Ethapolan synthesis by *Acinetobacter* sp. IMV B-7005 on the mixture of C₂-C₆-substrates and waste sunflower oil

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Abstract

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Introduction. The cultivation conditions of *Acinetobacter* sp. IMV B-7005, which would provide maximum synthesis of the microbial exopolysaccharide (EPS) ethapolan on the mixture of C₂-C₆-substrates (molasses, acetate, or ethanol) and waste sunflower oil was studied.

Materials and methods. Strain IMV B-7005 was grown in liquid mineral media, containing the mixture of C₂-C₆-substrates and sunflower oil of various quality (refined, waste oil after frying potatoes, meat, vegetables, or mixed one after frying various foods). 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. After replacement of refined sunflower oil in the mixture with molasses on various types of waste one (after frying potatoes, meat, vegetables, or mixed oil after frying various foods) the concentration of synthesized ethapolan was 10–14 g/l. Further neutralization of the hydrolyzed molasses with a simultaneous increase in the concentration of monosubstrates in the mixture to 3.0% was accompanied by an increase in the amount of synthesized EPS to 15.3–16.3 g/l, which remained at the same level regardless of the presence in the mixed substrate of different batches of mixed waste oil. Maximum indicators of ethapolan synthesis (15.6–15.9 g/l) on the mixture of ethanol and waste oil were achieved in the case of increasing in the culture medium content of magnesium cations (activator of key enzyme of C₂-metabolism in the producer of ethapolan) from 1.5 to 5 mM and fractional application of substrates (4 or 5 portions). Replacement of the refined substrate on mixed waste oil after frying various foods in the mixture with sodium acetate was accompanied by a slight decrease in the amount of synthesized ethapolan (from 17.3 to 16.4 g/l). However, EPS-synthesizing ability reached 7.3 g EPS/ g biomass, which was 3.7 and 2.2 times higher than the indicators obtained during the cultivation of ethapolan producer on the mixture of mixed waste oil with molasses and ethanol, respectively.

Conclusions. The obtained results are the basis for the development of universal technology for the ethapolan synthesis on mixed substrates, independent of the supplier and the quality of waste sunflower oil.

Introduction

Fierce competition in the global market of microbial polysaccharides greatly complicates the possibility of successful commercial implementation of new alternative exopolysaccharides (EPS) (Rana et al., 2020; Zayed et al., 2021). Thus, for decades, the most well-known representative of these biopolymers, which currently reaches nearly 6% of their world market, is xanthan. Several other polysaccharides have also become widespread: dextran, pullulan, levan, alginate, scleroglucan, velan, hyaluronic acid, etc. (Barcelos et al., 2020).

Although new polymers may have unique properties due to their high cost and low yield, they cannot compete with existing successful EPS.

The main ways to solve these problems are to intensify the synthesis of the target product and reduce the cost of the main components that affect its cost, in particular, the cost of carbon and energy sources.

Maximum EPS synthesis can be ensured by:

1. Increasing the efficiency of substrate conversion into the final product;
2. Optimization of cultivation conditions (Suryawanshi et al., 2019);
3. Improvement of producer strains by methods of metabolic and genetic engineering (Sun et al., 2021).

A promising approach that allows achieving a significant reduction in unproductive carbon and energy costs and intensifying the synthesis of secondary metabolites with minimal labor costs is the use of mixed substrates (Pidhorsky et al., 2010; Pirog et al., 2013; Liu et al., 2020).

Previous studies (Pidhorsky et al., 2010) have shown the possibility of intensifying the synthesis of exopolysaccharide ethapolan (producer of the strain *Acinetobacter* sp. IMV B-7005) on the combination of energy excess (ethanol and glucose, fumarate and glucose) and energy deficient (acetate and glucose) growth substrates. Subsequent studies have allowed to replace glucose in mixed C₂-C₆-substrates with molasses (by-product of sugar production) (Pirog et al., 2017).

Additional reduction of the cost of microbial polysaccharides is possible due to the replacement of classical expensive carbohydrate substrates on the cheap ones (Singh et al., 2019; Oet al., 2020).

One of such promising substrates is waste vegetable oil (sunflower, olive, corn, etc.), which due to its toxicity (contains carcinogens, toxic chemicals, etc. (0, 0)) cannot be reused in the food industry (food preparation, production of food additives) and agriculture (production of feed additives) (Oet al., 2019).

Unfortunately, most of this waste is currently drained uncontrollably into the sewer, which leads to a number of economic (rapid wear of pipelines) and environmental (1 liter of waste oil can contaminate up to 500 thousand liters of water) problems (Oet al., 2019; Loizides 0). At the same time, significant global volumes of waste oil generation (16.5 million tons per year) and the fact that its emissions to the environment are strictly regulated only in highly developed countries (USA, Japan, European countries etc.) makes the effective disposal and processing of this waste is an urgent problem (0; Mannu et al., 2020).

Currently, waste oil is usually used for biodiesel production and livestock fattening (0). Relatively new approaches of its utilization are the production of hydrogen gas, pyrolytic oil, rubber and electricity generation (Mannu et al., 2020). The main disadvantage of these approaches is the need for additional stages of pre-treatment that significantly increase the overall costs of waste oil utilization.

Another promising way to process this waste is its use in biotechnology as a substrate for the synthesis of practically valuable microbial products (surfactants, polyhydroxyalkanoates, organic acids, enzymes, vitamins, etc.) (Oet al., 2020; Orjuela et al., 2020).

It should be noted that to date, information about the use of oil-containing substrates for the microbial EPS biosynthesis is quite limited, and in the available literature, the concentration of the target product does not exceed a few grams per liter (Pirog et al., 2021).

Recently, due to the significant emulsifying properties and structural features of the microbial polysaccharide ethapolan (containing residues of palmitic, oleic, palmitoleic, lauric and stearic acids) the substrates base for its production was expanded through the use of oil-containing raw materials (refined and various types of waste sunflower, corn, olive, rapeseed oil, and their mixtures) (Voronenko et al., 2020).

The purpose of this work is to establish the cultivation conditions of *Acinetobacter* sp. IMV B-7005, which would provide maximum synthesis of the microbial polysaccharide ethapolan on the mixture of C₂-C₆-substrates (molasses, acetate, or ethanol) and waste sunflower oil.

Materials and methods

Materials

For the microbial polysaccharide ethapolan production the EPS-synthesizing 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 (Pidhorsky et al., 2010).

Medium composition and cultivation conditions

The strain was grown in such liquid mineral medium (g/l):

- *Medium 1 (basic)*: KH₂PO₄ – 6.8; KOH – 0.9; NH₄NO₃ – 0.2; MgSO₄×7 H₂O – 0.4 (1.6 mM); CaCl₂×2H₂O – 0.1; FeSO₄×7 H₂O – 0.001;
- *Medium 2* was similar to medium 1, but NH₄NO₃ was absent;
- *Medium 3* was similar to medium 1, but NH₄NO₃ (0.2 g/l) was replaced with KNO₃ (1.5 g/l);
- *Medium 4* was similar to medium 3, but the concentration of MgSO₄×7 H₂O was raised to 1.25 g/l (5.0 mM).
- *Medium 5* was similar to medium 1, but NH₄NO₃ (0.2 g/l) was replaced with NH₄Cl (1.5 g/l), KH₂PO₄ concentration was decreased to 3.4 g/l and KOH was absent.

Table 1

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

Medium number	Nitrogen source	Concentration, g/l			
		Nitrogen source	KH ₂ PO ₄	KOH	MgSO ₄ ×7 H ₂ O
1	NH ₄ NO ₃	0.2	6.8	0.9	0.4 (1.6 mM)
2		0	6.8	0.9	0.4 (1.6 mM)
3	KNO ₃	1.5	6.8	0.9	0.4 (1.6 mM)
4		1.5	6.8	0.9	1.25 (5.0 mM)
5	NH ₄ Cl	0.8	3.4	0	0.4 (1.6 mM)

An additional yeast autolysate (0.5%, v/v) was added into the medium, as well as the multivitamin complex “Complevit” (0.00085, w/w by pantothenate) (Pidhorsky et al., 2010).

The following mixed substrates were used as a carbon and energy source: the mixture of molasses (1.5-3.0%, w/w by carbohydrates) and oil (1.5-3.0%, v/v); ethanol (4.0%, v/v) and oil (1.2%, v/v); sodium acetate (3.0%, w/w) and oil (1.5%, v/v).

In some variants refined sunflower oil was replaced with various waste oils: after frying vegetables (obtained at home after three cycles of frying for 20 minutes each), after frying potatoes and meat (from “McDonald’s” restaurant network, Kyiv), and mixed waste oil after frying various foods (meat, potatoes, onions, and cheese; from “RockerPub”, Kyiv).

In some variants, fed-batch cultivation was carried out. For that purpose the initial concentration of acetate and oil in the medium was 1.0 and 0.5% respectively, and during the cultivation these substrates were fractionally added in two portions of 1.0% (acetate) and 0.5% (oil). In one variant, acetate was replaced with an equimolar concentration of acetic acid (0.35%).

In other variant, the initial concentration of ethanol and oil in the medium was 0.8-1.0% and 0.24-0.3% respectively. During the cultivation these substrates were fractionally applied in portions (total 3-4 portions) of 0.8-1.0% (ethanol) and 0.24-0.3% (oil).

Substrates were added every 24 hours from the beginning of the cultivation process until the final concentration of mixed substrate was reached.

As inoculum was used the culture in exponential growth phase, grown in the medium with monosubstrates: 0.5 % refined and waste sunflower oil, or 0.5 % ethanol. Concentration of inoculum was 10% (Pidhorsky et al., 2010).

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 (Pidhorsky et al., 2010).

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 (Pidhorsky et al., 2010).

The amount of EPS was determined gravimetrically (Pidhorsky et al., 2010). For this purpose, 1.5-2.0 volumes of isopropanol were added to a certain volume of the culture fluid (usually 10-15 ml). The polysaccharide precipitate was washed with pure isopropanol and dried at room temperature for 24 hours.

EPS-synthesizing ability was calculated as the ratio of the polysaccharide concentration to the biomass and expressed in g EPS/g biomass (Pidhorsky et al., 2010).

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 (Pidhorsky et al., 2010). Differences in average indicators were considered reliable at the level of significance $p < 0.05$.

Results and discussion

Influence of the type of waste oil in the mixture with molasses on the polysaccharide synthesis

One of the promising substrates for the ethapolan synthesis is the mixture of molasses and refined sunflower oil. In previous studies (Pirog et al., 2017) the possibility of synthesizing 10 g/l EPS on the mixture of molasses (1.5%, by carbohydrates) and oil (1.5%) was demonstrated.

To further reduce the cost of carbon and energy source, the possibility of replacing refined oil on the waste one in the mixture with molasses was investigated. In these studies, the inoculum was grown on the appropriate waste oil. It is noteworthy that such oil does not require sterilization and is currently much cheaper than molasses.

Experiments have shown that when replacing refined oil in the mixed substrate with different types of waste one (after frying potatoes, meat, vegetables, or mixed waste oil after frying various foods) the concentration of synthesized ethapolan (10–14 g/l) was the same as when using a refined substrate (10–12.5 g/l) (Table 2).

It should be noted that from a practical and economic point of view, for the inoculum preparation and EPS biosynthesis, it is most appropriate to use mixed waste oil, because before sending for disposal, different batches of oil after frying different foods are usually mixed.

Table 2

Ethapolan biosynthesis on mixture of different type of waste oil (1.5%) and molasses (1.5%)

Type of oil in the mixture with molasses	EPS, g/l	EPS-synthesizing ability, g EPS/g biomass
Refined (control)	10.09±0.50	3.60±0.18
Waste oil after frying potato (fries)	11.06±0.55	2.61±0.13
Waste oil after frying potato (wedges)	11.66±0.58	2.69±0.13
Waste oil after frying meat	12.41±0.62	3.42±0.17
Waste oil after frying vegetables	9.94±0.50	2.95±0.15
Mixed waste oil after frying various foods	13.92±0.70	3.49±0.17

Note. The inoculum was grown on medium 1, EPS biosynthesis on medium 2. The inoculum was grown on the refined or appropriate type of waste oil.

Since *Acinetobacter* sp. IMV B-7005 enzyme systems involved in sucrose catabolism have low activity in these studies molasses was hydrolyze before usage. The pH of the substrate was reduced to 4.0–4.5, which led to a decrease in the initial pH of the culture fluid to 5.8–6.0, which are suboptimal for the growth of the producer and the ethapolan synthesis (Pidhorskyy et al., 2010).

It was previously shown that the use of neutralized after hydrolysis molasses in the mixture with C₂-substrates (ethanol or acetate) allowed not only to avoid excessive decrease of the initial pH of the culture fluid, but was also accompanied by an additional increase in the synthesis of ethapolan (Pidhorskyy et al., 2010).

Therefore the effect of the method of molasses preparation on the synthesis of EPS during growth of strain IMV B-7005 on its mixture with waste oil was further investigated.

Experiments showed that regardless of the content of neutralized molasses and oil in the mixture (1.5–3.0%) the amount of synthesized polysaccharide was 1.15–1.25 times higher than the concentration obtained with the use of conventional hydrolyzed substrate (Table 3).

Table 3

Indicators of ethapolan synthesis on the mixture of molasses and oil depending on the method of molasses preparation

Method of molasses preparation	Concentration of monosubstrates in the mixture, %		pH _{end}	EPS, g/l	EPS-synthesizing ability, g EPS/g biomass
	Molasses	Oil			
Hydrolyzed without neutralization (control)	1.5	Refined, 1.5	6.9	10.09±0.50	3.60±0.18
Neutralized after hydrolysis	1.5	Refined, 1.5	7.0	12.15±0.61	2.25±0.11
	1.5	Mixed waste (1)*, 1.5	7.0	11.28±0.56	1.43±0.07
	1.5	Mixed waste (2)*, 1.5	7.2	11.73±0.59	2.16±0.11
	1.5	Mixed waste (3)*, 1.5	7.2	10.28±0.51	1.17±0.06
Hydrolyzed without neutralization (control)	2.0	Refined, 2.0	6.9	11.54±0.58	3.02±0.15
Neutralized after hydrolysis	2.0	Refined, 2.0	7.0	14.45±0.72	2.59±0.13
	2.0	Mixed waste (1)*, 2.0	7.1	12.23±0.61	1.29±0.06
	2.0	Mixed waste (2)*, 2.0	7.2	12.93±0.65	1.78±0.09
	2.0	Mixed waste (3)*, 2.0	7.3	13.51±0.68	1.39±0.07
Hydrolyzed without neutralization (control)	3.0	Refined, 3.0	6.8	13.09±0.65	3.11±0.16
Neutralized after hydrolysis	3.0	Refined, 3.0	7.3	16.25±0.81	1.95±0.10
	3.0	Mixed waste (1)*, 3.0	7.4	16.02±0.80	1.96±0.10
	3.0	Mixed waste (2)*, 3.0	7.6	15.28±0.76	1.47±0.07
	3.0	Mixed waste (3)*, 3.0	7.4	15.28±0.76	1.50±0.08

Note. The inoculum was grown on medium 1, EPS biosynthesis on medium 2. The inoculum was grown on the refined or mixed waste oil after frying various foods was used. * – the batch number of mixed waste oil after frying various foods was used is given in brackets.

It should be noted that the indicators of ethapolan synthesis on different batches of mixed waste oil after frying various foods did not change for the same concentration of monosubstrates in the mixture. A slight decrease in the amount of synthesized ethapolan and EPS-synthesizing ability compared to the use of refined substrate may be due to the presence in the waste oil of various toxic compounds (aldehydes, free radicals, etc.), which are formed during frying (0).

In general, the highest rates of ethapolan synthesis (concentration of EPS was 15.3–16.3 g/l, EPS-synthesizing capacity was 1.5–2.0 g EPS/g biomass) were observed with the use of 3.0% (by carbohydrates) neutralized molasses in the mixture with 3.0% of refined or mixed waste oil after frying various foods.

Note that various methods of molasses pre-treatment are widely used to increase the EPS synthesis on this substrate (Oet al., 2019; Ai et al., 2015).

It is known that molasses contains water, sucrose (45–55%), organic acids, amino acids, vitamins, and minerals, metal cations (Palmonari et al., 2020). Most of these compounds promote the growth of microorganisms and the synthesis of the final product, but, on the other hand, the presence of heavy metals in the culture medium can cause certain problems during biosynthesis. In particular, they are able to inhibit the growth of microorganisms and lead to the inactivation of enzymes involved in the synthesis of the target product (Ai et al., 2015). At the same time, such inhibitors can be partially removed by pre-treatment of molasses with acid, activated carbon, calcium phosphate, etc. (Li et al., 2020; Gojic-Cvijovic et al., 2019).

Replacement in the mixture with C₂-substrates of refined oil on mixed waste oil after frying various foods

It should be noted that during growth of the IMV B-7005 strain on the mixture of molasses and oil, there was a decrease in EPS-synthesizing capacity by almost 2 times compared to the use of oil as monosubstrate. We have suggested that this may be due to the insufficient C/N ratio due to the presence of additional nitrogen in the molasses.

Therefore, in the next stage, the possibility of ethapolan synthesis on the mixture of waste oil and substrate that do not contain nitrogen, in particular ethanol or acetate, was investigated. Due to the peculiarities of the pH change of the culture fluid during the cultivation of the IMV B-7005 strain on the mixture of refined oil and C₂-substrates (Pidhorskyi et al., 2010) fed-batch cultivation was carried out.

In subsequent experiments during refined oil replacement in the mixture with ethanol on mixed waste oil after frying various foods additional increase of the concentration of Mg²⁺ cations in the culture medium was applied. These cations are one of the activators of acetyl-CoA synthetase – key enzyme C₂-metabolism in *Acinetobacter* sp. IMV B-7005. Low activity of acetyl-CoA synthetase in the IMV B-7005 strain is one of the reasons of acetate metabolism limitation.

It was found that when the content of Mg²⁺ increased to 5 mM indicators of ethapolan synthesis (concentration of EPS was 15.6–15.9 g/l, EPS-synthesizing capacity was 3.1–3.3 g EPS/g biomass) on the mixture of ethanol and mixed waste oil after frying various foods did not depend on the method of fractional application of substrates (4 or 5 portions) and were higher than results obtained on the mixture of ethanol and refined oil (10–13.5 g/l) (Table 4).

At the same time, the additional introduction of Mg²⁺ in the medium with ethanol and refined oil led to a slight decrease in the synthesis of EPS. We suggest that this may be due to the different effects of these cations on the enzymatic systems responsible for the catabolism of fatty acids and other related components that are part of sunflower oil or formed during frying. Li et al. (2014) found that in lactic acid bacteria *Lactococcus lactis* ssp. *lactis* concentration of Mg²⁺, at which the maximum enzymatic activity is observed, differs for various enzymes of the β -oxidation system.

Table 4

Influence of magnesium cations concentration on the ethapolan synthesis on the mixture of ethanol (4.0%) and sunflower oil (1.2%) of different quality

Concentration of Mg ²⁺ in the medium, mM	Type of oil in the mixture with ethanol	Mode of fed-batch cultivation	pH _{end}	EPS, g/l	EPS-synthesizing ability, g EPS/ g biomass
1.6 (medium 3)	Refined (control)	Four portions*	5.9	11.88±0.59	3.08±0.15
		Five portions**	6.1	13.51±0.68	3.65±0.18
	Mixed waste oil after frying various foods	Four portions*	6.2	12.02±0.60	3.24±0.16
		Five portions**	6.1	10.10±0.51	2.73±0.14
5.0 (medium 4)	Refined (control)	Four portions*	6.1	10.37±0.52	2.58±0.13
		Five portions**	6.2	9.86±0.49	2.19±0.11
	Mixed waste oil after frying various foods	Four portions*	6.0	15.63±0.78	3.33±0.17
		Five portions**	5.9	15.95±0.80	3.11±0.16

Notes: The inoculum was grown on ethanol. Fractional application of substrates was carried out 4-5 times every 24 hours: * – four portions of 1.0% ethanol and 0.3% oil; ** – five portions of 0.8% ethanol and 0.24% oil.

Further experiments have shown (Table 5) that after the replacement of refined oil in the mixture with acetate on mixed waste oil after frying various foods the amount of final product reached 16.36 g/l, which was only slightly lower than that obtained using a refined substrate (17.27 g/l). Meanwhile, EPS-synthesizing capacity increased up to 7.34 g EPS/g biomass, that was 3.7 and 2.2 times higher than the indicators obtained on the mixture of mixed waste oil after frying various products and molasses or ethanol, respectively.

Table 5

Biosynthesis of ethapolan on the mixture of sodium acetate (3.0%) and sunflower oil (1.5%) of different quality

Type of oil in the mixture with sodium acetate	pH _{end}	EPS, g/l	EPS-synthesizing ability, g EPS/g biomass
Refined (control)	7.9	17.27±0.86	6.47±0.32
Mixed waste oil after frying various foods	7.7	16.36±0.82	7.34±0.37

Notes: Inoculate cultivation and EPS biosynthesis were carried out on medium 5. The inoculum was grown on the refined or mixed waste oil after frying various foods. Fractional application of substrates was carried out three times every 24 hours: two portions of 1.0% acetate and 0.5% of oil, and one portion of 0.35% acetic acid and 0.5% of oil.

Conclusion

Thus, as the result of that research:

1. The possibility of replacing refined sunflower oil with different types of waste one, in particular mixed waste oil after frying various foods, in the mixture with C₂-C₆-substrates (molasses, ethanol or sodium acetate) for the ethapolan synthesis was demonstrated;
2. Optimal cultivation conditions (concentration of substrates in the mixture of molasses and oil, method of fractional application of substrates during growth on the mixture of oil with ethanol or sodium acetate) providing the maximum synthesis of the final product on the corresponding mixed substrates were established;
3. It was found that regardless of the type of used C₂-C₆-substrates in the mixture with waste oil, the concentration of ethapolan reached 15.3–16.3 g/l;
4. Data obtained are the basis for the development of a universal technology for the ethapolan synthesis on mixed substrates, independent of the supplier and the quality of waste sunflower oil.

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