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EXPERIMENTAL ARTICLES

Exopolysaccharide Production and Peculiarities of C_6-Metabolism in *Acinetobacter* sp. Grown on Carbohydrate Substrates

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Abstract—An *Acinetobacter* sp. strain grown on carbohydrate substrates (mono- and disaccharides, molasses, starch) was shown to synthesize exopolysaccharides (EPS). Glucose catabolism proved to proceed via the Embden–Meyerhof–Parnas and Entner–Doudoroff pathways. Pyruvate entered the tricarboxylic acid cycle due to pyruvate dehydrogenase activity. Pyruvate carboxylation by pyruvate carboxylase was the anaplerotic reaction providing for the synthesis of intermediates for the constructive metabolism of *Acinetobacter* sp. grown on C_6-substrates. The C_6-metabolism in *Acinetobacter* sp. was limited by coenzyme A. Irrespective of the carbohydrate growth substrate (glucose, ethanol), the activities of the key enzymes of both C_2- and C_6-metabolism was high, except for the isocitrate lyase activity in glucose-grown bacteria. Isocitrate lyase activity was induced by C_2-compounds (ethanol or acetate). After their addition to glucose-containing medium, both substrates were utilized simultaneously, and an increase was observed in the EPS synthesis, as well as in the EPS yield relative to biomass. The mechanisms responsible for enhancing the EPS synthesis in *Acinetobacter* sp. grown on a mixture of C_2- and C_6-substrates are discussed.

Key words: exopolysaccharides, glucose metabolism, regulation, mixed substrates, intensification of biosynthesis.

The strain *Acinetobacter* sp. 12S is a producer of a complex polysaccharide EPS preparation, ethapolan [1]. We have previously developed a procedure for obtaining ethapolan with the use of ethanol as the carbon source and approaches to intensifying ethapolan synthesis and influencing its composition and physicochemical properties [1, 2]. However, strain *Acinetobacter* sp. 12S can grow and synthesize EPS not only on C_2- but also on C_3-, C_4-, and C_6-substrates [1]. This advantageous property distinguishes this *Acinetobacter* sp. strain from other microbial producers, which are only capable of synthesizing polysaccharides when grown on carbohydrates (*Xanthomonas campestris*, *Azotobacter vinelandii*, *Scleroticum glucanicum*) [1]. Since *Acinetobacter* sp. 12S synthesizes EPS when grown on C_2–C_6 substrates, it is possible to develop either a general and adaptable procedure for obtaining polysaccharides with the use of a wide range of carbon substrates or a complex of different methods to use the method that would be the most economical and would provide EPS with the definite required physicochemical properties.

In this work, we aimed to study the growth dynamics of strain *Acinetobacter* sp. 12S and the EPS synthesis by this strain during growth on carbohydrate substrates. We also aimed at determining the basic stages of the C_6-compound metabolism in *Acinetobacter* sp. to develop an approach to intensifying the EPS synthesis.

MATERIALS AND METHODS

Bacteria. The EPS-producing strain *Acinetobacter* sp. 12S described previously [1] and the mutant strain *Acinetobacter* sp. 1NG incapable of producing exopolysaccharides [3] were used in this study.

***Acinetobacter* sp. growth conditions.** Bacteria were grown in liquid mineral medium of the following composition (g/l): KH_2PO_4, 6.8; KOH, 1.8; KCl, 1.4; NH_4NO_3, 0.6; $MgSO_4 \cdot 7H_2$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.001. In addition, the medium contained 0.5% yeast autolysate and 0.0006% calcium pantothenate. Glucose and ethanol at concentrations of 1% served as the carbon and energy source. The ability of *Acinetobacter* sp. 12S to synthesize EPS was studied after the addition to the medium of mannose, fructose, arabinose, galactose, rhamnose, sucrose, maltose, lactose, hydrolyzed molasses, or starch DE-60 in amounts equivalent to the ethanol concentration (in terms of the carbon content in the medium). To hydrolyze molasses, 100 g of the latter was diluted in distilled water added to give a final volume of 200 ml; then, after the addition of 20 ml of 1N H_2SO_4, the solution was sterilized at

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112°C for 30 min. The DE-60 starch was enzymatically treated by amylase and glucoamylase as described in [1].

Bacteria were also cultivated in medium containing 1% glucose and, in addition, either ethanol at a concentration from 0.01 to 1% or acetate at a concentration of 0.02%. Acetate was added in the form of a 20% solution of sodium acetate.

Bacteria were grown at 30°C and pH 6.8–7.0 in shake flasks (220 rpm) for 16–96 h. A one-day culture grown on a mixture of nutrient agar and wort agar (1 : 1) served as inoculum.

To determine biomass concentration, the optical density of a cell suspension was measured to be converted to the dry cell biomass using a calibration curve.

The amount of synthesized EPS was determined by the weighing method [4].

Pyruvate concentration in the culture liquid was measured by the method described by Sloneker and Orentas [5].

Ethanol concentration was determined by gas-liquid chromatography on a Tsvet-4 chromatograph equipped with a flame-ionization detector and a 2-m column with Celite-545 as the solid carrier and 20% PEG-400 polyethylene glycol as the immobile liquid phase. The carrier gas was helium; the temperature was 150°C.

Glucose concentration was determined using glucose oxidase [6].

Cell-free extract preparation. Cell-free extracts were prepared from *Acinetobacter* sp. 1NG cells harvested at the mid-exponential growth phase (16 to 18 h of growth in liquid mineral medium containing glucose and/or ethanol as the carbon source). After centrifugation (4000 *g*, 15 min, 4°C) and washing the cell pellet with 0.05 M Tris–HCl buffer, pH 7.0, the bacterial cells were resuspended in 0.05 M Tris–HCl buffer, pH 7.0, and disrupted by sonication (22 kHz) on an UZDN-1 device (three 30-s sessions). The resulting homogenate was centrifuged (12000 *g*, 30 min, 4°C), and the pellet was discarded to use the supernatant as a cell-free extract.

Enzyme analyses. The activities of 6-phosphofructokinase (EC 2.7.1.11) [7], 6-phosphogluconate dehydratase (EC 4.2.1.12) [7], phosphoenolpyruvate carboxylase (EC 4.1.1.31) [8], and pyruvate carboxylase (EC 6.4.1.1) [9] were assayed from NADH oxidation measured at 340 nm.

The activity of glucose dehydrogenase (EC 1.1.1.118 and EC 1.1.1.119) was assayed from either NAD^+ or $NADP^+$ reduction measured at 340 nm [7]. Glucose dehydrogenase (EC 1.1.99.10) activity was assayed from dichlorophenolindophenol reduction in the presence of phenazine methosulfate, which was measured at 600 nm [10].

Table 1. Growth and exopolysaccharide production by *Acinetobacter* sp. 12S on various substrates

Substrate	Parameters			
	EPS, g/l	Biomass, g/l	EPS yield, g EPS/g biomass	pH (final value)
Glucose	2.25	0.85	2.65	5.5
Mannose	2.30	0.80	2.88	5.5
Fructose	2.30	0.85	2.71	5.6
Arabinose	2.00	0.75	2.67	5.4
Galactose	0.20	0.08	2.50	6.8
Rhamnose	0.20	0.08	2.50	6.8
Sucrose	0.90	0.35	2.57	5.8
Maltose	0.55	0.25	2.20	5.8
Lactose	0.50	0.20	2.50	5.8
Molasses	3.35	1.15	2.91	5.7
Starch	3.20	1.10	2.91	5.5
Ethanol	3.50	1.30	2.69	6.8

Note: Bacteria were grown on carbohydrate substrates without the addition of vitamin B_3 to the medium. Prior to the cultivation on media containing molasses and starch, these substrates were hydrolyzed.

The activities of pyruvate dehydrogenase (EC 1.2.2.2.) [11], α-ketoglutarate dehydrogenase (EC 1.2.4.2) [12], and decarboxylating malate dehydrogenase (EC 1.1.1.38) were determined from NAD^+ reduction measured at 340 nm.

The activities of alcohol dehydrogenase (EC 1.1.1.1), aldehyde dehydrogenase (EC 1.2.1.3 and EC 1.2.1.4) and isocitrate lyase (EC 4.1.3.1) were measured as described previously [14].

Enzymatic activities were determined at 28–30°C, a temperature optimal for the growth of *Acinetobacter* sp., and were expressed in nmol/(min mg protein). In cell-free extracts, the protein concentration was measured by Bradford's method [15].

Measurement of the rate of substrate oxidation by the intact cells of *Acinetobacter* sp. The rate at which ethanol, acetaldehyde, and potassium acetate were oxidized by intact cells of *Acinetobacter* sp. 1NG was determined polarographically from the rate of oxygen consumption at 28–30°C (a temperature optimal for the growth of *Acinetobacter* sp.) using a PPT-1 polarograph. The specific rate of oxygen consumption was expressed in nmol O_2/(min mg cells). The substrate concentration was 10 mM. Cells of *Acinetobacter* sp. 1NG grown for 16–18 h (exponential growth phase) in liquid mineral medium containing either 1% glucose or 1% ethanol were centrifuged (4000 *g*, 15 min, 4°C). The cell pellet was washed twice with 0.05 M K^+ phosphate buffer, pH 7.0, and resuspended in 0.05 M K^+

Table 2. Activity of the key enzymes of C_2–C_6-metabolism in *Acinetobacter* sp. 1NG grown on ethanol- or glucose-containing media

Enzymes	Enzymatic activities, nmol/(min mg protein) in cells grown on media containing	
	glucose (1%)	ethanol (1%)
6-Phosphofructokinase	696.9	226.1
6-Phosphogluconate dehydratase	161.0	45.3
NAD^+-dependent glucose dehydrogenase	0	ND
$NADP^+$-dependent glucose dehydrogenase	0	ND
PQQ-dependent glucose dehydrogenase	0.2	ND
Pyruvate dehydrogenase	207.2	0
α-Ketoglutarate dehydrogenase	380.0	340.3
Phosphoenolpyruvate carboxylase	0	ND
Pyruvate carboxylase	304.4	127.9
Malate dehydrogenase (decarboxylating)	0	ND
NAD^+-dependent alcohol dehydrogenase	135.1	339.1
$NADP^+$-dependent acetaldehyde dehydrogenase	176.2	263.8
NAD^+-dependent acetaldehyde dehydrogenase	54.3	85.7
Isocitrate lyase	4.1	130.0

Note: PQQ, pyrroloquinoline quinone; ND, not determined.

Table 3. Effect of pantothenic acid (vitamin B_3) on the growth of *Acinetobacter* sp. in glucose-containing medium and on exopolysaccharide synthesis

Acinetobacter sp. strain	B_3 concentration, %	Biomass, g/l	EPS g/l	pH (final value)	Pyruvate concentration in the culture liquid, M	Respiration rate of intact cells in the presence of pyruvate, nmol O_2/(min mg cells)	Activity, nmol/(min mg protein)	
							pyruvate dehydrogenase	α-ketoglutarate dehydrogenase
12S (EPS^+)	0	0.85	2.25	5.5	45	ND	ND	ND
	0.0006	1.20	3.50	6.9	0	ND	ND	ND
1NG (EPS^-)	0	0.80	0	5.4	40	78.2	98.2	205.6
	0.0006	1.45	0	6.9	0	155.5	207.2	380.0

Note: Glucose concentration in the culture medium was 1%. ND stands for "not determined."

phosphate buffer, pH 7.0. The rate of substrate oxidation was measured in cells incubated in Tris–HCl buffer (0.05 M, pH 7.0).

RESULTS AND DISCUSSION

Table 1 demonstrates that strain *Acinetobacter* sp. 12S can grow and synthesize EPS on many carbohydrate substrates, such as mono- and disaccharides, molasses, and starch. In these experiments, bacteria were grown on carbohydrate media containing no pantothenic acid (vitamin B_3), which is required for *Acinetobacter* sp. growth on C_2-substrates (*Acinetobacter* sp. is an auxotroph with respect to vitamin B_3 [1]). On rhamnose- and galactose-containing media, both the biomass yield and the level of EPS production were the lowest. When cultivated on media containing sucrose, lactose, or maltose, *Acinetobacter* sp. produced lower amounts of EPS than on media containing monosaccharides (glucose, fructose, mannose, or arabinose). This seems to be a result of a low activity of the enzymes hydrolyzing disaccharides. Therefore, in the experiments that involved the addition of starch or molasses as the carbon source, these substrates were subjected to preliminary hydrolysis. The strain studied showed similar levels of EPS synthesis when grown on carbohydrate substrates and on ethanol-containing medium, although the growth with C_6-compounds led to a decrease in the culture liquid pH to 5.5–5.7 (Table 1).

Analysis of the key stages of C_6-compound metabolism by *Acinetobacter* sp. allowed us to determine some factors responsible for the decrease in pH of the carbohydrate-containing cultivation medium. The mutant strain *Acinetobacter* sp. 1NG which incapable of synthesizing EPS was used for this analysis. The original EPS-synthesizing strain was unsuitable for these experiments because its cells are difficult to separate from the extremely viscous EPS with high molecular mass. The same approach was previously used to study C_2-metabolism by these bacteria [14], which allowed us to optimize the procedure for obtaining ethanol-derived ethapolan.

In *Acinetobacter* sp., glucose catabolism proceeded via the Embden–Meyerhof–Parnas pathway (glycolysis, 1.6-fructose-bisphosphate pathway). A portion of glucose can also be metabolized via the Entner–Doudoroff pathway (KDPG pathway) (Table 2). These conclusions are suggested by the high activity of 6-phosphofructokinase, the key enzyme of glycolysis, and 6-phosphogluconate dehydrogenase, the key enzyme of the KDPG-pathway. We did not study the enzymatic activities involved in the pentose-phosphate cycle, because the latter is not a major pathway of hexose catabolism in bacteria; it rather provides NADPH for the reactions of the constructive metabolism, as well as ribose for nucleotide synthesis [16, 17].

In some bacteria, glucose is known to be catabolized via gluconate under the influence of either $NAD(P)^+$- or pyrroloquinoline quinone–dependent glucose dehydrogenases [16, 18]. In the cell-free extract of *Acinetobacter* sp. 1NG, $NAD(P)^+$-dependent glucose dehydrogenase activity was not detected, and the activity of the pyrroloquinoline quinone-dependent glucose dehydrogenase was extremely low (no higher than 0.2 nmol/(min mg protein)) (Table 2).

Both *Acinetobacter* sp. strains, 12S and 1NG, are B_3-dependent [1, 3]. In the cell-free extract of the *Acinetobacter* sp. 1NG, pyruvate dehydrogenase activity was revealed (Table 2), and it was found to be higher in cells grown on B_3-limited carbohydrate media than in the presence of B_3 (Table 3). The reaction catalyzed by pyruvate dehydrogenase is known to require coenzyme A, whereas the B_3 vitamin is a precursor of the latter [16]. Thus, pyruvate accumulation may account for the decrease in the pH of the culture liquid of *Acinetobacter* sp. grown on carbohydrate media without B_3 (Table 1). In this case, pyruvate was indeed detected in the culture liquid at a concentration of 40 to 50 mM (Table 3). After the addition of exogenous vitamin B_3 to glucose-containing medium, both pyruvate accumulation and the decrease in the pH of the culture liquid were prevented. The rate of pyruvate-dependent respiration of intact cells grown on B_3-containing medium was substantially higher than that of cells grown without B_3 (Table 3). Thus, both C_6 and C_2 metabolisms in the bacteria studied are coenzyme A–limited.

The presence of α-ketoglutarate dehydrogenase activity in the cell-free extract of *Acinetobacter* sp. 1NG (Table 2) suggests operation of a complete tricarboxylic acid cycle.

Table 4. Respiration rate of intact ethanol- and glucose-grown cells of *Acinetobacter* sp. 1NG in the presence of C_2–C_6-substrates

Growth substrate	Respiration rate, nmol O_2/(min mg cells), in the presence of			
	ethanol	acetal-dehyde	potassium acetate	glucose
Ethanol (1%)	165.3	170.7	138.3	35.0
Glucose (1%)	60.0	71.6	30.6	66.8

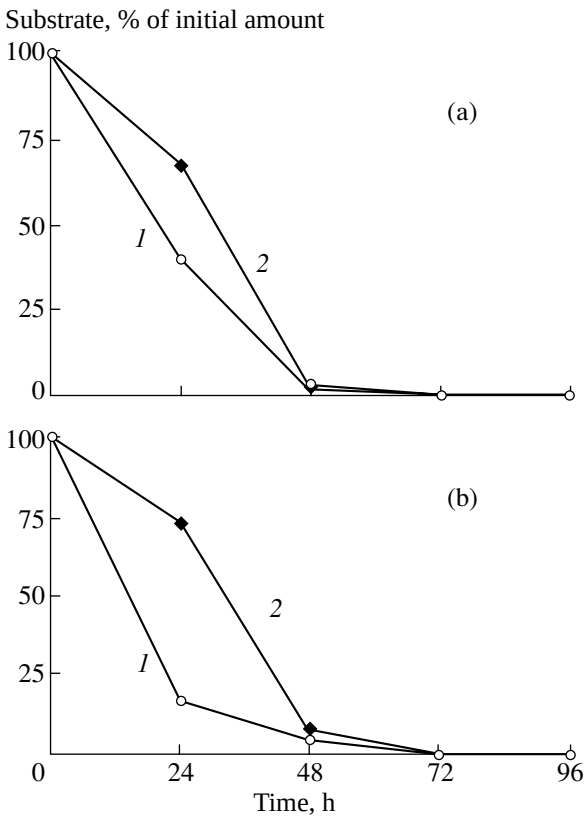
Table 5. Effect of various concentrations of C_2-compounds on enzymatic activities of anaplerotic pathways in glucose-grown cells of *Acinetobacter* sp. 1NG

Carbon source	Activity, nmol/(min mg protein)	
	isocitrate lyase	pyruvate carboxylase
Glucose	4.1	304.4
Glucose + ethanol, 0.01%	60.4	312.7
Glucose + ethanol, 0.1%	25.6	300.3
Glucose + ethanol, 0.5%	19.8	310.5
Glucose + ethanol, 1%	10.9	ND
Glucose + sodium acetate, 0.02%	52.9	309.2

Note: In all media, the glucose concentration was 1%. ND stands for "not determined".

Table 6. Synthesis of exopolysaccharides by *Acinetobacter* sp. 12S grown on glucose-containing medium in the presence of ethanol

Carbon source	Parameters		
	biomass, g/l	exopolysaccharides, g/l	EPS yield, g EPS/g biomass
Glucose, 1%	1.20	3.50	2.92
Glucose, 1.5%	1.30	4.00	3.08
Glucose, 1%, + ethanol, 0.01%	1.20	5.10	4.25
Glucose, 1%, + ethanol, 0.1%	1.35	5.60	4.15
Glucose, 1%, + ethanol, 0.5%	1.50	6.10	4.07



Consumption of (*1*) ethanol and (*2*) glucose by *Acinetobacter* sp. 1NG grown on a mixture of these substrates. Initial substrate concentrations were: (a) glucose 1%; ethanol, 0.5%; (b) glucose, 1%; ethanol, 0.1%.

In the cell-free extract of *Acinetobacter* sp. 1NG, we revealed a high activity of pyruvate carboxylase (Table 2), one of the three enzymes known to replenish the pool of intermediates of anabolic reactions in bacteria grown on C_6 substrates [16, 17]. The activities of phosphoenolpyruvate carboxylase and malate dehydrogenase (decarboxylating) were not detected. Due to the presence of pyruvate carboxylase activity in *Acinetobacter* sp., these bacteria are capable of growing on the medium containing pyruvate as the sole carbon and energy source [1]. In addition, strain *Acinetobacter* sp. 12S synthesizes EPS when grown on pyruvate-containing media [1]. In this regard, the experiments conducted with *Xanthomonas campestris* are of interest. As known from the literature, these bacteria show good growth on glucose and synthesize a large amount of the polysaccharide xanthan [19]. At the same time, poor growth of *X. campestris* was observed when pyruvate served as the sole carbon source and no xanthan synthesis occurred [20]. This does not, however, mean that this bacterium is unable to take up and utilize exogenous pyruvate, since it is implicated in the metabolism in the presence of glucose [21]. It is the absence or low activity of the metabolic branch ensuring gluconeogenesis that most probably accounts for the poor growth of *X. campestris* on pyruvate and the lack of EPS synthesis on this substrate.

In *Acinetobacter* sp., the activity of gluconeogenesis enzymes appears to be high: these bacteria show good growth and synthesize EPS on C_2–C_3 substrates [1]. The essence of gluconeogenesis is that a molecule of phosphorylated hexose, either glucose-6-phosphate or fructose-6-phosphate, is synthesized from two C_3 molecules [17]. In *Acinetobacter* sp. cells growing on C_2 compounds, the C_4 dicarboxylic acids (substrates for gluconeogenesis) are generated by the glyoxylate cycle [14], the key enzyme of which is isocitrate lyase.

Our further experiments showed that ethanol-grown intact cells of *Acinetobacter* sp. 1NG could oxidize glucose, whereas cells grown on glucose were capable of oxidizing ethanol, acetaldehyde, and acetate (Table 4). The key enzymes of glucose metabolism in ethanol-grown cells of *Acinetobacter* sp. 1NG displayed high activity, as well as the enzymes involved in ethanol transformation in glucose-grown cells (Table 2). The low activity of isocitrate lyase was an exception; probably, this enzyme is induced by C_2 compounds [16, 22]. Experiments with the addition of different amounts of ethanol to a medium containing 1% glucose were aimed at verifying this explanation. Under these conditions, the isocitrate lyase activity proved to be 2- to 15-fold higher, and it was the highest at low ethanol concentrations (Table 5). After the addition of acetate (0.02%) to glucose-containing medium, the isocitrate lyase activity also increased to 50–60 nmol/min per mg of protein. The activity of pyruvate carboxylase remained unchanged after the addition of C_2 compounds (Table 5).

It should be noted that no catabolite repression was observed in the bacteria grown on a mixture of ethanol and glucose; both substrates were utilized simultaneously (figure).

Proceeding from the data obtained, we tried to elucidate whether the EPS synthesis by *Acinetobacter* sp. 12S can be intensified. Both the overall amount of the produced EPS and their amount per unit of biomass increased after the addition of ethanol to medium containing 1% glucose (Table 6). The EPS production was intensified as the content of the C_2 substrate in the medium increased. Of interest is that the addition of acetate in low concentrations (0.02%) to the glucose-containing medium also stimulated the EPS synthesis. Like in the case of the mutant strain, no catabolite repression was observed in the EPS-producing strain grown on a mixture of substrates.

The intensification of EPS synthesis by *Acinetobacter* sp. 12S cells grown on a mixture of C_2 and C_6 substrates may be due to the following reasons: (1) After the addition of C_2 compounds to glucose-containing medium, the activities of enzymes of the glyoxylate

cycle, an additional anaplerotic succession of reactions, was increased to result in an enhanced synthesis of C_4-dicarboxylic acids (Table 5), which in turn might be involved into gluconeogenesis; i.e., C_2 compounds in the glucose-containing medium may induce gluconeogenesis. This mechanism is, most probably, responsible for intensification of the EPS synthesis at low concentrations of C_2 compounds (from 0.01 to 0.02% of either ethanol or acetate) in glucose-containing medium (in this case, the isocitrate lyase activity was almost 15-fold higher than that observed in the bacteria cultivated on medium containing only glucose). (2) At high ethanol concentrations (higher than 0.5%) in glucose-containing medium, ethanol may be used as an additional source of energy. In glucose-grown bacteria, about 40% of the substrate is known to be oxidized to CO_2 to generate energy required for the reactions of the constructive metabolism [16]. In the presence of ethanol, which is a high-energy substrate [14], a smaller amount of glucose is expended to generate energy. As a result, the transformation of this substrate into EPS becomes more efficient. In our further studies, we are going to verify both suggestions.

Thus, the results obtained in this work demonstrate that *Acinetobacter* sp. 12S can grow and synthesize EPS on C_6 compounds. Using the mutant strain *Acinetobacter* sp. 1NG unable to synthesize EPS, it was found that glycolysis and the KDPG-pathway are the major pathways of glucose catabolism. In *Acinetobacter* sp., the reaction catalyzed by pyruvate dehydrogenase is the bottleneck of glucose metabolism. Like the C_2-metabolism, the metabolism of C_6 compounds is coenzyme A–limited. When the strain studied is grown on C_2 compounds, pyruvate carboxylation is the anaplerotic reaction generating intermediates for the constructive metabolism. In ethanol-grown cells of *Acinetobacter* sp. 1NG, the activities of the key enzymes of glucose metabolism was found to be high, whereas in glucose-grown cells, the key enzymes of ethanol metabolism, except for isocitrate lyase exhibited high activity. The C_2 compounds are the inducers of isocitrate lyase in *Acinetobacter* sp. After the addition of C_2 compounds to the glucose-containing medium, the EPS production increased, as well as the EPS yield relative to biomass. No catabolite repression was observed when the bacteria were grown on a mixture of C_2 and C_6 substrates. The results obtained in this study will be helpful in developing a procedure for ethapolan production by the bacteria grown on either carbohydrates or on a mixture of C_2 and C_6 substrates.

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