ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 6, pp. 665–673. © Pleiades Publishing, Ltd., 2008.
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EXPERIMENTAL ARTICLES

Peculiarities of C_2 Metabolism and Intensification of the Synthesis of Surface-Active Substances in *Rhodococcus erythropolis* EK-1 Grown in Ethanol

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Received November 12, 2007

Abstract—Oxidation of ethanol, acetaldehyde, and acetate in *Rhodococcus erythropolis* EK-1, producer of surface-active substances (SAS), is catalyzed by *N,N*-dimethyl-4-nitrosoaniline (DMNA)-dependent alcohol dehydrogenase, $NAD^+/NADP^+$-dependent dehydrogenases (optimum pH 9.5), and acetate kinase/acetyl-CoA-synthetase, respectively. The glyoxylate cycle and complete tricarboxylic acid cycle function in the cells of *R. erythropolis* EK-1 growing on ethanol; the synthesis of phosphoenolpyruvate (PEP) is provided by the two key enzymes of gluconeogenesis, PEP carboxykinase and PEP synthetase. Introduction of citrate (0.1%) and fumarate (0.2%) into the cultivation medium of *R. erythropolis* EK-1 containing 2% ethanol resulted in the 1.5- and 3.5-fold increase in the activities of isocitrate lyase and PEP synthetase (the key enzymes of the glyoxylate cycle and gluconeogenesis branch of metabolism, respectively) and of lipid synthesis, as evidenced by the 1.5-fold decrease of isocitrate dehydrogenase activity. In the presence of fumarate and citrate, the indices of SAS synthesis by strain *R. erythropolis* EK-1 grown on ethanol increased by 40–100%.

Key words: *Rhodococcus erythropolis*, ethanol metabolism, glyoxylate cycle, gluconeogenesis, intensification of biosynthesis, surface-active substances.

DOI: 10.1134/S0026261708060039

Strain *Rhodococcus erythropolis* EK-1 was isolated from petroleum-contaminated soil samples [1]. *R. erythropolis* EK-1 was previously shown to produce surface-active substances (SAS) both on hydrophobic (hexadecane, liquid paraffins) and hydrophilic (glucose, ethanol) substrates [2], with much lower values of SAS synthesis on ethanol than on hexadecane. Since ethanol is a cheaper and more producible substrate than water-insoluble, hydrophobic compounds, its application for SAS biosynthesis may significantly enhance the efficiency of the SAS production technology. Although literature provides some data on the ability of *Rhodococcus* bacteria to assimilate ethanol as a carbon and energy source [3, 4], we did not find any information concerning SAS synthesis by rhodococci growing on this substrate.

One of the ways for intensification in the technologies of microbial synthesis is to reveal the possible sites of metabolic limitation and to develop approaches to their elimination based on the analysis of the peculiarities of energetic and constructive metabolism of producers of practically valuable metabolites. Earlier studies of C_2 metabolism regulation in *Acinetobacter* sp. IMV B-7005, a producer of the polysaccharide ethapolan, resulted in development of the method for its production on a nonbuffered medium with a fourfold decreas in content of salts (up to 2.95 g/l) [5].

Another approach that increases the efficiency of microbial biotechnologies is introduction of exogenous precursors into the cultivation medium. Previously we have proved the possibility for intensification of ethapolan synthesis by introduction of C_4-dicarboxylic acids, the intermediates of ethanol metabolism involved in gluconeogenesis [6]. Introduction of precursors into the cultivation medium is known to enhance the synthesis of macrolide antibiotics [7]. In the 80–90s of the 20th century, some researchers established the stimulating effect of sodium citrate on SAS formation by microorganisms [8–10]. Our studies showed that after introduction of fumarate (0.2%) and citrate (0.1%) into the medium with hexadecane in the beginning of the stationary growth phase of *R. erythropolis* EK-1 the values of SAS synthesis increased by 40–70% as compared with the analogous values at cultivation of the strain on the medium without these precursors [11].

The goal of this work was to study the peculiarities of ethanol metabolism in *R. erythropolis* EK-1 and to establish the possible mechanisms for enhancement of

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SAS synthesis on this substrate in the presence of precursors (fumarate and citrate).

MATERIALS AND METHODS

Object of research. The object of research was strain *R. erythropolis* EK-1 registered at the Depository of microorganisms of the Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine with accession number IMV Ac-5017.

Medium composition and cultivation conditions for *R. erythropolis* EK-1. Bacteria were grown in liquid mineral media. Medium 1 contained the following (g/l): KNO_3, 1.5; NaCl, 1.0; $Na_2HPO_4 \cdot 12H_2O$ – 0.6; KH_2PO_4 – 0.14; $MgSO_4 \cdot 7H_2O$, 0.1; pH 6.8–7.0. Medium 2 contained the following (g/l): KH_2PO_4, 6.8; NaOH, 1.0; NH_4NO_3 – 0.6; $MgSO_4 \cdot 7H_2O$ – 0.4; $CaCl_2 \cdot 2H_2O$ – 0.1; $FeCl_3 \cdot 6H_2O$, 0.01; pH 6.8–7.0. Ethanol (2%, vol/vol) was used as both a carbon and energy source. Medium 1 is a modified [1] Muenz medium [12], which is used for cultivation of hydrocarbon-oxidizing bacteria. Medium 2 was developed by us for the cultivation of biopolymer producers [5].

The precursors of SAS synthesis, sodium citrate and sodium fumarate (0.1 and 0.2%, respectively), were added to the medium with ethanol in one of the experimental variants. Precursors were introduced as 10% solutions in the beginning of cultivation and in the beginning of the stationary growth phase.

Citrate and fumarate are additional sources of carbon nutrition and their introduction into the medium results in the change of not only carbon concentration but also the C/N ratio. Hence, the content of the main carbon source (ethanol) in the control variants was corrected. The purpose of such a correction was to ensure equimolar amounts of carbon, to provide stability of the optimal carbon/nitrogen ratio in the cultivation medium.

Cultivation was performed in 750-ml flasks with 100 ml of the medium in a shaker (220 rpm) at 30°C for 24–68 h.

The daily culture grown on meat–peptone (MPA) or glucose–potato (GPA) agar and the culture in the exponential phase (48–72 h), grown on media 1 or 2 with 0.5% ethanol (vol/vol) in the presence or absence of SAS synthesis precursors, were used as inoculum. If the inoculum was grown in a liquid medium, its concentration was 5% of the volume of the inoculated medium.

Determination of growth and SAS synthesis characteristics. The biomass was assayed by the optical density of cell suspension followed by recalculation per absolutely dry weight according to the calibration diagram.

The ability for SAS synthesis was assessed by the following indices:

(1) Surface tension (σ_s) of cell-free culture liquid measured using a glass plate [1, 2].

(2) Express evaluation of the quantitative content of SAS in the culture liquid was performed using an index of conventional concentration of SAS (SAS*) defined as the degree of dilution of cell-free culture liquid (supernatant) to the MCC point (Micelle formation Critical Concentration). The dependence of surface tension σ_s on the logarithm of supernatant dilution was plotted as a diagram [1, 2]. The abscissa of the inflection point corresponds to the SAS* value. Conditional SAS concentration was expressed in dimensionless units.

(3) Index of culture liquid emulsification (E_{24}, %) was determined as described [1]. Sunflower oil was used as a hydrophobic substrate for emulsification.

(4) The quantity of synthesized SAS was determined by the gravimetric method of Bligh and Dyer [13] in our modification. Modification was necessary, because *R. erythropolis* EK-1 synthesizes a complex of polar and nonpolar lipids [1, 2], whereas the method of Bligh and Dyer allows extraction of mainly nonpolar lipids. In view of this fact, we modified the classical system of solvents (Folch mixture) by introducing 1 M HCl (chloroform: methanol: 1 M HCl = 4 : 3 : 2).

Obtaining the cell-free extracts. The bacterial suspension obtained after cultivation of *R. erythropolis* EK-1 in a liquid, mineral medium was centrifuged (4000 *g*, 15 min, 4°C). The cell precipitate was washed twice from the medium debris with 0.05 M K^+ phosphate buffer (pH 7.0) and centrifuged at 4000 *g* (15 min, 4°C). Washed cells were resuspended in 0.05 M K^+ phosphate buffer (pH 7.0) and sonicated (22 kHz) four times by 6 s at 4°C in an UZDN-1 apparatus. The resulting liquid was centrifuged (12000 *g*, 30 min, 4°C), the precipitate was removed, and the supernatant was used as a cell-free extract.

Cell-free extracts were obtained from cells in the early, middle, and late exponential growth phases (24, 48, and 72 h of cultivation, respectively).

Enzymatic analyses. The activities of alcohol dehydrogenase (EC 1.1.1.1, EC 1.1.1.2, and EC 1.1.99.8), acetaldehyde dehydrogenase (EC 1.2.1.3, EC 1.2.1.4, EC 1.2.1.10), acetate kinase (EC 2.7.2.1), acetyl-CoA-synthetase (EC 6.2.1.1), and isocitrate lyase (EC 4.1.3.1) were determined as described in [14]. The activities of nicotinoprotein (NAD(P)H-containing) alcohol dehydrogenase (EC 1.1.99. –) and acetaldehyde dehydrogenase (EC 1.2.99. –) were determined spectrophotometrically by *N*,*N*-dimethyl-4-nitrosoaniline (DMNA) reduction at 440 nm with ethanol, methanol, and acetaldehyde as electron donors, respectively [15]. The activity of alcohol oxidase (EC 1.1.3.13) was analyzed by H_2O_2 formation or dissolved oxygen consumption as described in [16]. The activity of acetaldehyde dehydrogenase (EC 1.2.99.3) was determined by the reduction of dichlorophenol indophenol in the presence of phenazine methosulfate at 600 nm [17].

Table 1. Activity of alcohol and acetaldehyde dehydrogenases in *R. erythropolis* EK-1 grown on ethanol

Enzymes		Activity (nmol min^{-1} mg^{-1} protein) when grown in	
		medium 1	medium 2
Alcohol dehydrogenase	NAD^+-dependent (pH 9.0)	8.91 ± 0.4	9.41 ± 0.3
	$NADP^+$-dependent (pH 9.0)	10.52 ± 0.5	8.57 ± 0.3
	PQQ-dependent	7.23 ± 0.3	3.74 ± 0.1
	DMNA-dependent	51.45 ± 2.5	40.16 ± 0.21
		(85.32 ± 3.9)	(155.92 ± 0.69)
	MNO	40.16 ± 2.1	10.93 ± 0.5
		(83.73 ± 4.0)	(145.89 ± 7.3)
Acetaldehyde dehydrogenase	NAD^+-dependent (pH 9.5)	96.72 ± 4.8	110.55 ± 5.5
	$NADP^+$-dependent (pH 9.5)	44.82 ± 2.4	29.71 ± 1.1
	PQQ-dependent	7.22 ± 0.3	3.15 ± 0.1
	DMNA-dependent	7.34 ± 0.2	4.38 ± 0.2
	Acylating	0	0

Note: The cells from the middle of exponential growth phase (48 h) were sampled. In brackets, the enzymatic activities in the cells from the early exponential growth phase (24 h) are given.

The activities of malate synthase (EC 4.1.3.2), citrate synthase (EC 4.1.3.7), aconitate hydratase (EC 4.2.1.3), isocitrate dehydrogenase (EC 1.1.1.41 and EC 1.1.1.42), 2-oxoglutarate dehyderogenase (EC 1.2.4.2), succinate dehydrogenase (EC 1.3.99.1), fumarate hydratase (EC 4.2.1.2), malate dehydrogenase (EC 1.1.1.37, EC 1.1.1.82), malate dehydrogenase (decarboxylating) (EC 1.1.1.38 and EC 1.1.1.40), phosphoenolpyruvate (PEP) synthetase (EC 2.7.9.2), and PEP carboxykinase (EC 4.1.1.49) were analyzed as described in [6].

Protein content in cell-free extracts was determined according to Bradford [18]. Enzyme activities were determined at 28–30°C, the temperature optimal for growth of *R. erythropolis* EK-1.

All experiments were carried out in three repeats; parallel measurements in the experiments were three to five. Statistical processing of experimental data was performed according to Lakin [19]. The experimental results, according to the Student's *t*-criterion, proved to be statistically reliable at a 5% level of significance.

RESULTS AND DISCUSSION

Oxidation of ethanol and acetaldehyde. Alcohol dehydrogenases oxidizing ethanol to acetaldehyde in microorganisms belong to three groups [20, 21]. The first group includes $NAD(P)^+$-dependent alcohol dehydrogenases that have been studied thoroughly. Representatives of the second group are $NAD(P)^+$-independent alcohol dehydrogenases that utilize pyrrole quinoline quinone (PQQ), heme (associated with PQQ), or factor F_{420} as a cofactor. The third group includes flavin adenine dinucleotide (FAD^+)-dependent alcohol oxidases catalyzing irreversible oxidation of alcohols.

In the 90s, of the 20th century, some Gram-positive bacteria (*Mycobacterium gastri, Rhodococcus rhodochrous, R. erythropolis, Rhodococcus* sp., and *Amycolatopsis methanolica*) were shown to have a new type of nicotinoprotein (NAD(P)H-containing) alcohol dehydrogenases revealed through the reaction with *N,N*-dimethyl-4-nitrosoaniline (DMNA) as an artificial electron acceptor [22]. Such enzymes are known as DMNA-dependent alcohol dehydrogenases. As an active site, they contain the bound NAD(P)H which is a cofactor but not a coenzyme of these dehydrogenases.

It is known from the literature that $NAD(P)^+$ and DMNA-dependent alcohol dehydrogenases function in the members of the genus *Rhodococcus* growing on ethanol [15, 22]. It is interesting to note that DMNA-dependent enzymes of ethanol-grown rhodococci are able to oxidize both ethanol and methanol [15, 22]. It is mentioned in these works that methanol and ethanol oxidation involves two different DMNA-dependent alcohol dehydrogenases, i.e. methanol: *N,N*-dimethyl-4-nitrosoaniline oxidoreductase (MNO) and alcohol: *N,N*-dimethyl-4-nitrosoaniline oxidoreductase (DMNA-ADH). The cells of *R. erythropolis* DSM 1069 growing on the medium with ethanol were shown to have the NAD^+-alcohol dehydrogenase activity with the optimum pH 9.0 and the DMNA-ADH and MNO activities [15].

The NAD^+, $NADP^+$, PQQ and DMNA-dependent enzymes as well as MNO were found in the cells of *R. erythropolis* EK-1 grown on ethanol (Table 1). The activities of PQQ- and $NAD(P)^+$-dependent alcohol dehydrogenases were not high (4–10 nmol min^{-1} mg^{-1} protein) and obviously could not be of essential significance for ethanol metabolism in the studied rhodococcus strain. These enzyme activities remained practi-

cally on the same level irrespective of the bacterial growth phase. Investigation of the activities of NAD^+- and $NADP^+$-dependent enzymes in the pH range from 7.0 to 9.5 showed that the optimal pH value was 9.0, when the activity did not exceed 10 nmol min^{-1} mg^{-1} protein (Table 1).

As can be seen from the data presented in Table 1, the cells of *R. erythropolis* EK-1 growing on ethanol possess both DMNA-ADH and MNO activities with the maximal values in the early exponential phase of bacterial growth. The activities of these alcohol dehydrogenases significantly decreased in the middle of the exponential phase and were only 4–5 nmol min^{-1} mg^{-1} protein by the end of the exponential phase.

It is known from the literature that DMNA-ADH activity determined in the cell-free extract of rhodococci grown on ethanol does not exceed 4–6 nmol min^{-1} mg^{-1} protein [15, 22]. It may be explained by the fact that DMNA-ADH activity decreases 2–20 times in the presence of adenylates, acetaldehyde, and many cations that are inhibitors of this enzyme [22]. The content of such inhibitors in cell-free extracts is rather high. The above assumption is supported by the fact that the activity of this enzyme increased by two orders of magnitude after its isolation and preliminary purification [22].

Thus, ethanol oxidation in *R. erythropolis* EK-1, like in other rhodococci, involves DMNA-dependent alcohol dehydrogenase.

On medium 1, DMNA-ADH and MNO activities in *R. erythropolis* EK-1 measured in the beginning of the exponential growth phase were almost two times lower than those obtained on medium 2 (84–85 and 146–156 nmol min^{-1} mg^{-1} protein, respectively). At the same time, by the middle of the exponential growth phase these enzymatic activities decreased only twice on medium 1 (to 40–50 nmol min^{-1} mg^{-1} protein) and 4–15 times on medium 2 (to 11–40 nmol min^{-1} mg^{-1} protein).

Acetaldehyde formed during ethanol oxidation is involved in metabolism with participation of acetaldehyde dehydrogenases. Most microorganisms have $NAD(P)^+$-dependent enzymes and acylating acetaldehyde dehydrogenase [23–25]. Recently, reports have appeared on acetaldehyde oxidation in bacterial cells by PQQ- and DMNA-dependent acetaldehyde dehydrogenases [15, 17]. The cell-free extract of *R. erythropolis* EK-1 was shown to contain several acetaldehyde dehydrogenases (Table 1). The activity of PQQ- and DMNA-dependent enzymes did not exceed 7–7.5 nmol min^{-1} mg^{-1} protein. The data presented in Table 1 demonstrate that acetaldehyde oxidation in *R. erythropolis* EK-1 is performed by NAD^+- and $NADP^+$-dependent acetaldehyde dehydrogenases. The study of the effect of the reaction mixture pH on these enzyme activities revealed the pH optimum at 9.5.

As is known from the literature, NAD^+-dependent acetaldehyde dehydrogenase, which can also oxidize formaldehyde, functions in *R. erythropolis* UPV-1 growing on ethanol [26]. As a result, strain UPV-1 can be used for removal of formaldehyde from industrial wastewater. Our preliminary experiments also demonstrated the possibility of formaldehyde oxidation by the cells of *R. erythropolis* EK-1. It should be noted that representatives of the genus *Rhodococcus*, including *R. erythropolis* strains, are characterized by a wide range of various enzymes including dehydrogenases. Hence, rhodococcus strains and their enzymes may be considered promising for application in different environment-protecting biotechnologies [27].

Central metabolism. The activity of the enzymes of central metabolism at cultivation of *R. erythropolis* EK-1 on ethanol is given in Table 2. Acetate formed by the acetaldehyde dehydrogenase reaction is oxidized by acetate kinase and acetyl-CoA-synthetase (280–300 and 45–55 nmol min^{-1} mg^{-1} protein, respectively). The role of the glyoxylate cycle as the anaplerotic sequence of reactions completing the pool of C_4-dicarboxylic acids in ethanol-grown *R. erythropolis* EK-1 is confirmed by the high activity of both isocitrate lyase and malate synthase (730–740 and 135–165 nmol min^{-1} mg^{-1} protein, respectively).

The cell-free extract of *R. erythropolis* EK-1 shows high activity of all the enzymes of the tricarboxylic acid cycle, with the exception of 2-oxoglutarate dehydrogenase: 10–20 nmol min^{-1} mg^{-1} protein. High activities of isocitrate dehydrogenase and isocitrate lyase and low activity of 2-oxoglutarate dehydrogenase at cultivation of *R. erythropolis* EK-1 on ethanol may demonstrate that the tricarboxylic acid cycle in these bacteria plays a mainly, biosynthetic role.

It should be noted that the activity of some enzymes of this cycle was practically the same at cultivation of strain EK-1 on media 1 and 2. At the same time, the activities of fumarate hydratase, NAD^+-dependent malate dehydrogenase, and $NADP^+$-dependent malate dehydrogenase (decarboxylating) were nearly 3 times higher and the activity of $NADP^+$-dependent isocitrate dehydrogenase was 1.5 times higher at cultivation of this rhodococcus strain on medium 1 than on medium 2 (Table 2).

At growth on ethanol, the synthesis of carbohydrates necessary for the formation of nucleic acids, polysaccharides, and a number of metabolites including surface active substances in *R. erythropolis* EK-1 is provided by the gluconeogenetic branch of metabolism, as demonstrated by the high activity of the two key enzymes of gluconeogenesis: PEP synthetase and PEP carboxykinase (Table 2).

It is notable that the activity of PEP synthetase at cultivation of *R. erythropolis* EK-1 on medium 1 was higher (nearly threefold) than the activity of this enzyme at cultivation on medium 2. At the same time, PEP carboxylase activity was practically the same at cultivation of the strain on both media. Since not only the PEP synthetase activity but also the activities of malate synthase, fumarase, NAD^+-dependent malate dehydrogenase and $NADP^+$-dependent malate dehy-

Table 2. Activity of the enzymes of central metabolism in *R. erythropolis* EK-1 cultivation on ethanol

Enzyme	Activity (nmol min^{-1} mg^{-1} protein) when grown in	
	medium 1	medium 2
Acetyl-CoA-synthetase	52.0 ± 2.6	44.6 ± 2.2
Acetate kinase	287.6 ± 14.3	302.2 ± 15.1
Isocitrate lyase	741.1 ± 37.0	733.0 ± 36.5
Malate synthase	164.8 ± 8.2	138.5 ± 6.9
Citrate synthase	216.4 ± 10.2	201.8 ± 10.0
Aconitase	152.4 ± 7.6	168.7 ± 8.4
NAD^+-dependent isocitrate dehydrogenase	10.2 ± 0.5	10.5 ± 0.5
$NADP^+$-dependent isocitrate dehydrogenase	608.6 ± 30.4	400.0 ± 20.0
2-Oxoglutarate dehydrogenase	20.4 ± 1.0	10.5 ± 0.5
Succinate dehydrogenase	99.3 ± 4.9	68.7 ± 3.4
Fumarate hydratase	776.9 ± 38.4	268.7 ± 13.4
NAD^+-dependent malate dehydrogenase	406.4 ± 20.0	148.4 ± 7.3
$NADP^+$ dependent malate dehydrogenase	50.8 ± 2.5	42.2 ± 2.1
NAD^+-dependent malate dehydrogenase (decarboxylating)	0	0
NAD^+-dependent malate dehydrogenase (decarboxylating)	304.8 ± 15.2	105.4 ± 5.2
Phosphoenolpyruvate carboxykinase	203.2 ±10.1	210.9 ± 10.5
Phosphoenolpyruvate synthetase	1307.1 ± 63.2	464.3 ± 23.2

Note: Cells from the middle of exponential growth phase (48 h) were sampled.

drogenase (decarboxylating) were higher at cultivation of *R. erythropolis* EK-1 on medium 1, a higher level of glycolipid synthesis could be expected under these conditions than on medium 2. However, the previous data [1] demonstrated that the amount of carbohydrate SAS synthesized during the growth of *R. erythropolis* EK-1 on medium 1 was somewhat less than that on medium 2. Elucidation of the causes of this phenomenon will be the subject of our further work. Nevertheless, the results of enzymatic studies demonstrate the existence of potential reserves for the increase of SAS synthesis on medium 1.

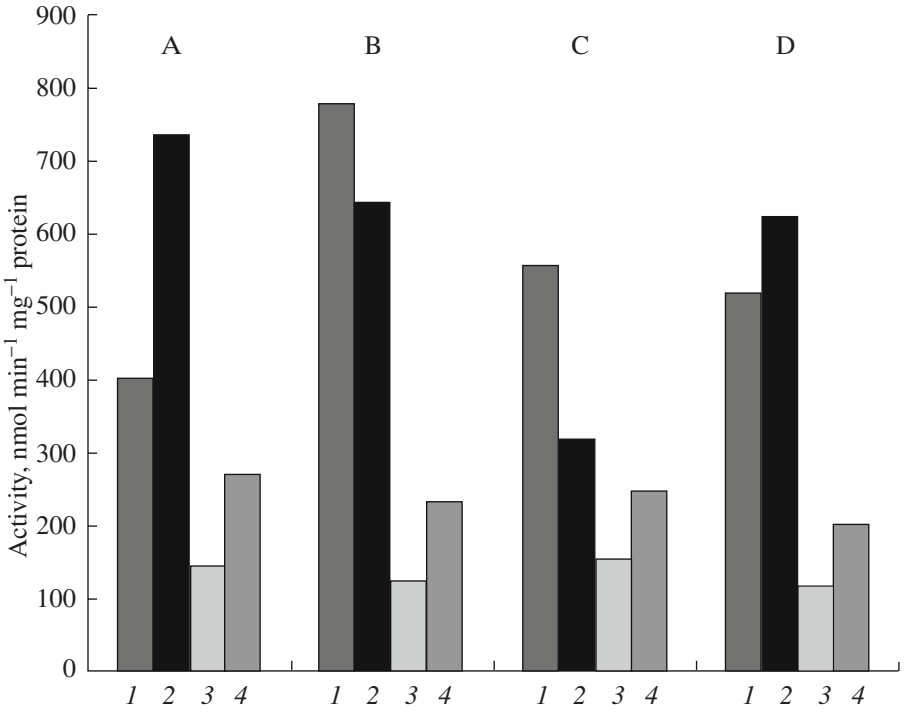
We believe that the differences in the activity of some enzymes at cultivation of *R. erythropolis* EK-1 on media 1 and 2 (see Tables 1 and 2) may be determined by the different mineral compositions of these media, in particular, different contents of potassium, sodium and ammonium cations, which are potential enzyme activators or inhibitors.

Mechanisms of intensification of SAS synthesis at cultivation of *R. erythropolis* EK-1 on ethanol in the presence of precursors. The SAS synthesized by *R. erythropolis* EK-1 in ethanol are a complex of glycolipids and neutral lipids with polysaccharide–protein compounds [1]. Hence, we have assumed that it is possible,similar to the case of cultivation on hexadecane [11], to increase the efficiency of SAS biosynthesis on C_2 substrates by an introduction into the medium of sodium citrate (the regulator of lipid synthesis) and C_4

Table 3. Formation of surface-active substances in the course of cultivation of *R. erythropolis* EK-1 on the medium with ethanol in the presence of biosynthetic precursors

Precursor	Indicators of synthesis		
	SAS*	SAS, g/l	E_{24},%
No precursors	3.0 ± 0.15	0.94 ± 0.04	60 ± 3.0
Citrate, 0.1%	3.0 ± 0.15	0.80 ± 0.03	90 ± 4.0
Fumarate, 0.2%	4.2 ± 0.21	1.4 ± 0.07	75 ± 3.0
Citrate, 0.1% + fumarate, 0.2%	4.8 ± 0.24	1.9 ± 0.09	80 ± 4.0

Note: Cultivation on medium 2; inoculum was grown on ethanol; fumarate and citrate were introduced in the beginning of the stationary growth phase.



Activity of the enzymes providing the involvement of exogenous fumarate and citrate in metabolism of *R. erythropolis* EK-1 grown on the medium with ethanol. Enzymes: *1*, $NADP^+$-dependent isocitrate dehydrogenase; *2*, isocitrate lyase; *3*, NAD^+-dependent malate dehydrogenase; *4*, fumarate hydratase. Cultivation conditions: A, without fumarate and citrate (control); B, citrate (0.1%); C, fumarate (0.2%); D, citrate (0.1%) + fumarate (0.2%). Cultivation was carried out on medium 2. The inoculum was grown on medium 2 with ethanol and respective precursors. The precursors were introduced in the beginning of cultivation. Enzyme activities were determined for cells from the middle of exponential growth phase.

dicarboxylic acids (fumarate), precursors of gluconeogenesis.

The experiments showed that simultaneous introduction of fumarate (0.2%) and citrate (0.1%) into medium 2 with ethanol in the beginning of the stationary phase of EK-1 growth resulted in an increase of the quantity of synthesized SAS (100%), index of emulsification (35%), and conventional SAS concentration (60%) as compared with the values for synthesis on the medium without fumarate and citrate (Table 3). Addition of fumarate and citrate (together or separately) in the beginning of *R. erythropolis* EK-1 cultivation was accompanied by an insignificant (10–15%) increase of the biomass level and a 15–25% decrease of SAS concentration, compared with the analogous values at cultivation on the medium with ethanol.

The analysis of the activities of the enzymes that involve fumarate and citrate in the metabolism of *R. erythropolis* EK-1 (figure) showed that introduction of citrate, fumarate, and citrate together with fumarate into the ethanol-containing medium in the beginning of the process resulted in an increase of isocitrate dehydrogenase activity (figure, *1*B, *1*C, and *1*D) and a decrease of isocitrate lyase activity (figure, *2*B, *2*C, and *2*D) as compared with cultivation on the medium without these precursors (figure, *1*A and *2*A, respectively). The activities of NAD^+-dependent malate dehydrogenase and fumarate hydratase were practically the same at cultivation on the medium with ethanol without fumarate and citrate and in the presence of these precursors (figure, *3*A–*3*D and *4*A–*4*D, respectively). In the above experiments, analogous media with the corresponding precursors were used to obtain inocula and for subsequent cultivation of strain EK-1.

The figure shows the enzyme activities for the cells in the middle of the exponential growth phase. Further experiments showed that the character for the changing of enzyme activities, in the presence of fumarate and citrate in the beginning of the stationary phase, was analogous to that presented in the figure. The results obtained, including an increase of isocitrate dehydrogenase activity and a decrease of isocitrate lyase activity in the presence of citrate and fumarate (as compared with cultivation of strain EK-1 on ethanol without precursors) may be evidence of a preferred direction for the carbon flow from substrates to the TCA cycle, rather than to the synthesis of SAS of glycolipid nature, under such cultivation conditions.

Later on, it was shown that a decrease of isocitrate dehydrogenase activity and an increase of isocitrate lyase activity were observed with the inoculum grown on ethanol in the absence of precursors, irrespective of the moment of fumarate and citrate introduction, as compared with cultivation of *R. erythropolis* EK-1 on

Table 4. Dependence of some enzyme activities on the moment of citrate and fumarate introduction into ethanol-containing cultivation medium for *R. erythropolis* EK-1

Moment of introduction of precursors	Precursors	Activity (nmol min^{-1} mg^{-1} protein)					
		$NADP^+$-dependent isocitrate dehydrogenase	Isocitrate lyase	Fumarate hydratase	NAD^+-dependent malate dehydrogenase	PEP carboxykinase	PEP synthetase
No precursors		632 ± 31	515 ± 25	256 ± 12	201 ± 10	603 ± 30	556 ± 27
In the beginning of cultivation	Citrate, 0.1%	417 ± 20	623 ± 31	96 ± 4.1	147 ± 7.3	238 ± 11	870 ± 43
	Fumarate, 0.2%	530 ± 26	735 ± 36	508 ± 25	300 ± 15	299 ± 14	1300 ± 65
	Citrate, 0.1% + fumarate, 0.2%	385 ± 19	598 ± 29	321 ± 16	255 ± 12	365 ± 18	1457 ± 72
In the end of the exponential growth phase	Citrate, 0.1%	368 ± 18	1063 ± 53	152 ± 7.6	159 ± 7.9	238 ± 11	1300 ± 65
	Fumarate, 0.2%	432 ± 21	840 ± 42	784 ± 39	354 ± 17	292 ± 14	1500 ± 75
	Citrate, 0.1% + fumarate, 0.2%	404 ± 20	717 ± 35	421 ± 21	297 ± 14	367 ± 18	1980 ± 99

Note: Inoculum was grown on ethanol; cultivation on medium 2; enzyme activities were determined for the cells from the early stationary growth phase (72 h).

ethanol (Table 4). The activities of fumarate hydratase and NAD^+-dependent malate dehydrogenase increased in the presence of fumarate, as well as fumarate and citrate. It is notable that the activities of isocitrate lyase, fumarate hydratase, and malate dehydrogenase increased more significantly, when the precursors were added in the end of the exponential growth phase, rather than in the beginning of the process and cultivation of the strain on the medium with ethanol without citrate and fumarate (Table 4).

At present, it is still unknown what exactly determines the different character of changes for the same enzymatic activities with the inocula grown on ethanol and on ethanol with the precursors (figure, Table 4). One may suppose a stimulating or inhibiting effect on some enzyme activities of sodium cations introduced into the medium together with the precursors in the case of inoculum preparation and EK-1 cultivation.

It was shown that PEP carboxykinase activity decreased 1.5–2 times and PEP synthetase activity increased more than three times in the presence of SAS synthesis precursors, as compared with cultivation of *R. erythropolis* EK-1 on the medium without fumarate and citrate (Table 4). The data of the enzymatic studies presented in Table 4 demonstrate the change in the direction of the metabolic processes toward biosynthesis, at cultivation of rhodococcus on ethanol, in the presence of fumarate and citrate.

We did not find data in the literature concerning enhanced SAS synthesis in the case of simultaneous presence of both citrate (regulator of lipid synthesis) and C_4-dicarboxylic acids (precursors of gluconeogenesis) in the medium. Besides, the mechanisms explaining an increase of the SAS level in the presence of biosynthetic precursors still remain virtually unstudied. It is known, that in the presence of citrate in the cultivation medium of *Bacillus subtilis,* an increase of surfactin synthesis correlates with a decrease of isocitrate dehydrogenase activity, which may indicate that the carbon flow from the substrate is directed to the pro-

cesses of constructive metabolism, i.e. to SAS synthesis [9]. At the same time, it has been shown for the yeast *Torulopsis apicola* that the mechanism of citrate action consists in pH maintenance on the level optimal for SAS synthesis as a result of citrate transport into the yeast cells via symport with proton [8]. Besides citrate, salts of other organic acids (succinate, tartrate, malonate) had the same effect.

Our studies showed that the increase of SAS synthesis rates in the presence of fumarate and citrate in ethanol-containing cultivation medium for *R. erythropolis* EK-1 resulted from the activation of gluconeogenesis and intensification of lipid synthesis, as demonstrated by an increase of isocitrate lyase and PEP synthetase activities 1.4–1.5 and 3.4–3.6 times, respectively, and by a decrease of isocitrate dehydrogenase activity 1.5–1.6 times.

The study of the peculiarities of C_2 metabolism in the strain *R. erythropolis* EK-1 is a basis for development of novel approaches to intensification of SAS biosynthesis and investigation of the potential applications of this strain for bioconversion and degradation of various xenobiotics.

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