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EXPERIMENTAL ARTICLES

Intensification of Exopolysaccharide Synthesis by *Acinetobacter* sp. on an Ethanol–Glucose Mixture: Aspects Related to Biochemistry and Bioenergetics

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Abstract—The possibility of intensifying the synthesis of microbial exopolysaccharides (EPS) by a strain of *Acinetobacter* sp. grown on a mixture of two substrates nonequivalent in terms of bioenergetics (ethanol + glucose) was shown. Based on theoretical calculations of the energy requirements for biomass and EPS synthesis from the energy-deficient substrate (glucose), the supplementary concentration of the energy-excessive substrate (ethanol) was determined that prevents the loss of glucose carbon that occurs when glucose is oxidized to CO_2 to obtain energy for the processes of constructive metabolism. This made it possible to increase the efficiency of conversion of the substrate carbon to EPS. The introduction of ethanol into glucose-containing medium at a molar ratio of 3.1 : 1 allowed the amount of the EPS synthesized to be increased 1.8- to 1.9-fold; their yield relative to biomass increased 1.4- to 1.7-fold, and the yield of EPS relative to the substrate consumed increased 1.5- to 2-fold as compared to growth of the producer on single substrates. These results form the basis for the development of new technologies for obtaining secondary metabolites of practical value with the use of mixed growth substrates.

Key words: exopolysaccharides, nonequivalent substrates, mixed substrates, energy requirements, intensification of synthesis.

Today, when the biosynthesis of products valuable for practical purposes is being realized on a large-scale basis, the problems of using inexpensive raw material, the economy of carbon substrates, reduction of energy expenditures (ATP and reducing equivalents) for the process of microbial synthesis, and more complete transformation of carbon substrates into valuable metabolites are becoming especially topical. The solution of these problems requires profound knowledge of the physiology, energetics, and biochemistry of producer microorganisms. The development of the principles of regulation of energy and constructive metabolism on the basis of this knowledge will allow biotechnological processes to be realized with maximum efficiency.

In natural habitats, microorganisms develop, as a rule, in the presence of several carbon substrates, whereas under laboratory conditions, single substrates are often used as the source of carbon and energy for their cultivation. A considerable part of the growth substrate is known to be oxidized to CO_2 to obtain the energy required for constructive metabolism (e.g., up to 40% in the case of glucose) [1]. However, there are works in which the ability of microorganisms to utilize mixtures of two (or more) substrates was shown, and some aspects of the regulation of such processes were investigated [2–5]. However, in these investigations, substrate mixtures were used only to increase the biomass yield. No data have been published on the possibility of obtaining secondary metabolites, microbial exopolysaccharides (EPS) in particular, on mixtures of growth substrates.

In our opinion, the auxiliary substrate concept proposed by Babel [6] in a work concerned with increasing biomass yield may serve as the theoretical basis of research aimed at increasing the synthesis of secondary metabolites on a mixture of several substrates. According to this concept, all growth substrates are divided into energy-excessive and energy-deficient [7]. Phosphoglyceric acid (PGA) is the key intermediate of the synthesis of all cellular components. If the amount of the ATP and reducing equivalents formed in the pathway from a substrate to PGA is sufficient for biomass synthesis, such a substrate is classified as energy-excessive. The substrates, a part of which has to be oxidized to CO_2 for obtaining the energy required for the synthesis of cellular components, are energy-deficient. Knowing the metabolic pathways of conversion of a carbon substrate to the key intermediate of biomass synthesis (PGA) will make it possible to determine the energy value of the substrate.

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It was shown that the combination of substrates nonequivalent in terms of bioenergetics (the introduction of an auxiliary energy-excessive substrate into the medium with an energy-deficient substrate) makes it possible (1) to avoid the unproductive losses of carbon and energy that occur when a single substrate is used and (2) to increase the efficiency of transformation of substrates to biomass [2–7]. In our opinion, such an approach can be used not only to increase the biomass yield but also to intensify the synthesis of microbial EPS. Verification of this supposition was the main aim of this investigation.

MATERIALS AND METHODS

The subjects of study. The subject of this study was strain *Acinetobacter* sp. 12S, a producer of the complex polysaccharide preparation ethapolan [8, 9]. Acylated polysaccharides (AP) and nonacylated polysaccharides (NAP) are identical in the molar ratio of D-glucose, D-mannose, D-galactose, L-rhamnose, D-glucuronic acid, and pyruvic acid (PA) (3: 2 : 1 : 1 : 1 : 1) and in the structure of the repeating carbon chain link. The difference between these EPS consists in that the acylated polysaccharide contains fatty acids (C_{12}–C_{18}): lauric, palmitic, palmitoleic, stearic, and oleic. The rheological properties of ethapolan solutions determining their practical significance depend on the AP to NAP ratio, as well as on the content of fatty acids in AP. Depending on the cultivation conditions of the producer, the ethapolan content of AP varies from 10 to 90% [9].

Cultivation of *Acinetobacter* sp. 12S. The bacterium was grown in Kodama liquid mineral medium [10] supplemented with 0.5 vol % yeast autolysate and 0.0006% calcium pantothenate. Ethanol at concentrations of 1.0 and 1.5 vol % and glucose at concentrations of 1.0 and 1.5%, as well as mixtures of these substrates at ratios of 1 : 2, 1 : 1, and 2 : 1 (vol % ethanol/% glucose), were used as the sources of carbon and energy. When the bacteria were grown on mixed substrate, the ethanol concentration was 0.5 or 0.75 vol % and the glucose concentration was 0.5 or 0.75%. The pH of the medium was 6.8 to 7.0.

Cultivation was performed in flasks on a shaker (220 rpm) at 30°C for 96 h. A 24-h culture grown on nutrient agar (NA) was used as the inoculum.

The biomass concentration was determined by measuring the optical density of the cell suspension and converting it to dry cell weight with the use of a calibration curve. The amount of EPS synthesized was determined by the gravimetric method [11]. The yield of EPS per substrate consumed was determined as described by Pirt [12]. The capacity for EPS synthesis was determined as the ratio of the amount of EPS synthesized to biomass and expressed in g EPS/g biomass.

The ethanol concentration was determined using a Tsvet-4 gas–liquid chromatograph equipped with a flame-ionization detector and a 2-m column packed with the Celit-545 solid carrier. The immobile liquid phase was polyethylene glycol PEG-400 (20%); the carrier gas was helium; the temperature was 150°C. The glucose concentration in the culture liquid was determined by the glucose oxidase method [13].

Determination of the energy expenditures for the synthesis of biomass and EPS. The energy expenditure for biomass formation was calculated as described in [7]. The energy requirements for EPS synthesis were determined as described in [14].

RESULTS AND DISCUSSION

Calculation of the ethanol/glucose concentration ratio for cultivation of growing *Acinetobacter* sp. 12S on their mixture. It was earlier shown that the oxidation of ethanol and acetaldehyde in *Acinetobacter* sp. is catalyzed by NAD^+- and $NADP^+$-dependent enzymes [15], and glucose catabolism is accomplished via the Embden–Meyerhof–Parnas pathway (glycolysis) and the Entner–Doudoroff (KDPG) pathway [16]. According to Babel's classification of substrates [7], ethanol is an energy-excessive substrate, whereas glucose is an energy-deficient substrate.

To determine the ethanol to glucose concentration ratios in the cultivation medium, it was necessary (1) to calculate the energy requirements for the biomass and EPS syntheses from the energy-deficient substrate and (2) to determine the concentration of the energy-excessive substrate that can prevent the loss of carbon of the energy-deficient substrate that occurs when it is oxidized to CO_2 to obtain the energy required for constructive metabolism.

To make such calculations, it is necessary to know the pathways of ethanol and glucose metabolism in *Acinnetobacter* sp. 12S and the structure of the repeating unit of the EPS carbohydrate chain (hereinafter, the EPS unit), as well as the P/O value.

When calculating the ethanol to glucose concentration ratio optimal for growth of *Acinetobacter* sp. 12S on a mixture of these substrates, we made the following assumptions: (1) ethanol is predominantly used as a source of energy, and the glucose carbon is used for the synthesis of biomass and EPS; (2) 50% glucose is catabolized via the Embden–Meyerhof–Parnas pathway and 50% is catabolized via the Entner–Doudoroff pathway; (3) the P/O value is equal to 2; (4) the EPS contains 50% AP and 50% NAP; and (5) the repeating AP unit contains two fatty acid residues. This assumption was made because we failed to elucidate the exact site of O-acylation when studying the structure of the repeating unit of the AP carbohydrate chain [9]. However, the resistance of glucose and galactose to the degradation according to Smith led us to suggest that at least these two monosaccharide residues are acylated. When performing calculations, we assumed that the AP unit contains residues of lauric ($C_{12}H_{24}O_2$) and palmitic ($C_{16}H_{32}O_2$) acids. NADPH formed in the catabolism of

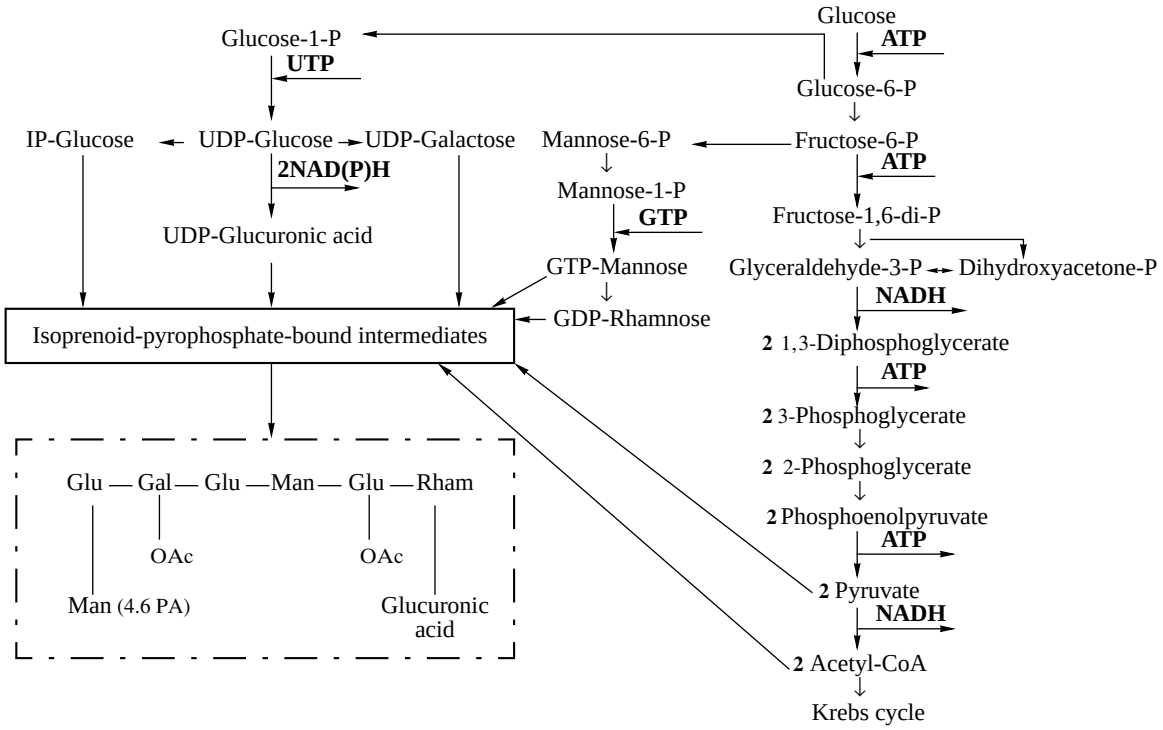

Fig. 1. Supposed scheme of the synthesis of the repeating unit of acylated polysaccharide by *Acinetobacter* sp. 12S via the glycolytic pathway of glucose catabolism. UDP, uridine diphosphate; GDP, guanosine diphosphate; IP, isoprenoid pyrophosphate; Glu, glucose; Gal, galactose; Man, mannose; Rham, rhamnose; Ac, acyl.

ethanol and glucose is a source of reducing equivalents, which are oxidized to water via the respiratory chain.

ATP requirement for ethapolan synthesis from glucose. Despite sufficiently intense studies in the field of microbial EPS, few works have been devoted to the study of the energy requirements of their syntheses. These works concerned the energetics of the syntheses of alginate [17] and xanthine [14] and of the synthesis of EPS synthesis from methanol [18].

Figures 1 and 2 show the supposed (by analogy with the scheme of xanthine synthesis proposed in [14]) schemes of the synthesis of the repeating unit of acylated polysaccharide in *Acinetobacter* sp, coupled to the glycolytic and the KDPG pathways of glucose catabolism, respectively. The ATP formed in these pathways is used for the synthesis of monosaccharides and fatty acids that are components of the AP unit.

1. Energy expenditure for the synthesis of monosaccharides. As follows from the schemes presented in Figs. 1 and 2, one mole of ATP (or its equivalent) is used for the formation of glucose-6-phosphate and one mole is used for the formation of nucleoside-diphosphate-saccharides (for example, in the reactions glucose-1-phosphate $\longrightarrow$ UDP-glucose or mannose-1-phosphate $\longrightarrow$ GDP-mannose). The energy of one more macroergic bond is used for adding the repeating unit to the EPS molecule in the process of EPS polymerization [14]. The AP unit includes residues of seven neutral monosaccharides and a residue of glucuronic acid. Their synthesis requires eight molecules of glucose. The synthesis of one monosaccharide component of the AP unit from glucose requires two molecules of ATP. The total ATP expenditure for the synthesis of the monosaccharide components of the repeating AP unit and for the addition of this unit to the EPS molecule constitutes $8 \times 2 + 1 = 17$ mol ATP (irrespective of whether the glycolytic or KDPG pathway of glucose catabolism is operative).

2. Energy expenditure for the synthesis of fatty acids from acetyl-CoA. The synthesis of fatty acids is accomplished in the following way [1]: malonyl-CoA is formed from acetyl-CoA and CO_2 by means of an ATP-dependent reaction, and then, as a result of three successive reactions, butyryl-CoA is formed, which interacts with a successive malonyl-CoA molecule with the formation of CH_3–$(CH_2)_4$-CO-SCoA. In the next turn of the cycle, CH_3–$(CH_2)_6$-CO-SCoA is formed. Thus, by sequential accretion of acetyl-CoA by a two-carbon fragment (by means of interaction with malonyl-CoA and subsequent loss of CO_2), higher fatty acids are synthesized in the form of their CoA derivatives. Consequently, the synthesis of lauric acid (C_{12}) requires five turns of the cycle and the formation of palmitic acid (C_{16}) requires seven turns of the cycle. One mole of ATP is used at each turn of the cycle. Thus, the energy expenditure for the synthesis of the component fatty acids of the repeating AP unit constitutes $5 + 7 = 12$ mol

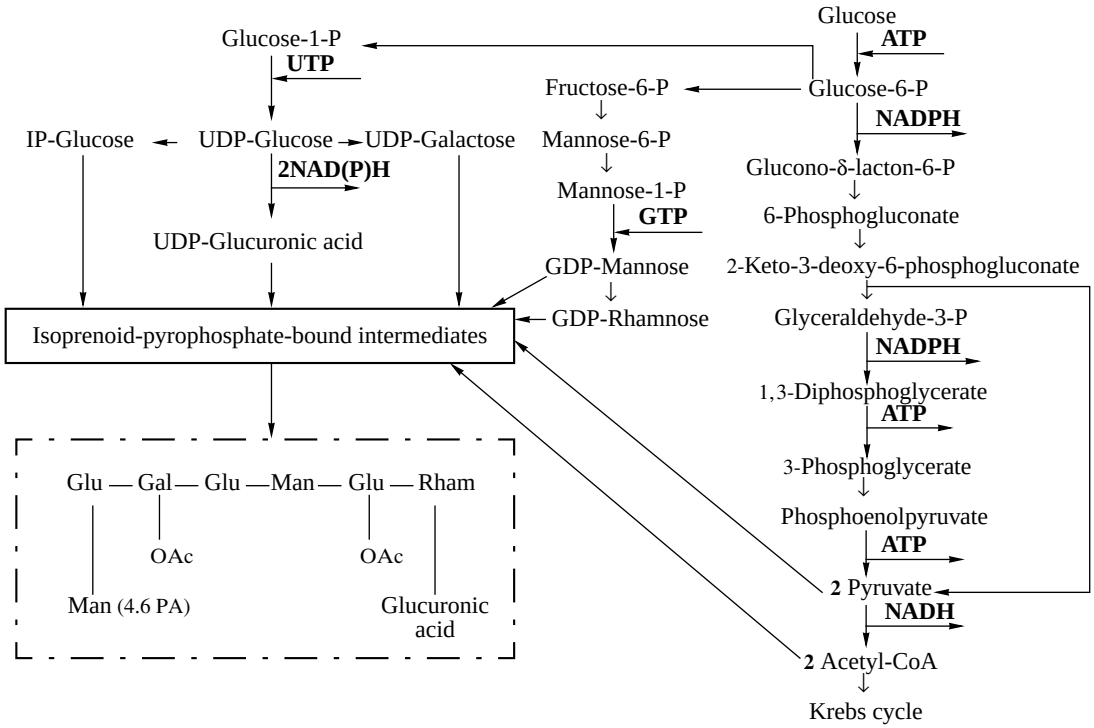

Fig. 2. Supposed scheme of the synthesis of the repeating unit of acylated polysaccharide by *Acinetobacter* sp. 12S via the KDPG pathway of glucose catabolism. UP, uridine diphosphate; GDP, guanosine diphosphate; IDP, isoprenoid pyrophosphate; Glu, glucose; Gal, galactose; Man, mannose; Rham, rhamnose; Ac, acyl.

ATP (irrespective of whether the glycolytic or the KDPG pathways of glucose catabolism is operative).

Energy generation in the ethapolan synthesis from glucose. Energy is generated in the synthesis of PA, glucuronic acid, and acetyl-CoA (the precursor of fatty acids).

1. PA synthesis. The overall reaction of PA formation from glucose can be represented in the form of the following equations:

$$\text{Glucose} \longrightarrow 2\text{PA} + 2\text{ATP} + 2\text{NADH (glycolysis)}, \quad (1)$$

$$\text{Glucose} \longrightarrow 2\text{PA} + \text{ATP} + \text{NADH} + \text{NADPH (KDPG pathway)}. \quad (2)$$

2. Formation of glucuronic acid. When glucose is oxidized to glucuronic acid (the repeating EPS unit includes one molecule of glucuronic acid), two molecules of NAD(P)H are formed:

$$\text{Glucose} \longrightarrow \text{Glucuronic asid} + 2\text{NAD(P)H}. \quad (3)$$

3. Acetyl-CoA synthesis. The overall reaction for acetyl-CoA formation from glucose can be represented in the form of the following equations:

$$\text{Glucose} \longrightarrow 2\text{Acetyl-CoA} + 2\text{ATP} + 4\text{NADH}, \quad \text{(glycolysis)} \quad (4)$$

$$\text{Glucose} \longrightarrow 2\text{Acetyl-CoA} + \text{ATP} + 3\text{NADH} + \text{NADPH}. \quad \text{(KDPG-pathway)} \quad (5)$$

For one mole of lauric and palmitic acids to be synthesized, 6 and 8 mol acetyl-CoA is required, respectively. The total requirement is 14 mol acetyl-CoA, whose formation consumes 7 mol glucose. The energy generation in this process is as follows:

$$7\text{Glucose} \longrightarrow 14\text{Acetyl-CoA} + 14\text{ATP} + 28\text{NADH}, \quad \text{(glycolysis)} \quad (6)$$

$$7\text{Glucose} \longrightarrow 14\text{Acetyl-CoA} + 7\text{ATP} + 21\text{NADH} + 7\text{NADPH}. \quad \text{(KDPG-pathway)} \quad (7)$$

The above data on the ATP requirements for the synthesis of the AP and NAP units, as well as the energy generation data ((1)–(7)), can be represented in the form of a table (Table 1).

The formation of the repeating AP unit requires 15.5 mol glucose (8 mol is required for the synthesis of

Table 1. Energy expenditure and generation in the formation of the repeating unit of acylated and nonacylated polysaccharides from glucose by *Acinetobacter* sp.

Pathway of glucose catabolism	EPS	Energy expenditure for the formation of an EPS unit, mol ATP		Energy generation in the formation of an EPS unit, mol ATP		
		monosaccharide synthesis	fatty acid synthesis from acetyl-CoA	PA synthesis	glucuronic acid synthesis	acetyl-CoA synthesis
Glycolysis	AP	17	12	3	4	70
	NAP	17	0	3	4	0
KDPG pathway	AP	17	12	2.5	4	63
	NAP	17	0	2.5	4	0

Note: P/O was taken to be equal to 2.

Table 2. Energy requirements for the synthesis of acylated and nonacylated polysaccharides from glucose by *Acinetobacter* sp.

Pathway of glucose catabolism	EPS	Glucose expenditure for the synthesis of an EPS unit, mol	Energy expenditure, mol ATP		Energy generation, mol ATP	
			for the EPS unit synthesis	per mol glucose used	for the EPS unit synthesis	per mol glucose used
Glycolysis	AP	15.5	29	1.87	77	4.97
	NAP	8.5	17	2	7	0.12
	AP + NAP	24	46	1.92	84	3.5
KDPG pathway	AP	15.5	29	1.87	69.5	4.48
	NAP	8.5	17	2.0	6.5	0.76
	AP + NAP	24	46	1.92	76	3.17

monosaccharides and glucuronic acid; 0.5 mol, for PA formation; 7 mol, for the formation of fatty acids). The synthesis of the NAP unit requires 8.5 mol glucose (8 mol is required for the synthesis of monosaccharides and glucuronic acid; 0.5 mol, for PA formation). The energy requirements for the synthesis of AP and NAP from glucose used are shown in Table 2. From the data of this table, it can be calculated that the net energy generation during the synthesis of the repeating AP and NAP units (AP + NAP) constitutes

3.5 – 1.92 = 1.58 mol ATP/mol glucose used (glycolysis);

3.17 – 1.92 = 1.25 mol ATP/mol glucose used (KDPG pathway).

According to our assumption, 50% glucose is catabolized via the glycolytic pathway and 50%, via the KDPG pathway. Hence, the energy generation during EPS synthesis from glucose constitutes 1.42 mol ATP/mol glucose used.

These results, as well as the data reported in [14], show that the higher the content of oxidized substitutes (uronic acids, pyruvate, fatty acids, acetate, succinate, etc.) in the EPS composition, the greater the energy generated during the synthesis of such polysaccharides. No energy generation occurs during the synthesis of neutral EPS (e.g., kurdlan).

Energy requirements for biomass synthesis. The energy requirements for biomass synthesis are reflected in the economic coefficient of ATP expenditure (Y_{ATP}): $Y_{ATP} = \Delta x/\Delta ATP$, where Δx is the amount of the biomass (g) synthesized with an energy expenditure (ΔATP) of 1 mol ATP. It was established that, for most microorganisms, the Y_{ATP} value is the same and constitutes approximately 10.5 g cells/mol ATP for biomass synthesis from PGA [19].

The biomass synthesis from PGA (when the ammonium source of nitrogen is used) can be represented in the form of an equation [7]:

Table 3. Ethapolan formation during growth of *Acinetobacter* sp. 12S on an ethanol–glucose mixture

Carbon source	Parameters of the process			
	biomass, g/l	EPS, g/l	EPS-synthesizing capacity, g EPS/g biomass	EPS yield from substrate, %
Ethanol 0.75% + glucose 0.75%	1.9	7.2	3.79	53.3
Ethanol 0.5% + glucose 0.5%	1.5	5.6	3.73	62.2
Ethanol 1% + glucose 0.5%	1.8	4.2	2.33	32.3
Ethanol 0.5% + glucose 1%	1.7	5.1	3.0	36.4
Ethanol 1%	1.5	3.2	2.13	40.0
Ethanol 1.5%	1.8	3.8	2.11	31.7
Glucose 1%	1.3	3.6	2.77	36.0
Glucose 1.5%	1.5	4.0	2.67	26.7

Note: The ethanol concentration is given in % by volume; the glucose concentration, in mass %.

$$4\text{PGA} + \text{NH}_3 + 29\text{ATP} + 5.5\text{NAD(P)H} \longrightarrow (\text{C}_4\text{H}_8\text{O}_2\text{N}_1)_3, \quad (8)$$

where $(C_4H_8O_2N_1)_3$ is the biomass formula.

The overall reactions of the conversion of ethanol and glucose to PGA are expressed by the following equations [7]:

Table 4. Biomass and exopolysaccharide content and the culture liquid viscosity after 24 h of *Acinetobacter* sp. 12S growth on single substrates and substrate mixtures

Carbon source	Parameters of the process		
	biomass, g/l	EPS, g/l	culture liquid viscosity, mm^2/s
Ethanol 0.75% + glucose 0.75%	0.95	1.50	87.6
Ethanol 0.5% + glucose 0.5%	0.90	1.25	75.9
Ethanol 1%	0.40	0.58	15.8
Ethanol 1.5%	0.45	0.70	16.3
Glucose 1%	0.35	0.75	18.4
Glucose 1.5%	0.40	0.80	20.1

Note: The ethanol concentration is given in % by volume; the glucose concentration, in mass %.

$$2\text{C}_2\text{H}_5\text{O}_6 + 3\text{ATP} \longrightarrow \text{PGA} + 6\text{NAD(P)H} + \text{FADH}_2 + \text{CO}_2, \quad (9)$$

$$\text{C}_2\text{H}_5\text{O}_6 \longrightarrow 3\text{PGA} + 2\text{NAD(P)H (glycolysis)}, \quad (10)$$

$$\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{ATP} \longrightarrow 2\text{PGA} + 2\text{NAD(P)H (KDPG-pathway)}. \quad (11)$$

Taking into account the assumption that 50% glucose is catabolized via the glycolytic pathway and 50% glucose is catabolized via the KDPG pathway, (10) and (11) can be combined:

$$\text{C}_6\text{H}_{12}\text{O}_6 + \text{ATP} \longrightarrow 2\text{PGA} + 2\text{NAD(P)H}. \quad (12)$$

For P/O equal to 2, (9) and (12) can be represented as follows:

$$2\text{C}_2\text{H}_5\text{OH} \longrightarrow \text{PGA} + 10\text{ATP}, \quad (13)$$

$$\text{C}_6\text{H}_{12}\text{O}_6 \longrightarrow 2\text{PGA} + 3\text{ATP}. \quad (14)$$

Proceeding from the equation of the biomass synthesis from PGA (8) and the equation of glucose catabolism to PGA (14), it can be calculated that, when *Acinetobacter* sp. is grown on glucose, the ATP requirement for the biomass synthesis is 17 mol per mol glucose. In our opinion, this energy can be obtained from ethanol. Given that the EPS synthesis from glucose generates 1.42 mol ATP per mol glucose used, 17 – 1.42 = 15.58 mol ATP should be obtained at the expense of ethanol. It follows from (13) that 3.12 mol ethanol is required for obtaining such an amount of ATP. Hence, the molar glucose/ethanol ratio in the medium should be 3.12 : 1. For example, at a glucose concentration in

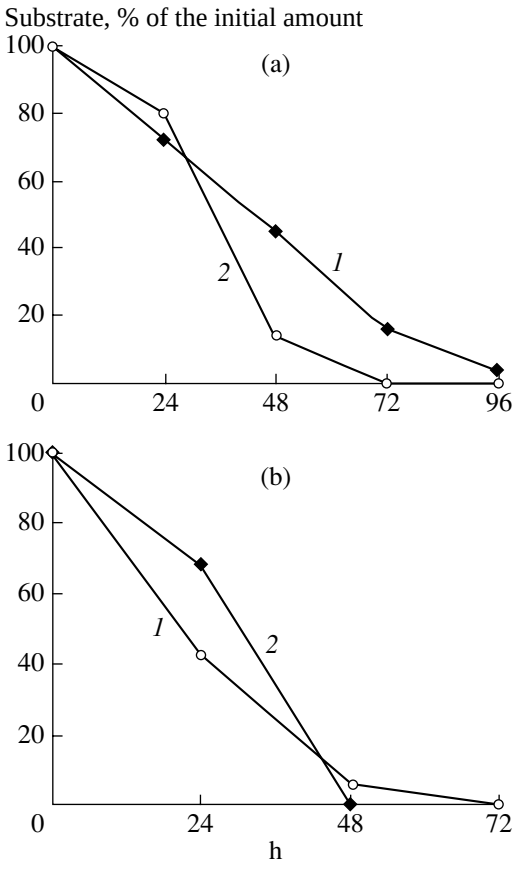

Fig. 3. Consumption of (*1*) ethanol and (*2*) glucose during the growth of *Acinetobacter* sp. 12S on a mixture of these substrates. The initial substrate concentrations were (a) glucose 0.75%, ethanol 0.75 vol % and (b) glucose 0.5%, ethanol 0.5 vol %.

the medium of 1 mass % (10 g/l, or 0.056 M), the ethanol concentration should be 0.175 M (8.04 g/l, or 1 vol %). Thus, the ratio of glucose mass to ethanol volume in the cultivation medium should be 1 : 1.

Ethapolan synthesis during the growth of *Acinetobacter* sp. 12S on a mixture of ethanol and glucose. Table 3 shows the data on the formation of ethapolan on a mixed substrate at different ethanol to glucose ratios. At an ethanol to glucose concentration ratio of 2 : 1, the EPS content virtually did not differ from that observed after growth on single substrates. Moreover, at ethanol to glucose concentration ratios of 2 : 1 and 1 : 2, the EPS-synthesizing capacity and the yield of EPS per substrate consumed did not substantially differ from the values recorded when the producer was grown on single substrates. It was only at the theoretically calculated concentration ratio of these substrates equal to 1 : 1 that a considerable increase was observed in the amount of the EPS synthesized and in their yield in relation to biomass and substrate. When the bacteria were cultivated on a mixed substrate, both substrates were used simultaneously (Fig. 3).

When *Acinetobacter* sp. 12S was grown on a mixture of ethanol and glucose at a ratio of 1 : 1, we observed not only an increase in the values shown in Table 3, but also an intensification of bacterial growth and EPS synthesis. Thus, after 24 h of growth on this substrate mixture, the biomass and EPS content was twofold higher than after growth on single substrates, and the culture liquid viscosity was four- to fivefold higher (Table 4).

Thus, our study is the first to show the possibility of intensifying the synthesis of secondary metabolites (microbial EPS) on a mixture of energy-nonequivalent substrates. These results form the basis for the development of new biotechnologies for obtaining secondary metabolites of practical value.

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