

## Biosurfactants of *Rhodococcus erythropolis* IMV Ac-5017: Synthesis Intensification and Practical Application

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**Abstract** Intensification of the surfactant synthesis by *Rhodococcus erythropolis* IMV Ac-5017 on different substrates, including industrial waste, as well as the use of surfactant preparations for oil degradation were studied. It was established that the addition of fumarate (0.2 %) and citrate (0.1 %) into the medium with ethanol, *n*-hexadecane, or glycerol (1–2 %) was accompanied by an increase of conditional surfactant concentration by 1.5–1.7 times compared to the indexes in the medium without organic acids. The intensification of surfactant synthesis in the presence of fumarate and citrate is caused by the increased activity of isocitrate lyase (by 1.2–15-fold) and enzymes of the surfactant biosynthesis (by 2–4.8-fold) compared to their activity in the medium without precursors. The possibility of surfactant synthesis intensification (by 3–4-fold) while cultivating of *R. erythropolis* IMV Ac-5017 in the medium with oil containing substrates (2 %) and glucose (0.1 %) was shown. The introduction of 0.01 mM Cu<sup>2+</sup> in the exponential growth phase of strain IMV Ac-5017 in the medium with ethanol accompanied by the increasing conditional surfactant concentration by 1.9 times. The highly efficient remediation (92–95 %) of oil (2–2.6 g/L) and Cu<sup>2+</sup> polluted water after treatment with surfactant preparations (native cultural liquid) at low concentrations (5 %) was determined.

**Keywords** *Rhodococcus erythropolis* IMV Ac-5017 · Surfactant · Biosynthesis intensification · Precursors · Oil degradation

### Introduction

Microbial surfactants (biosurfactants) are widely used in different fields of industry that is why the demand on synthetic surfactants is increasing constantly. At the same

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time, the pace of biotechnology development and increased environmental concerns has been of great interest to scientists using microbial surfactants as an alternative to chemical ones [1].

Biosurfactants have a number of advantages over synthetic substances: biodegradability (when they appear in the ecosystem, they degrade easily in simple substances; at the same time, the use of chemicals leads to great harm), synthesized by microorganisms from cheap raw materials (for example, the wastes after different production), the stability of properties in a wide range of pH and temperatures, no toxicity, and the complex chemical structure that is hardly produced by chemical synthesis.

Due to the amphiphilic structure of molecules, biosurfactants have different properties, which is why they are used in oil industry and mining, in chemical and food industry, agriculture, and also in environmentally safe technologies for bioremediation [1].

Despite the outstanding properties of biosurfactants and the advantages over the synthetic analogues, their industrial production is much lower than chemical ones. The high-cost of biosynthesis and the process of biosurfactants extraction from the cultural liquid, as well as the low concentration of biosurfactants in the cultural liquid, have restrained global industrial production of biosurfactants [2]. That is why the investigations devoted to the solution of these problems became topical and main in biotechnology of microbial surfactants.

In the previous work, the oil-oxidizing bacteria identified as *Rhodococcus erythropolis* IMV Ac-5017 were isolated from the oil-polluted samples of soil. The ability of the strain to synthesize the metabolites with surface-active and emulsifying activity during the cultivation on different hydrophobic (*n*-hexadecane, liquid paraffin) and hydrophilic (glucose, ethanol) substrates was determined [3].

The criteria of biosurfactant synthesis in medium with *n*-hexadecane, which were shown for *R. erythropolis* IMV Ac-5017, can be compared to the ones of the other *Rhodococcus* strains that were described in the publications [4, 5].

However, in comparison with the other representatives of genus *Rhodococcus*, the strain we have selected has the following advantages: (1) synthesizes biosurfactant in the medium with total salt content of 3.15 g/L (for the other *Rhodococcus*—up to 10 g/L); (2) does not need the trace elements and yeast extract in the medium; (3) produces biosurfactant with a higher yield from substrate. It was determined that the surfactant synthesized by *R. erythropolis* IMV Ac-5017 consists of glyco-, phospho-, and neutral lipids, which form the complexes with the substances of polysaccharide and peptide nature [3].

The aim of the present work—development of approaches allowing to increase the efficiency of biosurfactant synthesis by *R. erythropolis* IMV Ac-5017 on different carbohydrate substrates, and also to investigate the possibility of practical use of these products.

## Materials and Methods

### Object of Research

Object of research—*Rhodococcus erythropolis* EK-1 strain, registered in Microorganisms Depository of Institute of Microbiology and Virology, the National Academy of Sciences of Ukraine under the number IMV Ac-5017.

### Medium composition and conditions of *R. erythropolis* IMV Ac-5017 cultivation

Bacteria were grown in the liquid mineral medium (g/L distilled water):  $\text{NaNO}_3$ —1.3,  $\text{NaCl}$ —1.0,  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ —0.6,  $\text{KH}_2\text{PO}_4$ —0.14,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —0.1,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ —0.001, pH 6.8–7.0.

*n*-Hexadecane, ethanol, glycerol, and oil-containing substrates (waste of oil and fats manufacturing, fried sunflower oil) were used as the carbon and energy sources in concentration of 1–2 % v/v.

In one of the variants the organic acids (sodium citrate and sodium fumarate in concentration of 0.1 % and 0.2 %, respectively) or glucose (1–3 g/L) were added in the medium at the beginning of the stationary growth phase of strain IMV Ac-5017. Citric acid was also used in concentration of 0.08 % instead of sodium citrate. The concentrations of sodium citrate (0.1 %) and citric acid (0.08 %) were equimolar by carbon. The sodium fumarate, sodium citrate and citric acid were added into the medium as 10 % solutions.

Taking into account that fumarate and citrate (citric acid) were additional carbon sources and their presence in the medium led to the changing of carbon concentration, and also of the C/N ratio, which was corrected in the control flasks by adding higher concentrations of carbon sources.

In one of the variants only the fumarate was added into the medium with ethanol and glycerol at the beginning of the stationary growth phase of IMV Ac-5017 strain, and the neutralization of cultural liquid with the citric acid solution was conducted while the pH was increasing.

The  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Pb}^{2+}$  were added into the nutritive medium in the concentrations of 0.01 and 0.05 mmol/L at the beginning of the cultivation, at the exponential and stationary growth phases to test the influence of metals on the growth of *R. erythropolis* IMV Ac-5017 and the surfactant synthesis. These metals were added as 1 % solutions of salts— $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ , and  $\text{Pb}(\text{CH}_3\text{COOH})_4$ .

The culture in the exponential phase was used as the inoculum and added in concentration of 5 % of nutritive medium volume. The concentration of the corresponding carbon source in the medium for the inoculum obtainment was 0.5 % v/v. The cultivation was carried out in 750 mL flasks, containing 100 mL of medium, on a shaker (220 rpm) at 30 °C during 24–168 h.

The biomass was determined by a gravimetric method. The double treatment of cultural liquid with hexane or petrol ether was carried out to remove the residual *n*-hexadecane or oil, respectively, before the cell sedimentation (5,000×g, 30 min). The necessity of this operation was caused by the precipitation of *n*-hexadecane or oil with cells that overestimate the biomass level.

### Determination of Indexes of Growth and Biosurfactant Synthesis

The surfactant synthesizing capacity was estimated by adopting the following criteria.

- (1) Surface tension ( $\sigma_s$ ) of the cell-free cultural liquid, which was measured with a semi-automatic tensiometer (LAUDA TDIC, Germany). Before the indicator  $\sigma_s$  was defined, the supernatant of the cultural liquid was preliminarily washed with hexane from *n*-hexadecane residues or with petrol ether from oil residues, which have surface-active properties and significantly decrease the real value of surface tension.
- (2) For a rapid test of the quantitative content of surfactant in the cultural liquid, we used the index of conditional surfactant concentration (CSC), which was defined as the

**Table 4** Influence of  $\text{Cu}^{2+}$  on growth of *R. erythropolis* IMV Ac-5017 and surfactant synthesis

Moment of $\text{Cu}^{2+}$ addition	$\text{Cu}^{2+}$ concentration, mM	Biomass, g/L	CSC	$E_{24}$ , %
Beginning of process	0.01	0.18±0.009	1.9±0.10	40±2
	0.05	0.13±0.007	1.8±0.09	41±2
Exponential phase	0.01	0.25±0.013	5.6±0.28	47±2
	0.05	0.55±0.028	2.8±0.14	39±2
Stationary phase	0.01	0.67±0.033	2.8±0.14	45±2
	0.05	0.90±0.045	3.1±0.16	41±2
Control	0	0.75±0.038	3.0±0.15	43±2

The concentration of ethanol in nutritive medium was 2.0 vol.%. The emulsification index was determined for the native cultural liquid; duration of cultivation was 120 h

cluster of small dry flakes, some of which stayed on the water surface and the others settled down to the bottom of reservoirs after the mixing. The data on determination of concentration of residual oil after 30 days of the experiment is shown in Table 5.

At the next stage complex water pollution was modeled and the possibility of its treatment in the presence of surfactants was studied. Oil (2 g/L) and  $\text{Cu}^{2+}$  (0.01 and 0.05 mM) were added into the water (2 L), and then it was treated with surfactant preparation of *R. erythropolis* IMV Ac-5017 based on post fermentation cultural liquid (5 %, Table 6).

The data presented in Table 6 show that in the presence of  $\text{Cu}^{2+}$  the degree of oil destruction increased. Control of water microflora, conducted during the experiment, showed 1–2-fold increase in the total number of microorganisms in all samples treated with surfactant (Table 7).

## Discussion

The first articles that described the representatives of the genus *Rhodococcus* as producers of substances with surface-active properties appeared in 1970–1980s [20–22]. Later, the information about surfactants synthesis by *Rhodococcus* was summarized in reviews [4, 5], and has been extended [23–25]. Taking into account that there are many bacteria of

**Table 5** Efficiency of oil polluted water treatment with surfactants' preparations of *R. erythropolis* IMV Ac-5017

Surfactant preparation	Concentration of surfactant preparation, %	Number of treatments	Concentration of residual oil, g/L	Oil degradation degree, %
Cultural liquid	5	One	0.20±0.01	92.3±4.6
	10	Two	0.32±0.02	87.7±4.4
	15	One	0.36±0.02	88.2±4.4
	30	Two	0.28±0.01	89.2±4.5
Supernatant	5	One	0.44±0.02	83.1±4.2
	10	Two	0.32±0.02	85.7±4.3
	15	One	0.38±0.02	85.4±4.3
	30	Two	0.32±0.02	87.7±4.4
Without treatment (control)			2.6±0.13	0

**Table 6** Remediation of oil polluted (2 g/L) water by surfactant preparations of *R. erythropolis* IMV Ac-5017 in the presence of copper cations

Type of pollution	Concentration of residual oil, g/L	Oil degradation degree, %
Oil	1.0±0.050	50±2.5
Oil+Cu <sup>2+</sup> 0.01 mM	0.1±0.005	95±4.8
Oil+Cu <sup>2+</sup> 0.05 mM	0.5±0.025	75±3.8
Control (without surfactant)	1.82±0.091	9±0.5

The content of residual oil was determined at 20th day of the experiment

*Rhodococcus* genus known today as producers of surfactants, each new strain must have some advantages compared to the existing ones.

A significant advantage of the isolated strain IMV Ac-5017 is the ability to synthesize surfactant on a vast variety of hydrophilic (glucose, ethanol, glycerol) and hydrophobic (liquid paraffin, *n*-hexadecane, sunflower oil) substrates, while the majority of *Rhodococcus* strains form a surfactant mainly while growing on hydrocarbons. Strain IMV Ac-5017 grows and synthesizes surfactant in a simple mineral medium containing no trace elements and growth factors, and synthesizes mainly exocellular surfactant [19], while the other rhodococci—both associated with cells and exocellular surfactant [4, 5]. It should be noted that our work [3] was one of the first that reported on the synthesis of surfactant by rhodococci on ethanol.

One of the global environmental problems of today is the search for methods of disposal or the reuse of industrial wastes. Moreover, hazardous wastes are not only wastes containing toxic substances (such as phenol and its derivatives), but the wastes entering the environment in uncontrolled amounts, for example, oil-containing wastes (wastes after oil and fat production, fried oil after use in food establishments and etc.). Biotechnological methods allow industry to recycle wastes and to obtain practically valuable biologically active substances or biomass.

Despite the large number of publications on the synthesis of microbial surfactants on industrial waste [26–29], information about rodococci growing on such substrates is very limited. It is known that *R. erythropolis* 16 LM.USTHB synthesized surfactants in a medium containing 3 % of fried sunflower oil [30], and *Rhodococcus* sp. BS32—in a medium containing 20 g/L of rapeseed oil [31]. However, in these studies the authors did not show the quantitative determination of surfactant (in g/L), and therefore it is not possible to compare their results with data reported in this article. Our studies have shown that the rate

**Table 7** Control of microorganisms' quantity in water during the oil degradation process

Type of pollution	Quantity of microorganisms (CFU/mL) on the day			
	7th	14th	21st	28th
Oil	5.3·10 <sup>4</sup>	7.2·10 <sup>4</sup>	8.0·10 <sup>4</sup>	8.3·10 <sup>4</sup>
Oil+Cu <sup>2+</sup> 0.01 mM	1.6·10 <sup>5</sup>	2.6·10 <sup>5</sup>	2.8·10 <sup>5</sup>	3.3·10 <sup>5</sup>
Oil+Cu <sup>2+</sup> 0.05 mM	2.0·10 <sup>5</sup>	3.0·10 <sup>5</sup>	3.9·10 <sup>5</sup>	4.7·10 <sup>5</sup>
Control (without surfactant)	3.6·10 <sup>4</sup>	1.7·10 <sup>5</sup>	3.1·10 <sup>5</sup>	3.8·10 <sup>5</sup>

Quantity of microorganisms in the water before the contamination was 3.2·10<sup>3</sup> CFU/mL

presence of copper cations may occur in the activation of alkane hydroxylases—first catabolic enzymes of *n*-alkanes as in the strain IMV Ac-5017, and the indigenous oxidizing microorganisms. Thus, it is known [45, 46] that  $\text{Cu}^{2+}$  plays a key role in the physiology and activity of methanotrophs. Methane oxidation by methanotrophs is carried out by membrane bound and/or soluble methane oxygenase belonging to the class of alkane hydroxylases, as well as enzymes of catabolism of *n*-alkanes. The dependence of growth criteria (growth rate, biomass concentration, the economic factor) on the content of copper cations in the culture medium was determined for many methanotrophs [45]. Our future research will focus on studying of the influence of copper on the activity of alkane hydroxylase of strain IMV Ac-5017.

We established the possibility of increasing the efficiency of surfactant biosynthesis by *R. erythropolis* IMV Ac-5017 as a result of: (1) use of oil containing substrates as the carbon sources, including wastes of oil and fat industry and catering establishments; (2) introduction of biosynthesis precursors (glucose,  $\text{C}_4$ -dicarboxylic acid) and regulators of lipogenesis (citrate) into the medium with non-carbohydrate substrates; (3) introduction of copper cations (0.01 mM) into ethanol containing medium in the exponential growth phase. It was established that surfactants of *R. erythropolis* IMV Ac-5017 intensified processes of oil degradation in polluted water. The possibility of using surfactant preparations (native cultural liquid) in low concentrations (5 %) for the effective (83–95 % degradation of oil at the concentration of 2.0 g/L) treatment of water, containing heavy metals, was determined.

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