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EXPERIMENTAL ARTICLES

Enhanced Synthesis of the Exopolysaccharide Ethapolan by *Acinetobacter* sp. 12S Grown on a Mixture of Substrates

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Abstract—Enhanced synthesis of the exopolysaccharide ethapolan by *Acinetobacter* sp. 12S was observed when the bacterium was grown on a mixture of two energetically nonequivalent substrates (ethanol and glucose) taken in a molar proportion of 3.1 : 1. The efficiency of carbon transformation into EPSs was maximum when sodium ions were absent in the medium, the concentration of nitrogen source was reduced to 0.3–0.45 g/l, and the inoculum was grown on ethanol. Such conditions provided an increase in the maximum specific growth rate and its attainment in earlier cultivation terms. Molasses as a substitution for glucose was inefficient. The activities of the key enzymes of C_2 metabolism in *Acinetobacter* sp. 12S cells grown on the substrate mixture were 1.1 to 1.7 times lower than they were during growth on ethanol alone. The activity of isocitrate lyase in cells grown on the substrate mixture declined to an even greater extent (by 4–7 times), indicating that the role of the glyoxylate cycle in such cells is insignificant.

Key words: intensification of exopolysaccharide synthesis, mixed substrate, key enzymes of C_2 and C_6 metabolism.

Earlier, we found that the synthesis of the microbial exopolysaccharide (EPS) ethapolan by *Acinetobacter* sp. 12S can be enhanced by growing this bacterium on a mixture of ethanol and glucose [1]. The calculation of theoretical energy requirements for the synthesis of the biomass and the EPS allowed us to determine the concentration of the supplementary substrate ethanol that compensates for the shortage of carbon for constructive metabolism caused by the oxidation of glucose to CO_2 in energy-yielding pathways. Namely, the cultivation of *Acinetobacter* sp. 12S on ethanol and glucose in a molar proportion of 3.1 : 1 favored the maximum production of EPS and increased its yield with respect to the substrates consumed.

It should be noted that the cultivation of *Acinetobacter* sp. 12S on the substrate mixture was accompanied by the enhancement of the synthesis of not only the EPS but also the biomass. The aim of this work was to maximize the transformation of the substrate carbon into EPS. Among the factors studied that may influence the transformation efficiency were the effect of the inoculum, the concentration of carbon and nitrogen sources in the medium, the nature of the energy-deficient substrate, and the availability of sodium ions.

MATERIALS AND METHODS

Strains. The two bacterial strains used in this work were *Acinetobacter* sp. 12S (the ethapolan producer described earlier [2]) and the mutant strain *Acinetobacter* sp. 1NG, defective in the synthesis of this exopolysaccharide [3].

Cultivation conditions. The strains were grown in a liquid mineral medium [4] containing (g/l) KH_2PO_4, 6.8; NaOH, 0.9; NaCl, 1.1; NH_4NO_3, 0.6; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.001 or in the same medium in which NaOH and NaCl were substituted by equivalent amounts of KOH and KCl (these two media were designated K^+, Na^+ medium and K^+ medium, respectively). The media were supplemented with 0.5 vol % yeast autolysate and 0.0006% calcium pantothenate. The sources of carbon and energy were ethanol (0.5, 1.0, and 1.7 vol %), glucose (0.5, 1.0, and 1.35 wt %), and mixtures of ethanol and glucose in a molar proportion of 3.1 : 1 (1 : 1, vol/wt). In the latter case, the concentrations of ethanol and glucose in the medium were 0.5, 0.75, and 1 vol % and wt %, respectively. In some experiments, glucose in the K^+ medium was substituted by molasses in an amount equivalent in the carbon content. The molasses was preliminarily hydrolyzed as described elsewhere [4]. To investigate the effect of the nitrogen source (NH_4NO_3) in the medium on the synthesis of ethapolan, this salt was added at three different concentrations (0.3, 0.45, and 0.6 g/l).

Cells for inoculation were grown on nutrient agar for 2 days or in mineral K^+ medium with (a) 0.5 vol % ethanol, (b) 0.5 wt % glucose, or (c) 0.5 vol % ethanol + 0.5 wt % glucose for 16–24 h (i.e., to the exponential growth phase). The inoculum was grown at the NH_4NO_3 concentration 0.6 g/l. The inoculum size com-

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Table 1. The effect of inoculum on the biomass of *Acinetobacter* sp. 12S cells and EPS synthesis in media with different concentrations of ethanol–glucose mixtures

Carbon source in the cultivation medium	Carbon source for inoculum	Cultivation medium	Biosynthetic parameters			
			biomass, g/l	EPS, g/l	EPS-synthesizing capacity, g EPS/g biomass	EPS yield, % of the substrate consumed
0.5% Ethanol + 0.5% glucose	Nutrient agar	K^+,Na^+ medium	1.5	5.6	3.73	62.2
		K^+ medium	1.45	5.8	4.00	64.4
0.75% Ethanol + 0.75% glucose	Nutrient agar	K^+,Na^+ medium	1.9	7.2	3.79	53.3
		K^+ medium	1.8	7.7	4.28	57.0
1% Ethanol + 1% glucose	Nutrient agar	K^+,Na^+ medium	2.1	7.7	3.67	42.8
		K^+ medium	2.0	8.1	4.05	45.0
0.75% Ethanol + 0.75% glucose	0.5% Ethanol	K^+ medium	1.7	8.3	4.88	61.5
	0.5% Glucose	K^+ medium	1.8	7.6	4.22	56.3
	0.5% Ethanol + 0.5% glucose	K^+ medium	1.7	8.2	4.82	60.7

Note: Concentrations of ethanol and glucose are given in vol % and wt %, respectively.

Table 2. The respiration rate on various C_2 and C_6 substrates of intact *Acinetobacter* sp. 1NG cells grown on mixed substrates

Carbon source in the growth medium	Respiration rate (nmol O_2/(min mg cells)) during the oxidation of				
	ethanol	acetaldehyde	acetate	glucose	pyruvate
0.5% Ethanol + 0.5% glucose	76.0	81.5	62.1	61.5	59.3
1% Ethanol + 1% glucose	70.5	79.8	42.5	59.1	56.9
0.5% Ethanol + 0.5% molasses	39.5	50.4	39.3	48.7	45.3
1% Ethanol + 1% molasses	27.8	47.6	27.3	50.3	53.4

Note: The concentration of molasses is expressed in wt % of reducing sugars. Cells were washed with 0.05 M K phosphate buffer, pH 7.0. Cell respiration was measured in 0.05 M Tris–HCl buffer, pH 7.0.

prised 5 vol % of the cultivation medium. Cultivation was performed at 30°C at pH 6.8–7.0 on a shaker (220 rpm) for 16–96 h.

The efficiency of substrate transformation into EPS was estimated as the amount of EPS synthesized, the yield of EPS with respect to the substrate consumed, and the EPS-synthesizing capacity.

The biomass was evaluated from the optical density of cell suspensions and recalculated into the mass of dry cells using a calibration curve. The amount of EPS synthesized was determined gravimetrically [5]. The yield of EPS with respect to the substrate consumed and the specific bacterial growth rate were determined by the methods described in the handbook by Pirt [6]. The EPS-synthesizing capacity of the bacterium was expressed as the ratio of the EPS synthesized to the bacterial biomass accumulated. The concentrations of ethanol and glucose in the medium were determined as described elsewhere [1, 4].

The respiration rate of *Acinetobacter* sp. cells in the presence of ethanol, acetaldehyde, acetate, glucose, and pyruvate and the activities of the key enzymes of C_2 and C_6 metabolism in cell-free extracts were measured using the EPS-deficient mutant *Acinetobacter* sp. 1NG [4, 7]. The key enzymes were NAD^+-dependent alcohol dehydrogenase (EC 1.1.1.1), $NADP^+$-dependent acetaldehyde dehydrogenase (EC 1.2.1.4), acetyl-CoA synthetase (EC 6.2.1.1), acetate kinase (EC 2.7.2.1), isocitrate lyase (EC 4.1.3.1), 6-phosphofructose kinase (EC 2.7.11), and 6-phosphogluconate dehydratase (EC 4.2.1.12).

RESULTS AND DISCUSSION

Earlier, we showed that the utilization of acetate is a bottleneck stage in the ethanol metabolism of *Acinetobacter* sp. [7]. The activity of acetyl-CoA synthetase in cell-free extract and the respiration of intact cells in the presence of acetate were inhibited by sodium ions. Omitting sodium ions from the ethanol-containing cultivation medium led to a 2- to 2.5-fold increase in the activity of acetyl-CoA synthetase and somewhat raised the oxidation rate of acetate. This resulted in an

Table 3. The activity of the key enzymes of C_2 and C_6 metabolism in *Acinetobacter* sp. 1NG cells grown on ethanol, glucose, and their mixtures

Carbon source in the growth medium	Enzymatic activity, nmol/(min mg protein)						
	NAD^+-dependent alcohol dehydrogenase	$NADP^+$-dependent acetaldehyde dehydrogenase	Acetyl-CoA synthetase	Acetate kinase	Isocitrate lyase	6-Phosphofructokinase	6-Phosphogluconate dehydrogenase
0.5% Ethanol	296.8	197.9	198.5	22.2	165.5	272.1	24.7
0.5% Glucose	118.8	163.4	ND	ND	5.4	326.0	89.1
0.5% Ethanol + 0.5% glucose	170.4	176.8	143.6	19.5	40.3	231.8	34.9
1% Ethanol	339.1	263.8	139.5	18.9	130.0	226.1	45.3
1% Glucose	135.1	176.2	ND	ND	4.1	676.9	161.0
1% Ethanol + 1% glucose	213.9	248.4	85.2	15.7	19.7	276.0	69.0

Note: *Acinetobacter* sp. 1NG cells were grown in K^+ medium with 0.6 g/l NH_4NO_3. The inoculum was grown on nutrient agar. Cells were washed with 0.05 M K phosphate buffer (pH 7.0) and disintegrated in the same buffer. ND stands for "not determined."

enhanced synthesis of EPS and prevented the accumulation of acetate in the culture grown on ethanol.

Bearing this in mind, we evaluated the formation of ethapolan in K^+ and K^+,Na^+ media containing both ethanol and glucose (Table 1) and found that the amount and yield of EPS with respect to the substrate consumed, as well as the EPS-synthesizing capacity of *Acinetobacter* sp., were higher in the case of K^+ medium. For this reason, further experiments were performed using only this medium.

The parameters characterizing the efficiency of EPS synthesis were higher when the inoculum was grown in the presence of ethanol or ethanol + glucose than when it was grown on nutrient agar or glucose alone (Table 1). In further experiments, the inoculum was grown on ethanol.

Raising the concentrations of ethanol and glucose in the cultivation medium lowered the yield of EPS (Table 1), probably due to the inhibition of acetyl-CoA synthetase (the enzyme that metabolizes acetate [8]) by the intermediate products of ethanol oxidation, NADH and NADPH. Furthermore, as shown for the yeast *Saccharomyces cerevisiae* and the bacterium *Bacillus subtilis*, glucose can also suppress the biosynthesis of acetyl-CoA synthetase [9–12].

Thus, the inhibition of the activity and the suppression of the synthesis of acetyl-CoA synthetase may be responsible for the decreased efficiency of EPS synthesis in *Acinetobacter* sp. cells. Confirmation of this suggestion comes from the fact that the respiration rate on acetate of intact cells grown at high concentrations of glucose and ethanol decreased by about 1.5-fold as compared with growth at low concentrations of these substrates (Table 2). In this case, the oxidation rates of ethanol, acetaldehyde, glucose, and pyruvate virtually did not change.

The assay of the key enzymes of C_2 and C_6 metabolism in *Acinetobacter* sp. cells grown on different substrates (Table 3) confirmed the earlier inference [4] that the activity of these enzymes is high (with the exception of the activity of isocitrate lyase in cells grown on glucose) irrespective of the growth substrate used (glucose or ethanol).

In cells grown on glucose–ethanol mixtures, the activities of the enzymes of C_6 and C_2 metabolism were lower than they were in cells grown on monosubstrates (glucose and ethanol, respectively) (Table 3). The activity of isocitrate lyase in cells grown on glucose–ethanol mixtures was 4- to 7-fold lower than it was in ethanol-grown cells (the activity of this enzyme lowered with increasing concentrations of the substrates). According to earlier observations, there is a correlation between the activities of acetyl-CoA synthetase and isocitrate lyase during the growth of *Acinetobacter* sp. on ethanol [7]. The decline in isocitrate lyase activity at increasing concentrations of ethanol and glucose in the medium can be accounted for by the suppressing effect of glucose on the biosynthesis of acetyl-CoA synthetase and its inhibition by the intermediates of ethanol metabolism. This explanation is in agreement with the results of polarographic measurements (Table 2). Nevertheless, the activity of acetyl-CoA synthetase in cells grown on the ethanol–glucose mixtures remained sufficiently high (Table 3). Furthermore, the activity of acetate kinase was virtually the same during the growth of *Acinetobacter* sp. on mono- and mixed substrates (Table 3).

We believe that the decline in the activity of isocitrate lyase during bacterial growth on mixed substrates is due to the fact that the acetyl-CoA produced by acetyl-CoA synthetase and acetate kinase is utilized largely by the tricarboxylic acid cycle and not by the glyoxylate cycle. This problem, as well as the possible

Table 4. The effect of the nitrogen source in the cultivation medium on *Acinetobacter* sp. 12S biomass and EPS synthesis

Carbon source in the growth medium	NH_4NO_3 in the medium, g/l	Biosynthetic parameters			
		Biomass, g/l	EPS, g/l	EPS-synthesizing capacity, g EPS/g biomass	EPS yield, % of the substrate consumed
0.5% Ethanol + 0.5% glucose	0.30	0.85	6.45	7.59	71.7
	0.45	1.1	6.25	5.68	69.4
	0.60	1.3	6.2	4.77	68.9
0.75% Ethanol + 0.75% glucose	0.30	1.2	9.15	7.62	67.8
	0.45	1.5	9.28	6.19	68.7
	0.60	1.7	8.3	4.88	61.5
1% Ethanol + 1% glucose	0.30	1.2	8.1	6.75	45.0
	0.45	1.6	9.38	5.86	52.1
	0.60	2.0	8.45	4.23	46.9
1.7% Ethanol	0.30	1.4	6.3	4.5	46.3
1.35% Glucose	0.45	1.2	6.5	5.41	48.1
1.35% Glucose*	0.60	1.1	5.2	4.73	38.5

*The inoculum was grown on 0.5% glucose; in all other cases, it was grown on 0.5% ethanol.

suppressing effect of glucose on the biosynthesis of acetyl-CoA synthetase, is the subject of our further studies.

It is known from the literature that the proportion between carbon and nitrogen sources in the medium is important for the synthesis of EPS [2, 13–16], which is typically maximum under nitrogen limitation conditions. In the case of *Acinetobacter* sp. grown on glucose–ethanol mixtures, the degree of nitrogen limitation may control the direction of biosynthetic processes toward the synthesis of EPS (Table 4). Irrespective of the concentration of ethanol and glucose in the medium, nitrogen limitation increased the EPS-synthesizing capacity of cells and the yield of EPS with respect to the substrate consumed (Table 4). This was due to both the reduced level of biomass accumulated under nitrogen limitation conditions and the typically increased total amount of synthesized EPS.

The optimal concentrations of ammonium nitrate in the medium for the synthesis of EPS by *Acinetobacter* sp. cells grown on 0.5% ethanol + 0.5% glucose, 0.75% ethanol + 0.75% glucose, and 1% ethanol + 1% glucose were found to be 0.3, 0.3–0.45, and 0.45 g/l, respectively. It should be noted that, when the amount of synthesized EPS was maximum (cultivation on 1% ethanol + 1% glucose in the presence of 0.45 g/l ammonium nitrate), the yield of EPS reached only 50–52% of the substrates consumed (Table 4).

As compared with growth on monosubstrates, the *Acinetobacter* sp. cells grown on the glucose–ethanol mixtures produced 1.3–1.7 times more EPS and were characterized by 1.2–1.6 times greater EPS-synthesizing capacity and a higher yield of EPS with respect to the substrates consumed (Table 4).

Earlier, we found that ethanol–glucose mixtures are favorable both for growth and EPS synthesis by *Acinetobacter* sp. [1]. For instance, after 24 h of growth on the mixed substrates, the biomass was two times greater (and the viscosity of the culture liquid, as an indicator of the EPS content, was four to five times higher) than it was during growth on monosubstrates [1]. In this work, the mixed substrates augmented the maximum specific growth rate of *Acinetobacter* sp. 12S by 1.3–1.9 times (Table 5) and somewhat shortened the time of its achievement.

The growth of the inoculum on ethanol was favorable for the biosynthesis of EPS in glucose-containing media (Table 4). Earlier, we observed the same beneficial effect of low concentrations (0.01–0.02%) of ethanol or acetate added to the cultivation medium [4]. We believe that, in both cases (the use of the ethanol-grown

Table 5. The effect of cultivation conditions on the growth parameters of *Acinetobacter* sp. 12S

Carbon source in the growth medium	Maximum specific growth rate (μ_{max}), h^{-1}	Time of μ_{max} achievement, h
0.75% Ethanol + 0.75% glucose	0.21	4
1.7% Ethanol	0.11	28
1.35% Glucose	0.16	8
1.35% Glucose*	0.12	24

Note: The concentration of ammonium nitrate in the medium was 0.3 g/l.

*The inoculum was grown on 0.5% glucose; in all other cases, it was grown on 0.5% ethanol.

Table 6. The effect of molasses as an alternative to glucose on the *Acinetobacter* sp. 12S biomass and EPS synthesis

Carbon source in the growth medium	Biosynthetic parameters			
	Biomass, g/l	EPS, g/l	EPS-synthesizing capacity, g EPS/g biomass	EPS yield, % of the substrate consumed
1% Ethanol + 1% molasses	2.78	8.45	3.04	46.9
0.5% Ethanol + 0.5% molasses	1.65	5.95	3.60	66.1
1.8% Molasses	1.85	8.38	4.53	46.6
0.9% Molasses	1.4	5.8	4.14	64.4

Note: The inoculum was grown on nutrient agar. *Acinetobacter* sp. 12S was grown in K^+ medium with 0.3 g/l NH_4NO_3. The concentrations of ethanol and molasses are given in vol % and wt % of reducing sugars, respectively.

inoculum and the addition of ethanol or acetate in minor amounts to the glucose-containing cultivation medium), the biosynthesis of EPS is enhanced due to the activation of gluconeogenesis. Such a mechanism of enhancement of EPS synthesis is confirmed by the fact that the presence of low concentrations of ethanol or acetate in the glucose-containing cultivation medium led to a 15-fold increase in the activity of isocitrate lyase [4], which is one of the key enzymes of the glyoxylate cycle, an anaplerotic sequence of reactions replenishing the cellular pool of C_4-dicarboxylic acids, serving as precursors of gluconeogenesis [17]. The supposition that induced gluconeogenesis leads to the intensification of EPS biosynthesis in glucose-containing media inoculated with *Acinetobacter* sp. cultures grown on ethanol can be verified by measuring the activity of the key enzymes of gluconeogenesis under different cultivation conditions.

The use of molasses (an inexpensive alternative to glucose) instead of glucose in the mixed substrates led to the absence of the promoting effect of ethanol on EPS synthesis (Table 6). The rate of ethanol oxidation by *Acinetobacter* sp. cells grown on the molasses–ethanol mixtures was 2–2.5 times lower than it was in the case of cells grown on the glucose–ethanol mixtures (Table 2). These data suggest that some components of molasses are inhibitory to ethanol oxidation.

Thus, the maximum efficiency of conversion of the substrate carbon into EPS was observed when (a) the ethapolan producer was grown on ethanol and glucose taken in a molar proportion of 3.1 : 1; (b) the inoculum was grown on ethanol or an ethanol–glucose mixture; (c) the concentration of the nitrogen source (ammonium nitrate) in the cultivation medium was reduced to 0.3–0.45 g/l; and (d) sodium ions were omitted from the medium. Such conditions enhanced the maximum specific growth rate, shortened the time of its achievement, augmented the amount of synthesized EPS and the yield of EPS with respect to the substrates consumed, and increased the EPS-synthesizing capacity of *Acinetobacter* sp. cells.

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