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EXPERIMENTAL ARTICLES

Specific Features of the Synthesis of the Exopolysaccharide Ethapolan on a Mixture of Energy-Deficient Growth Substrates

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Abstract—Intensification of the synthesis of the microbial exopolysaccharide ethapolan by *Acinetobacter* sp. B-7005 was shown to occur on a mixture of energy-deficient growth substrates (acetate + glucose). When the bacterium grew on the substrate mixture, both substrates were utilized simultaneously; acetate was taken up by means of active transport at the expense of the energy of the proton-motive force. When acetate was present in the form of a sodium salt, the activities of acetyl-CoA synthetase and phosphoenolpyruvate synthetase (the key enzyme of gluconeogenesis) were tenfold higher than in the presence of potassium acetate, and the indexes of ethapolan synthesis were two times higher. The positive effect of Na^+ on ethapolan synthesis is supposed to consist in the creation of ion gradients on the membrane, necessary for the generation of the proton-motive force. Simultaneous functioning of the glyoxylate cycle and pyruvate carboxylase reaction, as well as an increase in the activity of isocitrate lyase, malate synthase, and phosphoenolpyruvate synthetase, provide evidence of increased gluconeogenesis in the presence of the acetate + glucose mixture (as compared to gluconeogenesis on the corresponding monosubstrates).

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Key words: energy-deficient substrate, intensification of biosynthesis, exopolysaccharides, gluconeogenesis, active transport.

Earlier, we showed the possibility of intensifying the synthesis of the microbial exopolysaccharide (EPS) ethapolan by cultivating the producer on a mixture of energetically nonequivalent growth substrates [1–3]. The combination of the accessory energy-excessive (ethanol) and energy-deficient (glucose) substrates allowed the unproductive carbon and energy losses occurring on the monosubstrates to be avoided and the effectiveness of the transformation of carbon substrates into EPS to be increased.

It is known that the "additional substrate effect" may be observed not only when microorganisms grow on a mixture of energetically nonequivalent substrates but also in the case of using two energy-deficient substrates, provided that they are assimilated simultaneously [4–6]. For example, in the case of simultaneous utilization of methanol and glycerol by the yeast *Pichia pinus*, the duration of the lag phase is shortened, and the growth rate and the biomass yield are increased compared to the yeast growth on the monosubstrate methanol [4].

In this connection, the aim of this work was to study the synthesis of the microbial polysaccharide ethapolan during cultivation of the producer on a mixture of energy-deficient growth substrates (acetate + glucose).

MATERIALS AND METHODS

The subject of study. The subject of this study was the EPS-synthesizing bacterial strain *Acinetobacter* sp. 12S [7], deposited in the Depositary of Microorganisms, Zabolotnyi Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, under the number B-7005.

Cultivation of the ethapolan producer. Cultivation was carried out in flasks on a shaker (220 rpm) at 30°C for 16–96 h in liquid medium of the following composition (g/l): KH_2PO_4, 3.4; KOH, 0.9; NH_4Cl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.001; yeast autolysate, 0.5% vol %; and calcium pantothenate (vitamin B_5; strain *Acinetobacter* sp. B-7005 is an auxotroph by this vitamin), 0.0009%; pH 6.8–7.8. In one of the variants, the magnesium and potassium cation concentration in the cultivation medium was increased to 5–10 and 100 mM, respectively. K^+ and Mg^{2+} were introduced into the medium in the form of a 20% KCl solution and a 10% $MgSO_4 \cdot 7H_2O$ solution.

A mixture of ethanol and glucose in a ratio of 1 : 1 (0.75 vol % and 0.75 wt %, respectively) or a mixture

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of sodium acetate (1.1 wt %) and glucose (0.75 wt %), or a mixture of potassium acetate (1.3 wt %) and glucose (0.75 wt %) were used as the source of carbon and energy. The concentrations of sodium acetate and potassium acetate were equimolar by carbon to the ethanol concentration (0.75 vol %).

The inoculum was an exponential-phase culture grown for 18–24 h in a medium with the following carbon and energy sources: ethanol (0.5% vol %), glucose (0.5 wt %), sodium acetate (0.7 wt %), or potassium acetate (0.8 wt %).

The biomass concentration and the effectiveness of transformation of carbon substrates into EPS (the amount of the EPS synthesized, the EPS/substrate ratio, the EPS-synthesizing capacity) were determined as described in [1]. The culture liquid concentration of glucose was determined by the glucose oxidase method [8]; the acetate concentration was determined by the enzymatic method using acetate kinase [9].

Preparation of cell-free extracts. An exponential-phase culture of *Acinetobacter* sp. B-7005 was subjected to ultrasonic (22 kHz) treatment for 60 s followed by centrifugation (5000 *g*, 10 min, 4°C). The sediment was washed three times with 0.05 M Tris–HCl buffer (pH 7.0). The ultrasonic treatment of the culture led to the cleavage of high-molecular EPS into low-molecular fragments. The culture liquid viscosity substantially decreased, which allowed the cells to be separated from the EPS fragments by centrifugation. The washed cells were resuspended in 0.05 M *Tris*–HCl buffer (pH 7.0) and disrupted by ultrasonic treatment (four 22-kHz 50-s bursts at 4 °C in a UZDN-1 apparatus (Russia)). The homogenate was centrifuged (12000 *g*, 30 min, 4°C), the sediment was discarded, and the supernatant was used as cell-free extract.

Enzyme assays. The activities of acetyl-CoA synthetase (EC 6.2.1.1), isocitrate lyase (EC 4.1.3.1), malate synthase (EC 4.1.3.2), isocitrate dehydrogenase (EC 1.1.1.42), 2-oxoglutarate dehydrogenase (EC 1.2.4.2), pyruvate dehydrogenase (EC 1.2.2.2), pyruvate carboxylase (EC 6.4.1.1), and phosphoenolpyruvate synthetase (PEP synthetase) (EC 2.7.9.2) were determined as described in [3].

Determination of the rate of acetate oxidation by intact cells of *Acinetobacter* sp. B-7005. The rate of acetate oxidation by intact bacterial cells (the respiratory rate in the presence of acetate) was determined from the rate of oxygen consumption from the reaction mixture using the polarographic method as described in [3]. The protonophore carbonylcyanide-*n*-trifluoromethoxyphenylhydrazone (FCCP) was introduced into the measuring cell as an isopropanol solution to attain a final concentration of 5 μM. The respiration rate was determined for strain *Acinetobacter* sp. B-7005 cells grown for 16–18 h (the exponential growth phase) in medium with sodium acetate (0.7%) or a mixture of sodium acetate (1.1%) and glucose (0.75%).

RESULTS AND DISCUSSION

In our previous experiments [1–3], increased ethapolan synthesis on a mixture of ethanol and glucose was observed when the producer was cultivated in a medium with a high buffer molarity (0.05 M) and a high salt concentration (up to 11 g/l). When the buffer molarity decreased due to the depletion of the content of phosphorus salts in the medium, we observed limitation of the C_2 metabolism determined by acetate accumulation, which was accompanied by a decrease in the EPS synthesis [10]. The study of the regulation of acetate metabolism during growth of the bacterium on a mixture of ethanol and glucose allowed us to realize ethapolan synthesis in a medium in which the buffer molarity (but not the salt concentration) was decreased twofold [10].

In the present work, the producer cultivation was carried out in a medium with a buffer molarity of 0.025 M and a total salt content of 5 g/l. As the nitrogen source, we used NH_4Cl at a concentration equimolar by nitrogen to that of ammonium nitrate in the medium used by us earlier [1–3, 10]. This substitution of the nitrogen source was motivated by the fact that, in the process of cultivation of the bacteria on a mixture of acetate and glucose, acetate consumption was accompanied by an increase in the culture liquid pH up to 9.5–10.0 (acetate is transported to the cells by symport with a proton [11]), and the pH optimum for ethapolan synthesis is 6.8–8.0. Since the culture liquid is acidified in the process of assimilation of ammonium nitrogen, the presence of acetate and ammonium chloride in the medium prevents its excessive alkalization and helps maintain the pH at a level required for ethapolan synthesis.

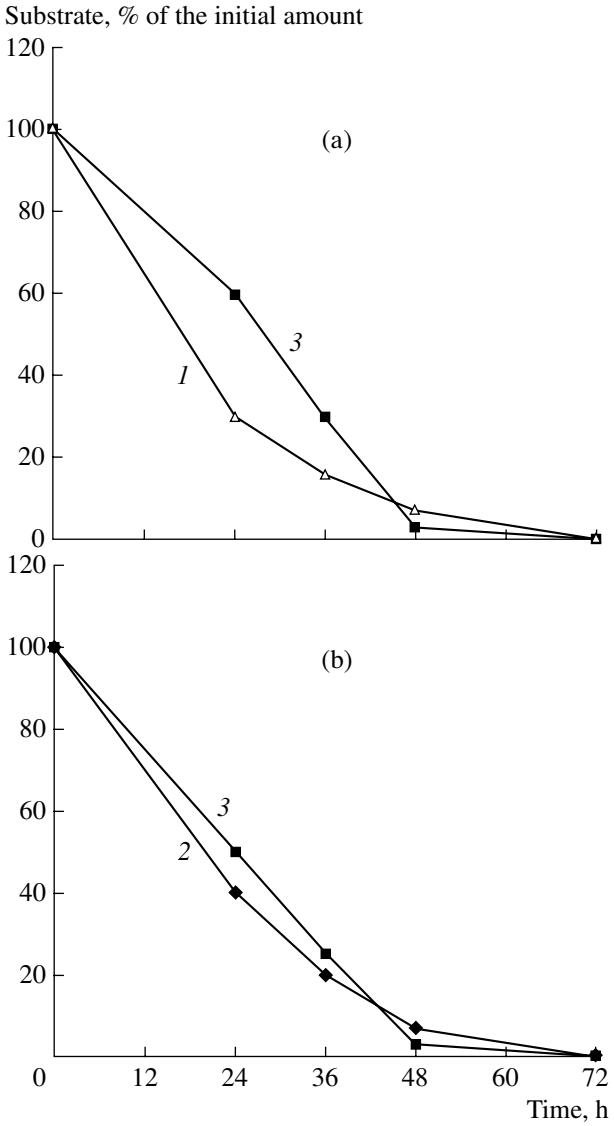
Data available in the literature give evidence that when microorganisms are grown on a mixture of energy-deficient substrates, their growth is intensified if both substrates are assimilated simultaneously, e.g., methanol and mannitol, methanol and glucose, oxalate and formate, acetate and formate, methanol and formate, methane and CO_2, or methanol and CO_2 [5]. During growth on an acetate + glucose mixture, both simultaneous and sequential consumption of the substrates by microorganisms may be observed [6, 12, 13]. Thus, when *Azotobacter vinelandii* was cultivated on a mixture of acetate and glucose, acetate was the first to be assimilated, and not until it was completely consumed was glucose utilized [13]. Depending on the cultivation conditions, the yeast *Zygosaccharomyces bailii* grown on a mixture of acetate and glucose may utilize these substrates either sequentially or simultaneously [12]. For *Corynebacterium glutamicum*, it was demonstrated that the intensification of the biomass synthesis takes place only when acetate and glucose are used simultaneously [6]. In this case, the acceleration of bacterial growth is determined by the fact that acetate is used for energy generation, while glucose is used in the anabolism.

Our experiments showed that when *Acinetobacter* sp. B-7005 was grown on a mixture of potassium acetate (or sodium acetate) and glucose, these substrates were assimilated simultaneously (figure); however, their consumption rates were somewhat lower than during growth on the corresponding monosubstrates. A similar phenomenon was established by us earlier when *Acinetobacter* sp. B-7005 was cultivated on a mixture of energetically nonequivalent substrates (ethanol + glucose) [1]. Despite the simultaneous assimilation of potassium acetate and glucose during the growth of the ethapolan producer on a mixture of these substrates, the EPS synthesis was not enhanced compared to cultivation of the bacterium on the monosubstrates (Table 1), irrespective of whether the inoculum was grown on ethanol or acetate; an increase in the concentration of Mg^{2+} (an activator of acetyl-CoA synthetase, an enzyme involving acetate in the metabolism in *Acinetobacter* sp. B-7005 [14]) in the culture medium also failed to enhance EPS synthesis. It should be pointed out that our previous studies established positive influence of magnesium cations and of sodium acetate–grown inoculum on the ethapolan synthesis by *Acinetobacter* sp. B-7005 grown on a mixture of ethanol and glucose [10].

It was quite unexpected that a substantial increase in the EPS synthesis occurred when potassium acetate in the mixed substrate was substituted with sodium acetate in an equimolar amount (Table 2). In this case, the amount of the EPS synthesized was greater not only compared to that observed on monosubstrates or a mixture of potassium acetate and glucose but also compared to the EPS synthesis on a mixture of ethanol and glucose. The use of an inoculum grown on sodium acetate and an increase in the concentrations of the acetyl-CoA synthetase activators (Mg^{2+} and K^{+}) [14] enhanced ethapolan synthesis on a mixture of sodium acetate and glucose (Table 2).

The data of growth experiments were confirmed by enzymological studies (Table 3). As seen from the data shown in Table 3, the activities of acetyl-CoA synthetase and the key gluconeogenesis enzyme PEP synthetase were more than ten times lower when *Acinetobacter* sp. B-7005 was grown on a mixture of potassium acetate and glucose than when it was grown on a mixture of sodium acetate and glucose. This regularity was observed irrespective of the nature of the carbon source (glucose, acetate, ethanol) used for obtaining the inoculum; however, the highest activity of both enzymes was recorded when an inoculum grown on sodium acetate was used.

Our previous studies [14] showed that sodium cations inhibit the activity of acetyl-CoA synthetase when *Acinetobacter* sp. B-7005 is cultivated on ethanol. However, the present work established the positive influence of Na^{+} on ethapolan synthesis during the growth of the producer on an acetate + glucose mixture (Tables 2, 3). In this connection, we assumed that, on a mixture of acetate and glucose, sodium cations may be involved in the transport of the C_2 substrate into *Acinetobacter* sp. B-7005 cells. All the subsequent experiments showed that, in the ethapolan producer, acetate is taken up via active transport at the expense of the proton-motive force energy, as has been established for many bacteria [11]. Our data on the rate of acetate oxidation by intact cells in the presence of the protonophore FCCP gave evidence in favor of this supposition (Table 4).



Acetate (*1, 2*) and glucose (*3*) consumption during growth of *Acinetobacter* sp. B-7005 on a mixture of these substrates. The initial concentrations were as follows: (a) potassium acetate, 1.3%; glucose, 0.75%; (b) sodium acetate, 1.1%; glucose, 0.75%.

Under the action of protonophores, the proton-motive force dissipates: these compounds eliminate the transmembrane proton and electric charge gradients due to the fact that the cytoplasmic membrane becomes

Table 1. Ethapolan synthesis during cultivation of *Acinetobacter* sp. B-7005 on a mixture of potassium acetate (1.3%) and glucose (0.75%)

Carbon source for obtaining inoculum	Mg^{2+} concentration in medium, mM	EPS, g/l	EPS-synthesizing capacity, g EPS/g ADB	EPS/substrate, %
Ethanol, 0.5%	1.6	4.3	8.6	28
	5	4.7	8.6	31
	10	5.4	9.0	36
Potassium acetate, 0.8%	1.6	5.5	9.2	36
	5	5.9	9.8	39
	10	6.1	11.1	40

permeable to protons in their presence. Since the action of protonophores is connected with their properties of weak acids, these compounds are ineffective at pH higher than 8.5 [11]. As seen from the data in Table 4, FCCP is not an inhibitor of acetate oxidation because no inhibition of acetate oxidation was observed at pH 8–9 (the conditions under which FCCP is deprotonated and does not act as a protonophore). These results indicate that the acetate uptake by *Acinetobacter* sp. B-7005 occurs by active transport at the expense of the energy of the proton-motive force. Since the generation of the proton-motive force requires the presence of ionic gradients across the membrane, it may be suggested that Na^+ ions are involved in the creation of such gradients in *Acinetobacter* sp. B-7005.

To establish the mechanism affording an increase in the ethapolan synthesis on a mixture of sodium acetate and glucose, the activity of the central metabolism enzymes was assayed when the producer was grown on the corresponding mono- and mixed substrates. The data on the activity of certain key enzymes of acetate and glucose metabolism (Table 5) confirm the results obtained earlier: irrespective of the nature of carbon nutrition, the *Acinetobacter* sp. B-7005 cells exhibit high activity of the C_2–C_6 metabolism enzymes [1, 3, 15]. The facts attracting attention were the substantial increase in the activity of the glyoxylate cycle enzymes (malate synthase, twofold; isocitrate lyase, tenfold) and a 1.2- to 1.4-fold decrease in the activity of pyruvate carboxylase during growth on a mixture of acetate and glucose (as compared to growth on the corresponding monosubstrates), as well as a certain decrease in the activity of isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase on mixed substrate as compared to growth on acetate. We found earlier [3] that the activity of isocitrate lyase and malate synthase was three to six times lower, and the activity of the tricarboxylic acid cycle enzymes was two to three times higher on a mixture of ethanol and glucose than on ethanol. An insignificant increase (as compared to growth on monosubstrates) in the activity of pyruvate carboxylase was observed on a mixture of ethanol and glucose [3]. Thus, the role of the glyoxylate cycle (but not of the tricarboxylic acid cycle) in the metabolism of *Acinetobacter* sp. B-7005 on a mixture of acetate and glucose is more significant than on a mixture of ethanol and glucose. It should be pointed out that, when the ethapolan producer was cultivated on a mixture of acetate and glucose, the pyruvate carboxylase activity, although decreased compared to growth on the monosubstrates, remained, nevertheless, sufficiently high—3314.8 nmol/(min mg protein) (Table 5). The increase in the isocitrate lyase and malate synthase activity, as well as

Table 2. Ethapolan synthesis during cultivation of *Acinetobacter* sp. B-7005 on a mixture of sodium acetate (1.1%) and glucose (0.75%)

Carbon source for obtaining inoculum	Mg^{2+} concentration in medium, mM	K^+ concentration in medium, mM	EPS, g/l	EPS-synthesizing capacity, g EPS/g ADB	EPS/substrate, %
Ethanol, 0.5%	1.6	35	7.6	15.2	50
	1.6	100	7.9	14.4	52
	5	35	8.3	15.1	55
	5	100	9.1	16.6	60
	10	35	9.0	16.4	60
	10	100	9.8	17.8	65
Sodium acetate, 0.7%	1.6	35	9.5	15.8	63
	1.6	100	9.7	16.2	64
	5	35	10.5	19.1	70
	5	100	10.5	19.1	70
	10	35	11.0	19.6	73
	10	100	10.7	19.5	71

Note: Here and in Table 1, the absolutely dry biomass (ADB) concentration in all of the variants was 0.5–0.6 g/l; during growth on monosubstrates, the EPS concentration was 4.5–6.5 g/l; on a mixture of ethanol and glucose, it was 7.0–8.4 g/l; when calculating the EPS/substrate value, the organic portion of the acetate molecule, CH_3COO^-, was taken as the substrate.

Table 3. Activity of acetyl-CoA synthetase and PEP synthetase during cultivation of *Acinetobacter* sp. B-7005 on a mixture of acetate and glucose

Carbon source in the cultivation medium	Carbon source for obtaining inoculum	Activity, nmol/min mg protein	
		acetyl-CoA synthetase	PEP synthetase
Potassium acetate, 1.3% + glucose, 0.75%	Glucose, 0.5%	35.7	134.0
	Potassium acetate, 0.8%	45.8	435.6
	Ethanol, 0.5%	37.2	179.9
Sodium acetate, 1.1% + glucose, 0.75%	Glucose, 0.5%	424.0	1614.0
	Sodium acetate, 0.7%	735.8	3977.0
	Ethanol, 0.5%	625.2	1734.9

Table 4. Effect of the protonophore FCCP on sodium acetate oxidation by intact *Acinetobacter* sp. B-7005 cells at different pH values

Carbon source in the cultivation medium	pH of the incubation mixture	Acetate oxidation rate, nmol/(min mg cells)	
		without FCCP	in the presence of FCCP
Sodium acetate, 0.7%	6.0	30.8	0
	7.0	106.0	0
	8.0	80.3	81.5
	9.0	63.9	63.9
Sodium acetate, 1.1% + glucose, 0.75%	6.0	23.5	0
	7.0	87.3	0
	8.0	66.9	61.4
	9.0	53.5	54.0

Note: The acetate oxidation rate was determined in 0.05 M Tris–phosphate buffer.

simultaneous functioning of two anaplerotic pathways (the glyoxylate cycle and the pyruvate carboxylase reaction), may give evidence of increased gluconeogenesis during *Acinetobacter* sp. B-7005 growth on a mixture of acetate and glucose. Indeed, the activity of PEP synthetase, the key enzyme of gluconeogenesis, was twice as high during growth on mixed substrate than during growth on acetate (Table 5). The results obtained indicate a change in the direction of the biosynthetic processes in *Acinetobacter* sp. B-7005 on mixed substrate toward carbohydrate formation.

The results presented in this work of studying the specific features of C_2–C_6 metabolism during growth of *Acinetobacter* sp. B-7005 on a mixture of acetate and glucose are different from those obtained for *Corynebacterium glutamicum* [6]. Thus, when the latter bacterium was cultivated on a mixture of acetate and glucose, acetyl-CoA was mainly formed from acetate, and only an insignificant part was formed in the pyruvate dehydrogenase reaction; glucose was predominantly utilized for generation of the precursors of biosynthesis of cell material [6]. When *C. glutamicum* was grown on mixed substrate, the glyoxylate cycle was the anaplerotic sequence of the reactions replenishing the pool of C_4-dicarboxylic acids, and the PEP carboxylase and pyruvate carboxylase reactions did not function.

Interestingly, the activities of PEP carboxylase and pyruvate carboxylase, the anaplerotic enzymes affording oxaloacetate synthesis, during growth on carbohydrates, were revealed not only when *C. glutamicum* was cultivated on glucose but also on acetate, despite the functioning of the glyoxylate cycle reactions [6]. Our previous investigations [3, 16] and the results of the present work also show that the activities of both glyoxylate cycle enzymes and pyruvate carboxylase were high when *Acinetobacter* sp. B-7005 was grown on ethanol or acetate. It should be mentioned that the ability of this enzyme to decarboxylate oxaloacetate is poorly documented in the literature to date [17, 18]. It is quite possible that, during the growth of *Acinetobacter* sp. B-7005 on C_2-substrates, pyruvate carboxylase may be involved in pyruvate formation (along with oxaloacetate decarboxylase).

Table 5. Activity of some key enzymes of C_2–C_6 metabolism during growth of *Acinetobacter* sp. B-7005 on sodium acetate (1.1%), glucose (0.75%), and a mixture of these substrates

Enzyme	Activity, nmol/(min mg protein), during growth on		
	acetate	glucose	acetate + glucose
Acetyl-CoA synthetase	1636.1	69.6	735.8
Isocitrate lyase	83.4	70.6	855.6
Malate synthase	148.4	154.1	295.9
Isocitrate dehydrogenase	3682.7	5381.5	3063.5
2-Oxoglutarate dehydrogenase	760.0	1479.1	508.6
Pyruvate dehydrogenase	ND	113.6	140.3
Pyruvate carboxylase	4557.3	4972.9	3314.8
PEP synthetase	2272.5	118.1	3977.0

Note: The inoculum was obtained on medium with sodium acetate (0.7%). ND stands for "not determined".

Thus, we have shown for the first time that the synthesis of the microbial polysaccharide ethapolan may be increased when the producer is grown on a mixture of energy-deficient substrates (sodium acetate + glucose), which are utilized simultaneously. Higher values of ethapolan synthesis on a mixture of sodium acetate and glucose (compared to potassium acetate and glucose) may be determined by fact that the sodium cations are involved in the creation of ionic gradients on the membrane, which are necessary for generating the proton-motive force used for the active transport of acetate into *Acinetobacter* sp. B-7005 cells. The increase in the EPS synthesis on a mixture of acetate and glucose is determined by an increase in gluconeogenesis, which is evidenced by simultaneous operation of the pyruvate carboxylase reaction and the glyoxylate cycle, as well as by an increase in the activity of isocitrate lyase, malate synthase, and PEP synthetase as compared to the producer growth on the monosubstrates.

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