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A N I N T E R N A T I O N A L J O U R N A L

Effect of Nutrient Sources on the Alginate Accumulation in the Culture Liquid of *Azotobacter vinelandii* D-05 and Obtaining Biocomposite Materials

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ABSTRACT

Using the classic biotechnological methods, the dependence of A. vinelandii D-05 culture alginate production from the media carbon and nitrogen content was investigated. The maximal alginate production was observed during cultivation bacterium in the medium with 2 to 4% of sucrose, but the maximal growth was found in the medium with 4% glucose. It was found that for the alginate production the optimal nitrogen contents could take from 0.05% yeast extract (carbon: nitrogen ratio 168:1). For the first time we demonstrated possibility the A. vinelandii growth during the cultivation in a medium with molasses (a by-product of sugar production) and the significant polysaccharide production (16.6 g/l) was obtained. It was established, that A. vinelandii culture broth could be used as a biological binder for obtaining the biocomposite materials.

Key words: *Azotobacter vinelandii*; alginate; nutrient medium; carbon: nitrogen ratio; biocomposite.

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INTRODUCTION

Currently, the microbial polysaccharides are widely used in the various fields of the human activity, such as medicinal[1], pharmaceutical[2], food, textile and chemical industries, oil production, and hydrometallurgy, to obtain the composite materials[3,4]. Despite advances in biotechnology, the number of commercially available microbial polysaccharides is extremely limited. The problems of determining both the new microbial polysaccharides producers and the efficient preparation methods for the various products remain to be resolved[5,6]. The optimization of the culture medium provides the key to the development of the modern methods for preparations of the microbial polysaccharides.

The alginates represent a family of the unbranched copolymers of β-D-mannuronic and α-L-guluronic acids, 1-4-linked and having differing composition and structure[7]. They are widely used in a medicine, in the food industry as gelling and the stabilizing agents, in the agriculture, in bio sorption of the metals, and in a number of areas[8,9,10,11]. *A. vinelandii* bacteria are considered as promising producers due to their ability to synthesize the alginates consisting of homopolymer blocks of the mannuronic and the guluronic acid as alternating units in the mixed blocks[7].

It is known that the polysaccharides possess good adhesive properties. The adhesives (binder), which include of the microbial origin polysaccharides (levan from *Azotobacter*, dextran from *Leuconostoc*) can be used to improve the environmental safety of wood composite materials instead of toxic synthetic resins and adhesives. The producers of these polysaccharides are *Leuconostoc*, *Azotobacter*, *Gluconoacetobacter* bacteria, grown on waste from beet sugar, starch, dairy and fermentation industries. In the process of bacterial growth, we found the accumulation of the high polymeric compounds - polysaccharides. They can be used as the main component in a bio glue and as a binder for the production of wood composites. Using the compression, we obtained biocomposite material based on the lignocellulosic materials and the levan and dextran containing culture broth[12,13,14,15].

The general idea of the current study was to optimize the media composition of the carbon and nitrogen nutrition for *Azotobacter vinelandii D-05* growth and alginate production and to investigate the possibility of alginate-containing culture broth as a biological binder for biocomposites.

MATERIALS AND METHODS

Strain and the culture media

The object of this study was bacterium A. vinelandii D-05 obtained from the VCIM. The culture was maintained on Ashby agar medium of the following composition (g/l): mannitol – 20, K_2HPO_4 – 0.2, NaCl – 0.2, K_2SO_4 – 0.1, $FeSO_4$ – 0.1, $CaCO_3$ – 5, agar-agar – 20, pH 6.8 – 7.2. Bacteria were grown on the agar medium in a thermostat at 28 ± 1°C for 5 days.

The various carbon compounds the bacterium consumption in culture was investigated using Hiss color media of the following composition, g / l: carbon source (carbohydrate or alcohol) - 10; peptone - 5.0; K_2HPO_4 - 1.0; bromothymol blue indicator – 2 ml of 1.6% alcohol solution per 1 liter of water.

In this study the carbon sources utilized were sucrose, fructose, xylose, glucose, arabinose, dulcitol, inositol, mannitol, lactose, and rhamnose. A background medium without the carbon source as a control was used.

The prepared medium was dispensed (9 ml) into tubes with the floats and sterilized at 0.5 atm during 20 min. The medium was inoculated with the microbial cell suspensions (0.2 ml) and was incubated at 30ºC during 4 days. After incubation, the

presence or absence of microbial growth in the carbon source medium was controlled by the medium revealing turbidity and precipitate formation. In addition, the indicators light intensities changes during the formation of acidic metabolic products and pH changes (at pH 7.6, the bromothymol blue indicator is blue, and at pH 6.0, it is yellow).
In the A. vinelandii growth and alginate production experiments, we used modified M22 medium[16] of the following composition (g/l): KH_2PO_4 – 0.011; Na_2HPO_4 - 0,189; $MgSO_4$ $7H_2O$ - 0,2; $CaSO_4$ $2H_2O$ - 0,02; $FeSO_4$ $7H_2O$ – 0,006; MoO_3 – 0,00005; NaCl – 0.01; $NaHCO_3$ – 0.05, pH 7.8.
Carbon sources (sucrose, glucose, lactose, and mannitol) were added at concentrations 1.5; 2; 4%. As the nitrogen source, yeast extract was added to the medium (0.5%).
The nitrogen source ($NaNO_3$, yeast extract, peptone, $(NH_4)_2SO_4$) (0.03, 0.04, 0.05, and 0.06%) effects on the growth and exopolysaccharide production (EPS) was investigated using the modified M22 medium with 2% sucrose.
Cultivation was also carried out on molasses medium (4% sucrose) of the following composition (g/l): molasses – 100 (40% sucrose), pH 6.8.

Inoculum preparation and the cultivation conditions
A two-day culture was used as an inoculum. The inoculum (5 mL) with the optical density at OD600 – 0.05 was added to flasks. Optical density of the culture broth using the UV-3600 -spectral-photometer (Shimadzu, Japan) for registration the light absorbance at 600 nm was measured. Samples were diluted if absorbance was above 0.05. The cultivation for 5 days at 28ºC on a shaking incubator (ES-20/60 (Biosan, Latvia) (220 rev/min) was carried out.

Analytical methods
The biomass and the culture broth (CB) polysaccharide contents after drying and weighing were determined. After the biomass fermentation, 10 mL culture broth was centrifuged at 8000 rpm for 30 min (Centrifuge CLn-16, Changsha Xiangzhi Centrifuge Instrument, China) and then the pellet for 24 hours at 100°C was dried. The cdw (g/L) is the coefficient was calculated as the weight difference. Alginate precipitation from the culture broth was performed by twice its volume of isopropanol[17]. The alginate was precipitated from the supernatant separated from the cells with 2 volumes of 96% cold isopropanol and separated by centrifugation at 2,500 × g for 15 min. After being dissolved in MilliQ water, the polymer was precipitated. This process was repeated two more times, and the polymer was finally dried.

Binder and biocomposite materials obtaining
To obtain the binder, we used M22 medium supplemented with 20 g/l sucrose and 0.50 g/l yeast extract. The cultivation was carried out for 4 days. The procedure of the press-mass preparing included next: the pine sawdust was mixed with a binder by extrusion method; the sawdust (100 g) was mixed with 100 ml *A. vinelandii* D-05 culture broth and dried at 60°C to a 6 to 8% moisture content; as a hydrophobizator, 1% of paraffin was added. The substrates (100 g) were placed into a mold (5×15 cm) and subjected to compression in a molding hydraulic press GT-7014-H (Gotech, Taiwan) at 180°C, pressure 26 MPa during 20 min, and followed by cooling. The obtained boards density was determined using automatic densitometer of high resolution H-200L (Gotech, Taiwan). According to Russian Federation standard, flexural strength was restarted using a tensile-testing machine UAI-7000 unit (Gotech, Taiwan). The values of water absorption and swelling were measured compare the samples weight and thickness after water immersion.

The results were subjected to statistical analysis using Microsoft Excel 2007 programs (n=10-15).

RESULTS AND DISCUSSION

To test the ability of *A. vinelandii* D-05 to utilize the carbohydrates and alcohols, in our experiments the cultivation was on Hiss colored media with the pH-depended light indicator, (bromothymol blue), which changes the medium color from yellow to blue in the interval from pH 6.0 - 7.6. It was found, that from the ten carbon sources tested, only the sucrose, glucose, lactose and the mannitol were changed pH because the of carbon sources was includes into the microorganism metabolism. One other side, the slight light change of pH indicator was observed during the bacterium cultivation in media with arabinose, fructose and rhamnose.

To study the effect of carbon sources on the growth of *A. vinelandii*, the cultivation was carried out on the media containing different mannitol concentrations (of 1.5 to 4%). It was shown that in all mannitol concentrations, the growth of *A. vinelandii* and the biomass increase was observed at up to three days of the cultivation, followed by it decrease, perhaps, the initiation of the cell lytic processes. At the bacterium growth in the 1.50% mannitol containing media (Fig. 1 A), the biomass production was insignificant. Increasing the carbon source concentration resulted in intensification of the growth processes. The best growth was observed at mannitol concentration 4% (5.6 g/l).

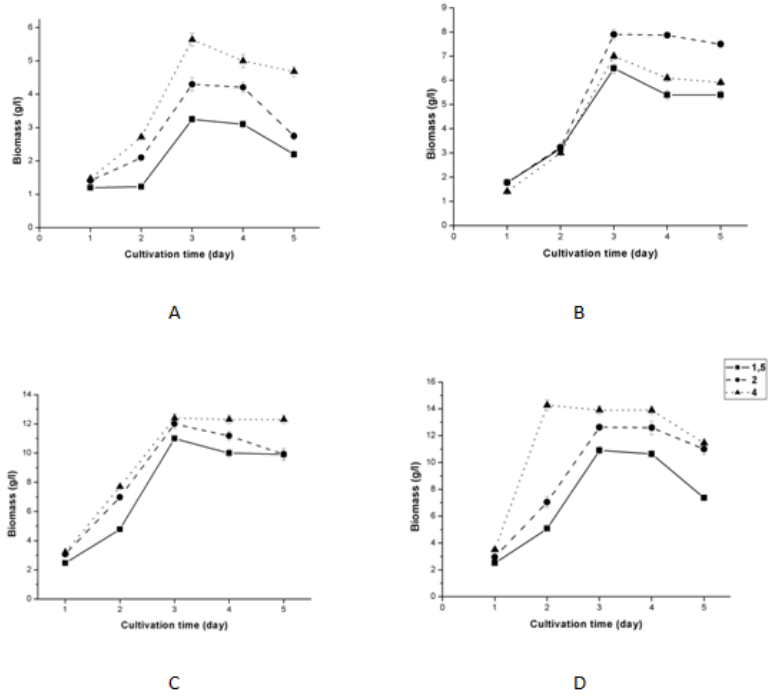

Fig.1. Influence of carbon source concentration in the medium on *A. vinelandii* growth:
A – mannitol; B – lactose; C – sucrose; D – glucose.
Key: Concentration (%): 1.5 (■), 2 (●), 4 (▲)

Using the lactose as a carbon source, similarly to mannitol, *A. vinelandii* grew up to 3 days was found (Fig. 1 B). The lowest of the biomass contents was at 1.5% lactose contained media, the 2% lactose stimulated the biomass production by 18% (7.9 g/ L) but at 4% lactose contained media the bacterium growth decreased, as in the data previously reported[18].

The substitution of the mannitol and lactose for sucrose led to improve *A. vinelandii* biomass level and the maximum biomass content of (12 g / l) was observed during cultivation at 2 - 4% sucrose contained media (Fig. 1 C).

The maximal of the *A. vinelandii* D - 05 culture growth was observed in glucose contained media. It was shown that the changes from 1.5 to 4% glucose concentration in the culture medium led to an increase of the biomass content and in 4% glucose contained media, the maximum biomass was observed as early as the second day (Figure 1 D).

It was found, that the existence of the carbon source in the culture medium changed not only the bacterium growth but also the synthesis of microbial metabolites including extracellular polysaccharides (EPS). (Fig. 2).

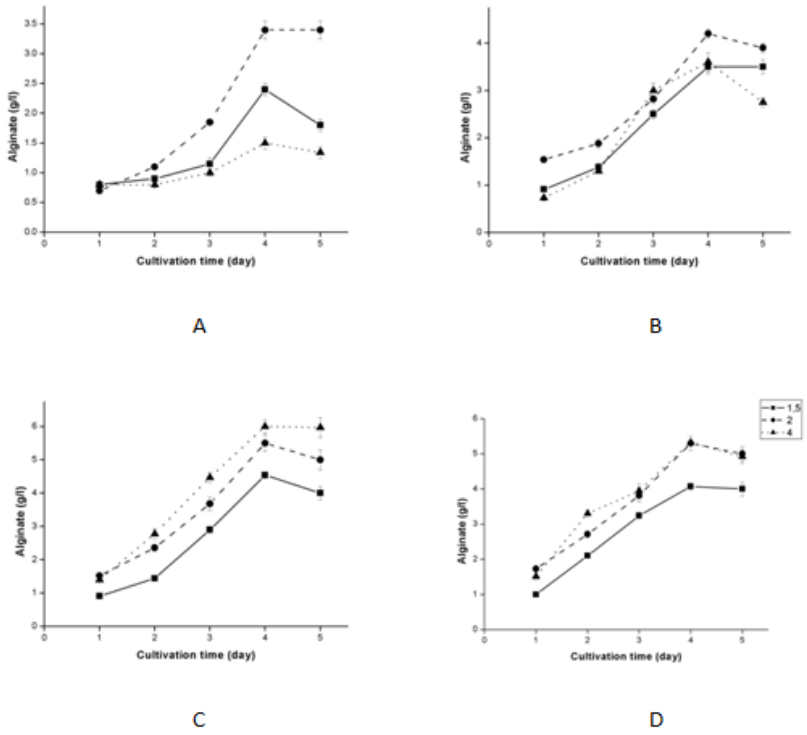

Fig. 2. Influence of carbon source concentration on the exopolysaccharide accumulation by *Azotobacter vinelandii*: A – mannitol; B – lactose; C – sucrose; D – glucose.
Key: Concentration (%): 1.5 (■), 2 (●), 4 (▲)

In all experiments the maximum EPS content in the culture broth (CB) was observed during four days (stationary phase of growth) and subsequently slightly decreased or remained at the control level. It is important that during cultivation the bacterium in mannitol contained media, the highest the alginate production was observed at 2% mannitol - and 3.4 g/l (Fig. 2 A), but at 4% the polysaccharide synthesis was blocked whereas the *A. vinelandii* biomass content was maximal (see Fig. 1 A).

During cultivation the bacterium with lactose the alginate biosynthesis was increased (in comparison with mannitol) (Fig. 2 B). The maximal of EPS contents was observed in 2% lactose, contained media, possible, directly depend on the biomass level.

As in the case of mannitol, increasing the lactose content in a medium to 4% inhibited the alginate biosynthesis and it fits the data that the concentrations more 1% lactose decrease the biosynthesis of alginate by *A. vinelandii* MTCC 2459[18].

The optimal sucrose concentration for the culture growth and alginate biosynthesis (Fig. 2 C) was 2% (5.5 g/l on the 4th day) and it increase to 4% did not lead to significant the polysaccharide biosynthesis.

It should be noted that the alginate production at *A. vinelandii* cultivation on a sucrose contained media was more than in medium with either mannitol or lactose. It was known[18], the sucrose was also an optimal carbon source for *A. vinelandii* MTCC 2459. It is known that for a number of various *Azotobacter* species and strains, cultivation at 2% glucose is one of the best substrates for alginate production[17,19,20].

However, in our experiments, the change of sucrose by glucose did not increase EPS production in the *A. vinelandii* D - 05 culture broth (Figure 2 D). For example, the alginate content in the experiment 1.5% glucose contained media was lower than at higher concentrations (2 - 4%).

In these experiments were found that during the bacteria *A. vinelandii* D- 05 cultivation it is possible using the different carbon sources for growth and alginate biosynthesis: mono-, disaccharides and polyhydric alcohol mannitol.

It is know, that the basic components of any culture medium are carbon and nitrogen compounds. Exactly these compounds and their ratios determine the specificity of the culture media[21]. Therefore, we investigated the effect of various concentrations of the nitrogen sources on the growth and the alginate production at 4% sucrose contained media. For this purpose, $NaNO_3$, and yeast extract were added to cultural medium in concentrations of 0.03% and 0.06%. The medium lacking a nitrogen source was used as a control. It is known that *Azotobacters* are capable to nitrogen fixation and require Mo ions for this process[22,23]. However, it has been observed that inorganic nitrogen sources enhance *Azotobacter* growth[20]. These bacteria can metabolize nitrogen in oxidized form as nitrate salts[18].

It was shown that the minimum of biomass formation in the control (to 2.5 g/l on day 3 was observed (Fig. 3). It is know, that bacterium use two enzymatic systems utilizing nitrates: the first in reducing nitrate to nitrite, the second – nitrate to ammonium. In experiments with minimum nitrate content (Fig. 3 A), the biomass was at the control level (2.5 g / l). The maximum biomass production was found at 0.05% nitrate contended media (C: N ratio 105:1; to 5.24 g/l). At increasing the $NaNO_3$ concentration in the medium to 0.06% the biomass contents was decreased.

It has been shown that the input of the reduced form of nitrogen, $(NH_4)_2SO_4$, into the medium increase the culture growth (Fig. 3 B). At concentration 0.04% $(NH_4)_2SO_4$,in the CB the biomass content increased to the maximal level - 8.3 g/l. and higher concentrations did not effected on the biomass contents, because decreasing the medium pH the bacterium development limited. So, at 0,04% ammonium sulfate contended media (C: N ratio 100:1) the optimal bacterium growth was found.

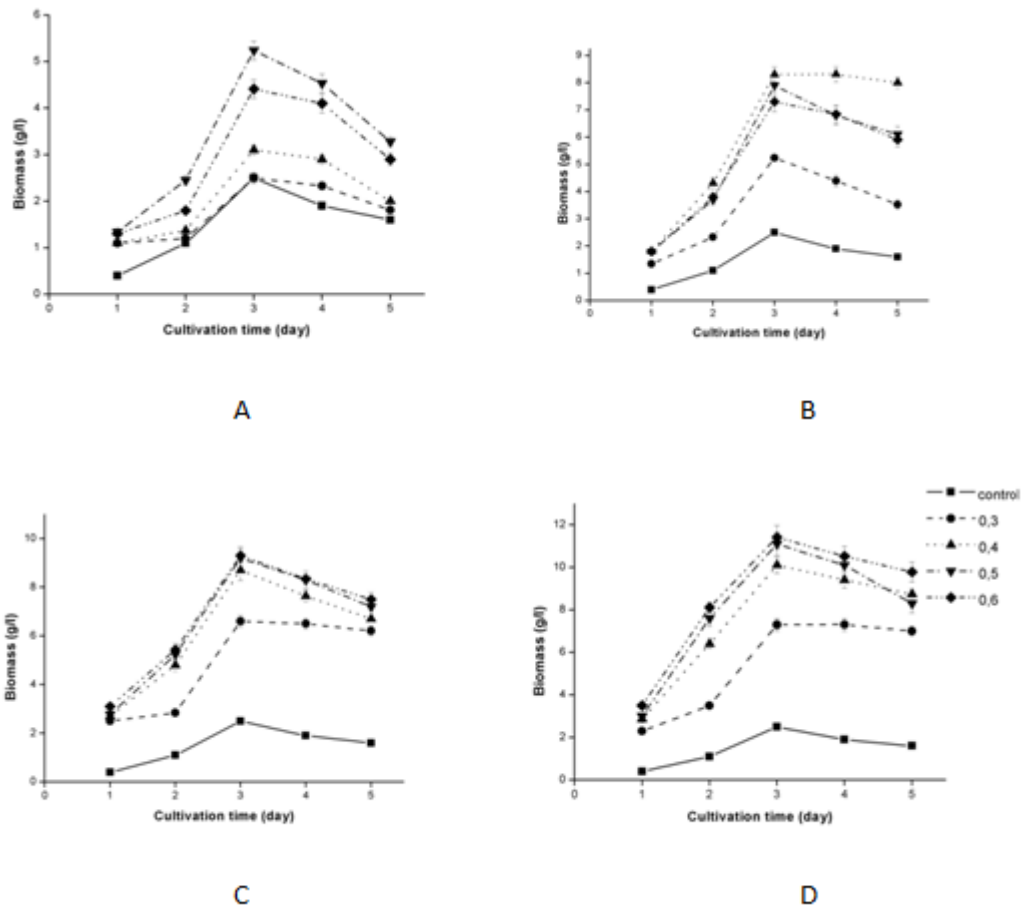

Fig. 3. Influence of nitrogen source concentration on the biomass accumulation:
A – $NaNO_3$; B – $(NH_4)_2SO_4$; C – peptone; D – yeast extract.
Key: Concentration (%): Control (■), 0.3 (●), 0.4 (▲), 0.5 (▼), 0.6 (♦)

Compare the inorganic forms of nitrogen and organic sources (such as peptone and yeast extract) added to the media, the maximal biomass contents on the medium with peptone (a product of incomplete digestion of proteins) was observed (at peptone concentrations of 0.05 - 0.06% (C: N ratio 100 - 120: 1) (Fig. 3 C).

The substitution of the peptone for yeast extract, the growth of the bacterium was increased (Figure 3 D). As in the case with other nitrogen sources, increasing the yeast extract concentration in the medium stimulated the biomass formation: 0.05% of yeast extract (C: N ratio 168: 1) increased the biomass up to 11.1 g / l. Further increase in this nitrogen source concentration to 0.06% no effected the bacterium growth.

So, it can be found that the maximal growth was observed in a medium containing 0.05 - 0.06% yeast extract (carbon / nitrogen ratio 168: 1).

The exopolysaccharide (EPS) formation, as the microorganism growth, is affected by the media composition. We have studied the changes in the amount of the EPS, alginate, depending on the nitrogen source and it concentrations. In all experiments, including the control, alginate biosynthesis was increased during 4 days, which corresponds to the stationary growth phase of the culture. (Figure 4).

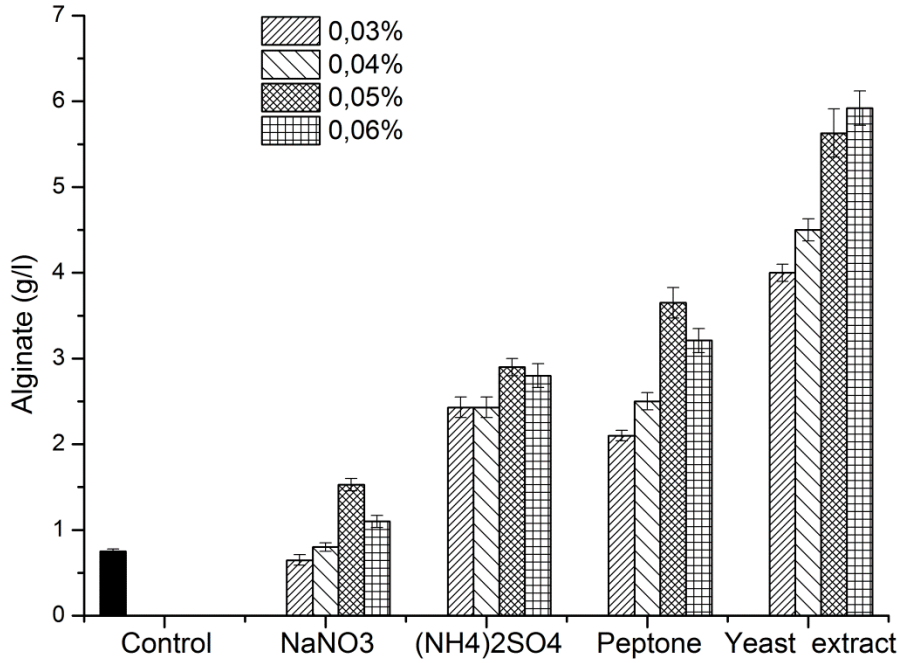

Fig. 4. Influence of the nitrogen source nature and concentration on the alginate biosynthesis (4 days of growth)

Compared to the control, the nitrates stimulated alginate biosynthesis. Addition only 0.05 – 0.06% nitrate slightly increased the polysaccharide content in CB in our experiments as in the literature data[18]. The effectivity of the reduced form of nitrogen ($(NH_4)_2SO_4$) contended media was more for alginate biosynthesis than the oxidized form (sodium nitrate), which is apparently due to the more rapid adaptation of the reduced form of nitrogen for the bacterium metabolism. In experiments with sulfate contended media, the highest EPS content was observed at a concentration 0.04% (carbon: nitrogen ratio 100: 1 and amounted to 3.2 g/l)/ In the reported data, it was found the optimal carbon: nitrogen ratio for alginate biosynthesis was in the range of 60 - 100:1[15] So, the microorganism cultivation and alginate biosynthesis increased at higher content of the ammonia nitrogen (0.1-0.2%) in cultivation medium[24]. When we are using the peptone, the EPS content generally depended on the ammonium sulfate concentration: at 0.05% peptone (carbon: nitrogen ratio 120: 1) the alginate production was on 13% higher than with sulfate. The maximum alginate content (5.63 g/l) was observed when 0.05% yeast extract (carbon: nitrogen ratio 168: 1) was used.

Tables 1 and 2 show the kinetics of cell growth and alginate production in media with various sources of carbon and nitrogen. It was found that the specific growth rate of biomass production was higher in 4% glucose contained media (0,297 h^{-1}) as a carbon source and with yeast extract (0,159 h-1, with 2 % sucrose) as a source of nitrogen. At the same time, the alginate $Y_{P/X}$ in relation to biomass was higher in the experiment with 1.5 - 2% mannite contained media (0,77-0,81 g/g) and the lactose contained media (0,6-0,65 g/g) but the biomass from glucose provided the lowest of alginate amount (0,38-0,42 g/g). Among the nitrogen sources, the highest value of alginate contents was found at 0,03 - 0.06% of yeast extract contained media (0,48- 0.56 g/g) and 0.03% ammonium sulfate contained media (0,55 g/g).

The biotechnology effect is based on the product EPS was calculated on a unit of substrate $Y_{P/S}$. It was found that the medium with 1% sucrose contained media made the highest $Y_{P/S}$ ratio (0.303 g/g); therefore, over 30% of the substrate is used to produce the polysaccharide. Increasing the concentration of the substrate up to 4% contained media, leads courses to a 2-fold decrease Yp/s, although the alginate

volumes production had increased. Higher amounts of yeast extract in the medium provide the highest biotechnology effect of all the sources of nitrogen contained media.

Table 1. Fermentation parameters of alginate produced of A.vinelandii with different carbon sources

Carbon source	Concentration, %	μ, h^{-1}	$Y_{P/S}$, g/g	$Y_{P/X}$, g/g
Mannitol	1,5	0,045±0.003	0,16±0.011	0,774±0.054
	2	0,06±0.004	0,170±0.052	0,808±0.06
	4	0,78±0.044	0,038±0.002	0,3±0.052
Lactose	1,5	0,09±0.005	0,233±0.017	0,648±0.041
	2	0,11±0.009	0,21±0.014	0,534±0.033
	4	0,078±0.005	0,09±0.006	0,6±0.041
Sucrose	1,5	0,153±0.008	0,303±0.016	0,454±0.032
	2	0,167±0.01	0,275±0.02	0,492±0.032
	4	0,172±0.009	0,15±0.009	0,488±0.029
Glucose	1,5	0,152±0.01	0,271±0.019	0,383±0.022
	2	0,175±0.012	0,265±0.018	0,421±0.034
	4	0,297±0.019	0,133±0.010	0,38±0.029

Table 2. Fermentation parameters of alginate produced of *A.vinelandii* with different carbon sources

Nitrogen source	Concentration, %	μ, h^{-1}	$Y_{P/S}$, g/g	$Y_{P/X}$, g/g
$NaNO_3$	0,03	0,035±0.002	0,033±0.002	0,279±0.02
	0,04	0,043±0.003	0,04±0.003	0,276±0.019
	0,05	0,073±0.005	0,077±0.005	0,338±0.022
	0,06	0,061±0.004	0,055±0.003	0,268±0.018
$(NH_4)_2SO_4$	0,03	0,073±0.005	0,122±0.009	0,552±0.044
	0,04	0,115±0.008	0,13±0.008	0,385±0.025
	0,05	0,11±0.009	0,145±0.010	0,426±0.035
	0,06	0,101±0.008	0,14±0.009	0,409±0.03
Peptone	0,03	0,092±0.006	0,105±0.007	0,323±0.021
	0,04	0,121±0.009	0,125±0.008	0,327±0.021
	0,05	0,128±0.008	0,183±0.013	0,44±0.03
	0,06	0,129±0.008	0,161±0.012	0,385±0.022
Yeast extract	0,03	0,101±0.007	0,2±0.015	0,547±0.039

0,04	0,14±0.009	0,225±0.017	0,478±0.031
0,05	0,154±0.01	0,282±0.021	0,557±0.04
0,06	0,159±0.011	0,296±0.022	0,563±0.041

One of the major tasks of any microbiological production is to reduce the product costs. Therefore, the next step involved *A. vinelandii* cultivation in molasses containing medium. Molasses is a by-product of sugar beet production, characterized by a relatively high content of sucrose, which is difficult to extract in the technological process. Besides sucrose molasses is rich in trace elements, vitamins, nitrogen. It therefore can serve as a cheap raw material for the culture media preparation. The results of *A. vinelandii* cultivation on molasses medium showed that the microorganism grows well and the alginate content in CB as compared for modified M22 medium increases 2.7-fold and reaches 16.6 g / l on 3 day of cultivation.

Currently, one of the promising biotechnological areas is in the production of biocomposite materials based on the polysaccharides that are used as a biological binder[4]. In this investigation, we used the alginate as a part of the CB for the wood chipboards production. In the conventional technological scheme, urea-formaldehyde, phenol-formaldehyde are used and it is toxic for people and animals. Therefore, the replacement of the synthetic binders by environmentally friendly bio-binders remains an urgent issue. For this purpose, in our work the sawdust was mixed with a culture broth containing the alginate. The mass was dried to a moisture content of 6 – 8% for the samples compression in a molding hydraulic press. It was shown that the chemical binders replacement with CB alginate, change the bioplastics parameters: the samples tensile strength during the pressing for 20 min amounted to 14.4 MPa (Table 3) that corresponds to with EN 312:2003 «Particle boards - Specifications», NEQ, the samples water absorption and the samples swelling had overstated values - to 22.6 and 28.6%, respectively.

Table 3. Physical and mechanical properties of biocomposite materials

Indicator	Biocomposite based on CB with 1% paraffin
Tensile strength, MPa	14.4±0.7
Density, kg / m^3	1185±59
Water absorption, %	22.6±1.1
Swelling in thickness, %	28.6±1.4

CONCLUSIONS

In this study was found, that the biomass production and the alginate biosynthesis are depended not only a type of carbon source but also by its concentration: the maximal alginate contents were observed during cultivation in a medium containing sucrose. It was shown that using *A. vinelandii* D-05 bacterium for the alginate biosynthesis requires additional the nitrogen source, and the organic forms are better consumed. The highest level of the polysaccharide productions observed in the 0.05% of yeast extract (carbon / nitrogen ratio 168:1) contained media. It was shown that the bacterium is able to grow on molasses medium providing the alginate yield of 16.6 g / l. The culture broth obtained can be used as a biological binder for the production of the ecological biocomposite materials.

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