (Fig. 4).

The results of obtaining hyaluronic acid from digestate CB are shown in Table 2.

The experimental results are shown in Figs. 5 and 6.

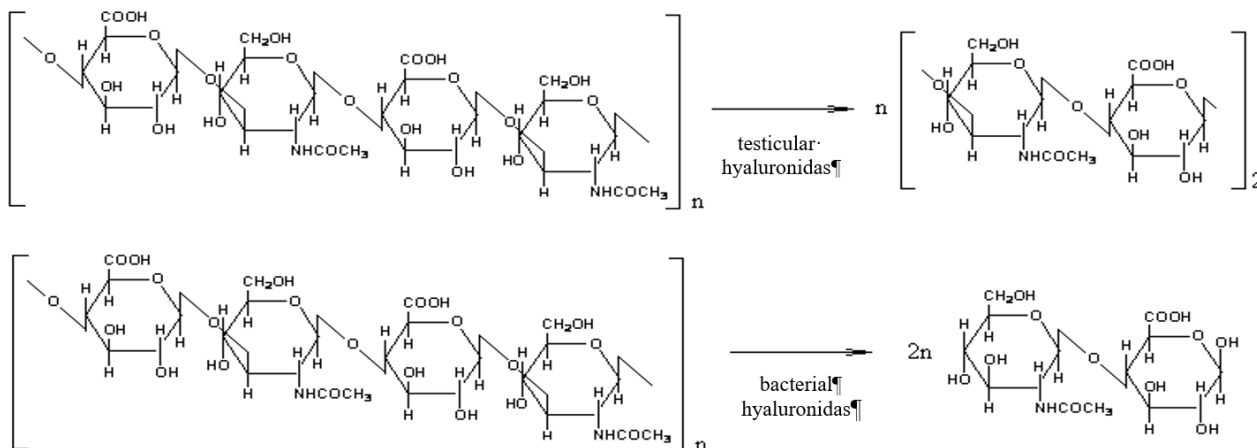


Fig. 4. Enzymatic biodegradation of hyaluronic acid¹⁶

Table 2. The results of obtaining hyaluronic acid from digestate CB

C NaCl, %	Hyaluronic acid mass, 10 ⁻³ g									
	Formation time of hyaluronic acid, hours									
	12	16	20	24	28	30	36	48	60	72
2	3.72	3.77	3.82	3.9	3.92	3.96	4.01	4.05	4.08	4.12
5	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
8	4.48	4.5	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
12	4.75	4.77	4.8	4.82	4.85	4.87	4.9	4.92	4.95	4.97
15	5	5.03	5.05	5.08	5.1	5.13	5.15	5.18	5.2	5.23
18	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.5
20	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
25	3.16	4.2	4.8	3.9	5.17	4.9	4.65	4.7	4.8	5.1

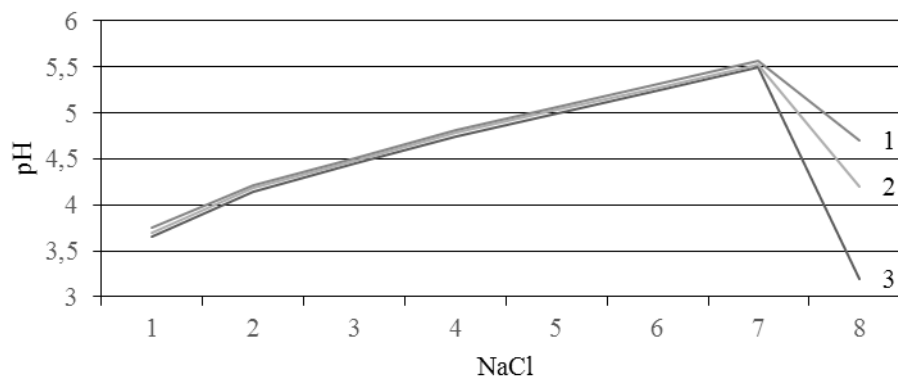


Fig. 5. The dependence of hyaluronic acid formation on the concentration of sodium chloride over time: 1 – 12 hours; 2 – 16 hours; 3 – 24 hours

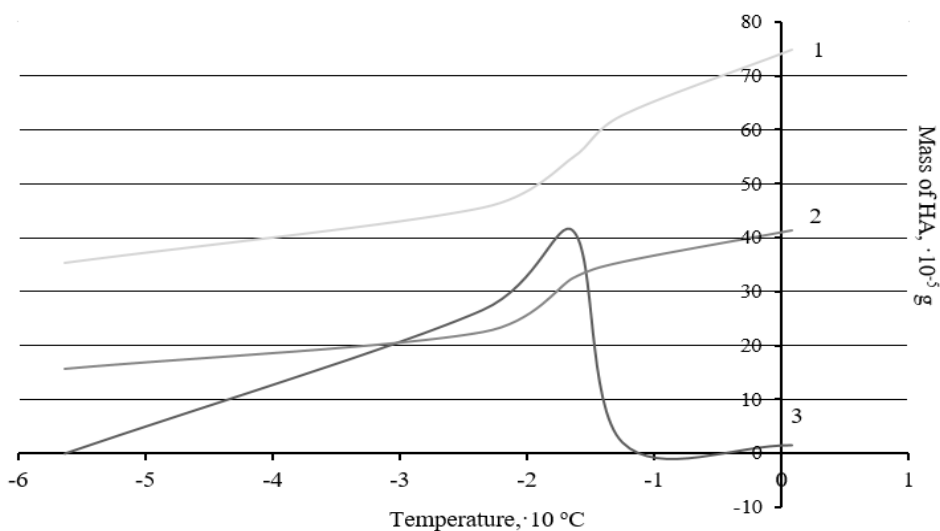


Fig. 6. The dependence of hyaluronic acid formation on the temperature of sodium chloride solution deposition: 1 – 20 %; 2 – 15 %; 3 – 25 %

The substance was identified by comparison with a hyaluronic acid product used in cosmetology. Thin-layer chromatography in the methanol: water: butanol (1:4:1) system showed similar *R* values in the range of 0.65-0.71. The obtained hyaluronic acid is not a pure product, as it may be a lipid conglomerate with hyaluronic acid capsules, coordination complexes with water molecules due to high sorption capacity, and organic substances of various structures that are present in natural waters as products of algae. Since hyaluronic acid is capable of forming salts with metal ions, an attempt was made to extract HA in the form of the corresponding salts with solutions of potassium chloride and sulfate, as well as calcium chloride. It was found that it was not possible to extract hyaluronic acid using potassium ions. More optimal are solutions of calcium chloride with a concentration of 5-20%. In this case, the consistency of

the resulting HA changes to a partially powdery consistency, although the gel-like properties are also preserved.

As can be seen from the obtained results, the cooling temperature of the solution significantly affects the formation of hyaluronic acid. A temperature above zero, especially above 10°C, promotes the breaking of oxygen-hydrogen bonds in the biopolymer and intermolecular hydrogen bonds. As a result, di- and monoglucose-amino glycans are formed (Fig. 3). The sudden decrease in HA output in the temperature range of -18°C at a calcium chloride solution concentration of 25% cannot be clearly explained (Fig. 7). It is possible that the so-called "glass transition" of molecules, which is typical for natural biopolymers, plays a role. At lower concentrations of calcium chloride solution, this effect is not observed, so the HA output also increases.

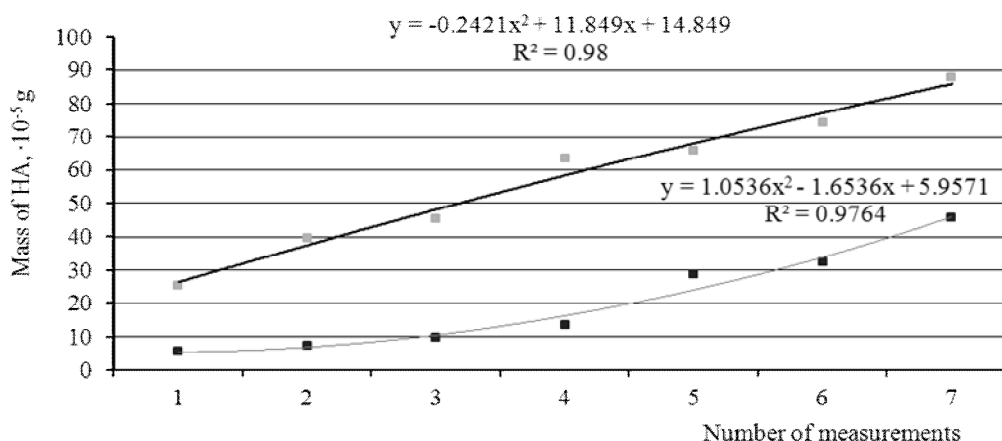


Fig. 7. The dynamics of hyaluronic acid output in calcium chloride solution

When sodium chloride is used instead of calcium chloride, the picture changes significantly. With an increase in the solution concentration from 15 to 25%, the HA output also increases. The nature of the curves can be described by the following dependence:

$$M_{HA} = \pm aC^2 \pm bC + c,$$

where C is the concentration of the calcium chloride solution (the accuracy of the approximation $R^2 = 0.97-0.98$).

The stability of calcium salts of hyaluronic acid can be explained in terms of the energy of the Ca-O ionic bond and changes in the spatial configuration of glucose rings. However, this point requires further research, since changes in the spatial arrangement of structural fragments can affect biological activity, either enhancing or neutralizing it.

4. Conclusions

The possibility of extracting hyaluronic acid from cyanobacterial biomass and its digestate as a component of natural surface waters during periods of intensive algal blooms has been experimentally proven. It has been shown that the main source of hyaluronic acid is the cyanobacterium *Microcystis aeruginosa* and cyanobacterial actinomycete associations *A. variabilis* and actinomycetes *Str. pluricorens* and *S. Cyano-fuscatus*. At the same time, no technological equipment is required at the stage of hyaluronic acid extraction.

The possibility of hyaluronic acid extraction has been experimentally proven. The dynamics of its quantitative characteristics is presented. A study was conducted to determine the dependence of hyaluronic acid extraction on the concentration of sodium and calcium chloride solutions. It is shown that the optimal conditions for the use of sodium chloride in the precipitation of hyaluronic acid are a concentration of 15-20% at a reaction medium temperature of (-5) to (-18) °C. The use of calcium chloride solutions increases the output of hyaluronic acid by 1.5-1.7 times in the temperature range of 0 - (-10°C).

The obtained samples of hyaluronic acid were compared with its true solutions used in cosmetics using thin-layer chromatography. In the process of processing cyanobacterial biomass, it was found that methanogenesis processes destroy molecules not only of hyaluronic acid but also the simplest glucose aminoglycans. To increase the output of hyaluronic acid from cyanobacterial biomass, neither laser treatment to extract lipids nor cavitation is suitable. This is due to the weakness of chemical bonds in the glucose-amino glycan chains of the hyaluronic acid molecule itself.

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МОЖЛИВІСТЬ ОТРИМАННЯ ГІАЛУРОНОВОЇ КИСЛОТИ З ЦІАНОБАКТЕРІЙ

Анотація. Представлено результати досліджень можливості отримання цінних речовин з біомаси ціанобактерій. Показано, що ціанобактерії природних вод *Microcystis aeruginosa* та ціанобактеріальні асоціації актиноміцетів є основним джерелом гіалуронової кислоти. Експериментально підтверджена можливість вилучення гіалуронової кислоти. Визначено динаміку її кількісних характеристик.

Ключові слова: гіалуронова кислота, ціанобактерії, біомаса, біотехнологія.