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BIOLOGICAL ACTIVITY OF *ACINETOBACTER CALCOACETICUS* IMV B-7241 SURFACTANTS SYNTHESIZED IN THE PRESENCE OF COMPETITIVE BACTERIA *BACILLUS SUBTILIS* BT-2

Currently, the effectiveness of technologies for microbial surfactants, which are characterized by a complex of practically valuable physicochemical and biological properties is lower than that of synthetic analogues. To reduce the cost of these products of microbial synthesis, industrial waste is used as substrates for their biosynthesis. In previous studies, it has been established that surfactants synthesized by Acinetobacter calcoaceticus IMV B-7241 on crude glycerol have lower antimicrobial activity compared to that obtained on purified glycerol. The main approaches to the regulation of the biological activity of microbial surfactants are their post-fermentation chemical modification, as well as the improvement of producer strains by methods of metabolic and genetic engineering. In recent years, numerous studies have appeared on the co-cultivation of producers of antimicrobial compounds with competitive microorganisms (biological inductors), in response to the presence of which the antimicrobial activity of the final product increases. ***Aim.*** *To study the effect of live and inactivated cells of Bacillus subtilis BT-2, as well as the corresponding supernatant, on the antimicrobial and anti-adhesive activity and the ability to destroy biofilms of A. calcoaceticus IMV B-7241 surfactants synthesized in a medium with glycerol of different degrees of purification.* ***Methods.*** *The A. calcoaceticus IMV B-7241 strain was grown in a liquid mineral medium with purified and crude glycerol, into which live and inactivated B. subtilis BT-2 cells as well as the supernatant after growing the B. subtilis BT-2 strain (2.5—10%, v/v) were added. Surfactants were extracted from the supernatant of the culture liquid with Folch's mixture. Anti-adhesive activity and the degree of destruction of biofilms were determined by the spectrophotometric method, and antimicrobial activity — by the indicator of the minimum inhibitory concentration. The activity of enzymes of surface-active aminolipids biosynthesis ($NADP^+$-dependent glutamate dehydrogenase) and glycolipids (phosphoenolpyruvate (PEP)-carboxylase, PEP-synthetase, PEP-carboxykinase,*

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trehalose-phosphate synthase) was analyzed in cell-free extracts obtained after cells sonication. ***Results.*** *It was established that the introduction of inactivated B. subtilis BT-2 cells and supernatant into the medium with both substrates did not affect the indicators of the surfactant synthesis, while in the presence of live cells of the B. subtilis BT-2 strain in the medium with purified glycerol, a decrease in the concentration of the final product by 1.5 times, and in the culture medium with crude glycerol — an increase by1.4 times were observed compared to the indicators with no inductor. The study of the antimicrobial activity of surfactants showed that the most effective of the used inductors (live, inactivated cells, supernatant) were live cells of B. subtilis BT-2. The introduction of B. subtilis BT-2 strain live cells into the culture medium with both substrates was accompanied by the formation of surfactants, the minimum inhibitory concentrations of which in relation to bacterial (Bacillus subtilis BT-2, Staphylococcus aureus BMS-1, Proteus vulgaris PA-12, Enterobacter cloacae C-8) and yeast (Candida albicans D-6, Candida tropicalis PE-2) test-cultures were 3—23 times lower than established for those synthesized on the medium with no inductor. Anti-adhesive activity of surfactants obtained on purified and crude glycerol in the presence of all types of inductors was higher compared to those synthesized in the culture medium without inductors (cells adhesion of bacterial and yeast test-cultures on polyvinyl chloride was 13—70 and 33—96%, respectively). Introduction of live and inactivated B. subtilis BT-2 cells or the supernatant into A. calcoaceticus IMV B-7241 cultivation medium was accompanied by the synthesis of surfactants, in the presence of which the disruption of bacterial biofilms was on average 10-20% higher compared to using surfactants synthesized without an inductor. In the presence of B. subtilis BT-2 in the medium, in the cells of the A. calcoaceticus IMV B-7241 strain, the activity of NADP$^+$-dependent glutamate dehydrogenase (a key enzyme of aminolipids biosynthesis) increased by 1.5—2 times, while the activity of biosynthesis of glycolipids enzymes remained practically at the same level as without an inductor. Such data indicate that the higher biological activity of surfactants obtained by A. calcoaceticus IMV B-7241 in the presence of biological inductors might be due to an increase in the content of aminolipids in their composition.* ***Conclusions.*** *This research has established the possibility of regulating the antimicrobial and anti-adhesive activity as well as the ability to disrupt biofilms of A. calcoaceticus IMV B-7241 surfactants by introducing competitive bacteria B. subtilis BT-2 into the culture medium. It is important that under such cultivation conditions, the antimicrobial activity of surfactants synthesized on toxic crude glycerol significantly increases.*

Keywords*: microbial surfactants, biological activity, competitive microorganisms.*

Nowadays, interest in microbial surfactants is due to the fact that they are not only able to decrease surface and interfacial tension and emulsify various substrates but also show high biological activity (antimicrobial and antiadhesive activity, including the ability to destroy biofilms) [1—5]. However, the disadvantages of biosurfactants are changes in these properties in different cultivation conditions and, accordingly, the high cost of such microbial synthesis products [1, 3]. One of the ways to increase the efficiency of microbial surfactant technology is the use available and accessible in large amounts of industrial waste, in particular, crude glycerol as substrates [4].

It was previously found that *Acinetobacter calcoaceticus* IMV B-7241 synthesizes microbial surfactants on glycerol of different degrees of purification. A study of the biological activity of biosurfactants synthesized on crude glycerol showed that such surfactants proved to be less effective antimicrobial agents compared to those formed on purified glycerol [5].

In recent years, the number of publications concerning the co-cultivation of producers of antimicrobial compounds with competitive microorganisms (biological inductors) has increased. This is considered to be an effective strategy to induce producing by microorganisms secondary metabolites with increased antimicrobial activity or/and will stimulate the production of bioactive secondary metabolites, which cannot be obtained in the corresponding pure culture [6].

Several studies have been carried out to establish the effect of inductors of different physiological states (live and inactivated cells, or supernatant) on the activity of antimicrobial metabolites [7, 8]. Researchers mainly use living cells of microorganisms as inductors, less often only thermally inactivated or autoclaved cells

[9], and very rarely supernatant after cultivation of inductor microorganisms.

In our previous studies, it has been established that the introduction of live cells of *Escherichia coli* IEM-1 and *Bacillus subtilis* BT-2 into the culture medium of *Nocardia vaccinii* IMV B-7405 and *Rhodococcus erythropolis* IMV Ac-5017 was accompanied by an increasing the biological activity of synthesized surfactants [10, 11].

Related to the above stated, the purpose of this work was to study the effect of live and inactivated *B. subtilis* BT-2 cells, as well as the corresponding supernatant, on the antimicrobial, anti-adhesive activity, and ability to destroy biofilms of *A. calcoaceticus* IMV B-7241 surfactants synthesized in a medium with glycerol of different degrees of purification.

Materials and methods. The main object of research was a strain of oil-oxidizing bacteria, isolated by us from an oil-contaminated soil sample, identified as *A. calcoaceticus* K-4 [12]. Strain K-4 is registered in the Depository of Microorganisms of the Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine under the number of IMV B-7241. By chemical nature, extracellular biosurfactants of *A. calcoaceticus* IMV B-7241 are a complex of amino- and glycolipids. Glycolipids are represented by trehalose mycolates [13].

Strains of bacteria (*Bacillus subtilis* BT-2, *Staphylococcus aureus* BMS-1, *Proteus vulgaris* PA-12, *Enterobacter cloacae* C-8) and yeast (*Candida albicans* D-6, *Candida tropicalis* PE-2) from the collection of live cultures of the Department of Biotechnology and Microbiology of the National University of Food Technologies (Ukraine) were used as test cultures in determining the biological activity of surfactants.

The cultivation of *A. calcoaceticus* IMV B-7241 was carried out in a liquid medium with the following composition (g/L): $(NH_2)_2CO$ — 0.35, NaCl — 1.0, $Na_2HPO_4 \cdot 12H_2O$ — 0.6, KH_2PO_4 — 0.14, $MgSO_4 \cdot 7H_2O$ — 0.1, distilled water — up to 1 L, pH 6.8—7.0. Yeast autolysate — 0.5% (v/v) and microelement solution — 0.1% (v/v) were added into the medium. The following carbon sources were used (%, v/v): purified glycerol — 3, crude glycerol — 5. Concentrations of glycerol of various qualities were equimolar by carbon.

The culture in the exponential phase, grown in a medium of the above composition with 0.5% of the corresponding substrate was used as an inoculum. The inoculum with the number of bacteria 10^4—10^5 cells/mL was added in the amount of 10% of the medium volume.

The bacterial strain *B. subtilis* BT-2 was used as a biological inductor in the form of live and inactivated cells, as well as the corresponding supernatant after BT-2 strain cultivation. of The inductor was introduced at the beginning of the cultivation process. *B. subtilis* BT-2, grown on MPA for 24 h, was suspended in 100 mL of sterile tap water, and 2.5 mL of the suspension was added into 100 mL of the culture medium of the surfactant producer. 10 mL of suspension of inactivated cells (autoclaving at 131 °C for 1 h) or 2.5 mL of the supernatant were introduced into 100 mL of the culture medium.

Cultivation of *A. calcoaceticus* IMV B-7241 in the presence of supernatant, live and inactivated cells of *B. subtilis* BT-2 and without inductors was carried out in 750 ml flasks containing 100 mL of the culture medium for 7 days at 30 ºC and 320 rpm.

To obtain cell-free extracts, the culture liquid was centrifuged (5000 g, 20 min, 4 ºC). The resulting cell precipitate was washed twice from the medium residues with 0.05 mol of K^+-phosphate buffer (pH 7.0) and centrifuged (4000 g, 15 min, 4 ºC). The washed cells were resuspended in 0.05 mol of K^+-phosphate buffer (pH 7.0) and destroyed by ultrasound (22 kHz) 3 times for 20 s at 4 °C on a UZDN-1 device. The resulting disintegrate was centrifuged (12000 g, 30 min, 4 ºC), the precipitate was separated, and the supernatant was used for further studies as a cell-free extract.

Enzyme activity was quantified by the method previously described in our work [14]. The

activity of phosphoenolpyruvate (PEP) synthase (EC: 2.7.9.2) was determined by the rate of pyruvate formation, which was analyzed through the oxidation of NADH at 340 nm in a coupled reaction with lactate dehydrogenase and phosphoenolpyruvate carboxykinase (EC: 4.1.1.49) — by the formation of PEP and pyruvate during the oxidation of NADH, glutamate dehydrogenase (EC: 1.4.1.4) — by the formation of glutamate during the oxidation of NADPH at 340 nm, and PEP-carboxylase (EC: 4.1.1.31) — by oxidation of NADH at 340 nm. The activity of trehalose-phosphate synthase (EC: 2.4.1.15) was analyzed through the formation of uridine diphosphate, which was determined spectrophotometrically by the oxidation of NADH at 340 nm in coupled reactions with pyruvate kinase and lactate dehydrogenase.

Enzyme activity is expressed as nmoles of the product generated per minute per mg of protein. The protein content in cell-free extracts was determined by the Bradford method. Enzyme activity was measured at 28—30 ºC (the optimal temperature for the *A. calcoaceticus* IMV B-7241 growth).

The amount of an extracellular surfactant was defined by weighing after the extraction from the culture liquid supernatant using a mixture of chloroform and methanol (2:1) as described in [10]. To obtain the supernatant, the culture liquid was centrifuged at 5000 g for 20 min.

In the research, solutions of surfactants with various concentrations were used as preparations. For this, the dry surfactant residue was dissolved in sterile phosphate buffer (0.1 M, pH 7.0) to the original volume (25 mL) and further diluted with this buffer to the required concentration. Surfactant solutions were sterilized in an autoclave at 112 °C for 30 min.

The antimicrobial activity of surfactants was analyzed by the minimum inhibitory concentration (MIC) as previously described in [10]. MIC was determined by the method of double serial dilutions in meat-peptone broth for bacteria and liquid wort for yeast. The results were evaluated visually by the turbidity of the medium: (+) — test tubes in which the turbidity of the medium was observed (growth of the test culture), (—) — no turbidity (no growth). The MIC of surfactant solutions was determined as the concentration of surfactant in the last test tube where growth was absent.

The anti-adhesive activity of surfactants was determined by the spectrophotometric method as described in our previous studies [10]. The number of adhered cells was determined as the ratio of the optical density of the suspension obtained after treatment of polyvinyl chloride with surfactant solutions to the optical density of the suspension obtained after surface treatment with phosphate buffer (control) and expressed as a percentage (%).

Determination of the effect of surfactants on biofilm destruction was carried out as described in our work [10]. The degree of biofilm destruction (%) was determined spectrophotometrically as the difference between cells' adhesion in untreated and surfactant-treated wells of a polystyrene plate.

All experiments were performed in three repetitions; the number of parallel determinations in the experiments was 3 to 5. Statistical processing of experimental data was carried out as described earlier [10, 13]. Differences in average indicators were considered reliable at the significance level of $P < 0.05$.

Results. The indicators of surfactant synthesis under *A. calcoaceticus* IMV B-7241 cultivation on glycerol of different degrees of purification in the presence of biological inductors are presented in Table 1.

The data listed in Table 1 indicate that during the cultivation of the surfactant producer on both substrates, the presence of inductors in the form of inactivated *B. subtilis* BT-2 cells and supernatant does not affect the concentration of the synthesized final product, which was 1.41—1.54 and 2, 32—2.52 g/L on purified and crude glycerol, respectively.

The introduction of live cells of *B. subtilis* BT-2 into the medium with purified glycerol was accompanied by a decrease in the amount of surfactant by 1.5 times compared to the indicators without an inductor. At the same time, in the presence of living cells of *B. subtilis* BT-2 in the medium with crude glycerol, a 1.4-fold increase in the amount of surfactant was observed compared to the cultivation of *A. calcoaceticus* IMV B-7241 on crude glycerol without an inductor.

The effect of surfactants synthesized by *A. calcoaceticus* IMV B-7241 in the presence of biological inductors on test cultures of bacterial strains and yeasts was studied during the following stage of the research (Table 2).

The data in Table 2 indicate that the most effective of the used inductors (live and inactivated cells, or supernatant) were live cells of *B. subtilis* BT-2: their introduction into the culture medium with both substrates had a great influence on the antibacterial activities of surfactants, the minimum inhibitory concentrations of which in relation to the tested test cultures were 3—23 times lower than those established for preparations obtained on the medium without corresponding inductor. After adding inactivated *B. subtilis* BT-2 cells into the culture medium of *A. calcoaceticus* IMV B-7241 with purified and crude glycerol, surfactants were synthesized, the MIC indexes of which against bacteria and yeast were 2—8 times lower than the values established for preparations obtained in the medium without the inductor.

The supernatant was the least effective of the considered inductors: the antimicrobial activity against most test cultures of surfactants synthesized in its presence was only two times lower than that of surfactants obtained without an inductor (see Table 2).

Table 3 shows the data on the number of cells of test cultures attached to polyvinyl chloride

Table 1. **Synthesis of surfactants under *A. calcoaceticus* IMV B-7241 cultivation on glycerol of different degrees of purification in the presence of biological inductors**

Biological inductor	Concentration of biosurfactants (g/L) synthesized	
	On purified glycerol	On crude glycerol
Control (no inductor)	1.42 ± 0.07	2.52 ± 0.12
Live cells of *Bacillus subtilis* BT-2	0.96 ± 0.04	3.56 ± 0.17
Inactivated cells of *Bacillus subtilis* BT-2	1.54 ± 0.07	2.32 ± 0.11
Supernatant	1.44 ± 0.07	2.36 ± 0.11

Table 2. **The effect of biological inductors on the antimicrobial activity of *A. calcoaceticus* IMV B-7241 surfactants**

Carbon source	Biological inductor	Minimum inhibitory concentrations (μg/mL) against					
		Bacillus subtilis BT-2	*Staphylococcus aureus* BSC-1	*Proteus vulgaris* PA-12	*Enterobacter cloacae* C-8	*Candida tropicalis* PE-2	*Candida albicans* D-6
Purified glycerol	Control (no inductor)	2.8	2.8	5.6	5.6	11.2	11.2
	Live cells	0.23	0.23	1.84	0.46	1.87	3.75
	Inactivated cells	1.4	1.4	1.4	0.7	2.8	5.6
	Supernatant	1.4	2.8	2.8	1.4	11.2	11.2
Crude glycerol	Control (no inductor)	9.8	4.9	9.8	19.6	19.7	19.7
	Live cells	0.85	0.85	1.7	0.85	3.5	7.0
	Inactivated cells	2.2	2.2	2.2	4.4	4.5	9.0
	Supernatant	4.6	2.3	4.6	18.4	9.2	18.4

When determining the antimicrobial activity, the error did not exceed 5%.

Table 3. **The effect of surfactants synthesized by *A. calcoaceticus* IMV B-7241 in the presence of biological inductor on the adhesion of test cultures to polyvinyl chloride**

Carbon source	Biological inductor	Adhesion, %					
		Bacillus subtilis BT-2	*Proteus vulgaris* PA-12	*Enterobacter cloacae* C-8	*Staphylococcus aureus* BMS-1	*Candida tropicalis* PE-2	*Candida albicans* D-6
Purified glycerol	Control (no inductor)	90	76	62	83	33	86
	Live cells	30	20	28	33	18	23
	Inactivated cells	60	25	46	38	19	26
	Supernatant	70	50	56	40	24	28
Crude glycerol	Control (no inductor)	n.d.	96	75	33	64	68
	Live cells	n.d.	40	32	17	15	13
	Inactivated cells	n.d.	70	39	17	24	17
	Supernatant	n.d.	70	57	25	42	18

The concentration of surfactant when determining the adhesion of bacteria was 96 μg/mL, yeast — 12 μg/mL; n.d. — not defined; when determining adhesion, the error did not exceed 5%.

Table 4. **Destruction of bacterial biofilms under the action of surfactants synthesized by *A. calcoaceticus* IMV B-7241 on purified glycerol in the presence of *B. subtilis* BT-2**

Test culture	Biological inductor	Destruction of biofilm (%) under the action of surfactants at a concentration (μg/mL)					
		240	120	60	30	15	7,5
Bacillus subtilis BT-2	Control (no inductor)	55	54	43	40	38	35
	Supernatant	54	52	41	40	36	36
	Live cells	64	60	58	50	50	48
	Inactivated cells	59	58	50	45	43	40
Staphylococcus aureus BMS-1	Control (no inductor)	38	33	31	30	30	28
	Supernatant	56	52	48	40	34	33
	Live cells	65	65	50	46	40	36
	Inactivated cells	55	53	48	40	35	33
Enterobacter cloacae C-8	Control (no inductor)	55	55	50	40	35	30
	Supernatant	60	60	55	55	45	40
	Live cells	80	75	73	70	70	65
	Inactivated cells	76	68	65	60	55	51
Proteus vulgaris PA-12	Control (no inductor)	38	38	38	36	32	29
	Supernatant	47	45	45	40	40	36
	Live cells	58	55	55	50	43	42
	Inactivated cells	58	52	50	48	40	40

When determining the destruction of the biofilm, the error did not exceed 5%.

treated with solutions of surfactants synthesized by *A. calcoaceticus* IMV B-7241 on glycerol of various degrees of purification in the presence of biological inductors. These data indicate that the antiadhesive activity of surfactants obtained on purified and crude glycerol in the presence of all types of inductors was higher than those synthesized in the culture medium without an inductor. Meanwhile, the effect of using the supernatant as an inductor was lower compared to live and inactivated *B. subtilis* BT-2 cells.

Listed in Table 4 data show that during the producer cultivation on purified glycerol in the presence of both live and inactivated cells, as well as the supernatant, the synthesis of surfactants was observed, under the influence of which bacterial biofilm disruption increased on average by 10—20% compared to the indicators for the effects of surfactants synthesized without an inductor.

The most effective inductor under *A. calcoaceticus* IMV B-7241 cultivation on purified glycerol was live *B. subtilis* BT-2 cells: surfactants synthesized in their presence were characterized by a higher ability to destroy more bacterial biofilms than those obtained using inactivated inductor cells or supernatant.

The following studies showed that the given in Table 4 regularities were also observed for surfactants synthesized by *A. calcoaceticus* IMV B-7241 on crude glycerol in the presence of biological inductors.

At the final stage of experiments, the influence of live and inactivated *B. subtilis* BT-2 cells, as well as the supernatant in a medium with glycerol of various degrees of purification on the activity of biosynthesis enzymes of surface-active amino- and glycolipids in *A. calcoaceticus* IMV B-7241 was analyzed (Table 5).

It was established that in the presence of live and inactivated cells of the inductor in the culture medium of IMV B-7241 strain in its cells, the activity of $NADP^+$-dependent glutamate dehydrogenase increased up to by 1.5—2 times compared to that during the cultivation without *B. subtilis* BT-2, while as in the case of using the supernatant, the activity of this enzyme did not change. Regardless of the used inductor, the activity of PEP-carboxykinase, PEP-synthetase, and trehalose phosphate synthase remained almost at the same level as that without *B. subtilis* BT-2.

Discussion. Taking *B. subtilis* BT-2 as a competitive microorganism (inductor) was due to the following reasons. In works [15, 16], it is noted that during co-cultivation, imitation of natural conditions took place, and in the fight for the substrate, «silent» genes are activated, re-

Table 5. **The activity of key enzymes in surfactants biosynthesis in the presence of biological inductors in the *Acinetobacter calcoaceticus* IMV B-7241 culture medium**

Growth substrate	Inductor	Activity (nmol min^{-1} mg^{-1} of protein)			
		$NADP^+$-dependent glutamate dehydrogenase	PEP-synthase	PEP-carboxylase	Trehalose phosphate synthase
Purified glycerol	Control (no inductor)	670 ± 33	21459 ± 1073	980 ± 49	25 ± 2
	Live cells	1178 ± 59	20373 ± 1018	1178 ± 59	27 ± 1
	Inactivated cells	986 ± 49	20111 ± 1006	1107 ± 55	26 ± 1
	Supernatant	655 ± 32	19682 ± 984	955 ± 48	25 ± 2
Crude glycerol	Control (no inductor)	370 ± 18	22555 ± 1127	386 ± 19	110 ± 5
	Live cells	730 ± 36	23333 ± 1166	370 ± 18	100 ± 5
	Inactivated cells	570 ± 28	20945 ± 1047	370 ± 18	105 ± 5
	Supernatant	370 ± 18	21204 ± 1060	370 ± 18	116 ± 6

sponsible for the synthesis of compounds with antimicrobial activity against competitive microorganisms. This was true primarily for microorganisms isolated from common habitats. Therefore, as competitive microorganisms or inductors to increase the antimicrobial activity of antibiotics synthesized by soil streptomycetes, the researchers chose *B. subtilis* strains, which are typical inhabitants of such biotopes [9]. It should be noted that *A. calcoaceticus* IMV B-7241 (the surfactant producer) was also isolated by us from the soil [12] as a representative of *Bacillus* genus.

At the first stage of research, the effect of live, inactivated *B. subtilis* BT-2 cells and supernatant on the synthesis of surfactants by *A. calcoaceticus* IMV B-7241 was analyzed. A 1.5-fold decrease in the number of surfactants in the presence of live cells of the inductor in the medium with purified glycerol compared to the indicators without *B. subtilis* BT-2 might be due to the competition between the inductor and the surfactant producer for the substrate and the higher growth rate of *B. subtilis* BT-2 under these cultivation conditions. The increase in the synthesis of surfactants after the introduction of live *B. subtilis* BT-2 cells into the culture medium with crude glycerol compared to the cultivation of *A. calcoaceticus* IMV B-7241 on this substrate without an inductor can be explained as follows. In our opinion, toxic impurities in the composition of crude glycerol inhibited the growth of the inductor, while methanol, ethanol, triglycerides, and fatty acids served as additional carbon sources for the surfactant producer.

The study of the antimicrobial activity of surfactants synthesized in the presence of biological inductors showed that the minimum inhibitory concentrations for bacterial and yeast test cultures differ depending on the physiological state of the inductor (live, inactivated cells, supernatant) (Table 2). Similarly, the anti-adhesive activity (Table 3), as well as the ability to destroy biofilms (Table 4) of surfactants synthesized with the addition of inductors, depended on the form in which the inductor was introduced into the culture medium. Live cells of *B. subtilis* BT-2 were the most effective inductor whereas the supernatant was the least effective.

However, the results of our previous studies [10] have shown that the antimicrobial activity of *R. erythropolis* IMV Ac-5017 surfactants do not depend on the physiological state of the inductors (live or inactivated cells). Similar results have been obtained by us during research on the effect of live and inactivated cells of *E. coli* IEM-1 and *B. subtilis* BT-2 on the biological activity of surfactants synthesized by *N. vaccinii* IMV B-7405 [11].

It should be mentioned that literature data on the effectiveness of using live, inactivated cells, or the corresponding supernatants as inductors are very different. In [7], it is noted that the spectrum of metabolites synthesized by actinobacteria in the presence of live inductor cells is wider than in the case of using cells inactivated by heat treatment. At the same time, the synthesis of phenazine by *Pseudomonas aeruginosa* increases almost equally regardless of the physiological state (live or inactivated cells) of inductors *Escherichia coli*, *B. subtilis*, and *Saccharomyces cerevisiae* [17]. The use of the supernatant of *Streptomyces bullii* C2 as an inductor did not have a positive effect on the synthesis of antimicrobial compounds by the fungus *Aspergillus fumigatus* MBC-F1-10, while in the presence of live cells of the inductor, the synthesis of nine new antimicrobial metabolites that were not produced by the monoculture of the producer was observed [8]. Wang et al. [18] found that the supernatant of *Penicillium chrysogenum* AS 3.5163 proved to be a more effective inductor of the synthesis of the antibiotic natamycin by the strain *Streptomyces natalensis* HW-2 than the cells of the fungus inactivated by autoclaving (an increase in the concentration of the antibiotic was by 3.4 and 1.4 times, respectively). Such data indicate various mechanisms for increasing the synthesis or activity of antimicrobial compounds synthesized under the action of inductors.

In the vast majority of studies on the influence of competitive microorganisms on the antimicrobial activity of metabolites, living cells were used as biological inductors [19—21]. In some works [7, 17], the role of both living and inactivated cells of inductors in the regulation of antimicrobial activity of products of microbial synthesis was investigated. Of course, in technology, it is easier to use inactivated cells of inductors. Therefore, it is not surprising that some scientists investigated the effect of only inactivated cells of competitive microorganisms on the synthesis and antimicrobial activity of metabolites [9, 22—25].

So, Leães et al. [24] established that after introduction into the culture medium of lipopeptide producer *Bacillus amyloliquefaciens* P11 *inactivated by heat treatment* of *S. aureus* subsp. *aureus* ATCC 25923 or *Aspergillus parasiticus* (strain number not given) cells synthesized surfactants, the antimicrobial activity of which against *Listeria monocytogenes* ATCC 7644 increased by 3—3.5 times. Under such cultivation conditions, the level of expression of genes responsible for the synthesis of iturin and fengycin increased. It is interesting that using inactivated cells of *E. coli* ATCC 25922, *Bacillus cereus* ATCC11788, *L. monocytogenes* ATCC 7644, and fungi *Fusarium oxysporum* f. *lycopersici* and *Fusarium graminearum* (numbers not given) was not accompanied with an increase in the antimicrobial activity of synthesized surfactants. In work [24], the authors did not explain such a «selective» effect of the type of inductors on the biological activity of surfactants. Note, that the authors of the research [24] studied the effect of inactivated cells of inductors only on the antimicrobial activity of synthesized surfactants, although these products of microbial synthesis have an anti-adhesive activity as well.

Our results showed that synthesized in the presence of inductors by *A. calcoaceticus* IMV B-7241 surfactants were characterized by high both antimicrobial (see Table 2) and antiadhesive (see Table 3) activity, as well as the ability to destruction of biofilms (see Table 4).

It should be noted, that there were little literature data on increasing the ability of microbial synthesis products synthesized in the presence of biological inductors (living or inactivated cells or supernatant) to disrupt biofilms.

In work [26], it was noted that in the presence of supernatant after the co-cultivation of *B. subtilis* together with *Lactobacillus plantarum*, the degree of *S. aureus* biofilm destruction reached up to 61%, while the supernatant after the cultivation of the *Lactobacillus plantarum* monoculture inhibited the formation of the *S. aureus* biofilm only by 40% after 24 h. The authors established that the increase in the biofilm disruption due to the action of the supernatant after the co-cultivation of the two strains was caused by the synthesis of antimicrobial lipopeptides of *B. subtilis* under such conditions.

Mohamed et al. [27] established that during the co-cultivation of two bacterial strains *Micromonospora* sp. UR56 and *Actinokineospora* sp. EG49, there were a few new metabolites, which were not typical for monocultures. The induced metabolites were characterized as phenazine derivatives and showed the ability to destroy biofilms of *B. subtilis, S. aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. Phenazine derivatives at a concentration of 10 μl destroyed 24-h biofilms of *E. coli* by an average of 54, *S. aureus* — 50, *B. subtilis* — 18, and *P. aeruginosa* — 42%.

The data we obtained regarding the higher efficiency of using live cells as an inductor compared to inactivated cells or the supernatant (Tables 2—4) may indicate that the inducing factor is connected to the cells, and induction requires both chemical and biological interaction between the surfactant producer and the inductor. The process of autoclaving inductor cells leads to the denaturation of proteins and other macromolecules, partially inhibiting potential biochemical interactions.

Similar data were presented in other scientific papers. Onaka et al. [28] established that the inductors of synthesis of secondary metabolites (including antibiotics) by representatives of the genus *Streptomyces* were bacteria that contained mycolic acids in the composition of the cell wall (*R. erythropolis, Corynebacterium glutamicum, Tsukamurella pulmonis*). However, later [23], it was found that inactivated cells of bacteria *T. pulmonis, R. erythropolis* and *Rhodococcus opacus* do not induce pigment synthesis by the *Streptomyces lividans* strain. Apparently, mycolic acids localized in the outer layer of bacterial cells affected the secondary metabolism of streptomycetes as a result of direct interaction between inductor bacteria and bacteria of the genus *Streptomyces*.

The last stage of our research was devoted to determining the activity of key enzymes in the biosynthesis of surface-active amino- ($NADP^+$-dependent glutamate dehydrogenase) and glycolipids (PEP-carboxykinase, PEP-synthetase, and trehalose-phosphate synthase) in *A. calcoaceticus* IMV B-7241. The need to conduct such research was due to the following reasons. Firstly, aminolipids are characterized by higher antimicrobial activity compared to glycolipids [3]. Secondly, one of the mechanisms underlying the anti-adhesive activity and ability to disrupt biofilms of microbial surfactants is their antimicrobial activity [29]. Therefore, we assumed that the introduction of biological inductors into the culture medium of *A. calcoaceticus* IMV B-7241 will be accompanied by an increase in the activity of the key enzyme of aminolipid biosynthesis. The performed enzymatic studies (Table 5) confirmed our assumptions. Consequently, the higher biological activity of surfactants obtained by the cultivation *A. calcoaceticus* IMV B-7241 in the presence of biological inductors, compared to that of preparations synthesized without them, may be due to the higher content of aminolipids in their composition.

Therefore, as a result of the work carried out, it was established the possibility of regulating the antimicrobial and anti-adhesive activity as well as the ability to disrupt biofilms of *A. calcoaceticus* IMV B-7241 surfactants by introducing the producer of competitive bacteria *B. subtilis* BT-2 into the culture medium. It is important that under such cultivation conditions, the antimicrobial activity of surfactants synthesized on toxic crude glycerol significantly increased.

REFERENCES

1. Bhadra S, Chettri D, Kumar Verma A. Biosurfactants: secondary metabolites involved in the process of bioremediation and biofilm removal. Appl Biochem Biotechnol. 2022; doi: 10.1007/s12010-022-03951-3.
2. De Giani A, Zampolli J, Di Gennaro P. Recent trends on biosurfactants with antimicrobial activity produced by bacteria associated with human health: different perspectives on their properties, challenges, and potential applications. Front Microbiol. 2021;12:655150. doi: 10.3389/fmicb.2021.655150.
3. Pirog TP, Kliuchka LV, Shevchuk TA, Muchnyk FV. [Interrelation of Chemical Composition and Biological Properties of Microbial Surfactants]. Mikrobiol Z. 2019; 81(3):84—104. doi: https://doi.org/10.15407/microbiolj81.03.084. Ukrainian.
4. Sharma P, Gaur VK, Gupta S, Varjani S, Pandey A, Gnansounou E, et al. Trends in mitigation of industrial waste: global health hazards, environmental implications and waste derived economy for environmental sustainability. Sci Total Environ. 2022; 811:152357. doi: 10.1016/j.scitotenv.2021.152357.
5. Pirog TP, Lutsay DA, Kliuchka LV, Beregova KA. Antimicrobial activity of surfactants of microbial origin. Biotechnologia Acta. 2019; 12(1):39—57. https://doi.org/10.15407/biotech12.01.039.
6. Chen J, Zhang P, Ye X, Wei B, Emam M, Zhang H, et al. The structural diversity of marine microbial secondary metabolites based on co-culture strategy: 2009—2019. Mar Drugs. 2020; 18(9):449. doi: 10.3390/md18090449.
7. Liang L, Wang G, Haltli B, Marchbank DH, Stryhn H, Correa H, et al. Metabolomic comparison and assessment of co-cultivation and a heat-killed inducer strategy in activation of cryptic biosynthetic pathways. J Nat Prod. 2020; 83(9):2696—705. doi: 10.1021/acs.jnatprod.0c00621.

8. Rateb ME, Hallyburton I, Houssen WE, Bull AT, Goodfellow M, Santhanam, R, et al. Induction of diverse secondary metabolites in *Aspergillus fumigatus* by microbial co-culture. RSC Adv. 2013; 3(34):14444—50. https://doi.org/10.1039/C3RA42378F
9. Liang L, Sproule A, Haltli B, Marchbank DH, Berrué F, Overy DP, et al. Discovery of a new natural product and a deactivation of a quorum sensing system by culturing a «producer» bacterium with a heat-killed «inducer» culture. Front Microbiol. 2019; 9:3351. doi: 10.3389/fmicb.2018.03351.
10. Pirog T, Kluchka L, Skrotska O, Stabnikov V. The effect of co-cultivation of *Rhodococcus erythropolis* with other bacterial strains on biological activity of synthesized surface-active substances. Enzyme Microb Technol. 2020; 142:109677. doi:10.1016/j.enzmictec.2020.109677.
11. Pirog TP, Skrotska OI, Shevchuk TA. [Influence of Biological Inducers on Antimicrobial, Antiadhesive Activity and Biofilm Destruction by *Nocardia vaccinii* IMB V-7405 Surfactants]. Mikrobiol Z. 2020; 82(3):35—44. Ukrainian. doi: https://doi.org/10.15407/microbiolj82.03.035
12. Pirog TP, Shevchuk TA, Voloshina IN, Gregirchak NN. Use of claydite-immobilized oil-oxidizing microbial cells for purification of water from oil. Appl Biochem Microbiol. 2005; 41(1):51—5. https://doi.org/10.1007/s10438-005-0010-z.
13. Pirog T, Sofilkanych A, Konon A, Shevchuk T, Ivanov S. Intensification of surfactants' synthesis by *Rhodococcus erythropolis* IMV Ac-5017, *Acinetobacter calcoaceticus* IMV B-7241 and *Nocardia vaccinii* K-8 on fried oil and glycerol containing medium. Food Bioprod Process. 2013; 91(2):149—57. http://dx.doi.org/10.1016/j.fbp.2013.01.001.
14. Pirog TP, Shevchuk TA, Beregova KA, Kudrya NV. [Peculiarities of glucose and glycerol metabolism in *Nocardia vaccinii* IMB B-7405]. Ukr Biochem J. 2015; 87(2):66—75. Ukrainian.
15. Abdel-Wahab NM, Scharf S, Ozkaya FC, Kurtan T, Mandi A, Fouad MA, et al. Induction of secondary metabolites from the marine-derived fungus *Aspergillus versicolor* through co-cultivation with *Bacillus subtilis*. Planta Medica. 2019; 85 (6):503—12. doi: 10.1055/a-0835-2332.
16. Buijs Y, Zhang SD, Jørgensen KM, Isbrandt T, Larsen TO, Gram L. Enhancement of antibiotic production by co-cultivation of two antibiotic producing marine *Vibrionaceae* strains. FEMS Microbiol Ecol. 2021; 97(4):fiab041. doi: 10.1093/femsec/fiab041.
17. Luti KJK, Yonis RW. Elicitation of *Pseudomonas aeruginosa* with live and dead microbial cells enhances phenazine production. Rom Biotechnol Lett. 2013; 18(6):8769—78.
18. Wang D, Yuan J, Gu S, Shi Q. Influence of fungal elicitors on biosynthesis of natamycin by *Streptomyces natalensis* HW-2. Appl Microbiol Biotechnol, 2013; 97(12):5527—34. doi: 10.1007/s00253-013-4786-0.
19. Li B, Li Q, Xu Z, Zhang N, Shen Q, Zhang R. Responses of beneficial *Bacillus amyloliquefaciens* SQR9 to different soilborne fungal pathogens through the alteration of antifungal compounds production. Front Microbiol. 2014; 5. doi: 10.3389/fmicb.2014.00636.
20. Alves AR, Sequeira AM, Cunha Â. Increase in bacterial biosurfactant production by co-cultivation with biofilm-forming bacteria. Lett Appl Microbiol. 2019; 69(1):79—86. doi: 10.1111/lam.13169.
21. Hifnawy M, Hassan HM, Mohammed R, Fouda M, Sayed AM. Induction of antibacterial metabolites by co-cultivation of two red-sea-sponge-associated Actinomycetes *Micromonospora* sp. UR56 and *Actinokinespora* sp. EG49. Mar Drugs. 2020; 18 (5):243—54. doi: 10.3390/md18050243.
22. Luti KJ, Mavituna F. Elicitation of *Streptomyces coelicolor* with dead cells of *Bacillus subtilis* and *Staphylococcus aureus* in a bioreactor increases production of undecylprodigiosin. Appl Microbiol Biotechnol. 2011; 90(2):461—6. doi: 10.1007/s00253-010-3032-2.
23. Asamizu S, Ozaki T, Teramoto K, Satoh K, Onaka H. Killing of mycolic acid-containing bacteria aborted induction of antibiotic production by *Streptomyces* in combined-culture. PLoS One. 2015; 10(11):e0142372. doi: 10.1371/journal.pone.0142372.
24. Leães FL, Velho RV, Caldas DG, Ritter AC, Tsai SM, Brandelli A. Expression of essential genes for biosynthesis of antimicrobial peptides of *Bacillus* is modulated by inactivated cells of target microorganisms. Res Microbiol. 2016; 167(2):83—9. doi: 10.1016/j.resmic.2015.10.005.
25. Elsherbiny G, Moghannem S, Kalaba M. Enhancement of *Streptomyces* sp. Mh-133 activity against some antibiotic resistant bacteria using biotic elicitation. Al Azhar Bulletin of Science. 2017; 9:275—88.
26. Kimelman H, Shemesh M. Probiotic bifunctionality of *Bacillus subtilis*-rescuing lactic acid bacteria from desiccation and antagonizing pathogenic *Staphylococcus aureus*. Microorganisms. 2019; 7(10):407. doi: 10.3390/microorganisms7100407.

27. S Hifnawy M, Hassan HM, Mohammed R, M Fouda M, Sayed AM, A Hamed A, et al. Induction of antibacterial metabolites by co-cultivation of two red-sea-sponge-associated actinomycetes *Micromonospora* sp. UR56 and *Actinokinespora* sp. EG49. Mar Drugs. 2020;18(5):243. doi: 10.3390/md18050243.
28. Onaka H, Mori Y, Igarashi Y, Furumai T. Mycolic acid-containing bacteria induce natural-product biosynthesis in *Streptomyce*s species. Appl Environ Microbiol. 2011;77(2):400—6. doi: 10.1128/AEM.01337-10.
29. Sharma D, Misba L, Khan AU. Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrob Resist Infect Control. 2019; 8:76. doi: 10.1186/s13756-019-0533-3.

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БІОЛОГІЧНА АКТИВНІСТЬ ПОВЕРХНЕВО-АКТИВНИХ РЕЧОВИН A*CINETOBACTER CALCOACETICUS* ІМВ В-7241, СИНТЕЗОВАНИХ ЗА НАЯВНОСТІ КОНКУРЕНТНИХ БАКТЕРІЙ *BACILLUS SUBTILIS* БТ-2

На теперішній час ефективність технологій мікробних поверхнево-активних речовин, які характеризуються комплексом цінних фізико-хімічних та біологічних властивостей, є нижчою, ніж синтетичних аналогів. Для зниження собівартості цих продуктів мікробного синтезу використовують промислові відходи як субстрати для їх біосинтезу. У попередніх дослідженнях встановлено, що поверхнево-активним речовинам, синтезованим *Acinetobacter calcoaceticus* ІМВ В-7241 на відходах виробництва біодизелю, притаманна нижча антимікробна активність порівняно з утвореними на очищеному гліцерині. Основними підходами до регуляції біологічної активності мікробних поверхнево-активних речовин є їхня постферментаційна хімічна модифікація, а також вдосконалення штамів-продуцентів методами метаболічної та генетичної інженерії. Останніми роками з'являється все більше публікацій про спільне культивування продуцентів антимікробних сполук з конкурентними мікроорганізмами (біологічними індукторами), у відповідь на наявність яких відбувається підвищення антимікробної активності цільового продукту. **Мета.** Дослідити вплив живих та інактивованих клітин *Bacillus subtilis* БТ-2, а також відповідного супернатанту на антимікробну, антиадгезивну активності та здатність до руйнування біоплівок поверхнево-активних речовин *A. calcoaceticus* ІМВ В-7241, синтезованих на гліцерині різного ступеня очищення. **Методи.** Штам *A. calcoaceticus* ІМВ В-7241 вирощували у середовищах з очищеним гліцерином та відходами виробництва біодизелю, в які вносили живі та інактивовані клітини *B. subtilis* БТ-2, а також супернатант після вирощування штаму *B. subtilis* БТ-2 (2,5—10%, об'ємна частка). Поверхнево-активні речовини екстрагували з супернатанту культуральної рідини сумішшю Фолча. Антиадгезивну активність та ступінь деструкції біоплівок визначали спектрофотометричним методом, антимікробну активність — за показником мінімальної інгібуючої концентрації. Активність ферментів біосинтезу поверхнево-активних аміноліпідів (НАДФ$^+$-залежна глутаматдегідрогеназа) та гліколіпідів (фосфоенолпіруват(ФЕП)-карбоксилаза, ФЕП-синтетаза, ФЕП-карбоксикіназа, трегалозофосфатсинтаза) аналізували у безклітинних екстрактах, одержаних після руйнування клітин ультразвуком. **Результати.** Встановлено, що внесення у середовище з обома субстратами інактивованих клітин *B. subtilis* БТ-2 і супернатанту не впливало на показники синтезу поверхнево-активних речовин, у той час як за наявності живих клітин штаму *B. subtilis* БТ-2 у середовищі з очищеним гліцерином спостерігали зниження концентрації цільового продукту в 1,5 рази, а у середовищі з відходами виробництва біодизелю — підвищення в 1,4 рази порівняно з показниками без індуктора. Дослідження антимікробної активності поверхнево-активних речовин показало, що найефективнішими з використовуваних індукторів (живі, інактивовані клітини, супернатант) виявилися живі клітини *B. subtilis* БТ-2. Внесення живих клітин штаму *B. subtilis* БТ-2 у середовище з обома субстратами супроводжувалося утворенням поверхнево-активних речовин, мінімальні інгібуючі концентрації яких щодо бактеріальних ((*Bacillus subtilis* БТ-2, *Staphylococcus aureus* БМС-1, *Proteus vulgaris* ПА-12, *Enterobacter cloacae* С-8) і дріжджових (*Candida albicans* Д-6, *Candida tropicalis* РЕ-2) тест-культур були в 3—23 рази нижчими, ніж встановлені для синтезованих на середовищі без цього індуктора. Антиадгезивна активність поверхнево-активних речовин, одержаних

на очищеному гліцерині і відходах виробництва біодизелю за наявності усіх типів індукторів була вищою, ніж синтезованих у середовищі без індукторів (адгезія клітин бактеріальних і дріжджових тест-культур на полівінілхлориді становила відповідно 13—70 і 33—96 %). Внесення в середовище культивування *A. calcoaceticus* ІМВ В-7241 як живих, так і інактивованих клітин *B. subtilis* БТ-2, а також і супернатанту супроводжувалося синтезом поверхнево-активних речовин, під впливом яких деструкція бактеріальних біоплівок була в середньому на 10—20 % вищою порівняно з показниками за дії препаратів, синтезованих без індуктора. За наявності *B. subtilis* БТ-2 у середовищі в клітинах штаму *A. calcoaceticus* ІМВ В-7241 в 1,5—2 рази підвищувалася активність НАДФ$^{+}$-залежної глутаматдегідрогенази (ключовий фермент біосинтезу аміноліпідів), у той час як активність ферментів біосинтезу гліколіпідів залишалася практично на тому ж рівні, як і без індуктора. Такі дані свідчать про те, що вища біологічна активність поверхнево-активних речовин, утворених *A. calcoaceticus* ІМВ В-7241 за наявності біологічних індукторів може бути зумовлена підвищенням вмісту аміноліпідів в їхньому складі. **Висновки.** Встановлено можливість регуляції антимікробної та антиадгезивної активності, а також здатності до руйнування біоплівок поверхнево-активних речовин *A. calcoaceticus* ІМВ В-7241 внесенням у середовище культивування продуцента клітин конкурентних бактерій *B. subtilis* БТ-2. Важливо, що за таких умов культивування суттєво підвищуються антимікробна активність поверхнево-активних речовин, синтезованих на токсичних промислових відходах виробництва біодизелю.

Ключові слова: *мікробні поверхнево-активні речовини, біологічна активність, конкурентні мікроорганізми.*