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Alginate Biosynthesis by *Azotobacter* Bacteria

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Abstract—The ability of representatives of various species of the bacterial genus *Azotobacter* (*A. chroococcum* 7B, *A. chroococcum* 12B, *A. chroococcum* 12BS, *A. agile* 12, *A. indicum 8*, *A. vinelandii* 17, and *A. vinelandii* 5B) to alginate synthesis has been studied. It has been shown that all tested bacterial strains have this ability to different extents. Capsular alginate comprises from 2.6 to 32% of the total amount of synthesized alginate in various bacterial species. Strains that are able to active synthesis of alginate have been selected; the effect of the medium composition on their biosynthesis has been studied. The optimal conditions for alginate synthesis by the *A. chroococcum* 12BS producer strain include the presence of mannitol (40 g/L), yeast extract (1%), and low concentration of phosphates (KH_2PO_4—0.008 g/L, K_2HPO_4—0.032 g/L) in the medium; alginate production under these conditions is 4.5 g/L. The effect of aeration on polymer biosynthesis has been revealed: an increase in aeration causes an increase in alginate synthesis, while its decrease promotes the synthesis of poly-3-hydroxybutirate. It has been shown by IR spectroscopy that alginates obtained under various conditions of cultivation contain different ratios of residues of mannuronic and guluronic acids (M/G from 70/30 to 80/20) in the polymer chain and also differ in the amount of acetyl groups (from 10 to 25%) in the polyme structure.

Keywords: biosynthesis, biopolymers, exopolysaccharides, alginates, polyhydroxyalkanoates polymer, *Azotobacter*

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INTRODUCTION

Alginates (**ALG**s) are polyuronic acids consisting of β-D-mannuronic and α-L-guluronic acid copolymers in various ratios that are bound by the 1–4 glycosidic linkage. ALGs are produced by some species of brown algae (Phaeophyceae) and synthesized as an exopolymer by bacteria *Pseudomonas sp*. and *Azotobacter sp*. [1]. ALGs of bacterial origin differ from ALGs produced by algae in the presence of acetyl groups at C_2 and C_3 atoms in the residues of mannuronic acids and higher molecular weight [2]. The molecular weight of algae ALG ranges from 50 to 200 kDa, while the molecular weight of bacterial ALG can reach 4000 kDa. Acetylation and high molecular weight are directly connected with the rheological properties of ALG solutions and define the possibility of their application in food and pharmaceutical industries [3]. ALG bacterial synthesis enables regulation of the molecular composition and molecular weight of the polymer. It is practically impossible in algae, because the ALG monomeric composition and molecular weight depend on algae species and growth conditions, and it is difficult to regulate such factors as temperature, light intensity, etc. upon growth under natural conditions [4].

Azotobacter bacteria produce two classes of biotechnologically important polymers: poly(3-hydroxyalkanoates) (PHAs) that are a bacterial storage material, and ALGs, which play a protective role. Protection from various unfavorable factors is one of the main functions of bacterial ALG. It is considered that ALG is necessary in the cell for the creation of a diffusion barrier against O_2 diffusion and the protection of nitrogenase from it. Moreover, ALGs performing this function have a high content of guluronic acid [5]. Clementi et al. have shown that, due to the high affinity to calcium ions, ALGs protect the cell from the toxic effect of heavy metals and unfavorable conditions of the environment: drying, high temperature, etc. [6].

The use of *Azotobacter* bacteria for ALG biosynthesis is more advantageous than the work with *Pseudomonas* bacteria. First, *Pseudomonas* bacteria are pathogenic or opportunistic; this complicates their application. Second, the ALGs synthesized by them have lower viscosity, since their polymer chain con-

tains less residues of guluronic acid, which are responsible for the formation of cross-links between polymer chains [7].

Aeration is the main parameter that affects bacterial growth and their synthesis of polymers. It has been shown that the addition of phosphates decreases the amount of ALGs, while the addition of nitrogen sources increases synthesis [3, 8].

ALGs from algae have been used for many years as a material for studies in various areas of biology and medicine and in medical practice, for example, as drug carriers, wound coverings, stomatological materials, and, in some cases, in GIT diseases. One of the advantages of ALG application as peroral preparations is their ability to maintain the gel state even under conditions of low gastric acidity. These peculiarities allow ALGs to perform efficient protection of GIT upon gastritis and other diseases [9]. The study of the possibility of ALG production by *Azotobacter* bacteria is a currently important problem.

The purpose of the work is to study the effect of culture conditions of *Azotobacter chroococcum* on the synthesis and physico-chemical composition of ALG.

METHODS

An object of study. The work was carried out with the following strains from the *Azotobacter* bacterial collection: *A. chroococcum* 12B, *A. chroococcum* 12BS (with enhanced slime production), *A. chroococcum* 7B, *A. agile* 12, *A. indicum* 8, *A. vinelandii* 17, and *A. vinelandii* 5B isolated from soddy-podzolic soils of the Moscow region. The collection is maintained at the Laboratory of the Biochemistry of Nitrogen Fixation and Metabolism of the Bach Institute of Biochemistry, Federal Research Centre "Fundamentals of Biotechnology" of the Russian Academy of Sciences. The strains had the following morphological features: large, slimy colonies; oblong, motile (with flagella), Gram-negative cells that reside individually or are arranged in pairs and chains; and strict aerobes.

Bacterial cultures were maintained in solid Ashby's medium with the following composition (g/L): K_2HPO_4—0.2, $MgSO_4$—0.2, NaCl—0.2, Na_2MoO_4—0.006, $CaCO_3$—5.0, sucrose—20, and agar—20.

Conditions of *A. chroococcum* cultivation. Cultivation of bacteria in liquid medium is traditionally performed in liquid Burk's medium of the following composition (g/L): KH_2PO_4—0.2, K_2HPO_4—1.05, $MgSO_4$—0.4, NaCl—0.1, $FeSO_4$—0.01, Na_2MoO_4—0.06, $CaCl_2$—0.1, Na citrate—0.5, and sucrose—20.

In order to study the effect of culture conditions on ALG synthesis, variants of Burk's medium were used. Various sucrose concentrations were used as a carbon source; sucrose was substituted with mannitol; the concentration of phosphates in medium varied; and different concentrations of yeast extract were added as a nitrogen source.

Inoculum preparation. *A. chroococcum* was grown in liquid Burk's medium in flasks with a volume of 750 mL with 200 mL of medium at 250 rpm. The initial pH of the medium was 7.2. The cultivation temperature was 28°C. The inoculum was 1 day old. The volume of added inoculum was 4%.

In order to study ALG synthesis, *A. chroococcum* was cultivated under the same conditions as inoculum culture but with a change in medium composition. The duration of fermentation was 72 h. Fermentation was performed in 750 mL flasks (the volume of culture medium of 200 mL) on an Innova 43 microbiological shaker (New Brunswick Scientific, United States) at different mixing rates: 200, 280, and 340 rpm. The optical density of culture medium was determined by nephelometry at 520 nm. The morphology of *Azotobacter* bacteria was studied by light microscopy in a Biomed-1 microscope (Biomed, Russia) with a digital camera. Cells and alginate were visualized by fuchsine staining. Determination of poly(3-hydroxybutyrate) (PHB) content in bacterial cells were carried out by extraction with chloroform [2].

Determination of ALG accumulation by *A. chroococcum* cultures. In order to break capsules and decrease the viscosity of the solution, 10 mL of 0.1 M EDTA solution and 10 mL of 1.0 M NaCl were added to 100 mL of culture. After that the samples were centrifuged for 30 min at 4500 *g*. The pellet was separated, washed with water, and dried at 60°C to a bone-dry state. In order to determine the ALG, the supernatant was supplemented with a threefold volume of isopropanol or ethanol. The pellet was collected by centrifugation (20 min at 2000 *g*), dried, and dissolved in water. This procedure was repeated three times; the pellet was then washed with distilled water and dried. The process resulted in water-soluble dry sodium ALG. All experiments and measurements were performed in triplicate.

The qualitative reaction for ALG was performed with $CaCl_2$ [4]. For this purpose 10 mL of 10% $CaCl_2$ solution were added to 1 mL of culture medium. The reaction was accompanied by the formation of calcium ALG, which formed as water-insoluble flake-like pellet.

Determination of ALG viscosity. The molecular weight of ALG was determined by viscometry. The viscosity of ALG solution in 0.2 M NaCl was measured at 25°C.

The specific viscosity was calculated according to the formula

$$\eta_{sp} = (t - t_0)/t_0,$$

where t_0 is the flow time of the solvent (s) and t is the flow time of the polymer solution (s).

The molecular weight was calculated according to the Mark-Houwink equation [4] $[\eta] = K(M)^a$ with the following coefficients: $K = 7.3 \times 10^{-5}$; $a = 0.92$;

Table 1. ALG production by *Azotobacter* bacteria upon cultivation on solid Ashby's medium

Species, strain	Biomas, g/L	ALG, g/g of biomass	Capsular ALG, g/g of biomass	Portion of capsular ALG of the total ALG, %
A. chroococcum 7B	8.75	5.88	1.88	32.0
A. chroococcum 12B	4.56	8.32	1.35	16.2
A. chroococcum 12BS	4.52	8.20	1.10	13.4
A. agile 12	9.34	5.07	0.15	3.0
A. indicum 8	5.27	8.48	2.31	27.2
A. vinelandii 17	0.89	5.61	0.15	2.6
A. vinelandii 5B	1.06	8.23	1.40	17.0

$$[\eta] = 7.3 \times 10^{-5} \times (M)^{0.92},$$

where M is the molecular weight and $[\eta]$ is viscosity [10].

IR spectroscopy. IR spectra were registered in the reflection mode in a Hyperion-2000 IR microscope connected with an IFS-66 v/s FTIR spectrometer (Ge crystal, resolution of 2 cm^{-1}, a range of 4000–600 cm^{-1}, scan.-50) (Bruker, United States). For comparison, this method was also applied for the study of a commercial ALG sample isolated from algae (Sigma-Aldrich, Germany).

RESULTS AND DISCUSSION

We estimated the capability of various *Azotobacter* bacterial species for ALG synthesis. The work was performed with strains capable of PHB synthesis that were available from the collection of nitrogen-fixing microorganisms of the Bach Institute of Biochemistry, Federal Research Centre "Fundamentals of Biotechnology" of the Russian Academy of Sciences (Russia): *A. chroococcum* 7B, *A. chroococcum* 12B, *A. chroococcum* 12BS, *A. agile* 12, *A. indicum* 8, *A. vinelandii* 17, and *A. vinelandii* 5B.

All strains of different *Azotobacter* species were capable of ALG synthesis (Table 1); however, the amount of the synthesized polymer varied in different species. It was shown that the fraction of capsular ALG in different species ranged from 2.6 to 32% of the total ALG. Capsular ALG usually contains larger amounts of guluronic units in the polymer chain and therefore has a higher capacity for gel formation, which is functionally important for the cell upon the formation of the protective capsule. We selected potential ALG producers—strains that are able to active synthesis of the polymer.

For further studies we selected *A. chroococcum* 12B and 12BS, because these strains are capable of active growth and active ALG synthesis with a large portion of capsular ALG.

It is known that the fermentation conditions define the monomeric composition of the chain, molecular weight, and acetylation level of bacterial ALG. The composition of culture medium is crucial for the synthesis of bacterial ALG. In particular, carbon and nitrogen sources and the content of molybdenum, iron, magnesium, potassium, sodium, sulfates, and especially calcium and phosphates in the medium affect both bacterial growth and the capacity for polymer synthesis [2, 3].

We decreased the phosphate concentration in liquid Burk's medium used for cultivation of bacteria by four times [11]. Furthermore, nitrogen-free Burk's medium was supplemented with yeast extract in concentrations of 1.0 and 3.0 g/L [8]. The carbon source, sucrose, was also changed for mannitol [1].

Table 2 represents the results of biomass yields and ALG synthesis by *A. chroococcum* 12B and 12BS in media with various compositions. Figure 1 shows the growth curves of *A. chroococcum* cultures and ALG synthesis in selected Burk's media with sucrose and molasses as the main carbon sources with the addition of yeast extract. As can be seen from the presented data, there were no remarkable differences in culture growth in all of the tested media. The best growth of 12B and 12BS cultures was observed upon the addition of yeast extract; this can be explained by the addition of a nitrogen source into the medium.

It was also shown that both cultures synthesized the maximum ALG amounts (3.7 and 4.5 g/L, respectively) upon cultivation for 72 h in medium with mannitol as a carbon source, the addition of 1 g/L of yeast extract, and a low phosphate concentration (0.008 g/L of KH_2PO_4 and 0.032 g/L of K_2HPO_4). In standard culture medium (Burk's medium), both cultures synthesized the minimum ALG amounts. The use of other modifications of the medium resulted in higher yields of the polymer. The qualitative test for ALG with $CaCl_2$ (the formation of calcium ALG pellet) was performed for all the variants, and the pellet formation was noted in all cases.

Aeration is the main parameter affecting bacterial growth and polymer synthesis [3]. Cultivation of *A. chroococcum* 12BS under various aeration condi-

Table 2. Biosynthesis of ALG by *Azotobacter chroococcum* 12B and *Azotobacter chroococcum* 12BS in modified Burk's media with different compositions

Burk's medium	Carbon source (40 g/L)	Yeast extract, g/L	Phosphates, g/L	Biomass yield, g/L	ALG production, g/L
Azotobacter chroococcum 12B					
1	Sucrose	No	KH_2PO_4—0.008, K_2HPO_4—0.032	4.3	2.5
2.1	Mannitol	No	KH_2PO_4—0.2, K_2HPO_4—1.05	4.2	2.2
2.2	″	No	KH_2PO_4—0.008, K_2HPO_4—0.032	4.2	2.5
3.1	Sucrose	1.0	KH_2PO_4—0.008, K_2HPO_4—0.032	4.2	3.1
3.2	″	3.0	KH_2PO_4—0.008, K_2HPO_4—0.032	4.7	2.8
4.1	Mannitol	1.0	KH_2PO_4—0.008, K_2HPO_4—0.032	4.2	3.8
4.2	″	3.0	KH_2PO_4—0.008, K_2HPO_4—0.032	5.2	3.2
5	Sucrose	No	KH_2PO_4—0.2, K_2HPO_4—1.05	4.4	0.8
Azotobacter chroococcum 12BS					
1	Sucrose	No	KH_2PO_4—0.008, K_2HPO_4—0.032	4.3	2.2
2.1	Mannitol	No	KH_2PO_4—0.2. K_2HPO_4—1.05	4.1	1.5
2.2	″	No	KH_2PO_4—0.008, K_2HPO_4—0.032	3.4	2.7
3.1	Sucrose	1.0	KH_2PO_4—0.008, K_2HPO_4—0.032	5.0	3.5
3.2	″	3.0	KH_2PO_4—0.008, K_2HPO_4—0.032	5.7	2.0
4.1	Mannitol	1.0	KH_2PO_4—0.008, K_2HPO_4—0.032	4.7	4.5
4.2	″	3.0	KH_2PO_4—0.008 K_2HPO_4—0.032	5.0	3.8
5	Sucrose	No	KH_2PO_4—0.2, K_2HPO_4—1.05	4.9	0.9

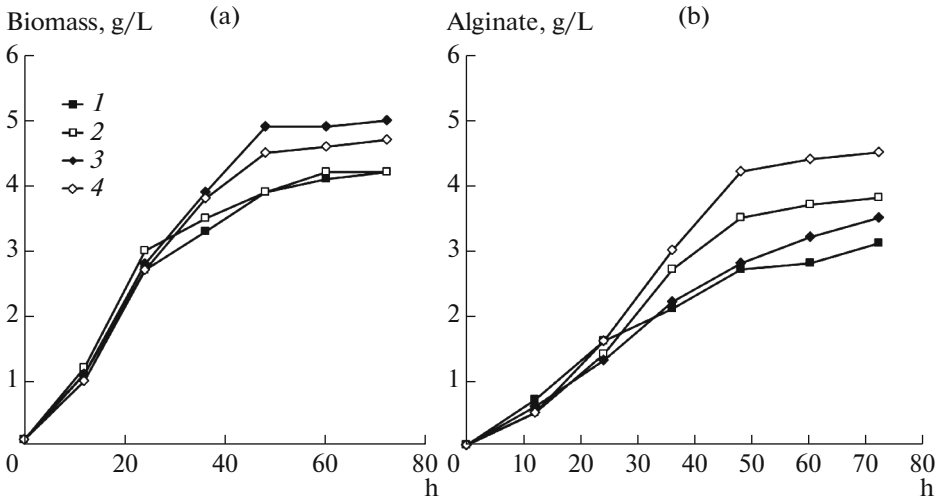

Fig. 1. Dynamics of (a) biomass growth and (b) alginates production by *A. chroococcum* 12B and *A. chroococcum* 12BS in Burk's media 3.1 and 4.1. *1—A. chroococcum* 12B, Burk's medium 3.1; *2—A. chroococcum* 12B, Burk's medium 4.1; *3—A. chroococcum* 12BS, Burk's medium 3.1; *4—A. chroococcum* 12BS, Burk's medium 4.1. Designations and characteristics of Burk's media are provided in Table 2.

tions (shaker rpm) was performed for 3 days in Burk's medium with sucrose as a carbon source. It can be seen from the results presented in Table 3 that the maximum bacterial growth occurred at 280 rpm. The biomass yield reached 5.1 g/L. The highest yield of PHB (3.2 g/L) was also noted for this variant of the experiment. An increase in aeration to 340 rpm decreased biomass production by two times, to 2.6 g/L. Maximum aeration was also accompanied by the lowest PHB yield; in the first instance, this is connected with low biomass yield under these conditions. At the same time, ALG is actively produced under these aeration conditions (2.2 g/L). A decrease in aeration to 200 rpm slightly decreased the yield of biomass and PHB and did not affect ALG yield. Consequently, aeration intensification resulted in an increase in ALG synthesis while aeration diminution, on the contrary, promotes PHB synthesis. The results are in accordance with the literature data [2, 5, 8, 12].

Table 4 shows the results of the effect of medium composition on the molecular weight of the polymer. Under experimental conditions the molecular weight in different variants changed insignificantly and did not surpass 98 kDa. Alginate with a relatively low molecular weight (168 kDa) was also obtained from *A. vinelandii* NCIMB 9068 in [13]; in this case, also, no

Table 3. Effect of aeration on the synthesis of polymers in *A. chroococcum* 12BS—ALG and PHB upon cultivation in liquid Burk's medium with sucrose (40 g/L)

Mixing rate, rpm	Biomass, g/L	ALG, g/L	PHB, g/L	ALG:biomass, %	PHB:biomass ratio, %
340	2.6	2.2	1.5	83.3	77.6
280	5.1	1.1	3.2	21.2	81.5
200	4.4	1.1	2.5	25.8	78.3

Table 4. Effect of medium composition on the molecular weight and intrinsic viscosity of ALG produced by *A. chroococcum* 12BS in various media

Ashby's medium	Carbon source (40 g/L)	Yeast extract	Phosphate, g/L	Intrinsic viscosity, cm^3/g	Molecular weight, kDa
1	Sucrose	No	0.2	2.2	75
2	Sucrose	No	0.05	2.3	98
3	Sucrose	1 g/L	0.05	2.25	94
4	Mannitol	No	0.05	2.4	88

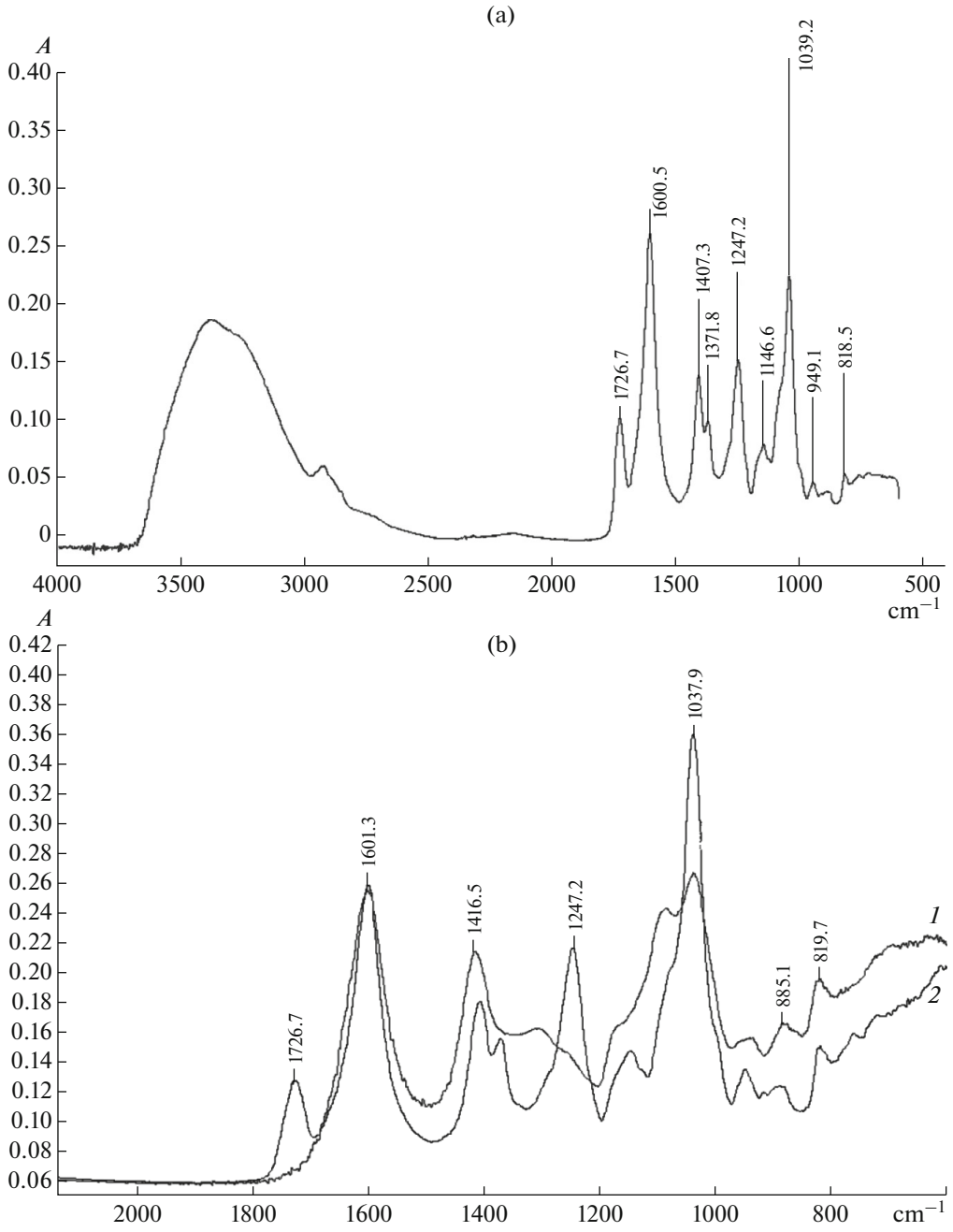

Fig. 2. (a) Total IR spectrum of ALG obtained upon cultivation of *A. chroococcum* 12BS in Ashby's medium. (b) IR spectra of (*1*) algae ALG and (*2*) bacterial ALG obtained upon cultivation of *A. chroococcum* 12BS in Ashby's medium in a range of 2000–500 cm^{-1}.

remarkable difference in molecular weight was observed upon a change in the composition of culture medium for the producer. A decrease in the concentration of phosphates in medium from 0.2 to 0.05 g/L was accompanied by a 20–25% increase in the molecular weight. The addition of a nitrogen source (yeast extract) to the medium hardly changed the molecular weight of synthesized ALG.

ALG synthesized by *A. chroococcum* 12BS under various conditions and medium compositions was studied by IR spectroscopy. The obtained IR spectra of bacterial ALG are shown in Fig. 2, and all of the results are in Table 5. The spectra of synthesized bacterial ALG are similar and have common absorption bands with different intensities of bands at 1600 cm^{-1} (COO^-), 1720 cm^{-1} ($COCH_3$), 819 cm^{-1} (M-blocks), and 765 cm^{-1} (G-blocks). The commercial preparation

Table 5. Effect of medium composition on the content of mannuronic and guluronic acids and the acetylation level of studied ALG determined by absorption bands in IR spectra

ALG sample (obtained)	D_{1600}/D_{1726}	Acetylation degree, %	D_{819}/D_{765}	M/G
From algae	88.6**	1	4.4***	80/20
In Ashby's medium 1*	3.0	25	2.6	72/28
In Ashby's medium 2	9.3	10	4.3	81/19
In Ashby's medium 3	2.9	26	3.1	75/25
In Ashby's medium 4	3.6	22	2.4	70/30

* The media characteristics are provided in Table 4.
** A band at 1726 cm^{-1} was absent in this spectrum; that is why the general absorption background was taken for the intensity at 1726 cm^{-1}.
*** A band at 787 cm^{-1} appeared in this spectrum instead of a band at 765 cm^{-1}.

of algae ALG was studied simultaneously by the same method. Figure 2b shows the comparison of two spectra of ALG synthesized by *A. chroococcum* 12BS upon cultivation in Ashby's medium and isolated from algae. The spectra are similar and have common absorption bands. However, there are differences, which include the presence of bands at 1720 and 1245 cm^{-1} in bacterial ALG and the intensification of a band at 1030 cm^{-1}. These particular signals are characteristic of absorption bands of acetyl groups (1720 and 1245 cm^{-1}) [4]. It is known that bacterial ALG are acetylated by positions 2 and/or 3 of D-mannuronic acid residues, and ALG containing more than 55% of mannuronic residues are characterized by the content of acetyl groups of more than 22% [14].

The presence of mannuronic acid in the polymer chain is seen in the IR spectrum from the presence of a band of valence vibrations of the pyranose ring of mannuronic acid (**M**)—819 cm^{-1}; the presence of guluronic acid (**G**) is confirmed by an absorption band at 765 cm^{-1}. The relative intensities of these bands and their percentage ratio are shown in Table 5.

The ratio of bands at 1600 and 1720 cm^{-1} defines the percentage of acetylated residues in the polymer, while the ratio of bands at 819 cm^{-1} and 765 cm^{-1} defines the ratio between the amounts of mannuronic and guluronic residues (M/G). The ratio of mannuronic and guluronic acids in the ALG chain can vary depending on the polymer source. It is exactly the amount of guluronic residues and their distribution in the chain that defines the capacity of ALG for gel formation [4]. In practically all of the samples, we observed higher contents of guluronic residues as compared to algae ALG, except the sample obtained upon cultivation in medium with a low phosphate content. This sample also showed a low acetylation percentage. The results are in good accordance with the data of Hoefer et al. [15], who studied ALG synthesis by *A. vinelandii* ATCC 9046 and showed that the ratio between residues in the obtained polymer was M/G = 60/40, and the observed acetylation ranged from 30% to 60%. It should be noted that Loginov et al. obtained ALG with a predominance of guluronic acid over mannuronic acid in the polymer chain (M/G = 30/70) upon fermentation of *A. vinelandii* IB1 in Fedorov's medium, the composition of which is close to the used Burk's medium [1]; therefore, we can conclude that the species and strain of the ALG producer used is very important.

CONCLUSIONS

Consequently, we obtained ALG with different contents of mannuronic and guluronic acids in the polymer chain. We showed the presence of acetylation of uronic acids in the chain of bacterial ALG in contrast to the polymer from algae. A change in conditions of cultivation affected the ratio of mannuronic and guluronic acids in the polymer chain and the acetylation level.

Thus, we estimated the capacity of *Azotobacter* bacteria from the collection of the Bach Institute of Biochemistry, Federal Research Centre "Fundamentals of Biotechnology" of the Russian Academy of Sciences, for ALG synthesis. We revealed potential ALG producers. We showed the possibility of ALG biosynthesis regulation via variation in conditions of cultivation. It was shown by IR spectroscopy that ALGs obtained under various conditions of cultivation had different contents of mannuronic and guluronic residues (M/G varied from 70/30 to 80/20) in the polymer chain and differed in the presence of acetyl groups (from 10 to 25%) in the molecular structure. It was shown that variation in fermentation parameters allows directed control of the structure of the synthesized ALG chain, namely, ratio of mannuronic and guluronic residues, molecular weight, and acetylation level of polymers.

In further studies we plan to investigate the effect of various fermentation parameters on the production and composition of alginates and PHA and to develop

new fermentation strategies, which can be applied in order to increase the polymer production by *Azotobacter* bacteria.

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