

Influence of cobalt chloride and ferric citrate on purple non-sulfur bacteria *Rhodopseudomonas yavorovii*

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Heavy metals that enter the environment due to natural processes or industrial activities, when accumulated, have a negative impact on organisms, including microorganisms. Microorganisms have developed various adaptations to heavy metal compounds. The aim of our work was to investigate the influence of ferric citrate and cobalt (II) chloride on biomass accumulation, indicators of free radical damage and activity of enzymes of the antioxidant defense system of bacteria *Rhodopseudomonas yavorovii* IMV B-7620, that were isolated from the water of Yavorivske Lake (Ukraine, Lviv region), which was formed as a result of flooding of a sulfur quarry. We used cultural, photometric methods, and statistical processing of the results was performed using two-way ANOVA and factor analysis. It was found that ferric citrate at a concentration of 1–12 mM causes inhibition of the accumulation of biomass of bacteria *Rh. yavorovii* IMV B-7620 up to 44.7%, and cobalt (II) chloride at a concentration of 1–15 mM – up to 70.4%, compared with the control. The studied concentrations of ferric citrate and cobalt (II) chloride cause free radical damage to lipids and proteins of *Rh. yavorovii* IMV B-7620. As a result of two-way ANOVA we found that under the influence of ferric citrate statistically significant changes in biomass accumulation, lipid hydroperoxides and thiobarbiturate reactive species content, superoxide dismutase activity were predetermined by increasing the concentration of metal salts as well as increasing the duration of cultivation of bacteria, while the content of diene conjugates and catalase activity changed with increasing duration of cultivation. Under the influence of cobalt (II) chloride, statistically significant changes in all studied indicators were found both due to the increase in the concentration of metal salts and with increasing duration of bacterial cultivation. The studied parameters of *Rh. yavorovii* IMV B-7620 cells under the influence of ferric citrate and cobalt (II) chloride are combined into two factors, that explain 95.4% and 99.2% of the total data variance, respectively. Under the influence of ferric citrate, the first latent factor included diene conjugates, thiobarbiturate reactive species, carbonyl groups in proteins, which are closely linked by a direct bond and inversely related to the content of lipid hydroperoxides and catalase activity. The second latent factor included duration of cultivation of bacteria, biomass accumulation, and superoxide dismutase activity, which are inversely related to lipid hydroperoxide content and catalase activity. Under the influence of cobalt (II) chloride, the first latent factor included the content of lipid hydroperoxides, carbonyl groups in proteins, as well as catalase and superoxide dismutase activities, which are inversely related to bacterial biomass.

Keywords: free radical damage; lipid peroxidation; heavy metals; oxidative modification of proteins; antioxidant defense system; cobalt ions; ferric ions.

Introduction

Most heavy metals (Fe, Zn, Mn, Ni, Cr, Co etc.) in appropriate concentrations are necessary for the normal functioning of organisms (Barra Caracciolo & Terenzi, 2021). In addition to natural processes (weathering of minerals, etc.), sources of heavy metal pollution include industry, agricultural fields, sewage sludge, and waste treatment plants (Kapahi & Sachdeva, 2019). Accumulating in the environment, heavy metals cause negative effects for biocenoses (Li et al., 2022). Toxicity of heavy metals to living organisms is due to participation in oxidation reactions, catalysis of Fenton-type reactions (which involve transition metals such as copper, nickel, iron), Haber–Weiss reaction (with the formation of reactive oxygen species (ROS)), inhibition of transport processes (due to influence on activity of specific membrane transporters), disappearance of the transmembrane proton potential of membranes (Prabhakaran et al., 2016) etc. Toxicity of metals depends on their bioavailability and concentration. The bioavailability of metals is due to the pH of the medium, temperature, redox potential of the medium and the presence of various organic substances (Ayangbenro & Babalola, 2017; Presentato et al., 2019). The toxicity of some metals is due to the displacement of biologically essential metals from their native binding sites or the interaction of ligands (Prabhakaran et al., 2016).

Increasing the concentration of unbound iron ions causes oxidative stress mediated by the Fenton reaction (Wang et al., 2004; Vasylyv &

Hnatush, 2013). Fe^{2+} can damage photosystems and inhibit photosynthesis. Fe^{2+} and Fe^{3+} can affect the transport of other elements essential for cells (Barra Caracciolo & Terenzi, 2021). The mechanism of cobalt toxicity is unknown. This metal is characterized by a high affinity for sulfhydryl groups, which can lead to inhibition of important enzymes. Also, the toxicity of cobalt for different organisms may be due to the substitution of divalent cations in the ionic center of metal-activated enzymes; its antagonistic effect on Ca^{2+} channels; with probable inhibition of Ca^{2+} entry, Ca^{2+} signaling and competition with Ca^{2+} ions for intracellular Ca^{2+} -binding proteins; by generating ROS in cells (e.g. by Fenton-like reactions) that cause oxidative damage to DNA, proteins and lipids (Simonsen et al., 2012). Cobalt ions alter the conformation of biomolecules (proteins or nucleic acids) and metabolic reactions by binding to reactive groups – hydroxyl, carboxyl and sulfhydryl (Singh et al., 2016). In addition, metals at high concentrations can damage cell membranes, alter enzyme specificity, disrupt cellular functions, and damage DNA structure (Mohammed et al., 2011).

Mechanisms of resistance of microorganisms to metals are: intra- and extracellular binding, active efflux, enzymatic reduction and alteration of sensitivity of cellular targets to metal ions, etc. (Mohammed et al., 2011; Prabhakaran et al., 2016; Yin et al., 2018). Different combinations of these mechanisms give preference to microorganisms in metal-contaminated environments (Mohammed et al., 2011). Microorganisms interact with metal ions through binding to the cell wall, intracellular accumulation,

synthesis of siderophores, extracellular transformation reactions, extracellular mobilization or immobilization of metal ions, conversion of metals into volatile forms (Ayangbenro & Babalola, 2017; Yin et al., 2018). Deposition of metals on the surface of bacteria is associated with the interaction of metal cations with negatively charged groups of the cell wall surface of microorganisms (hydroxyl, amine, carboxyl, sulfhydryl, phosphate, sulfate, thioether, thiol) (Ayangbenro & Babalola, 2017).

Some microorganisms use metals for respiration, some bacteria in the process of evolution have formed mechanisms of detoxification of heavy metals. Microbial interactions with metals play a significant role in the biogeochemical cycles of toxic heavy metals, as well as in cleaning the environment from metal pollution. The transformation of metals by microorganisms can be divided into two broad categories: redox transformations of inorganic forms and transformations from inorganic to organic form and vice versa. Microorganisms can produce energy by oxidizing iron, manganese or arsenic. Dissimilative reduction is also possible with the use of metals as ultimate electron acceptors for anaerobic respiration (Mohammed et al., 2011).

Purple non-sulfur bacteria, which are common in waters of various types, including wastewater, are characterized by flexible metabolism (Monroy & Buitrón, 2020), which is a prerequisite for the use of individual representatives for biotechnological purposes (Delgado-Sarmiento, 2020; Asif et al., 2021). Strains of genera *Rhodopseudomonas*, *Rhodobacter*, *Rhodocyclus* are producers of single cell protein (Garimella et al., 2017), hydrogen (Sagir & Alipour, 2021), carotenoids (Wang et al., 2017; Reaksputi et al., 2019), polyhydroxyalcanoates (Monroy & Buitrón, 2020; Montiel-Corona & Buitrón, 2021), 5-aminolevulinic acid (Bunraksa et al., 2020). It is known that *Rhodobacter* sp. GSKRLMBKU-03 are resistant to 0.05 mM chromate ions and reduce 0.014–0.045 mM, depending on conditions of reaction (Rajyalaxmi et al., 2019). Arsenic-resistant strains of *Rhodopseudomonas* and *Rhodobacter* species are resistant to 0.5 mM CoCl_2 , PbCl_2 , NiCl_2 under aerobic and anaerobic growing conditions, up to 0.5 mM Na_2SeO_3 under anaerobic growing conditions; individual strains are resistant to 0.5 mM CuCl_2 (Mohsin et al., 2019). In recent years, biotechnologies for treatment of wastewater of various origins with the involvement of purple non-sulfur bacteria have been developed (Bunraksa et al., 2020; Cerruti et al., 2020; Lu et al., 2021). Because industrial processes are sources of various toxic substances that are by-products of production, it is important to understand the effects of these compounds on the cells of microorganisms. One of the main components of wastewater are heavy metal compounds (Chanpiwat et al., 2010; Huang et al., 2019; Malovanyy et al., 2019) and organic complexes (Kaushik et al., 2018; da Silva Brito et al., 2019; Li et al., 2020; Yang et al., 2021). Therefore, it is advisable to study the adaptations of bacterial strains to the effects of such compounds.

The aim of our work was to investigate the effect of ferric citrate and cobalt (II) chloride on biomass accumulation, indicators of free radical damage and activity of enzymes of the antioxidant system of bacteria *Rhodopseudomonas yavorovii* IMV B-7620 (Tarabas et al., 2021) which were isolated from the water of Yavorivske Lake (Ukraine, Lviv region), which was formed as a result of flooding of a sulfur quarry.

Materials and methods

Bacteria *Rhodopseudomonas yavorovii* IMV B-7620 were grown in ATCC 1449 medium (g/L): NH_4Cl – 0.4, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 0.32, K_2HPO_4 – 0.6, CaCl_2 – 0.05. Sodium citrate at a concentration of 12 mM was added as a carbon source. To study the effect of heavy metal ions, 1, 5, 10, 15 mM cobalt (II) chloride or 1, 3, 6, 9, 12 mM ferric citrate were added to the medium. The control was a medium without metal salts. When ferric citrate was added, the concentration of sodium citrate in the medium was proportionally reduced.

To obtain cell-free extracts, the precipitated cells were washed twice with 0.9% NaCl solution and resuspended in 0.05 M potassium phosphate buffer (pH 7.0) with phenylmethylsulfonyl fluoride (10^{-5} M) and ethylenediaminetetraacetic acid (10^{-5} M). Cells were disrupted with ultrasonic disintegrant UZDN-2T (22 kHz, 5 min, 0 °C). Cell fragments were precipitated by centrifugation (8000 g, 30 min, 4 °C). The protein concentration was determined by the Bradford method (Bredford, 1976).

The processes of lipid peroxidation were studied by changes in the content of diene conjugates, lipid hydroperoxides, thiobarbiturate reactive species (TBARS) in the cell-free extract of bacteria during cultivation. To determine the content of diene conjugates, to 0.2 mL of cell-free extract 1.8 mL of a mixture of n-heptane and isopropyl alcohol was added in a ratio of 1:1. The obtained mixture was shaken and left in closed tubes for 15 min, then centrifuged (2000 g, 10 min). The supernatant was collected in tubes, which were previously made 0.05 mL of double-distilled water. To 0.5 mL of the obtained heptane phase 2.0 mL of ethanol was added and the absorbance measured at $\lambda = 233$ nm. The control was a solution containing 0.5 mL of n-heptane and 2.0 mL of ethanol.

To 0.2 mL of cell-free extract 2.8 mL of ethanol and 0.05 mL of 50% trichloroacetic acid solution were added, the obtained mixture was shaken for 5 minutes. 1.5 mL of the supernatant was taken and 1.2 mL of ethanol, and 0.02 mL of concentrated HCl, and 0.03 mL of a 1% solution of Mohr's salt in a 3% solution of HCl were added, the obtained mixture was shaken and 0.2 mL of 20% ammonium thiocyanate solution was added after 30 s. Absorption was measured at a wavelength of 480 nm on a spectrophotometer SF-46. The content of lipid hydroperoxides was determined by the difference between the test sample and the control, in which instead of the cell-free extract the appropriate amount of double-distilled water was added. The concentration of lipid hydroperoxides was expressed in conventional units per 1 g of protein.

To determine TBARS 1 mL of trichloroacetic acid was added to 1 mL of cell-free extract to a final concentration of 10% and centrifuged (2500 g, 10 min). The resulting supernatant was mixed with 1.5 mL of saturated thiobarbituric acid solution in 0.1 M HCl solution (pH 2.5). The mixture was boiled in a water bath for 20 minutes. In the control sample, double-distilled water was added instead of the supernatant. After rapid cooling, 3 mL of butanol was added to the samples, the mixture was stirred vigorously and centrifuged in the previous mode. The concentration of TBARS in the butanol layer was determined at a wavelength of 535 nm.

Carbonyl groups (CG) in proteins were investigated by reaction with 2,4-dinitrophenylhydrazine. To 0.5 mL of cell-free extract 1 mL of trichloroacetic acid was added to a final concentration of 10% and centrifuged (5000 g, 5 min). To the resulting precipitate 1 mL of a 10 mM solution of 2,4-dinitrophenylhydrazine in 2 M HCl solution was added. 1 mL of 2 M HCl solution was added to the control solution instead. The mixture was stirred and incubated for 1 h at room temperature, then centrifuged (7000 g, 5 min). The precipitate was washed twice with 1 mL of a mixture of ethanol and ethyl acetate (1:1) and centrifuged (7000 g, 5 min). The washed precipitate was dissolved for 30 min in 6 M guanidine hydrochloride solution. Undissolved material was separated by centrifugation (8000 g, 15 min). In the supernatants, the extinction of CG in proteins was determined at a wavelength of 370 nm.

To determine the specific superoxide dismutase activity, reagent C was prepared (100 mL of 0.08 mM EDTA solution and 100 mL of 0.1 M potassium phosphate buffer (pH 7.8) and the pH was adjusted to 10 with concentrated N,N,N,N-tetramethylethylenediamine solution). Quercetin at a concentration of 1.4 μM was dissolved in dimethyl sulfoxide and brought to a liquid state by immersion in hot water. Immediately before determination, the resulting quercetin solution was diluted 10 times with distilled water. 1 mL of reagent C, 2.4 mL of H_2O , 0.1 mL of quercetin were added to the test tube. To the test tube was added 1 mL of reagent C, 2.3 mL of H_2O , 0.1 mL of cell-free extract, 0.1 mL of quercetin. Measurements were performed using DS-11+ (DeNovix Inc., USA, 2018) spectrophotometer at a wavelength of 406 nm at zero time (immediately after the addition of quercetin) and after 20 minutes.

The reaction mixture for determining catalase activity contained 2.8 mL of 0.5% H_2O_2 solution and 0.1 mL of cell-free extract diluted n times. After 5 min of incubation, the reaction was stopped by adding 1.0 mL of 6% $(\text{NH}_4)_2\text{MoO}_4$ solution. The control was a sample that contained H_2O instead of cell-free extract. Measurements were performed on a DS-11+ spectrophotometer (DeNovix Inc., USA, 2018) at a wavelength of 410 nm immediately after the addition of $(\text{NH}_4)_2\text{MoO}_4$.

Statistical processing of the results was performed in OriginPro 8.5 (OriginLab Corporation, USA, 2010). The values of different groups were compared using ANOVA followed by Bonferroni test. The two-way

analysis of variables (ANOVA) followed by Bonferroni test was used to compare the effects of concentrations of ferric citrate, cobalt (II) chloride and duration of cultivation on biomass accumulation, free radical damage and activity of enzymes of the antioxidant system. Factor analysis by principal components analysis was performed using Statistica 8.0 (StatSoft, Inc, USA, 2008) to establish relationships between the studied indicators.

Results

Ferric citrate at concentration of 1–6 mM did not cause inhibition of *Rh. yavorovii* IMV B-7620 biomass accumulation during the 7th day of culture growth. Reduction of the biomass accumulation by 10–15% under the influence of these concentrations of ferric citrate occurred by increasing of the duration of cultivation of bacteria up to 14 days. The intensity of the damaging action also increased with an increase in the concentration of metal salt in the cultivation medium. Ferric citrate at concentration 9–12 mM caused inhibition of biomass accumulation up to 44.7%, compared with control.

In order to study the influence of cobalt ions, cobalt (II) chloride in concentrations 1, 5, 10, 15 mM was added into the medium. Cobalt (II)

chloride in concentration of 1 mM did not significantly influence the accumulation of biomass of *Rh. yavorovii* IMV B-7620 for 14 days of growth. It was found that 5–15 mM cobalt (II) chloride affected inhibition of biomass accumulation by 60.0–70.4% for 14 days of bacterial growth.

Studied concentrations of ferric citrate predetermined inhibition of biomass accumulation by 15.0–44.7% (Fig. 1a), and cobalt (II) chloride – by 5.2–70.4% (Fig. 1b). Selected concentrations of metal salts enabled us to investigate the properties of bacteria during growth with damaging action.

Investigation of indicators of lipid peroxidation, oxidative modification of proteins, catalase and superoxide dismutase activity was performed at 7th, 10th and 14th days of cultivation of bacteria.

At all studied concentrations of ferric citrate, the content of diene conjugates in the cells of *Rh. yavorovii* IMV B-7620 was higher than in the control. The highest content of diene conjugates under the influence of all studied concentrations of ferric citrate was found for the 10th day, which was 1.6–7.5 times higher than the content of diene conjugates in the cells of bacteria not exposed to this metal (Fig. 2a). High levels of diene conjugates were found in the cells of bacteria grown with 10 and 15 mM cobalt (II) chloride on the 14th day of cultivation (197 and 295 nmol/mg protein, accordingly, Fig. 2b).

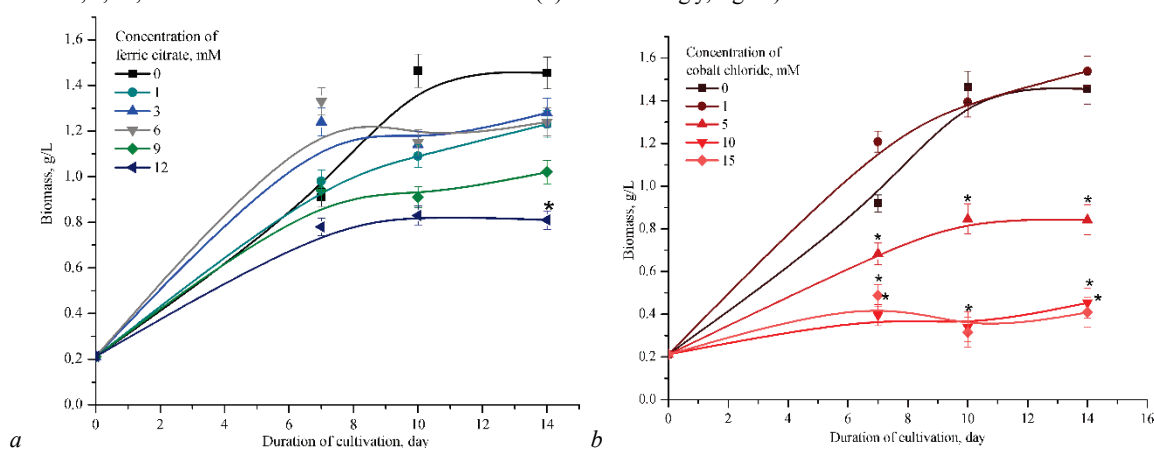


Fig. 1. The effect of different concentrations of ferric citrate (a) and cobalt (II) chloride (b) on the accumulation of biomass of bacteria *Rhodospseudomonas yavorovii* IMV B-7620: $x \pm SD$, $n = 3$, * – $P < 0.05$ – probable changes compared to control

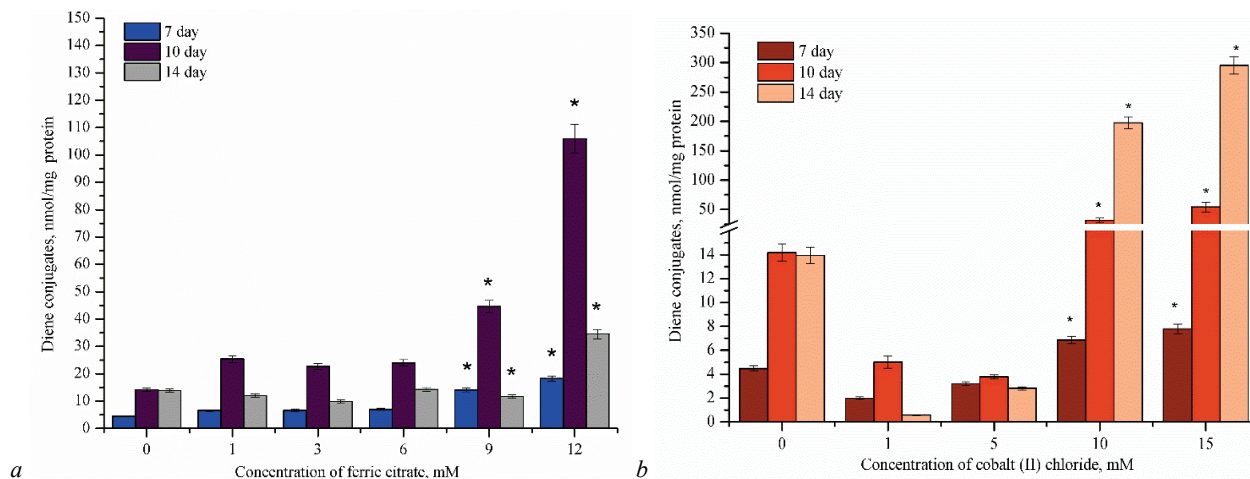


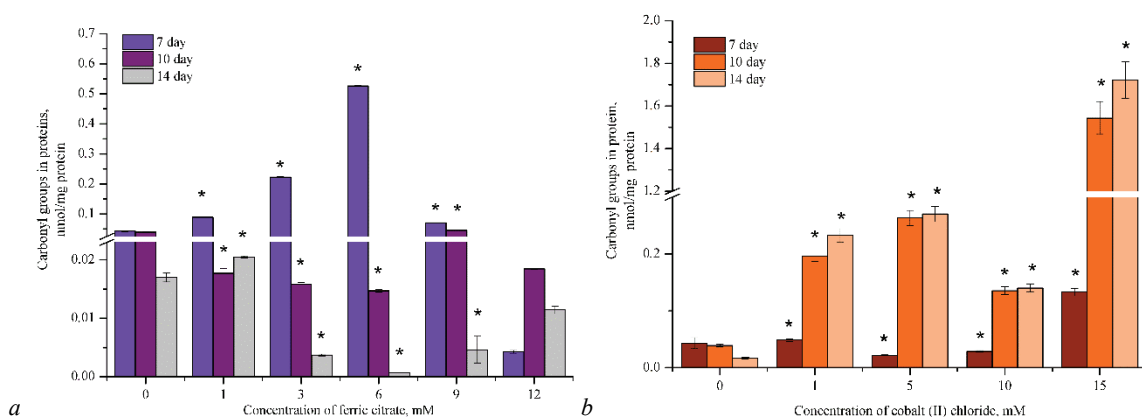
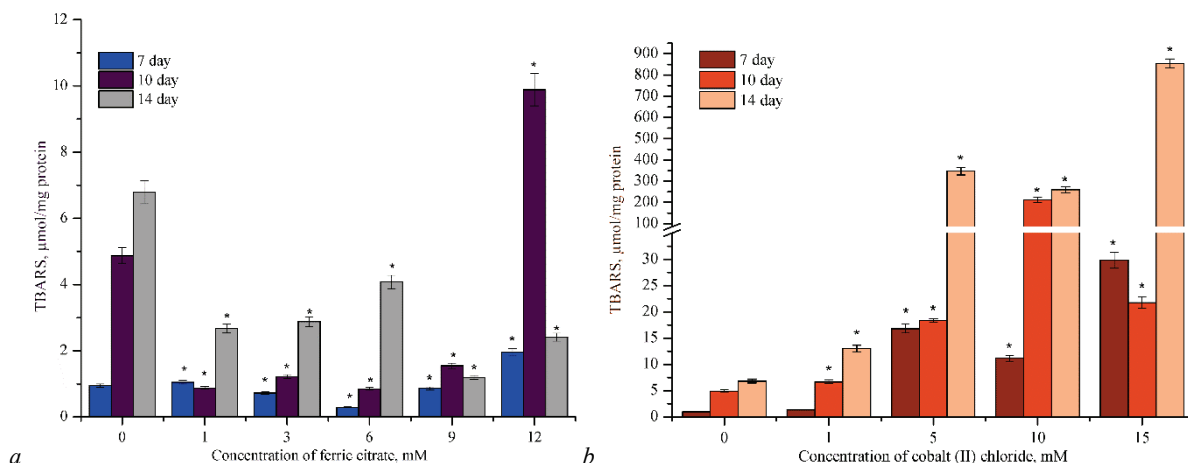
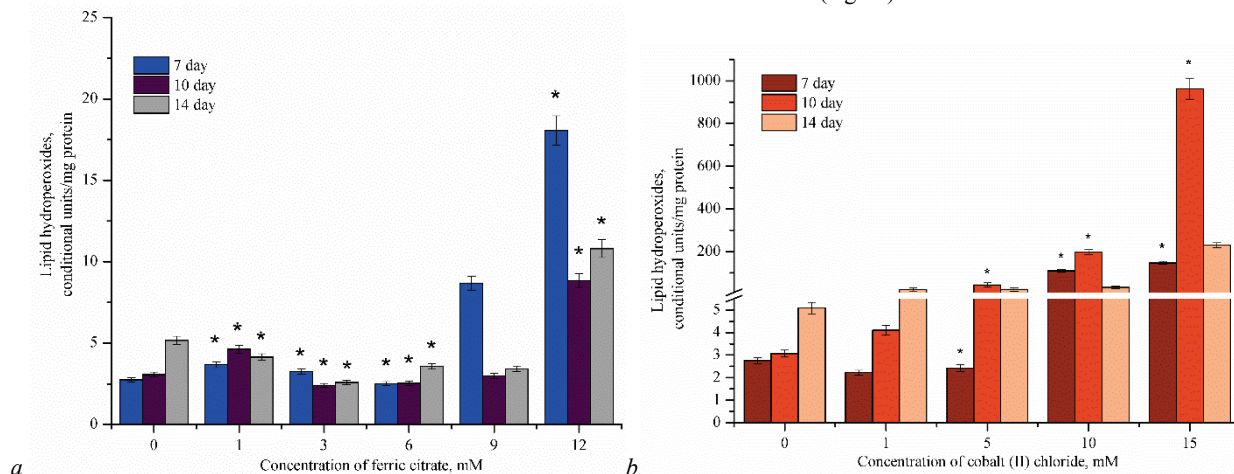
Fig. 2. Content of diene conjugates in bacteria *Rhodospseudomonas yavorovii* IMV B-7620 under the influence of ferric citrate (a) and cobalt (II) chloride (b): $x \pm SD$, $n = 3$, * – $P < 0.05$ – probable changes compared to control

The highest content of lipid hydroperoxides (18.1 ± 0.01 conditional units/mg protein) in the cells of *Rh. yavorovii* IMV B-7620 under the influence of ferric citrate was on the 7th day of cultivation at a concentration of 12 mM (Fig. 3a). Under the influence of cobalt (II) chloride, the content of lipid hydroperoxides also increased compared to the control. When 5–15 mM cobalt (II) chloride was added into the culture medium of *Rh. yavorovii* IMV B-7620, the maximum content of lipid hydroperoxides was detected on the 10th day of cultivation (Fig. 3b). Under the influence of ferric citrate in concentrations of 1–9 mM, the content of primary

and secondary products of lipid peroxidation differed slightly or was lower compared to the control. An increase in the content of lipid peroxidation products was observed under the influence of 12 mM ferric citrate (Fig. 4a). At all studied concentrations of cobalt (II) chloride, the content of TBARS in the cells of bacteria was significantly higher than in the control and exceeded the content of these products when exposed to 12 mM ferric citrate (Fig. 4b).

Ferric citrate and cobalt (II) chloride in selected concentrations caused the formation of carbonyl groups in proteins, indicating free radical da-

mage to proteins of *Rh. yavorovii* IMV B-7620 (Fig. 5). The dynamics of formation of carbonyl groups in proteins under the influence of the investigated salts of metals was different. Under the influence of ferric citrate, the content of carbonyl groups in proteins was the highest during the 7th day of growth at all investigated concentrations of metal salt, except for a concentration of 12 mM. Under the influence of cobalt (II) chloride, the content of carbonyl groups in proteins of *Rh. yavorovii* IMV B-7620 increased with an increase in the duration of cultivation of bacteria and metal concentration in the medium. We assume that free radical damage to proteins of *Rh. yavorovii* IMV B-7620 under the influence of the iron or cobalt can affect the processes of cell metabolism and adaptation reactions under these conditions.



Under the influence of the studied metal salts, the catalase activity of *Rh. yavorovii* IMV B-7620 was higher than in the control. Under the influence of ferric citrate, catalase activity was highest on the 7th day of bacterial cultivation and exceeded the activity in the control 41 times. During further cultivation, the catalase activity of *Rh. yavorovii* IMV B-7620 differed slightly from the control (Fig. 6a). Under the influence of cobalt (II) chloride, catalase activity increased up to 10 days of *Rh. yavorovii* IMV B-7620 culturing. Under the influence of 5 and 10 mM cobalt (II) chloride, the maximum values of catalase activity were almost the same as under the influence of 12 mM ferric citrate, when the concentration of cobalt (II) chloride increased to 15 mM, catalase activity increased 1.3 times (Fig. 6b).

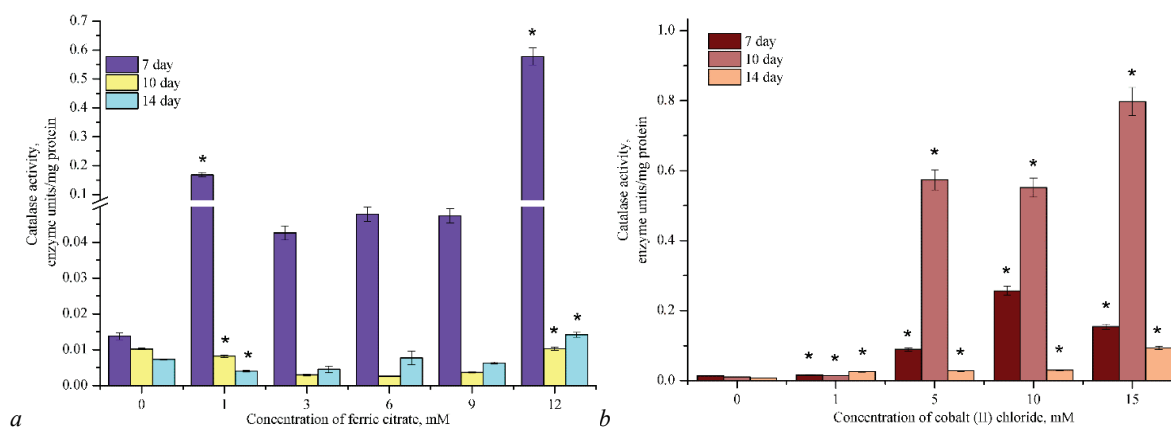


Fig. 6. Catalase activity of bacteria *Rhodopseudomonas yavorovii* IMV B-7620 cells under the influence of ferric citrate (a) and cobalt (II) chloride (b): $x \pm SD$, $n = 3$, * – $P < 0.05$ – probable changes compared to control

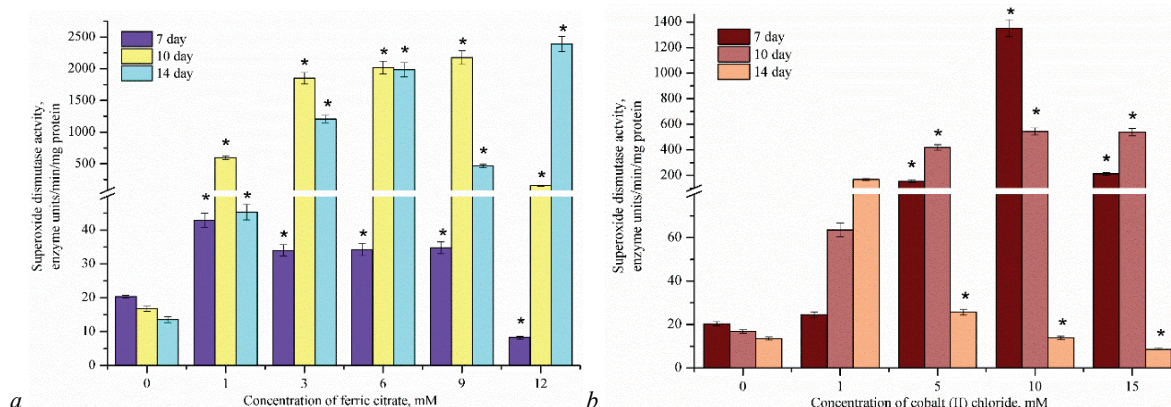


Fig. 7. Superoxide dismutase activity of *Rhodopseudomonas yavorovii* IMV B-7620 cells under the influence of ferric citrate (a) and cobalt (II) chloride (b): $x \pm SD$, $n = 3$, * – $P < 0.05$ – probable changes compared to control

The addition of ferric citrate and cobalt (II) chloride into the culture medium of *Rh. yavorovii* IMV B-7620 caused an increase in superoxide dismutase activity by 1.25–176 times. Under the influence of all studied concentrations of ferric citrate, the values of superoxide dismutase activity were higher than under the influence of cobalt (II) chloride at concentrations of 1–15 mM (Fig. 7, a, b).

Under the influence of ferric citrate, the increase in the concentration of metal salts caused statistically significant changes in the accumulation of biomass, the content of hydroperoxides of lipids and TBARS, superoxide dismutase activity. The content of diene conjugates and catalase activity

did not depend on the concentration of the metal salt, but changed with increasing duration of bacterial cultivation. There are also relationships between changes in the concentration of ferric citrate and the duration of cultivation on the studied indicators (Table 1). Increasing the concentration of cobalt (II) chloride from 1 to 15 mM caused statistically significant changes in all studied indicators. Also, significant changes in the accumulation of biomass, indicators of lipid peroxidation and oxidative modification of proteins, the activity of antioxidant enzymes were caused by prolongation of the duration of bacterial cultivation. Relationships between these two factors have been identified (Table 1).

Table 1

Two-way ANOVA analysis of the parameters measured in *Rh. yavorovii* IMV B-7620 under the influence of ferric citrate and cobalt (II) chloride

Indicator	Factor	F (DFn, DFd) value under the influence of		P value under the influence of	
		ferric citrate	cobalt (II) chloride	ferric citrate	cobalt (II) chloride
Biomass	concentration	F (5, 13) = 273.77	F (4, 10) = 923.94	<0.0001*	<0.0001*
	day of cultivation	F (2, 6) = 103.97	F (2, 6) = 71.92	<0.0001*	<0.0001*
	interactions	F (10, 43) = 42.76	F (8, 36) = 38.04	<0.0001*	<0.0001*
Lipid hydroperoxides	concentration	F (5, 13) = 1062.59	F (4, 10) = 1818.07	<0.0001*	<0.0001*
	day of cultivation	F (2, 6) = 257.26	F (2, 6) = 967.64	<0.0001*	<0.0001*
	interactions	F (10, 43) = 128.52	F (8, 36) = 648.14	<0.0001*	<0.0001*
Diene conjugates	concentration	F (5, 13) = 807.96	F (4, 10) = 1233.80	<0.0001*	<0.0001*
	day of cultivation	F (2, 6) = 1934.33	F (2, 6) = 1808.59	<0.0001*	<0.0001*
	interactions	F (10, 43) = 259.39	F (8, 36) = 741.18	<0.0001*	<0.0001*
Thiobarbiturate reactive species	concentration	F (5, 13) = 748.22	F (4, 10) = 854.46	<0.0001*	<0.0001*
	day of cultivation	F (2, 6) = 1207.82	F (2, 6) = 2197.05	<0.0001*	<0.0001*
	interactions	F (10, 43) = 560.13	F (8, 36) = 768.13	<0.0001*	<0.0001*
Carbonyl groups in proteins	concentration	F (5, 13) = 789.01	F (4, 10) = 1966.23	<0.0001*	<0.0001*
	day of cultivation	F (2, 6) = 2537.88	F (2, 6) = 850.42	<0.0001*	<0.0001*
	interactions	F (10, 43) = 877.84	F (8, 36) = 417.91	<0.0001*	<0.0001*
Catalase activity	concentration	F (5, 13) = 570.13	F (4, 10) = 1128.63	<0.0001*	<0.0001*
	day of cultivation	F (2, 6) = 1464.70	F (2, 6) = 1268.46	<0.0001*	<0.0001*
	interactions	F (10, 43) = 520.85	F (8, 36) = 606.91	<0.0001*	<0.0001*
Superoxide dismutase activity	concentration	F (5, 13) = 772.94	F (4, 10) = 707.79	<0.0001*	<0.0001*
	day of cultivation	F (2, 6) = 2251.04	F (2, 6) = 902.64	<0.0001*	<0.0001*
	interactions	F (10, 43) = 624.59	F (8, 36) = 1095.60	<0.0001*	<0.0001*

Note: * – significant differences within one of three variables – concentration of ferric citrate or cobalt (II) chloride, day of cultivation, or their interactions.

To establish the relationship between the processes of free radical damage to lipids and proteins, the activity of enzymes of the antioxidant system, the accumulation of *Rh. yavorovii* IMV B-7620 biomass and the concentration of ferric citrate or cobalt (II) chloride in the environment the factor analysis of principal components was performed. As a result of the analysis, the data were reduced, where 8 variables were combined into two factors, the variance of which according to the Kaiser and Cattell criteria was greater than 1 (Table 2).

Table 2
Eigenvalues of factors of bacteria *Rh. yavorovii* IMV B-7620 under the influence of ferric citrate and cobalt (II) chloride

Salt of metal	Value	Eigenvalue	Total variance, %	Cumulative eigenvalue	Cumulative, %
Ferric citrate	1	4.86	60.81	4.86	60.81
	2	2.76	34.54	7.63	95.35
Cobalt (II) chloride	1	4.43	55.42	4.43	55.42
	2	3.50	43.77	7.93	99.19

Analysis of the matrix of factor loadings of the studied indicators under the influence of ferric citrate revealed two latent factors that explain 95.4% of the total data variance, and under the influence of cobalt (II) chloride – 99.2% (Table 2). This result indicates that a large number of parameters that significantly affect the functionality of the system are taken into account.

Under the influence of ferric citrate on *Rh. yavorovii* IMV B-7620 cells, the first latent factor included diene conjugates, TBARS, carbonyl groups in proteins, which are closely linked by a direct bond and inversely related to the content of lipid hydroperoxides and catalase activity (Table 3, Fig. 8a). The variance of the first factor is 51.5%, which indicates

the high importance of these indicators. The second latent factor included the duration of bacterial cultivation, biomass accumulation, content of lipid hydroperoxides, catalase and superoxide dismutase activity. In particular, duration of cultivation of bacteria, biomass accumulation, and superoxide dismutase activity are inversely related to lipid hydroperoxide content and catalase activity. The variance of this factor is 43.8%.

Table 3
Matrix of factor loadings of studied indicators of bacteria *Rh. yavorovii* IMV B-7620 under the influence of different concentrations of ferric citrate and cobalt (II) chloride

Variable	Ferric citrate		Cobalt (II) chloride	
	Factor 1	Factor 2	Factor 1	Factor 2
Day	–	–0.978	–	0.998
Biomass	–	–0.845	–0.921	–
Lipid hydroperoxides	–0.817	0.565	0.997	–
Diene conjugates	0.997	–	–	0.972
Thiobarbiturate reactive species	0.982	–	–	0.923
Carbonyl groups in proteins	0.961	–	0.530	0.845
Catalase	–0.692	0.713	0.975	–
Superoxide dismutase	–	–0.953	0.861	–0.505
Expl. Var	4.12	3.51	4.02	3.92
Pp. Totl, %	51.5	43.8	50.2	49.0

Under the influence of cobalt (II) chloride, the first latent factor included the content of lipid hydroperoxides, carbonyl groups in proteins, as well as catalase and superoxide dismutase activities, which are inversely related to bacterial biomass accumulation (Table 3, Fig. 8b). The variance of this factor is 49.0%. The obtained results show that under the influence of cobalt (II) chloride the influence of both factors on the system is equal and important.

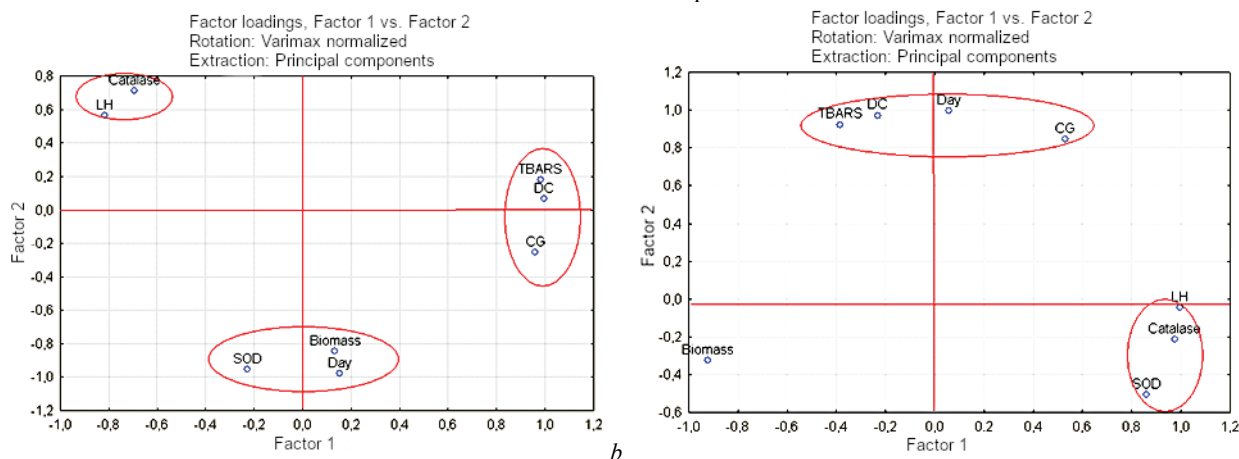


Fig. 8. Distribution of factor loadings of the studied parameters of *Rhodospseudomonas yavorovii* IMV B-7620 cells under the influence of different concentrations of ferric citrate (a) and cobalt (II) chloride (b): LH – lipid hydroperoxides, DC – diene conjugates, TBARS – thiobarbiturate reactive species, CG – carbonyl groups in proteins, SOD – superoxide dismutase

Discussion

The effect of metal compounds on the cells of microorganisms is due to the chemical properties of ions, which are formed as a result of the dissociation of these compounds. Because sodium citrate was used as a carbon source for the growth of *Rh. yavorovii* IMV B-7620 in the study, the citrate ion concentration was left unchanged (12 mM) for the study of oxidative processes under the influence of ferric citrate, which led to the choice of the maximum concentration of ferric citrate. It was found that despite the similar chemical properties of cobalt and iron ions, the biological effect on the bacteria *Rh. yavorovii* IMV B-7620 differed.

Despite the large number of studies on the effects of cobalt ions on the cells of microorganisms, the mechanism of damaging effects of cobalt ions is debatable. It is known that cobalt ions are involved in the formation of ROS because they are in the redox states, mainly Co (II) and Co (III), but also under certain circumstances Co (I) (Barras & Fontecave, 2011). In addition to catalyzing the reactions in which ROS are formed, cobalt ions affect the homeostasis of iron and sulfur ions, disrupt the organization of Fe/S clusters in enzymes and the electron transport chain, have geno-

toxic effects, etc. (Barras & Fontecave, 2011; Kumar et al., 2017; Liu et al., 2020). Kumar et al. (2017) found that the toxicity of cobalt ions to *Escherichia coli* BW25113 is due to ROS-independent DNA damage, decelerating the replication fork progression, inhibition of the activity of enzymes involved in the SOS response, in particular inhibition of RecBCD exonuclease functions to suppress SOS response. In response to the stress caused by cobalt ions, the cells of sulfur-reducing bacteria *Geobacter sulfurreducens*, that accumulate significant concentrations of cobalt ions, undergo extensive transcriptional reprogramming, during which, along with overexpression of metal efflux systems from cytoplasm and periplasmic space, specific histidine kinases and transcriptional regulators of metabolism, there is an increase in the level of transcripts of periplasmic glutaredoxin, which restores oxidized cysteine residues in proteins, and digemine cytochrome c peroxidase, which restores H₂O₂ (Dulay et al., 2020). One of the reasons for the toxicity of LiCoO₂ nanoparticles to *Bacillus subtilis* is the accumulation of cobalt ions in cells, which is accompanied by ROS generation and increased expression of genes whose products provide antioxidant protection, transport and DNA repair (Gari et al., 2021).

In cells of *Rh. yavorovii* IMV B-7620 at high concentrations of ferric citrate and all studied concentrations of cobalt (II) chloride there is an increase in the content of diene conjugates and lipid hydroperoxides, indicating a free radical mechanism of damage to cellular lipids of *Rh. yavorovii* IMV B-7620 under the influence of these salts. Under these conditions, oxidative damage to protein molecules also occurs, which reflects the increase in the content of carbonyl groups in protein molecules. After increasing the duration of cultivation of bacteria, the content of carbonyl groups in proteins decreased, which may be due to proteolysis of oxidized proteins or, conversely, the formation of a significant number of carbonyl groups in protein molecules, and makes their detection impossible (Grune et al., 2004). To determine the reasons for reduction in the content of carbonyl groups in proteins of *Rh. yavorovii* IMV B-7620 under the influence of ferric citrate during different phases of culture growth, further studies are needed. However, the formation of these groups in proteins under the influence of heavy metal compounds is an indicator of free radical damage to the proteins of *Rh. yavorovii* IMV B-7620. Catalase and superoxide dismutase provide neutralization of the formed free radical compounds in *Rh. yavorovii* IMV B-7620 cells under the influence of ferric citrate and cobalt (II) chloride. Bacteria of the genus *Rhodospseudomonas* are known to have two types of catalases: manganese (WP_114358207.1) and catalase/oxidase (WP_092681558.1). There are *Rhodospseudomonas palustris* (ADU42423.1), *Rh. pseudopalustris* (SEP24300.1), *Rh. pentothentaxigens* (RED38428.1), *Rh. thermotolerans* (REG06013.1), *Rh. rhenobacensis* (WP_184258932.1) amino acid catalase sequences in GenBank. Cu/Zn (WP_114359954.1) and Fe/Mn (WP_013503506.1) superoxide dismutases were detected in cells of bacteria of the genus *Rhodospseudomonas*. Amino acid sequences of Fe/Mn-SOD *Rh. palustris* (ADU45394.1, WP_011440573.1, WP_011439508.1, WP_012495261.1, WP_011665231.1, WP_011665231.1, WP_011504010.1, ACF00416.1) and Cu/Zn-SOD *Rh. palustris* (RIA03292.1), *Rh. pentothentaxigens* (RED28570.1, SSW92512.1), *Rh. faecalis* (PYF02344.1), *Rh. pseudopalustris* (SEP35666.1), *Rh. thermotolerans* (REF91489.1) are in the GenBank database. Since cobalt is able to replace iron in enzyme reaction centers, FeS clusters, etc., lower values of superoxide dismutase activity under the influence of cobalt (II) chloride may be due to inactivation of this enzyme as a result of substitution and free radical damage, as evidenced by carbonyl groups in proteins.

The increase in the content of TBARS under the influence of ferric citrate and cobalt (II) chloride is probably due to the intensive formation of ROS and the spread of free radical reactions.

The results of factor analysis under the influence of both studied metal salts are similar. In particular, in two cases, close links were found between the content of lipid hydroperoxides and catalase activity. Close links have also been established between diene conjugates, TBARS and carbonyl groups in proteins content. Therefore, we assume that at the early stage of exposure to the studied salts, hydroperoxides of lipids are formed, and probably other peroxides. Catalase activity plays an important role in the neutralization of these compounds. Since classical catalases do not have peroxidase activity, we assume the presence of several enzymes with catalase activity which protect cells from peroxides of organic and inorganic origin.

Under the influence of ferric citrate, no relationship was found between the products of lipid peroxidation, oxidative modification of proteins and superoxide dismutase activity, and under the influence of cobalt (II) chloride an inverse relationship between these indicators was established. This is probably due to damage to the enzyme molecules themselves. In the case of exposure to cobalt (II) chloride, catalase and superoxide dismutase activity are among the first factors. Probably more free radicals of different origins are formed, due to the different chemical properties of cobalt ions, which cells try to neutralize most effectively.

Conclusions

The studied concentrations of ferric citrate and cobalt (II) chloride caused free radical damage to lipids and proteins of *Rh. yavorovii* IMV B-7620. As a result of two-way ANOVA we found that under the influence of ferric citrate statistically significant changes in biomass accumulation, lipid hydroperoxides and thiobarbiturate reactive species content, super-

oxide dismutase activity were predetermined by increasing the concentration of metal salts as well as increasing the duration of cultivation of bacteria, while the content of diene conjugates and catalase activity changed with increasing duration of cultivation. Under the influence of cobalt (II) chloride, statistically significant changes in all studied indicators were found both due to the increase in the concentration of metal salts and with increasing duration of bacterial cultivation. The studied parameters of *Rh. yavorovii* IMV B-7620 cells under the influence of ferric citrate and cobalt (II) chloride are combined into two factors, that explain 95.4% and 99.2% of the total data variance, respectively. Under the influence of ferric citrate, the first latent factor included diene conjugates, thiobarbiturate reactive species, carbonyl groups in proteins, which are closely linked by a direct bond and inversely related to the content of lipid hydroperoxides and catalase activity. The second latent factor included duration of cultivation of bacteria, biomass accumulation, and superoxide dismutase activity, which are inversely related to lipid hydroperoxides content and catalase activity. Under the influence of cobalt (II) chloride, the first latent factor included the content of lipid hydroperoxides, carbonyl groups in proteins, as well as catalase and superoxide dismutase activities, which are inversely related to bacterial biomass.

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