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S. Komplikevych [1], O. Maslovska [1], T. Moravska [1], I. Yarmoliuk [1], N. Biront [2], Y. Zaritska [2], S. Hnatush [1, *]

[1] Ivan Franko National University of Lviv, Lviv, 79005, Ukraine
[2] State Research Control Institute of veterinary medicinal products and feed additives, Lviv, 79019, Ukraine

*** Corresponding author:** shnatush1965@gmail.com

Adaptations of the antarctic bacterium *Paenibacillus tundrae* IMV B-7915 to copper (II) chloride exposure

Abstract. Heavy metals are common in Antarctic habitats. However, the adaptations of Antarctic microorganisms to heavy metals are poorly understood. One of the mechanisms of toxicity of transition metals is the formation of free radicals which damage the cell macromolecules. In 2020, we isolated the bacteria *Paenibacillus tundrae* IMV B-7915 from a sample containing moss, soil, and underground parts of *Deschampsia antarctica* (Berthelot Islands, Maritime Antarctic). The aim of the study was to investigate the influence of copper (II) chloride on the specific growth rate, the content of products of free radical damage to lipids and proteins, the activity of antioxidant defense system enzymes, and the synthesis of extracellular polymeric substances in *P. tundrae* IMV B-7915. The bacteria were incubated for an hour in Tris-HCl buffer with 2–8 mM copper (II) chloride, then washed and inoculated into the tryptic soy broth. The bacteria were cultured for 72 hours. The content of copper in the cells was determined by atomic absorption spectrometry. The content of indicators of lipid peroxidation (diene conjugates, lipid hydroperoxides, thiobarbituric acid-reactive substances), oxidative modification of proteins (carbonyl groups in proteins), the activity of the antioxidant defense system enzymes (catalase, superoxide dismutase, glutathione peroxidase, glutathione S-transferase, glutathione reductase), total thiols, exopolymeric compounds (exopolysaccharides and proteins) were determined photometrically. Within an hour, cells accumulate 1.5–3.4 mg Cu/g of biomass, leading to a decrease in biomass accumulation and specific growth rate within 24 hours. In cells, copper ions induce free radical reactions of damage to cell macromolecules, reflected in the increase in the content of primary lipid peroxidation products and carbonyl groups in proteins. Cell division is inhibited. In response, *P. tundrae* IMV B-7915 cells activate efflux systems, as evidenced by a significant decrease in copper content during prolonged cultivation, and enzymes of antioxidant defense and synthesis of exopolysaccharides. The complex of the studied adaptation reactions ensures the detoxification of copper accumulated in cells, reflected in the restoration of the specific growth rate.

Keywords: antioxidant defense, extracellular polymeric substances, free radicals, heavy metals, lipid peroxidation, protein damage

1 Introduction

Heavy metals, particularly copper, are found in different substrates of the Maritime Antarctic (Parnikoza et al., 2017; Artemenko et al., 2019; Bedernichek et al., 2020; Komplikevych et al., 2023). They are one of the factors of environmental stress affecting the local microbiota. Copper mainly exists in two redox states: Cu (I) and Cu (II); Cu (I) predominates in the cytoplasm, while in an oxidizing environment, both states can occur (Zuily et al., 2022). The high redox potential of the Cu (II)/Cu (I) pair (+160 mV) pro-

motes oxidation processes (Andrei et al., 2020). During the oxidation of cellular macromolecules, Cu^{2+} is reduced to Cu^{+} and then is easily re-oxidized by interaction with O_2 and H_2O_2. The hydroxyl radicals produced in this way are highly reactive and cause further damage to cellular components (Imlay, 2018; Zuily et al., 2022). Free radical formation intensifies at low temperatures (Tribelli & Lopez, 2018).

Oxidative damage to proteins disrupts the structure of the polypeptide backbone and amino acid side groups, forming amino acid derivatives (Stadtman & Levine, 2003; Hawkins & Davies, 2019). The toxic effect of protein radicals involves the protein-mediated initiation of lipid peroxidation (Schöneich, 2011), i.e., the formation of cytotoxic compounds known to covalently modify cellular proteins, lipids, and nucleic acids. These agents affect the activity of cellular enzymes and fluidity of the cytoplasmic membrane and cause DNA mutations (Repetto et al., 2012; Semchyshyn & Lushchak, 2012; Pizzimenti et al., 2013; Ayala et al., 2014). The cell's response to protein radicals and lipid peroxidation products depends on metabolic conditions and reparative potential (Pradenas et al., 2013; Ayala et al., 2014; Ezraty et al., 2017). Bacteria show significant adaptive potential to the influence of stress agents, including reactive oxygen species (ROS). In cells of both anaerobic and facultatively anaerobic bacteria, the most common systems under oxidative stress are superoxide dismutases–catalases/peroxidases for aerobes and superoxide reductases-rubberitrin – for anaerobes (Holmes et al., 2009; Mishra et al., 2020). The glutathione redox system and glutathione-dependent enzymes detoxify ROS, terminate free radical chain reactions, detoxify toxic products of ROS-mediated damage to cellular macromolecules, and maintain the cell's redox status (Fu et al., 2007; Allocati et al., 2009; Yan et al., 2013).

Several microorganisms resistant to heavy metals were isolated from different substrates of Antarctica (Havryliuk et al., 2020). Adaptations of Antarctic microorganisms to heavy metal compounds need to be studied more. Studying adaptations occurring in psychrophilic and psychrotolerant microorganisms to the effects of copper ions may be important for understanding cellular resistance mechanisms to heavy metals in general.

The genus *Paenibacillus* is found in different climatic conditions, from polar regions to the tropics and from aquatic environments to the driest deserts (Dsouza et al., 2014). Interest in these bacteria is due to the fact that among the genus *Paenibacillus*, there are plant growth-promoting strains (acting through phosphate solubilization, indole-3-acetic acid biosynthesis, and siderophore production) (Kumari & Thakur, 2018; Xue et al., 2023), heavy metals-resistant strains (Kumari & Thakur, 2018), and bacteria capable of biosorption of copper (Prado Acosta et al., 2005). There are some approaches for creating copper-detecting environmental biosensors based on *Paenibacillus* sp. (Abdu et al., 2017).

We aimed to analyze the products of free-radical damage to lipids and proteins, the activity of antioxidant defense system enzymes, and the synthesis of extracellular polymeric substances in *Paenibacillus tundrae* IMV B-7915 under the influence of copper (II) chloride. The results will help to elucidate the mechanism through which the polar microorganisms adapt to the effects of heavy metals.

2 Materials and methods

2.1 The cultivation of bacteria

Paenibacillus tundrae IMV B-7915 (GenBank accession number: MW362265.1) was isolated at the Department of Microbiology of Ivan Franko National University of Lviv. The strain was isolated from a sample containing moss, soil, and underground parts of *Deschampsia antarctica* E. Desv., 1854 (Berthelot Islands, Maritime Antarctica), collected during the XXIII Ukrainian Antarctic Expedition in February-March 2019 (Hnatush et al., 2020). Bacteria were grown at + 27 ... + 30 °C in 250 ml flasks filled with 100 ml of tryptic soy broth (TSB) (Merck, USA).

To investigate the effect of copper ions on cells, *P. tundrae* IMV B-7915 was incubated for one hour in 50 mM Tris-HCl buffer (pH 7.5) containing 2, 4, 5.5, 7, or 8 mM $CuCl_2$. After incubation, the cells were washed and transferred (0.1 g/l) to metal-free TSB. Bacteria were cultivated for 72 hours. The pretreatment stage was necessary because adding the salt directly to TSB, which is optimal for the bacteria (Bos-

mans et al., 2017), would lead to the formation of the insoluble $Cu(OH)_2$. It is also known that the optimal pH value for the biosorption of copper by microbial cells is pH 6 (Prado Acosta et al., 2005; Ghaed et al., 2013; Liu et al., 2013), which is determined by the charge of carboxyl, hydroxyl, amino groups and other functional groups that bind the ions. However, we did not use this pH value, as it is not optimal for *P. tundrae* IMV B-7915 and may affect the physiology of the bacteria.

The testing was done at 1, 24, 48, and 72 hours of cultivation (HoC) unless stated otherwise.

2.2 Copper content determination

To prepare samples for analysis of copper, 0.45 g of cells were weighted into Teflon glass, after which acid decomposition in autoclaves of the START D microwave laboratory system (Milestone, Italy) was carried out. The system keeps the sample during microwave decomposition in 69% nitric acid (Merck, USA), its transition into the solution, and subsequent removal of solvent vapors. After cooling, the hydrolysates were quantitatively transferred for subsequent dissolution in deionized water (Milli-Q system, Merck Millipore, USA) to a concentration within the calibration curve. Copper was measured using atomic absorption spectrometry (Zeeman Atomic Absorption Spectrometer AA240Z Varian with GTA 120 Graphite Tube Atomizer, Australia). Argon flow was 0.3 L/min, the ashing temperature was +800 °C, and the temperature of the atomization stage was +2300 °C. Detection of copper was carried out at 327 nm.

2.3 Biochemical methods of determination of cell damage indicators

To obtain cell-free extracts, the cells were washed twice with saline solution and resuspended in 0.05 M potassium phosphate buffer (pH 7.0) with phenylmethylsulfonyl fluoride (10^{-5} M) and ethylenediaminetetraacetic acid (EDTA) (10^{-5} M). Cells were disrupted by 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 (Bradford, 1976).

The processes of lipid peroxidation were studied by changes in the content of diene conjugates, lipid hydroperoxides, and thiobarbituric acid reactive substances (TBARS) in the cell-free extract of bacteria during cultivation.

To determine the content of diene conjugates, 0.1 ml of cell-free extract was added to 0.9 ml of a mixture of n-heptane and isopropyl alcohol (1:1). The obtained mixture was shaken and left in closed tubes for 30 min, then centrifuged (2000 g, 10 min). The supernatant was collected in tubes, and 0.05 ml of double-distilled water was added. To 0.5 ml of the obtained heptane phase, we added 2.0 ml of ethanol and measured the absorbance at $\lambda = 233$ nm. The control was a solution containing 0.5 ml of n-heptane and 2.0 ml of ethanol (Oleksiuk & Yanovych, 2010).

The content of lipid hydroperoxides. 2.8 ml 96% ethanol and 0.05 ml 50% trichloroacetic acid were added to 0.2 ml of cell-free extract. The mixture was shaken for 5 minutes. 1.5 ml of the supernatant was mixed with 1.2 ml of ethanol. Then 0.02 ml of concentrated HCl and 0.03 ml of a 1% solution of Mohr's salt in 3% HCl were added. The obtained mixture was shaken for 30 s, and 0.2 ml of 20% ammonium thiocyanate solution was added. Absorption was measured with the application of DeNovix DS-11+ at 480 nm. The content of lipid hydroperoxides was determined by the difference between the test sample and the control, to which we added the appropriate amount of double-distilled water instead of the cell-free extract. The concentration of lipid hydroperoxides was expressed in conventional units per 1 g of protein (Oleksiuk & Yanovych, 2010).

To determine TBARS, 1 ml of cell-free extract was added to 1 ml of trichloroacetic acid (at a final concentration of 10%) and centrifuged (2500 g, 10 min). The supernatant was mixed with 1.5 ml of saturated thiobarbituric acid solution in 0.1 M HCl (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 as

before. The concentration of TBARS in the butanol layer was determined at 535 nm (Lushchak et al., 2004; Holovchak et al., 2012).

Carbonyl groups (CG) in proteins were investigated by the reaction with 2,4-dinitrophenylhydrazine. To 0.5 ml of cell-free extract, 1 ml of trichloroacetic acid (at a final concentration of 10%) was added and centrifuged (5000 g, 5 min). To the resulting precipitate, we added 1 ml of a 10 mM solution of 2,4-dinitrophenylhydrazine in 2 M HCl. To the control, we added 1 ml of 2 M HCl 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 ethanol and ethyl acetate (1:1) and centrifuged (7000 g, 5 min). The washed precipitate was dissolved for 30 min in a 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 370 nm (Lushchak et al., 2004).

2.4 Determination of the activity of enzymes of the antioxidant defense system

To determine the activity of **specific superoxide dismutase** (EC 1.15.1.1), we prepared reagent C (100 ml of 0.08 mM EDTA solution was combined with 100 ml of 0.1 M potassium phosphate buffer (pH 7.8), and the pH was adjusted to 10.0 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 ten times with distilled water. To prepare the control mixture, 1 ml of reagent C, 2.4 ml of H_2O, and 0.1 ml of quercetin were added. To the test tube, 1 ml of reagent C, 2.3 ml of H_2O, 0.1 ml of cell-free extract, and 0.1 ml of quercetin were added. Measurements were done at 406 nm at zero time (immediately after adding quercetin) and after 20 minutes (Holovchak et al., 2012).

The reaction mixture for determining **catalase activity** (EC 1.11.1.7) 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, we added 1.0 ml of 6% $(NH_4)_2MoO_4$ solution to stop the reaction. The control was a sample that contained H_2O instead of a cell-free extract. Measurements were performed at 410 nm immediately after adding the $(NH_4)_2MoO_4$ (Holovchak et al., 2012).

To determine the activity of **glutathione peroxidase** (EC 1.11.1.9) (μmol reduced glutathione $min^{-1} \cdot mg^{-1}$ protein), 0.1 ml of cell-free extract was incubated for 10 min at +28 °C with 0.9 ml of 0.1 M Tris-HCl buffer (pH 8.5) containing 6 mM EDTA, 12 mM NaN_3, and 4.8 mM reduced glutathione. After incubation, 0.1 ml of 20 mM tert-Butyl hydroperoxide was added and incubated for 5 min. The reaction was stopped by 0.2 ml of 20% trichloroacetic acid. The precipitated proteins were removed by centrifugation (2500 g, 15 min). To 0.1 ml of the supernatant, 3 ml of 0.1 M Tris-HCl buffer (pH 8.5) and 0.1 ml of 10 mM Elman's reagent were added. After 5 min, the samples were measured at 412 nm (Holovchak et al., 2012).

Glutathione-S-transferase (EC 2.5.1.18) activity (μmol glutathione-S-2,4-dinitrobenzene $min^{-1} \cdot mg^{-1}$ protein) was determined by the rate of formation of reduced glutathione conjugate in the reaction with 1-chloro-2,4-dinitrobenzene. For this purpose, 1 ml of 0.1 M potassium phosphate buffer (pH 8.5) with 2 mM reduced glutathione and 1 ml of 2 mM 1-chloro-2,4-dinitrobenzene dissolved in 0.1 M potassium phosphate buffer (pH 8.5) was added to 0.1 ml of cell-free extract. The measurements were done at 340 nm immediately after the addition of 1-chloro-2,4-dinitrobenzene and after 3 min (Holovchak et al., 2012).

Glutathione reductase (EC 1.8.1.7) activity (μmol $NADPH_2$ $min^{-1} \cdot mg^{-1}$ protein) was determined by the decrease in $NADPH_2$ content within 1 min by measuring the optical density at 340 nm. For this, 2.9 ml of assay solution (12.5 μM oxidized glutathione, 20 μM $NADPH_2$, 200 μM potassium phosphate buffer (pH 8.0)) was added to the spectrophotometer cuvette and the optical density was measured against the control using potassium phosphate buffer. 0.1 ml of cell-free extract was added into the assay solution in the cuvette, and the decrease in optical density at 340 nm was measured for 5 min at 1 min intervals. To calculate the activity of glutathione reductase, the difference in the extinction of the sample at zero and at 5 minutes was used (Holovchak et al., 2012).

2.5 Determination of the total thiol content

We added 1 ml of 0.5 mM Elman's reagent in 100 mM potassium phosphate buffer (pH 7.4) to 50 µl of cell-free extract. To the control solution, 1 ml of potassium phosphate buffer (pH 7.4) was added instead of Elman's reagent. A sample containing 50 µl of water and 1 ml of Elman's reagent was used as a blank solution. The solutions were mixed and incubated in the dark for 30 min at room temperature. The optical density was measured at 412 nm. The differences between the test, control, and blank samples were used for calculations (Hawkins et al., 2009).

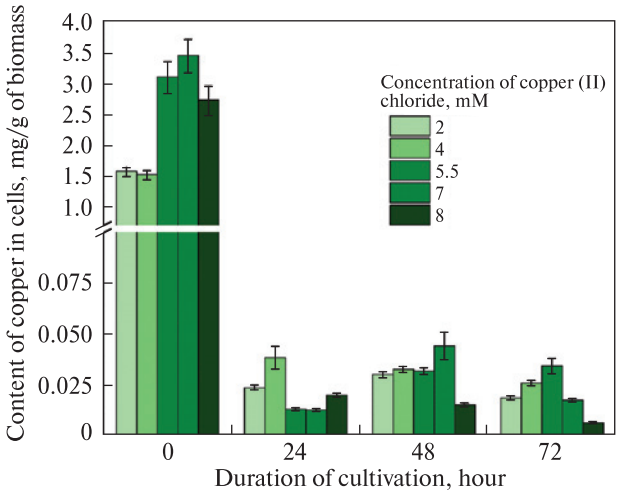

Figure 1. The content of copper in *Paenibacillus tundrae* IMV B-7915 cells after incubation in Tris-HCl buffer with different $CuCl_2$ content and after cultivation in metal-free tryptic soy broth

2.6 Extracellular polymeric substances

To extract extracellular substances, an equal volume of EDTA (2 %) was added to the bacterial cell suspension and incubated at 4 °C for 3 h. After that, the mixture was centrifuged at 8300 g for 20 min at 4 °C (Pan et al., 2010).

In the supernatant with extracted extracellular substances, the content of exopolysaccharides was determined using anthrone (Frølund et al., 1996). To do this, 1.6 ml of anthrone solution in sulfuric acid (0.125 % anthrone in 94.5 % H_2SO_4) was added to 0.8 ml of the supernatant. The reaction mixture was mixed and kept in a water bath at 100 °C for 14 min and cooled at 4 °C for 5 min. The optical density was determined at 625 nm. The calibration curve for calculations used glucose as a standard.

The content of extracellular proteins was determined by the Bradford method (Bradford, 1976) in the supernatant with extracted extracellular substances.

2.7 Data analysis

The results are presented as the average values with standard deviations (x ± SD). The influence of $CuCl_2$

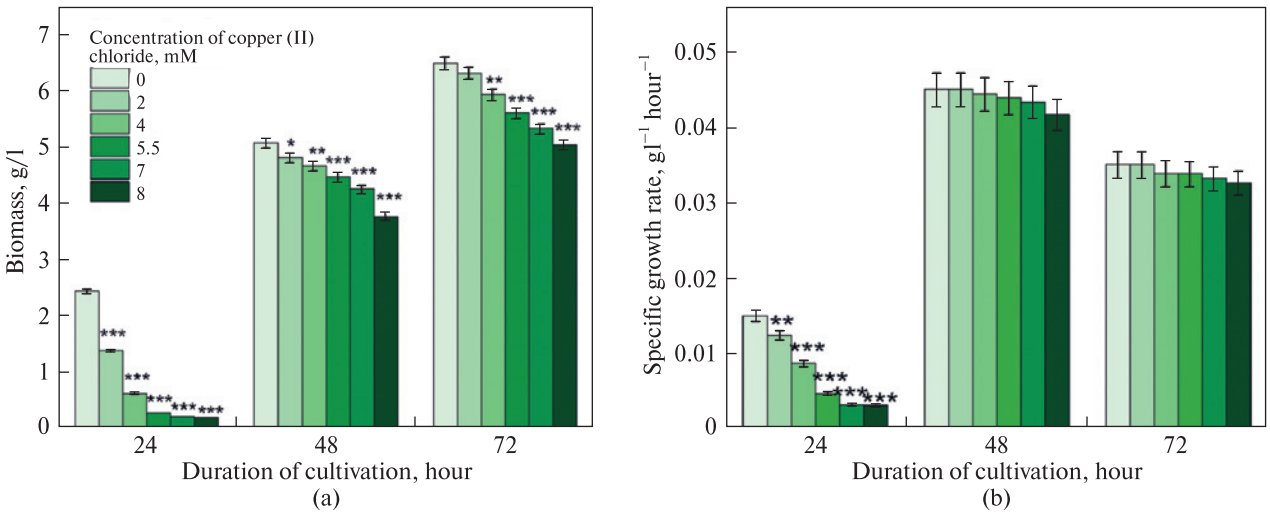

Figure 2. The effect of copper on biomass accumulation (a) and specific growth rate (b) of *Paenibacillus tundrae* IMV B-7915 (x ± SD, * – P ≥ 0.95, ** – P ≥ 0.99, *** – P ≥ 0.999)

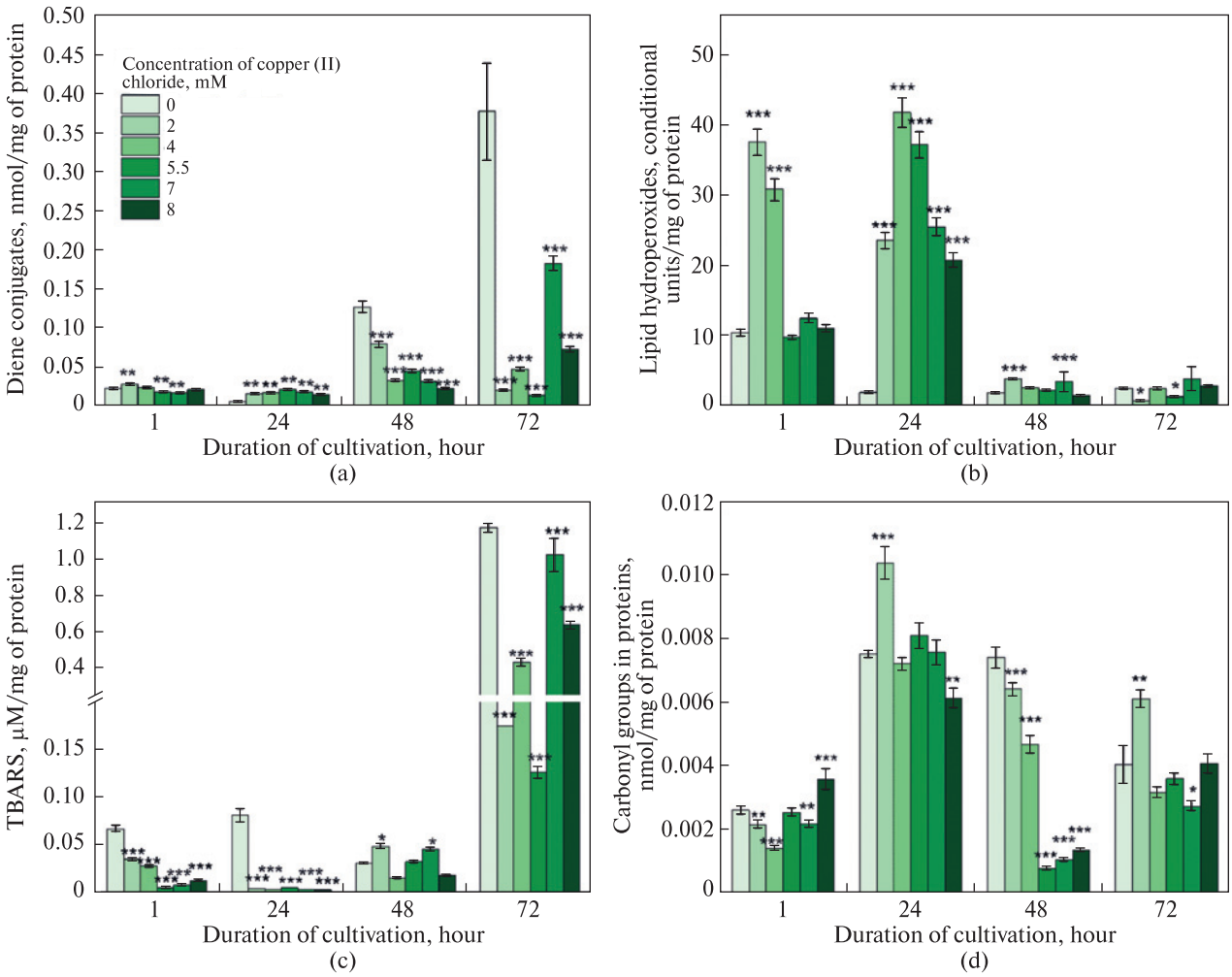

Figure 3. The content of diene conjugates (a), lipid hydroperoxides (b), thiobarbituric acid reactive substances (c), and carbonyl groups in proteins (d) of *Paenibacillus tundrae* IMV B-7915 bacteria under the influence of copper (x±SD, * – $P \geq 0.95$, ** – $P \geq 0.99$, *** – $P \geq 0.999$)

on the indicators of interest was estimated using the t-test (p-value < 0.05). The graphs were built using OriginPro 8.5 (OriginLab Corporation, USA, 2010).

3 Results

The influence of different concentrations of copper on biomass accumulation and specific growth rate

After incubation of *P. tundrae* IMV B-7915 in 50 mM Tris-HCl buffer (pH 7.5) for 1 hour, copper ions were accumulated in bacterial cells. At 2 mM and 4 mM $CuCl_2$ in the buffer, the cells contained 1.5 ± 0.08 mg Cu/g of biomass. With an increase in $CuCl_2$ concentration (5.5–8 mM), the content of copper in bacterial cells also increased (2.7 ± 0.2–3.4 ± 0.3 mg Cu/g). After incubation, the bacteria were grown in a TSB medium without the metal. Under these conditions, within 24–72 hours, the content of copper in the cells did not exceed 0.060 mg Cu/g of biomass (Fig. 1).

As a result of copper accumulation in the cells, the biomass of *P. tundrae* IMV B-7915 decreased by 56–92 % compared to the control at 24 hours of cultivation. At 48 and 72 HoC, the bacterial biomass was 5–26 % lower than the control (Fig. 2, a). The ac-

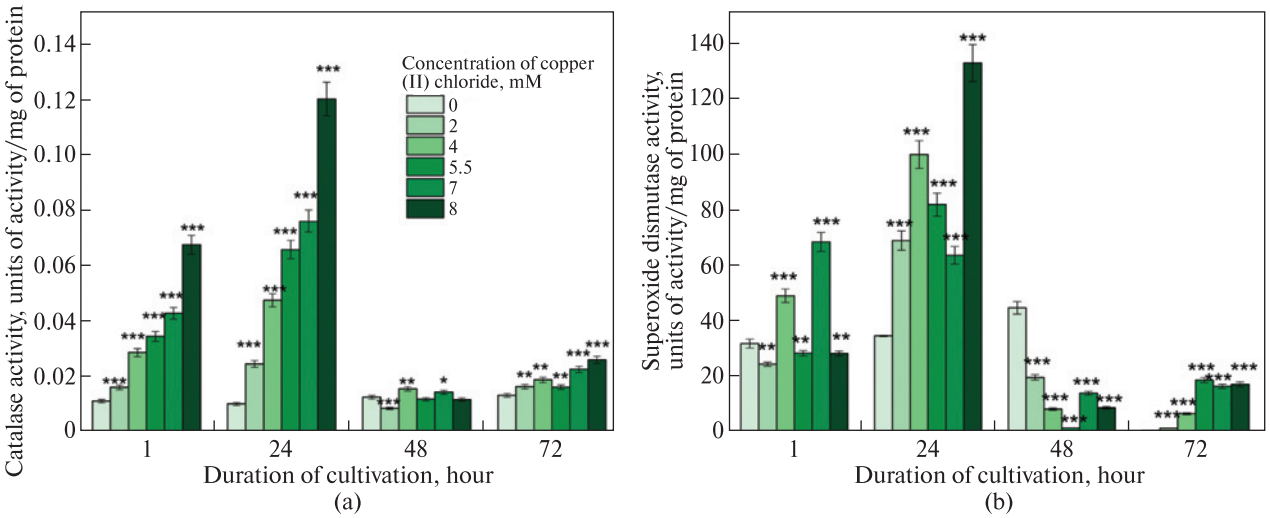

Figure 4. Catalase (a) and superoxide dismutase (b) activities of *Paenibacillus tundrae* IMV B-7915 bacteria under the influence of copper (x ± SD, * – P ≥ 0.95, ** – P ≥ 0.99, *** – P ≥ 0.999)

cumulation of copper in *P. tundrae* IMV B-7915 cells caused a decrease in the specific growth rate at 24 HoC. The specific growth rate decreased with an increase in the accumulated copper. At 48 and 72 HoC, the specific growth rate of pretreated cells did not differ from the control (Fig. 2, b).

Lipid peroxidation and oxidative modification of proteins

Within 1 HoC, the content of diene conjugates changed slightly compared to the control. Under the influence of all studied concentrations, the content of diene conjugates at 24 HoC was 2.7–3 times higher compared to the control. At 48 HoC, the content of diene conjugates decreased with increasing concentration of $CuCl_2$. After further cultivation, the content of these products was significantly lower compared to the control (Fig. 3, a).

The content of lipid hydroperoxides increased after 1 HoC under the influence of 2–4 mM $CuCl_2$, and at higher concentrations, it did not differ significantly from the control. Within 24 hours, under the influence of all studied concentrations, the content of lipid hydroperoxides significantly exceeded the content of these LPO products in the control. At 48 and 72 HoC, the content of lipid hydroperoxides decreased compared to 24 HoC and did not differ significantly from the control (Fig. 3, b).

The content of TBARS exceeded its level in control only at 48 HoC (Fig. 3, c).

The content of carbonyl groups in the bacterial proteins after 1 HoC after exposure to 2–7 mM $CuCl_2$ was lower or slightly different from the control. After exposure to 8 mM, it was higher. At 24 HoC, the content of carbonyl groups exceeded the control under the influence of 2 mM $CuCl_2$, and with increasing concentration, their content decreased. A similar tendency was observed at 48 and 72 HoC (Fig. 3, d).

Adaptations of Paenibacillus tundrae IMV B-7915 to copper

Under the influence of $CuCl_2$, the activity of the enzymes of the antioxidant system of *P. tundrae* IMV B-7915 was intensified. In particular, at 1 and 24 HoC, superoxide dismutase and catalase activities were 15.7–28.4 and 2.4–11.9 times higher than the control. With further cultivation for up to 48 hours, superoxide dismutase and catalase activity decreased and only slightly differed from the control (Fig. 4). At 72 HoC, catalase activity increased with increasing concentration of $CuCl_2$ and was 1.2–2.0 times higher than the control (Fig. 4, a). Superoxide dismutase activity increased 3.4–43.0 times compared to the control (Fig. 4, b).

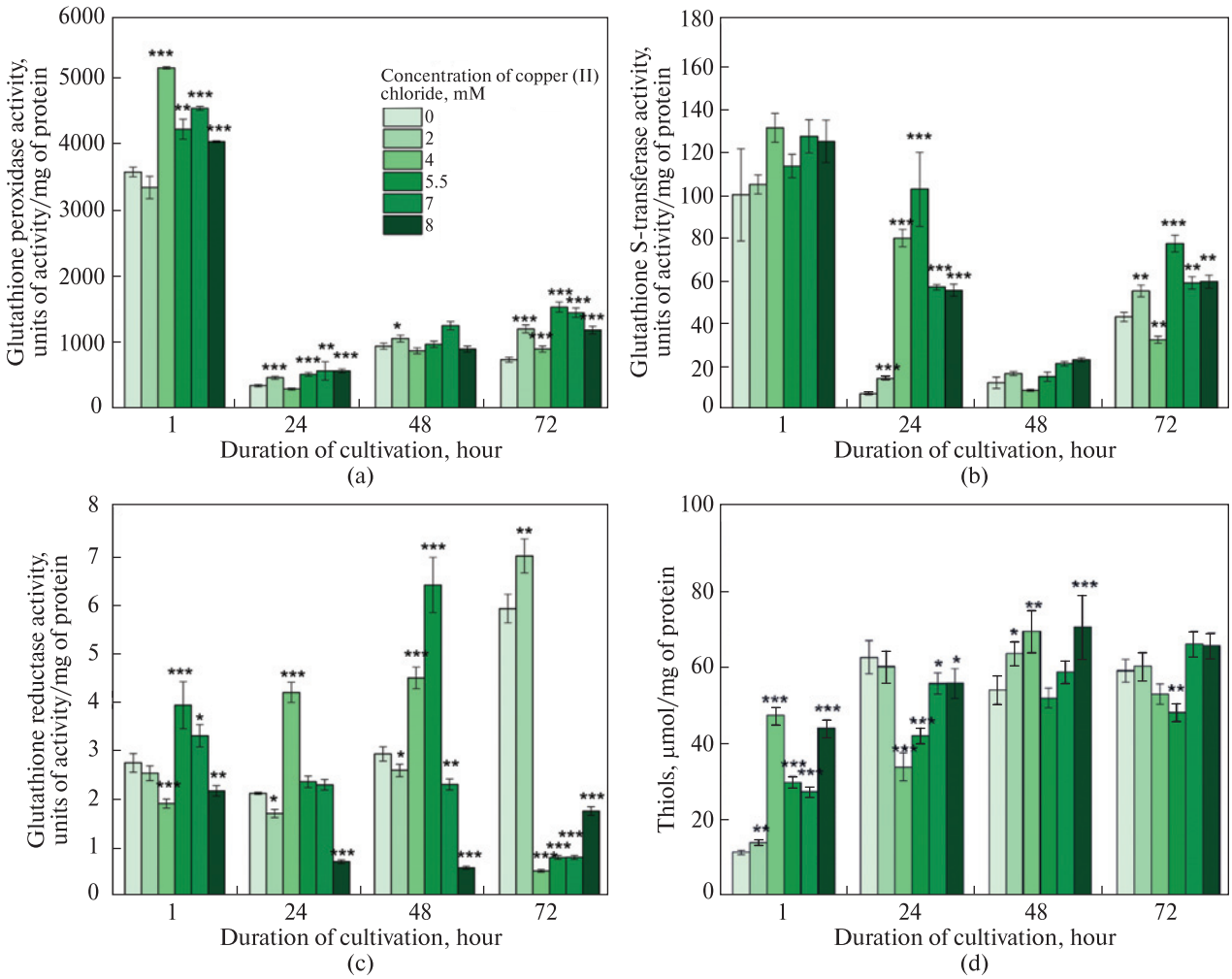

Figure 5. Glutathione peroxidase (a), glutathione S-transferase (b), glutathione reductase (c) activity, and total thiol content (d) in *Paenibacillus tundrae* IMV B-7915 bacteria under the influence of copper (II) chloride (x ± SD, * – P ≥ 0.95, ** – P ≥ 0.99, *** – P ≥ 0.999, n = 3 – probable changes compared to control)

The glutathione peroxidase and glutathione S-transferase activities of *P. tundrae* IMV B-7915 in the control and under the influence of $CuCl_2$ were the highest during the first hour of growth (Fig. 5, a, b). At 24 and 48 HoC, glutathione peroxidase activity increased slightly compared to the control. An increase in glutathione peroxidase activity was detected at 72 hours (Fig. 5, a). Glutathione S-transferase activity increased by 1.9–13.3 times at 24 HoC compared to the control. At 48 and 72 HoC, glutathione-S-transferase activity decreased compared to its activity at the 24-hour mark but was slightly higher than the control (Fig. 5, b). An exception is the glutathione-S-transferase activity at 4 mM $CuCl_2$ because at 48 and 72 HoC, it was lower than the control. The glutathione peroxidase activity at 4 mM $CuCl_2$ was also lower than at other concentrations and the control at 24 and 48 HoC (Fig. 5, a, b).

The glutathione reductase activity under the influence of 2 mM $CuCl_2$ was not significantly different from the control over the whole period of cultivation. When the salt concentration increased to 7–8 mM, the enzymatic activity decreased (Fig. 5, c). The total thiol content in cells increased significantly within

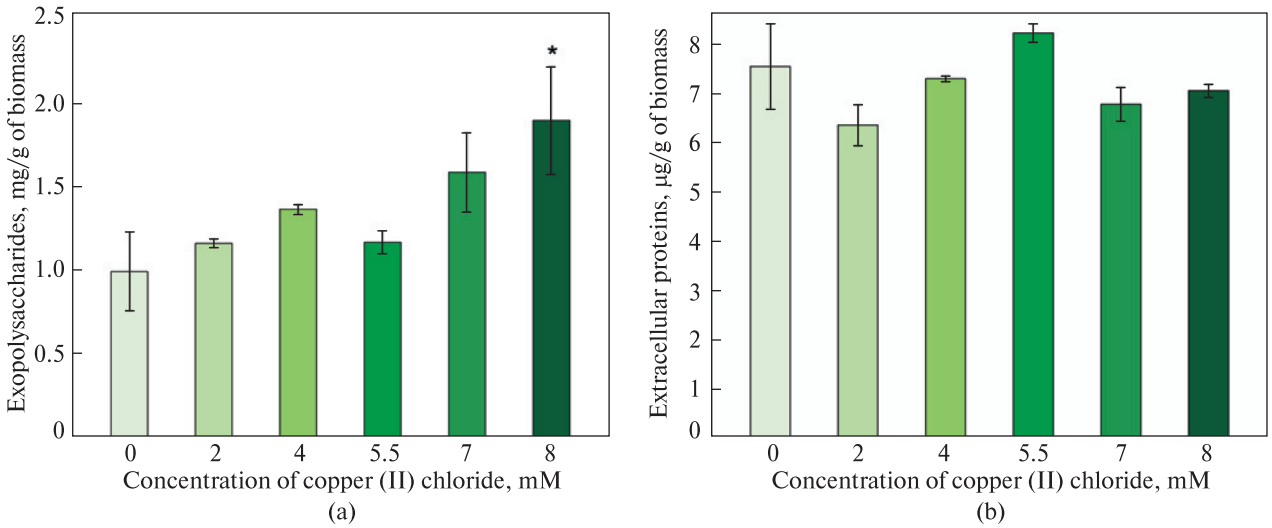

Figure 6. The content of exopolysaccharides (a) and extracellular proteins (b) synthesized by *Paenibacillus tundrae* IMV B-7915 bacteria under the influence of copper (II) chloride (x ± SD, * – $P \geq 0.95$, n = 3 – probable changes compared to control)

the first hour at all studied $CuCl_2$ concentrations. During further cultivation, it was slightly lower than the control or did not differ (Fig. 5, d).

The content of exopolysaccharides during 72 HoC increased with increasing concentration of $CuCl_2$, and the content of extracellular proteins did not differ from the control (Fig. 6).

4 Discussion

Since copper ions have a damaging effect, bacterial cells that are resistant to it exhibit adaptation reactions. These reactions were studied in this work.

As a result of incubation in the buffer with $CuCl_2$ for 1 hour, the cells accumulated up to 3.4 ± 0.3 mg of Cu/g biomass. It is known that Gram-positive bacteria accumulate significantly higher levels of heavy metal compounds than Gram-negative bacteria (Nanda et al., 2019). *Paenibacillus polymyxa* accumulated up to 150 mg of Cu/g cells (Prado Acosta et al., 2005), *Rhodococcus erythropolis* – 68 mg of Cu/g cells (Baltazar et al., 2019), *Pseudomonas stutzeri* LA3 – 1.62 mg of Cu/g biomass (Palanivel et al., 2020).

In this study, we aimed to decipher the effect of Cu(II) ions on *P. tundrae* IMV B-7915 cells and their adaptations under these conditions. It was found that already after 24 hours of cultivation, the content of copper in cells significantly decreased, probably via efflux. The content of free copper in the cytoplasm of bacteria is known to be insignificant (approximately 10^{-21} M) (Solioz, 2018). Therefore, during the growth of *P. tundrae* IMV B-7915, the efflux of copper ions from the cell is insufficient since the remaining content is also toxic. There are several reasons for the incomplete pumping of such a toxic metal: the formation of complexes with reduced glutathione or with carboxyl, hydroxyl, and other functional groups in proteins, cell wall components, or exopolysaccharides, replacement of Fe ions in FeS clusters of cytochromes and other metals in the active sites of enzymes, or insufficient efficiency of efflux systems as this process is energy-consuming. The accumulation of copper in cells negatively affected the specific growth rate during the first 24 hours of growth. It is assumed that the metal ions induced certain reactions that made cell growth and division impossible; however, during further growth, the bacteria managed to reduce the ions' toxicity and eliminate the damage that occurred.

We think the lipid damage might be related to such reactions that occur under the influence of copper. The content of primary lipid peroxidation products

in cells increased significantly compared to the control at 24 hours of growth, and during further growth, was lower or not significantly different from the control. The formation of secondary products probably does not occur, which is reflected by the low content of TBARS and is due to the high activity of glutathione S-transferase which is known to effectively reduce lipid hydroperoxides without the need for hydrolysis of the damaged lipid by phospholipase. We did not detect a significant increase in carbonyl groups in proteins of *P. tundrae* IMV B-7915 cells under the influence of copper; however, we assume that there may be other modifications of protein molecules or amino acid residues. In addition, it is known that under certain conditions of copper damage to proteins, protein aggregates are formed, which makes it impossible to detect carbonyl groups (Zuily et al., 2022).

The increased content of diene conjugates and lipid hydroperoxides under the influence of copper leads to the idea that free radical reactions of cell macromolecular damage occur due to ions catalyzing Fenton and Haber-Weiss reactions. To remove excess O_2^- and H_2O_2, bacteria express enzymes that neutralize harmful oxidants, preventing damage to DNA, membrane lipids, and proteins (Gout, 2019). We observed the activity of catalase, superoxide dismutase, and glutathione peroxidase increase at the first 24 hours of cultivation and decrease later.

We assume that the copper ions remaining in the cells induce new reactions of free radical oxidation and that the cells pass into a stationary phase of growth, also accompanied by damage to lipids and other components. It is known that under the influence of copper, it is important to maintain intracellular redox homeostasis, provided by low-molecular-weight thiols (Gout, 2019). The most abundant low-molecular-weight thiols in bacteria include glutathione, cysteine, bacillitiol, mycothiol, and coenzyme A (Gout, 2019). They are structurally diverse and differ by expression profile, biophysical and biochemical properties, and cellular functions. Among the major low-molecular-weight thiols, Cys and CoA are widely represented in all bacterial species. Glutathione is detected in millimolar concentrations in Gram-negative and only some Gram-positive bacteria, while the formation of bacillitiol and mycothiol is limited to *Firmicutes* and *Actinomycetes*, respectively (Gout, 2019; Ulrich & Jakob, 2019). We suppose that low-molecular-weight thiols are involved in the adaptation of *P. tundrae* IMV B-7915 and ensure cell survival during the first hour of growth since their content increased compared to the control during 1 hour of bacterial cultivation. A decrease in the content of low-molecular-weight thiols during the 24th hour of growth of *P. tundrae* IMV B-7915 may be due to the oxidation of these groups or the formation of complexes with metal ions. Glutathione reductase maintains the required content of reduced glutathione (Yan et al., 2013).

One of the ways that *P. tundrae* IMV B-7915 adapts to the effects of copper is the synthesis of extracellular polymeric substances such as exopolysaccharides and proteins. Their role in the resistance of bacteria to copper and other heavy metals is described in anammox granular sludge, *Pseudoalteromonas*, *Shewanella*, *Winogradskyella, Bacillus* sp.*, Paenibacillus polymyxa* (Raza et al., 2011; Zeng et al., 2020; Nagar et al., 2021; Pagliaccia et al., 2022). *Paenibacillus tundrae* IMV B-7915 are able to synthesize exopolysaccharides and secrete proteins on the cell surface. Exopolysaccharides play an important role in protecting against the effects of copper, as their content increased 2-fold when the bacteria were incubated at 8 mM $CuCl_2$.

5 Conclusions

Thus, penetrating into *P. tundrae* IMV B-7915 cells, copper ions induce free radical damage of cell macromolecules, resulting in an increase in the content of diene conjugates and lipid hydroperoxides. This causes inhibition of cell division processes. In response to the effect of copper, *P. tundrae* IMV B-7915 activate efflux systems, as evidenced by a significant decrease in the copper content during prolonged cultivation, as well as activate antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase, and glutathione S-transferase) and synthesize exopolysaccharides. The complex of these adaptation reactions ensures the detoxification of copper accumulated in the cells, reflected in the restoration of the specific growth rate.

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С. Комплікевич[1], О. Масловська[1], Т. Моравська[1], І. Ярмолюк[1], Н. Біронт[2], Є. Заріцька[2], С. Гнатуш[1,*]

[1] Львівський національний університет імені Івана Франка, м. Львів, 79005, Україна
[2] Державний науково-дослідний контрольний інститут ветеринарних препаратів та кормових добавок, м. Львів, 79019, Україна

***Автор для кореспонденції:** shnatush1965@gmail.com

Адаптації антарктичних бактерій *Paenibacillus tundrae* IMB B-7915 до впливу Купрум (II) хлориду

Реферат. Важкі метали поширені в біотопах Антарктики. Однак адаптації антарктичних мікроорганізмів до важких металів недостатньо з'ясовані. Одним із механізмів токсичності металів із змінною валентністю є утворення вільних радикалів, які пошкоджують макромолекули клітини. Бактерії *Paenibacillus tundrae* IMB B-7915 виділені нами у 2020 році зі зразка, що містив мох, ґрунт, підземні частини *Deschampsia antarctica* (о. Берселот, Морська Антарктика). Метою роботи було дослідити вплив Купрум (II) хлориду на питому швидкість росту, вміст продуктів вільнорадикального пошкодження ліпідів та білків, активності ензимів системи антиоксидантного захисту, синтез позаклітиннних полімерних речовин бактерій *P. tundrae* IMB B-7915. Бактерії інкубували упродовж години у трис-HCl буфері із 2–8 мМ Купрум (II) хлориду, після чого відмивали та інокулювали у триптон-соєвий бульйон. Культивували бактерії упродовж 72 годин. Вміст Купруму у клітинах визначали методом атомно-абсорбційної спектрометрії. Вміст маркерів перекисного окиснення ліпідів (дієнових кон'югатів, гідропероксидів ліпідів, тіобарбітуратактивних продуктів), окисної модифікації білків (карбонільних груп у білках), активність ензимів антиоксидантної системи захисту (каталази, супероксиддисмутази, глутатіонпероксидази, глутатіон-S-трансферази, глутатіонредуктази), загальний вміст тіолів, екзополімерних сполук (екзополісахаридів та білків) визначали фотометрично. Упродовж години клітини нагромаджують 1.5–3.4 мг Cu/г біомаси, що призводить до зниження нагромадження біомаси та питомої швидкості росту упродовж 24 годин. У клітинах йони Купруму індукують вільнорадикальні реакції пошкоджень макромолекул, що відображається у зростанні вмісту первинних продуктів перекисного окиснення ліпідів та карбонільних груп у білках. Це призводить до пригнічення поділу клітини. У відповідь на дію Купруму, клітини *P. tundrae* IMB B-7915 активують системи ефлюксу, про що свідчить значне зниження вмісту Купруму за вирощування, а також активують ензими антиоксидантного захисту і синтезують екзополісахариди. Встановлено, що комплекс досліджених реакцій адаптації забезпечує детоксикацію нагромадженого у клітинах Купруму, що відображається у відновленні питомої швидкості росту.

Ключові слова: антиоксидантний захист, важкі метали, вільні радикали, перекисне окиснення ліпідів, позаклітинні полімерні речовини, пошкодження білків