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PRODUCTION OF EXOPOLYSACCHARIDE ETHAPOLAN UNDER *Acinetobacter* sp. IMV B-7005 CULTIVATION ON THE MIXTURE OF ACETATE AND SUNFLOWER OIL

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The aim of the work was to establish the cultivation conditions of *Acinetobacter* sp. IMV B-7005 for providing the maximum indicators of the exopolysaccharide ethapolan synthesis on the mixture of acetate and sunflower oil, as well as to study the possibility of replacing the refined oil in the mixture with acetate on a waste one.

The optimal molar ratio of concentrations for refined sunflower oil and acetate in the mixture was calculated theoretically according to Babel's concept of "auxiliary substrate". The EPS concentration was determined gravimetrically after precipitation with isopropanol. The EPS-synthesizing ability was calculated as the ratio of the EPS concentration to the concentration of biomass and expressed in g EPS/g biomass.

Based on theoretical calculations of energy requirements for EPS synthesis and biomass of *Acinetobacter* sp. B-7005 on energy-deficient substrate (acetate) it was found that molar ratio for the concentrations of sodium acetate and oil in the mixture, at which the maximum EPS synthesis was achieved, should be 1:0.13. It was experimentally confirmed that at this ratio of monosubstrate concentrations and the use of the inoculum grown on refined oil, the synthesis rates of ethapolan were higher than at the other ratios of acetate and oil concentrations in the mixture. However, the assimilation of sodium acetate through the symport with proton led to an increase in pH of the culture liquid to 9.0–9.3, which is not optimal for EPS synthesis. Decrease of the medium alkaline component and fractional introduction of substrates enabled not only to stabilize pH at the level of 7.8–7.9, but to increase the amount of synthesized ethapolan to 16–17 g/l, which was achieved regardless of the type of used oil (refined or mixed waste) in the mixture with acetate.

Key words: *Acinetobacter* sp. IMV B-7005, mixed substrates, waste oil, fractional substrate introduction, ethapolan.

Every year new microorganisms are discovered in the world and already known microorganisms capable of synthesizing exopolysaccharides (EPS) are studied in depth [16]. Due to the unique physical and chemical properties (ability to gelling, emulsifying and altering rheological characteristics of water systems, etc.) and biological properties (immunomodulatory, bactericidal, anti-inflammatory, antitumor activity, etc.) these polymers are promising for the use in various industries (food, medical, petroleum, etc.) [4, 6, 8, 13].

At the same time, only some of the studied exopolysaccharides are commercially successful. Thus, xanthan takes 6% of the world market of microbial polysaccharides, which reaches hundreds of billions of dollars [4]. The rest market is divided among dextran, gelan, alginate, levan, pululuan, velan, scleroglucan, hyaluronic acid and some other less-known EPS.

This is mainly due to the fact that the new EPS, in addition to its practically valuable properties, have high production costs determined by low level of polysaccharides synthesis and the use of expensive hydrocarbon substrates for their production. It should be noted that substrate costs can be up to 50% of the final cost of the final product [8].

Therefore, it is not surprising that in the recent years there is more and more information about the synthesis of EPS on cheap substrates, which are usually industrial waste (molasses, milk whey, technical glycerol, wastewater from various industries, etc.) [9].

In our previous work [12] the possibility of synthesis of microbial polysaccharide etapolan (produced by *Acinetobacter* sp. IMV B-7005) on mixtures of molasses and different types of waste oil has been shown. High efficiency of the use of such mixed substrate was achieved by establishing an optimal molar ratio of monosubstrate concentrations in the mixture. Thus, the highest rates of EPS synthesis (EPS concentration 14 g/l, EPS-synthesizing ability 3.5 g EPS/g biomass) were observed in molar ratio of molasses and mixed waste oil 1:1.1, as closely as possible to theoretically calculated (1:0.9). However, under such cultivation conditions, there was an increase not only in EPS concentration, but in biomass as well, and so the EPS synthesizing capacity was 2 times lower than that on oil monosubstrate. We suggested that this problem could be solved by replacing molasses in a mixture with oil on another energy-deficient substrate that did not contain nitrogen, particularly acetate. Thus, it was previously shown that EPS-synthesizing ability under cultivation of *Acinetobacter* sp. IMV B-7005 on a mixture of acetate and glucose reached almost 20 g of EPS/g of biomass [8].

In connection with the above, the aim of this work was to establish the cultivation conditions of *Acinetobacter* sp. IMV B-7005 for the maximum indicators of the exopolysaccharide ethapolan synthesis on the mixture of acetate and sunflower oil, as well as to study the possibility of replacing the refined oil in the mixture with acetate on a waste one.

Materials and Methods

Objects of research. The study object is EPS-synthesizing strain *Acinetobacter* sp. 12S, deposited in the Depository of the Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine under the number IMV B-7005.

Composition of medium and cultivation conditions. The IMV B-7005 strain was grown in liquid medium of the following composition (g/l): medium 1 (base): KH_2PO_4 — 6.8; KOH — 0.9; $MgSO_4 \cdot 7\ H_2O$ — 0.4; $CaCl_2 \cdot 2\ H_2O$ — 0.1; NH_4Cl — 0.8; $FeSO_4 \cdot 7\ H_2O$ — 0.001; medium 2 is similar to medium 1, but the concentration of KH_2PO_4 is halved; medium 3 is similar to medium 2, but KOH concentration is halved; medium 4 is similar to medium 3, but KOH is absent; medium 5 is similar to medium 4, but KH_2PO_4 concentration is halved. Description of the media is given in Table 1.

An additional 0.5% (v/v) of yeast autolysate was added to the medium, as well as the multivitamin complex "Complevit" at a concentration of 0.00085% (w/w by pantothenate).

A mixture of sodium acetate (1–3%, w/w) and refined sunflower oil (0.3–1.75%, v/v) was used as a carbon source. In one of the variants refined oil was replaced with mixed waste oil (after roasting meat, potatoes, onions, and cheese; from "RockerPub", Kyiv).

In one variant, the initial concentration of acetate in the medium was 0.5–1.5 and oil 0.25–0.75%, and during the cultivation process these substrates were fractionally added (fertilization) in portions of 0.5–1.5% (acetate) and 0.25–0.75% (oil). If before fertilization the pH of the culture liquid exceeded 8.0–8.5, acetic acid was applied into the equimolar carbon concentration (0.35%, v/v) instead of acetate.

The culture in exponential growth phase, grown in a medium with oil (0.5%), sodium acetate (0.5%), or a mixture of acetate (0.25%) and oil (0.25%) was used as inoculum. Concentration of inoculum was 10%.

Cultivation of IMV B-7005 strain was carried out in the flasks (750 ml) with 100 ml of medium in shaker (320 rpm) at 30 °C for 120 hours.

Growth and EPS synthesis indicators. The concentration of biomass was determined by optical density of cell suspension with subsequent recalculation to dry biomass in accordance with the calibration curve. The amount of synthesized ethapolan was determined gravimetrically. For this purpose, 1.5–2 volumes of isopropanol were added to a certain volume of culture liquid (usually 10–15 ml). The EPS precipitate was washed with pure isopropanol and dried at room temperature for 24 hours. The EPS-synthesizing ability was calculated as the ratio of the EPS concentration to the concentration of biomass and expressed in g EPS/g biomass.

The theoretical yield of EPS relative to the substrate was calculated considering following assumptions: 1) 50% of substrate carbon is

Table 1. **Characteristics of *Acinetobacter* sp. IMV B-7005 growth medium**

Medium number	Concentration, g/l	
	KH_2PO_4	KOH
1	6.8	0.9
2	3.4	0.9
3	3.4	0.45
4	3.4	0
5	1.7	0

oxidized to produce energy ("idle oxidation"); 2) 50% of carbon is included in biomass and ethapolan [8]; 3) refined sunflower oil contains 50% linoleic and 50% oleic higher fatty acids [14]; 4) nitrogen content in biomass is 10%.

Thus, for example, with 1% (10 g/l) of sodium acetate (carbon content of 2.93 g/l) and 0.5% (4.6 g/l) of oil (carbon content of 3.54 g/l), taking into account "idle oxidation", a total of 6.46 g/l of EPS and biomass can be obtained. Considering, that when using 0.8 g/l of NH_4Cl (nitrogen content 0.21 g) the level of biomass is 2.1 g/l, the maximum concentration of EPS is 4.36 g/l.

To determine the optimal molar ratio of the concentrations of substrates (sodium acetate and refined sunflower oil) in the mixture, appropriate theoretical calculations were made, which are based on determination of the energy requirements for the EPS and biomass synthesis on the energy-excessive substrate (acetate) with subsequent determination of the concentration of the energy-deficient substrate (sunflower oil), which will ensure "coverage" of energy costs for this process, as described in our previous work [12].

Energy costs for ethapolan synthesis from acetate were determined on the basis of the activity of Krebs cycle, glyoxylate cycle and gluconeogenesis enzymes of the strain *Acinetobacter* sp. IMV B-7005 [8]. Energy generation in the catabolism of linoleic and oleic acids was calculated as described earlier [12].

Statistical data processing. All experiments were conducted in triplicate, the number of parallel definitions in the experiments was from three to five. Statistical processing of experimental data was carried out as described earlier [8, 12]. Differences in average indicators were considered to be statistically significant $P < 0.05$.

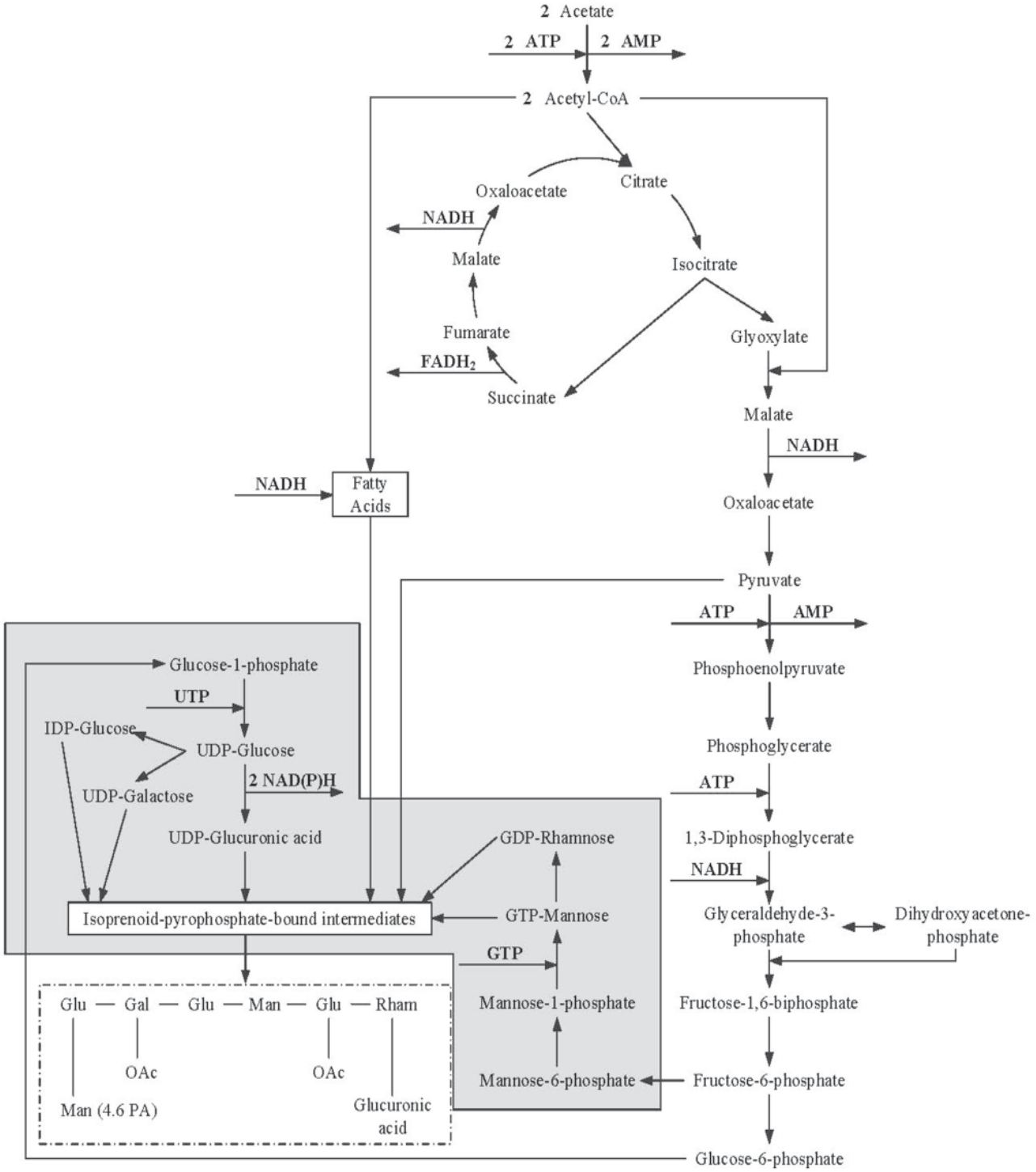
Results and Discussion

Determination of the optimal molar ratio of monosubstrate in a mixture is a complex task, which solution requires theoretical calculations with subsequent experimental testing.

According to Babel's energy classification of substrates [3], acetate is an energy-deficient substrate and oil is an energy-excessive one. Such separation of the substrates is based on the amount of the energy generated during their catabolism to the central carbon precursor — phosphoglyceric acid (PGA). The energy required for the synthesis of cellular components from this precursor is constant and amounts to 1 ATP molecule per 10.5 g of dry biomass [3].

To calculate the optimal ratio of concentrations of acetate and sunflower oil, we acepted the same assumptions as in the work [12] for cultivation of *Acinetobacter* sp. IMV B-7005 on the mixture of oil and molasses (sucrose): refined sunflower oil contains 50% linoleic and 50% oleic acids; EPS contains 50% of acylated (AP) and 50% of non-acylated polysaccharide (NAP); AP contains two residues of fatty acids (lauric and palmitic); NADPH, formed in the catabolism of substrates, is a source of reducing equivalents that are oxidized to water through the respiratory chain; P/O ratio is 2.

ATP requirement for ethapolan synthesis from acetate. The AP repeating unit consists of neutral monosaccharides, some of which, unlike NAP, are acylated and contain glucuronic and pyruvic acid residues. The synthesis of these components from acetate is inextricably bound up with expenditures (synthesis of monosaccharides, fatty acids) and generation of energy (formation of glucuronic and pyruvic acids (PA)) (Figure).



Synthesis scheme of repetitive units in the process of acetate catabolism (literature data are grayed out)

Energy expenditure for the synthesis of monosaccharides. The synthesis of carbohydrate part of ethapolan could be divided into 3 stages:

1. *Phosphoryl carbohydrate formation* (glucose-6-phosphate and fructose-6-phosphate). From the scheme shown in Figure, it can be seen that the total equation of 1 mol of glucose-6-phosphate obtaining from acetate can be expressed as follows:

$$2\ \text{Acetate} + 4\ \text{ATP} + \text{NADH} \rightarrow \rightarrow \text{Glucose-6-phosphate} + 2\ \text{NADN} + + \text{FADN}_2 \quad (1)$$

At P / O = 2, the equation takes form:

$$2\ \text{Acetate} + \text{ATP} \rightarrow \text{glucose-6-phosphate} \quad (2)$$

2. *Synthesis of EPS precursors.* The formation of each precursor is accompanied by the consumption of 1 mole of GTP or UTP.

3. *EPS polymerization.* At the end, the repeating unit is attached to a polysaccharide molecule. During this process, the energy of one macroergic bond is consumed.

The repetitive unit of EPS consists of the remains of 7 neutral monosaccharides and the residue of glucuronic acid. For their synthesis 8 mole of glucose-6-phosphate (fructose-6-phosphate) are needed, which are synthesized from 16 mole of acetate. Thus, the total expenditure of ATP during the synthesis of monosaccharides, which are the part of the repetitive AP units and the addition of this unit to the EPS molecule, is $8 \cdot 2 + 1 = 17$ mole of ATP.

At this stage, an additional 1 mole of NAD(P)H is generated by the formation of glucuronic acid, resulting in 2 mole of ATP. So, energy expenditure for monosaccharides synthesis is $17 - 2 = 15$ mole of ATP.

Energy expenditure for the synthesis of fatty acids. It is known [14] that the formation of higher fatty acids in the form of corresponding acyl-CoA occurs cyclically and is accompanied by the expenditure of 1 mole of ATP per cycle. In addition, 1 mole of ATP is consumed during conversion of acetate to acetyl-CoA (Figure).

Thus, in order to obtain lauric (C_{12}) and palmitic (C_{16}) acids it is necessary to carry out 5 and 7 cycles of synthesis involving 6 and 8 moles of acetyl-CoA, respectively. Thus, energy requirements for fatty acids synthesis, which are the part of the repeated AP unit, are $(5 + 7) + (6 + 8) = 26$ mole of ATP.

Energy generation for PA synthesis. The total reaction of PA formation from acetate can be expressed by the following equation:

$$2\ \text{Acetate} + 2\ \text{ATP} \rightarrow \rightarrow \text{PA} + 2\ \text{NADH} + \text{FADN}_2 \quad (3)$$

Thus, the generation of ATP is:

$$2\ \text{Acetate} \rightarrow \text{PA} + 3\ \text{ATP} \quad (4)$$

Summary data on energy requirements (equation 1–4) for the microbial synthesis of AP and NAP units in terms of one mole of used acetate can be presented in the form of a table (Table 2).

Thus, the total energy expenditure for the synthesis of the repetitive unit of AP and NAP (AP + NAP) is: 1.12 — 0.20 = 0.92 mole of ATP/mol of used acetate.

Energy generation in the catabolism of linoleic and oleic fatty acids. According to calculations given in [12], energy generation in the synthesis of ethapolan from sunflower oil (linoleic and oleic fatty acids) is 39.5 mole of ATP/mole of used oil (equation 5):

$$0.5\ C_{17}H_{31}COOH + 0.5\ C_{17}H_{33}COOH \rightarrow \rightarrow 4.5\ \text{PGA} + 39.5\ \text{ATP} \quad (5)$$

Energy requirements for biomass biomass synthesis. Synthesis of biomass from PGA (using an ammonium nitrogen source) can be represented by the equation [8]:

Table 2. **Energy requirements for the synthesis of acylated and nonacylated polysaccharides from acetate**

EPS	Acetate expenditure for the synthesis of an EPS unit, mol	Energy expenditure, mol ATP		Energy generation, mol ATP	
		For the EPS unit synthesis	Per mol of used acetate	For the EPS unit synthesis	Per mol of used acetate
AP	32	41	1.28	5	0.16
NAP	18	15	0.83	5	0.28
AP + NAP	50	56	1.12	10	0.20

$$4\ PGA + NH_3 + 29\ ATP + 5.5\ NAD(P)H \rightarrow \rightarrow (C_4H_8O_2N_1)_3, \quad (6)$$

where $(C_4H_8O_2N_1)_3$ is the formula of one biomass mole.

The overall transformation of acetate to PGA is expressed in the following equations [3, 8]:

$$2\ Acetate + 3\ ATP \rightarrow \rightarrow PGA + 2\ NAD(P)H + FADN_2. \quad (7)$$

For P / O 2, the equation takes form:

$$2\ Acetate \rightarrow PGA + 2\ ATP. \quad (8)$$

On the basis of the equation for biomass synthesis from PGA (Equation 6) and the equation of acetate catabolism to PGA (Equation 8), it can be calculated that under cultivation on acetate the ATP requirements for the biomass synthesis (per mole of acetate) is 4 mole. It is assumed that this energy can be obtained from fatty acids of oil. Taking into consideration that during EPS synthesis 0.92 mole of ATP/mol of used acetate is generated, it is necessary to obtain 4 + 0.92 = 4.92 mole of ATP out of the oil. It follows from Equation 5 that 0.13 mole of oil fatty acids are required to produce this amount of energy.

So, the molar ratio of acetate and refined sunflower oil in the medium should be 1:0.13. For example, at a sodium acetate concentration of 1% (w/w, 10 g/l, or 0.12 mol), the oil concentration should be 0.016 mol, or 4.5 g/l, or 0.5% (v/v). Thus, the ratio of sodium acetate (w/w) and refined sunflower oil (v/v) in the medium should be 1.0:0.5, or 1.0:0.4 when using potassium acetate.

Synthesis of ethapolane under cultivation of Acinetobacter sp. IMV *B-7005 on a mixture of sodium acetate and refined sunflower oil.*

All theoretical calculations should be accompanied by appropriate experimental studies, thus at the next stage of work the synthesis of ethapolan at different molar ratios of concentrations of sodium acetate and sunflower oil in the mixture was investigated. In these experiments, inoculum was grown on mono- (acetate, oil) and mixed substrates.

Since it was established earlier [8] the positive effect of Na^+ cations on the synthesis of ethapolan during the growth of IMV B-7005 strain on a mixture of acetate and glucose, sodium acetate was used in these studies in the mixture with oil. We have suggested that Na^+ cations are used to generate proton-motive force during the active transport of acetate to cells of the EPS producer.

It has been found that regardless of the nature of the carbon source in the medium for inoculum preparation, the indicators of the ethapolan synthesis were maximum for the thoretically calculated ratio of monosubstrates in the mixture (1:0.13) (Table 3). At the same time, when using oil-grown inoculum, there was an increase in the concentration of synthesized ethapolan and EPS-synthesizing ability to 4.17 g/l and 1.7 g EPS/g biomass, respectively. In this connection, in further experiments inoculum was grown on the refined oil.

The data given in Table 3 indicate that regardless of the concentration of substrates in the mixture, by the end of cultivation there was observed an increase in pH of the culture liquid to 9.0–9.3, which is not optimal for the synthesis of EPS (optimal pH 7.0–8.0 [8]). We assume that this is due to the presence of a sufficiently high concentration of sodium acetate in the mixture (1.0%), which is during the transportation to the cells of producer through the symport with proton led to an increase in pH of the culture liquid [8]. This influence of sodium acetate is partially compensated by assimilation of ammonium chloride, which transport takes place by antiport with proton and is accompanied by acidification of culture liquid [8]. However, the amount of nitrogen source available in the medium is insufficient to stabilize pH at an optimal level for the synthesis of ethapolan. However an increasing the content of ammonium chloride in the medium is inexpedient, as this will reduce the ratio of C/N, that negatively affects the synthesis of EPS [8].

In our opinion, it is possible to reduce the pH of culture liquid during growth of ethapolan producer on the mixture of acetate and oil by reducing an alkaline component in the nutrient medium. For this purpose at the next stage we studied the synthesis of EPS in the modified medium 1–5 with a reduced content of KH_2PO_4 and KOH (Table 1).

Our experiments have shown that cultivation of *Acinetobacter* sp. IMV B-7005 in the modified medium 4 (without KOH and with concentration of KH_2PO_4 3.4 g/l) was accompanied by maintenance of culture liquid pH at the level of 7.8–7.9 (Table 4). Under such conditions, the concentration of ethapolan (4.7 g/l) and EPS-synthesizing ability (2.0 g EPS/g biomass) reached the maximum

Table 3. **Indicators of the ethapolan synthesis on the mixture of sodium acetate and refined sunflower oil depending on the method of inoculum preparation**

Substrate for inoculum growth,%	Concentration of substrates in the medium for biosynthesis,%		pH_{end}	EPS, g/l	EPS-synthesis ability, g of EPS/g biomass
	Acetate	Oil			
Acetate, 0,5	1.0	0.3	9.2	2.51 ± 0.13**	0.72 ± 0.04***
	1.0	0.5*	9.2	3.42 ± 0.17	1.06 ± 0.05
	1.0	0.7	9.3	2.98 ± 0.15**	0.80 ± 0.04***
Oil, 0,5	1.0	0.3	9.0	3.24 ± 0.16**	1.10 ± 0.06***
	1.0	0.5*	9.1	4.17 ± 0.21	1.70 ± 0.09
	1.0	0.7	9.2	3.66 ± 0.18**	1.48 ± 0.07***
Acetate, 0,25, and oil, 0,25	1.0	0.3	9.2	1.71 ± 0.09**	0.38 ± 0.02***
	1.0	0.5*	9.2	2.35 ± 0.12	0.82 ± 0.04
	1.0	0.7	9.3	2.03 ± 0.10**	0.56 ± 0.03***

Notes: * — oil concentration at which the theoretically calculated molar ratio of monosubstrates concentration in the mixture is achieved — 1: 0.13; ** — $P \le 0.05$ relative to control (the EPS concentration by the theoretically calculated molar ratio of monosubstrates concentration in the mixture); *** — $P \le 0.05$ relative to control (the EPS-synthesizing ability at the theoretically calculated molar ratio of monosubstrates concentration in the mixture).

Table 4. **Synthesis of ethapolan on modified medium with sodium acetate and refined oil**

Concentration of sodium acetate and oil in the mixture,%		Molar ratio of sodium acetate and oil	pH_{end}	EPS, g/l	EPS-synthesized ability, g of EPS/g of biomass
Acetate	Oil				
1.0	0.3	1:0.08	7.9	4.03 ± 0.20**	1.78 ± 0.09***
1.0	0.5	1:0.13*	7.8	4.70 ± 0.24	2.00 ± 0.10
1.0	0.7	1:0.18	7.8	4.18 ± 0.21**	1.65 ± 0.8***

Notes: Cultivation was carried out in the modified medium 4; * — optimal molar ratio of concentrations of monosubstrates in the mixture; ** — $P \le 0.05$ relative to control (the concentration of EPS by the theoretically calculated molar ratio of concentration of monosubstrates in the mixture); *** — $P \le 0.05$ relative to control (EPS-synthetized ability by the theoretically calculated molar ratio of monosubstrates concentration in the mixture).

possible level for the given substrate concentration, but they remained lower in comparison with parameters on the mixture of acetate and glucose (molasses) [8, 11].

Evidently to further intensify the synthesis of ethapolan it is necessary to increase the content of monosubstrates in the mixture with simultaneous support of pH at a level optimal for EPS formation.

One of the ways to solve this problem is to reduce the initial concentration of substrates followed by their fractional introduction during the cultivation. So, for example, the use of such approach together with maintenance of pH values of the culture liquid at a level of 7.5 under cultivation of IMV B-7005 strain on a mixture of acetate and molasses was accompanied by 10–45%

Table 5. **Effect of fractional substrate introduction on the ethapolan biosynthesis on the mixture of acetate and oil**

Monosubstrate concentration in the mixture,%	Fractional substrate addition mode,%	pH_{end}	EPS, g/l	EPS-synthesized ability, g of EPS/g of biomass
Acetate, 1,5 + refined oil, 0,75	Without fractional addition (control)	8.7	4.02 ± 0.20	1.07 ± 0.05
	Three portions of 0.5% acetate and 0.25% oil	6.4*	5.67 ± 0.28**	2.00 ± 0.10***
Acetate, 3,0 + refined oil, 1,5	Without fractional addition (control)	9.5	2.84 ± 0.14	0.68 ± 0.03
	Three portions of 1.0% acetate and 0.5% oil	7.8*	13.82 ± 0.69**	4.53 ± 0.23***
	Two portions of 1.0% acetate and 0.5% of oil, the third portion is 0.35% of acetic acid and 0.5% of oil	7.9*	17.27 ± 0.86**	6.47 ± 0.32***
Acetate, 3,0 + waste oil, 1,5	Without fractional addition (control)	9.6	2.31 ± 0.12	0.55 ± 0.03
	Two portions of 1.0% acetate and 0.5% of oil, the third portion is 0.35% of acetic acid and 0.5% of oil	7.7*	16.36 ± 0.82**	7.34 ± 0.37***

Notes: Cultivation was carried out in the modified medium 4. Molar ratio of monosubstrates in the mixture is 1: 0.13; * — $P \leq 0.05$ relative to control (pH without fractional addition of substrates); ** — $P \leq 0.05$ relative to control (concentration of EPS without fractional addition of substrates); *** — $P \leq 0,05$ relative to control (PH without fractional addition).

increase of the indicators of the ethapolan synthesis in comparison with the initial technology [11].

Subsequent experiments showed that reducing the initial concentration of sodium acetate and refined oil in the mixture to 1/3 of their total content, followed by the fractional introduction of three portions of substrates during the cultivation to the final concentration of acetate 1.5–3.0% and oil 0, 75–1.5% enabled to maintain the pH of culture liquid during cultivation at a level of 6.4–7.8 and to increase the ethapolan synthesis rates compared with a single introduction of the corresponding concentration of substrates (Table 5).

It should be noted that after the second addition of 1.0% acetate and 0.5% refined oil the pH of culture liquid increased to 8.0–8.2. Therefore in the third portion of substrates, sodium acetate was replaced with acetic acid in equimolar to carbon quantity. Such approach enabled not only to stabilize pH of the culture liquid at the level optimal for EPS synthesis, but was accompanied by 1.25-fold increase in concentration of the synthesized ethapolan (up to 17.27 g/l, Table 5) as well.

In our previous works [10, 12] the possibility of replacement of refined oil in mono- and mixed substrates with different types of waste oil was demonstrated. At the same time, high rates of ethapolan synthesis were achieved when using mixed sunflower waste oil as a substrate, which is usually formed by mixing different overcooked oil before being sent for utilization.

Therefore, in the next stage the synthesis of ethapolan on the mixture of acetate and waste mixed oil was investigated (Table 5). It was found that replacement of refined oil in the mixture with acetate with mixed waste oil was accompanied by only a slight decrease in the concentration of synthesized ethapolan compared with the use of the refined substrate (from 17.27 to 16.36 g/l), which may be due to the presence of toxic compounds in it (aldehydes, heterocyclic amines, free radicals, etc.) [5, 20]. At the same time EPS-synthesizing ability increased, on the contrary, by 1.13 times and reached 7.34 g of EPS/g of biomass. In general, such features are typical for the synthesis of ethapolan on waste oils [12, 19].

In our previous works [9, 12] we noted that data on the synthesis of EPS on industrial

wastes (both monosubstrates and mixed components) are extremely limited, and the situation has not changed practically to date. Thus, after the publication of the review [8] only a few reports on the use of waste for the production of microbial polysaccharides have appeared in the literature [2, 15, 17, 18].

Asgher et al. [2] found that the mutant *Bacillus licheniformis* MS3 strain synthesized 15.6 g/l EPS under conditions of solid phase cultivation on crushed mango skins. Under cultivation of *Aureobasidium pullulans* MTCC 2013 on a mixture of hydrolyzed kitchen waste, contained 46 and 31 g/l of reducing sugars and glucose, respectively, the concentration of pullulan was 24.77 g/l [15]. It should be noted that the choice of cheap carbon sources for the production of pullulan is especially critical, because its cost is higher ($ 25 per kg) than most other EPS [1]. The review [18] summarizes the data available for the last two decades on the synthesis of pullulan on agricultural wastes (molasses, syrups, sugar cane and sugar beet squeezes, processed seeds and husks, etc.). The concentration of EPS can vary from 6.5 to 90 g/l, depending on the type and concentration of the used waste.

Sengupta et al. [17] established the ability of *Ochrobactrum pseudintermedium* C1 strain to synthesize EPS on different types of waste mineral (lubricant-cooling, hydraulic and compressor) and food (mustard and palm) oils, as well as on molasses. However, the concentration of the final product on all substrates did not exceed 1–2 g/l, which indicates the need for further optimization of cultivation conditions.

An et al. [1] reported the possibility of replacing sucrose with cheaper potato starch hydrolyzate (PSH) for pullulan production by *A. pullulans* 201253. It was found that when cultivating the 201253 strain on monosubstrate PSH the maximum concentration of EPS was reached for 110 h of cultivation, and when using a mixture of PSH (80 g/l) and sucrose (20 g/l) — 60 h. The concentration of pullulan was 54.57 g/l and was almost the same as in the case of the cultivation in the medium with 100 g/l of sucrose. The authors [1] suppose that insignificant amount of sucrose in the mixture can stimulate the activity of enzymes responsible for EPS synthesis and increase the conversion efficiency of PSH. Substitution of sucrose in the mixture with equal parts of glucose and fructose led to a decrease in the synthesis of the final product.

Similar features were also found by Maalej et al. [7] during the optimization of EPS synthesis by *Pseudomonas stutzeri* AS22. It was determined that the addition of small amounts of mannose (1 g/l) under growth of bacteria on starch (50 g/l) allowed increasing the concentration of the synthesized polysaccharide by 32% (up to 10.2 g/l) in comparison with the cultivation on monosubstrate starch. At the same time, the AS22 strain almost did not synthesize EPS on monosubstrate mannose, although it is part of this polymer. In our opinion, this may be due to the low activity of a system of mannose transport into *P. stutzeri* AS22 cells.

In works [1, 7] the ratio of substrate concentrations in a mixture was chosen empirically. It was not always accompanied by finding of optimal cultivation conditions for EPS biosynthesis. At the same time, our previous studies [8, 12] of the intensification of ethapolan synthesis and the results of this work point to the expediency of preliminary theoretical calculations, which allow reducing the volume of experimental works and to establish almost exactly the optimal ratio of monosubstrate concentrations in the mixture that ensures the maximum bioconversion of carbon sources in the final product.

In this paper, in order to intensify the synthesis of ethapolan, firstly, it was theoretically calculated and experimentally confirmed the optimal molar ratio of concentrations of sodium acetate and oil in the mixture (1: 0.13), and secondly, it was modified the composition of the nutrient medium (excluded KOH from the composition and reduced the content of KH_2PO_4 to 3.4 g/l), thirdly, it was carried out a fractional introduction of the substrates. To reduce the cost of the final product it was additionally carried out replacement of refined oil in the mixture with acetate to mixed waste one. In total under such cultivation conditions the concentration of ethapolan and EPS-synthesizing ability was 16.36 g/l and 7.34 g EPS/g biomass, respectively, which is 4 times higher than in the basic medium containing 1.0% acetate and 0.5% refined oil (4.14 g/l EPS, 1.7 g EPS/g biomass, Table 3).

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УТВОРЕННЯ ЕКЗОПОЛІСАХАРИДУ ЕТАПОЛАНУ ЗА КУЛЬТИВУВАННЯ *Acinetobacter* sp. IMB B-7005 НА СУМІШІ АЦЕТАТУ ТА СОНЯШНИКОВОЇ ОЛІЇ

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Метою роботи було встановити умови культивування *Acinetobacter* sp. IMB B-7005, які б забезпечували максимальні показники синтезу мікробного екзополісахариду (ЕПС) етаполану на суміші ацетату та соняшникової олії, а також дослідити можливість заміни рафінованої олії в суміші з ацетатом на відпрацьовану.

Оптимальне молярне співвідношення концентрацій рафінованої соняшникової олії та ацетату в суміші розраховували теоретично згідно з концепцією «допоміжного субстрату» Бабеля. Концентрацію ЕПС визначали ваговим методом після осадження ізопропанолом, ЕПС-синтезувальну здатність — як відношення концентрації ЕПС до біомаси та виражали у г ЕПС/г біомаси.

На основі теоретичних розрахунків енергетичних потреб синтезу ЕПС і біомаси *Acinetobacter* sp. IMB B-7005 на енергетично дефіцитному субстраті (ацетат) встановлено, що молярне співвідношення концентрацій ацетату натрію та олії в суміші, за якого досягається максимальний синтез ЕПС, має становити 1:0,13. Експериментально підтверджено, що за даного співвідношення концентрацій моносубстратів та з використанням інокуляту, вирощеного на рафінованій олії, показники синтезу етаполану були вищими, ніж за інших співвідношень концентрацій ацетату та олії в суміші. Проте асиміляція ацетату натрію симпортом з протоном призводила до підвищення pH культуральної рідини до 9,0–9,3, що є неоптимальним для синтезу ЕПС. Зниження вмісту лужної складової середовища та дробне внесення субстратів дало змогу не лише стабілізувати pH на рівні 7,8–7,9, а й підвищити кількість синтезованого етаполану до рівня 16–17 г/л, якого було досягнено незалежно від типу використаної олії (рафінованої або змішаної відпрацьованої) в суміші з ацетатом.

Ключові слова: *Acinetobacter* sp. IMB B-7005, змішані субстрати, відпрацьована олія, дробне внесення субстратів, етаполан.

ОБРАЗОВАНИЕ ЭКЗОПОЛИСАХАРИДА ЭТАПОЛАНА ПРИ КУЛЬТИВИРОВАНИИ *Acinetobacter* sp. ИМВ B-7005 НА СМЕСИ АЦЕТАТА И ПОДСОЛНЕЧНОГО МАСЛА

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Целью работы было установить условия культивирования *Acinetobacter* sp. ИМВ B-7005, обеспечивающие максимальные показатели синтеза микробного экзополисахарида (ЭПС) этаполана на смеси ацетата и подсолнечного масла, а также исследовать возможность замены рафинированного масла в смеси с ацетатом на отработанное.

Оптимальное молярное соотношение концентраций рафинированного подсолнечного масла и ацетата в смеси рассчитывали теоретически согласно концепции «вспомогательного субстрата» Бабеля. Концентрацию экзополисахарида устанавливали весовым методом после осаждения изопропанолом, ЭПС-синтезирующую способность — как отношение количество синтезированного полисахарида к биомассе и выражали в г ЭПС/г биомассы.

На основе теоретических расчетов энергетических потребностей синтеза экзополисахарида и биомассы *Acinetobacter* sp. ИМВ B-7005 на энергетически дефицитном субстрате (ацетат) установлено, что молярное соотношение концентраций ацетата натрия и масла в смеси, при котором достигается максимальный синтез экзополисахарида, должно составлять 1:0,13. Экспериментально подтверждено, что при данном соотношении концентраций моносубстратов и использовании инокулята, выращенного на рафинированном масле, показатели синтеза этаполана были выше, чем при других соотношениях концентраций ацетата и масла в смеси. Однако ассимиляция ацетата натрия симпортом с протоном приводила к повышению pH культуральной жидкости до 9,0–9,3, что является неоптимальным для синтеза экзополисахарида. Снижение содержания щелочной составляющей среды и дробное внесение субстратов позволило не только стабилизировать pH на уровне 7,8–7,9, но и повысить количество синтезированного этаполана до уровня 16–17 г/л, который достигался независимо от типа использованного масла (рафинированного или смешанного отработанного) в смеси с ацетатом.

Ключевые слова: *Acinetobacter* sp. ИМВ B-7005, смешанные субстраты, отработанное масло, дробное внесение субстратов, этаполан.