ISSN 0003-6838, Applied Biochemistry and Microbiology, 2009, Vol. 45, No. 1, pp. 50–55. © Pleiades Publishing, Inc., 2009.
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The Effect of Cultivation Conditions on the Physicochemical Properties of the Exopolysaccharide Ethapolan

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Received August 19, 2007

Abstract—The physicochemical properties of the complex exopolysaccharide ethapolan (EPS) produced by *Acinetobacter* sp. 12S during growth on media with various C/N ratios and different concentrations of mineral components and phosphate buffer were studied. Irrespective of the cultivation conditions, the concentrations of carbohydrates (38–44%) and pyruvic acid (3.2–3.7%) in the total EPS, as well as in the acylated (AP) and nonacylated (NAP) polysaccharides obtained from them, were practically the same. The EPS, AP, and NAP were also identical in their monosaccharide composition: the molar ratio of glucose, mannose, galactose, and rhamnose was 3 : 2 : 1 : 1. The polysaccharides contained different concentrations of mineral salts (6–28%), uronic acid (3.7–22.0%), and fatty acids (5.8–15.4%); they also differed in the ratio of acetylated and nonacetylated polysaccharides. Due to the differences in the chemical composition and molecular mass (500 kDa – 1.5 MDa), the viscosities of the EPS solutions (in the presence of 0.1 M KCl, in the H^+–form, and in Cu^{2+}–glycine system) were different as well. The mechanisms responsible for changes in the physicochemical properties of the total EPS, AP, and NAP synthesized on various media are discussed.

DOI: 10.1134/S0003683809010098

Strain *Acinetobacter* sp. 12S is a producer of the complex exopolysaccharide ethapolan. Due to the unique properties of ethapolan water solutions, ethapolan preparations could be used for the enhancement of oil recovery, as well as in mining, food, chemical, and cosmetic industries [1].

In our previous works [2-4], we studied the principal stages of C_2-metabolism in *Acinetobacter* sp. 12S in order to detect and eliminate limitations in the metabolic pathway. It was established that, in the case of *Acinetobacter* sp. 12S, ethanol metabolism is limited by the rate of acetate assimilation. Our previous studies of the regulation of the acetyl-CoA synthetase activity, as well as on the effect of exogenic acetate on ethapolan production, enabled us to eliminate factors that limit C_2-metabolism and to refine the technology of ethapolan production on ethanol. We have developed a number of new nutrient media with the reduced contents of mineral salts and phosphate buffer (which are two and four times lower, respectively) [3, 4]. This has allowed us to reduce both the duration of the producer cultivation and ethapolane production costs.

According to the results obtained and the literature data, not only the EPS synthesis (the amount of synthesized polysaccharides, rate of their formation, EPS yield calculated per substrate, etc.), but also the physicochemical properties of polysaccharide preparations (EPS chemical composition, molecular mass, and proportions of polysaccharides) depend on the cultivation conditions, which affects the rheological properties of EPS and, consequently, their practical value [5–9].

The purpose of this work is to study the physicochemical properties of ethapolan synthesized on various ethanol-containing media.

MATERIALS AND METHODS

Strains. In our study, we used strain *Acinetobacter* sp. 12S, an EPS producer that was previously described in [1].

Cultivation of *Acinetobacter* sp. Bacteria were grown in liquid mineral media containing the following (g/l): (**Medium 1**) KH_2PO_4, 6.8; NaOH, 0.9; NaCl, 1.05; NH_4NO_3, 0.6; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.01; phosphate buffer, 0.05 M; (**Medium 2**) KH_2PO_4, 6.8; KOH, 1.8; KCl, 1.4; NH_4NO_3, 0.6; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.01; phosphate buffer, 0.05 M; (**Medium 3**) KH_2PO_4, 3.4; KOH, 0.9; NH_4NO_3, 0.3; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.01; phosphate buffer, 0.025 M; and (**Medium 4**) KH_2PO_4, 1.7; KOH, 0.45; NH_4NO_3, 0.3; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.01; phosphate buffer, 0.0125 M. The media were additionally supplemented with 0.5 vol % yeast autolysate and 0.0009% calcium pantothenate. Ethanol at a concentration of 1.0 vol % (media 1, 2, and 3), as well as potassium ace-

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tate (0.1%) and ethanol (1%) (medium 4) served as the carbon and energy source. Potassium acetate was added in the form of a 20% solution.

To obtain the inoculum, exponential phase cultures (16–18 h) were grown on the above-described mineral media. The inoculum grew on the above-mentioned compounds, which served as a carbon and energy source. Also, 24-h cultures grown on meat–peptone agar were used as the inoculum, which was introduced in a dose of 5%.

Bacteria were grown for in flasks on a shaker (220 rpm) at 30°C for 120 h; the pH of the medium was 6.8–7.0.

Isolation of native (total) ethapolan. The ethapolan-containing culture liquid was dialyzed against distilled water for five days, diluted three- to fivefold with distilled water, and centrifuged at 12000 *g* for 40 min to separate the producer cells. The supernatant was concentrated in a vacuum (50°C) to the initial volume; ethapolan was then precipitated by the addition of 1.5 volumes of isopropanol. The EPS precipitate was washed in pure isopropanol and dried at room temperature.

Separation of EPS into acetylated (AP) and nonacetylated (NAP) components. To isolate the fatty acid-containing AP, native EPS solutions (with carbohydrate concentrations ranging from 0.01 to 0.02%) were used. The solutions were transformed into the H^+ form by the KU-2-8 (H^+) cationite treatment (500 mg of resin per 100 ml of EPS), which was conducted until constant pH was attained. The resin was separated by centrifugation. Then, 130 ml of the EPS solution in the H^+ form was placed in a 500-ml cylindrical separatory funnel and supplemented with 130 ml of chloroform. The funnel was sealed with a ground glass stopper, and the contents were agitated (emulsified) for 1–7 min. The obtained mixture was left in the funnel for 24 h to allow for the complete separation of the phases. The emulsion and aqueous phase were then discharged separately, and the lower phase was centrifuged at 12000 *g* for 30 min to remove the remnants of another phase. The aqueous phase was evaporated in a rotary evaporator to remove chloroform and to concentrate the EPS solution; the emulsion supplemented with three volumes of distilled water was treated the same way. Polysaccharides contained in the aqueous phase and in the chloroform emulsion were precipitated by the addition of two volumes of isopropanol. The EPS precipitate was washed in pure isopropanol and dried at room temperature (stage I of separation).

The similar chloroform-based procedure was performed 2–4 times for the polysaccharide solutions isolated from the aqueous phase (stages II, III, IV, and V of separation), and the APs obtained from the emulsion at all separation stages were combined. NAP was isolated from the aqueous phase at the last stage of separation (Fig. 1).

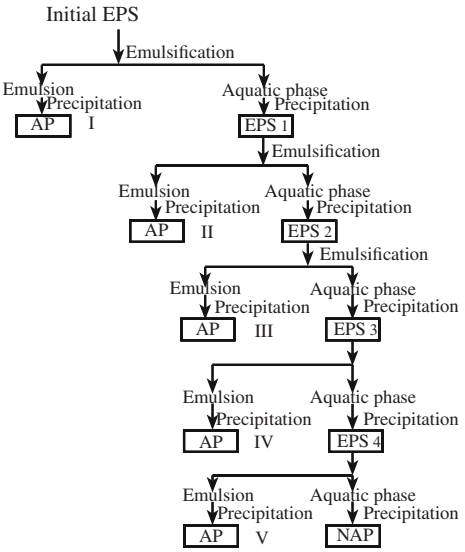

Fig. 1. Scheme of ethapolan separation into acylated and nonacylated components.

Preparation of deacylated ethapolan. Sodium borane and solid NaOH (10 and 500 mg, respectively, per 100 mg EPS) were added to the 0.15% solutions of native polysaccharides; the mixture was kept for 48 h at room temperature with periodical agitation. After neutralization of the solution with concentrated hydrochloric acid, fatty acids were extracted from the solution five times with hexane.

To obtain deacylated ethapolan, the water solution obtained after fatty acid extraction was dialyzed against distilled water for five days and concentrated in a vacuum. Deacylated EPS was then precipitated with isopropanol. The precipitate was washed in pure isopropanol and dried at room temperature.

Analysis of the chemical composition of ethapolan. The chemical composition of mineral components (**MC**) in ethapolan, as well as the contents of carbohydrates, pyruvic acid (**PA**), uronic acids (**UA**), fatty acids (**FA**), and the monosaccharide composition in the total EPS, AP, NAP, and their deacylated derivatives, were determined as described previously [6, 10].

The contents of carbohydrates, PA, UA, FA, and MC in EPS were expressed as a percentage calculated per weight of dry substance, which was defined as the substance retaining its weight after drying in a vacuum at 40°C.

Determination of the EPS molecular mass. Analysis of the EPS molecular mass composition was performed by the method of analytical gradient centrifugation that we developed previously [11]. The amount of components of a certain molecular mass was determined from the amount of carbohydrates in the relevant fractions and expressed as a percentage of the total (initial) amount of carbohydrates. The content of carbohy-

Table 1. Chemical composition of native and deacylated ethapolan synthesized on various media

Medium	Component content, % of the weight of dry EPS								
	native					deacylated			
	carbohydrates	PA	UA	FA	MC	carbohydrates	PA	UA	MC
1	44.5	3.35	7.5	6.5	24.3	60.0	4.83	20.0	3.5
2	45.0	3.7	7.7	6.8	28.6	59.1	4.4	22.0	3.9
3	46.7	3.37	11.4	8.8	7.2	58.6	4.73	20.6	N/D
4	45.9	3.22	13.8	5.88	6.19	55.7	4.79	N/D	N/D

Note: PA, pyruvic acid; UA, uronic acids; FA, fatty acids; MC, mineral components; and N/D, not determined.

drates was determined in the reaction with phenol and sulfuric acid [12]. The average molecular mass of EPS was calculated from the proportions of EPS components with different molecular masses.

To determine the EPS molecular mass, evaporated EPS concentrates and polysaccharides obtained by isopropanol precipitation and drying were used.

Analysis of the rheological properties of ethapolan solutions. Rheological properties of ethapolan solutions (0.03% by carbohydrates) were determined by measurement of their viscosity in the presence of 0.1 M KCl during the EPS transformation into the H^+ form, as well as in a Cu^{2+}–glycine system, as described in [5]. The practical value of EPS depends on these rheological properties [1].

The relative viscosity difference in the ethapolan solution viscosities was considered the criterion for evaluation of the rheological properties of the ethapolan solutions synthesized under different growth conditions. This difference was calculated using the following formula:

$$\text{Relative viscosity difference} = \frac{\eta_1 - \eta_0}{\eta_0} \times 100\%,$$

where η_1 is the EPS solution viscosity under given conditions (in the presence of 0.1 M KCl, in the H^+ form, and in a Cu^{2+}–glycine system), and a η_0 is the viscosity of the EPS solution in distilled water. Viscosity was measured using an Ostwald glass capillary viscosimeter at 20°C.

The results were statistically examined according to Lakin [13]. Student's *t*-test showed the results to be statistically significant at a 5% significance level.

RESULTS AND DISCUSSION

Chemical composition of native ethapolan. The study of the chemical composition of native and deacylated ethapolan synthesized on media with different concentrations of mineral salts demonstrated that, irrespective of the cultivation conditions, native EPS contained the same amounts of carbohydrates and pyruvic acid (Table 1). The contents of fatty acids in the native ethapolan synthesized on the media 1, 2, and 4 were practically the same (5.8–6.3%), whereas the content of fatty acids was higher in the EPS synthesized on medium 3 (8.8%). In the polysaccharide obtained on the media 3 and 4, the concentration of mineral salts was four times lower as compared to the EPS synthesized on the media 1 and 2. The low content of mineral salts in these polysaccharides can be attributed to the low contents of univalent cations (e.g., potassium cations) in the media 3 and 4. For instance, we have previously demonstrated that, during the cultivation of the ethapolan producer, structuring of the synthesized EPS is caused by univalent cations contained in the nutrient medium [1].

The native polysaccharides synthesized on the media 3 and 4 were characterized by the higher content of uronic acids (11–14%) as compared to the native polysaccharides synthesized on the media 1 and 2 (7–8%) (Table 1). In our previous studies, we demonstrated that the real content of UA in native EPS can be assessed only after polysaccharide deacylation [6]. In fact, the content of uronic acids in deacylated EPS was two to three times higher than in the similar preparations before deacylation and reached 20–22% in all the studied polysaccharides. High concentrations of uronic acids after EPS deacylation can be attributed to the fact that the dissociation of high-molecular EPS conglomerates occurs during deacylation. The conformation of polysaccharide molecules and the EPS spatial structure change as well, so that uronic acids become less accessible to various reagents. A similar explanation may apply to the increased contents of pyruvic acid and carbohydrates in deacylated EPS.

It is well known that the major carbohydrate chain of the polysaccharide remains, as a rule, unchanged under various growth conditions, whereas the side chains experience the biggest changes [1, 7]. Analysis of the chemical compositions of the native EPS synthesized on different media revealed that all the investigated polysaccharides were identical in their monosac-

charide composition (Table 2). In the EPS analyzed, the molar ratio of glucose, mannose, galactose, and rhamnose was 3 : 2 : 1 : 1.

Chemical composition of acetylated and nonacetylated components of ethapolan. It has been previously demonstrated that ethapolan consist of two components: acylated (in which the carbohydrate chain is etherified by fatty acids) and nonacylated (which does not contain any fatty acids) [10]. The ethapolan properties are determined by the AP/NAP ratio and the content of fatty acids in AP, as well as by the conditions of the producer cultivation [14–16]. In this connection, fractionation of native EPS into the acylated and nonacetylated components was performed at the next stage; the chemical composition of AP and NAP was then determined (Table 3).

The three stages of separation of the EPS synthesized on the media 1 and 2 revealed that the AP content in both EPSs was 68–72%. The content of fatty acids in the EPS isolated from the aqueous phase (nonacetylated polysaccharide) was 0.6–1.0% (Table 3). Two additional stages of separation resulted in a decrease (to 0.3–0.5%) in the content of fatty acids in nonacetylated polysaccharides. We believe that the presence of small amounts of fatty acids in nonacetylated polysaccharides may be explained by the fact that the two polysaccherides cannot be completely separated by the technique described in Materials and Methods; therefore, NAP preparations may contain small amounts of acetylated polysaccharides. Another reason lies in the fact that, during extraction of hydrophobic EPS component with chloroform, trace amounts of fatty material, including cell fragments, may get into NAP solutions.

The ethapolan synthesized on the media 3 and 4 was subjected to three-stage separation; as a result, the EPS isolated from the aqueous phase contained about 5% of fatty acids. In this connection, two additional separation stages have been included. Nevertheless, after the five-stage separation, the content of fatty acids in NAP was 3.2–2.2 % (Table 3). Further fractionation has not resulted in any decrease in the fatty acid content in the EPS isolated from the aqueous phase (NAP). Thus, it may be concluded that the polysaccharides synthesized on the media 3 and 4 are completely acylated with varying degrees of acylation (2.2–15.9%). It should be noted that, for the first time, we obtained a completely acylated polysaccharide.

EPS molecular mass. Table 4 shows data on the molecular mass of ethapolan synthesized under various conditions. The average molecular mass of the evaporated concentrates of all the studied EPS ranged from 1400 to 1600 kDa. In these EPSs, the concentrations of fractions with molecular masses below 2×10^6 were similar (24–32%). Somewhat different results were obtained when determining the molecular masses of EPS after precipitation and drying. For instance, the average molecular mass of the EPS synthesized on the media 1 and 2 was three times lower that that before precipitations, whereas the average molecular mass of the EPS obtained on the media 3 and 4 remained practically unchanged (Table 4). The content of fractions with molecular masses below 2×10^6 in the dry EPS synthesized on the media 1 and 2 reached 70–80%. In the EPS synthesized on the media 3 and 4, the content of fractions with molecular masses below 2×10^6 did not change after precipitation and drying of polysaccharide preparations. We believe that the stability of molecular masses during the isolation and purification of the EPS synthesized on the media 3 and 4 can be attributed to the complete acylation of these polysaccharides. It is obvious that the solid structure of EPS, which remains unchanged during the treatment of ethapolan solutions with organic solvents, is a result of the carbohydrate chain acylation.

We have previously demonstrated that, in the course of cultivation of the ethapolan producer in the presence of formaldehyde (30 μg/ml), a 1.5- to 1.7-fold decrease in the molecular mass of the ethapolan after precipitation and drying was observed [5]. In this work, we demonstrated the possibility of obtaining a high-molecular-weight ethapolan whose molecular mass does not change during isolation and purification.

Table 2. Monosaccharide composition of ethapolan synthesized on various media

Medium	Molar ratio			
	glucose	mannose	galactose	rhamnose
1	3.3	2.3	1.0	1.0
2	3.1	2.0	0.9	0.9
3	3.2	2.1	1.1	1.0
4	3.0	1.9	0.9	0.9

Table 3. Chemical composition of acylated and nonacylated polysaccharides synthesized under various conditions of the *Acinetobacter* sp. 12S cultivation

Medium	Component content, % of the weight of dry EPS					
	AP			NAP		
	PA	UA	FA	PA	UA	FA
1	2.77	n. d.	12.4	3.31	N/D	1.0
2	n. d.	n. d.	11.7	3.6	N/D	0.6
3	3.61	5.85	15.9	3.9	14.7	3.2
4	2.27	3.65	10.2	3.4	9.2	2.2

Note: PA, pyruvic acid; UA, uronic acids; FA, fatty acids; MC, mineral components; and N/D, not determined. Three stages of the separation of the EPS synthesized on the media 1 and 2 and five stages of the separation of EPS synthesized on the media 3 and 4 were performed.

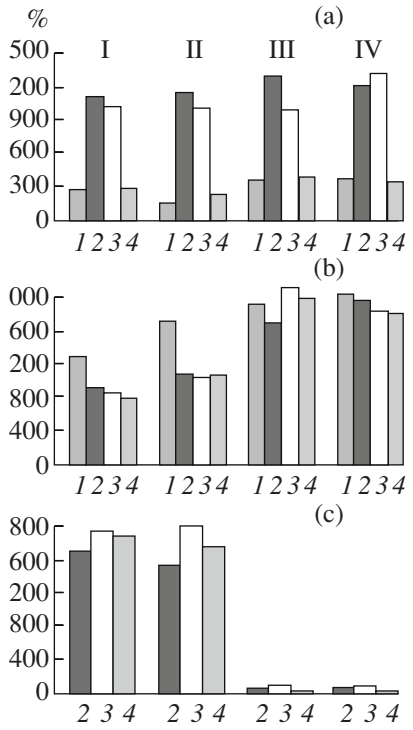

Fig. 2. Changes in the viscosity of 0.03% EPS solutions synthesized (a) in the presence of 0.1 M KCl, (b) in the Cu^{2+}–glycine system, and (c) in the H^+ form on (I) medium 1, (II) medium 2, (III) medium 3, and (IV) medium 4: *1*, culture liquid before dialysis; *2*, culture liquid after dialysis; *3*, evaporated concentrate; and *4*, EPS after precipitation and drying.

Rheological properties of ethapolan solutions. The rheological properties of ethapolan solutions (cation-induced structuring, increased viscosity at low pH and in a Cu^{2+}–glycine system, etc.) are determined by the ratio between the acylated and nonacylated EPS, as well as by the content of fatty acids in the acylated polysaccharide [14–16]. Deacylated EPS did not exhibit the above-listed properties [16]. Moreover, the rheological properties of ethapolan solutions may depend on the molecular mass of EPS and other (aside from fatty acids) substituents found in EPS, such as PA and UA.

The study of the rheological properties of ethapolan synthesized on media with different concentrations of mineral salts has shown that the water solutions of the EPS synthesized on media 3 and 4 exhibited higher viscosity in the presence of 0.1 M KCl and in the Cu^{2+}–glycine system; however, after transformation into the H^+ form, this property was lost (Fig. 2). This phenomenon was observed at all the stages of isolation and purification of EPS preparations. The inability of the EPS solutions synthesized on the media 3 and 4 to increase viscosity after transformation into the H^+ form results from the low (30–40 mM) contents of univalent cations in the cultivation media and, consequently, by the low contents of mineral salts in EPS (Table 1).

The higher (as compared to the EPS synthesized on the media 1 and 2) viscosity of the EPS solutions synthesized on the media 3 and 4 in the presence of univalent cations and in the Cu^{2+}–glycine system can be attributed to the high molecular mass of polysaccharides, as well as to the fact that these EPS were completely acylated.

It has been previously demonstrated that the medium used for the cultivation of the ethapolan producer must contain no less than 100 mM of K^+, which is a prerequisite for the synthesis of acylated polysaccharides [16]. The results of the present work, as well as the data presented in [4], demonstrate that a high concentration of potassium cations in the medium is not a prerequisite for the synthesis of ethapolan whose solutions exhibit the rheological properties of practical value.

The absence of nonacylated components in the EPS synthesized on the media 3 and 4 with a low content of univalent cations (30–40 mM) and a high content of fatty acids in the AP isolated from these EPS preparations can be attributed to the fact that we were able to eliminate metabolism-limiting factors associated with the production of acetyl-CoA, a precursor of fatty acid synthesis [2–4].

Hence, we have demonstrated that high-molecular-weight acylated ethapolan with improved rheological properties (determining its practical value) was synthesized during cultivation of strain *Acinetobacter* sp. 12S on moderately deficient media, in which the concentrations of mineral salts and phosphate buffer were two and four times lower as compared to the initial medium.

Table 4. Molecular mass of ethapolan synthesized under various cultivation conditions

Medium	Evaporated EPS concentrate		EPS after precipitation and drying	
	Average molecular mass, kDa	Fractions with molecular mass below 2 MDa, %	Average molecular mass, kDa	Fractions with molecular mass below 2 MDa, %
1	1441.0	31.7	479.8	82.7
2	1437.3	29.9	498.3	71.2
3	1606.95	23.7	1536.04	25.7
4	1573.3	25.4	1483.0	27.8

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