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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

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ABSTRACT

Searching for the ways to process waste has become very topical today. Biotechnology is one of the most environmentally attractive methods, which has the ability to solve the problem of waste utilization and to produce the valuable microbial products, for example biosurfactants. We concluded that fried sunflower oil, oil-containing wastes (soapstock) and glycerol can be used for biosurfactant production by *Rhodococcus erythropolis* IMV Ac-5017, *Acinetobacter calcoaceticus* IMV B-7241 and *Nocardia vaccinii* K-8. Glucose addition (0.1%) into the medium with fried oil (2 vol.%) led to a 4-fold increase of final surfactant concentration (6.8 g/L). The simultaneous addition of fumarate and citrate (0.01–0.2%) into the IMV B-7241 and K-8 strains' cultivating medium was accompanied by an increase of the exocellular biosurfactant quantity by 2–2.5-fold compared to the cultivation without organic acids. An increase in surfactant concentration of IMV B-7241 strain was the result of the simultaneous functioning of two anaplerotic pathways, also resulting in a 3–5-fold increase in activity of biosynthesis enzymes. Cultivating on a mixture of glycerol and *n*-hexadecane (0.5–1.0 vol.%) led to a 1.5–3-fold increasing surfactant synthesis. Biosurfactant preparations of IMV B-7241 (0.15–0.22 mg/mL) and IMV Ac-5017 (0.61–2.1 mg/mL) strains were effective against *Escherichia coli* IEM-1 (67% of cell loss), and vegetative (45–100% of cell loss) and spore (75% of spore loss) cells of *Bacillus subtilis* BT-2.

Keywords: *Rhodococcus erythropolis* IMV Ac-5017; *Acinetobacter calcoaceticus* IMV B-7241; *Nocardia vaccinii* K-8; Biosurfactant; Wastes; Biosynthesis intensification

1. Introduction

Surfactants are widely used in different fields of industry, and so henceforth the demand for synthetic surfactants has been increasing constantly. At the same time, the pace of biotechnology development and environmental safety concerns has caused a great deal of interest to scientists to study microbial surfactants (biosurfactants) as an alternative to chemical ones (Banat et al., 2010). Biosurfactants have a number of advantages over synthetic substances (Shavandi et al., 2011): biodegradability; the stability of properties in wide range of pH and temperature; no toxicity.

Due to their physico-chemical properties, the use of microbial surfactants has been proposed for various industrial applications, as additives in foods, cosmetics and detergent formulations (Banat et al., 2010). In the food industry, the most useful property is the ability to form stable emulsions, which improves the texture and creaminess of dairy products. Biosurfactants are also used to retard staling, solubilize flavor oils and improve organoleptic properties in bakery and ice cream formulations and as fat stabilizers during cooking of fats. Although the addition of rhamnolipids has been suggested to improve dough characteristics of bakery products, the use as food ingredients of compounds derived from an

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Table 1 – Production of biosurfactants during the cultivation of *R. erythropolis* IMV Ac-5017 on waste after oil production and fried sunflower oil.

Substrate	Substrate concentration (vol.%)	Indexes of surfactants' synthesis		
		pH_{final}	E_{24} (%)[a]	CSC*
Oil-containing wastes (soapstock)	1.0	7.9 ± 0.3	37 ± 2	4.8 ± 0.24
	2.0	7.9 ± 0.3	40 ± 2	10.0 ± 0.50
Fried sunflower oil	1.0	7.5 ± 0.2	40 ± 2	4.0 ± 0.20
	2.0	7.7 ± 0.3	65 ± 3	4.8 ± 0.24
n-Hexadecane (control)	2.0	7.5 ± 0.2	44 ± 2	3.8 ± 0.19

[a] E_{24}, % (the emulsification index) – here and in Table 2 was determined for the native cultural liquid.

opportunistic pathogen such as *Pseudomonas aeruginosa* is not practically feasible. Instead, it has been suggested to use biosurfactants obtained from yeasts or Lactobacilli, which are generally recognized as safe and are already involved in several food-processing technologies (Nitschke and Costa, 2007).

Despite the outstanding properties and advantages of biosurfactants, there are factors that have restrained their global industrial production (Mukherjee et al., 2006), such as: (1) the high-cost biosynthesis (raw material, energy); (2) the high-cost process of biosurfactants extraction from cultural liquid; (3) the low concentration of biosurfactants. One of the ways to reduce the cost of biosynthesis is with the use of low-cost growth substrates, such as wastes of other industries (Octave and Thomas, 2009).

Millions of tons of hazardous and non-hazardous waste is produced annually all over the world. The treatment and disposal costs occupy a significant place in the budget of enterprises. However, a rational approach to waste management includes the concept of reduce, reuse and recycle. Waste of processing of agricultural crops (soybeans, sugar beets, potatoes, straw and bran, fruits) and oil production can be used as industrial substrates in biotechnology (Morita et al., 2007; Müller et al., 2010; Nitschke et al., 2010). The utilization of waste glycerol is becoming very important, because the amount of waste has been increasing year by year through the increased production of biodiesel (Easterling et al., 2009). On the other hand, glycerol has been successfully used for different microbial productions (da Silva et al., 2009; Silva et al., 2010).

The oil-oxidizing bacteria were isolated from the oil-polluted samples of soil and identified as *Rhodococcus erythropolis* IMV Ac-5017, *Acinetobacter calcoaceticus* IMV B-7241 and *Nocardia vaccinii* K-8. The ability of these strains to synthesize the metabolites with surface-active and emulsifying properties was determined during the cultivation in medium with hydrophobic (*n*-hexadecane, liquid paraffin) and hydrophilic (glucose, ethanol) substrates (Pirog et al., 2004, 2009).

The aim of present work – development of approaches for increasing biosurfactant synthesis by *R. erythropolis* IMV Ac-5017, *A. calcoaceticus* IMV B-7241 and *N. vaccinii* K-8 on wastes.

2. Materials and methods

2.1. Objects of research

R. erythropolis EK-1 and *A. calcoaceticus* K-4 strains, registered in the Microorganisms Depositary of the Institute of Microbiology and Virology, the National Academy of Sciences of Ukraine under the numbers IMV Ac-5017 and IMV B-7241, respectively, and also the strain *Nocardia vaccinii* K-8, were studied.

Antimicrobial properties of surfactants were identified against some test-cultures of microorganisms: *Escherichia coli* IEM-1, *Bacillus subtilis* BT-2, *Saccharomyces cerevisiae* OB-3, *Aspergillus niger* P-3, *Fusarium culmorum* T-7. These strains are being saved in the Microorganism collection of Biotechnology and Microbiology Department of the National University of Food Technologies (Kyiv, Ukraine).

2.2. Composition of media and cultivation conditions

R. erythropolis IMV Ac-5017 was grown on a liquid mineral medium, which contained (g/L): $NaNO_3$ – 1.3; $MgSO_4 \cdot 7H_2O$ – 0.1; NaCl – 1.0; Na_2HPO_4 – 0.6; KH_2PO_4 – 0.14; $FeSO_4 \cdot 7H_2O$ – 0.01; pH 6.8–7.0.

For cultivation of *A. calcoaceticus* IMV B-7241 the nutrient medium with the following composition was used (g/L): $(NH_2)_2CO$ – 0.35; $MgSO_4 \cdot 7H_2O$ – 0.1; NaCl – 1.0; Na_2HPO_4 – 0.6; KH_2PO_4 – 0.14; pH 6.8–7.0; the yeast autolysate – 0.5 vol.% and trace elements solution (Pirog et al., 2009) – 0.1 vol.% were also added.

N. vaccinii K-8 strain was grown on the synthetic nutrient medium containing (g/L): $NaNO_3$ – 0.5; $MgSO_4 \cdot 7H_2O$ – 0.1; $CaCl \cdot 2H_2O$ – 0.1; KH_2PO_4 – 0.1; $FeSO_4 \cdot 7H_2O$ – 0.1, yeast autolysate – 0.5 vol.%.

Ethanol, *n*-hexadecane, glycerol or oil-containing wastes (wastes of oil production, fried sunflower oil) at a concentration of 0.5–2 vol.%, and a mixture of *n*-hexadecane and glycerol at a concentration of 0.5–1.0 vol.% were used as substrates.

The organic acids (sodium citrate and sodium fumarate at concentration of 0.01–0.2%) were added into some samples at the beginning of the stationary growth phase. Sodium citrate and sodium fumarate were added into the medium as a 10% solution. Glucose (1–3 g/L) was added in some samples during cultivation on the oil-containing medium.

The inoculum – culture from the middle of exponential growth phase (48–60 h), cultivated on the medium of the aforementioned composition. Glycerol, ethanol, *n*-hexadecane at a concentration of 0.5 vol.% and a mixture of *n*-hexadecane (0.25 vol.%) and glycerol (0.25 vol.%) were used as sources of carbon and energy for inoculum cultivation. The inoculum quantity was 5% of the total medium volume (10^4–10^5 cells/mL). The cultivation of bacteria took place in the 750 mL Erlenmeyer flasks with 100 mL of the medium on rotor shaker (320 rpm) at 28–30 °C during 120 h.

1.5–3.0 mL BP of *A. calcoaceticus* IMV B-7241 (the conditional surfactant concentration was 2.5). The following experiments have shown that BP of IMV Ac-5017 strain at the concentration of 1.5 mg/mL, and BP of IMV B-7241 strain at the concentration of 0.3 mg/mL did not show antimicrobial activity against the micromycetes *A. niger* P-3 and *F. culmorum* T-7. Obviously, the higher concentrations of BP or longer treatment are required for the suppression of growth of these organisms.

The first experimental data about antimicrobial properties of surfactants of *N. vaccinii* K-8 against different bacterial strains, including phytopathogens, was obtained (data not shown).

4. Discussion

Our experiments showed that the use of industrial wastes for biosurfactant synthesis by IMV Ac-5017 strain was effective. The highest rates of biosynthesis were obtained by using oily substrates, namely, oil-containing wastes (CSC* = 10) and fried sunflower oil (CSC* = 4.8). Assuming that *R. erythropolis* IMV Ac-5017 synthesizes a surfactant of lipid nature (mainly trehalose mycolates), then the presence of fatty acids in the nutrient medium stimulates the formation of surfactants. The use of various oils as substrates for the surfactants' synthesis is widely described in the literature.

Thus, the use of sunflower oil for the cultivation of *Tsukamurella* sp. DSM 44370 in flasks on shakers allowed to obtain up to 5 g/L of glycolipids (Vollbrecht et al., 1999). Significant increases of the surfactants yield (39 g/L) was achieved by Müller et al. (2010) using the same substrate for the cultivation of *P. aeruginosa* EMS1. These scientists studied the biosynthesis process in a bioreactor with fractional oil addition (150 g/L).

Morita et al. (2007) determined that the addition of the so-called "secondary carbon source" (mannose or erythritol) into the medium with glycerol is aimed to increase (by 30–50%) the surfactant synthesis by *Pseudozyma rugulosa* NBRC 10877. The presence of glucose in the nutrient medium of other strains, that produced mannosylerythritol lipids (*Pseudozyma siamensis* CBS 9960 and *Pseudozyma hubeiensis* KM-59), led to the increase of 50% of surfactant quantity (Konishi et al., 2008; Morita et al., 2008). Sunflower oil and glycerol were used as growth substrates for strains CBS 9960 and KM-5. It should be noted that the concentration of glucose in the oil-containing medium was quite high – 4% (as much as the main growth substrate). Obviously, glucose was the additional carbon source in this case, and the term "biosynthesis precursor" is hardly appropriate. Our study has shown that the addition of glucose (0.1%) into the medium with fried oil at the beginning of the cultivation process resulted in an increase of 400% of surfactant synthesized by the strain IMV Ac-5017. Considering that glucose concentration is more than an order of magnitude lower than the concentration of the carbon source (oil), in this case glucose was the biosynthesis precursor (precursor of the trehalose mycolates), but not the additional substrate.

Since annually there has been an increase in the quantities of glycerol – a byproduct of biodiesel production – the problem of its processing is highly relevant (da Silva et al., 2009). The range of substances derived by microbial conversion of glycerol is currently vast. Among them are organic acids (propionate, succinate, pyruvate, citrate) (da Silva et al., 2009; Rywinska and Rymowicz, 2010; Zhu et al., 2010), alcohols (ethanol, 1,3-propanediol) (da Silva et al., 2009), ketones (dihydroxyacetone) (da Silva et al., 2009), amino acids (phenylalanine) (Khamduang et al., 2009), polyesters (polyhydroxyalkanoates) (Ciesielski et al., 2010), pigments (astaxanthin, prodigiozin) (da Silva et al., 2009), and surface active substances (da Silva et al., 2009; Morita et al., 2007; Silva et al., 2010).

The concentration of surfactants that have been synthesized by microorganisms on glycerol is usually much lower than on traditional hydrophobic substrates. Thus, *Pseudozyma antarctica* JCM 10317^T produced 3.5 g/L of surfactant from 10 vol.% of glycerol (Morita et al., 2007). A slightly higher rhamnolipids concentration of 8 g/L was observed on the medium with 3 vol.% of glycerol that was used for *P. aeruginosa* UCP0992 cultivation (Silva et al., 2010).

In the available literature we failed to find information about the ability of *Nocardia* species to synthesize surfactants when cultivated on glycerol. Moreover, data on the surfactant synthesis by *Nocardia* are very limited. Our study showed that the isolated strain of *N. vaccinii* K-8 synthesized neutral, amino- and glycolipids (mainly trehalose mycolates) complex. Thus, our data is the first demonstrating the ability of *Nocardia* species to synthesize an unusual chemical composition of surfactants on a glycerol-containing medium. *N. vaccinii* K-8 synthesized surfactant on the medium containing 1.5 vol.% of glycerol (Pirog et al., 2011) – this is lower than what had previously been thought of from literature (between 2 and 10 vol.%). Taking into account that *N. vaccinii* K-8 synthesizes more surfactant than the other well-known producers (Das et al., 2009; Morita et al., 2007; Rooney et al., 2009; Silva et al., 2010) the product yield from the substrate quantity is significantly higher for strain K-8.

Taking into account that *A. calcoaceticus* IMV B-7241 and *N. vaccinii* K-8 had synthesized the neutral, amino- and glycolipids complex (Pirog et al., 2009), we assumed that it would be possible to stimulate the surfactant synthesis by the introduction of fumarate and citrate into the nutrient medium. Such approach was successful for *R. erythropolis* IMV Ac-5017 (Pirog et al., 2010). Fumarate, like other C_4-dicarboxylic acids, is a precursor of gluconeogenesis that provides carbohydrates synthesis and, consequently, glycolipids synthesis (trehalose mycolates).

Established regularities concerning the influence of organic acids on the surfactant synthesis by *A. calcoaceticus* IMV B-7241 differ from those of *R. erythropolis* IMV Ac-5017 (Pirog et al., 2010). Firstly, the optimal concentration of fumarate and citrate for IMV B-7241 strain was 10 times lower. Secondly, the effect of the simultaneous introduction of fumarate and citrate into the glycerol-containing nutrient medium of *A. calcoaceticus* IMV B-7241 was more significant. It was determined that the surfactant synthesis intensification, while *A. calcoaceticus* IMV B-7241 cultivation on the medium with glycerol, fumarate and citrate, was caused by the 3–5-fold increased activity of enzyme of surface-active amino- and glycolipids biosynthesis; as well as by the simultaneous functioning of two anaplerotic pathways (glyoxylate cycle and PEP-carboxylase reaction) in comparison with the cultivation of bacteria on a medium without organic acids.

A promising field of practical application of microbial surfactants is within the creation of modern antimicrobial agents for use in food and pharmaceutical industries, medicine and agriculture (Parisien et al., 2008; Raaijmakers et al., 2010). Currently the most investigated surfactants, which inherent antimicrobial action, are lipopeptides. This is also clear since they are actually polypeptide antibiotics (Banat et al., 2010; Das et al., 2008; Singh and Cameotra, 2004). The antimicrobial properties of surface-active glycolipids sophorolipids

(produced by *Candida bombicola* ATCC 22214) (Kim et al., 2002), mannosylerythritol lipids (produced by *Candida antartica* T34, yeasts of *Pseudozyma*, i.e. *P. siamensis* SBS 9960) (Morita et al., 2008), rhamnolipids (produced by bacteria of *Pseudomonas*) (Abdel-Mawgoud et al., 2010) were determined. Besides, the search for antimicrobial preparations, effective against spore microorganisms is relevant now. We have shown the effectiveness of surfactant of *A. calcoaceticus* IMV B-7241 against the vegetative and spore cells of *B. subtilis* BT-2, and the antimicrobial action of the BP against the spores was stronger.

We assume that the different antibacterial effect of BP of *R. erythropolis* IMV Ac-5017 and *A. calcoaceticus* IMV B-7241 was caused by the differences in their chemical composition. Thus, the effect of surfactant of *R. erythropolis* IMV Ac-5017 was similar to the one of surfactant N1, which was effective only against Gram-positive bacteria (Singh and Cameotra, 2004), and surfactant of *A. calcoaceticus* IMV B-7241 – to lipopeptide of *Bacillus circulans* (Das et al., 2008), which was more effective against different *Bacillus* species, then *E. coli*.

It is known that the mechanism of antimicrobial action of surfactants consists in violation of cytoplasmic membrane integrity of test-cultures and loss of cell viability (Singh and Cameotra, 2004). We assume that differences in the antimicrobial influence of surfactants of *R. erythropolis* IMV Ac-5017 and *A. calcoaceticus* IMV B-7241 on various pro- and eukaryotic microorganisms can be caused by numerous reasons: different chemical composition of cell wall and plasma membrane in the test-cultures, the presence or absence of surface structures of the cell wall (mucosa, outer membrane), various mechanisms of cell protection from antimicrobial agents, etc. In this study we used sterile supernatant of cultural liquid to investigate the antimicrobial activity of surfactants synthesized by *R. erythropolis* IMV Ac-5017 and *A. calcoaceticus* IMV B-7241. We understand that the influence investigation of the solutions of purified surfactants in equal concentrations shall be used for correctly comparing antimicrobial properties of surfactants of *R. erythropolis* IMV Ac-5017 and *A. calcoaceticus* IMV B-7241. This will be the aim of our next research.

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