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ПОВЕРХНЕВО-АКТИВНІ КОМПЛЕКСИ ДЛЯ ШТАМУ *PSEUDOMONAS AERUGINOSA JRV-L*

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Показано, що штам *Pseudomonas aeruginosa* JRV-L синтезує екстрацелюлярні поверхнево-активні речовини рамноліпідної природи, полімер полісахарид альгінат та пігмент піовердин, що має властивості сидерофора та елісатора. Встановлено, що вказані продукти біосинтезу *P. aeruginosa* JRV-L мають властивість утворювати супрамолекулярні комплекси у культуральній рідині. Отримані супрамолекулярні комплекси рамноліпід-альгінат JRV-L-1 і рамноліпід-альгінат-піовердин JRV-L-2 мають високу емульгуючу, піноутворювальну активність і вищий біологічний ефект порівняно з очищеними рамноліпідами. Крім того, присутність пігменту піовердіна з антиоксидантною активністю у складі супрамолекулярного комплексу JRV-L-2 значно посилює вплив на клітини рослин і тварин і захищає препарати від окиснення і сприяє їх довготривалій стабільності.

Ключові слова: біогенні поверхнево-активні речовини, рамноліпіди, полісахариди.

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SURFACE-ACTIVE COMPLEXES OF THE STRAIN *PSEUDOMONASAERUGINOSAJRV-L*

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It was shown that the strain *Pseudomonas aeruginosa* JRV-L synthesizes extracellular surfactants of rhamnolipid nature, the polymer polysaccharide alginate and pigment pyoverdine which has the properties of siderophore and plant elicitor. It was established that these biosynthesis products of *P. aeruginosa* JRV-L have the ability to form supramolecular complexes in the culture liquid. The resulting supramolecular complexes rhamnolipid-alginate JRV-L-1 and rhamnolipid-alginate-pyoverdine JRV-L-2 with high emulsifying, foaming activity and have higher biological activity compared to purified rhamnolipids. In addition, the presence of pigment pyoverdine with antioxidant activity as part of supramolecular complex JRV-L-2 significantly enhances the effect on the cells of plants and animals, protects the product from oxidation and promotes its long-term stability.

Key words: biosurfactants, rhamnolipids, polysaccharides.

Problem statement. The development of new ecologically safe products is of great importance both for protecting the environment and for reducing the risk to human health associated with the widespread use of chemical products in industry, agriculture and medicine.

Analysis of recent searches and publications. Rhamnolipids are glycolipids that are synthesized by certain types of soil microorganisms, such as *Pseudomonas aeruginosa* strains. Microorganisms usually produce rhamnolipid mixtures containing mono- and dirhamnolipids, the molecules of which have one or two units of rhamnose, as well as lipid chains of varying length (one or two residues of β -oxydecanoic acid).

The patent EP153634 describes a composition of the mixture with the balanced weight ratio of mono- and dirhamnolipids [1].

The patent EP0499434 discloses in Example 3 rhamnolipid mixtures which contain balanced in a weight ratio of two components: mono-rhamnolipids and dirhamnolipids [2].

The characteristics of the surface activity of mono-rhamnolipids and dirhamnolipids and mixtures thereof are described, for example, in Karpenko et al. (1996) [3].

Patent DE 19628454 B4 describes the isolation of the complex formed by rhamnolipids and polymer alginate, which are the products of the biosynthesis of the strain *Pseudomonas aeruginosa*. Rhamnolipids are a mixture of mono- and dirhamnolipids. Isolation of the complex is carried out by concentrating the supernatant or freeze-drying or using special reagents. The resulting complex is proposed for the use in washing of various fabrics, cleaning surfaces and cleaning animals and birds from oil contamination as a result of oil spills [4].

U. S. Patent 5656747 discloses a method of concentrating and purifying glycolipids, one stage of which is carried out by acidifying the glycolipid solution (culture liquid supernatant of the strain *Pseudomonas aeruginosa*) to $\text{pH} \leq 5.0$ followed by heating to 60–130 °C and cooling to ≤ 50 °C. The precipitate was kept par excellence at the temperature 20-30 °C. Subsequently rhamnolipids were extracted from the obtained precipitate with organic solvents [5].

The patent CA 2457993 A1 proposes the prevention of the oxidation of lipids in foods using compositions comprising of the effective amounts of antioxidants-siderophores (including pyoverdine) and organic acids [6].

The disadvantage of the invention, described in the patent DE 19628454 B4 is the absence of evidence of rhamnolipids-alginate complex formation, as well as the absence of safety studies for the rhamnolipids-alginate complex to human health and the environment; the possibility of its wide application, including in the various preparations was not shown [4].

The aim of the work was the investigation of surface-active products of the strain *Pseudomonas aeruginosa* JRV-L for the determination of physico-chemical and biological properties of supramolecular complexes.

Experimental part. The strain *P. aeruginosa* JRV-L synthesizes rhamnolipids, alginate polymer and pigment pyoverdine, which are the target products of the microbial synthesis, on the mineral nutrient medium with glycerol.

Cultivation of the strain was carried out in the fermenter with volume 1000 liters for 125 h, cultivation temperature – 30 °C.

The composition of the nutrient medium (g/l): NaNO_3 – 0,4; $\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$ – 0,2; KH_2PO_4 – 0,12; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ – 0,5; sodium citrate – 5,0; glycerol – 50,0; yeast extract – 1,0; pH – 6,8-7,0.

Depending on the cultivation conditions the ratio of mono- and dirhamnolipids in culture liquid may vary from 50:50 to 90:10, which allows the production of a preparation with the desired properties.

Pyoverdine content in the culture liquid ranges from 50 to 700 mg/l, depending on the cultivation conditions. The pyoverdine content was determined according to the method [7].

Depending on the cultivation conditions alginate content in the culture liquid of the strain *P. aeruginosa* JRV-L amounts from 1 to 5 g/l.

Depending on the fermentation conditions the culture liquid of *P. aeruginosa* JRV-L comprises of 50 to 100 g/l of supramolecular complex rhamnolipids-alginate JRV-L-1.

Depending on the fermentation conditions the culture liquid of *P. aeruginosa* JRV-L comprises of 50 to 100 g/l of supramolecular complex rhamnolipids-alginate-pyoverdine JRV-L-2.

Surface-active biocomplex was isolated from the cell-free culture liquid by acid precipitation (10 % HCl solution) at pH 3,0. The precipitate was kept for 12 hours at 4 °C, separated by centrifugation (8000 rpm, 20 min.), dried under vacuum to constant weight.

Rhamnolipids were isolated via extraction with Folch mixture (chloroform / methanol 2: 1) from biocomplex, followed by the evaporation of solvents from the extract under vacuum [8].

The content of polysaccharides was determined according to DuBois method [9].

Surface tension and critical micellar dilution of culture liquid was determined by the method [10] with a platinum Wilhelmy plate.

For statistical analysis of the reliability of experimental data the methods of variation statistics [11] were used.

Presentation of basic material and discussion of results. Composition and structure of the supramolecular complex rhamnolipids-alginate JRV-L-1 and supramolecular complex rhamnolipids-alginate-pyoverdine JRV-L-2. In the native culture liquid rhamnolipid surfactants, alginate polymer and pigment pyoverdine form complex intermolecular structures: supramolecular complex rhamnolipids-alginate JRV-L-1 and supramolecular complex rhamnolipids-alginate-pyoverdine JRV-L-2.

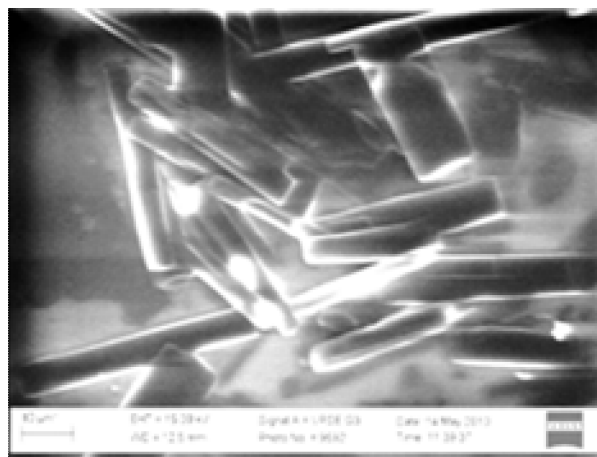
Rhamnolipids as part of a supramolecular complex JRV-L-1 (JRV-L-2) are responsible for the manifestation of surface and interfacial activity, the formation of fine emulsions of “oil in water”. Polymer-alginate provides a concentration of the active compounds (rhamnolipids, pyoverdine) by adsorption of molecules on its surface due to hydrogen and Van der Waals bonds, and acts as an emulsion stabilizer. The presence in the supramolecular complex of pigment-antioxidant pyoverdine protects the rhamnolipid-based compositions from oxidation, which ensures long-term stability of the developed products.

In the process of isolation of the supramolecular complex JRV-L-1 from the culture liquid supernatant of the strain *P. aeruginosa* JRV-L (at pH 2-4) crystalline structures are formed, as identified by light and scanning electron microscopy (Figura).

Unlike purified rhamnolipids, which form small amorphous structures in an acidic medium, rhamnolipid-alginate complex forms large rectangular lamellar crystals. The molecular weight of supramolecular complex JRV-L-1 is $7,5-9,0 \times 10^3$ kDa.

The composition of the supramolecular complex rhamnolipids-alginate JRV-L-1 is dominated by the more polar dirhamnolipid consisting of two rhamnose residues and two molecules of β -oxidecanoic acid. The content of other homologous rhamnolipids is about 10 times lower. There are traces of nonpolar lipids and protein.

Pyoverdine, which is also a product of biosynthesis of the strain *P. aeruginosa* JRV-L, due to its structure forms intermolecular complexes with mono- and dirhamnolipids and alginate through their functional groups. Thus, hydroxyl groups of pyoverdine condense with the carboxyl groups of rhamnolipids and alginate to form esters. Especially active is a chromophore group of pyoverdine due to the presence of the positively charged nitrogen in cycles, as well as the sidechain due to amino groups and the carbonyl group. The structures of complexes formed should have a folded configuration with minimum surface resulting in maximum energy effect of the formed complexes.



SEM-images of crystals of the supramolecular complex rhamnolipids-alginate JRV-L-1

A comparative analysis of the spectra obtained from IR- and UV-spectroscopy also indicate the presence of non-covalent bonds between rhamnolipids and alginate, as well as rhamnolipids with pyoverdine in supramolecular complexes JRV-L-1 and JRV-L-2. This analysis revealed a shift of the absorption bands of functional groups, and –CO and –OH in the IR spectrum of the supramolecular complex JRV-L-1 and JRV-L-2 relative to the spectrum of rhamnolipids.

One of the benefits of the obtained supramolecular complexes JRV-L-1 (JRV-L-2) is their high functional activity. A comparative analysis of some of the functional properties of the supramolecular complexes JRV-L-1 and JRV-L-2 and rhamnolipids is shown in Table 1.

Table 1

Properties of rhamnolipids and supramolecular complex JRV-L-1 and supramolecular complex JRV-L-2 from the strain *P. aeruginosa* JRV-L

Substance	Surface tension, mN/m	Interfacial tension (n-heptane), mN/m	Index of emulgation, E24, %	Foaming capacity (foam expansion)
Dirhamnolipid	28,8	0,02	70	6
Supramolecular complex JRV-L-1	29,5	0,17	85	9
Supramolecular complex JRV-L-2	30,5	0,18	85	9

The functional role of pyoverdine in the supramolecular complex JRV-L-2 is stipulated by its antioxidant properties, as well as antimicrobial and eliciting activity.

Production of the supramolecular complex rhamnolipids-alginate JRV-L-1 and supramolecular complex rhamnolipids-alginate-pyoverdine JRV-L-2.

Isolation of the supramolecular complex rhamnolipids-alginate JRV-L-1. The cell biomass was separated from the culture liquid of the strain *P. aeruginosa* JRV-L, obtained from the fermentation of bacteria by centrifugation or membrane filtration. The supernatant was decanted to separate it from the biomass and acidified to pH 2,0–3,0. Acidification was carried out with acid solutions (or concentrated acids), common laboratory practice (e. g. H₂SO₄, HCl, H₃PO₄ etc.).

The resulting solution was heated to a temperature 60-130 °C under stirring and maintained for 10–30 minutes, preferably 15–20 minutes.

The resulting mixture is settled with the formation of precipitate, which can be separated by centrifugation, filtration or decantation of the supernatant. The sedimentation is carried out at room temperature, preferably at 4 °C.

For rapid precipitation and more complete isolation of the supramolecular complex JRV L-1 potassium alum in an amount of 0,15 g/l can be added to the supernatant.

For the production of high purity supramolecular complex JRV-L-1 the resulting precipitate was washed with sterile water with pH 2.0–3.0 and passed through a membrane filter to remove fine contamination fractions.

The resulting high-purity supramolecular complex JRV-L-1 contains no more than 0,5-1 % of impurities.

Isolation of the supramolecular complex rhamnolipids-alginate-pyoverdine JRV-L-2. Supramolecular complex rhamnolipids-alginate-pyoverdine JRV-L-2 according to the invention can be prepared by separation of low molecular weight components of the culture liquid supernatant using membrane technology.

Supramolecular complex JRV L-2 according to the invention can also be prepared by two-fold freezing at –15 °C followed by thawing, which allows the separation of the low molecular weight fractions.

Supramolecular complexes JRV-L-2 according to the invention can also be obtained by mixing a supramolecular complex rhamnolipids-alginate JRV-L-1 with purified pyoverdine.

Pyoverdine isolation (toxicology testing). Individual rhamnolipids have a significantly higher toxicity than supramolecular complex rhamnolipids-alginate JRV-L-1 or rhamnolipids-alginate-pyoverdine JRV-L-2. This has been proven in tests for cytotoxicity and *Vibrio fischeri* bioassay.

Thus, at concentrations 0,013, 0,025 and 0,050 g/l supramolecular complexes JRV-L-1 and JRV-L-2, unlike rhamnolipids, showed no cytotoxicity with respect to cell culture TCCD50, mice fibroblast cell line L929 (ATCC CCL-1) and human lung adenocarcinoma cell line A549 (ATCC CCL 185).

At the concentration 0,01-0,02 g/l the rhamnolipid solutions promoted *V. fischeri* bacterial luminescence inhibition on 50 %, but in the case of supramolecular complex JRV L-1, these concentrations were 10 times greater – 0,1–0,2 g/l (Table 2).

Table 2

The maximum non-toxic dose of rhamnolipids and supramolecular complexes JRV-L-1 and JRV-L-2

Tested substance	For cell culture A549, g/l	For cell culture L929, g/l	For cell culture CHEB, g/l	Inhibition of <i>Vibrio fischeri</i> luminescence EC50, g/l	For cell culture TCCD ₅₀ g/l
Rhamnolipids	0,013	0,014	0,010	0,011	0,013
Supramolecular complex JRV-L-1	0,119	0,188	0,100	0,110	0,120

Cytotoxicity of the supramolecular complex JRV-L-1 and rhamnolipids was also tested on embryonic swine kidney cell line. The maximum non-toxic doses of supramolecular complex JRV-L-1 exceed the respective doses of rhamnolipids in 10 times.

Table 3

Rhamnolipids MTC (maximal tolerable concentrations) toward eukaryotic cell lines, HepG2 cell line

Rhamnolipids MTC (maximal tolerable concentrations) toward Cytotoxic concentrations (MTC)	Eukaryotic cell lines	Concentration Cytotoxicity % (Abs492) ± Std. dv HepG2 cell line
Mono-rhamnolipids	~ 100 µg/ mL	Jiang L. et al. x
Dirhamnolipids	~ 150 µg/ mL	Jiang L. et al. x
Rhamnolipid B	100 µg/ mL	Jensen et al. -
<i>Pseudomonas</i> sp. 17	64 µg/mL	Remichkova et al. -
JRV-L-1 JRV – LV3700	1400 µg/mL RL	80 % 81.4 ± 0.3
	280 µg/mL RL	- Not applicable
JRV-L-1 JRV – LV4900	1700 µg/mL RL	Not applicable 61.8 ± 3.5
	340 µg/mL RL	Not applicable 61.0 ± 0.003

Notes:

JRV – LV3700 – sterilized culture liquid, complex isolated with alginate by heating, concentration of rhamnolipids – 70 %.

JRV – LV4900 – culture liquid, purified through a membrane filter, complex isolated with alginate by heating, concentration of rhamnolipids – 85 %.

Determination of lethal (CL₅₀) concentrations for the aerosols. When exposed to the maximum concentration of the supramolecular complex, which can be achieved in the chamber (1000 mg/m³), the death of white rats was recorded. When intragastric administration of supramolecular complexes JRV-L-1

was applied to white rats the respective LD₅₀ amounted to 10,000 mg/kg; when the tested agents were applied to the skin no irritation; when applied to the eye mucosa it does not cause irritation.

According to the results of toxicological and hygienic studies there are no limitations for the application of supramolecular complexes JRV-L-1 (JRV-L-2) in environmentally safe technologies for water and soil remediation, agriculture, production of household chemicals, food, pharmaceutical, oil industry.

Thus, the effect of reduction of the toxicity of supramolecular complex rhamnolipids-alginate JRV-L-1 if compared with di- and monorhamnolipids was shown. This phenomenon is connected with the increase of the molecular weight of supramolecular complexes and the length of the carbon chain due to the formation of bonds of rhamnolipids with alginate. The literature describes the effect of the reduction of the toxicity of substances with increasing length of the carbon chain [Barmantlo et al., 2015], which is most likely related to the decrease in the permeability of cell membranes to such materials.

Applications. Depending on the field of application the supramolecular complexes JRV-L-1 and L 2-JRV with different degrees of purification can be used:

- Supramolecular complex rhamnolipids-alginate JRV-L-1, characterized in that it has a high degree of purification (95-99 %). The impurity content is no more than 0.5–1.0 %.
- Supramolecular complex rhamnolipids-alginate JRV-L-1, characterized in that it is a crude form of product, the impurity content is not more than 1-5 %;
- Supramolecular complex rhamnolipids-alginate-pyoverdine JRV-L-2, characterized in that it contains 0,1-2,0 % of pigment-antioxidant pyoverdine. The impurity content is not more than 1–5 %.
- The culture fluid supernatant of the strain *Pseudomonas aeruginosa* JRV-L, containing supramolecular complex JRV-L-2 in native form, the content of a supramolecular complex JRV L-2 is from 5 to 10 %.

Supramolecular complexes JRV-L-1 and JRV-L-2 can be used in a variety of fields:

1. Supramolecular complexes JRV L-1 and JRV L-2 can be used for the wound treatment (including burns, venous and non-healing wounds) in animals and humans.
2. Supramolecular complex JRV-L can be used for the treatment of skin diseases of different etiologies: psoriasis, eczema, vitiligo.
3. JRV L-1 can be used as antibacterial or antifungal drug as well as to enhance the action of biocides and antibiotics.
4. JRV-L-1 can be used for increasing the activity of enzymes.
5. Both complexes JRV-L-1 and JRV-L-2 can be used in compositions of shampoos for animals or humans, detergent compositions, cosmetic creams, compositions, soap, household chemicals, personal care composition, toothpaste, mouthwash.
6. Complexes JRV-L-1 and JRV-L-2 can be used for the pre-sowing seed treatment or foliar treatment of vegetative parts of plants as standalone agents or in combination with the macro-, micronutrients and pesticides, phytohormones and natural minerals.
7. JRV-L-1 and JRV-L-2 can be used for the improvement of the efficiency of biofertilizers based on associative and symbiotic diazotrophs in pre-sowing seed treatment of plants.
8. JRV-L-1 and JRV-L-2 can be used for the improvement of the digestion of feed for cattle and poultry (chickens, ducks, etc.), to stimulate the growth of animals and improve meat quality.
9. JRV-L-1 and JRV-L-2 can be used as antiviral agents.
10. JRV-L-1 and JRV-L-2 can be used for the treatment of phytopathogen-induced diseases of plants, caused by bacteria, fungi and viruses.
11. JRV-L-1 and JRV-L-2 can be used for control of zoospores and microsclerotia of phytopathogenic fungi.
12. JRV-L-1 and JRV-L-2 can be used as corrosion inhibitors.
13. JRV-L-1 and JRV-L-2 can be used in the methods of bioremediation and phytoremediation of contaminated soils
14. JRV-L-1 and JRV-L-2 can be used in enhanced oil recovery.

Conclusions. The proposed supramolecular complexes rhamnolipids-alginate JRV-L-1 and rhamnolipids-alginate-pyoverdine JRV-L-2 have the following advantages:

- Supramolecular complexes JRV-L-1 and JRV-L-2 have high foaming capacity and foam stability in aqueous solutions in comparison to the purified rhamnolipids.
- Complexes have higher emulsifying activity of hydrophobic substances compared to purified rhamnolipids.
- They have good compatibility with conventional thickeners, emulsion stabilizers, vegetable oils and animal fats, as well as anionic, nonionic, amphoteric surfactants in various detergents or cosmetic compositions.
- The complexes have high capacity for washing the hair and skin, as well as a variety of fabrics and surfaces.
- Comparative tests on living objects have shown that supramolecular complexes JRV-L-1 and JRV-L-2 are non-allergic, and have a lower toxicity than purified rhamnolipids.
- The advantage of supramolecular complexes JRV-L-1 and JRV-L-2 compared with purified rhamnolipids is its high stimulant and eliciting effect on plants, which manifests itself in an increase in the biomass and productivity of agricultural plants after pre-sowing seed treatment and foliar treatment of vegetative plant parts.

1. *European Patent EP0153634B1. Fritz Prof. Dr. Wagner, Christoph Dr. Syltak, Uwe Matulowic, Hans-Jürgen Dr. Hofmann, Kai-Udo Dr. Sewe, Walter Dr. Lindörfer. Process for the biotechnological production of rhamnolipids and rhamnolipids with only one beta-hydroxydecanecarboxylic acid moiety in the molecule. Publication date 1989-08-23.* 2. *European Patent EP0499434B1. Peter John Unilever Research & Engineering Hall Johan Unilever Research & Engineering Haverkamp Michael Unilever Research & Engineering Schmidt Kralingen Cornelis Gerhard Unilever Research Van. Publication date 1992-08-19.* 3. *E. V. Karpenko, A. N. Shulga, R. I. Vildanova-Marzishin, S. A. Elyseev, A. A. Turovsky, N. S. Shcheglova, Surface-active compounds of Pseudomonas sp. S-17 strain, Microbiological Journal. 52 (1996) 78-82.* 4. *Deutsch Patent DE19628454B4. Valentina Dr. Bolochovskaja Serguei Dr. Eliseev Elena Dr. Karpenko Alexander Dr. Shulga. Rhamnolipid-Alginatpolymer-Komplex, mikrobiologisches Verfahren zuseiner Herstellung, Rhamnolipid-Alginatpolymer-Komplex-Zubereitungen und deren Verwendungen. Publication date 2006-02-16.* 5. *United States Patent US5656747A. Johann Mixich Reinhardt Rothert Dieter Wullbrandt. Process for the quantitative purification of glycolipids. Publication date 1997-08-12.* 6. *Canadian Patent CA2457993C. Cheryl Baldwin Ahmad Akashe Robert Dinwoodie Rathna Koka Leslie G. West Olaf Kortum. Use of siderophores and organic acids to retard lipid oxidation. Publication date 2011-12-06.* 7. *Sierra L. Hartney, Sylvie Mazurier, Maëva K. Girard, Samina Mehnaz, Edward W. Davis II, Harald Gross, Philippe Lemanceau, Joyce E. Loper. Ferric-Pyoverdine Recognition by Fpv Outer Membrane Proteins of Pseudomonas protegens Pf-5. Journal of Bacteriology. – 2013. – Vol. 195, No. 4. – P. 765–776.* 8. *Sutherland I. W. Biofilm exopolysaccharides: a strong and sticky framework // Microbiology. – 2001. – Vol. 147. – P. 3–9.* 9. *M. DuBois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, F. Smith. Colorimetric Method for Determination of Sugars and Related Substances // Anal. Chem. – 1956. – 28 (3). – Pp. 350–356.* 10. *Абрамзон А. А., Зайченко Л. П., Райнгольд С. И. Поверхностно-активные вещества. Синтез, анализ, свойства, применение. – Л.: Химия, 1987. – 200 с.* 11. *Лакин А. Н. Курс вариационной статистики / А. Н. Лакин. – К.: Вища шк., 1990. – 116 с.*