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Production of Surfactants by *Rhodococcus erythropolis* Strain EK-1, Grown on Hydrophilic and Hydrophobic Substrates

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Abstract—The ability of *Rhodococcus erythropolis* strain EK-1 to produce surfactants when grown on hydrophilic (ethanol and glucose) and hydrophobic (liquid paraffins and hexadecane) substrates was studied. The strain was found to produce surfactants with emulsifying and surface-active properties. The production of surfactants depended on the composition of the nutritive medium, nature and concentration of the sources of carbon and nitrogen, and duration of cultivation. Chemically, surfactants produced by *Rhodococcus erythropolis* EK-1 grown on ethanol are a complex of lipids with polysaccharide–proteinaceous substances. The lipids include glycolipids (trehalose mono- and dicorynomycolates) and common lipids (cetyl alcohol, palmitic acid, methyl *n*-pentadecanoate, triglycerides, and mycolic acids).

In the last two or three decades, extensive theoretical and applied studies have been dedicated to microbial surfactants. This is related to their potential in oil recovery; mining; the chemical, drug, and food industries; agriculture; and environment remediation. Microbial surfactants are no less efficient than synthetic ones. These advantages, in addition to biodegradability and lack of toxicity, encourage their use in environmentally safe technologies.

Oil-oxidizing bacteria were isolated by us from oil-polluted soil and water samples and identified as *Acinetobacter calcoaceticus* K-4, *Nocardia vaceinii* K-8, *Rhodococcus erythropolis* EK-1, and *Mycobacterium* sp. K-2 [1]. It was found that water could be purified from oil (100 mg/l) by *R. erythropolis* EK-1 and *N. vaceinii* K-8 cells immobilized on expanded clay aggregate. The degree of water purification was shown to depend on the water flow rate, aeration, and nitrogen and phosphorus sources. The degree of water purification by immobilized *R. erythropolis* EK-1 cells at a high water flow rate (up to 0.68 l/h), low aeration (below 0.1 l/(l min)), and batch supply of 0.01% diammonium phosphate was 99.5–99.8%.

The ability of microorganisms to consume hydrocarbon substrates is often determined by surfactant production [2–6].

In the present study, we describe the production of surfactants by *Rhodococcus erythropolis* strain EK-1 and its features during the growth of the rhodococci on hydrophobic and hydrophilic substrates; physicochemical properties of the surfactants and the potential for increasing their production by *R. erythropolis* EK-1 are also discussed.

MATERIALS AND METHODS

Cultivation of Rhodococcus erythropolis EK-1. Bacteria were grown in liquid mineral media of variable composition (g/l). Medium A: KH_2PO_4 (6.8), NaOH (1.0), NH_4NO (0.6), $MgSO_4 \cdot 7H_2O$ (0.4), $CaCl_2 \cdot 2H_2O$ (0.1), $FeSO_4 \cdot 7H_2O$ (0.001), pH 6.8–7.0. Medium B: KNO_3 (1.0), NaCl (1.0), Na_2HPO_4 (0.6), KH_2PO_4 (0.14), $MgSO_4 \cdot 7H_2O$ (0.1), pH 6.8–7.0. The following carbon and energy sources were used: ethanol (1 and 2 vol %), liquid paraffins (*n*-alkanes C_{10}–C_{16}, 0.5 and 1 vol %), hexadecane (1 and 2 vol %), and glucose (1%). In one variant of the experiment, NH_4NO_3 was replaced by KNO_3 in medium A (1.0 and 1.5 g/l).

Bacteria were shaken (220 rpm) in flasks at 30°C for 48–168 h. The flasks were inoculated with a 2-day culture grown on glucose–potato agar.

The biomass growth was determined from the optical density of the cell suspension (followed by normalizing to the dry cell weight from a calibration plot) and a gravimetrically.

Determination of surfactant-production indices. The ability of cultures to produce surfactants was judged by the following indices:

(1) Surface tension (σ_s) of a cell-free culture liquid, measured with platinum and glass plates using the Wilhelmy hanging-plate method [7]. The σ_s value is a qualitative index of the presence of surfactants in the culture liquid.

(2) For proximate evaluation of surfactant content in a culture liquid, the index of conditional surfactant con-

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centration was used (Surf*). The index is determined as the dilution of the cell-free culture liquid at the surface tension decrease point on the curve of σ_s vs. dilution. The X coordinate of the inflection point corresponds to the conditional surfactant concentration Surf*. It is expressed in dimensionless units. The use of this approach for estimating surfactant concentration in a culture liquid is described in [8].

(3) Emulsifying activity, determined as described in [9]. Vaseline oil, sunflower oil, light crude oil (density 0.80 g/cm^3), and hexadecane were used as substrates to be emulsified. Five milliliters of a substrate were added to five milliliters of a solution under study containing a surfactant (culture liquid, supernatant of culture liquid, or cell suspension) and shaken for 2 min. The thickness of the emulsion layer (in percentages of the total height of the liquid column) after 24 h was taken to be equal to the emulsification index (A_{24}). To obtain a supernatant, the culture liquid was centrifuged at 5000 *g* for 20 min. Cell suspension was obtained by suspending the sedimented cells in a volume of distilled water equal to the volume of the starting culture liquid from which the cells were sedimented.

(4) Oil-washing properties of culture liquids, culture liquid supernatants, and cell suspensions. To estimate the oil-washing activity, a thin layer of heavy crude oil (density 0.99 g/cm^3) was applied to a glass plate (4 × 2 cm), the plate was kept in a surface-active solution under study for one hour, and the clean area of the glass surface was determined. The oil-washing activity was calculated as the area of the clean glass surface expressed as a percentage of the total glass surface.

(5) Content of carbohydrates in a culture liquid, assayed with phenol and sulfuric acid [10]. The choice of this index for quantitative surfactant determination was determined by the ability of rhodococci to produce glycolipids, including trehalose lipids [2–4].

(6) Total lipid content in a culture liquid, determined gravimetrically after extraction using the Folch procedure (chloroform–methanol 2 : 1, v/v) followed by solvent evaporation *in vacuo* (40°C).

Chemical composition of lipids. Lipids were extracted from the culture liquid, supernatant, or cell suspension with chloroform–methanol 2 : 1, v/v. Qualitative assay was performed by TLC on DC-Alufolien Kieselgel 60 plates (Merck, Germany). The plates were developed with various solvent systems: polar system: chloroform–methanol–water (85 : 15 : 1); nonpolar system I: hexane–diethyl ether (2 : 1); and nonpolar system II: hexane–diethyl ether–acetic acid (90 : 10 : 1). Lipids were visualized with the following reagents: common lipids, with an ethanol solution of phosphomolybdic acid and iodine vapors; peptidyl lipids, with ninhydrin; and glycolipids, with anisaldehyde and the anthrone reagent [11].

Chemical composition of extracellular surfactants. Extracellular surfactants were also isolated by another method. A culture liquid was centrifuged (5000 *g*, 20 min) to separate rhodococcus cells. The supernatant was dialyzed against distilled water for three days and concentrated *in vacuo* (40°C). Neutral monosaccharides, uronic acids, total protein, and fatty acids were assayed in the concentrated supernatant.

For assaying neutral monosaccharides and uronic acids, the concentrated supernatant was hydrolyzed with 2 N trifluoroacetic acid at 121°C for 2.5 h in sealed ampoules. The contents of neutral monosaccharides and uronic acids were determined with a Biotronik LC-2000 carbohydrate analyzer (Germany), in a 0.38 × 12.5 cm column with Dionex Ax8-II resin. Elution was performed with 0.5 M sodium borate buffer pH 8.0 at 60°C and with 0.04 M phosphate buffer pH 2.4 at 70°C, respectively. Detection was performed at 570 nm after reaction with copper 2.2^1-bicinchoninate.

Total protein was assayed with a Biotronik LC-2000 amino acid analyzer (Germany), in a 0.4 × 22 cm column with Ostion LG AN resin. Elution was performed with sodium borate–citrate buffer at 80°C after hydrolysis with 4 N hydrochloric acid at 100°C for 16 h. Detection was performed at 570 nm after reaction with ninhydrin.

Methyl esters of fatty acids were assayed after hydrolysis with 2 N trifluoroacetic acid followed by methanolysis with 0.5 N HCl in methanol at 80°C for 2 h. Separation was performed with a Hewlett-Packard 5890 GL chromatograph (USA) equipped with a flame ionization detector and glass capillary column (0.2 mm × 25 m) with OV-1 sorbent.

RESULTS AND DISCUSSION

Bacteria of the genus *Rhodococcus* respond to the presence of *n*-alkanes in the culture medium by the production of surface-active lipids [2, 3, 12, 14]. Surfactants of rhodococci are glycolipids, in particular, trehalose lipids (trehalose mono- and dicorynomycolates). The physiological function of the surfactants produced by rhodococci grown on hydrocarbons is the solubilization of hydrophobic substrates in cells. For example the surface tension of the supernatant of the culture liquid obtained by growing *R. erythropolis* AP-25 on hexadecane was σ_s = 30.3 mN/m, and on glucose, about 50 mN/m [13]. The surfactants produced on glucose lacked trehalose monocorynomycolate, and the lipids were less diverse than on hexadecane [13].

The amount of surfactants produced by rhodococci on hydrophobic substrates varies over a wide range, from 0.5 to 30 g/l. It depends on the concentration of the carbon source, carbon : nitrogen ratio, nature of the nitrogen source, pH, concentration of dissolved oxygen, etc. [2, 3, 12]. Batch cultivation of rhodococci in flasks in a medium containing 2% hexadecane yields mainly 1–3 g/l surfactant.

Production of surfactants by Rhodococcus erythropolis EK-1 grown on various carbon substrates. Our experiments showed that the amount of surfactants

Table 1. Effect of cultivation conditions on the production of surfactants by the *Rhodococcus erythropolis* EK-1 strain

Carbon source	Carbon source concentration, %	Duration of growth, h	Medium	pH_{final}	Biomass, g/l	Carbohydrates, g/l	Surf*	Emulsification index, %
Ethanol	1.0	48	A	6.3	0.80	0.50	n.d.	n.d.
			B	7.8	0.56	0.40	n.d.	n.d.
	1.0	96	A	5.5	1.30	0.90	1.1	70
			B	4.5	0.85	0.70	1.1	67
	2.0	96	A	5.3	2.00	0.86	1.2	88
			B	4.8	1.90	0.70	1.2	75
Liquid paraffins	0.5	96	A	6.5	0.62	0.48	n.d.	29
			B	7.9	0.40	0.16	n.d.	32
	1.0	96	A	6.5	2.05	0.60	1.5	23
			B	7.9	1.65	0.32	1.4	22
	1.0	120	A	6.1	1.80	1.05	2.7	31
			B	7.7	1.55	0.63	2.5	26
Hexadecane	1.0	120	A	5.7	1.60	1.00	4.4	50
			B	6.7	1.50	1.08	5.4	54
	2.0	120	A	5.8	1.90	1.35	4.3	54
			B	6.6	1.85	1.23	4.2	56
	2.0	168	A	5.6	1.70	1.65	5.2	50
			B	6.7	1.60	1.60	6.0	53
Glucose	1.0	120	A	6.7	0.95	0.70	1.1	69
			B	7.4	1.40	0.63	1.1	68

Note: Vaseline oil was used as a substrate for emulsification.
n.d., not determined.

produced by *R. erythropolis* EK-1 varied from 0.2 to 1.65 g/l (according to the phenol–sulfuric acid method). It depended on the composition of the medium, nature and concentration of the carbon source, and duration of cultivation (Table 1). The lowest surface tension values (σ_s 30–39 mN/m) and the highest Surf* values (2.5–6.0) were recorded in the supernatant of the culture liquid obtained with hydrophobic substrates (hexadecane or liquid paraffins). With hydrophilic substrates (ethanol or glucose), σ_s increased to 50–55 mN/g, and Surf* decreased to 1.1–1.2. The maximum amount of surfactants (up to 1.65 g/l) was obtained with hexadecane (Table 1).

However a higher index of culture liquid emulsification in the presence of vaseline oil and sunflower oil (60–88%) was obtained when *R. erythropolis* EK-1 was grown on ethanol and glucose, whereas with hydrophobic substrates it varied within 23–56% (Tables 1 and 2). It is also worth noting that the ability to emulsify hexadecane was detected only in the culture liquid obtained by cultivation on ethanol.

The decrease in the emulsifying activity of a culture liquid after the removal of cells (emulsification index of the supernatant of the culture liquid, see Table 2) suggests that part of the surfactants produced by *R. erythropolis* EK-1 is associated with cells. According to data from the literature, surfactants produced by rhodococci are mainly associated with cells. Only a minor portion of the surfactants is released to the ambient [2, 3].

The presence of a fairly high emulsifying activity in both the culture liquid and the supernatant in experiments with *R. erythropolis* EK-1 grown on ethanol was surprising. It is known that culture liquids of rhodococci grown on hydrophilic substrates have no emulsifying activity [13]. Note also that the supernatant and culture liquid after *R. erythropolis* EK-1 cultivation on both hydrophobic and hydrophilic substrates demonstrated an oil-washing activity (40–90%).

Analysis of the data presented in Tables 1 and 2 suggests that *R. erythropolis* EK-1 grown on any of the substrates investigated produces a complex of substances having emulsifying and surface-active properties. The presence of hydrophilic substrates favored the predominance of emulsifiers, as evidenced by the high emulsification index, and hydrophobic substances caused the predominance of substances with surface-active properties, which was confirmed by lower σ_s values and higher Surf* values.

Table 2. Effect of cultivation conditions on the emulsifying properties of the culture liquid of *Rhodococcus erythropolis* EK-1

Carbon source	Carbon source concentration, %	Duration of growth, h	Emulsification index of the culture liquid (%) with the presence of			Emulsification index of the supernatant of the culture liquid, %
			vaseline oil	sunflower oil	hexadecane	
Ethanol	1.0	96	70	81	52	53
	2.0	96	88	82	50	52
Glucose	1.0	120	69	60	n.d.	49
Liquid paraffins	1.0	96	23	31	0	18
	1.0	120	31	31	0	27
Hexadecane	2.0	120	54	47	0	35
	2.0	168	50	51	0	38

Note: Vaseline oil was used as a substrate for emulsification with the supernatant of the culture liquid.
n.d., not determined.
Bacteria were grown in medium A.

Table 3. Production of surfactants by *Rhodococcus erythropolis* EK-1 in medium A with ethanol

Ethanol concentration, %	Nitrogen source	Nitrogen source concentration, g/l	Growth time, h	Surf*	pH_{final}	Biomass, g/l	Carbohydrates, g/l	Surfactant concentration, g/l	Emulsification index of the culture liquid with vaseline oil, %			Oil-washing activity of the culture liquid, %	
									not diluted	1 : 10	1 : 50	not diluted	1 : 10 or 1 : 50
1.0	NH_4NO_3	0.6	96	1.1	5.5	1.3	0.9	0.40	70	50	43	90	n.d.
2.0		0.6	96	1.2	5.3	2.0	0.86	0.43	88	56	48	75	n.d.
1.0	KNO_3	1.0	96	1.3	6.4	1.5	0.85	0.6	75	55	45	90	95 (1 : 10)
1.0		1.5	96	1.3	6.8	1.5	0.9	0.7	70	52	48	85	100 (1 : 10)
2.0		1.0	120	1.6	6.4	1.75	1.25	0.7	78	50	45	95	90 (1 : 10)
2.0		1.5	120	3.0	6.8	1.90	1.5	1.0	90	60	50	90	90 (1 : 10)
2.0		1.0	168	1.8	6.6	1.9	1.4	0.85	75	50	45	50	95 (1 : 10)
2.0		1.5	168	3.3	6.7	2.2	1.8	1.2	85	68	60	50	95 (1 : 50)

Note: n.d., not determined

In subsequent experiments, bacteria were grown in medium A with ethanol. These cultivation conditions were chosen for the following reasons:

1. There were no data in the literature on the production of surfactants by rhodococci grown on ethanol.

2. The yield of surfactants in medium A was greater than in medium B (Table 1).

3. Cultivation of *R. erythropolis* EK-1 in medium A with ethanol gives rise to a homogeneous culture liquid. Cells do not aggregate or adhere to flask walls. This facilitates the utilization of the liquid.

4. Cultivation in an ethanol-containing medium is accompanied by a pH decrease to 5.0–5.5, whereas the optimum pH value for surfactant production is, as a rule, close to neutral [4, 15, 16]. Thus the production of surfactants by *R. erythropolis* EK-1 can be increased.

Intensification of surfactant production by *R. erythropolis* EK-1 grown on ethanol. Experiments showed that replacement of the nitrate–ammonium nitrogen source in the cultivation medium by an equimolar amount of nitrate (KNO_3, 1 g/l) resulted in pH stabilization during cultivation at 6.4–6.5. Simultaneously Surf* increased to 1.3, and the amount of surfactant, by a factor of 1.5, according to data obtained with chloroform–methanol extracts, although the content of carbohydrates remained at the same level (Table 3). Obviously the increase in surfactant production was determined by the maintenance of pH close to neutral as a result of bacterial consumption of nitrogen in the nitrate form. This pH level is most favorable for most surfactant producers [4, 15, 16]. In addition, the carbon : nitrogen ratio is of great importance for surfactant production [4, 15, 17]. Our experiments showed that an

Table 4. Composition of the lipids produced by *Rhodococcus erythropolis* EK-1 grown on ethanol

Cultivation conditions	Location of the lipids	Qualitative composition of common lipids	Qualitative composition of glycolipids
1% ethanol 0.6 g/l NH_4NO_3	Culture liquid	Cetyl alcohol, palmitic acid, methyl *n*-pentadecanoate	Trehalose monocorynomycolate Trehalose dicorynomycolate
	Supernatant	″	″
	Cells	″	–
2% ethanol 1.5 g/l KNO_3	Culture liquid	″ + triglyceride, mycolic acids	Trehalose monocorynomycolate Trehalose dicorynomycolate
	Supernatant	Cetyl alcohol, palmitic acid, methyl *n*-pentadecanoate	″
	Cells	″ + triglyceride, mycolic acids	±
2% ethanol 1.0 g/l KNO_3	Culture liquid	Cetyl alcohol, palmitic acid, methyl *n*-pentadecanoate, triglyceride	Trehalose monocorynomycolate Trehalose dicorynomycolate

Note: –, not detected; ±, traces.

increase in the ethanol concentration to 2% and of KNO_3, to 1.5 g/l with cultivation lasting for 7 days increased the content of surfactants to 1.2 g/l, carbohydrates, to 1.8 g/l, and Surf*, to 3.3 (Table 3). The culture liquid obtained under such conditions had a high emulsification index (85%), which remained at the level of 60% when the culture liquid was diluted fiftyfold. It is noteworthy that the oil-washing activity of the culture liquid increased after such dilution. This pattern was also observed for the culture liquid obtained under other cultivation conditions in a medium with ethanol (Table 3).

Chemical composition of the lipids produced by *R. erythropolis* EK-1 grown on ethanol. The data presented in Table 4 indicate that all samples of the culture liquid and supernatant contained trehalose mono- and dicorynomycolates. Trehalose mycolates were either absent from or present (trace amounts) in cell-associated surfactants, although they are typical of cellular surfactants of rhodococci. In our opinion this is related to the fact that *R. erythropolis* EK-1 was grown on a hydrophilic substrate, ethanol, whereas trehalose mycolates, which facilitate the consumption of hydrophobic compounds, are present mainly in the walls of cells cultivated on paraffins, oil, hexadecane, etc. [2, 3].

The qualitative composition of common lipids was the same in the culture liquid and cells (Table 4). Of note is the appearance of triglyceride and mycolic acids in the surfactants extracted from the culture liquid and cells grown in the medium with KNO_3. These compounds, however, were not detected in the corresponding supernatants. The study of the chemical composition of the lipids confirmed our suggestion that *R. erythropolis* EK-1 produced both free and cell-associated surfactants.

Measurement of the surface-active properties of the lipid fraction extracted from the culture liquid of bacteria grown in a medium with 2% ethanol and 1.5 g/l KNO_3 yielded σ_s = 40 mN/m and Surf* = 4.3. For the culture liquid, these indices were 50 mN/m and 3.3, respectively. After extraction and drying, the lipids were virtually insoluble in water or 0.1 M phosphate-buffered saline, pH 7.0. They were dissolved (incompletely) after alkalization of the buffer to pH 9.0. Moreover, in most cases during of the culture liquid extraction with chloroform-methanol, the emulsion was very stable. The presence of this emulsion phase and the difficult dissolution of the organic extract may indicate that lipids form a complex with other compounds (e.g., proteins and/or polysaccharides). This suggestion was confirmed by visualization of chromatograms with ninhydrin and by analysis of extracellular surfactants (Isolation is described in *MATERIALS AND METHODS*.). Such samples contained neutral monosaccharides and glucuronic acid, as well as protein (3.4%) and fatty acids identified as C_{16}, C_{18}, C_{20}, C_{22}, and higher, which manifested themselves as a series of peaks eluted at equal time intervals.

Here are the carbohydrate compositions of extracellular surfactants produced by *R. erythropolis* EK-1 grown in medium A with ethanol:

Carbohydrate Content	Percentage of the total amount of surfactants
Glucose	2.7
Mannose	2.3
Galactose	1.6
Rhamnose	0.7
Ribose	traces
Glucuronic acid	1.5

These data confirm our suggestion that *R. erythropolis* EK-1 grown on ethanol produces a set of substances with surface-active and emulsifier properties (“surfactant” and “emulsifier”). Extraction of lipids from culture liquid with chloroform–methanol (2 : 1) is

accompanied by the transition of part of the emulsifier to the organic phase and the transition of part of the surfactant to the emulsion phase. The assay of lipids by extraction with organic solvents is imprecise, since it results in underestimation. The contents of carbohydrates in the culture liquid are also underestimated. Both the surfactant and emulsifier contain only part of the carbohydrates. Obviously, the most appropriate indices for correct evaluation of the contents of the surfactant and emulsifier produced by *R. erythropolis* EK-1 are the Surf* of the culture liquid and the emulsification index of the liquid determined for a series of dilutions.

To sum up, our study demonstrates that *R. erythropolis* strain EK-1, grown on various carbon sources, produces a set of free and cell-associated biological substances that possess surface-active and emulsifying properties. When grown on hydrophilic substrates, the strain produces mainly the emulsifier; on hydrophobic substrates, substances with surface-active properties. We optimized the conditions of cultivation of the strain in a medium with ethanol (the nature and concentration of the nitrogen source, ethanol concentration, and duration of cultivation). This allowed the yield of surfactants to be increased threefold. Glycolipids and common lipids were detected among the surfactants produced on ethanol. They formed a complex with compounds of polysaccharide and protein nature.

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