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Physicochemical and Biological Properties of Polysaccharides Contained in Biofilms Formed by *Psseudomonas alcaligens.*

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ABSTRACT

The article provides the results of investigations of physicochemical and biological properties of microbial polysaccharides. The representatives of the genus *Pseudomonas*, namely non-fermentative ubiquitous bacteria having specific metabolic cycles, were used as natural producers of exopolysaccharides. In the result of comparative study there was determined a range of monosaccharides, oligosaccharides as well as of free -COOH - OCH_3 groups and oxyacids in the composition of metabolites of geographically heterogeneous *Pseudomonas alcaligens* strains. There was assessed the activity of biosynthesis in the presence of hydrated fullerene in terms of ability to form biofilm on the surface of polystyrene plates and in terms of a set of lytic ferments. There were obtained data on antagonist properties of polysaccharides in regard to pathogenic and opportunistic pathogens. The results of experiments are of great value for the following assessment of a perspective of microbial exopolysaccharides use as new-generation nanomaterials.

Keywords: biofilms, biosynthesis, biotechnology, nanomaterials, polysaccharides, producers, *Pseudomona alcaligens*

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INTRODUCTION

The most of modern materials are represented by various nanocompounds with a definite range of functional properties and this situation can explain intense interest of the investigators to study of performance characteristics of modified natural polymers. Microbial polysaccharides synthesized by unicellular organisms in the course of their growth and development under diverse environmental unbalance conditions may be named as an example of such compounds. The fact that mucus produced by a lot of microorganisms may have a carbohydrate nature was already known at the times of Pasteur [25]. However microbial polysaccharides became a subject of detailed study only at the beginning of the twenties of the last century when it became known that the substances which determined serospecificity of pnemococci referred to polysaccharides. By the present time the following properties of exopolysaccharides were discovered: high viscosity and gelling ability, compatibility with a range of salts under high variety of pH and temperature and high water solubility [4, 22]. It was found that free hydroxyl groups of polysaccharides are acidylable and alkylable and that glycosidic linkages of polysaccharides are hydrolysable under the influence of acids and specific ferments [1, 7, 8]. Currently microbial polysaccharides are widely used by different industry sectors, medicine and knowledge-intensive production. For example as a matrix for tissue engineering, for obtaining surgical and non-woven fabrics, elements for osteosynthesis, vascular implants, drug delivery systems, foods preparation technologies and many other applications [9, 12, 16]. Intensive development of various technologies which use extracellular polysaccharides for their own purposes resulted in high volumes of industrial production of such polysaccharides in foreign developed countries (USA, FRG, France, Japan etc.). Annual global increase of production of microbially-derived polymers makes 10% at the average. Requirement for such compounds constantly grows but the demand for them is not met to the full extent [31, 32].

The vast majority of microbial polysaccharides have a unique structure which is specific for this genus or for the genus serogrup and as a rule is represented by a mixture of molecules with different molecular weight but with similar chemical composition [2, 11, 13]. They are represented by macromolecular carbohydrates with a general formula $C_nO_nH_{2m}$, which do not contain non-carbohydrate components and are built from residues of monosaccharides. Since polysaccharides of microorganisms may be composed of one or several types of monosaccharides, polysaccharides are divided into homopolysaccharides and heteropolysaccharides. Basic chain of xanthan (core) is built similar to cellulose (1-4-(3-glycopyranose), and the core branches contain trisaccharide composed of beta-D-mannose, beta-D-glucuronic acid and alpha-D-mannose. Glucuronic acid residues and acidulous pyroracemic groups provide xanthan molecules with anionic nature. It is necessary to point out that molecules of dextrans are represented by branched chains the linear part of which contains 1:6-linkages and small quantity of 1:3-linkages, some rare-occurring dextrans have alternating 1:6- and 1:3-linkages. Branches in dextran molecule are formed by means of 1:2-, 1:3- or 1:4-linkages. An individual dextran usually has one or two types of branching linkages. As a rule side branches of a molecule are composed of one or two glucose residues, side chains with bigger length are rarely-occurring. In industry this polymer is produced by growing of the abovenamed microorganisms with use of saccharose. Most of polysaccharides are products of intracellular synthesis however at time of dextran formation substrate does not penetrate into a cell [6, 18, 23]. Dextrans are classified depending on the ratio of each of three types of linkages as well as on water solubility. Relative molecular mass of native dextrane reaches hundreds of millions of daltons. Native dextran is subject to hydrolyse which should result in obtaining of a preparation with a set molecular mass distribution:

- I group — low-molecular dextrans (30000 — 40000 D) — polyoxidinum;
- II group — medium-molecular dextrans (50000 — 70000 D) — polyglucin, polyglusol.

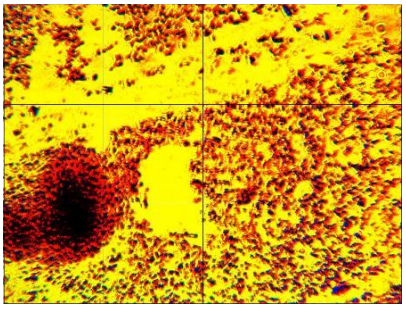
Dextransaccharase is an extracellular enzyme acting as a catalyst in dextran synthesis, it is inducible but may by synthesized within a logarithmic phase of growth. This enzyme releases fructose and transports glucose residues to an enzyme-linked acceptor molecule. The growing dextran chain remains in close linkage with the enzyme. The investigations of immobilized dextransaccharase showed that it generates products with narrow range of molecular masses while within a soluble system very high-molecular dextrans are synthesized. The organisms synthesizing dextrans produce large quantities of this enzyme in a soluble form or in a cell-linked state. Moreover microbial glycans often contain previously unknown monosaccharides which are not found both in plants and in animals [15, 17, 26]. Such high polymers have not only a primary spatial structure but also advanced spatial structures. These are due to weak intramolecular interactions main part in which is being played by hydrogen bonds and complex formation. Since microbial exopolysaccharides are considered to

be very important for biotechnology and biomedicine study of these compounds as contained in the genus *Pseudomonas alcaligens* microorganisms which have high potential from the point of view of biotechnology would be of current concern. These are non-fermentative aerobic bacteria with characteristic ubiquitous natural occurrence and complex metabolic cycles within the trophic chains of biocenoses [2, 14, 24]. Notwithstanding high variety of works dedicated to Pseudomonas biology and ecology biomolecular peculiarities of their metabolism have not still been thoroughly studied. There is information available on infectious value of cellular and extracellular biopolymers of pathogenic Burkholderia [19, 20] and Pseudomonas [2, 21] however data on polysaccharides properties of individual types of the genus *Pseudomonas* are scarcely ever given.

Study of physicochemical and biological properties of *Pseudomonas alcaligens* polysaccharides contained in biofilms were performed by the Laboratory of preclinical studies, cytopathology and bioregulation of the Institute of High Biomedical Technologies of Petrozavodsk State University as a part of the University Strategic Development Program. The experiments were carried out with use of the standard bacteriological methods of investigation [28, 33]. 15 *Pseudomonas alcaligens* wild strains with different geographical origin were used as exopolysaccharides producers.

BIOFILM-FORMING ACTIVITY OF BIOFILM PRODUCERS - *PSEUDOMONAS ALCALIGENS*

The ability of strains-producers of polysaccharides to form a biofilm on the surface of 96-cup polystyrene plates was studied with use of the G.O. Toole and R. Kolter method [29]. Activity of biofilm formation was assessed by the level of a coloring agent absorption by ethanol which was measured in optical-density units (OD630) with use of a universal single-beam spectrophotometer LEKI SS2107 at the wavelength of 630 nm. For interpretation of the obtained data the ability of strains to form a biofilm was determined according to the criteria developed by Stepanovic S. et al. [30]: when the values of OD_{630} were lower then 0.090 it was considered that the strains did not have the ability to form a biofilm; with the range of values from $0.090 < OD_{630} \leq 0.180$ the strains had weak ability; with the range of $0.180 < OD_{630} \leq 0.360$ the strains had medium ability; with the range of $OD_{630} \leq 0.360$ the strains had high ability to form a biofilm. Digital images of the fields of vision were obtained by means of a microscope MOTIC and modular software ZEN ("Carl Zeiss", Germany). The digital images of the preparations were used for measurement of an area of a field of vision and an area occupied by single adherent cells and microcolonies. There were calculated the shares of single adherent cells and microcolonies in the field of vision area.


a

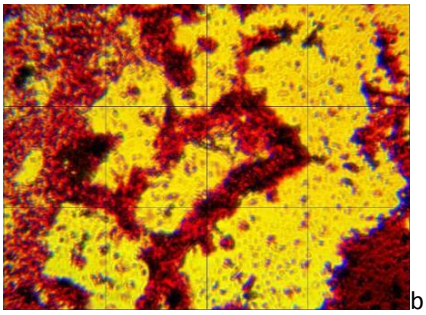

b

Figure 1: Correlation of shares of microcolonies formed by the *P. Alcaligens* strains and their associations obtained in vitro on the glass surface after 48 hours:

a – in the presence of hydrated fullerene; b – in the presence of agarose.

The most active polysaccharides synthesis was observed with *P. alcaligens* strains in the presence of hydrated fullerene (II group) as evidenced by medium values of OD_{630} (0.352-0.457 o.d.u.). *P. alcaligens* strains immobilized with collagen and agarose (I group) were characterized by low ability to form a biofilm which is consistent with data on high rate of natural carriers biodegradation. At the average intensiveness of a biofilm formation by *P. alcaligens* strains in the presence of a synthetic polymer was by 2.3 times higher as compared to the level of a biofilm formation by pure strains immobilized with collagen and agarose. The *P. alcaligens* strains stabilized by synthetic polymers demonstrated more intensive adhesion of single cells the share of which in the field of view area constituted from 33 to 55% and exceeded the share of the Pseudomonas cells stabilized by natural polymers. There were also registered differences in the structure of biofilms which is

determined by the size and share of microcolonies in the area of the field of view. For example after 24 hour in the biofilms formed on the polystyrene surface by the I group of strains microcolonies with the size up to 8 μm^2 were dominating and in the biofilms of the II group of strains the major part of area was occupied by colonies with the size from 8 to 120 μm^2. Besides microcolonies with the size from 1000 to 10 000 μm^2 were detected in the biofilms of the II group of strains. After 48 hours all of the experiemental variants demonstrated growth of the share of microcolonies, the ability of association of microorganisms to form a biofilm remained the same which can be exlpained by change of correlation of microcolonies with different sizes (Fig. 1).

PHYSICOCHEMICAL PROPERTIES OF *PSEUDOMONAS ALCALIGENS* POLYSACCHARIDES CONTAINED IN BIOFILMS

Depending on their localization polysaccharides of microorganisms are divided into intracellular and extracellular. Usually the intracellular ones include polysaccharides of cytoplasm, cell membranes and walls and the extracellular include polysaccharides of capsules and sheathes (parietal structures) and extracellular fibers not adjoining a cell wall. Polysaccharides located to the outside of a cell membrane are referred to the extracellular group. As a rule these are cell walls polysaccharides. Microbial polysaccharides are joint into different functional groups: reserve ones taking part in active transportation; supporting ones participating in intercellular interaction and protective ones [3, 5, 10].

In order to obtain preparations with general cellular proteins the cells were lyzed in the presence of sodium dodecyl sulphate (SDS) as described by Laemmli U.K [27]. When a bacterial mass dried with acetone was used preliminary ultrasound disintegration was carried out. The Pseudomonas superficial biopolymers were obtained according to procedure recommended by N.N. Piven [19].

The content of monosaccharides was determined by a densitometric method after chromatography of hydrolyzates in the thin layers of an adsorbent in the solvent system n-butanol-acid and acetic water in the ratio of 3:1:1. The presence of free carboxyl and methoxy groups in the producers was evaluated by means of a titrimetric method and the presence of oxyacids by means of a modification of the Mellor method based on insolubility of oxyacids in petroleum-ether. The carried out experiments resulted in detection of general monosaccharides and monosaccharides as a part of pectic substances, core oligosaccharides, free carboxyl groups, free methoxy groups and oxyacids in the *P. alcaligens* strains. The results of investigation of the Pseudomonas intracellular factors are given in Tables 1 and 2.

Table 1: The range of mono- and oligosaccharides of the investigated strains of *Pseudomonas alcaligenes*

No.	Monosaccharides contained in pectic substances		General monosaccharides					Core oligo-sacchari-des
	galactose	rhamnose	galactose	arabinose	xylose	rhamnose	GA	
1	+	+	+	+	+	+	–	+
2	+	+	+	–	+	–	+	–
3	+	+	+	+	+	+	+	+
4	+	+	+	–	+	+	+	–
5	+	+	–	+	+	+	+	–
6	–	+	+	–	+	+	+	+
7	+	+	+	+	+	+	+	+
8	+	+	–	+	–	+	+	–
9	–	–	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+
11	–	–	–	+	+	+	+	+
12	+	+	+	–	+	+	–	–
13	+	–	+	+	–	+	+	+
14	–	–	+	+	+	+	+	–
15	+	+	+	+	+	+	+	–

In the *Pseudomonas alcaligens* hydrolyzates there were detected 5 compounds with monosaccharide content: galactose, arabinose, xylose, rhamnose and galacturonic acid (GA); in the pectic substances hydrolyzates there were identified 2 monosaccharides: galactose and rhamnose. In the structure of core

oligosaccharides besides rhamnose there was identified α-alanine which is a typical aliphatic aminoacid. 5 strains among 15 demonstrated a full set of mono- and oligosaccharides. Strain No. 4 didn't have xylose synthesis and didn't contain core oligosaccharides. The other strains showed lack of 2 or 3 mono- and oligosaccharides.

Table 2: The range of carboxyl and methoxy groups and oxyacids of the investigated strains of *Pseudomonas alcaligenes*

№	- COOH	- OCH_3	oxyacids
1	+	+	+
2	–	–	–
3	+	+	+
4	+	–	+
5	–	+	–
6	+	–	+
7	+	+	+
8	–	+	–
9	+	+	+
10	+	+	+
11	–	+	–
12	+	–	+
13	+	+	–
14	+	+	+
15	+	+	+

The whole range of carboxyl and methoxy groups and oxyacids was detected in seven *Pseudomonas alcaligenes* strains. Three strains demonstrated absence of the methoxy group. Absence of the carboxyl group correlated with lack of oxyacids. Strain No. 2 proved to be incomplete of all carboxyl, methoxy groups and oxyacids. Presence of the carboxyl group in the composition of the *Pseudomonas alcaligenes* intracellular compounds allows explaining solubility in water and high boiling temperature of metabolites. Presence of the metoxy group means presence of C-effect in metabolites due to which electron density in ortho- and para-locations of intracellular compounds of the Pseudomonas is increased. This facilitates electrophylic substitution of the *Pseudomonas alcaligenes* strains polysaccharides (it is also necessary to note that the methoxy group can substitute both the carboxyl one and side-chain hydrogen).

Presence of oxyacids in some of the investigated *Pseudomonas alcaligenes* strains can be explained by participation of these compounds in a range of specific and important biochemical reactions. It should be noted that oxyacids are stronger as compared to the corresponding carboxylic acids. This is connected with existence of intramolecular hydrogen bond between OH and COOH groups in α- and β-oxyacids, a stronger hydrogen bond is formed by carboxylate-anion obtained in the process of oxyacids dissociation. An inductive effect of the OH group also facilitates acidity rise. The obtained results correspond to the properties characteristic for the genus *Pseudomonas* representatives.

BIOLOGICAL PROPERTIES OF *PSEUDOMONAS ALCALIGENS* POLYSACCHARIDES CONTAINED IN BIOFILMS

Biological properties of the *P. alcaligens* contained in biofilms were assessed on the basis of morphophysiological indices and antagonist properties of polysaccharides producers. The results of experiments show that biological peculiarities of a microbial producer of polysaccharides are determined by origin of a strain, age of a culture and nature of a used carbon source. With the optimal growth parameters (the ratio of inoculum to growing medium volume – 5%, pH = 6.8, temperature – 58^{o}C) rich carbohydrate-protein media ensured high level of biomass accumulation, promoted growth of the investigated microorganisms in all respects. In order to determine an interrelation between the indices we've established a correlation relationship which is a special case of statistical relationship which supposes correspondence of different values of one variable to different average values of other variable. During study of the dynamics of an exopolysaccharide producer growth a pair correlation allowing to explore correlation relationship between two variables was used. Based on the correlation relationship theory and the obtained data on the *P. alaligens* morphophysiological properties there was established a direct linear correlation relationship between such indices as "Time of generation" and "Diameter of colonies on beef-extract agar (BEA)" (Figure 2).

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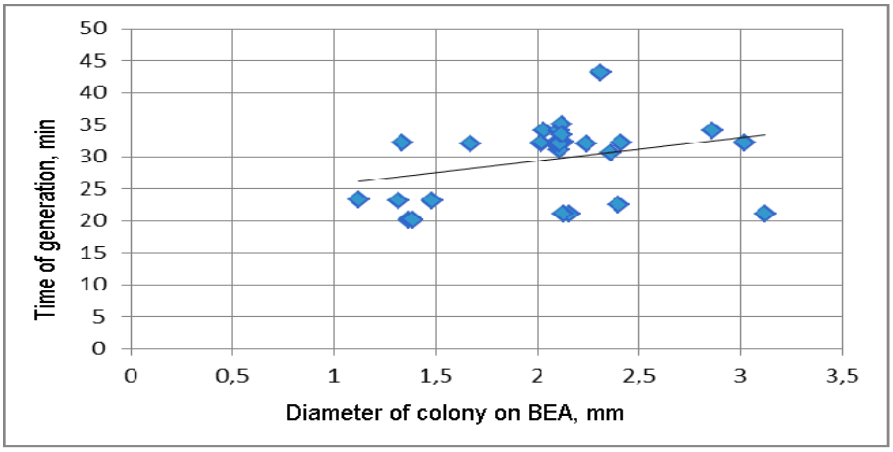

Figure 2: Linear direct relationship between the indices "Time of generation" and "Diameter of colonies on BEA, mm".

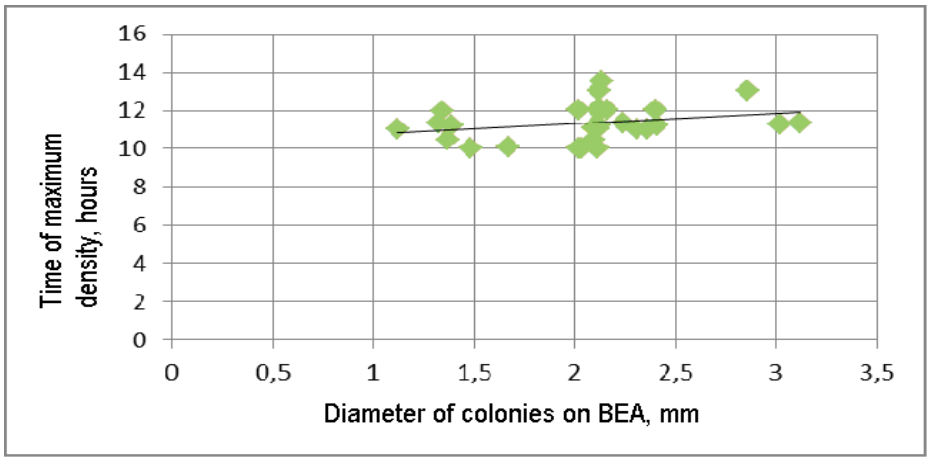

Figure 3: Linear relationship between the indices "Time of maximum density, hours" and "Diameter of colonies on BEA, mm"

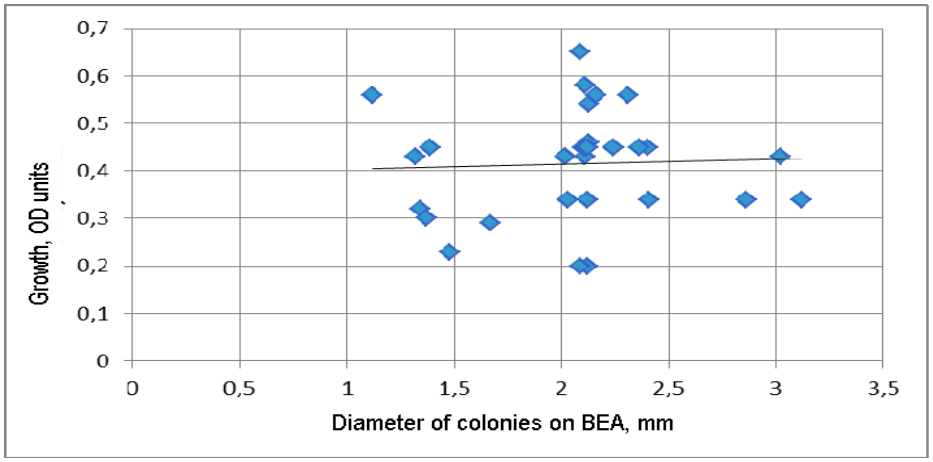

Figure 4: Correlation relationship between the indices "Characteristic of growth, OD units" and "Diameter of colonies on BEA, mm"

There were described the strains producing exopolysaccharides and able to ensure active growth on plates of beef-extract agar. They are capable of ensuring high-concentration product biosynthesis and to be used as industrial producers of biologically active substances. There were determined 8 cultures of Pseudomonas having the generation time over 25 minutes and not capable to adapt to a nutritious substrate

and produce secondary metabolites. Linear relationship was established between the time of achievement of the maximum population density and the size of colonies (Fig.3).

Experimental simulation of the growth conditions of polysaccharides producers population allowed to prove heterogenicity of the Pseudomonas based on the following investigated characteristics: the cells with the colonies sized from 1 to 1.5 mm are able to reach the maximum population density within 12 hours; the cells with the colonies sized from 2.8 to 3.2 mm reach the maximum population density within 13 hours; the Pseudomonas with diameter of colonies from 2 to 2.5 mm are able to reach the maximum population density within 14 hours.

The major part of the investigated strains forming colonies with the size not exceeding 2.5 mm (Fig. 4) in the process of growth reached the optical density of 0.2 – 0.5 OD units. But during investigation there were found out the strains able to form colonies with the diameter of three millimeters and over at that their growth index in OD units was within 0.4 units. Study of data on dynamic characteristics of the Pseudomonas alcaligens populations growth is necessary for the following opportunity to control bacterial culture growth. Owing to joint cultivation of the producers with the laboratory cultures of *S. aureus* and *E. coli* there were obtained data on antagonist properties of the *Pseudomonas alcaligens* polymers. The direct antagonism methods allowed to establish that variability for percent inhibition of strains was determined by antibiotic properties of a polymer.

CONCLUSION

The obtained data enable statement on presence of evident strain specificity of the *P. alcaligens* polysaccharides in regard to physicochemical and biological properties as well as on cultivation conditions influence on ratio of fractions and on antibiotic properties of the synthesized polymers. The resulting material is of great importance for the following assessment of microbial exopolysaccharides use as new-generation nanomaterials.

Conflict of Interests

The authors confirm that the presented data do not contain any conflict of interests.

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