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EXPERIMENTAL ARTICLES

Intensification of Biosurfactant Synthesis by *Acinetobacter calcoaceticus* IMV B-7241 on a Hexadecane–Glycerol Mixture

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Abstract—The possibility of enhanced biosurfactant (BS) synthesis by the cultivation of *Acinetobacter calcoaceticus* IMV B-7241 on a mixture of energetically nonequivalent substrates (hexadecane and glycerol) was shown. Based on theoretical calculations of the energy requirements for biomass production and the synthesis of surface-active trehalose monomycolate from the energy-deficient substrate (glycerol), the concentration of the energy-excessive substrate (hexadecane), which increased the efficiency of the substrate carbon conversion to BS, was determined. The synthesis of extracellular BS on a mixture of hexadecane and glycerol in a molar ratio of 1 : 7 at C/N ratio of 30 increased 2.6–3.5-fold compared to that on single-substrate media. Increased BS synthesis by *Acinetobacter calcoaceticus* IMV B-7241 grown on a hexadecane–glycerol mixture was accompanied by a 1.3–2.4-fold increase in activities of the enzymes involved in their biosynthesis, as well as by simultaneous functioning of two anaplerotic pathways (the glyoxylate cycle and the phosphoenolpyruvate carboxylase reaction).

Keywords: Acinetobacter calcoaceticus IMV B-7241, biosurfactants, intensification of biosynthesis, energetically nonequivalent substrates, mixed substrates

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Surface-active substances (surfactants, SAS) are widely applied in various industrial processes; therefore, demand for synthetic surfactants is constantly increased. At present, intensive development of biotechnology and the environmental safety requirements dictate great interest in microbial surfactants as an alternative to the chemical analogues [1].

Biosurfactants (BS) of microbial origin have some advantages over synthetic compounds, such as biodegradability, stability of properties at a wide range of pH and temperature, and nontoxicity.

Earlier, we showed that strain *Acinetobacter calcoaceticus* IMV B-7241 isolated from petroleum-contaminated soils produced low-molecular-weight surfactants when grown on hydrophobic (hexadecane and liquid paraffins) and hydrophilic (ethanol and glucose) substrates [2]. It should be noted that most representatives of the genus *Acinetobacter* synthesize high-molecular-weight BS which possess emulsifying but not surface-active properties [3, 4]. Chemically, these compounds are complexes of extracellular polysaccharides and proteins. Only recently, members of the genus *Acinetobacter* were reported to produce low-molecular-weight surfactants when grown on hydrophobic substrates [5]. We selected strain *A. calcoaceticus* IMV B-7241, which produced BS of unusual composition containing a complex of neutral, amino-, and glycolipids [2]; the latter were represented by trehalose mycolates typical of the genus *Rhodococcus* but not of *Acinetobacter* [4]. The ability of *A. calcoaceticus* IMV B-7241 to synthesize trehalose mycolates was also confirmed by enzymatic studies [6].

The organization of industrial BS production requires economic evaluation of this process. At present, the production cost of BS remains higher than that of their chemical analogues due to considerable expenditures for biosynthesis and extraction of the end product. Therefore, increasing efficiency of microbial surfactant production is a key aspect of the investigations [7]. One of the ways to solve this problem is application of industrial waste products as growth substrates [7, 8].

At present, glycerol, which is formed in large amounts as a by-product of biodiesel production, is considered a promising substrate for biotechnological processes [9, 10]. The production of 100 L of biodiesel resulted in formation of almost the same amount of crude glycerol. From 2004 to 2006, the market price of glycerol decreased more than tenfold. The known consumers of glycerol, the fragrance and cosmetic industries and the military-industrial establishment, are unable to utilize its supplies [9].

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The application of mixed substrates for the cultivation of producers is one of the methods for optimization of microbiological processes. Literature data and our studies showed that the use of a mixture of growth and nongrowth substrates increased both the growth of microorganisms and the synthesis of valuable metabolites, in particular, of the microbial exopolysaccharide ethapolan [11–14]. Such approach makes it possible to avoid the unproductive loss of carbon and energy occurring in the case of cell growth on monosubstrates and to increase the efficiency of carbon transformation from substrates into products.

The goal of the present work was to study the possibility of increasing BS synthesis by cultivation of *A. calcoaceticus* IMV B-7241 on a mixture of growth substrates (hexadecane and glycerol).

MATERIALS AND METHODS

Subject of research. The study was carried out with strain *Acinetobacter calcoaceticus* K-4 deposited in the Depositary of Microorganisms, Institute of Microbiology and Virology, National Academy of Sciences of Ukraine under the number IMV B-7241.

Medium composition and cultivation conditions for *A. calcoaceticus* IMV B-7241. Bacteria were grown in a modified [2] Munz liquid mineral medium of the following composition (g/L): NaCl, 1.0; $Na_2HPO_4 \cdot 12H_2O$, 0.6; $(NH_2)_2CO$, 0.35; KH_2PO_4, 0.14; $MgSO_4 \cdot 7H_2O$, 0.1; pH 6.8–7.0. In one of the variants, to maintain the appropriate C/N ratio, concentrations of $(NH_2)_2CO$ of 0.32, 0.39, 0.42, 0.45, and 0.63 g/L were used.

The medium was supplemented with yeast autolysate, 0.5% (vol/vol) and the trace element solution, 0.1% (vol/vol) [2].

Glycerol, (0.5–1.3%, vol/vol), *n*-hexadecane (0.5–1.25%, vol/vol), and a mixture of *n*-hexadecane (0.5%, vol/vol) and glycerol (0.3, 0.4, 0.5, 0.6, 0.7, and 0.8%, vol/vol) at a molar ratio of 1 : 3, 1 : 4, 1 : 5, 1 : 6, 1: 7, and 1 : 8, respectively, were used as the sources of carbon and energy.

The mid-exponential-phase culture (48 h) grown on the above-described medium was used as inoculum. The inoculum medium contained glycerol or *n*-hexadecane (both 0.5%), or a mixture of *n*-hexadecane (0.25%) and glycerol (0.25%) as the carbon and energy sources, and was supplemented with yeast autolysate and the trace element solution.

The amount of inoculum was 5% of the medium volume (10^4–10^5 cells/mL). Cultivation was performed in 750-mL flasks with 100 mL of the medium on a shaker (220 rpm) at 30°C for 24–120 h.

Determination of the BS synthesis indices. The capability for BS synthesis was assessed by the following indices: surface tension (σ_s) of the cell-free culture liquid, conditional concentration of BS (BS*, dimensionless unit), index of emulsification of the culture liquid (E_{24}, %), and the quantity of extracellular BS (g/L) determined as described earlier [2, 15].

Obtaining of the cell-free extracts. The cells of *A. calcoaceticus* IMV B-7241 grown in liquid mineral medium were collected by centrifugation (4000 *g*, 15 min, 4°C), washed twice from the medium with 0.05 M K^+-phosphate buffer (pH 7.0), and centrifuged (4000 *g*, 15 min, 4°C). The washed cells were resuspended in 0.05 M K^+-phosphate buffer (pH 7.0) and sonicated (22 kHz) three times for 40–60 s at 4°C on an UZDN-1 apparatus. The resulting liquid was centrifuged (12000 *g*, 30 min, 4°C), the pellet was removed, and the supernatant was used as a cell-free extract.

Enzymatic analyses. The activity of NAD^+-dependent glycerol dehydrogenase (EC 1.1.1.6) [16] was determined spectrophotometrically by NAD^+ reduction at 340 nm with glycerol as an electron donor.

The activities of pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (EC 1.1.2.8) [17] and PQQ-dependent glycerol dehydrogenase (EC 1.1.99.22) [18] were assayed by dichlorophenolindophenol reduction in the presence of phenazine methosulfate at 600 nm with glycerol as an electron donor.

The activities of dihydroxyacetone kinase (EC 2.7.1.29) [19] and glycerol kinase (EC 2.7.1.30) [19, 20] were determined by formation of dihydroxyacetone phosphate and glycerol-3-phosphate, respectively, which were analyzed spectrophotometrically by oxidation (reduction) of NADH (NAD^+) in a coupled reaction with glycerol-3-phosphate dehydrogenase.

The activity of flavin adenine dinucleotide (FAD)-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.5.3) [21] was determined by reduction of 3-(4,5-dimethylthiazolyl-2-)2,5-diphenyltetrazolium bromide in the presence of phenazine methosulfate at 570 nm with glycerol-3-phosphate as an electron donor.

The activity of NAD^+-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) [22] was analyzed spectrophotometrically by NADH oxidation at 340 nm with dihydroxyacetone phosphate as an electron donor.

The activities of alcohol dehydrogenase (EC 1.1.99.36), isocitrate lyase (EC 4.1.3.1), glutamate dehydrogenase (EC 1.4.1.2), phosphoenolpyruvate (PEP) synthase (EC 2.7.9.2), PEP carboxykinase (EC 4.1.1.49), and PEP carboxylase (EC 4.1.1.31) were determined as described earlier [6, 15].

Protein content in the cell-free extracts was determined according to Bradford [23]. Enzyme activities were determined at 28–30°C, the temperature optimal for growth of *A. calcoaceticus* IMV B-7241.

All the experiments were carried out in three replicates with three to five parallel measurements in each experiment. The data were statistically processed as

Table 1. Activities of the enzymes involved in glycerol metabolism in *A. calcoaceticus* IMV B-7241

Enzymes	Activity (nmol min^{-1} mg^{-1} of protein) in cell-free extracts obtained from the cells of the:	
	early exponential phase	mid-exponential phase
Dihydroxyacetone pathway		
NAD^+-dependent glycerol dehydrogenase (EC 1.1.1.6)	0	0
PQQ-dependent glycerol dehydrogenase (EC 1.1.99.22)	149 ± 7	107 ± 5
NDMA-dependent alcohol dehydrogenase (EC 1.1.99.36)	101 ± 5	32 ± 1.6
PQQ-dependent alcohol dehydrogenase (EC 1.1.2.8)	0	0
Dihydroxyacetone kinase (EC 2.7.1.29)	750 ± 3	336 ± 16
Glycerol-3-phosphate pathway		
Glycerol kinase (EC 2.7.1.30)	ND	780 ± 39
NAD^+-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.1.8)	373 ± 18	159 ± 8
FAD^+-dependent glycerol-3-phosphate dehydrogenase (1.1.5.3)	0	0

Note: ND stands for "not determined".

described in [24]. Differences between the mean values were considered to be reliable at a significance level $p < 0.05$.

RESULTS AND DISCUSSION

Since both bacterial growth and the product synthesis on mixed substrates were shown to depend on the inoculum quality [2], in the first series of experiments, the effect of carbon source in the inoculum medium on the BS synthesis by *A. calcoaceticus* IMV B-7241 was studied. It was revealed that the value of conditional BS concentration observed in bacteria grown on a mixture of hexadecane and glycerol was maximal (4.0 ± 0.2) when the inoculum was grown on hexadecane and decreased in the case of inoculum cultivation on glycerol or on mixed substrates (2.7 ± 0.13 and 2.2 ± 0.11, respectively).

In further experiments, inocula were grown on hexadecane.

Glycerol metabolism in *A. calcoaceticus* IMV B-7241. To achieve the maximum conversion of the substrate carbon to the end product for microorganisms cultivated on mixed substrates, it is essential to determine the optimal molar ratios of the concentrations of monosubstrates in the mixture [11–14, 25]. For this purpose, it was necessary to make theoretical calculations of the energy required for BS synthesis and biomass formation on the energy-deficient substrate and to determine the concentration of the energy-excessive substrate which supplies energy for these processes, as had been determined earlier in our studies on the production of the microbial polysaccharide ethapolan [11–14]. To make such calculations, it was necessary to know the pathways of metabolism of the relevant monosubstrates in BS producers. It is known that *n*-hexadecane is always an energy-excessive substrate, whereas glycerol is an energy-deficient one independent of the pathways of their metabolism in various microorganisms [25].

Two pathways of glycerol assimilation are known in microorganisms utilizing it as the carbon and energy source [26, 27]. The first route starts with ATP-dependent phosphorylation of glycerol catalyzed by glycerol kinase to form glycerol 3-phosphate, which is subsequently oxidized to dihydroxyacetone phosphate by glycerol 3-phosphate dehydrogenase or glycerol-3-phosphate oxidase (the glycerol 3-phosphate pathway). Dihydroxyacetone phosphate is an intermediate of glycolysis, and is subsequently metabolized via this route. In the second pathway, glycerol is initially oxidized to dihydroxyacetone by glycerol dehydrogenases (NAD^+- or PQQ-dependent), which is then phosphorylated to dihydroxyacetone phosphate by dihydroxyacetone kinase (the dihydroxyacetone pathway).

Our studies showed that in *A. calcoaceticus* IMV B-7241, catabolism of glycerol to dihydroxyacetone phosphate (intermediate of glycolysis) could proceed via both the glycerol-3-phosphate pathway (activity of glycerol kinase was 740–820 nmol min^{-1} mg^{-1} of protein) and the dihydroxyacetone pathway (Table 1). Glycerol oxidation to dihydroxyacetone in strain IMV B-7241 was catalyzed by both PQQ-dependent glyc-

erol dehydrogenase and NDMA-dependent alcohol dehydrogenase (Table 1).

Calculation of hexadecane–glycerol ratios in the medium for *A. calcoaceticus* IMV B-7241 cultivation. When calculating the optimum ratio of hexadecane and glycerol concentrations, we made the following assumptions: (1) trehalose monomycolates are the major components of BS; (2) hexadecane is predominantly used as a source of energy, whereas glycerol carbon is used for the synthesis of biomass and trehalose mycolates; (3) glycerol catabolism proceeds via formation of dihydroxyacetone phosphate with the involvement of glycerol kinase; (4) the mycolic acid in trehalose mycolates is 3-hydroxy-2-dodecanoyldocosanoic acid containing 34 carbon atoms (similar to that in the trehalose lipids of *Rhodococcus erythropolis*) [4]; and (5) the P/O ratio is 2.

The tentative scheme of trehalose monomycolate synthesis from glycerol (figure) is based on the results of this work (Table 1) and previous studies on metabolism of noncarbohydrate substrates in strain IMN B-7241 [6].

Energy expenditures for the synthesis of trehalose phosphate. As can be seen from the scheme, the synthesis of 1 mol trehalose phosphate requires 8 mol of glycerol (4 mol for the glyoxylate production and 4 mol for the synthesis of acetyl-CoA which interacts with glyoxylate to produce malate). Thus, 8 mol ATP are necessary for production of glycerol 3-phosphate from glycerol; 8 mol, for the formation of PEP from pyruvate; 4 mol, for the synthesis of 1,3-diphosphoglycerol from phosphoglyceric acid (PGA); and 8 mol (4 NADH), for the transformation of 1,3-diphosphoglycerol to triosephosphate. Therefore, the energy expenditures amount to 28 mol of ATP. Moreover, one mol ATP is required for the formation of nucleoside-diphosphate-sugar (glucose-6-phosphate → UDP-glucose), which is necessary for the synthesis of trehalose-6-phosphate. Therefore, the overall energy expenditures for the synthesis of trehalose-6-phosphate from glycerol amount to 30 mol ATP.

Energy expenditures for the synthesis of mycolic acid. Taking into account the pathway of fatty acid biosynthesis from acetyl-CoA described earlier [12], it can be calculated that the formation of 3-hydroxy-2-dodecanoyldocosanoic acid containing 34 carbon atoms requires 17 mol of acetyl-CoA, which requires 17 mol ATP for synthesis from glycerol. Given that the mycolic acid synthesis from acetyl-CoA proceeds in 16 cycles, the overall energy expenditures amount to 16 + 17 = 33 mol ATP.

ATP generation in the synthesis of trehalose monomycolate from glycerol. Energy is generated in the acetyl-CoA synthesis as follows:

$$\text{Glycerol} \rightarrow \text{Acetyl-CoA} + \text{ATP} + 2\text{NADH}. \quad (1)$$

Since the synthesis of mycolic acid and trehalose phosphate requires 17 and 8 mol of acetyl-CoA, respectively, equation (1) can be represented as follows:

$$25\ \text{Glycerol} \rightarrow 25\text{Acetyl-CoA} + 25\text{ATP} + 50\text{NADH}. \quad (2)$$

Given that the P/O ratio is 2, it follows from equation (2) that the synthesis of trehalose monomycolate from glycerol generates 25 + 100 = 125 mol ATP or 5 mol ATP per mol glycerol used. The overall energy expenditure for the synthesis of trehalose phosphate and mycolic acid from glycerol is 30 + 33 = 63 mol ATP or 2.52 mol ATP per mol glycerol utilized.

Therefore, energy generation during trehalose monomycolate synthesis is 5 – 2.52 = 2.48 mol ATP/mol glycerol.

Energy expenditures for biomass synthesis. Biomass production from phosphoglyceric acid, a key intermediate in the synthesis of all cellular components, can be represented as follows [25]:

$$4\text{PGA} + \text{NH}_3 + 29\text{ATP} + 5.5\text{NAD(P)H} \rightarrow (\text{C}_4\text{H}_8\text{O}_2\text{N})_3, \quad (3)$$

where $(C_4H_8O_2N)_3$ is the formula of mol biomass.

The overall reactions of conversion of glycerol and hexadecane to PGA are expressed by the following equations:

$$\text{Glycerol} \rightarrow \text{PGA} + \text{NAD(P)H}. \quad (4)$$

$$\text{Hexadecane} + 5\text{ATP} \rightarrow 4\text{PGA} + 16\text{NAD(P)H} + 11\text{FADH}_2 + \text{CO}_2. \quad (5)$$

For P/O equal to 2, equations (4) and (5) can be represented as follows:

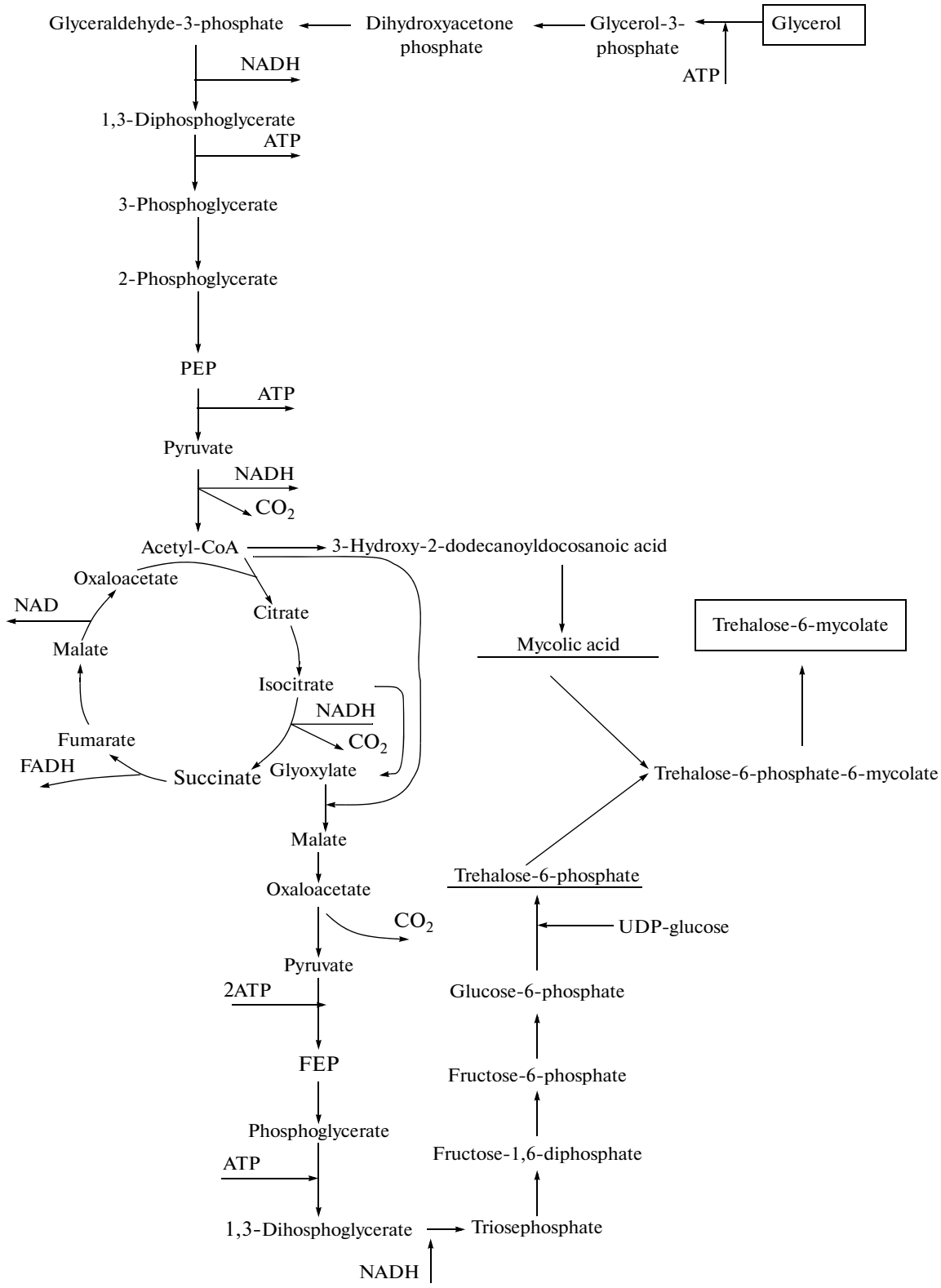
$$\text{Glycerol} \rightarrow \text{PGA} + 2\text{ATP}, \quad (6)$$

$$\text{Hexadecane} \rightarrow 4\text{PGA} + 38\text{ATP}. \quad (7)$$

Proceeding from the equation (3) of biomass synthesis from PGA and the equation (6) of glycerol catabolism to PGA, it may be calculated that biomass synthesis requires 8 mol ATP per mol glycerol. In our opinion, this energy can be obtained from hexadecane. Taking unto account that trehalose monomycolate synthesis from glycerol generates 2.48 mol ATP per mol glycerol used, 8 – 2.48 = 5.52 mol ATP should be generated at the expense of hexadecane. It follows from (7) that 0.145 mol hexadecane is required for obtaining such an amount of ATP. Hence, the molar hexadecane/glycerol ratio in the medium should be 0.145 : 1 or 1 : 6.9.

Effect of the molar ratio of hexadecane and glycerol concentrations on BS synthesis. The data on the BS synthesis by *A. calcoaceticus* IMV B-7241 at different molar ratios of hexadecane and glycerol concentrations are presented in Table 2. The maximum values of the conditional BS concentration (4.8) and the emulsification index (55%) were observed at the theoretically calculated molar ratios of the monosubstrates.

Effect of the C/N ratio on BS synthesis by *A. calcoaceticus* IMV B-7241 grown on a mixture of hexadecane and glycerol. Since C/N ratios varied with changes in the molar ratios of substrate concentra-



Tentative scheme of trehalose monomycolate synthesis from glycerol in *A. calcoaceticus* IMV B-7241.

Table 2. BS production by *A. calcoaceticus* IMV B-7241 depending on the molar ratio of hexadecane and glycerol concentrations

Molar ratio of hexadecane and glycerol in the mixture	C/N ratio	BS*	Emulsification index, %
1 : 3	24	2.8 ± 0.14	46 ± 2.3
	30	3.0 ± 0.15	43 ± 2.1
1 : 4	27	3.8 ± 0.18	40 ± 2.0
	30	3.9 ± 0.19	45 ± 2.2
1 : 5	30	4.0 ± 0.20	47 ± 2.4
1 : 6	33	4.2 ± 0.21	43 ± 2.1
	30	4.2 ± 0.21	43 ± 2.1
1 : 7	36	4.3 ± 0.21	52 ± 2.6
	30	4.8 ± 0.24	55 ± 2.7
1 : 8	39	4.1 ± 0.20	42 ± 2.1
	30	4.2 ± 0.21	43 ± 2.1

Table 3. Effect of the C/N ratio in the hexadecane–glycerol medium on the synthesis of extracellular BS by *A. calcoaceticus* IMV B-7241

C/N ratio	BS, g/L	BS (g/L), % of concentration obtained on the monosubstrate	
		glycerol	hexadecane
20	1.7 ± 0.08	170 ± 8	150 ± 7
30	2.5 ± 0.12	350 ± 17	265 ± 13
40	2.3 ± 0.11	288 ± 14	200 ± 10

Note: In Tables 3 and 4, the molar ratio of hexadecane to glycerol was 1 : 7.

tions, in this series of experiments, we maintained C/N ratio at a constant level of 30; under these conditions, the maximum BS synthesis by *A. calcoaceticus* IMV B-7241 occurred at the molar ratio of hexadecane and glycerol concentrations of 1 : 7 (Table 2).

To confirm the dependence of BS synthesis by *A. calcoaceticus* IMV B-7241 both on the C/N ratio and on the molar ratio of substrate concentrations, we varied C/N ratios in the media maintaining the molar ratio of hexadecane and glycerol concentrations at a level of 1 : 7 (Table 3). The concentration of extracellular BS (g/L), which was determined gravimetrically after extraction with organic solvents, was taken as an index of BS synthesis. This index was chosen since the graph of the surface tension dependence on the logarithm of dilution of the cell-free culture liquid of strain IMV B-7241 differed from those for other BS producers by the presence of several peaks of surface tension, so that it was therefore impossible to correctly determine the conditional BS concentration (BS*).

As may be seen from Table 3, the C/N ratio of 30 was optimal for BS synthesis by *A. calcoaceticus* IMV B-7241: the amount of BS reached 350 and 265% of the levels obtained on glycerol and hexadecane, respectively.

Mechanisms of enhancing BS synthesis in *A. calcoaceticus* IMV B-7241 grown on a mixture of hexadecane and glycerol. To determine the mechanisms responsible for intensified BS synthesis, we analyzed activities of the enzymes involved in anaplerotic pathways (PEP carboxylase and isocitrate lyase), biosynthesis of surface-active glycolipids (PEP carboxykinase and PEP synthetase), and formation of aminolipids ($NADP^+$-dependent glutamate dehydrogenase) on mono- and mixed substrates (Table 4). The activity of PEP carboxylase, an enzyme filling up the C_4-dicarboxylic acid pool in the carbohydrate-growing microorganisms, was rather high. Earlier, we discussed the physiological role of this enzyme in the studies on cul-

Table 4. Activities of the enzymes involved in anaplerotic pathways and BS biosynthesis in *A. calcoaceticus* IMV B-7241 grown on mono- and mixed substrates

Substrate concentration, %	Activity (nmol min^{-1} mg^{-1} of protein)				
	isocitrate lyase	$NADP^+$-dependent glutamate dehydrogenase	PEP carboxylase	PEP carboxykinase	PEP synthetase
Glycerol, 1.20	45 ± 2	322 ± 16	1608 ± 80	448 ± 22	2894 ± 144
Hexadecane, 1.15	0	620 ± 31	2571 ± 129	667 ± 33	3456 ± 172
Hexadecane, 0.5 + glycerol, 0.7	494 ± 24	769 ± 38	1724 ± 86	923 ± 46	4483 ± 224

Note: Enzyme activities were determined in cell-free extracts of the mid-exponential-phase cells (48 h).

tivation of strain IMV B-7241on a medium with ethanol and urea; it was shown that PEP carboxylase was involved in detoxification of CO_2 produced in urease reaction that was accompanied by increasing the C_4-dicarboxylic acid pool, stimulating gluconeogenesis, and enhancing synthesis of surface-active glycolipids [31].

It should be mentioned that activities of all the studied enzymes (except for PEP carboxylase) were higher on a mixture of hexadecane and glycerol than on monosubstrates (Table 4). At the same time, both anaplerotic pathways operated on mixed substrates, and activity of isocitrate lyase was tenfold higher than on the monosubstrate glycerol. As may be seen from Table 4, when *A. calcoaceticus* IMV B-7241 was grown on a hexadecane–glycerol mixture in a ratio of 1 : 7, enhanced synthesis not only of the surface-active glycolipids (which was expected on the basis of theoretical calculations), but also of aminolipids was observed.

There is information concerning BS synthesis on a mixture of the so-called "primary" (basic) and "secondary" (supplementary) carbon sources [28–30]. The supplementary substrates used for the production of surface-active glycolipids were often carbohydrates, which were added into the medium together with hydrophobic compounds (usually plant oils) both at the beginning of cultivation and at different phases of the producer growth (mostly in the stationary phase).

In particular, addition of glucose (4%) into the medium for cultivating the producer of mannosylerythritol lipids increased BS synthesis by 50% as compared with cultivation on safflower oil or glycerol [30]. The concentration of the primary growth substrate was the same as that of glucose (4%). Addition of glucose into the medium with rapeseed oil resulted in a 1.8-fold increase in sophorolipid synthesis by *Candida bombicola* ATCC 22214 [29]. It was shown that the maximum synthesis of sophorolipids by the strain *C. bombicola* NRRL Y-17069 (33 g/L) was in the medium containing (g/L): deproteinized wheat, 90; glucose, 10; and oleic acid, 100. When oleic acid was replaced by soybean oil or olive oil, the BS synthesis decreased to 5.6–6.2 g/L [28].

In some publications [30], additional substrates are called "precursors of biosynthesis," which is an incorrect statement since the concentrations of primary (oil) and secondary (carbohydrates) carbon sources were similar and rather high (4%). Mannose and erythritol, however, may indeed act as precursors of surface-active mannosylerythritol lipids. In some studies [28–30], monosubstrates and their concentrations in the mixture were established empirically; it should be noted that monosubstrate concentrations were extremely high (100–200 g/L), which resulted in a rather low level of substrate conversion into BS (10–15%).

The results of the present work confirm our previous findings [11–14] that the application of a mixture of energetically nonequivalent substrates is a promising way to increase the synthesis of secondary metabolites; high efficiency of substrate mixtures may be achieved in the case of a correct choice of monosubstrates and determination of the molar ratios of their concentrations.

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