

Intensification of microbial exopolysaccharide ethapolan synthesis on the mixture of energy-excessive substrates

Andrii Voronenko, Tetyana Pirog

National University of Food Technologies, Kyiv, Ukraine

Abstract

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Introduction. The cultivation conditions of *Acinetobacter* sp. IMV B-7005, providing maximum synthesis of exopolysaccharide (EPS) ethapolan on the mixture of ethanol and sunflower oil were studied, as well as the possibility of replacing refined oil in the mixture with ethanol on a waste one was demonstrated.

Materials and methods. Strain IMV B-7005 was grown in liquid mineral media, containing the mixture of ethanol and sunflower oil of various quality, as well as appropriate monosubstrates. The optimal molar ratio of the concentrations of substrates in the mixture was calculated theoretically according to Babel's concept. The EPS concentration was determined gravimetrically after precipitation with isopropanol, the EPS-synthesizing ability – as the ratio of the EPS concentration to the concentration of biomass and expressed in g EPS/g biomass.

Results and discussion. The highest rates of ethapolan synthesis were observed with the molar ratio of concentrations of ethanol and refined sunflower oil in the mixture of 1:0.056, as close as possible to the theoretically calculated (1:0.076), and the use of inoculum grown on ethanol. Further increasing of the concentrations of ethanol and oil led to a decrease in pH of the culture fluid to a suboptimal level for the EPS synthesis (4.5-4.8). To ensure the synthesis of ethapolan on a medium with high concentrations of ethanol (4%) and oil (1.2%) ammonium nitrate was replaced with an equimolar amount of nitrogen KNO₃ (0.8 g/l), which is transported into cells by the symport with proton; fractional introduction of substrates in five equal portions during cultivation was carried out and was increased the concentration of Mg²⁺ cations, which are one of the activators of acetyl-CoA synthetase in *Acinetobacter* sp. IMV B-7005 affecting the enzymatic activity of systems responsible for the catabolism of fatty acids. Under such cultivation conditions, regardless of the type of used sunflower oil (refined or mixed waste) in the mixture with ethanol, the concentration of ethapolan reached 13.5-16.0 g/l, and EPS-synthesizing ability – 3.1-3.7 g EPS/g of biomass, which were respectively 3.2-3.8 and 1.6-1.9 times higher than before optimization.

Conclusions. Based on determining the optimal molar ratio of monosubstrate concentrations in the mixture, modification of the medium composition (replacement of ammonium nitrate with potassium nitrate, increasing the content of magnesium cations, replacement of refined oil on a mixed waste one) and fractional addition of substrates the possibility of intensification of ethapolan synthesis on the mixture of energy-excessive substrates (ethanol and sunflower oil) was established.

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Corresponding author:

Andrii Voronenko
E-mail:
voronenkoandr@gmail.com

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Introduction

Production of microbial exopolysaccharides (EPS) is growing every year worldwide (Rana *et al.*, 2020). This is mainly due to the physicochemical and functional properties of these polymers (gelling and emulsifying abilities, ability to retain a large amount of water and alter rheological properties of water systems, anticancer, antioxidant, antimicrobial, antiviral, anti-inflammatory, and immunomodulatory activities, etc.), which make possible their use in various industries, from food to petroleum (Barcelos *et al.*, 2020).

Nevertheless, the same commercially successful EPS (xanthan, dextran, gelatin, alginate, levan, pullulan, welan, scleroglucan, emulsan, hyaluronic acid, etc.), some of which have been known since the first half of the twentieth century, continue to be used in most industries for decades (Donot *et al.*, 2012).

At the same time, most of the new microbial polysaccharides continue to be only at the stage of fundamental research (Zayed *et al.*, 2021). Nowadays, the presence of unique properties is not enough for the industrial implementation of these polymers (Fukuda *et al.*, 2021; Rana *et al.*, 2020). In addition, they must be cheap, which in the biotechnological production of microbial EPS is mainly determined by the final concentration of the target product, the cost, and efficiency of consumption of carbon and energy sources (Barcelos *et al.*, 2020).

One approach to the intensification of microbial synthesis and increasing the efficiency of substrates transformation into biomass and secondary metabolites is the use of a mixture of growth substrates (Babel *et al.*, 1985; Pidhorskyi *et al.*, 2010). In our previous studies, this approach has been successfully used to increase the polysaccharide ethapolan synthesis (produced by *Acinetobacter* sp. IMV B-7005) (Pidhorskyi *et al.*, 2010).

The mixture usually consists of a combination of two substrates, which depending on the amount of energy generated during their catabolism to the central carbon precursor – phosphoglyceric acid (PGA), are divided into energy-excessive and energy-deficient (Babel *et al.*, 1985; Pidhorskyi *et al.*, 2010).

If the amounts of ATP and reducing equivalents produced in the transformation of the substrate to PGA are sufficient for the synthesis of cellular components, such substrate is energy-excessive (Babel *et al.*, 1985; Pidhorskyi *et al.*, 2010). The substrates which must be partially oxidized to CO₂ in order to obtain the energy necessary for constructive metabolism are energy-deficient (Babel *et al.*, 1985; Pidhorskyi *et al.*, 2010).

It should be noted that according to the classical concept of the auxiliary substrate, the use of a mixture of two energy-excessive substrates is not possible (Babel *et al.*, 1985). Meanwhile, it is known about the possibility of using mixed substrates for both energy needs and biomass synthesis (Pidhorskyi *et al.*, 2010).

We suggested that it can be possible to intensify the synthesis of ethapolan by using a mixture of two energy-excessive substrates. Ethanol and sunflower oil were selected as substrates for the studies, because during the growth on them high rates of ethapolan synthesis by strain IMV B-7005 were observed (Pidhorskyi *et al.*, 2010).

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 ethanol and sunflower oil, as well as to study the possibility of replacing the refined oil in the mixture with a waste one.

Materials and methods

Materials

The strain *Acinetobacter* sp. 12S, deposited in the Depository of Institute of Microbiology and Virology, National Academy of Sciences of Ukraine under the number IMV B-7005 was used for microbial polysaccharide ethapolan synthesis (Pidhorskyi *et al.*, 2010).

The complex polysaccharide preparation ethapolan consists of one neutral and two acidic EPS, one of which is acylated (AP). The acylated and non-acylated (NAP) polysaccharides are identical in molar ratios of *D*-glucose, *D*-mannose, *D*-galactose, *L*-rhamnose, *D*-glucuronic acid and pyruvic acid (3: 2: 1: 1: 1: 1) and the structure of repeated unit of the carbohydrate chain. The difference between those EPS is that the acylated polysaccharide contains fatty acids (C₁₂-C₁₈) (Pidhorskyi *et al.*, 2010).

Medium composition and cultivation conditions

The IMV B-7005 strain was grown in such liquid mineral medium (g/l): *medium 1* (*basic*): KH₂PO₄ – 6.8; KOH – 0.9; NH₄NO₃ – 0.6; MgSO₄×7 H₂O – 0.4 (1.6 mM); CaCl₂×2H₂O – 0.1; FeSO₄×7 H₂O – 0.001 (Pidhorskyi *et al.*, 2010); *medium 2* is similar to medium 1, but NH₄NO₃ was replaced with KNO₃ (1.5 g/l); *medium 3* is similar to medium 2, but the concentration of MgSO₄×7 H₂O was 1.25 g/l (5.0 mM).

An additional 0.5% (v/v) of yeast autolysate was added into the medium, as well as the multivitamin complex “Complevit” at a concentration of 0.00085 (w/w by pantothenate) (Pidhorskyi *et al.*, 2010; Pirog *et al.*, 2020).

The following types of substrates were used as a carbon and energy source: *monosubstrates* – ethanol 1.66% (v/v), refined sunflower oil 1.0% (v/v); *mixed substrates* – the mixture of ethanol (1.0-4.0%) and refined oil (0.2-1.2%). In one variant refined oil was replaced with the mixed waste one (after frying meat, potatoes, onions, cheese from “RockerPub”, Kyiv).

In some variants the initial concentration of ethanol in the medium was 0.66-2.0% and oil 0.2-0.6%, respectively. During the cultivation process every 24 h these substrates were fractionally applied (fed-batch) in portions (total 1-4 portions) of 0.66-2.0% (ethanol) and 0.2-0.6% (oil).

The culture in exponential growth phase, grown in a medium with ethanol (0.5%), oil (0.5%) or the mixture of ethanol (0.25%) and refined oil (0.25%) was used as inoculum. Concentration of inoculum was 10% (Pidhorskyi *et al.*, 2010; Pirog *et al.*, 2020).

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 (Pidhorskyi *et al.*, 2010; Pirog *et al.*, 2020).

Biomass and ethapolan estimation

Biomass concentration was determined by optical density of cell suspension with subsequent recalculation to dry biomass in accordance with the calibration curve (Pidhorskyi *et al.*, 2010).

The amount of EPS was determined gravimetrically (Pidhorskyi *et al.*, 2010). For this purpose, 1.5-2 volumes of isopropanol were added to a certain volume of the culture fluid (usually 10-15 ml). The EPS precipitate was washed with pure isopropanol and dried at room temperature for 24 hours.

EPS-synthesizing ability was calculated as the ratio of the EPS concentration to the

biomass and expressed in g EPS/g biomass (Pidhorskyi *et al.*, 2010).

Determination of the optimal molar ratio of substrates concentrations

Determination of the optimal molar ratio of substrates concentrations (ethanol and refined sunflower oil) in the mixture was based on the corresponding theoretical calculations (Pirog *et al.*, 2020). Taking into account that during the metabolism of each energy-excessive substrate to PGA a sufficient amount of ATP and reducing equivalents for the synthesis of cell components is produced, it is necessary to: 1) calculate the energy generation during the EPS synthesis on each substrate; 2) determine the energy distribution of each substrate that occurs after the biomass synthesis.

Determination of energy expenditures for the formation of ethapolan from ethanol was calculated on the basis of the activity of Krebs cycle enzymes, glyoxylate cycle and gluconeogenesis of the strain *Acinetobacter* sp. IMV B-7005, as well as on the calculations as described earlier (Pidhorskyi *et al.*, 2010). Energy generation in the catabolism of linoleic and oleic acids was calculated as described earlier (Pirog *et al.*, 2020).

Since the ethapolan contains residues of fatty acids in its composition, during calculations it was additionally assumed that part of fatty acids of the oil can be used without significant transformations for the esterification of the carbohydrate chain of the EPS. Thus, after the biomass synthesis from fatty acids of oil and ethanol is generated 25 and 75% of total amount of energy, respectively. Other assumptions were similar to those described in the paper (Pirog *et al.*, 2020).

Statistical data processing

All experiments were conducted in three repetitions; the number of parallel definitions in the experiments was from three to five. Statistical processing of experimental data was carried out as described earlier (Pidhorskyi *et al.*, 2010; Pirog *et al.*, 2020). Differences in average indicators were considered reliable at the level of significance $p < 0.05$.

Results and discussion

Theoretical calculation of the optimal molar ratio of ethanol and sunflower oil in the mixture

From the schemes shown in Figures 1 and 2, it is possible to calculate the amount of energy generated during the synthesis of the repeating unit of EPS from ethanol and fatty acids (oleic and linoleic) that prevail in the composition of sunflower oil. The corresponding summary data are represented in Table 1 and 2.

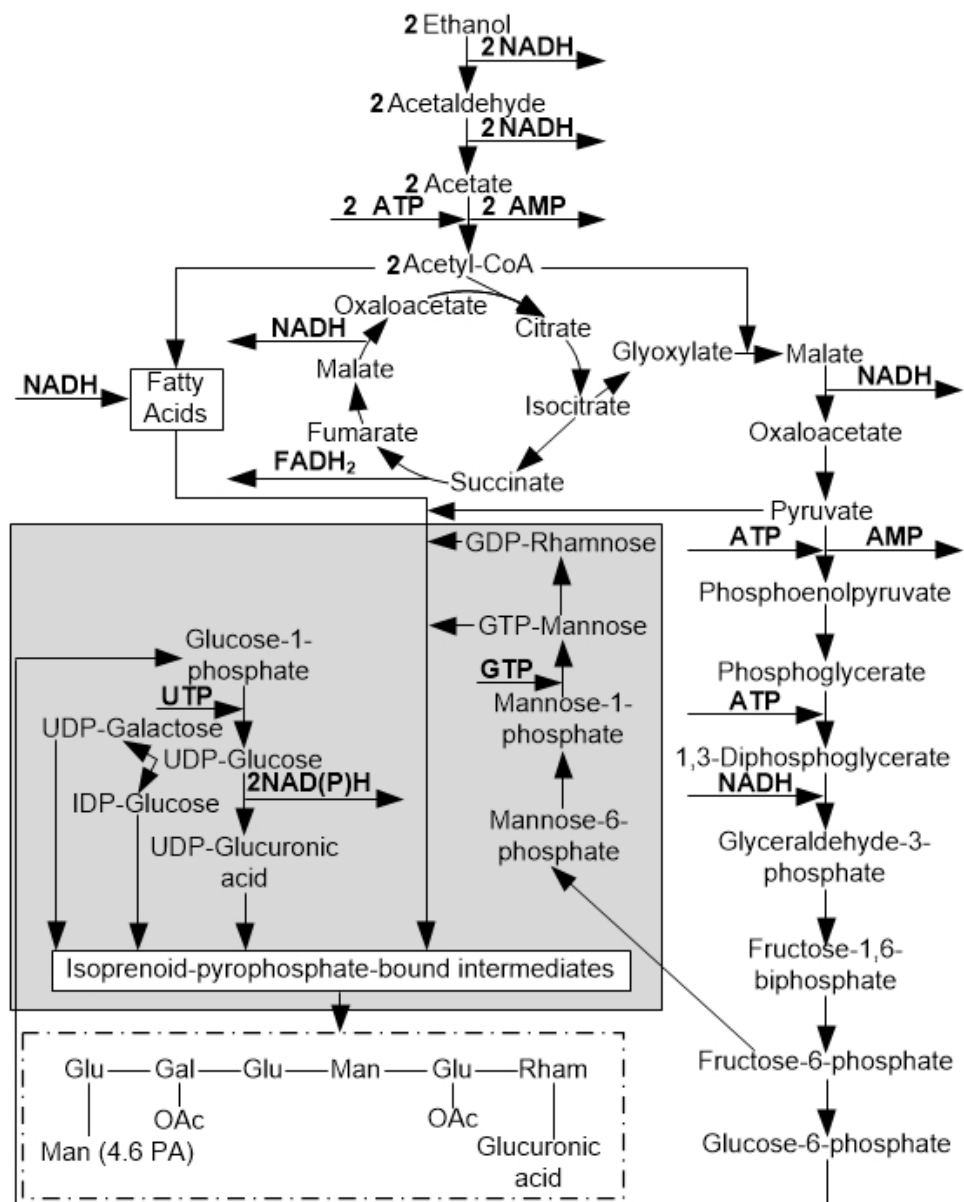


Figure 1. Synthesis scheme of repetitive units in the process of ethanol catabolism (literature data are grayed out)

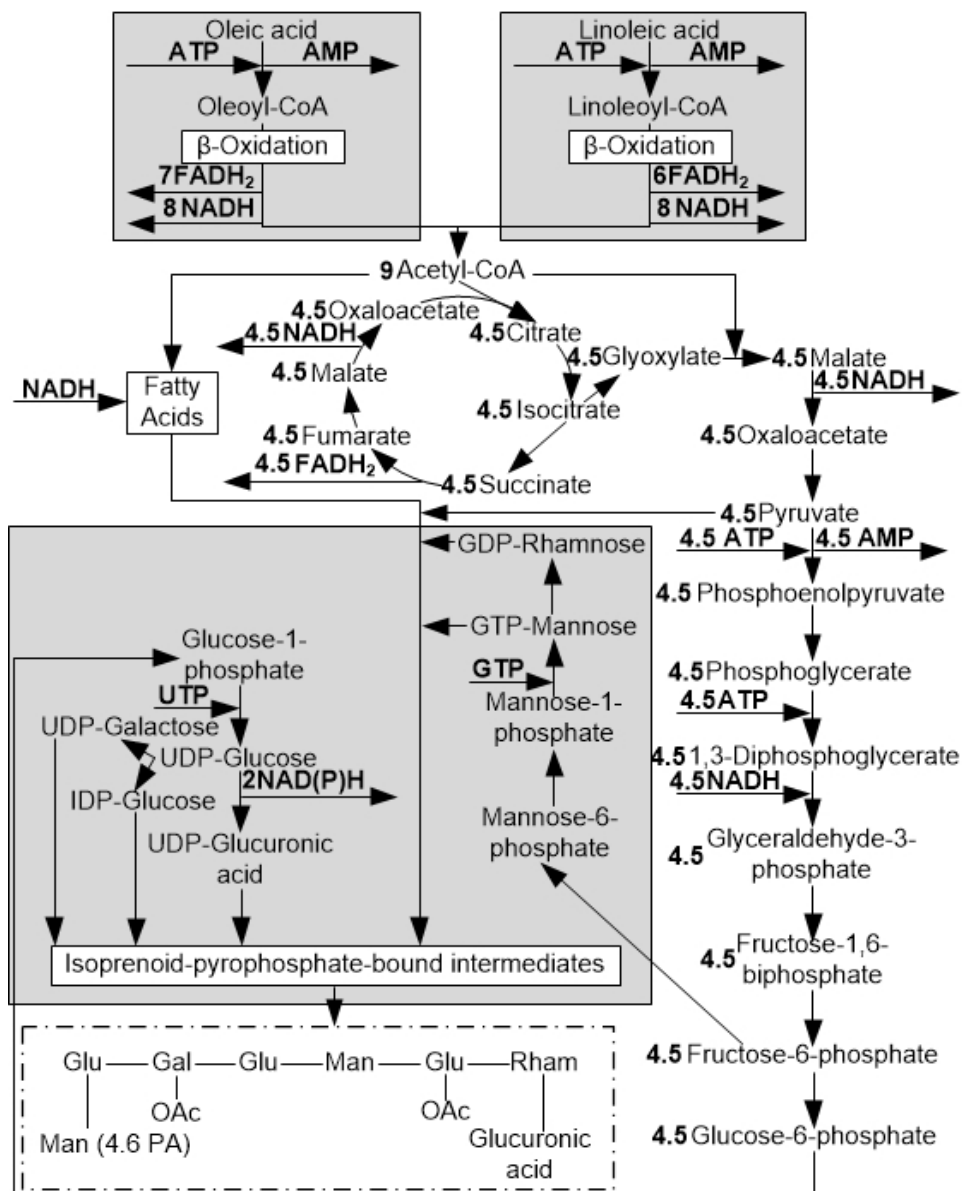


Figure 2. Synthesis scheme of repetitive units in the process of oleic and linoleic acids catabolism (literature data are grayed out)

Table 1
Energy generation during synthesis of the EPS components from ethanol and sunflower oil

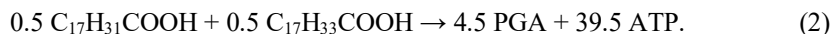
Substrate for the EPS biosynthesis	Stage	Consumption of substrate for the synthesis of the EPS unit, mol	Energy generation, mol ATP
Ethanol	Synthesis of monosaccharides	16.00	51.00
	Synthesis of fatty acids	14.00	29.00
	Synthesis of pyruvate	2.00	11.00
Sunflower oil	Synthesis of monosaccharides	1.78	41.28
	Synthesis of fatty acids	2.00	12.00
	Synthesis of pyruvate	0.22	9.78

Table 2
Energy expenditures of acylated and non-acylated polysaccharides synthesis from ethanol and sunflower oil

Substrate for the EPS biosynthesis	EPS	Consumption of substrate for the synthesis of the EPS unit, mol	Energy generation, mol ATP	
			for the EPS unit synthesis	per mol of used acetate
Ethanol	AP	32	91.00	2.84
	NAP	18	62.00	3.40
	AP + NAP	50	152.00	3.04
Sunflower oil	AP	4	63.06	15.77
	NAP	2	51.06	25.53
	AP + NAP	6	114.12	19.02

Thus, the total energy expenditure for the synthesis of the repetitive unit of AP and NAP (AP + NAP) is: $3.04 + 19.02 = 22.06$ mol ATP/mol of used substrate.

According to the calculations presented in (Pidhorskyi *et al.*, 2010; Pirog *et al.*, 2020), the energy generation during the conversion of ethanol and oil to PGA is 5.0 and 39.5 mol of ATP/mol of the used substrate, respectively (equations 1 and 2).



Synthesis of biomass from PGA (using an ammonium nitrogen source) can be represented by the equation (Pidhorskyi *et al.*, 2010):



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

On the basis of the equation for biomass synthesis from PGA (Equation 3) and the equation of ethanol and oil catabolism to PGA (Equation 1 and 2), as well as the information shown in Table 2, it can be calculated that under cultivation on these substrates after synthesis of biomass and EPS remains 3.04 and 13.52 mol ATP/mol of used substrate, respectively, or 16.56 mol ATP in total.

Assuming that 75% and 25% of excess energy are generated from ethanol and oil, respectively, 4.09 mol of ethanol and 0.31 mol of oil are required to generate 16.56 mol of ATP, which corresponds to a molar ratio of 1:0.076.

For example, at an ethanol concentration of 1% (v/v, 10 ml/l or 8 g/l, 0.12 mol), the oil concentration should be 0.013 mol, or 3.66 g/l, or 3.98 ml/l, or 0.4% (v/v).

Experimental verification of the molar ratio of concentrations of monosubstrates in the mixture

It has been found that regardless of the method of inoculum preparation, the indicators of the ethapolan synthesis were 1.3-2.1 times higher than those obtained on the corresponding monosubstrates (Table 3). In further studies, the inoculum was grown on ethanol, because when used the concentration of the final product was maximum (3.18 g/l).

Table 3

Synthesis of ethapolan depending on the method of inoculum preparation

Concentration of substrates in the medium for		EPS concentration, g/l	EPS-synthesizing ability, g EPS/g biomass
biosynthesis, %	inoculum preparation, %		
Ethanol, 1.0, and oil, 0.4*	Ethanol, 0.5	3.18±0.16	1.85±0.09
	Oil, 0.5	2.14±0.11	1.39±0.07
	Ethanol, 0.25, and oil, 0.25	2.00±0.10	0.84±0.04
Ethanol, 1.66	Ethanol, 0.5	1.49±0.07	0.93±0.05
Oil, 1.00	Oil, 0.5	1.63±0.08	1.10±0.06

Notes. 1. Cultivation was carried out in the medium 1. 2. * – molar ratio of concentrations of ethanol and sunflower oil in the mixture was 1:0.076.

Since theoretical calculations, due to several assumptions, allow only to approximately determining the molar ratio of substrates in the mixture, the synthesis of polysaccharide at different molar ratios of ethanol and oil concentrations in the mixture was further investigated.

It was shown that the highest indicators of the ethapolan synthesis (EPS concentration 4.2 g/l, EPS-synthesizing ability – 2.0 g EPS/g biomass) were observed for the 1:0.056 molar ratio of monosubstrates in the mixture, maximally approximated to the theoretically calculated (1:0.076) (Table 4). In our opinion, this deviation from the theoretical ratio is due to the uneven involvement of each substrate in energetic and constructive metabolism.

Table 4

Influence of molar ratio of ethanol and refined oil in the mixture on the ethapolan synthesis

Concentration of substrates in the mixture, %		Molar ratio of ethanol and oil	EPS concentration, g/l	EPS-synthesizing ability, g EPS/g biomass
ethanol	oil			
1.0	0.2	1:0.036	2.10±0.11	0.49±0.02
1.0	0.3	1:0.056	4.23±0.21	2.00±0.10
1.0	0.4	1:0.076	3.18±0.16	1.85±0.09
1.0	0.5	1:0.096	2.94±0.15	1.20±0.06
1.0	0.6	1:0.116	2.67±0.13	0.79±0.04

Notes. 1. Cultivation was carried out in the medium 1. 2. Inoculum was grown on ethanol.

Intensification of ethapolan synthesis on the mixture of refined sunflower oil and ethanol

One of the important indicators of the effectiveness of technologies for obtaining practically valuable microbial metabolites that directly affects the possibility of their industrial implementation is the concentration of the target product (Fukuda *et al.*, 2021).

It should be noted that the indicators of EPS synthesis on mixed substrates depend not only on the optimal molar ratio of their concentration in the mixture, but also on the total concentration of monosubstrates in the mixture (Pidhorsky *et al.*, 2010).

Experiments have shown that increasing the concentrations of ethanol and oil in the mixture in two-folds to 2.0% and 0.6%, respectively, was not only led to an increase in the amount of synthesized ethapolan by 1.2 times, but also was accompanied by a decrease in the pH of the culture fluid from 7.2 to 5.5 (optimum for the ethapolan synthesis 7.0-8.0). Further increase in the concentration of ethanol (3.0-4.0%) and oil (0.9-1.2%) in the cultivation media led to a drastic decrease in the indicators of EPS synthesis (EPS concentration 1.9-2.4 g/l, EPS-synthesizing ability – 1.0 g EPS/g biomass) and pH of the cultured fluid (4.5-4.8).

This decrease in pH can be due to several reasons, including the consumption of NH₄NO₃ through the antiport with proton and limitation of acetate metabolism due to low activity of acetyl-CoA synthetase, which catalyzes the assimilation of this substrate in the strain IMV B-7005. To solve this problem, the nitrogen source in the culture medium was replaced by an equimolar nitrogen concentration of KNO₃, the assimilation of which is carried out by the symport with proton and is accompanied by an increase in pH of the cultural fluid, as well as fed-batch was applied.

It was found that when replacing the nitrogen source, regardless of the mode of fractional application of substrates, the pH of the culture fluid during cultivation was maintained at an acceptable level for the synthesis of EPS (Table 5). The highest rates of ethapolan synthesis (EPS concentration 13.5 g/l, EPS-synthesizing ability – 3.5 g EPS/g biomass) were observed when reducing the initial concentration of monosubstrates in the mixture to 1/5 of their total content, followed by their fractional application during the cultivation to the final concentration of ethanol 4.0% and oil 1.2%.

Table 5

Influence of nitrogen source replacement and fractional application of substrates on ethapolan biosynthesis on the mixture of ethanol and refined oil

Nitrogen source	Concentration of monosubstrates in the mixture, %	Fractional substrate addition mode*	pH_{end}	EPS concentration, g/l	EPS-synthesizing ability, g EPS/g biomass
NH ₄ NO ₃ (medium 1)	Ethanol, 2.0, and oil, 0.6	Without fractional application (control)	5.5	5.01±0.25	1.95±0.10
		Two portions of 1.0% ethanol and 0.3% oil	5.6	8.02±0.40	1.81±0.09
		Three portions of 0.66% ethanol and 0.2% oil	5.4	9.45±0.47	2.65±0.13
	Ethanol, 4.0, and oil, 1.2	Two portions of 2.0% ethanol and 0.6% oil	5.1	6.82±0.34	1.40±0.07
		Three portions of 1.33% ethanol and 0.4% oil	5.2	8.07±0.40	1.25±0.06
	KNO ₃ (medium 2)	Ethanol, 2.0, and oil, 0.6	Without fractional application (control)	7.0	5.16±0.26
Two portions of 1.0% ethanol and 0.3% oil			6.4	9.27±0.46	2.10±0.11
Three portions of 0.66% ethanol and 0.2% oil			6.6	8.36±0.42	2.17±0.11
Ethanol, 4.0, and oil, 1.2		Two portions of 2.0% ethanol and 0.6% oil	5.7	7.45±0.37	1.18±0.06
		Three portions of 1.33% ethanol and 0.4% oil	5.9	11.38±0.57	1.59±0.08
		Four portions of 1.0% ethanol and 0.3% oil	5.9	11.88±0.59	3.08±0.15
		Five portions of 0.8% ethanol and 0.24% oil	6.0	13.51±0.68	3.65±0.18

Notes:

1. Inoculum was grown on ethanol.

2. * – during the cultivation process fractional addition of substrates was carried out every 24 hours

Replacement of the refined oil in the mixture with ethanol on a waste one

It is known that substrate costs can be up to 50% of the final cost of the target product (Fukuda *et al.*, 2021). To further reduce the cost of ethapolan, the refined oil in the mixture with ethanol was replaced on a mixed waste one, which is usually formed by mixing various fried oils before sending for utilization. It should be noted that these experiments were carried out with an additional increase in the cultivation medium of the concentration of Mg^{2+} cations, affecting the enzymatic activity of systems responsible for fatty acid catabolism and are one of the activators of acetyl-CoA synthetase in the producer of EPS.

It was established that with increasing content of Mg^{2+} up to 5 mM in the medium with ethanol and waste oil, regardless of the mode of fractional addition of substrates (total 4 or 5 portions), an increase in the amount of synthesized polysaccharide to 15.5-16 g/l was observed, which is higher than the synthesis indicators on the basic medium with ethanol and refined oil (10-13.5 g/l) (Table 6).

Table 6
Indicators of ethapolan synthesis on the mixture of ethanol (4.0%) and sunflower oil (1.2%) of different quality

Content of Mg^{2+} in the medium, mM	Quality of oil in the mixture with ethanol	Fractional substrate addition mode*	pH _{end}	EPS concentration, g/l	EPS-synthesizing ability, g EPS/g biomass
1.6 (medium 2)	Refined oil	Four portions of 1.0% ethanol and 0.3% oil	5.9	11.88±0.59	3.08±0.15
		Five portions of 0.8% ethanol and 0.24% oil	6.1	13.51±0.68	3.65±0.18
	Mixed waste oil	Four portions of 1.0% ethanol and 0.3% oil	6.2	12.02±0.60	3.24±0.16
		Five portions of 0.8% ethanol and 0.24% oil	6.1	10.10±0.51	2.73±0.14
5.0 (medium 3)	Refined oil	Four portions of 1.0% ethanol and 0.3% oil	6.1	10.37±0.52	2.58±0.13
		Five portions of 0.8% ethanol and 0.24% oil	6.2	9.86±0.49	2.19±0.11
	Mixed waste oil	Four portions of 1.0% ethanol and 0.3% oil	6.0	15.63±0.78	3.33±0.17
		Five portions of 0.8% ethanol and 0.24% oil	5.9	15.95±0.80	3.11±0.16

Notes.

- Inoculum was grown on ethanol.
- * – during the cultivation process fractional addition of substrates was carried out every 24 hours.

It turned out to be unexpected, but the additional introduction of Mg^{2+} into the medium with ethanol and refined oil led to a slight decrease in the synthesis of EPS. This may be due to the different effects of magnesium cations on the enzymatic systems responsible for the catabolism of fatty acids and other components of sunflower oil. For example, in *Lactococcus lactis* ssp. *lactis* concentration of Mg^{2+} , which provides maximum enzymatic activity, differs for various enzymes of the β -oxidation system (Li *et al.*, 2014).

According to literature data, the use of mixed substrates is primarily associated with the production of various microbial metabolites (enzymes, ethanol, methane, lipids, etc.) from lignocellulosic biomass (wood, straw, pulp, etc.) (Chukwuma *et al.*, 2021). At the same time, information on the use of lignocellulose to obtain microbial EPS is limited (Liu *et al.*, 2020; Wang *et al.*, 2020; Wu *et al.*, 2021).

Wu *et al.* (Wu *et al.*, 2021) established the ability of *Sphingomonas sanxanigenens* NX02 to synthesize microbial EPS sanxan on a mixture of glucose and xylose. Regardless of the ratio of substrates in the mixture (7:3, 5:5, 3:7) their consumption rate, EPS concentration and biomass level in the first 24 h of cultivation were 1.1-2.3 times higher than those obtained on the corresponding monosubstrates. Meanwhile, at the end of cultivation the indicators were at the same level regardless of the type of used substrate (mono- or mixed). It should be noted that the replacement of refined carbohydrates with 4% (w/w by carbohydrates) of corn straw hydrolyzate (ratio of glucose and xylose 3.48:1) was accompanied by an additional increase in the amount of synthesized sanxan to 13.4 g/kg.

Other researchers (Liu *et al.*, 2020) report the ability of *Aureobasidium melanogenum* TN2-1-2 on the medium containing 11% (w/w by carbohydrates) of wheat straw hydrolyzate (glucose to xylose ratio 78%:22%) to synthesize 55.1 g/l of pullulan. During cultivation on corresponding monosubstrates (glucose and xylose) the EPS concentration was 58.3 and 50.2 g/l, respectively.

Note that the above producers due to the functioning of independent metabolic systems of glucose and xylose consumption grown in mixotrophic conditions.

Recently, new information about the EPS synthesis on mixture of waste substrates has appeared. Fathiyah *et al.* (Fathiyah *et al.*, 2021) demonstrated the ability of *Acetobacter xylinum* to accumulate 4.5 g/l of bacterial cellulose during growth on a mixture of oil palm frond juice and coconut water (optimal ratio 60:40). Under cultivation in a medium containing 8% (w/w by carbohydrates) of mixed fruit waste the strain *Bacillus* sp. SRA4 synthesized 25.1 g/l EPS for 72 h (Vaishnav *et al.*, 2020).

In the work (Gao *et al.*, 2020) it was shown that additional application of 5.09% (w/w by carbohydrates) of cane molasses powder during solid-state cultivation of *Kosakonia cowanii* TL-1 strain on the medium, which contains a mixture of cane bagasse and broadbean seed capsule (optimal 2:1 ratio), was accompanied by an increase in the EPS yield in 7.16 times (up to 0.42 g of EPS/g of substrate).

One of the features of the microbial polysaccharide ethapolan, which distinguishes it from other known EPS, is the ability to use various oil-containing (refined and waste sunflower, olive oil, etc.) and C_2 - C_6 -substrates (carbohydrates, ethanol, acetate, organic acids, etc.), as well as their mixtures for its production (Pidhorskyi *et al.*, 2010; Pirog *et al.*, 2020).

It should be noted that nowadays information about the use of oil-containing substrates for microbial EPS is very limited, and in available data the concentration of the target product does not exceed a few grams per liter (Pirog *et al.*, 2021; Pirog *et al.*, 2016).

At the same time, this work demonstrated the possibility of obtaining 16 g/l EPS under cultivation of strain IMV B-7005 on the mixture of ethanol and mixed waste oil.

Conclusion

Thus, during the studies the possibility of microbial EPS ethapolan synthesis on a mixture of energy-excessive substrates (ethanol and sunflower oil) was established. To intensify the EPS synthesis, a comprehensive approach was used, which included:

1. Calculation and experimental confirmation of the optimal molar ratio of ethanol and refined sunflower oil concentration in the mixture (1:0.056);
2. Stabilization of pH of the medium at an acceptable level for the EPS synthesis while using high concentrations of ethanol and refined oil in the mixture (4% and 1.2%, respectively) by replacing the nitrogen source (NH_4NO_3 with KNO_3), establishing the optimal mode of fractional addition of substrates (5 equal portions during cultivation) and increasing the concentration of Mg^{2+} cations to 5.0 mM (acetyl-CoA-synthetase activator);
3. Replacement of refined oil in the mixture with ethanol on a mixed one.

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