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A Two-Stage Cultivation Technique for Producing Microbial Exopolysaccaride Ethapolan with Improved Rheological Properties

T. P. Pirog, Yu. R. Malashenko, and S. K. Votselko

Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kiev, 03143 Ukraine

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Abstract—A two-stage technique was proposed for cultivating producers of microbial exopolysaccharide ethapolan. The practical value of ethapolan is determined by its rheological properties. The use of a formaldehyde-supplemented medium at the second stage of cultivation improved the rheological properties of ethapolan without reducing its yield. This effect of formaldehyde was due to its binding to the exopolysaccharide, which altered the molecular-weight characteristics of the latter and protected cells against the toxic action of formaldehyde. At all stages of its purification, ethapolan had improved rheological properties, suggesting that it was tightly bound to formaldehyde.

Despite numerous studies, in which technologies for producing diverse microbial exopolysaccharides (EPSs) are developed, and despite the fact that some EPSs (xanthan, emulsan, and scleroglucan) are produced on an industrial scale, the production of biopolymers that have a constant composition and stable properties remains an important problem in microbial EPS technology. The goal of developing a strategy for obtaining EPSs with desirable properties was pursued in this study using the production of ethapolan as an example. Ethapolan is a complex ethanol-based EPS synthesized by *Acinetobacter* sp. In the strategy proposed, the following approaches were used to control the composition of EPSs, their physicochemical properties, and synthesis rates [1].

(1) Identification of the functional groups that determined the physicochemical properties of EPSs and the conditions required for synthesis of an EPS carrying the necessary functional groups.

(2) Determination of the stage in which EPSs with the target physicochemical properties were synthesized by studying how their composition and properties varied during producer cultivation.

(3) Identification of the rate-limiting steps in EPS synthesis by analyzing the putative metabolic pathways and the ways to increase their rates were proposed.

At present, there is no reason to expect that the mechanisms whereby the EPS is synthesized from ethanol and from carbohydrates are very different. However, with ethanol as a growth substrate, gluconeogenesis is needed to generate one phosphorylated hexose from two three-carbon (C_3) molecules. Therefore, it is obvious that the overall efficiency of EPS synthesis by microorganisms growing on ethanol largely depends on how efficiently the C_2 substrate is converted into a C_3 intermediate of constructive metabolism (anabolism). The reaction sequence leading to the key intermediate synthesis may contain a rate-limiting step (the slowest reaction in the sequence, which is either highly endothermic or results in the loss of substrate carbon). An analysis of the pathways of C_2 metabolism not only revealed the rate-limiting steps in the ethapolan synthesis but also allowed us to outline approaches for obtaining higher ethapolan yields.

(4) Examination of the relationships between rheological properties and protective functions of the EPS and determination of the cultivation conditions needed to stimulate the latter.

Note that the role of EPSs in cell protection remains out of the focus of biotechnologists' attention. However, knowing how the producer itself benefits from EPS synthesis may help to view many problems of biotechnology, including the production of EPS with desirable properties, from a new perspective. As shown by our previous studies, cell-protecting properties of ethapolan vary with cultivation conditions. The ethapolan synthesized by bacterial cells grown under unfavorable conditions was most efficient at protecting these cells against stressors. A correlation was found between the rheological and cell-protecting properties of ethapolan [1, 2]. The results described made a basis for the development of a two-stage technique for cultivating an ethapolan producer, with favorable growth conditions at the first stage and unfavorable at the second stage. This technique made it possible to improve the rheological properties of ethapolan that determine its practical value without reducing its yield [1].

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The goal of this study was to perform a comprehensive analysis of ethapolan synthesis at each cultivation stage using formaldehyde (FA) as a toxic supplement of the culture medium to make the growth conditions at the second stage unfavorable for the producer. We also studied the mechanisms whereby FA affected the rheological properties of ethapolan.

METHODS

We used *Acinetobacter* sp. 12S resistant to streptomycin (up to 1000 μg/ml), which was described in our previous study [1].

Cultivation conditions. Bacterial cells were grown in Kodama's liquid synthetic medium [4] containing 1 vol % ethanol as a source of carbon and energy. The medium was additionally supplemented with yeast autolysate (0.5 vol %) and 0.0003% calcium pantothenate. At a total univalent ion ($K^+ + Na^+$) concentration in the culture medium of 0.065 M (0.4 M Na^+ + 0.025 M K^+), ethapolan was synthesized in the nonacylated form; its acylated form was produced at 0.090 M (0.4 M Na^+ + 0.050 M K^+) [5, 6].

Cultures were grown either in flasks on a shaker (220 rpm) or in an AK-210 fermenter (working volume, 4 l). Suspensions of 2-day-old cultures grown on potato–dextrose agar were used to seed the flasks. Flask-grown stationary-phase cultures were used to inoculate the fermenter (at a 1 : 20 volume ratio). In the fermenter, cells were grown at 30°C and a pH of 6.8–7.0; dissolved oxygen was maintained at 30–40% by varying the air supply and stirring rates.

Conditions favorable for the growth and EPS synthesis were usually maintained until the end of exponential growth (24–30 h in flasks or 12–16 h in the fermenter). Subsequent cultivation proceeded in the presence of FA (20, 30, or 40 μg/ml). In one variant, FA was added at the lag phase, midexponential phase, or stationary phase of the growth.

The amount of biomass was estimated by measuring the optical density of cell suspensions and converting the result into dry weight using a calibration plot. The culture liquid was assayed for EPS using a reaction with phenol in the presence of sulfuric acid [7].

Ethapolan isolation and purification. The ethapolan-containing culture liquid was dialyzed against distilled water for five days, then diluted three- to fivefold with distilled water, and centrifuged at 12 000*g* for 40 min to sediment producer cells. After the supernatant was vacuum evaporated at 50°C to the initial volume, isopropanol (1.5 vols) was added to precipitate the EPS. The EPS precipitate was washed in absolute isopropanol and then dried at room temperature.

The acylated and nonacylated EPS forms (APS and NAPS, respectively) were separated based on the emulsifying properties of the APS using the method that we developed previously [8]. The fatty acid content in the APS was quantified as described elsewhere [8].

Molecular weight determination. In fractionation experiments, both evaporated EPS concentrates and dried isopropanol precipitates were studied. The molecular weights of EPSs were determined by our method of analytical centrifugation in a NaCl gradient [9] using dextrans (Fluka) with molecular weights of 13.5, 20, 40, 70, 110, 500, and 2000 kDa as standards. Carbohydrates in 1-ml fractions isolated by centrifugation were assayed by their reaction with phenol and sulfuric acid [7].

Evaporated EPS concentrates and preparations precipitated with isopropanol and then dried were used for calculating the molecular weight.

The carbohydrate content in each fraction was expressed as the percentage of total carbohydrate (assessed before fractionation). Knowing the percentages of EPS components with different molecular weights, we calculated the mean molecular weight.

The mean molecular weights of the EPS complexes with lectin were determined similarly. The EPS–lectin complexes were obtained by coincubating their 0.1% solutions mixed at a 1 : 1 or 10 : 1 ratio at room temperature for 20 h. The glucuronic-acid-specific lectin purified from *Bacillus subtilis* was a gift from Dr. E.A. Kovalenko (Institute of Microbiology and Virology, National Academy of Sciences of Ukraine). Ethapolan for these experiments was obtained by cultivating the producer in the Kodam's medium at the univalent ion concentration of 0.09 M as described above.

Rheological characterization of ethapolan. Rheological properties of ethapolan (in the H^+-form) solutions were determined at pH 4.0–4.5 from changes in their viscosity either in the presence of 0.1 M KCl or in the Cu^{2+}–glycine system. Note that the practical value of ethapolan is determined by its rheological properties [1]. Ethapolan is unique in its ability to increase the viscosity of the Cu^{2+}–glycine system. Inorganic copper salts precipitate ethapolan from solution. The precipitate dissolves in the presence of the chelating agent glycine [3]. Applications have been developed that take advantage of this property of ethapolan: gelling ethapolan-based formulations in oil production [11] and a technique for isolating EPS from culture liquid [3].

Ethapolan was studied as a 0.03% carbohydrate solution at all four purification stages (first as a culture liquid before dialysis, then after dialysis, after dialysis and evaporation, and finally after dialysis, evaporation, isopropanol precipitation, and drying). When describing the results below, we referred to this 0.03% solution as a dilute culture liquid.

To transfer ethapolan to the H^+-form, 0.03% EPS solution (15 ml) was treated with KU-2-8 cationite in the H^+-form (300 mg) as described elsewhere [5]. In experiments with Cu^{2+}–glycine systems, we first added 0.003 M of $CuSO_4 \cdot 5H_2O$ and then 0.015 M of glycine to a continuously stirred ethapolan solution. The mixture was heated to 80°C, kept at this temperature for 5 min, and then cooled.

Table 1. Effect of FA on the *Acinetobacter* sp. 12S biomass and ethapolan yield*

FA addition	FA, μg/ml	Biomass, g/l	EPS, g/l
Lag	20	0.1	0.1
	30	0.1	0.1
	40	0.1	0.1
Mid-exponential	20	1.3	1.2
	30	1.25	1.1
	40	1.25	1.1
Late exponential	20	2.0	1.95
	30	1.95	1.95
	40	1.95	1.9
Stationary	20	1.95	2.0
	30	2.0	1.9
	40	1.95	1.9
Control (no FA added)	0	2.0	1.9

* Cultures grown in flasks for 120 h.

The rheological properties of EPSs synthesized under different growth conditions were compared based on the relative viscosity change η_{rel} defined as

$$\eta_{rel} = \frac{\eta_1 - \eta_0}{\eta_0} \times 100\%,$$

where η_0 is the viscosity of the EPS in distilled water and η_1 is the EPS solution viscosity under the conditions studied (H^+ form, 0.1 M of KCl, or Cu^{2+} of glycine). The viscosity of ethapolan solutions was measured at 20°C by means of an Ostwald glass capillary viscometer.

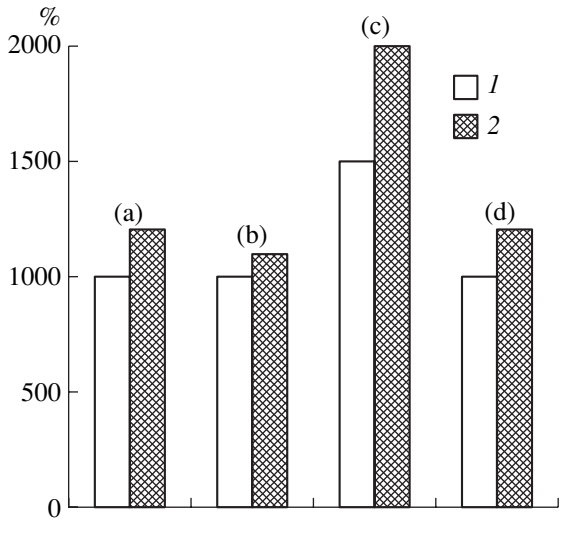

Fig. 1. Relative changes in the viscosity (η_{rel}, %) of the dilute culture liquid of *Acinetobacter* sp. 12S grown (a) in the absence or in the presence of (b) 20, (c) 30, or (d) 40 μg/ml FA: (*1*) 0.1 M KCl vs. distilled water and (*2*) Cu^{2+} + glycine vs. distilled water.

The rheological properties of EPS–lectin complexes were studied similarly.

Statistical processing of the results was performed as described by Lakin [12]. The data proved statistically significant at $p = 0.05$ (Student's *t*-test).

RESULTS AND DISCUSSION

Ethapolan, a complex polysaccharide synthesized by *Acinetobacter* sp. 12S, consists of one neutral and two acidic components. One of the acidic components has an acyl chain. The APS and NAPS contain D-glucose, D-mannose, D-galactose, L-rhamnose, D-glucuronic acid, and pyruvic acid at the same 3 : 2 : 1 : 1 : 1 : 1 molar ratio; in addition, their carbohydrate chains contain an identical repeated motif. The only distinction between them is the presence of fatty acids (C_{12}–C_{18}) in the APS [1, 8].

The practical use of ethapolan is based on its rheological properties. Ethapolan possesses an emulsifying capacity; its viscosity increases with decreasing pH in the presence of univalent or divalent cations; high viscosity values are observed at low shear rates and in the Cu^{2+}–glycine system. The rheological properties of ethapolan depend on its component (APS/NAPS) ratio and the fatty acid content in the APS [5]. The APS synthesis and acylation depend on the univalent cation (K^+ and Na^+) concentration in the culture medium [6].

In a previous study [1], *Acinetobacter* sp. 12S was cultivated at the second stage (unfavorable growth conditions) of the two-stage process at a relatively low temperature of 24°C and an alkaline pH (8.0). In this study, we used FA to provide unfavorable growth conditions, because ethapolan is capable of protecting the cells against this biocide [13]. First, we had to determine the concentration at which FA improves the rheological properties of ethapolan without reducing its yield. Table 1 shows the amounts of biomass formed by *Acinetobacter* sp. 12S in the presence of FA, which was added at various concentrations during the lag, exponential, or stationary growth phase. Whatever the FA concentration, neither the biomass nor EPS yield decreased if FA was added at the end of exponential growth or during the stationary phase. In further experiments, we added FA in the late exponential phase.

Studies of the rheological properties of ethapolan at varying FA concentrations showed that, if bacterial cells were grown in the presence of 30 μg/ml FA, their dilute culture liquid responded with a significant increase in viscosity to addition of KCl or a Cu^{2+}–glycine combination (Fig. 1). Its initial viscosity was also higher than that of the dilute culture liquid of control cells grown without exposure to FA. Conceivably, FA bonding with EPS molecules altered their conforma-

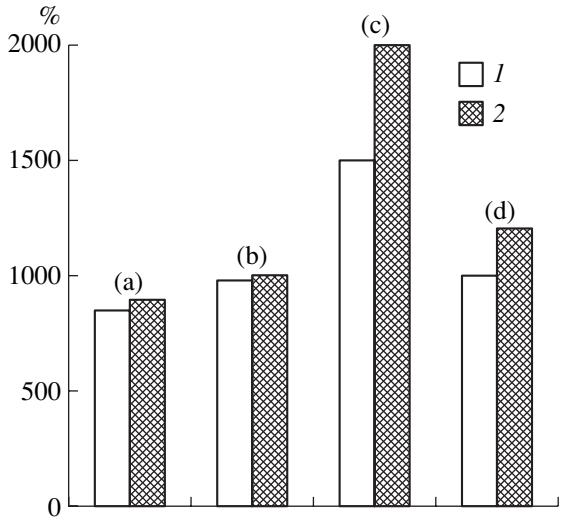

Fig. 2. Timing of FA treatment and relative changes in the viscosity (η_{rel}, %) of the dilute culture liquid: (*1*) 0.1 M of KCl vs. distilled water and (*2*) Cu^{2+} + glycine vs. distilled water. (a) FA (30 μg/ml) added directly into the dilute culture liquid; (b) 30 μg/ml FA added at the stationary phase; (c) 30 μg/ml FA added at the late exponential phase; and (d) control (dilute culture liquid of cells grown without exposure to FA).

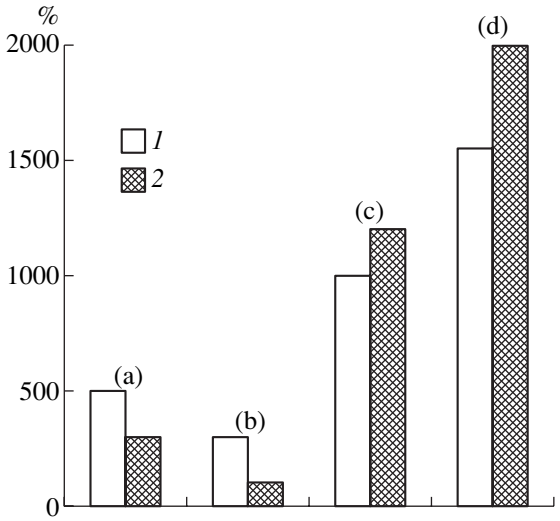

Fig. 3. Relative changes in the viscosity (η_{rel}, %) of the dilute culture liquid of *Acinetobacter* sp. 12S grown at a univalent ion concentration of (a, b) 0.65 M or (c, d) 0.9 M in the (a, c) absence or (b, d) presence of 30 μg/ml FA: (*1*) 0.1 M of KCl vs. distilled water and (*2*) Cu^{2+} + glycine vs. distilled water.

tion and thereby changed their rheological properties. It was reasonable to question whether addition of FA to the culture liquid when the producer stopped growing would also improve the rheological properties of ethapolan. However, the effect was observed only when FA was added to growing cells (Fig. 2), suggesting that the molecules were cross-linked during their synthesis.

Our previous studies [1, 3, 5] showed that the fatty acid content in ethapolan determines the rheological properties of its solutions. FA added to the culture medium affected neither the APS to NAPS ratio nor the fatty acid content in the APS (Table 2). Only at high univalent ion concentrations in the culture medium did addition of FA improve the rheological properties of the EPS (Fig. 3). The presence of univalent ions at high concentrations in the culture medium favored APS synthesis: the APS content in ethapolan increased up to 79%. Therefore, it is conceivable that FA interacted with highly acylated EPSs.

The rheological properties of ethapolan were studied at all stages of its isolation and purification. These experiments were expected to test the suggestion on chemical (or physicochemical) interaction between EPSs and FA by demonstrating whether or not their binding was strong. If FA were loosely bound to EPS molecules, it would become free during dialysis and the precipitation steps of EPS purification. Such a phenomenon was observed previously when we studied interactions of ethapolan with cations [3, 6, 14]. In the presence of cations, links were formed between the polysaccharide molecules, as judged by increases observed in the molecular weights of EPSs and in the viscosity of EPS solutions. However, during dialysis and then during precipitation with inorganic solvents, cations dissociated from their complexes with EPSs. This process adversely affected the rheological properties of the purified ethapolan.

It is reasonable to assume that, if FA binds loosely to polysaccharide molecules, the purified ethapolan and the ethapolan synthesized by cells grown in the absence of FA would have similar rheological properties. However, at all stages of purification, ethapolan (whether APS or NAPS) synthesized in the presence of FA had a higher viscosity in the Cu^{2+}–glycine system than ethapolan synthesized in the absence of FA (Fig. 4). Similar results were obtained when 0.1 M of KCl was used instead of the Cu^{2+}–glycine system or when the EPS was studied in the H^+-form. These results suggested that FA was strongly bound to EPS molecules. Taking into account the data shown in Fig. 3, we expected FA

Table 2. Chemical composition of ethapolan synthesized by cells grown in the presence of FA

Univalent ion concentration, M	FA concentration, μg/ml	APS content, %	Fatty acid content in the APS, %
0.065	without FA	40	4.0
	FA (30 μg/ml)	40	4.2
0.09	without FA	70	12.4
	FA (30 μg/ml)	73	12.0

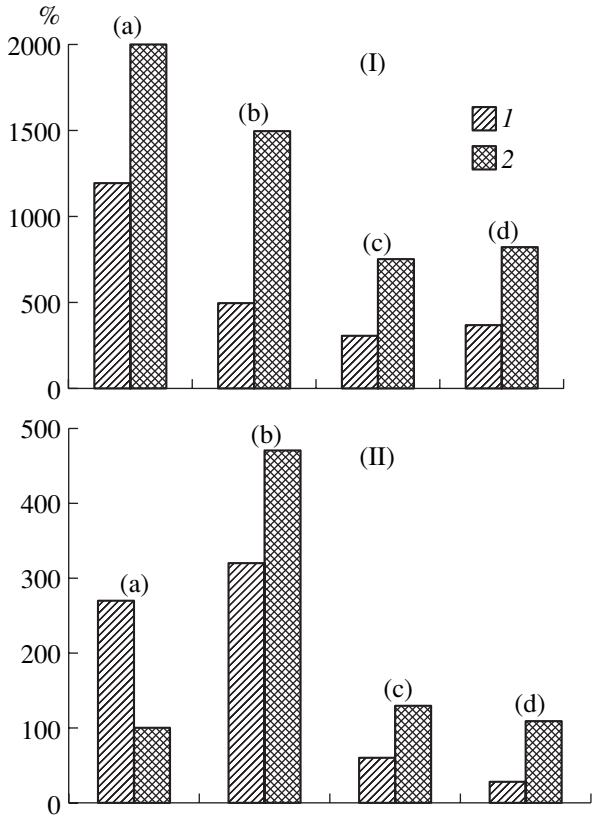

Fig. 4. Relative change (η_{rel}, %) in the ethapolan (0.03% carbohydrate solution) viscosity in the Cu^{2+}–glycine system (vs. distilled water) at different stages of purification from the culture liquid of cells grown at a univalent ion concentration of (I) 0.65 M or (II) 0.9 M in the (*1*) absence or (*2*) presence of 30 μg/ml FA: (a) before dialysis, (b) after dialysis, (c) after evaporation, and (d) after isopropanol precipitation and drying.

to bind only to highly acylated EPS molecules. However, the weakly acylated EPS purified from the FA-containing culture liquid also improved the rheological properties (Fig. 4), suggesting that FA could bind not only to highly acylated but also to weakly acylated EPS molecules. The effect of FA on the rheological properties of EPSs depended on their fatty acid content: the higher the content, the more favorable the effect.

To provide evidence of FA binding to EPS molecules, we studied their molecular weights. The mean molecular weights of all the EPSs studied, irrespective of the extent of their acylation, decreased 1.5- to 3.0-fold after precipitation (Table 3). However, this decrease was consistently lower for EPSs synthesized in the presence of FA than for those synthesized in the absence of FA (34–39% vs. 58–66%). The EPSs synthesized in the presence of FA at different univalent ion concentrations did not differ in the percentage of fractions with molecular weights below 2000 kDa (Table 3). These results demonstrate that the two-stage cultivation technique, with the use of FA at the second stage, is especially expedient in practical applications of ethapolan.

Analysis of the relationship between the EPS components of different molecular weights showed that the EPSs synthesized in the presence of FA contained fewer low-molecular-weight (13, 20, and 40 kDa) fractions and more high-molecular-weight (110–500 kDa) fractions (Fig. 5). Presumably, FA linked low-molecular-weight fragments of EPSs into larger molecules, which may account for the observed improvement of the rheological properties of such EPSs.

In experiments designed to study the effect of glucuronic-acid-specific lectin on the physicochemical properties of ethapolan solutions, we found that FA did not interact with reactive functional groups of the EPSs (at least with glucuronic acid residues). In fact, although lectin binding to glucuronic acid residues of EPS molecules resulted in the formation of their complex with a higher molecular weight, the viscosity of solutions decreased (Table 4). In addition, the viscosity of lectin–EPS complex solutions did not change in the presence of cations, in the Cu^{2+}–glycine system, or after conversion into the H^+-form.

Unlike lectin, FA interacts with EPSs without impairing their rheological properties. Moreover, the presence of FA during EPS synthesis improved their rheological properties. Hence, FA, unlike lectin, does not block the reactive functional groups of EPSs.

Table 3. Molecular weights of EPSs synthesized by *Acinetobacter* sp. 12S grown in a FA-containing medium

Univalent ion concentration, M	FA concentration, μg/ml	Evaporated EPS		Precipitated EPS		Relative decrease in mean MW after precipitation of EPS, %
		mean molecular weight, kDa	percentage of EPS fractions with MWs below 2000 kDa	mean molecular weight, kDa	percentage of EPS fractions with MWs below 2000 kDa	
0.065	without FA	926.0	58.1	386.6	88.3	58.3
	FA*	864.2	61.5	522.5	71.2	39.3
0.090	without FA	1441.0	31.7	479.8	82.7	66.7
	FA*	1016.7	53.7	667.7	74.2	34.4

* 30 μg/ml FA.

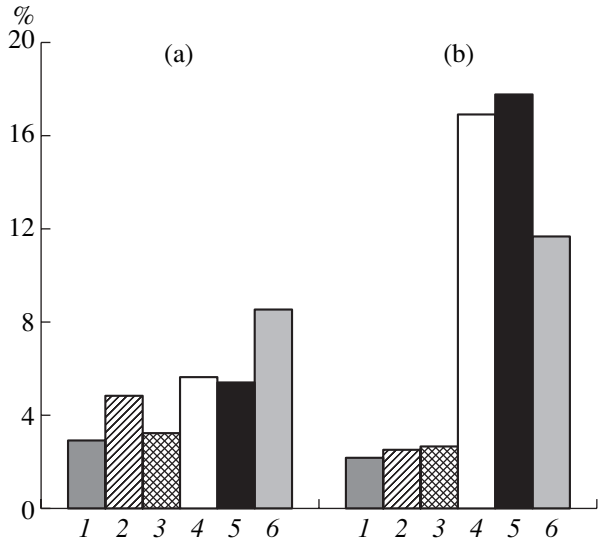

Fig. 5. Molecular-weight analysis of ethapolans synthesized (a) in the absence and (b) in the presence of FA: (*1*) 13.5, (*2*) 20, (*3*) 40, (*4*) 70, (*5*) 110, and (*6*) 500 kDa. Ordinate: percentage of fractions.

Thus, we analyzed the two-stage process of ethapolan synthesis with addition of the toxic factor FA at the second stage. Note that the known two-stage cultivation techniques for microbial EPS production [15, 16] are based on a different approach: the conditions of the first stage are favorable for the growth and those at second stage facilitate the EPS synthesis. A distinctive feature of our technique is that favorable conditions for bacterial growth and EPS synthesis at the first stage were followed by unfavorable conditions at the second stage in order to fully stimulate the cell-protecting functions of microbial EPSs. In response to unfavorable conditions, bacterial cells synthesized EPSs with altered rheological properties. Hence, when such a two-stage technique is used to cultivate bacterial cells, the rheological properties of EPSs are regulated by the enhancement of their cell-protecting effects.

Table 4. Physicochemical characteristics of the EPS–lectin complex

Polysaccharide	Mean molecular weight, kDa	Percentage of fractions with MMs below 2000 kDa	Viscosity of 0.1% solution, mm^2/s
EPS (without lectin)	479.0	82.7	11.7
EPS + lectin (1 : 1)	560.7	81.0	8.8
EPS + lectin (10 : 1)	525.9	84.7	6.5

REFERENCES

1. Pirog, T.P., Regulation of THE Composition and Physical-Chemical Properties of Exopolysaccharides Synthesized by *Acinetobacter* sp., *Doct. Sci. (Biol.) Dissertation*, Kiev: Inst. Microbiology and Virology NAN Ukraine, 1999.
2. Pirog, T.P., *Mikrobiol. zh. (Kiev)*, 1999, vol. 61, no. 5, pp. 64–71.
3. Grinberg, T.A., Pirog, T.P., Malashenko, Yu. R., and Pinchuk, G.E., *Mikrobnyi sintez ekzopolisakharidov na C_1–C_2-soedineniyakh* (Microbial Synthesis of Exopolysaccharides in C_1 and C_2-Compounds), Kiev: Naukova Dumka, 1992.
4. Kodama, T., Nakakhara, T., Omori, T., Binkh, N.T., Khoshino, K., and Minoda, I., Abstracts of Papers, *Simposium po rostu mikroorganizmov na C_1-soedineniyakh*, (Symp. on Growth of Microorganism in C_1 Compounds), Pushchino: NTsBI AN SSSR, 1977, pp. 213–215.
5. Pirog, T.P., Grinberg, T.A., Senchenkova, S.N., and Malashenko, Yu.R., *Mikrobiologiya*, 1995, vol. 65, no. 4, pp. 527–532.
6. Pirog, T.P., *Mikrobiologiya*, 1996, vol. 65, no. 5, pp. 639–643.
7. Dubois, M., Gilles, K., Hamilton, J., Rebers, P., and Smith, F., *Anal. Chem.*, 1956, vol. 28, no. 3, pp. 350–356.
8. Pirog, T.P., Grinberg, T.A., Pinchuk, G.E., Senchenkova, S.N., and Malashenko, Yu.R., *Mikrobiologiya*, 1994, vol. 63, no. 5, pp. 840–846.
9. Votselko, S.K., Pirog, T.P., Malashenko, Y.R., and Grinberg, T.A., *J. Microbiol. Methods*, 1993, no. 18, pp. 349–356.
10. Podgorskii, V.S., Kovalenko, E.A., and Simenko, I.A., *Lektiny bakterii* (Bacterial Lectins), Kiev: Naukova Dumka, 1992.
11. USSR Inventor's Certificate no. 1 726 732, 1992.
12. Lakin, G.F., *Biometriya* (Biometry), Moscow: Vysshaya shkola, 1990.
13. Pirog, T.P., Grinberg, T.A., and Malashenko, Yu.R., *Mikrobiologiya*, 1997, vol. 66, no. 3, pp. 335–340.
14. Pirog, T.P., Grinberg, T.A., Buklova, V.N., Votselko, and S.K., Malashenko, *Mikrobiologiya*, 1995, vol. 64, no. 1, pp. 51–54.
15. UK Patent 2090847, 1982.
16. European Patent 0112661, 1984.