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SYNTHESIS OF EXOPOLYSACCHARIDE ETHAPOLAN DURING CULTIVATION OF AUXOTROPH ACINETOBACTER SP. IMV B-7005 IN THE MEDIUM WITH SUNFLOWER OIL

Ivahniuk Mykola Oleksandrovich

student

Pirog Tetyana Pavlivna

doctor of biological sciences

Penchuk Yuriy Mykolayovich

candidate of technical sciences
National University of Food Technologies, Kyiv (Ukraine)

author@apriori-journal.ru

Abstract. The possibility to use the multivitamin complex «Complevit» as a source of calcium pantothenate by auxotrophic strain *Acinetobacter* sp. IMV B-7005 – the producer of microbial exopolysaccharide ethapolan was shown. The cultivation conditions for the producer were established to provide the synthesis of 6.6-6.7 g/l of the desired product in the medium with high (4-5 %) content of sunflower oil – cheap and available substrate for microbial technologies.

Key words: exopolysaccharides; sunflower oil; growth factors; calcium pantothenate; intensification of biosynthesis.

СИНТЕЗ ЭКЗОПОЛИСАХАРИДА ЭТАПОЛАНА ПРИ КУЛЬТИВИРОВАНИИ АУКСОТРОФА *ACINETOBACTER* SP. IMB B-7005 В СРЕДЕ С ПОДСОЛНЕЧНЫМ МАСЛОМ

Ивахнюк Николай Александрович

студент

Пирог Татьяна Павловна

д-р биол. наук

Пенчук Юрий Николаевич

канд. тех. наук
Национальный университет пищевых технологий, Киев (Украина)

Аннотация. Показана возможность использования поливитаминного препарата «Комплевит» в качестве источника пантотената кальция для ауксотрофного штамма *Acinetobacter* sp. IMB B-7005 – продуцента микробного экзополисахарида этаполана. Установлены условия культивирования продуцента, обеспечивающие синтез 6,6-6,7 г/л целевого продукта в среде с повышенным (4-5 %) содержанием подсолнечного масла – дешевого и доступного субстрата для микробных технологий.

Ключевые слова: экзополисахарид; подсолнечное масло; факторы роста; пантотенат кальция; интенсификация биосинтеза.

Microbial exopolysaccharides (EPS) – high molecular polymers, which are widely used in food, perfume, oil and textile industries due to its ability to modify the rheological properties of aqueous systems [1].

Complex microbial exopolysaccharide ethapolan, synthesized by *Acinetobacter* sp. IMV B-7005, consists of a neutral and two acid polysaccharides, one of them being acylated. The neutral polysaccharide is a minor component: its content is about 5 %. The acylated fraction contains residues of fatty acids C_{10}-C_{18}. The complexity of ethapolan properties, such as (i) its high viscosity enhancing capacity, (ii) unique capability to increase viscosity sharply in the presence of Cu^{2+} -glycine system, as well as other inorganic salts (iii) its solution pseudoplasticity, (iy) its thermal stability, (y) its resistance to the action of acids, and (yi) its emulsifying capacity, could make it a competitive industrial polysaccharide, in the first line for food and oil industries [2].

Previously, it was found that producer of ethapolan is auxotroph for calcium pantothenate (vitamin B_5) [2; 3]. But for today industrial production of this vitamin is stopped in Ukraine and Russia too, so it is important to find preparations that would become its substitute to provide needs of auxotrophic strain IMV B-7005.

At work [2] it was shown, that a wide range of mono- and mixed C_2-C_6-substrates (ethanol, acetate, propanol, pyruvate, C_4-dicarboxylic acids, carbohydrates − mono- and disaccharides, starch, molasses, etc.) can be used for the synthesis of ethapolan. Further studies have shown the possibility to use sunflower oil as a source of carbon and energy for the synthesis of this microbial polysaccharide [3].

Note, that the literature data about synthesis of microbial EPS on any industrial waste (not just oil-containing) is extremely limited. Thus, *Cellulomonas flavigena* UNP3 was described as the strain, which is able to synthesize kurdlan-like EPS in the medium with vegetable oil or appropriate waste [4]. It is known that *Xanthomonas campestris* ATCC 13951 synthesized 28 g/l of xanthan under cultivation in the medium containing partially hydrolyzed mo-

lasses (the concentration of lactose, galactose, glucose was 4.7; 17.8, 17.8, respectively) as the carbon source [5]. It was determined that *Pseudomonas oleovorans* NRRLB-14682 synthesized EPS (12.18 g/l) on the medium with crude glycerol (by-product of biodiesel production) [6]. *Acinetobacter* sp. DR1 under cultivation in the medium with diesel oil (2 %) synthesized about 5 g EPS/g biomass [7].

However, in earlier studies, the concentration of oil in the cultivation medium was low (only 1 %, v/v). As for the synthesis of ethapolan we supposed to use fried oil as a substrate, so its content in the medium has to be more higher.

The purpose of this work – to study the possibility of the deficient calcium pantothenate replacement on multivitamin complex «Complevit» which contains pantothenate in its composition. And, also, intensification of microbial polysaccharide ethapolan synthesis in medium with the maximum concentration of sunflower oil.

Research Methods

EPS-synthesized strain of bacteria *Acinetobacter* sp. 12S, which is deposited in the Depository of Institute of Microbiology and Virology, National Academy of Sciences of Ukraine by the number of IMV B-7005 was used as the object of research.

Cultivation of *Acinetobacter* sp. IMV B-7005 was carried out in a liquid mineral medium of such composition (g/l): KH_2PO_4 − 6.8; KOH − 0.9; $MgSO_4 \times 7H_2O$ − 0,4; $CaCl_2 \times 2H_2O$ − 0,1; NH_4NO_3 − 0,4; $FeSO_4 \times 7H_2O$ − 0,001. In one variant, the concentration of ammonium nitrate in the medium was increased to 0.6 and 0.8 g/l.

Sunflower oil (1-5 %, v/v) was used as a source of carbon and energy. In additionally yeast autolysate (0.5 %, v/v), calcium pantothenate (0.0006 %) and multivitamin complex «Complevit» (0.0006-0.00095 %) were added to the medium as growth promoters and sources of pantothenate, respectively.

Culture from the exponential phase, grown in the medium with 0.5 % of sunflower oil was used as the inoculum. Quantity of inoculum was 10 % from the volume of the medium.

Cultivation of *Acinetobacter* sp. IMV B-7005 was carried out in flasks (750 ml) with 100 ml of medium in shacker (320 rpm) at 30 ºC for 120 hours.

Growth of the strain and EPS synthesis were evaluated by the following parameters.

Biomass concentration was determined by optical density of the cell suspension with the following recalculation on the absolutely dry biomass (ADB) according to the calibration curve. Quantity of synthesized ethapolan was determined gravimetrically. For this, 1.5-2 volumes of isopropanol were added to a certain amount of culture liquid (usually 10-15 ml), the precipitate of EPS was washed by clean isopropyl alcohol and dried at room temperature for 24 h. EPS-synthesizing ability was determined as the ratio of the EPS concentration to the concentration of ADB and was expressed in g EPS/g ADB.

Statistical analysis of experimental data were performed according to Lakin [8]. The results of the experiment in accordance with the Student t-test were statistically significant at the 5 % significance level.

Results and discussions

During the searching of vitamins that could become substitute of calcium pantothenate for cultivation of ethapolan's producer the main selection criteria were: high content of pantothenate, low cost and absence of potential inhibitors of microbial growth (metal ions).

«Complevit» was selected for further work because it contained the largest concentration of pantothenate (20 mg per capsule) and the cost of the drug was low. In addition, metal concentration was low in it (Fe^{3+} – 1.4 mg, Zn^{2+} – 1.7 mg, Cu^{2+} – 0.3 mg).

On the next stage the optimal concentration of pantothenate in the «Complevit» was established for synthesis of ethapolan in the medium with sunflower oil (1 %, v/v) (Table 1).

As it shown in the data, the amount of synthesized exopolysaccharide reached to 5.1 g/l, and EPS-synthesizing ability – to 5.6 gEPS / gADB, with the content of pantothenate in the «Complevit» 0.00085 %. Adding of pantothenate in concentrations above 0.00095 % led to the decreasing synthesis rate.

Low concentrations of EPS (1 g/l, see table 1) after using of usual calcium pantothenate can be explained by a partial loss of its physiological properties during long storage term (over 5 years).

Thus, it was shown that *Acinetobacter* sp. IMV B-7005 synthesized 5 g/l of EPS in the medium with 1 % of sunflower oil and 0.00085 % of «Complevit». Further studies demonstrated that increasing of sunflower oil content in the IMV B-7005 strain medium to 2-3 % was accompanied by increasing of synthesized ethapolan concentration to 5.8-6.3 g/l, but the EPS-synthesizing ability was slightly decreased (Table 2).

Table 1

Dependence of ethapolan synthesis on the concentration of pantothenate in the medium with sunflower oil

Source of pantothenate	Concentration of pantothenate, %	Indices of synthesis		
		ADB, g/l	EPS, g/l	g EPS / g ADB,
Calcium pantothenate	0.0006	1.98 ± 0.10	1.0 ± 0,05	0.5 ± 0.03
«Complevit»	0.0006	3.26 ± 0.16	4.0 ± 0.20	1.3 ± 0.07
	0.00075	2.27 ± 0.11	4.6 ± 0.23	2.1 ± 0.10
	0.00085	0.89 ± 0.05	5.1 ± 0.26	5.6 ± 0.32
	0.00095	0.69 ± 0.03	3.6 ± 0.20	5.2 ± 0.30
	0.00105	0.7 ± 0.04	2.9 ± 0.15	4.3 ± 0.20

Indices of EPS synthesis decreased with the higher substrate concentration (4-5 %) and the highest EPS-synthesizing ability (5 g EPS/g ADB) was

observed under *Acinetobacter* sp. IMV B-7005 cultivation in the medium with 1 % of sunflower oil (Table 2).

As in case of increasing of carbon's concentration in the medium, C/N ratio changes, that significant impacts on synthesis of microbial polysaccharides [2], so on the next stage we increased concentration of nitrogen source simultaneously with enhancing of oil content (Table 3).

Results presented in Table 3, show that increasing ammonium nitrate concentration to 0.8 g/l in a medium containing 3-5 % of sunflower oil promotes degrease of synthesized ethapolan concentration and EPS-synthesizing ability compared with those in the medium with lower (0.4 g/l) concentration of nitrogen sources (see Table 2 and 3).

Table 2

Depending ethapolan synthesis on the concentration of sunflower oil in the cultivation medium of *Acinetobacter* sp. IMV B-7005

Concentration of sunflower oil in the medium, %	EPS, g/l	EPS-synthesizing ability, g EPS/g ADB
1	5.0 ± 0.25	5.0 ± 0.25
2	5.8 ± 0.29	4.7 ± 0.23
3	6.3 ± 0.31	4.0 ± 0,20
4	5.0 ± 0.25	3.7 ± 0.19
5	4.9 ± 0.24	3.6 ± 0.18

Note. The concentration of pantothenate in the medium was 0.00085 %, ammonium nitrate − 0.4 g/l.

Results presented in Table 3, show that increasing ammonium nitrate concentration to 0.8 g/l in a medium containing 3-5 % of sunflower oil promotes degrease of synthesized ethapolan concentration and EPS-synthesizing ability compared with those in the medium with lower (0.4 g/l) concentration of nitrogen sources (see Table 2 and 3). However, concentration of synthesized ethapolan in the medium with 4 and 5 % of sunflower oil and 0.6 g/l of NH_4NO_3 was 5.6 and 6.4 g/l, respectively. That is higher than in

medium with 0.4 g/l of ammonium nitrate (5.0 and 4.9 g / l, see Table 2 and 3). EPS-synthesizing ability also increased under such cultivation conditions of IMV B-7005 strain. Thus, parameters of ethapolan synthesis were improved by increasing NH_4NO_3 concentration to 0.6 g/l with increase of oil content to 4-5 % in the medium.

Table 3

The influence of the nitrogen source concentration on the synthesis of ethapolan under *Acinetobacter* sp. IMV B-7005 cultivation on sunflower oil

Concentration of ammonium nitrate, g/l	Concentration of sunflower oil in the medium, %	EPS, g/l	EPS-synthesizing ability, g EPS/g ADB
0.6	3	4.6 ± 0.23	4.1 ± 0.21
	4	5.6 ± 0.28	4.2 ± 0.21
	5	6.4 ± 0.32	3.9 ± 0.19
0.8	3	3.2 ± 0.16	3.0 ± 0.15
	4	3.4 ± 0.17	2.9 ± 0.14
	5	3.6 ± 0.18	2.7 ± 0.13

Note. The concentration of pantothenate in the medium was 0.00085 %.

The concentration of pantothenate in the medium is another factor that may affect on synthesis of ethapolan, as *Acinetobacter* sp. IMV B-7005 is auxotroph for calcium pantothenate [2]. Therefore, on the next stage concentration of pantothenate in the cultivation medium of IMV B-7005 strain was increased with enhancing sunflower oil and nitrogen source content (Table 4).

Thus, increasing of pantothenate content to 0.00095 % in medium with 0.4 g/l of ammonium nitrate and 5 % of sunflower oil allowed to enhance the concentration of EPS in 1.4 times, comparing with results in the medium with lower amount of pantothenate.

However, no positive effect on the synthesis of ethapolan with higher concentrations of pantothenate and NH_4NO_3 (0.6 g/l) in the medium was observed (Table 4).

Table 4

Synthesis of ethapolan depending on the concentration of pantothenate in medium with sunflower oil

Concentration in the medium			EPS, g/l
of ammonium nitrate, g/l	of pantothenate, %	of sunflower oil, %	
0.4	0.00085	4	4.8 ± 0.24
		5	4.9 ± 0.24
	0.00095	4	5.6 ± 0.28
		5	6.7 ± 0.33
0.6	0.00085	4	5,6 ± 0.28
		5	6,4 ± 0.32
	0.00095	4	5,5 ± 0.27
		5	6,6 ± 0.33

Conclusions

As a result of this work, the possibility of using vitamin complex «Complevit» as a calcium pantothenate substitute was shown in the cultivation medium of auxotrophic strain *Acinetobacter* sp. IMV B-7005 – the producer of microbial polysaccharide ethapolan. The optimum concentration of pantothenate in «Complevit» composition was 0.00085 %. Increasing content of ammonium nitrate to 0.6 g/l and/or pantothenate concentration to 0.00095 % allowed to increase the concentration of ethapolan up to 6.6-6.7 g/l synthesized in medium with 5 % of sunflower oil, which in 1.3-1.4 times higher than in basic medium with the same concentration of the substrate but lower content of NH_4NO_3 (0.4 g/l) and pantothenate (0.00085 %).

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