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ANTIMICROBIAL ACTIVITY OF SURFACTANCES OF BACTERIA *NOCARDIA*, *RHODOCOCCUS* AND *ACINETOBACTER* GENERA

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It was found that the minimum inhibitory concentrations against bacteria and yeast of *Acinetobacter calcoaceticus* IMV B-7241, *Rhodococcus erythropolis* IMV Ac-5017 and *Nocardia vaccinii* IMV B-7405 surfactants, synthesized on traditional substrates, were 9–120 μg/ml and were within the limits defined for the surfactants known in the world. It was for the first time established that surfactants synthesized by the study strains on wastes of biodiesel production and fried sunflower oil were characterized by high antimicrobial activity against bacteria and yeast (minimum inhibitory concentrations 0.45–120 and 1.9–142 μg/ml respectively). It was found that the added of both live and inactivated *Escherichia coli* IEM-1 and *Bacillus subtilis* BT-2 cells in *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV B-7405 medium cultivation was accompanied by synthesis of surfactants, minimum inhibitory concentrations of which were several times lower than those showed for surfactants synthesized without competitive microorganisms. The obtained results indicate the possibility of using the studied surfactants as effective antimicrobial agents.

Key words: microbial metabolites, antimicrobial agents, minimum inhibitory concentrations, wastes of biodiesel production, fried sunflower oil, competitive microorganisms.

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АНТИМІКРОБНА АКТИВНІСТЬ ПОВЕРХНЕВО-АКТИВНИХ РЕЧОВИН БАКТЕРІЙ РОДІВ *NOCARDIA*, *RHODOCOCCUS* ТА *ACINETOBACTER*

Встановлено, що мінімальні інгібуючі концентрації щодо бактерій і дріжджів поверхнево-активних речовин *Acinetobacter calcoaceticus* IMB B-7241, *Rhodococcus erythropolis* IMB Ac-5017 та *Nocardia vaccinii* IMB B-7405, синтезованих на традиційних субстратах, становили 9–120 мкг/мл і перебували в межах визначених для відомих у світі поверхнево-активні речовини. Вперше встановлено, що поверхнево-активні речовини, синтезовані досліджуваними штамами на відходах виробництва біодизелю та пересмаженій соняшниковій олії, характеризувалися високою антимікробною активністю щодо бактерій та дріжджів (мінімальна інгібуюча концентрація 0,45–120 та 1,9–142 мкг/мл відповідно). Встановлено, що внесення як живих, так і інактивованих клітин *Escherichia coli* IEM-1 та *Bacillus subtilis* у середовище культивування *R. erythropolis* IMB Ac-5017 та *N. vaccinii* IMB B-7405 супроводжувалося синтезом поверхнево-активних речовин, мінімальні інгібуючі концентраціїяких були у кілька разів нижчими порівняно з показниками, встановленими для поверхнево-активних речовин, синтезованими у середовищі без сторонніх мікроорганізмів. Одержані результати засвідчують можливість використання досліджуваних ПАР як ефективних антимікробних агентів.

Ключові слова: мікробні метаболіти, антимікробні агенти, мінімальні інгібуючі концентрації, відходи виробництва біодизелю, пересмажена соняшникова олія, конкурентні мікроорганізми.

The study is a fragment of the research project "Complex microbial preparations for multifunctional purposes: from the regulation of biosynthesis and properties to the prospects for practical use", state registration No. 0116U001530.

Over the past decades, the number of multidrug-resistant extremely antibiotic resistant pathogenic bacteria has been increasing, which is primarily due to the uncontrolled use of these drugs and non-compliance with therapeutic doses. [2]. Some experts predict that in 2050, the death rate from infectious diseases caused by antibiotic-resistant pathogens may reach 10 millions people. [8]. This situation creates

the need to search for new antimicrobial compounds alternative to antibiotics, among which bacteriocins, surfactants of microbial origin, lectins, essential oils and bacteriophages attract the greatest attention [5].

Compared to known antimicrobial compounds, microbial surfactants have a number of advantages: biodegradability and non-toxicity, which prevents environmental pollution and the manifestation of allergic reactions, as well as the possibility of using in a wide range of pH, temperature and other external factors, due to the stability of physical and chemical properties; at the same time, the mechanism of action, which consists in disrupting the integrity of the cytoplasmic membrane, reduces the possibility of the emergence of resistant forms of microorganisms [4]. There are several dates in the literature about the possibility of enhancing the antimicrobial effect of surface-active lipopeptides under of producer co-cultivation with other microorganisms (inductors) [4, 10].

Previously, the strains Acinetobacter calcoaceticus IMV B-7241, Rhodococcus erythropolis IMV Ac-5017 and Nocardia vaccinii IMV B-7405 were isolated and their ability to synthesize surfactants both on traditional substrates and industrial waste was establised [12, 13].

The purpose of the work is to study the antimicrobial activity of surfactants synthesized under cultivation of A. calcoaceticus IMV B-7241, R. erythropolis IMV Ac-5017, and N. vaccinii IMV B-7405 on various carbon substrates, as well as the possibility of its increase under of producer strains co-cultivation with other microorganisms.

Materials and methods. The main objects of research were the Rhodococcus erythropolis IMV Ac-5017, Acinetobacter calcoaceticus IMV B-7241, and Nocardia vaccinii IMV B-7241 strains. By chemical nature, surfactants synthesized by strains are a complex of glyco-, amino- and neutral lipids [12]. The R. erythropolis IMV Ac-5017, A. calcoaceticus IMV B-7241 and N.vaccinii IMV B-7405 strains were grown in a liquid medium, as a carbon source used ethanol, n-hexadecane, purified glycerol, refined sunflower oil and sunflower oil after frying potatoes and meat, waste of biodiesel production at a concentration of 2.0 % (v/v).

In one of the variants, in the lag- and exponential growth phase of IMV Ac-5017 and IMV B-7405 strains, a suspension of live or inactivated (sterilized in an autoclave at 131° C for 1 h) E. coli IEM-1 or B. subtilis BT-2 cells was added in the amount of 2.5 and 10 ml per 100 ml medium cultivation, respectively. The bacteria were cultivated in flasks of 750 ml with 100 ml of medium on the shaker (320 rpm) at 30ºC during 5 days

The amount of extracellular surfactants was determined using modified by us Bligh and Dyer method [12]. To obtain the supernatant, the culture liquid was centrifuged at 5000 g for 20 minutes. In a 100 ml cylindrical separatory funnel was placed 25 ml of the supernatant and extracted surfactant according to the advanced procedure below. Firstly, 5 ml of 1M HCl was added and shaken for 5 min, then 20 ml of a modified Folch mixture (16 ml of Folch reagent and 4 ml of 1M HCl) was added immediately and shaken again for 5 min. The mixture obtained after extraction was left in a separating funnel to separate the phases, then the lower fraction was drained (organic extract 1) and the aqueous phase was re-extracted. After re-extraction, 25 ml of the modified Folch mixture was added to the aqueous phase again (but at once 16 ml of Folch reagent and 9 ml of 1M HCl) and extracted with shaking for 5 min. After phase separation, the lower fraction was poured off to obtain organic extract 2. The extraction was repeated once more using a standard Folch mixture (chloroform: methanol = 2:1), and organic extract 3 was obtained. The extracts 1–3 were combined and evaporated on an IP1– M2 rotary evaporator (Russia) at 50 °C and an absolute pressure of 0.4 atm to constant weight.

The antimicrobial activity of surfactants was determined by index of the minimum inhibitory concentration (MIC) [6]. The MIC was determined by the method of two-fold serial dilutions in meat-peptone broth (MPB) for bacteria and liquid wort for yeast. The results were evaluated visually by the clouding of the medium: (+) – test tubes in which the medium became cloudy (the test culture grew), (–) – no clouding occurred (no growth). The minimum inhibitory concentration of surfactant solution was determined as the value of the surfactant concentration in the first test tube, where growth was absent.

All experiments were performed in three replications, the number of parallel determinations in experiments ranged from three to five. Statistical processing of the experimental data was performed by Lakin as in previous papers [12, 13]. The average difference was considered significant at $P < 0.05$ The graphs were constructed using the Microsoft Office Excel computer program; the calculations were performed using the Mathcad15 program.

Results of the study and their discussion. Due to the unique mechanism of action, microbial surfactants are competitive in the market for antimicrobial compounds. Nevertheless, their use is limited due to the high cost of production and purification, which can be reduced by using industrial waste as substrates

At the first stage, the antimicrobial activity of surfactants synthesized on traditional substrates and industrial waste was studied (table 1).

Table 1

Minimum inhibitory concentrations of surfactants synthesized by IMV Ac-5017, IMV B-7241 and IMV B-7405 strains on traditional substrates and industrial waste

Studied strains	Substrate	MIC (μg/ml) against			
		Escherichia coli IEM-1	Bacillus subtilis BT-2 (spore)	Pseudomonas sp. MI-2	Staphylococcus aureus BMS-1
IMV B-7405	purified glycerol	90	22.5	90	90
	waste of biodiesel production	30	15	60	30
	oil after meat frying	35	140	140	280
	oil after frying potatoes	8	64	128	128
IMV B-7241	ethanol	18	9	37.5	75
	n-hexadecane	27	27	54	54
	purified glycerol	36	36	36	72
	waste of biodiesel production	3.8	15.3	n.d.	7.6
	oil after frying potatoes	0.9	28.8	0.45	0.45
IMV Ac-5017	ethanol	15	60	240	n.d
	waste of biodiesel production	125	62.5	62.5	125

Notes: Tables 1–2: when determining the antimicrobial activity, the error did not exceed 5 %; n.d. – not determined.

It was found that R. erythropolis IMV Ac-5017, A. calcoaceticus IMV B-7241, and N. vaccinii IMV B-7405 surfactants showed high antimicrobial activity against all tested test cultures; however, MIC depended on the producer strain and test culture. Thus, the highest antimicrobial activity against most test cultures (MIC 9–75 μg/l) was establishmed for the A. calcoaceticus IMV B-7241 surfactants.

Studies have shown that surfactants synthesized by IMV Ac-5017, IMV B-7241 and IMV B-7405 strains on fried oil and waste of biodiesel production were also characterized by high antimicrobial activity. Note, that surfactants A. calcoaceticus IMV B-7241, synthesized on industrial waste, proved to be effective antimicrobial agents compared to surfactants synthesized by N. vaccinii IMV B-7405 and R. erythropolis IMV Ac-5017.

Thus, the MIC of surfactants, synthesized by studied strains on traditional substrates, are comparable, and in some cases lower than those established for the lipopeptides and rhamnolipids described in the literature. Studies have also shown that high antimicrobial activity is also inherent for surfactants synthesized by R. erythropolis IMV Ac-5017, A. calcoaceticus IMV B-7241, and N. vaccinii IMV B-7405 on industrial waste. The data given in table 1 show that the antimicrobial activity of *N. vaccinii* IMV B-7405 surfactants depended on the quality of the fried oil in medium cultivation.

At the next stage, the antimicrobial activity against Candida genus yeast of surfactants synthesized by the IMV Ac-5017, IMV B-7241 and IMV B-7405 strains on different substrates was investigated (table 2).

Table 2

Antifungal activity of surfactants synthesized by strains IMV Ac-5017, IMV B-7241 and IMV B-7405 on different substrates

Strain	Substratt	MIC (μg/ml) against		
		Candida albicans D-6	*Candida tropicalis* PE-2	*Candida utilis* BVS-65
N. vaccinii IMV B-7405	purified glycerol	11.5	22.5	11.5
	waste of biodiesel production	15	30	15
	refined oil	42	84	84
	oil after meat frying	71	71	35,5
	oil after frying potatoes	33	66	16,5
A.calcoaceticus IMV B-7241	purified glycerol	1.9	3.8	1,9
	waste of biodiesel production	15.2	7.6	7.6
R. erythropolis IMV Ac-5017	ethanol	125	n.d.	125
	purified glycerol	250	n.d.	250
	waste of biodiesel production	250	n.d.	125
	oil after frying potatoes	40	40	40

Regardless of the carbon source nature in N. vaccinii IMV B-7405 and A. calcoaceticus IMV B-7241 medium cultivation were synthesized surfactants with high antifungal activity (MIC 1.9–84 μg/ml).

The R. erythropolis IMV Ac-5017 surfactants showed a low antimicrobial effect against Candida genus representatives: MIC 125–250 μg/ml.

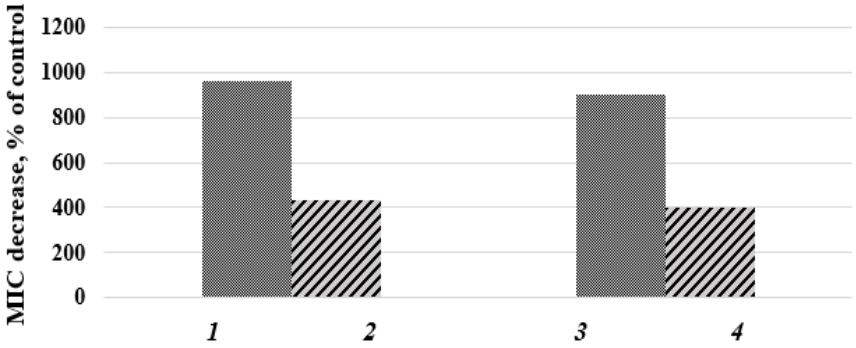

Fig 1. Minimum inhibitory concentration against *E. coli* IEM-1 of N. vaccinii IMV B-7405 surfactants, synthesized in the presence of E. coli IEM-1 (*1, 2*) and B. subtilis BT-2 (*3, 4*) cells

1, 3 – living cells, *2, 4* – inactivated cells. Control (100%) – the MIC of surfactants synthesized in medium without inducers.

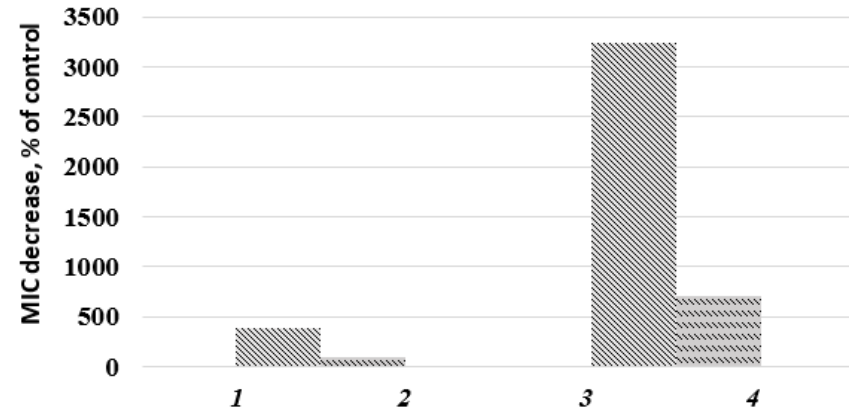

Fig 2. Minimum inhibitory concentration against *B. subtilis* BT-2 (spore cells) of R. erythropolis IMV Ac-5017 surfactants, synthesized in the presence of *E. coli* IEM-1 (*1, 2*) and B. subtilis BT-2 (*3, 4*) cells

1, 3 – living cells, 2, 4 – inactivated cells. Control (100%) – the MIC of surfactants synthesized in medium without inducers.

Fig. 1 shows an antimicrobial activity of surfactants synthesized by introducing in medium cultivation of IMV Ac-5017 and IMV B-7405 strains competitive microorganisms (inductors).

It was found that in the presence of both live and inactivated E. coli IEM-1 or B. subtilis BT-2 cells, were formed surfactants MIC of which was several times lower than those established for surfactants synthesized in a medium without inducers (B. subtilis BT-2 and *E. coli* IEM-1) (fig. 2).

There are a large number of literature works about antimicrobial activity of surfactants synthesized on such traditional substrates as glucose and purified glycerol [1, 3, 6, 7, 9], however, we were unable to find data on the antimicrobial activity of surfactants synthesized on ethanol. Thus, in [1] it was found that the MIC of rhamnolipids Lysinibacillus sp. BV152.1 against Pseudomonas aeruginosa PAO1, P. aeruginosa DM50, S. aureus ATCC 25923, S. aureus MRSA was 500 μg/ml. At the same time, the minimum inhibitory concentrations of rhamnolipids synthesized by P. aeruginosa W10 against S. aureus ATCC 43300 (MRSA), Staphylococcus capitis SH6, and Bacillus licheniformis CAN55 were higher: 37500–18750 μg/ml [6]. The MIC of the lipopeptide paenibacterin (synthesized by Paenibacillus thiaminolyticus OSY-SE) against *E. coli* K-12, P. aeruginosa ATCC 999, S. aureus ATCC 6538 was only 8–16 μg/ml [9]. Dalili et al. [7] found that the lipopeptide coryxin, synthesized by Corynebacterium xerosis NS5, was characterized by low antimicrobial activity against gram-negative bacteria E. coli and P. aeruginosa (MIC 3120 and 10000 μg/ml, respectively); however, MIC against S. aureus strains was significantly lower (190 μg/ml). Aneurinifactin (produced by Aneurinibacillus aneurinilyticus SBP-11 [3]) showed higher than coryxin antimicrobial activity: MIC against E. coli MTCC 443, S. aureus MTCC 96, P. aeruginosa MTCC was 8–424 μg/ml.

In our previous studies [12], we noted that after frying, the oil changes its composition, depending on the type of food prepared, the method of frying and the frequency of oil use. It is likely that the compounds formed during frying of meat are potential inhibitors of certain surfactant complex components responsible for their antimicrobial properties. In the literature there is no information about antimicrobial activity of surfactants synthesized on industrial waste, and there are only few data of surfactants biological properties obtained on refined oils [14, 15]. The first reports of surfactants antimicrobial activity synthesized on such substrates date back to 1999 [15]. Strain Tsukamurella spec. DSM 44370 in a medium with refined sunflower oil synthesized a complex of glycolipids GL1, GL2 and GL3, and only the GL2 component showed low antimicrobial effect against *E. coli* [15]. In [14], was found that in the presence of lipopeptides synthesized by P. aeruginosa MA-1 on olive oil in concentration 0.5–5 g/l, the growth inhibition zones of S. aureus ATCC 43300 did not exceed 7–9.5 mm. The available in the literature data relate only to antimicrobial activity against Candida, surfactants synthesized on glucose [9, 11]. Under the

action of B. amyloliquefaciens ST34 surfactin (concentration 260 μg/ml), the growth inhibition zones of various C. albicans and Cryptococcus neoformans strains were within 13–15 mm [11]. Lipopeptides surfactants synthesized by Paenibacillus sp. MSt1 showed a high antimicrobial activity: the minimum inhibitory concentration against C. albicans IMR was 12.5 μg/ml [9].

Now in the literature data on enhancing of surfactants antimicrobial activity in the presence of competitive microorganisms concern mainly lipopeptide surfactants [4, 10]. Thus, in [4] it was shown that in under co-cultivation producer of lipopeptides B. amyloliquefaciens LBM 5006 with *E. coli* ATCC 25922, increase the antimicrobial activity of surfactants synthesized was observed. Li et al. [10] found that in response to the presence of competitive phytopathogenic fungus in the medium, were accompanied by changes in the composition of the lipopeptide complex synthesized by *B.* amyloliquefaciens SQR9: in the presence of Fusarium oxysporum, the main component of the complex was bacilomycin D; Verticillium dahliae kleb, Fusarium oxysporum were inductors of fengicin synthesis, Sclerotinia sclerotiorum, Rhizoctonia solani – surfactin, bacillibactin was synthesized in the presence of each studied phytopathogenic fungi.

Conclusion

So, our data indicate that A. calcoaceticus IMV B-7241, N. vaccinii IMV B-7405, and R. erythropolis IMV Ac-5017 surfactants, synthesized on a wide range of substrates, including industrial waste, are characterized by high antimicrobial activit against bacteria and yeast, compared to that for the known in the world rhamno- and lipopeptides. The possibility of increasing several times the antimicrobial activity of surfactants under co-cultivation producers with competitive microorganisms has been established.

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