Reviews

Ethapolan As A microbial exopolysaccharide of multifunctional INVOLVeMENT

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The review represents the data of the study of microbial exopolysaccharide (EPS) ethapolan (producer – Acinetobacter sp. B-7005) concerning the synthesis processes activation, the influences on its physical and chemical properties as well as its practical applications. Ethapolan consists of both non-acylated and acylated (contains the C_{10}-C_{18} fatty acids) components, which are identical as for the content of carbohydrates, pyruvic and glucuronic acids. The presence of fatty acids moiety in this molecule determines unique properties of the EPS solutions such as ability to emulsify; to increase viscosity in the presence of the cations, and also at low pH values and using Cu^{2+}-glycin system. Due to these properties the ethapolan can be exploited in the petroleum production, chemical, food industry and agriculture. The using of as much as one ton of EPS in petrolium industry enables yielding additional 240 tons of petrolium. The review summarizes the data of the technological peculiarities of this EPS biosynthesis on different carbon substrates (ethanol, carbohydrates, mixture of growth C_2-C_6-compounds), this allows decreasing the salts concentration in nutrient medium by 3-4 fold (up to 2.95g/l) and elevating by 2-5 fold the quantity of EPS synthesized by the produces used. Also, the EPS composition of the properties needed depending on the field of its exploitation could be properly regulated.

Key words: *exopolysaccharides, ethapolan, intensification of synthesis, regulation of metabolism, physical and chemical properties, mixture of growth substrates, biosynthesis*

Both intracellular and exogenous microbial polysaccharides are of great interest to researchers – microbiologists, biochemists, molecular biologists, and biotechnologists. Practical importance of EPS used in small amountsis caused by their ability to change significantly rheological characteristics of water systems, This is important for EPS usage in petroleum, food, chemical industry, agriculture and medicine.

The ability to EPS synthesis was revealed in many microorganisms, though the synthesis level of these polymers fluctuates in a broad range for different EPS producers as well as for the same producer at different conditions of its cultivation. The elaboration of highly effective technologies of obtaining metabolites, important for practical application, is based on the purposeful regulation of biosynthesis process. This requires deep knowledge of physiology, biochemistry and genetics of producers.

A strain of *Acinetobacter sp*. bacteria which is a producer of ethapolan, a complex polysaccharide preparation, was selected in the Institute of Microbiology and Virology, NAS of Ukraine. Due to its unique physical and chemical properties this biopolymer may be

considered as a polysaccharide of multifunctional application.

The characteristics of ethapolan producer. *Acinetobacter sp*. strain was deposited with the Ukrainian microorganisms collection as B-7005 number. The strain was isolated from EPS-producing accumulating culture, obtained as a result of several subsequent reinoculations of a sample of activated sludge of the biological purification station of sewage water of Nadvornyansky petroleum refinery (Ukraine) on the mineral medium containing ethanol [1, 2].

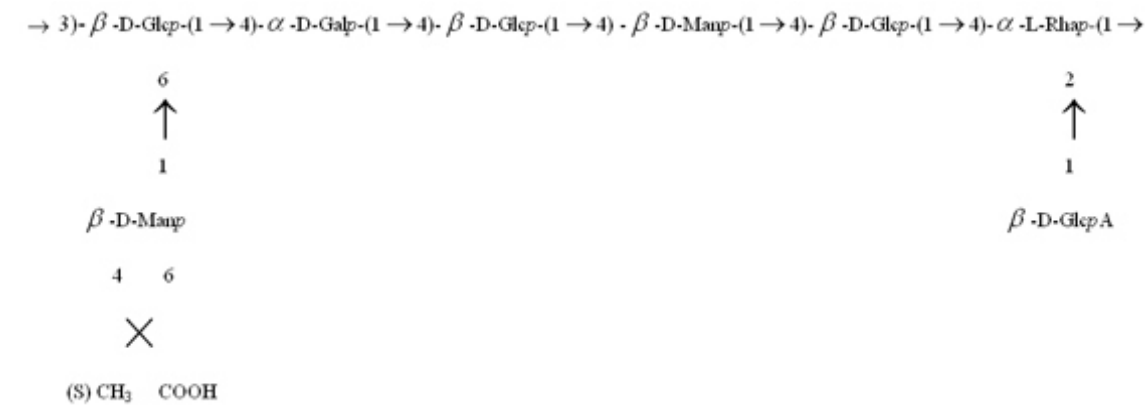
The investigation of morphologic-culture properties of this strain showed that in the exponential phase of growth the cells are rod-like (thick short rods), and in the stationary phase they are coccus-like, located in pairs (diploforms) or in small chains. The cells having the size of (0.95-1.5) x (1.2-2.0) micrometer do not produce any spores. Cells reproduction occurs by binary division. The cells are gram-negative and not motile. On agarosed wort medium they produce smooth lustrous muculent colonies of cream colour. The size of a three-day colony is 4-5 mm at diameter. On agarosed mineral medium containing acetate, ethanol, or sucrose the colonies are lustrous, mucoid, with the size of 1-2 mm; on the meat infusion agar they are smooth, white, protuberant, mucoid, shown the size of 3 mm at the diameter. During the cultivation on liquid media the cell population produces a homogenous suspension. The strain is an obligate aerobe, which is catalase-positive and oxidase-negative. It grows on complex organic media, and acetate is accumulated at the growth on mineral medium containing ethanol lacking growth factors in culture liquid [2].

Ethapolan producer is a natural auxotroph, which needs for its growth pantothenic acid and non-identified growth factor that is in yeast extract [2].

Taxonomic study of ethapolan producer. On the basis of the investigation of morphological, physiological investigations, conducted in late 80s of the previous century, ethapolan producer was placed among *Acinetobacter* sp. According to the 9th edition of Bergey's manual of systematic bacteriology [3], all the strains of *Acinetobacter* sp. bacteria originate from of one kind of *Acinetobacter calcoaceticus*. Due to the fact that the investigated strain differed from *A. calcoaceticus* in some properties, it was identified as *Acinetobacter sp*. [1]. It should be mentioned that after the publication of the abovementioned edition on systematic bacteriology, several dozens kinds of *Acinetobacter* sp. were described [4], but ethapolan producer was different from them as well. A great volume of information concerning B-7005 strain properties caused the necessity of defining its taxonomic status. Therefore a series of molecular biological researches of this strain were conducted, the analysis of 16S rRNA, in particular. Gene 16S rRNA sequencing proved that B-7005 strain is the closest to the members of *Rhodobacter* and *Paracoccus* species [5].

Thus, the strain of *Acinetobacter* sp. B-7005 is a member of a single cluster with a photosynthetic strain of *Rhodobacter capsulatus* ATCC 11166 (D16428) revealing the similarity coefficient as 95.7%. The strain of *Acinetobacter* sp. B7005 is also close to photosynthetic *Rhodobacter massiliae* and *R. sphaeroides* bacteria (the similarity level achieves 94.6-94.3%) and non-photosynthetic *Paracoccus* bacteria (the similarity level – 95.2-95.0%). Considering the results obtained, nucleotide sequences of 16S rRNA genes of several strains of *R. capsulatus* and *Paracoccus denitrificans* were compared using the ClastalX program (1.81 version).

It was determined that differences between 16S rRNA genes of these kinds are located in the following positions: 36 non-concurrent nucleotides are distributed practically equally in different loci of 16S rRNA gene and 26 non-concurrent nucleotides are concentrated in one of 16S rRNA gene fragments (which corresponds to the nucleotide positions from 935 to 980). In the same fragment 16S rRNA *Acinetobacter* sp. B-7005 gene is practically identical to *R. capsulatus*. At the same time in other positions (where there are 36 non-concurrent nucleotides in *R. capsulatus* and *P. denitrificans*) 16S rRNA gene of *Acinetobacter* sp. B-7005 strain is identical to *P. denitrificans*. In this work the strain name is given according to data available at the Depositary of microorganisms of the Institute of Microbiology and Virology, NAS of Ukraine (*Acinetobacter* sp. B-7005); it could be seen that the mentioned discrepancies of nucleotide sequence in different 16S rRNA gene fragments did not allow the identification of B-7005 strain as a member of *Rhodobacter* or *Paracoccus* species.



The preparation characteristics of ethapolan, a microbial exopolysaccharide. Ethapolan, a complex polysaccharide material, consists of neutral and acid polysaccharides [2, 6-8]. A neutral polysaccharide, containing glucose, manose, galactose (3:2:1) is a minor component – because its content does not exceed 5-6%. According to the data of gas-liquid chromatography, the residues of glucose, manose, galactose and rhamnose are found in the composition of acid EPS in the molar correlation of 3:2:1:1. The presence of uronic and pyruvic acids is determined in acid EPS.

In the process of alkaline processing of acid EPS the mixture of fatty acids was determined. The main components turned out to be dodecanoic acid, hexadecane acid, hexadecenic acid, octadecanoic acid and cis-octadecenic acid in the as 10:29:12:7:20 [1, 8]. It is appropriate to mention that the presence of fatty acids, etherifying a carbon chain, is not characteristic for microbial EPS.

40-50% of carbohydrates were revealed in acid EPS content, protein was not found at all. This was proved by both negative coloured reactions of **Lori and Bradford**, and the data of ^{13}C-NMR-spectroscopy. In ^{13}C-NMR spectra of this EPS the signals with the chemical shift of 55-57 m.d., characteristic of C-N-bond are absent [9]. Acid EPS also contains 20-30% of mineral components [8, 10].

It was established that depending on the nature of the carbon source and potassium cations concentration used in the cultivation medium of *Acinetobacter* sp. B-7005 the content of PVK residues in EPS varies (in % to the mass of relatively dry substance) from 3.0 to 4.3; for uronic acids it is 15.3 to 22.5; for fatty acids it is from 1.8 to 6.5 [2, 8, 11].

Our further researches showed that the acid component of ethapolan consists both of acylated (AP) and non-acylated (NAP) polysaccharides [7]. For this reason a simple method to separate the EPS into acylated and non-acylated components was developed. It was based on the emulsifying properties of acylated EPS solutions [7]. The need to develop such method was caused by the fact that the methods polysaccharides subdividing, so the one could contain fatty acids, were unknown. The approaches, available at that time, allowed separating polysaccharides into components of different molecular weight (e.g. the methods of gel-chromatography [12] and gradient centrifugation [13]); as well as fractioning acid and neutral polysaccharides (on cellulose anionites – DEAE and ECTEOLA or at treating with bromide cetylthreemetylamonium) [14-16]. The available method of the components, as a hydrophobic part, was fractionation using the column with hydrophobic carriers. However, its usage to separate EPS in large amounts was time and labor-consuming.

The authors of the work [17] used emulsification of carbohydrate in EPS solution at the isolation of acylated polysaccharide emulsane, synthesized *Acinetobacter calcoaceticus* RAG-1. Our experiments showed that this approach can also be successfully used for the separation of polysaccharides, one of which contains residues of fatty acids.

The comparative analysis of the chemical composition of yielded EPS, AP and NAP material, isolated from it, didn't differ in molar relation of D-glucose, D-manose, D-galactose, L-rhamnose, D-glucuronic and pyruvic acids (3:2:1:1:1:1). The differences be-

tween these polysaccharides cover only the fact that acylated EPS contains fatty acids (C_{10}-C_{18}) [7, 18, 19].

The same structure of a repeated unit of a carbohydrate chain of non-acylated polysaccharide and acylated EPS after the extraction of fatty acids was determined on the basis of chemical EPS modifications, solvolysis of EPS by water-free anhydrous hydrogen fluoride, degradation by Smith, ^{1}H- and ^{13}C-NMR-spectroscopy [19] (a scheme).

The resistance of glucose and galactose at degradation according to Smith allowed supposing that at least these two monosaccharide residues are acylated, though the exact place of O-acylation of AP was not determined [19].

The analysis of rheological properties of native and disacylated ethapolan solutions showed that the solutions of disacylated EPS are not structured by cations, their viscosity does not increase at low shift speeds, as well as at pH decrease and in Cu^{2+}-glycin system [2, 8]. It is worth mentioning that these characteristics of ethapolan solutions determine its practical value [8]. Thus, rheological properties of ethapolan solutions are caused by the presence of fatty acids in its composition.

It was found that depending on the cultivation conditions the average molecular weight (m.w.) of ethapolan is 926.0-1441.0 kDa [20]. The analysis of molecular weight composition of EPS showed the presence of components with m.w. margins from 13.5 to 2000 kDa, though the main component of the preparation was composed of a fraction having m.w. of more than 2000 kDa. After EPS precipitation by ethanol (isopropanol, acetone) the average m.w. decreased 2-3 fold and achieved 400-500 kDa [20]. In our opinion, it can be explained by the destruction of the ethapolan solutions structure in the process of their treatment with organic dissolvents. Such a phenomenon is characteristic of the majority of microbial EPS [2].

Ethapolan synthesis on different carbon substrates. *Ethapolan synthesis on ethanol*. It was shown that in the growth process of *Acinetobacter* sp. B-7005 in the mineral medium with ethanol without growth factors the level of biomass and EPS amounted to 0.3-0.5 g/l, and acetate compounds were accumulated in the cultural medium [2, 21, 22]. The introduction of yeast extract into the cultivation medium was accompanied by the biomass and EPS increase to 2-3 g/l. As it is known yeast extract is a source of aminoacids, vitamins, peptides, purines, pyrimidines, and a series of other factors [23]. Therefore we investigated the influence of these materials on the growth of *Acinetobacter* sp. B-7005. It was found that the strain does not need aminoacids, and as for vitamins we observed the increase of biomass and EPS level only in the presence of calcium pantothenate [2, 21].

Nevertheless, in some subsequent reinoculations of *Acinetobacter* sp. B-7005 in the medium with ethanol and calcium pantothenate we observed gradual decrease of biomass, EPS and washing-out of the culture. The introduction of yeast extract (0.5%) into the medium with ethanol additionally to B_5 vitamin resulted in growth stabilization and high-viscosity EPS synthesis (4.5-5.0 g/l). It proved that besides calcium pantothenate the strain also needs other growth factors that are available in yeast extract.

The further experiments allowed excluding purine (adenine, guanine) and pyrimidine (thymine, cytosine, uracil) bases as well as corresponding nucleotides (mono- and triphosphates) out of the group of the possible growth factors.

The process of obtaining ethapolan on ethanol in the presence of C4-dicarboxylic acids. To intensify the process of EPS biosynthesis on ethanol we used the so called “metabolic” approach, the sense of which was in theoretical definition of possible “narrow places” of culture metabolism and the search of the ways of their removing [2, 24, 25].

While solving this task we proceeded from the fact that ethanol metabolism in heterotrophic bacteria may be performed due to its oxidation through acetaldehyde to acetate. Acetate is introduced to further metabolism due to the mediation of acetyl-coenzyme A (CoA) [26, 27]. At the growth on ethanol bacteria use acetyl-CoA mainly for the synthesis of fatty acids, 2-oxoglutarate, C_4-dicarboxylic acids, from which then C_3-intermediates, carbohydrates, aminoacids, nucleotides are formed. The synthesis of C_4-dicarboxylic acids in microorganisms, which grow on ethanol, acetate, fatty acids or carbohydrates, occurs in glyoxylate cycle [26, 27].

As for CoA synthesis *Acinetobacter* sp. B-7005 bacteria need exogenous calcium pantothenate – the antecedent to coenzyme A [2, 25], this branch of metabo-

lism is evidently limited by acetyl-CoA. Hence, it was suggested to introduce C_4-dicarboxylic acids into the medium with ethanol to intensify gluconeogenesis and to increase EPS synthesis. It was shown that while cultivating *Acinetobacter* sp. B-7005 in the medium containing ethanol, calcium pantothenate and C_4-dicarboxylic acids, the EPS output from the substrate used increased 2-fold, and the quantity of EPS increased by 30% [2, 24, 25]. For further experiments potassium fumarate was selected as C_4-blocks; the optimum concentration of fumarate which was applied to the cultivation medium, amounted to 0.2%. Adding fumarate in the exponential phase of *Acinetobacter* sp. B-7005 growth at the cultivation in the medium with ethanol and calcium pantothenate depressed cells growth completely and increased the EPS synthesis by them considerably.

These researches showed that exogenous potassium fumarate is used for the EPS formation *i.e.* fumarate addition allows regulating the direction of processes of ethapolan biosynthesis of *Acinetobacter* sp. B-7005 [2, 24, 25].

It was found in further experiments that the addition of fumarate in the beginning of the stationary phase of bacteria growth resulted in the increase of EPS-synthesis ability, and exogenous fumarate was metabolized in EPS stoichiometrically. The process of fumarate assimilation which is transported into cells together with proton, was accompanied by the increase of pH value of the medium, which reached maximum at complete fumarate usage, after which it decreased to the primary level. Reaching the pH neutral value served as a "signal" of the necessity of introducing the following portion of C_4-dicarboxylic acids. As at the repeated addition of fumarate there was a gradual decrease of pH value to the primary level, its following portions were introduced after the acidation of the culture liquid to pH 7.0. It allowed decreasing the duration of the cultivation process of *Acinetobacter* sp. B-7005 due to the periodicity reduction of the addition of C_4-dicarboxylic acid.

It was found in the following experiments that separate introduction of potassium fumarate (in portions of 0.2%) allowed increasing the number of synthesized EPS 4-5 fold (to 10-15 gr/l), and EPS output from the used substrate (ethanol+fumarate) amounted to 60-80% [2, 24, 25].

Enzymological researches of the *Acinetobacter* sp. B-7005 strain, which were conducted later, proved our theoretical assumption concerning possible ways of ethanol metabolism in these bacteria. Thus, it was determined that the "bottle neck" of C_2-metabolism of *Acinetobacter* sp. B-7005 is acetate assimilation, which was proved by the fact that sodium ions inhibit both acetate oxidation in intact cells, and acetyl-CoA-synthetase activity in cell-free extract, as well as the limitation of C_2-metabolism by coenzyme A [28, 29]. At the introduction of C_4-dicarboxylic acids (potassium fumarate) into the medium with ethanol there was 1.5-2 fold increase of the activity of glyoxylate cycle enzymes, as well as fumarate hydrase, malate dehydrogenase and phosphoenolpyruvate synthetase (PEP-synthetase). In these conditions the increase of PEP-carboxykinase activity was more evident (almost 7.5 fold) [30]. Thus, the increase of level of ethapolan synthesis during producer cultivation on ethanol in the presence of C_4-dicarboxylic acids is caused by gluconeogenesis enhancing.

Ethapolan synthesis on carbohydrates substrates. We found the possibility of ethapolan synthesis during the cultivation of the *Acinetobacter* sp. B-7005 strain on carbohydrate substrates (mono- and disaccharides, molasses, starch) [31]. In the course of producer growth on C_6-substrates without calcium pantothenate the number of synthesized EPS amounted to 2-3 g/l, though pH value of the cultural liquid decreased to 5.5-5.7 till the end of cultivation [31, 32]. To find out the reasons, causing the decrease of pH level of the culture liquid, we investigated the main stages of C_6-compounds metabolism of *Acinetobacter* sp. B-7005. It was shown that glucose catabolism in the mentioned bacteria occurs by the pathways of Embden-Mayerhof-Parnas and Entner-Dudorov, which is proved by high activity of key enzymes of these pathways [31]. The presence of 2-oxoglutaratedehydrogenase activity pointed out to the fact of the complete cycle of tricarboxylic acids (CTA) functions in *Acinetobacter* sp. B-7005.

It was found that the "bottle neck" of glucose metabolism in ethapolan producer is the reaction which is catalysed by pyruvate dehydrogenase, *i.e.* C_6-metabolism in these bacteria is limited by CoA. The replenishment of intermediates pool for constructive metabolism

reactions takes place with the participation of pyruvate carboxylase.

Irrespective of the source of carbon nutrition (glucose, ethanol) high activity of key enzymes of C_2- and C_6-metabolism was determined in the *Acinetobacter* sp. B-7005 cells. At the bacteria growth on glucose the activity of isocytratliase amounted to 4.3 nmol•min^{-1}•mg^{-1} of protein. It was found that C_2-compounds (ethanol, acetate) are inducters of this enzyme in ethapolan producer. The introduction of C_2-compounds in low concentrations (0.01-0.1%) into the medium with glucose was accompanied by simultaneous consumption of two substrates, the increase of the number of synthesized EPS (5.1-6.1 gr/l) and EPS-synthesis ability (4.07-4.25gr EPS to 1gr of biomass) [31]. The results obtained became the basis for the elaboration [32] of the technology of obtaining ethapolan on carbohydrates, as well as on the mixture of C_2-C_6 compounds.

The improvement of ethapolan synthesis using the mixture of growth compounds. The technology of synthesis intensification of microbial EPS ethapolan on the mixture of energy-wise non-equal growth substrates is principally new [32-36].

In the natural places of existence microorganisms develop in the presence of several carbon substrates while in laboratory conditions monosubstrates are used as the only source of carbon and energy for their cultivation. At the same time it is known that a considerable part of some substrates is spent on the oxidation to CO_2 to obtain the energy, necessary for constructive metabolism (e.g. on glucose – 40%). Besides, there are works, proving the ability of microorganisms to use the mixtures of two (or more) substrates and investigating some aspects of such processes regulation [27, 37-40]. Nevertheless, the mentioned investigations concern the usage of mixed substrates only for the increase of biomass output.

It is also known that the combination of energy-wise non-equal substrates allows increasing the transformation effectiveness of substrates carbon into biomass [39, 40]. We supposed that such an approach may be used not only for the increase of biomass yield, but for the enhancing the secondary metabolites synthesis (with the consideration of additional power inputs for their formation).

The identification of the ways of energetic and constructive metabolism of C_2-C_6 compounds in ethapolan producer allowed determining which of these compounds are surplus energy-wise, and which are deficit energy-wise [28, 31]. It was found that oxidation of ethanol and acetaldehyde in these bacteria is made by NAD^+- and $NADF^+$-dependent dehydrogenases [28], so this substrate is classified as surplus energy-wise. Glucose catabolism in the *Acinetobacter* sp. B-7005 strain (ethapolan producer) occurs according to the method of Embden-Mayerhof-Parnas and Entner-Dudorov [31]. According to the energetic substrates classification of Babel [39], glucose is a deficit substrate energy-wise.

As a result of theoretical calculations of energy needs of EPS and biomass yield of the *Acinetobacter* sp. B-7005 strain the correlation of ethanol and glucose concentrations was found which allows avoiding non-productive losses of carbon and energy, taking place at the usage of monosubstrates, and increasing the transformation effectiveness of substrates carbon into EPS [33, 35]. The introduction of ethanol into the medium with glucose in molar relation 3.1:1 allowed increasing the number of synthesized EPS 1.8-1.9 fold (to 7.5-8.0 gr/l), EPS-synthesis ability – 1.4-1.7 fold (to 3.8g EPS per 1g of biomass), and EPS output from the substrate – 1.5-2 fold (to 62-65%) in comparison with the producer growing on monosubstrates.

The investigations, the results of which are shown in works [32-34, 36] proved that during cultivating *Acinetobacter* sp. B-7005 on the mixture of ethanol and glucose both substrates are assimilated at the same time, the maximum specific growth speed increases by 1.3-1.4 fold and the time of reaching this result decreases considerably.

Nevertheless, during the cultivation of ethapolan producer on the mixture of ethanol and glucose, not only EPS but also biomass was often observed.

Therefore, in further investigations the conditions of bacteria cultivation were determined, which provide complete conversion of carbon of both substrates into EPS itself to the maximum [32, 36]. It was determined that while growing ethapolan producer on the mixture of ethanol and glucose the highest transformation effectiveness of substrates carbon into EPS was observed in the following conditions: 1) the usage of sowing material, grown on ethanol or the mixture of ethanol and glu-

cose; 2) the concentration decrease of nitrogen nutrition source (ammonium nitrate) in the medium to 0.3-0.45 g/l; 3) the absence of sodium cations in the cultivation medium.

The study of the key enzymes activities of C_2-metabolism (alcohol- and acetaldehyde dehydrogenases, acetate kinase, and acetyl-CoA-synthetase) and C_6-metabolism (phosphofructokinase and phosphogluconate dehydratase) at the ESP producer growing on the mixture of ethanol and glucose showed simultaneously that their values were somewhat lower in comparison to growing on corresponding monosubstrates [36, 41]. The decrease of isocytratliase and malate synthase activity while growing on mixed substrate was more significant (in comparison with the cultivation on ethanol). Thus, we supposed that acetyl-CoA, which is formed from ethanol in acetate kinase and acetyl-CoA-synthetase reactions, is introduced to further metabolism mostly through CTA.

It was shown that during growing bacteria on the mixture of ethanol and glucose the CTA enzymes activity was higher than on ethanol only (especially the activity of isocytratdehydrogenase). Probably, the role of glyoxylate cycle in ethapolan producer metabolism on the mixed substrate is not so important as that on ethanol. Moreover, the activity increase of pyruvate carboxylase, which is an enzyme of anaplerotic reaction, was observed in the conditions of mixotrophic bacteria growth which provides replenishment of C_4-dicarboxylic acids pool while growing on carbohydrates. At the same time simultaneous functioning of two anaplerotic routes (glyoxylate cycle and pyruvate carboxylase reaction) may be accounted for the reinforcement of gluconeogenesis in the conditions of mixotrophic producer growth. Actually, the activity of PEP-synthetase, which is a key enzyme of gluconeogenetic branch of metabolism on mixed substrate, was 3 and 1.5-fold higher than on glucose and ethanol respectively.

Thus, the results of enzymologic researches could be explained by the change of biosynthesis processes direction on the mixture of growth C_2-C_6 compounds towards EPS formation in comparison with bacteria growing on monosubstrates.

The data obtained are the basis for creating fundamentally new technologies preparation of valuable secondary metabolites on the mixture of energy-wise non-equal growth substrates.

C_2-metabolism regulation in Acinetobacter sp. B-7005 and improving ethapolan biosynthesis technology on ethanol. The drawback of the technology of obtaining ethapolan on ethanol, which was elaborated previously, was the necessity of keeping neutral pH value in the process of producer cultivation, which was accomplished by the introduction of salts in high concentrations (11 g/l) into the liquid to create sufficient high-capacity phosphate buffer (0.05 M). While growing *Acinetobacter* sp. B-7005 on the medium with ethanol, which does not contain buffer, pH value lowered to 4-5 inhibitions of bacteria growth and EPS increasing synthesis [2, 28, 29].

Study of peculiarities of energetic and constructive metabolism in ethapolan producer, allowed the approaches to regulate C_2-metabolism and to find the ways of regulation of EPS biosynthesis process.

It is known that oxidation of ethanol and acetaldehyde is carried out by NAD^+- and $NADF^+$-dependent dehydrogenases, acetate is included into the metabolism due to acetyl-CoA-synthetase activity [28]. Anaplerotic reaction sequence, which replenishes C_4-dicarboxylic acids pool in *Acinetobacter* sp. B-7005 cells, is glyoxylate cycle, and CTA has a biosynthetic role mainly. Phosphoenolpyruvate synthesis is provided by two key gluconeogenesis enzymes namely PEP-carboxynase and PEP-synthetase [30].

It was found that the “bottle neck” of ethanol metabolism of *Acinetobacter* sp. B-7005 is acetate assimilation. This reaction is catalyzed by acetyl-CoA-synthetase and limited in speed-limited [28, 29]. Thus, in the cells of *Acinetobacter* sp. B-7005 bacteria, which grow on ethanol, acetate is formed at a higher speed than it is being included into further metabolism.

The following investigations were directed to the search of factors which promote the same speed of formation and further metabolism of acetate in *Acinetobacter* sp. B-7005 cells, grown on ethanol. It was found that the inhibitors of acetyl-CoA-synthetase are sodium ions as well as products of ethanol and acetaldehyde oxidation – NADN and NADFN. Enzyme activators are pantothenic acid, cations of potassium and magnesium [29]. The decrease of the primary etha-

nol concentration from 1.0 to 0.5% (with subsequent introduction of 0.5% ethanol in the middle of exponential growth phase), the absence of sodium ions and the presence of 100mM K^+ in the medium of ethapolan producer cultivation conditioned practically the same speed of ethanol, acetaldehyde and acetate oxidation in intact bacteria cells, and the activity of acetyl-CoA-synthetase in cell-free extract increased 3 fold. It allowed implementing the ethapolan synthesis process in the medium, the molarity of which is 2 fold decreased. Obtaining an analogous result at the primary ethanol concentration of 1.0% was provided (on the background of the absence of sodium salts and presence of 100mM K^+) at the increase of pantothenic acid and Mg^{2+} concentration in the medium to 0.0009-0.0012% and 5mM respectively. Nevertheless, at further decrease of salts concentration and buffer capacity of the medium there was a drop of ethapolan synthesis level and acetate accumulation in the cultural liquid. In such conditions of ethapolan producer cultivation the activity of acetyl-CoA-synthetase was 2-fold lower [29].

The literature data testify to the fact that acetyl-CoA-synthetase, which functions in cells of many pro- and eukaryotes, is an inducible enzyme [42, 43], and acetate is an inducer of this synthesis [44]. Our experiments showed that the introduction of exogenous acetate into the medium with ethanol increases the activity of acetyl-CoA-synthetase in *Acinetobacter* sp. B-7005 cells [45].

On the basis of the investigation of C_2-metabolism regulation the method of obtaining ethapolan was developed. The key elements of the method are the absence of sodium cations in the cultivation medium, the increase of calcium pantothenate concentration in it to 0.0009%, as well as the presence of 0.1% of potassium acetate during cell subculturing and polysaccharide biosynthesis. It allowed performing the process of ethapolan accumulation in the medium (without decrease of synthesis indicators), in which the salts content was decreased 4 fold (from 11 to 2.95 g/l).

Regulation principles of physical and chemical properties of ethapolan. It is known that producer cultivation conditions influence not only EPS synthesis, but also physical and chemical properties of the final product [46-48]. In different cultivation conditions the chemical composition of EPS, their molecular weight, correlation of several polysaccharides may change, which influences EPS rheological properties, determining the practical value of these polymers. Therefore, an important and necessary condition of developing microbial EPS biotechnologies is the stability of physical and chemical properties of polysaccharides as well as the possibility of regulating polymers properties depending on the fields of their practical use. It was found that *Acinetobacter* sp. B-7005 cells subcultured periodically cultivation of the composition of ethapolan of AP/NAP change [6, 49, 50]. The synthesis of both polysaccharides started in the first hours of bacteria growth and occurs parallelly. At the moment of reaching maximum viscosity of 0.02% solutions of ethapolan, the AP content in its composition was the highest and amounted to 65-68%. The content of NAP increased to the end of the periodic process in EPS composition (to 60%) which was accompanied by viscosity decrease of its solutions [6]. Besides, viscosity did not increase in EPS solutions with high NAP content in the presence of cations, in the area of low shift speeds, at pH level decrease, as well as in Cu^{2+}-glycin system, i.e. they did not show rheological properties which determine practical value of ethapolan [49]. Thus, the properties of ethapolan solutions depend on the correlation of AP and NAP in its composition [50]. To obtain EPS, the solutions of which are characterized by necessary rheological properties, the producer cultivation process should be stopped before the stationary growth phase, which leads to the decrease of synthesized EPS quantity.

The following investigations showed that at the 2 fold increase (from 0.025 to 0.05M) of K^+ concentration in the medium, there was gradual viscosity increase of synthesized EPS solutions in the course of all the cultivation process. In these conditions AP content did not change much and amounted to 70-75%, and its formation occurred in the stationary growth phase as well [51]. Analogous patterns were found while growing the producer in the medium, containing 0,10M K^+. In this case AP content in EPS amounted to 90-95%. Obtained data showed that while cultivating *Acinetobacter* sp. B-7005 in the medium with increased K^+ content it is possible to obtain EPS with necessary rheological properties (irrespective of process duration). The analysis of EPS chemical composition, synthesized in the media

with different K^+ concentration (0.025-0.1M) showed that they differ in AP content as well as in fatty acids content in AP [50]. The solutions of investigated EPS were characterized by different viscosity in the presence of cations, at low pH values, as well as in Cu^{2+}-glycin system. Thus, rheological properties of ethapolan solutions are defined not only by the AP and NAP relation in its composition, but also by the content of fatty acids in AP [6, 49-51]. It was found that the synthesis of acylated EPS depended on the content of monovalent cations (K+ and Na+) in the medium of producer cultivation. To form the highly acylated AP, containing 12-16% of fatty acids, the concentration of monovalent cations (K^+ and Na^+) in the medium should be not less than 0.09M [49, 52].

To make ethapolan a commercial product, it was necessary to develop a controlled technology of its obtaining which would provide high EPS output possessing certain predetermined properties. Previously [2, 24, 25] it was found that there is a possibility of EPS synthesis increasing of *Acinetobacter* sp. B-7005 due to the introduction into the medium of C_4-dicarboxylic acids that are gluconeogenesis antecedents.

It is known that AP or NAP is synthesized depending on the concentration of monovalent cations in the medium with potassium fumarate [49]. Thus, together with the increase of monovalent cations concentration in the medium, AP content in the composition of synthesized EPS increased which resulted in the improvement of rheological properties of their solutions. Nevertheless, in the course of the process the content of fatty acids decreased from 7.2% (after the introduction of the 1st fumarate portion) to 3.5% (after the addition of the 4th portion). It was accompanied by viscosity decrease of EPS solutions, synthesized at the end of the process in the presence of cations in H^+-form and in Cu^{2+}-glycin system [49].

The following researches showed that the decrease of fatty acids content in AP, synthesized after the introduction of the last fumarate portion added, is caused by insufficient quantity of univalent cations in the bacteria cultivation medium. At the increase of the primary concentration of monovalent cations in the medium to 0.14M AP with high content of fatty acids was synthesized from potassium fumarate (7.5-8.5%) which was constant in the course of the whole cultivation process. At the same time the properties of EPS solutions, synthesized after the introduction of each of four fumarate portions, did not change and were analogous to EPS properties, formed in the medium lacking fumarate [8, 49].

The following experiments showed that the synthesis of highly acylated ethapolan is also possible in the course of growing the producer in the media with ethanol, which contain only 20-40mM K^+. Thus, as a result of investigation of C_2-metabolism regulation in *Acinetobacter* sp. B-7005 it was found that bacteria cultivation in the conditions lacking the metabolic limitations, connected with the formation of acetyl-CoA (fatty acids antecedent), is accompanied by the synthesis of mostly acylated EPS with high m.w. (1.5 mln Da) which does not decrease in the process of isolating and purifying the preparation [45].

It was found that ethapolan protects producer cells from unfavourable environmental factors namely, the action of toxic oxygen derivatives, heavy metals (Cu^{2+}, Pb^{2+}, Cr^{3+}, Zn^{2+}), biocide (formaldehyde), detergent (sodium dodecyl sulfate), high and low pH values, heating, drying, and freezing [53-56]. It was shown that ethapolan implements protective functions not only concerning producer cells, but also concerning microorganisms cells which are in trophic relations with *Acinetobacter* sp. B-7005 [55].

The researches, the results of which are stated in the works [8, 57], showed that at non-optimal cultivation conditions of *Acinetobacter* sp. B-7005 (at the change of temperature, pH of environment, dissolved oxygen) EPS quantity decreased, but EPS-synthesis ability remained stable and amounted to 1g EPS per 1g of biomass. EPS solutions, synthesized at non-optimal conditions, were characterized by different viscosity in the presence of cations, in H^+-form and in Cu^{2+}-glycin system. Obtained results allowed supposing that the effectiveness of protective functions of such EPS is different, it correlates with rheological properties of ethapolan and is implemented under non-optimal conditions of *Acinetobacter* sp. B-7005 cultivation the most. Nevertheless, at bacteria cultivation in non-optimal conditions the quantity of synthesized EPS decreased. In this connection we made a supposition that it is possible to increase EPS concentration and improve ethapolan solutions properties at the same time

due to growing the producer in a two-stage process. On the first stage of the bacteria growth conditions are used optimal for the EPS growth and synthesis, while on the second stage non-optimal conditions are created (that is temperature decrease to 24°C, pH increase to 8.0, addition of formaldehyde into the medium – 30 mkg/ml). It was found that while cultivating *Acinetobacter* sp. B-7005 in two-stage mode, the quantity of EPS synthesized reached the level, corresponding to that achieved at optimal conditions of growth [20]. EPS solutions were characterized by a higher viscosity in the presence of cations in H^+-form and in Cu^{2+}-glycin system, than polysaccharides, synthesized in optimal conditions.

Practical application of ethapolan. Ethapolan is EPS which can be used in different spheres of industry [8, 58-62] and agriculture as plant protective preparations [63].

Petroleum industry. Such physical and chemical properties of ethapolan solutions as the ability to emulsify, the viscosity increase in the presence of cations, at low shift speeds, at pH decrease and in Cu^{2+}-glycin system attracted attention of petroleum industry specialists to this EPS as to an petroleum-sweeping agent.

It was shown that the usage of ethapolan containing 70-95% AP with the acylation degree of 5-12% is reasonable for the intensification of petroleum production.

At present such microbial EPS as xanthane (producer *Xanthomonas campestris*), scleroglucane (producer *Sclerotium glucanicum*, *S. rolfsii*), emulsane (producer *Acinetobacter calcoaceticus*) are considered the most perspective to be used in petroleum production in the world. Ethapolan is considerably different from these polysaccharides by significant increase of solution viscosity in mineralized media. An important peculiarity of ethapolan solutions is the increase of their viscosity at oxidation which allows using this EPS to prolong acid treatment of bottomhole formation zones. Ethapolan solutions are more thermostable that xanthane and scleroglucane solutions which allows using them in the fields with high strata temperatures [59].

Due to the presence of a lipophilic moiety in ethapolan molecule it can stabilize emulsions of water and petroleum or other carbohydrates. Ethapolan solutions are characterized by higher ability (in comparison with known biopolymers) of increasing viscosity in the areas of low shift speeds (0.1-1.0 c^{-1}). Ethapolan is able to form gel-like systems while interacting with metal ions and other cross-linking agents. At the same time the viscosity increase of modified ethapolan solutions is observed more than 10 fold and there is stabilization of obtained solution at long-term keeping [2, 59].

It is possible to use ethapolan in petroleum industry as a cultural liquid which excludes the necessity of isolating and purifying the preparation previously. The application of such commercial form of ethapolan has an additional advantage in the fact that in it polysaccharides are stabilized by which are of the components of cultural liquid composition. Besides, it does not have microgel clots. This explains light dissolubility of ethapolan even in mineralized media and its compatibility with other chemical reagents while preparing appropriate formulations.

The compositions of ethapolan and polyacrylamide, as well as ethapolan and sodium silicate, which were developed in the cooperation with the specialists of "Soyuznefteotdacha" Institute (Russia) and "VNIIneft'" (Russia), were used in 1988-1990 for the improvement of petroleum output on exploited petroleum wells of VO "Bashneft'" (Bashkortostan). The production of experimental lots of ethapolan was also performed on Bashkyrsky biochemical plant and on VO "Enzyme" (Ukraine).

The method of isolating strata water input using gel-forming compositions on the basis of ethapolan, non-organic copper salt and glycin, providing highly effective isolation of permeable seams, accompanied by the increase of petroleum yield and significant decrease of petroleum flooding, was developed in the cooperation with the workers of "VNIIneft'" and ENTO "ITIN" Institute (Russia) [64]. The developed method of strata water input isolation was used in 1991-1992 for hydroisolation of exploited petroleum wells in Prylutsky petroleum field (Chernigiv oblast). Production lots of ethapolan were made on experimental mobile plant, developed together with ENTO "ITIN" in the conditions of Trypilsky biochemical plant VAT "Stirolbiotech" (Ukraine).

The use of much as 1 tone of ethapolan in petroleum industry enables to yield 240 petroleum tones in addition.

Household chemical goods and cosmetology. Ethapolan preparations, the solutions of which are characterized by high emulsifying activity, are used in household chemical goods and cosmetology [8]. Ethapolan, containing 70% AP with the acylation degree of 12-16%, meets these requirements. A technical detergent BIMS-1 was developed on ethapolan basis, the sale volume of which amounted to 1000 tones of the products in 1989-1990. A technology of producing cosmetic creams with the general name "Ekol" was elaborated on the basis of ethapolan [8].

Ethapolan with high content of non-acylated polysaccharide may be used as a thickening agent in alkaline media (e.g. while producing some detergents, washing pastes etc). In such conditions the use of ethapolan with high content of fatty acids is not reasonable as alkaline medium (with pH 10 and higher) may cause partial disacylation of EPS which results in viscosity decrease of its solutions, and therefore, in effectiveness decrease of EPS use [8].

Food industry. The researches, performed in the Ukrainian State University of Food Technologies (UDUHT, since 2002 – the National University of Food Technologies) under the leadership of Corresponding Member of UAAS V.I. Drobot, proved the possibility and appropriateness of using ethapolan in bread and bread products production [60-62].

Technological regimes and parameters of producing "Zhytomyrsky" and "Polisky" bread were developed, providing stable rhythm of dough formation and high quality of ready products. The technological dose of ethapolan for bread baking was defined, it amounts to 0.3-0.5% of flour weight. Economic effect of ethapolan use is in the possibility of using flour of the 2^{nd} and 3^{rd} sort (flour with low content of gluten), flour economy on the condition of ethapolan addition to it and in slowing down hardening of bread products.

Complex additives K-1, K-2 and K-3 were worked out in UDUHT on ethapolan basis for the use in bread production from wheat flour with low content of gluten. Production testing in bread production with complex additives K-, K-2, and K-3 was conducted in a bread-baking plant #2 (Kyiv). The volume of experimental lots of bread products, produced on the basis of K-1 additives, amounted to 21.4 tones; K-2 – 24.5 tones; K-3 – 21.7 tones.

The researches, conducted in Science Research Institute of Nutrition Hygiene of the Ministry of Health of Ukraine, showed the absence of mutagenicity, embryotoxicity, teratogenicity and allergenicity of ethapolan, and determined the complex-forming ability of ethapolan concerning salts of heavy metals (on the example of lead). Due to the ability of ethapolan to absorb salts of heavy metals and take them out of the organism, bread products with it use may be recommended for preventive nutrition.

Ethapolan with low content of fatty acids (40-50% AP with acylation degree of 3-5%) may be used in food industry, unlike to petroleum industry and cosmetology.

Protection of plants from viral and infectious diseases. It is known that microbial polysaccharides of different structure have antiviral properties [64]. Heteropolysaccharides attract the greatest attention as a significant quantity of uronic acids is found in their composition together with neutral monosaccharides [8, 32]. Such polymers have polyanion structure and due to this fact they are able to induce organism resistance to viral infections *de novo*. Taking into consideration the abovementioned we aimed at investigating the antiviral activity of these preparations on phytoviruses models in particular.

The testing of preparations in contact experiments *in vitro* showed that irrespective of carbon source in the medium of producer cultivation (ethanol, glucose, ethanol+glucose) EPS depressed the infection of the virus of tobacco mosaic and X-virus of potatoes in the plants of stramonium (*Datura stramonium*) and gomphrena (*Gomphrena globosa L.*) in a wide concentration range. It is interesting to mention that both native and disacylated ethapolan preparations had the same activity. It showed that at least fatty acids were not the main factors of antiphytoviral properties of this EPS [64].

It was found that ethapolan preparations at the concentration of 500-2000 mkg/ml are able to induce resistance of super-sensitive tobacco plants of Immune sort 580, *Nicotiana sanderae* and stramonium to VTM-infection.

Thus, a great variety of substrates (ethanol, C_4-dicarboxylic acids, carbohydrates – mono- and disaccharides, starch, molasses) can be used for ethapolan synthesis. Such a property distinguishes

ethapolan producer favourably among known microbial synthesizers which mainly synthesize EPS only while growing on carbohydrates (*Zanthomonas campestris, Azotobacter vinelandii, Sclerotium glucanicum, Aureobasidium pullulans* etc). It should be mentioned that researches, conducted in the 70-80s of the 20th century, demonstrated the possibility of extending the source of raw materials for microbiological EPS production due to the usage of non-nutritive substrates – methane, lower alcohols, ethylene glycol, carbohydrates and petroleum products [48]. The representative of a new generation of microbial EPS is emulsane (producer *Acinetobacter calcoaceticus*), obtained in production scale on ethanol basis [17]. Nevertheless, there are no literature data on microorganisms, able to synthesize EPS actively on both carbohydrate and non-carbohydrate substrates.

The ability of *Acinetobacter* sp. B-7005 to form EPS on C_2-C_6 compounds allowed usto develop a complex of different technologies on obtaining ethapolan on the basis of a wide range of carbon substrates. Each of technology could be used depending on economic appropriateness, presence and availability of a certain substrate, and the necessity of obtaining EPS with certain physical and chemical properties.

Ethapolan synthesis was increased using the following approaches:

- the determination of totality of optimal external factors (the nature and concentration of growth factors, sources of carbon and nitrogen nutrition, C/N correlation, the way of substrate giving etc);
- the introduction of precursors of polysaccharides biosynthesis antecedents into the cultivation medium;
- the use of the mixture of energy-wise non-equal growth substrates;
- revealing “bottle neck” of metabolism and the development of approaches of their removal.

Physical and chemical properties of ethapolan were regulated, using the following methods:

- revealing functional groups in the EPS composition, determining their rheological properties, as well as the search of factors, providing EPS synthesis with necessary functional groups;
- the investigation of EPS composition and properties change in the producer cultivation process and the determination of the growth phase in which EPS synthesis with necessary physical and chemical properties occurs;
- the study of interplay between rheological EPS properties and their protective functions as well as the determination of producer cultivation conditions, necessary to reveal protective functions.

Some of these approaches concerning EPS synthesis increase and their physical and chemical properties regulation were used by us for the first time.

Thus, the literature data concerning the use of mixed substrates by microorganisms, known at present, show only to the increase of biomass synthesis level on the mixture of growth and non-growth substrates. We were the first to show the possibility of enhancing of the secondary metabolites synthesis (on the example of microbial exopolysaccharide ethapolan) on the mixture of energy-wise non-equal growth substrates. On the basis of experimental data the technology of obtaining ethapolan was developed, enabling to increase almost 2-fold the maximum speed of producer growth, the quantity of synthesized EPS, their output relating to biomass and EPS output from the substrate, as well as to shorten the cultivation duration and improve rheological properties of EPS solutions, defining its practical value.

There are no literature data concerning rheological properties regulation of microbial EPS due to the effectiveness change of their protective functions. At present the researches, concerned with the study of physiological functions of microbial EPS, are disregarded by biotechnologists. In our opinion finding out reasons, causing the necessity of EPS synthesis for the producer itself, will allow to view the series of problems of microbial polysaccharides biotechnology from new angles, including EPS obtaining with predetermined properties.

It is worth mentioning that the methods of obtaining microbial EPS in two-stage process are known [65, 66], but they are characterized by the fact that on the first stage there are optimal conditions for producer growth, and on the second – optimal conditions for EPS synthesis. The distinguishing feature of our approach is the fact that on the first stage of the process the producer is grown at conditions that are optimal for both growth and EPS synthesis, and on the second stage – there are

non-optimal (or even stress) conditions, created for the producer, in which protective functions of microbial EPS are revealed at the most. In response to non-favourable environment polysaccharides are synthesized with changed rheological characteristics. The literature data highlight the stimulation action of organic acids (pyruvate, citrate, succinate, ?-ketoglutarate) on xanthane formation by *Xanthomonas campestris* bacteria [67]. The authors think that the influence of organic acids is connected with the determination of pH value, favourable for xanthane synthesis. The analogical effect takes place at the introduction of fumaric acid into the cultivation medium of *X. campestris* [68]. In this case there is an increase of xanthane output from 6.0 to 9.1 g/l. The mechanism of exogenous fumarate action at the growth of ethapolan producer on C_2-compounds is different, as in this case C_4-dicarboxylic acids are intermediates of ethanol metabolism, which are directly included to gluconeogenesis.

The approaches mentioned to synthesis increasing and ethapolan properties regulation may be used while elaborating technologies of not only microbial polysaccharides, but also any practically valuable secondary metabolites.

Unique rheological properties of ethapolan solutions (the ability to emulsify, viscosity increase in the presence of mono- and bivalent cations, at pH decrease, in the area of low shift speeds, in Cu^{2+}-glycin system), the totality of which is not characteristic of any of already known microbial EPS, allow considering it as polysaccharide of multifunctional assignment, which may be used in petroleum, household, chemical, perfumery-cosmetic, as well as in food industry and agriculture.

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Этаполан-микробный экзополисахарид мультифункционального назначения

Резюме

Суммированы опубликованные экспериментальные данные, касающиеся интенсификации синтеза, регуляции физико-химических свойств и практического использования микробного экзополисахарида (ЭПС) этаполана (продуцент–Acinetobacter sp. B-7005). Приведены характеристики этаполана и штамма продуцента, анализ разработанных технологий биосинтеза этого ЭПС на разных углеродных субстратах (в том числе и на смеси ростовых C_2-C_6-соединений), преимущества этаполана по сравнению с известными ЭПС, а также новые подходы к регуляции синтеза и свойств этаполана.

Ключевые слова: экзополисахариды, этаполан, интенсификация синтеза, регуляция метаболизма, физико-химические свойства, смесь ростовых субстратов, биосинтез.

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