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Industrial wastes as substrates for synthesis of surfactants with antiadhesive activity by *Rhodococcus erythropolis* IMV Ac-5017

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Abstract

Introduction. Microbial surfactants can affect the degree of microorganism's adhesion to different surfaces and lead to biodegradation of the already formed biofilms. They are also non-toxic to the environment. These properties allow to use the microbial surfactants in medicine and food industry.

Material and methods. Surfactants were obtained by cultivation of the *Rhodococcus erythropolis* IMV Ac-5017 on waste from biodiesel production, used sunflower oil after frying meat, potatoes, onions, and cheese. The surfactants were extracted from the supernatant of cultural liquid by a Folch mixture. The anti-adhesive activity and the degree of destruction of biofilms were determined by spectrophotometric method.

Results and discussion. It was found that surfactants obtained by cultivation of *R. erythropolis* IMV Ac-5017 on waste mixed sunflower oil reduce the adhesion of yeast *Candida albicans* D-6, *Candida utilis* BVS-65, *Candida tropicalis* PE-2 and bacterial strains *Escherichia coli* IEM-1, *Bacillus subtilis* BT-2, *Pseudomonas* sp. MI-2 to abiotic surfaces (tiles, steel and glass) by 51–73% and 57–86%, respectively. The effectiveness of the anti-adhesive activity of surfactants, obtained on waste from biodiesel production as a substrate against the studied yeast and bacterial cultures was lower (44-77%). The ability of surfactants to destroy of bacterial biofilms by 44–73% was achieved at low concentrations of surfactants (8−15 μg/ml) synthesized on different waste oil. Surfactants were equally effective in destroying biofilms (72–80%) of *Candida* genus and their activities were not depended on the nature of carbon source in the culture medium of *R. erythropolis* IMV Ac-5017.

Conclusion. The obtained results show the possibility of using surfactants of *R. erythropolis* IMV Ac-5017, gained on industrial waste, as effective anti-adhesive and antibiofilm agents.

Introduction

There is an increasing interest to microbial surfactants as alternative to synthetic analogues due to such advantages as biodegradability, lack of toxicity, stable physical and chemical properties in a wide range of temperatures and pH (De Almeida, 2016) and unique biological properties (Naughton et al., 2019; Vecino et al., 2018). Thus, the possibility of using surfactants of microbial origin in the oil and mining, chemical, food industries, agriculture, and environmental technologies has already been proved.

Since the 90s of the XX century surfactants of microbial origin are actively studied as alternative agents for the destruction of biofilms formed on various materials used in medicine and the food industry (Velraeds et al., 1996). It is known that the colonization of abiotic surfaces by microorganisms is a very dangerous phenomenon, which causes not only spoilage of products, but also the spread of infectious diseases. Many studies supported the use of inorganic compounds, anti-adhesive chemicals, antibiotics and bacteriophage therapy to prevent various infections. However, the increasing resistance of microorganisms to antibiotics and other biocides, high cost of current methods for preventing of formation and further destruction of biofilms leads to search for new substances with appropriate properties – and such substances are surfactants of microbial origin.

Although at present the effectiveness of biotechnology methods for obtaining surfactants is low due to the high cost of the products of microbial synthesis. A possible solution is to use industrial waste (that are available in large quantities) as substrates (Singh et al., 2019).

In our previous studies (Pirog et al., 2013; 2015; 2021) a possibility of synthesis of surfactants by *Rhodococcus erythropolis* IMV Ac-5017 on waste from biodiesel production and refried sunflower oil was shown. However, to date, an anti-adhesive activity of surfactants synthesized on these toxic wastes has not been studied.

Therefore, the aim of this work is to study surfactants, synthesized by *R. erythropolis* IMV Ac-5017 on waste from biodiesel production and refried sunflower oil, influence on the attachment of microorganisms to abiotic surfaces and destruction of biofilms.

Material and methods

Study objects

Strain *R. erythropolis* IMV Ac-5017 is the main object of research, isolated from oil-contaminated soil samples and registered in the Depository of Microorganisms of the Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine.

Bacterial strains (*Escherichia coli* IEM-1, *Bacillus subtilis* BT-2, *Pseudomonas* sp. MI-2) and yeast (*Candida albicans* D-6, *Candida utilis* BVS-65, *Candida tropicalis* PE-2) from the collection of living cultures of the Department of Biotechnology and Microbiology of the National University of Food Technologies (Kyiv, Ukraine) were used as a test cultures for the determination of biological activity of the surfactants.

Cultivation conditions

R. erythropolis IMV Ac-5017 was grown in a liquid mineral medium of the following composition (g/l): $NaNO_3$ – 1.3; $MgSO_4 \times 7H_2O$ – 0.1; NaCl – 1.0; Na_2HPO_4 – 0.6; KH_2PO_4 – 0.14; $FeSO_4 \times 7H_2O$ – 0.01; pH 6.8–7.0.

The following compounds were used as a carbon source (2% volume fraction): refined glycerol; waste from biodiesel production (Komsomol Biofuel Plant, Poltava region, Ukraine), refined sunflower oil (TM «Oleyna», Ukraine), waste mixed sunflower oil after frying meat, potatoes, onions, cheese (from RocketPub fast food restaurants, Kyiv, Ukraine).

A culture from the end of the exponential growth phase (48 h), grown in the media as described above with a suitable carbon source (0.5%, volume fraction) was used as the inoculum. The amount of inoculum was 5% of the volume of the culture medium.

Bacteria were cultured in 750 ml flasks with 100 ml of medium on a shaker (320 rpm) at 30 °C for 120 hours.

Determination of the concentration of surfactants

The surfactant concentration in the culture fluid (g/l) was determined by the weight method after their extraction from the supernatant with a modified Folch mixture as described in our previous work (Pirog et al., 2020)

Determination of the anti-adhesive activity

The study of anti-adhesive properties of surfactants was carried out as described previously (Pirog et al., 2014). Purified plates of studied materials (tiles, steel and glass) of equal size were sterilized at 112 °C for 40 min. One-day test-cultures of bacteria and yeast were suspended in 100 ml of sterile tap water. Pre-treated with preparations of surfactants with different concentration and untreated (control) materials were added to the suspension, kept in a thermostat for 2 h at 30 °C, and then rinsed with 10 ml of sterile tap water to remove unattached cells.

Further the degree of cell adhesion was determined using a spectrophotometric method. For this purpose, the plates of materials were treated with methanol (99%) for 15 min to fix the adherent cells and dried at room temperature; then placed in 1% solution of gentian violet for 5 min and rinsed with tap water. After drying, the materials were treated with 10 ml of 33% acetic acid solution, and optical density of the resulting cell suspension was measured. The optical density of the suspensions was measured by photoelectric colorimeter at a wavelength of 540 nm. The number of adhered cells (adhesion) was defined as the ratio of the optical density of the suspension obtained from the treated surfactant samples to the optical density of the control samples and expressed as a percentage (Pirog et al., 2014).

Determination of the ability to destroy microbial biofilms

Study on the effect of surfactants on the destruction of biofilms was carried out as described in (Gomes et al., 2012). For biofilm formation, 180 µl of meat-peptone broth or liquid wort and 20 µl of one-day test culture suspension were added to the polystyrene microplates and incubated for 24 h at the optimum temperature for the culture test. Then the culture fluid was drained and 180 µl of fresh meat-peptone broth (or liquid wort) and 20 µl of test culture suspension was added for further incubation for the next 24 hours. After 48 h, the culture fluid was drained, and 200 µl of surfactants of different concentrations (0.0078–1 mg/ml) were added to the wells of the microplate (with the test film biofilm previously formed on them).

Sterile tap water (200 µl) was added into the control variants (wells) instead of surfactants. After 24 h of exposure, the wells were washed three times with 200 µl of distilled

water and the number of adherent cells was determined by spectrophotometric method as described above.

All experiments were performed in 3 repeats, the number of parallel measurements in the experiments was 3-5. Statistical processing of experimental data was performed as described previously (Pirog et al., 2013; Pirog et al., 2015). Difference in the average values was considered reliable at $p < 0.05$ significance level.

Results and discussion

Anti-adhesive activity of surfactants synthesized by *R. erythropolis* IMV Ac-5017 grown on industrial waste

The data given in Table 1, show that the adhesion of bacteria and yeast to abiotic surfaces treated with solutions of surfactants of *R. erythropolis* IMV Ac-5017, depends on the nature of the carbon source in the culture medium, concentration of a surfactant and a type of test cultures.

Table 1

Influence of surfactants of *R. erythropolis* IMV Ac-5017 on attachment of test cultures to abiotic surfaces

Substrate for surfactant synthesis	Abiotic surface	Adhesion,%				
		Escherichia coli IEM-1	*Bacillus subtilis* BT-2	*Pseudomonas* sp. MI-2	*Candida utilis* BVS-65	*Candida albicans* D-6
Refined glycerol	Tile	62	75	83	54	68
	Steel	78	60	60	77	80
	Glass	67	56	58	86	78
Waste from biodiesel production	Tile	45	63	78	45	49
	Steel	66	52	50	68	65
	Glass	58	46	48	77	60
Refined sunflower oil	Tile	25	50	50	65	81
	Steel	47	62	42	48	51
	Glass	24	74	65	68	52
Waste refried sunflower oil	Tile	14	41	33	49	27
	Steel	26	42	40	41	33
	Glass	14	43	40	40	34

Notes: The effective surfactant concentration, which ensured minimal adhesion of bacterial and yeast test cultures, was 6–12 and 24–48 μg/ml, respectively.

Thus, the most effective anti-adhesive agents were surfactants synthesized on the oil waste: after treatment with such agents of the studied surfaces, the adhesion of microorganisms on them was only 14–49%. At the same time for the surfactants synthesized

on refined oil the adhesion increased to 24–81%. Replacement of refined glycerol in the culture medium of *R. erythropolis* IMV Ac-5017 with waste from biodiesel production was accompanied by the formation of surfactants with higher antiadhesive activity: the number of attached bacteria and yeast cells on abiotic materials was by 5–19% lower comparing to those from purified substrates. In general, surfactants synthesized on refined and waste oils reduced the adhesion of test cultures more effectively than those formed on refined glycerol and waste from biodiesel production.

In order to ensure minimal adhesion of yeast cells a higher concentration of surfactants is required compared to that of bacteria (24−48 and 6−12 μg/ml, respectively).

In order to ensure minimal adhesion of yeast cells a higher concentration of surfactants is required compared to that of bacteria (24−48 and 6−12 μg/ml, respectively).

Analysis of the literature showed that information on the anti-adhesive activity of surfactants of the representatives of genus *Rhodococcus* is limited; available data is related to surfactants synthesized only on hexadecane (Janek et al., 2018; Kuyukina et al., 2016). In addition, an effective reduction in adhesion was achieved by treating surfaces with such surfactants in high concentrations (100–1000 μg/ml). In the study (Janek et al., 2018) it was found that the adhesion of *E coli* 17-2, *E. coli* ATCC 10536, *Staphylococcus epidermidis* KCTC 1917 and *C. albicans* ATCC 10231 did not exceed 28% after treatment of the wells of polystyrene tablets and silicone surfaces with surfactants of *Rhodococcus fascians* BD8 at a concentration of 500 μg/ml. Number of attached cells of *B. subtilis* ATCC 6613 and *E. coli* K-12 to the wells of polystyrene tablets treated with surfactants of *Rhodococcus ruber* IEGM 231 (100–1000 μg/ml) reduced to 20% (Kuyukina et al., 2016).

It should be noted that there are no publications regarding to anti-adhesive properties of microbial surfactants synthesized on waste from biodiesel production; there is also a limited information regarding to substrates containing oil. The study (Rufino et al., 2011) reported that Rufisan synthesized by *C. lipolytica* UCP 0988 on waste soybean oil reduced the adhesion of bacteria *Streptococcus* and *Lactobacillus* on polystyrene plates. Even at the minimum studied surfactant concentration (0.75 mg/l) the degree of adhesion of test cultures was 61–91%. As the surfactant concentration in solution increased to 12 mg/l, Rufisan reduced the number of attached *E. coli* and *C. albicans* cells by 21–51%.

Probiotic strain *Propionibacterium freudenreichii* subsp. *freudenreichii* PTCC 1674 under conditions of growth on various substrates, including sunflower oil waste, synthesized surfactant, which at a concentration of 10 mg/ml reduced the number of attached cells of *E. coli* to the plate by 13% and for *Staphylococcus aureus* by 37% (Hajfarajollah et al., 2014).

Data listed in the Table 1 shows that surfactants of *R. erythropolis* IMV Ac-5017, synthesized on either refined or waste oil, are more effective anti-adhesive agents than Rufisan and surfactants of *P. freudenreichii* subsp. *freudenreichii* PTCC 1674. They reduce the adhesion of test cultures by 19–86% at a concentration of only 6–12 μg/ml.

Destruction of bacterial and yeast biofilms under the action of surfactants synthesized by strain *R. erythropolis* IMV As-5017 grown on industrial waste

In the Tables 2 and 3, the data on effect of surfactants of *R. erythropolis* IMV Ac-5017 on the destruction of bacterial and yeast biofilms is shown.

Table 2

Destruction of bacterial biofilms under the action of *R. erythropolis* IMV Ac-5017 surfactants

Substrate for surfactant synthesis	Biofilm destruction,%		
	***Escherichia coli* IEM-1**	***Bacillus subtilis* BT-2**	***Pseudomonas* sp. MI-2**
Refined glycerol *	48	71	72
Waste from biodiesel production*	44	73	69
Refined sunflower oil**	42	60	44
Waste refried sunflower oil**	65	76	63

Notes: * – The concentration of surfactant is 8 mg/ml, ** – concentration of surfactant is 15 mg/ml.

Table 3

Influence of surfactants synthesized by *R. erythropolis* IMV Ac-5017 on industrial waste, on destruction of yeast biofilms

Test culture	Destruction of biofilms (%) under the action of surfactants synthesized on			
	Refined glycerol	**Waste from biodiesel production**	**Refined sunflower oil**	**Waste refried sunflower oil**
Candida albicans D-6	72	74	79	80
Candida utilis BVS-65	76	73	72	77
Candida tropicalis PE-2	75	78	75	78

Note: The surfactant concentration is 30 μg/ml.

The ability of surfactants to destroy the biofilms, as well as their anti-adhesive activity, depended on the nature of the growth substrate, the concentration of surfactants and the type of test culture.

The degree of destruction of bacterial biofilms by surfactants synthesized on both refined glycerol and waste from biodiesel production was almost the same. While surfactants synthesized on waste oil destroyed such biofilms more effective than those obtained on refined oil (see Table 2). It should be noted that the destruction of bacterial biofilms by 42−76% was achieved at low concentrations of surfactants (8−15 μg/ml) synthesized on all studied substrates. Surfactants were equally effective in destroying biofilms (72–80%) of *Candida* (see Table 3) and not depended on the nature of carbon source in the culture medium of *R. erythropolis* IMV Ac-5017. The concentrations of surfactants were 2–4 times higher than bacterial biofilms.

In (Janek *et al.*, 2018) it was found that the destruction of bacterial biofilms was achieved at a surfactant of *R. fascians* BD8 concentration 250 μg/ml, which is up in the order of magnitude greater than the surfactant of the IMV strain Ac-5017 (see Table 2). In addition,

the authors of (Janek et al., 2018) analyzed an efficiency of destruction of biofilms by surfactants only visually using confocal laser scanning microscopy.

Das et al. (Das et al., 2014) showed that rhamnolipids of *Pseudomonas aeruginosa* IMP67 synthesized on glycerol destroyed the biofilms of *B. subtilis*, *E. coli* and *Staphylococcus aureus* by 50% at a concentration of 64–128 μg/ml, which is higher in comparison with surfactants of *R. erythropolis* IMV Ac-5017.

In addition, there is virtually no published data regarding to the destruction of biofilms in the presence of surfactants synthesized on oil-containing substrates. In 2017, there was a publication (Kiran et al., 2017) regarding to the effect of lipopeptide synthesized by actinobacteria *Nesterenkonia* sp. MSA31 (isolated from the sea sponge *Fasciospongia cavernosa*) on destroying biofilms of *S. aureus* strain MSA31 was grown on a medium with 10% olive oil, the lipopeptide was extracted with organic solvents (ethyl acetate, methanol, petroleum ether, dichloromethane) from the supernatant, previously pre-acidified to pH 2.0. To determine the role of lipopeptide in the destruction of the biofilm, surfactant solutions in the concentration range of 25–150 μg/ml were used. It was established that the maximum degree of destruction of a biofilm of *S. aureus* (90%) was achieved in the presence of lipopeptide at a concentration of 125 μg/ml (Kiran et al., 2017).

It should be noted that he destruction of yeast biofilms of the genus *Candida* is an urgent problem today, because most modern biocides, including some surfactants, are not effective enough (Gulati and Nobile, 2016). For example, surfactants synthesized by *Lactobacillus jensenii* P6A and *Lactobacillus gasseri* P65 at a concentration of 180 μg/ml destroyed yeast biofilms only by 25–35% (Morais et al., 2017).

C. albicans biofilms are resistant to most known antifungal drugs that complicates the control of infections caused by these yeasts. In (Shinde et al., 2012) it was shown that these yeasts easily colonize the surfaces of prostheses (laryngeal, knee, heart valves), implants (especially thoracic), endotracheal tubes, which causes the spread of infection throughout the body. Azoles and polyenes are not effective against biofilms of *C. albicans.* This reduces the number of potential agents for treating these infections and underlines the need for new effective agents (Gulati and Nobile, 2016).

Conclusions

As a result of this work it was found that surfactants synthesized by *R. erythropolis* IMV Ac-5017 on toxic industrial waste are characterized by high anti-adhesive activity and able to destroy bacterial and yeast biofilms. This set of biological properties makes the surfactant strain IMV Ac-5017 promising for practical use. Also, bioconversion of waste from biodiesel production and waste oil into microbial surfactants will help solving several problems: reduce the cost of surfactants production by using cheap raw materials as a substrate; increase the profitability of biodiesel production by disposing of a by-product – glycerol; protect the environment from uncontrolled emissions of toxic waste.

References

Das P., Yang X.P., Ma L.Z. (2014), Analysis of biosurfactants from industrially viable *Pseudomonas* strain isolated from crude oil suggests how rhamnolipids congeners affect emulsification property and antimicrobial activity, *Frontiers in Microbiology*, 5, 696.

De Almeida D.G., Soares Da Silva R.C., Luna J.M., Rufino R.D., Santos V.A., Banat I.M., Sarubbo L.A. (2016), Biosurfactants: promising molecules for petroleum biotechnology advances, *Frontiers in Microbiology*, 7, pp. 1718.

Gomes M-Z.V., Nitschke M. (2016), Evaluation of rhamnolipids surfactants as agents to reduce the adhesion of *Staphylococcus aureus* to polystyrene surfaces, *Letters in Applied Microbiology*, 49(1), pp. 960–965.

Gulati M., Nobile C.J. (2016), *Candida albicans* biofilms: development, regulation, and molecular mechanisms, *Microbes and Infection*, 18(5), pp. 310–321.

Hajfarajollah H., Mokhtarani B., Noghabi K.A. (2014), Newly antibacterial and antiadhesive lipopeptide biosurfactant secreted by a probiotic strain, *Propionibacterium freudenreichii, Applied Biochemistry and Biotechnology*, 174, pp. 2725–2740.

Jane T., Krasowska A., Czyżnikowska Ż., Łukaszewicz M. (2018), Trehalose lipid biosurfactant reduces adhesion of microbial pathogens to polystyrene and silicone surfaces: an experimental and computational approach, *Frontiers in Microbiology*, 9, 2441.

Kiran G.S., Priyadharsini S., Sajayan A., Priyadharsini G.B., Poulose N., Selvin J. (2019), Production of lipopeptide biosurfactant by a marine *Nesterenkonia* sp. and its application in food industry, *Frontiers in Microbiology*, 8, 1138.

Kuyukina M.S., Ivshina I.B., Korshunova I.O., Stukova G.I., Krivoruchko A.V. (2016), Diverse effects of a biosurfactant from *Rhodococcus ruber* IEGM 231 on the adhesion of resting and growing bacteria to polystyrene, *AMB Express*, 6(1), 6, 14.

Morais I.M.C., Cordeiro A.L., Teixeira G.S., Domingues V.S., Nardi R.M.D., Monteiro A.S., Alves R.J., Siqueira E.P., Santos V.L. (2017), Biological and physicochemical properties of biosurfactants produced by *Lactobacillus jensenii* P6A and *Lactobacillus gasseri* P65, *Microbial Cell Factories*, 16, 155.

Naughton P.J., Marchant R., Naughton V., Banat I.M. (2019), Microbial biosurfactants: current trends and applications in agricultural and biomedical industries, *Journal of Applied Microbiology*, 127(1), pp. 12–28.

Rufino R.D., Luna J.M., Sarubbo L.A. (2011), Antimicrobial and anti-adhesive potential of a biosurfactant Rufisan produced by *Candida lipolytica* UCP 0988, *Colloids and Surfaces B: Biointerfaces*, 84(1), pp. 1–5.

Pirog T.P., Konon A.D., Beregovaya K.A., Shulyakova M.A. (2014), Antiadhesive properties of the surfactants of *Acinetobacter calcoaceticus* IMB B-7241, *Rhodococcus erythropolis* IMB Ac-5017, and *Nocardia vaccinii* IMB B-7405, *Microbiology*, 83(6), pp. 732–739.

Pirog T., Kluchka L., Lytsai D., Stabnikov V. (2021), Factors affecting antibiofilm properties of microbial surfactants, *Scientific Study & Research: Chemistry & Chemical Engineering, Biotechnology, Food Industry*, 21 (1), pp. 27-37.

Pirog T.P., Savenko I.V., Lutsay DA. (2016), Microbial surface-active substances as antiadhesive agents, *Biotechnologia Acta*, 9(3), pp. 7–22.

Pirog T.P., Sofilkanych A.P., Shulyakova M., Shevchuk T.A. (2015), Biosurfactant synthesis by *Rhodococcus erythropolis* IMV Ac-5017, *Acinetobacter calcoaceticus* IMV B-7241 and *Nocardia vaccinii* IMV B-7405 on byproduct of biodiesel production, *Food and Bioproducts Processing*, 93, pp. 11–18.

Pirog T., Sofilkanych A., Konon A., Shevchuk T., Ivanov S. (2013), 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 and Bioproducts Processing*, 91(2), pp. 149–157.

Shinde R.B., Raut J.S., Karuppayil M.S. (2012), Biofilm formation by *Candida albicans* on various prosthetic materials and its fluconazole sensitivity: a kinetic study, *Mycoscience*, 53(3), pp. 220–226.

Singh P., Patil Y., Rale V. (2019), Biosurfactant production: emerging trends and promising strategies, *Journal of Applied Microbiology*, 126(1), pp. 2–13.

Vecino X., Rodríguez-López L., Ferreira D., Cruz J.M., Moldes A.B., Rodrigues L.R. (2018), Bioactivity of glycolipopeptide cell-bound biosurfactants against skin pathogens, *International Journal of Biological Macromolecules*, 109, pp. 971–979.

Velraeds M.M., van der Mei H.C., Reid G., Busscher H.J. (1996), Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates, *Applied and Environmental Microbiology*, 62(6), pp. 1958–1963.