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Effect of Environmental Factors on the Synthesis and Properties of *Acinetobacter* sp. Exopolysaccharides

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Abstract—The effects of external factors on the synthesis and physicochemical properties of *Acinetobacter* sp. exopolysaccharides (EPSs), which determine the biological functions of this microorganism, were studied. The cultivation temperature, medium pH, and oxygen concentration in the medium (pO_2) affected the viscosity of EPS solutions in the presence of monovalent cations in the H^+-form and in a Cu^{2+}–glycine system. All of the EPSs studied were precipitated with heavy metal ions (Cr^{3+}, Cu^{2+}, Pb^{2+}, Cd^{2+}, etc.). No changes in the EPS yield were observed under unfavorable environmental conditions. At high pO_2 values (up to 80% saturation), the maximum specific rates of bacterial growth and EPS synthesis increased. It was suggested that *Acinetobacter* sp. EPSs perform different biological functions under optimal and nonoptimal conditions.

The capacity for exopolysaccharide (EPS) synthesis offers producer microorganisms certain advantages over nonproducers [1, 2]. In particular, EPSs protect microbial cells against the harmful action of environmental factors. We suggested that not only EPS production but also the ability to alter the composition and physicochemical properties of EPSs are required for normal functioning of cells under various environmental conditions. Thus, changes in physicochemical properties of EPSs enable these molecules to fulfill their biological functions upon alterations in the environmental conditions and, therefore, to enable the survival of microbial populations.

To reveal external factors that affect the properties of synthesized EPSs, one should consider the ecological niche that the microorganism studied inhabits. Since *Acinetobacter* sp. was isolated from sewage, the most important external factors that may vary within this ecological niche and affect the properties of EPSs are temperature, pH, oxygen concentration in water, and concentrations of cations, heavy metal ions, nitrocompounds, chlorine-containing substances, dyes, etc.

We believe the following physicochemical properties of EPSs to be responsible for fulfilling the biological functions: viscosity of EPS solutions and its increase at low pH values, formation of regular structures in the presence of cations, precipitation with ions of bivalent and trivalent metals, etc. [3]. It should be noted that these properties also determine the practical importance of EPSs. Therefore, studies of properties of EPSs synthesized by *Acinetobacter* sp. under different cultivation conditions may extend the area of possible application of these polymers.

In light of this fact, this work was designed to study the effects of external factors on the synthesis and physicochemical properties of *Acinetobacter* sp. EPSs.

MATERIALS AND METHODS

Acinetobacter sp. was cultivated on Kodama's mineral medium [4] containing 1 vol % ethanol as the only energy and carbon source, 0.0003% calcium pantothenate, and 0.5 vol % yeast autolysate. The concentrations of K^+ and Na^+ in the medium were 50 and 40 mM, respectively (K^+ was added to the medium as KCl).

Periodical cultivation of *Acinetobacter* sp. was performed in flasks on a shaker or in an AK-210 fermenter as described earlier [5, 6].

The effects of temperature, pH, and oxygen concentration in the medium (pO_2) on the synthesis and properties of *Acinetobacter* EPSs sp. were studied.

The following conditions were found to be optimal for *Acinetobacter* sp. growth: 28–30°C, pH 6.8–7.0, and pO_2 of 30–40% saturation.

The bacteria were cultivated at 24, 30, 37, or 42°C at pH 5.0, 6.0, 7.0, or 8.0. The concentration of oxygen was 5–10, 30–40, 50–60, or 70–80% saturation.

When studying the effect of a particular factor, the other two factors were maintained at their optimal levels.

pH was adjusted with 6% HCl or 6% NaOH.

The oxygen concentration in the medium was maintained by regulating the rate of medium shaking (100–700 rpm) and the air flow rate (0.2–3.0 l per l medium per min).

The effect of high oxygen concentrations on *Acinetobacter* sp. growth and EPS synthesis was studied under conditions of fed-batch cultivation in an AK-210

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Table 1. Effects of external factors on the biomass yield and EPS synthesis in *Acinetobacter* sp.

Factor		Concentration, g/l		EPS yield, g/g DCM
		biomass	EPS	
Temperature, °C	24	1.90	1.85	0.97
	30	2.20	2.10	0.95
	37	0.96	1.00	1.04
	42	0.65	0.70	1.08
pH	5.0	1.10	1.00	0.91
	6.0	1.80	1.70	0.94
	7.0	2.20	2.10	0.95
	8.0	1.80	1.75	0.97
Oxygen concentration in the medium, % of saturation	5–10	1.70	1.80	1.06
	30–40	2.20	2.10	0.95
	50–60	2.00	1.90	0.95
	70–80	2.00	1.90	0.95

fermenter. At the first stage, the bacteria were cultivated at the optimal oxygen concentration, and the bacterial culture grown in flasks on a shaker was used for inoculation. After cultivation, the culture liquid (80–85% of total volume) was decanted, and a fresh medium was added. At the second stage, the bacteria were cultivated at pO_2 of 50–60 or 70–80% saturation.

The biomass weight was determined from the optical density of the bacterial culture and calculated as the dry cell mass (DCM) using the calibration curve.

The amount of synthesized EPSs was calculated from the total content of carbohydrates determined colorimetrically from the reaction with phenol and sulfuric acid [7]. The calibration curve was plotted using standard solutions of glucose.

The EPS yield was determined as a ratio of the amount of synthesized EPSs (g/l) to the biomass yield (g/l).

The maximum rates of *Acinetobacter* sp. growth (μ_{max}) and EPS synthesis (Q_{max}) were estimated by the method described in [8].

EPSs were isolated and purified, and acylated and nonacylated EPS components were fractionated as described in [9].

The kinematic viscosity of EPS solutions was measured with a glass Ostwald viscosimeter at 20°C in distilled water in the presence of cations (K^+, Na^+, or NH_4^+), at low pH values (upon transition into the H^+-form), and in a Cu^{2+}–glycine system.

A relative increase in the viscosity was determined as the ratio of the difference between the values of the kinematic viscosity under experimental conditions and in distilled water to the viscosity of the EPS solution in distilled water and expressed in percents.

EPS solutions for viscosimetry were prepared as follows. EPS solutions obtained after dialysis of the culture liquid, separation of cells, and concentration in the vacuum were diluted with distilled water to the concentration of 0.03% (with respect to carbohydrates). KCl, NaCl, or NH_4Cl was then added to the concentrations of 0.1 or 0.4 M, respectively. EPSs were converted to the H^+-form with KU-2-8 cationite (H^+) (300 mg of resin per 15 ml of EPS solution). To study the behavior of EPS solutions in the Cu^{2+}–glycine system, 3 mM $CuSO_4 \cdot 5H_2O$ and 15 mM glycine were added to the EPS solution; the mixture was heated to 80°C, incubated at this temperature for 5 min, and then cooled to 20°C.

RESULTS AND DISCUSSION

When *Acinetobacter* sp. was cultivated under the optimal conditions, the biomass yield was 2.2 g/l, and the EPS yield was 2.10 g/l (Table 1). EPSs synthesized by *Acinetobacter* sp. grown under the same conditions in the presence of 90 mM monovalent cations contained 70% acylated polysaccharides with the extent of acylation of 12.4%. Relative increases in the viscosity of 0.03% (with respect to carbohydrates) EPS solutions were 350–400% in the presence of 0.1 M K^+; 1000–1100% in the H^+-form; and 900–1000% in the Cu^{2+}–glycine system [10].

Changes in temperature, pH, and pO_2 did not affect the EPS yield (1 g/g DCM) (Table 1). At low pO_2 and pH values or at increased temperatures, the duration of the lag phase increased by 3–5 h. It was found earlier that the biomass and EPS yields remained constant upon *Acinetobacter* sp. cultivation at different concentrations of monovalent cations (0.065–0.140 M) [6].

When *Acinetobacter* sp. was grown at high pO_2 values (70–80% of saturation), the maximum specific rates of bacterial growth and EPS synthesis increased; moreover, they were achieved at an earlier period of growth (Table 2). It should be noted that at early stages of cultivation, *Acinetobacter* sp. is sensitive to high oxygen concentrations in the medium [3]. Thus, when the bacteria were grown in a fermenter (after inoculation with the seeding material from the flasks) at pO_2 over 50%, the culture growth was inhibited. When the bacteria were grown by fed-batch cultivation, no growth inhibition was observed at even higher pO_2 values (up to 80% saturation). In the latter case, the cells were protected from the toxic action of oxygen by to the high viscosity of the culture liquid used as seeding material and the high amount of the EPS-containing inoculum (15–20%). We consider this phenomenon to be a manifestation of the adaptive mechanism that allows the population to survive under unfavorable conditions.

Data shown in Table 1 suggest that, in our experiments, *Acinetobacter* sp. cells responded to unfavorable cultivation conditions by changing the properties of the synthesized EPSs.

Earlier, we found that physicochemical properties of *Acinetobacter* sp. EPSs (structuration in the presence

of cations, viscosity increase at low pH values or in the Cu^{2+}–glycine system, etc.) are determined by the ratio between acylated and nonacylated polysaccharides and the extent of their acylation [5, 10–12]. Solutions of *Acinetobacter* sp. EPSs with a high content of nonacylated polysaccharides (over 50%) did not display the properties mentioned above. The formation of acylated polysaccharides depends on the monovalent cation (K^+ and Na^+) concentration in the cultivation medium [10–12]. Thus, EPSs synthesized by *Acinetobacter* sp. grown in the presence of 65 mM monovalent cations contained only 40% acylated polysaccharides with a low extent of acylation (4%). The content of acylated polysaccharides increased to 70–95% with the increase in the monovalent cation concentration in the medium up to 90–140 mM [10].

When the cultivation temperature was increased to 37 or 42°C, *Acinetobacter* sp. synthesized EPSs whose solutions were not virtually structured with cations. The viscosity of these solutions only slightly increased in the Cu^{2+}–glycine system or upon conversion into the H^+-form (Fig. 1). We found that mainly nonacylated polysaccharides (90–95% of the total polysaccharide content) were synthesized, probably due to disorders in the activity of enzymatic systems involved in fatty acid synthesis or excretion at high temperatures.

At the cultivation temperature of 24°C, *Acinetobacter* sp. synthesized EPSs whose solutions increased their viscosity up to 800–900% in the presence of K^+ and Na^+ (versus 350–400% observed for EPSs synthesized at 30°C) (Fig. 1). Similar properties were characteristic of EPSs synthesized at pH 5.0, 6.0, or 8.0 or at low pO_2 values (Figs. 2 and 3). It should be noted that in the presence of NH_4^+, the viscosity of these solutions increased to a lesser extent (Figs. 1–3).

Table 2. Effect of oxygen concentration in the medium on the rates of growth and EPS synthesis by *Acinetobacter* sp.

Oxygen concentration in the medium, % of saturation	μ_{max}, h^{-1}	Q_{max}, h^{-1}	Time from the beginning of cultination, h	
			μ_{max}, h^{-1}	Q_{max}, h^{-1}
30–40	0.12	0.06	12	16
70–80	0.20	0.10	4	8

In contrast to EPSs synthesized under the optimal conditions, less pronounced increases in the viscosity in the Cu^{2+}–glycine system of solutions of EPSs synthesized at 24°C; pH 5.0, 6.0, or 8.0; or at low pO_2 values were observed (Figs. 1–3). This phenomenon may be explained as follows. EPS acidic groups (residues of glucuronic and pyruvic acids) are involved in the reaction with Cu^{2+} and the structuring of EPS solutions by monovalent cations. EPSs are structured during their synthesis. It appears that under nonoptimal conditions, *Acinetobacter* sp. synthesizes EPSs with a high capacity for the formation of regular structures in the presence of monovalent cations. Solutions of such EPSs should only slightly increase their viscosity in the Cu^{2+}–glycine system, because all acidic groups in EPSs are occupied with monovalent cations.

Solutions of EPSs synthesized by *Acinetobacter* sp. at decreased temperatures; pH 5.0, 6.0, or 8.0; or low pO_2 values were characterized by similar viscosity increase in the presence of monovalent cations (Figs. 1–3). The viscosity in the Cu^{2+}–glycine system of solutions of EPSs synthesized at pH 5.0 and 6.0 was lower than that of other EPS. The low viscosity in the Cu^{2+}–glycine system of solutions of EPSs synthesized at low pH values may be explained by the low contents of glucuronic and/or pyruvic acids in these EPSs.

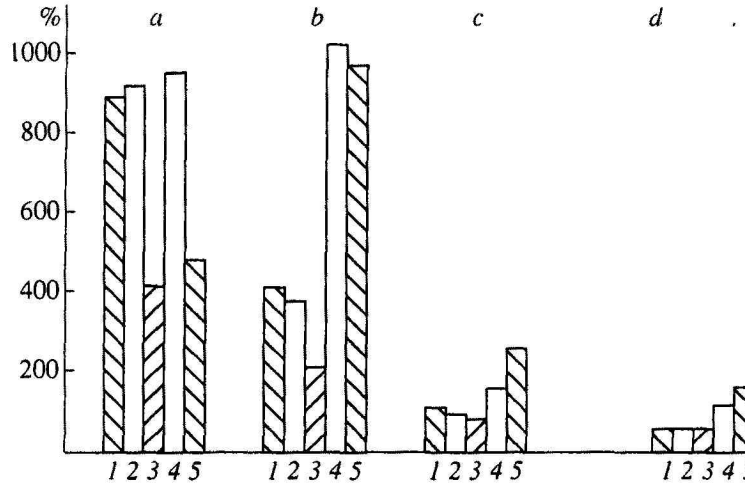

Fig. 1. Effect of cultivation temperature on the viscosity increase in *Acinetobacter* sp. EPS solutions in the presence of (*1*) 0.1 M KCl, (*2*) 0.1 M NaCl, and (*3*) 0.4 M NH_4Cl; (*4*) in the H^+-form; (*5*) in the Cu^{2+}–glycine system. Cultivation temperature: (*a*) 24; (*b*) 30; (*c*) 37; and (*d*) 42°C.

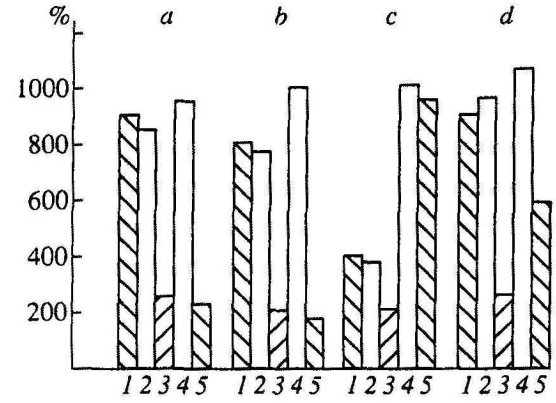

Fig. 2. Effect of medium pH on the viscosity increase in *Acinetobacter* sp. EPS solutions in the presence of (*1*) 0.1 M KCl, (*2*) 0.1 M NaCl, and (*3*) 0.4 M NH_4Cl; (*4*) in the H^+-form; (*5*) in the Cu^{2+}–glycine system. Medium pH: (*a*) 5.0; (*b*) 6.0; (*c*) 7.0; and (*d*) 8.0.

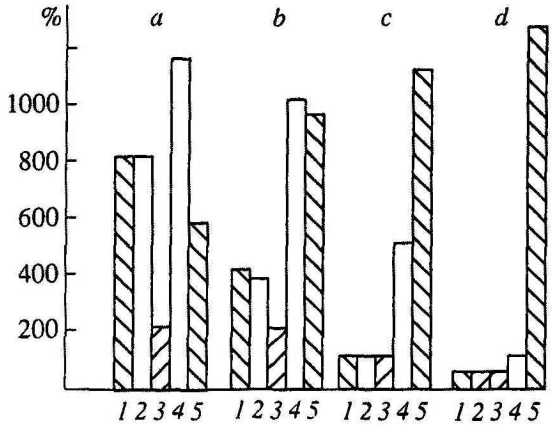

Fig. 3. Effect of oxygen concentration in the medium on the viscosity increase in *Acinetobacter* sp. EPS solutions in the presence of (*1*) 0.1 M KCl, (*2*) 0.1 M NaCl, and (*3*) 0.4 M NH_4Cl; (*4*) in the H^+-form; (*5*) in the Cu^{2+}–glycine system. Oxygen concentration in the medium (%): (*a*) 5–10; (*b*) 30–40; (*c*) 50–60; and (*d*) 70–80.

When the oxygen concentration in the medium increased to 50–60 or 70–80%, the properties of the EPS solutions altered. Thus, no structuring effect of monovalent cations was observed. At pO_2 of 70–80%, EPSs formed solutions, which did not increase their viscosity upon the transition into the H^+-form (Fig. 2) because of the predominant formation of nonacylated polysaccharides (over 90% of the total polysaccharide content). The viscosity of such solutions considerably increased in the Cu^{2+}–glycine system due, probably, to the fact that these solutions were not structured by monovalent cations. However, solutions of EPSs synthesized at 37 or 42°C, which contained mainly nonacylated polysaccharides, were also not structured by cations, but their viscosity did not increase in the Cu^{2+}–glycine system (Fig. 1). Therefore, EPSs with high contents of glucuronic and/or pyruvic acids are synthesized at high pO values.

Earlier, we demonstrated that EPSs synthesized by *Acinetobacter* sp. under different cultivation conditions (in particular, in the presence of C_4 dicarbonic acids or various monovalent cation concentrations in the medium) are characterized by the same ratios between the contents of neutral monosaccharides, glucuronic acid, and pyruvic acid [10, 12, 13]. The data obtained in this study allows the suggestion that upon *Acinetobacter* sp. cultivation at low pH values or in the presence of high oxygen concentrations in the medium, the content of acidic groups in EPSs may vary. Such variation in the EPS composition, as well as changes in the content of acylated polysaccharides and the extent of their acylation [10–12], may result in the changes in certain physicochemical properties of EPS solutions.

It should be noted that all the EPSs studied (i.e., those synthesized at different temperatures, pH, pO_2 values, and concentrations of monovalent cations in the cultivation medium) precipitated in the presence of Cr^{3+}, Fe^{3+}, Al^{3+}, Pb^{2+}, Cd^{2+}, and Cu^{2+}. The capacity of *Acinetobacter* sp. EPSs to absorb heavy metal ions upon precipitation appears to be the major factor in the cell resistance to toxic metals.

In conclusion, changes in the external factors of *Acinetobacter* sp. cultivation result in the synthesis of EPSs whose solutions exhibit different physicochemical properties. The results obtained allowed us to suggest that the biological functions of these EPSs differ and to reveal new areas of EPS application.

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