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Bacterial Cellulose and Pullulan from Simple and Low Cost Production Media

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Abstract

In this study, the production rate of both water-insoluble EPS, bacterial cellulose, and water-soluble EPS, P, was improved through cultivation of their producers on a nutrient media containing industrial wastes, and their material properties were analyzed. The growth rate and productivity of *Gluconoacetobacter xylinus* C3 strain on media with industrial wastes was investigated. An optimal nutrient medium based on molasses was selected for the bacterial cellulose producer. The nutrient medium contains 2% molasses, 1% yeast extract and peptone in a 1: 1 ratio, 0.3% sodium hydrogen phosphate, 0.1% citric acid and 1% ethanol. Cultivation of *Gluconoacetobacter xylinus* C3 strain on this medium for 7 days at 25–30 °C ensures its high productivity – 8.21 g/L. The composition of the optimized medium with molasses provides high mechanical properties (tensile strength – 37.12 MPa and relative elongation at break – 3.28%) of bacterial cellulose and does not affect the polymer microfibrillar structure. A modified Czapek-Dox medium with 10% molasses and 1% peptone is preferable for the exopolysaccharide accumulation by *A. pullulans* C8 strain. The optimized media has an advantage over the traditionally used media in terms of the efficiency of exopolysaccharide accumulation and cost reduction. The pullulan yield in media was 10.08 g/l, that is 1.5 times higher than in a standard Czapek-Dox medium. The surface morphology and microstructure of the pullulan samples obtained on different media showed minor changes. Therefore, the replacement of carbon source for molasses in a Czapek-Dox media for pullulan production did not alter the polymer content and viscosity.

1. Introduction

Polysaccharides are very important materials in nature. Many microorganisms possess the ability to synthesize extracellular polysaccharides (EPS), which are secreted out of cells either as soluble or insoluble polymers. EPS have been found to be extremely useful in many diverse applications in food, pharmaceutical, and other industries [1, 2]. Recently, two microbial polysaccharides have attracted a lot of attention from researchers and manufacturers: Bacterial cellulose (BC) and Pullulan (P). BC, a water-insoluble EPS produced by *Acetobacter xylinum*, chemically pure polymer, has a high degree of crystallinity, mechanical strength and an extremely high absorption capacity [3]. It is characterized by a number of advantages, such as biological compatibility, lack of toxicity and allergenicity [4]. BC has been used in the food industry for applications such as low-calorie desserts, salads, and fabricated foods [5, 6]. It has also been used in the paper manufacturing industry to enhance paper strength, the electronics industry in acoustic diaphragms for audio speakers, the pharmaceutical industry as filtration membranes, in cosmetics and drug delivery, in the medical field as wound dressing and artificial skin material [7, 8].

P, a water soluble EPS synthesized by a yeast-like fungus *Aureobasidium pullulans*, is often described as an 1.6 linked maltotriose polymer. With this unique linkage pattern, pullulan demonstrates distinctive physical properties, such as adhesive ability, the capacity to form fibers, and the ability to be formed into thin and biodegradable films which are transparent and impermeable to oxygen.

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As a result, pullulan has been used for a wide range of applications in food, pharmaceutical, chemical, and environmental remediation applications. P is capable of forming resilient, strong films and fibers that are used as packaging material for food products, thickener to sauces and for increasing the shelf life of fresh meat and fish [9].

One of the problems that limits the production of EPS on an industrial scale is the high cost of the final product. The low-yield production have limits its commercial applications and industry potentials.

To reduce the polymer cost, producers can be grown on cheap media. The cost of fermentation medium is 30–50% of the total value of target product and plays a crucial role in microbial fermentation. One of the most important components of the nutrient medium, affecting the yield, productivity and synthesis rate of EPSs is a carbon source used. The use of industrial waste and by-products of some industries as a source of carbon in fermentation media can increase the profitability of BC and P production. Therefore, researches have focus on agriculture waste and industrial by-product as new cost-effective carbon sources.

In this regard, many studies have focused on the development of fermentation media based on food industry wastes for BC production: dry brandy extract [10], fruit juices [11], maple syrup [12], sugar cane juice, wastewater from rice wine production [13], grape oil cake [14], wastewater from confectioneries, molasses, corn liqueur, milk and soy whey [15]. In a number of research works agricultural wastes were tested: wheat straw [16], fiber sludge [17], spruce hydrolyzate [18], technical cellulose hydrolyzates; as well as waste from biodiesel production, such as crude glycerin and acetone-butanol-ethanol fermentation by-products [19].

There are various reports on the production of P from different sources such as sweet potato [20], potato starch waste [21], coconut by-products [22], agro-industrial wastes such as starch waste, olive oil waste effluents and beet molasses [23, 24], brewery wastes [25], jaggery which is a concentrated sugar cane juice [26]. Thus, the range of agricultural and industrial wastes that could be used in fermentation media for the growth of producers and formation of BC and P by them is wide enough.

The Republic of Kazakhstan has a well-developed dairy and sugar production, which wastes are whey and molasses. One of the ways to use them is to develop simple and cheap nutrient media based on them. In addition, in large quantities there are by-products of cereal crops processing – shells of spring wheat, rice, oats, barley grains, which, after enzymatic hydrolysis of saccharification, can also be used as a carbon source in production media. Fruit shells are annually reproducible raw materials that are distributed globally and have a zero cost, since the cultivation costs are included in the cost of grain. Glycerin is one of the main components of organic waste in the production of biofuels. A significant amount of glycerin is formed by industrial distillation of alcohol and the production of bioethanol from plant materials. Waste disposal is one of the key positions in the organization of environmentally friendly and cost-effective production.

The research team has active producers of BC (*Komagataeibacter xylinus* C-3) and P (*Aureobasidium pullulans* L-25) and successful experience in their cultivation laboratory as well as semi-industrial conditions. Despite the fact that there are many publications about using cheap sources of raw materials to obtain a BC and P, the technological parameters for each producer and a specific strain should be specified. It is impossible to transfer the optimal conditions identified for one strain to another, since it is always necessary to take into account the biosynthetic features of a particular producer.

In this regard, the aim of the study was to develop a nutrient media from industrial wastes for both BC and P producers and comparative analysis of the obtained materials' structural and mechanical properties. It was supposed, that such an approach will reduce the cost of these polysaccharides and offer a way of these wastes disposal.

2. Experimental

2.1. Obtaining enzymatic cellulose hydrolyzate

Technical cellulose (TC) was obtained from the waste from the processing of cereals by the culture-acidic method, consisting in the sequential processing of raw materials with dilute solutions of nitric acid and sodium hydroxide. To determine the chemical composition of the raw material it was pre-crushed with scissors. Determination of the mass fraction of extractives was carried out according to standard methods of plant materials analysis [27]. TC had the following composition (%): lignin – 0.67; ash – 2.20, a-cellulose – 95.10; pentosans – 2.03.

Enzymatic hydrolysis of the TCs was carried out in a fermenter in an aqueous medium at 50 °C for 72 h using the multienzyme composition of industrial enzyme preparations "Cellolux-A" (0.04 L/kg substrate) and "Bryuzyme BGX" (0.1 L/kg substrate) [28]. The resulting hydrolyzate was filtered from the substrate residues under vacuum. After filtration, the enzymatic hydrolyzate was a clear straw-colored liquid.

2.2. Pretreatment of raw molasses

The molasses was diluted with water in an amount of 1:10 and suspended solids were removed by centrifuging at 6000 rpm for 20 min; kept for 24 h in a 1N solution of H_2SO_4, centrifuged again at 6000 rpm for 20 min; and treated with 1% $Ca_3(PO_4)_2$ solution [29].

2.3. Estimation of reducing sugars by dinitrosalicylic acid method

The total amount of reducing sugars was determined spectrophotometrically using 3,5-dinitrosalicylic acid. In the cellulose hydrolyzate, the amount of reducing sugars was 43.8 g /L, in molasses – 119.2 g/L, in whey – 47.4 g/L.

2.4. Preparation of bacterial cellulose gel-film

The synthesis of cellulose was carried out by surface cultivation of *Gluconoacetobacter xylinus* C-3 strain on a classical nutrient medium Hestrin-Schramm and medium based on industrial wastes, pH 6.5–7.0. A 48-hour culture of acetic acid bacteria grown on a medium containing yeast extract and beer wort in a 1: 1 ratio with 2 wt.% glucose 1 vol.% ethanol.

In parallel, the productivity of the strain on nutrient media containing milk whey, cellulose-containing hydrolysates and molasses as carbon and nitrogen sources was evaluated.

Cultivation was carried out at 29–30 °C for 6–7 days, after which the cellulose was separated and periodically washed with 0.5–1% aqueous NaOH solution at boiling until the cells were removed. Then the cellulose film was washed with distilled water, 0.5% acetic acid solution and again with distilled water until neutral reaction. The resulting cellulose was stored as a gel-film in distilled water at 5 °C.

The biomass of the BC films was determined after preliminary drying in a dry-heat thermostat at 80 °C to a constant mass of the sample.

2.5. Pullulan isolation from the cultural liquid

The separation of microbial cells from the cultural liquid was carried out on the 7th day by centrifuging for 15 min at 6000 rpm. The exopolysaccharide was isolated by precipitating it from the supernatant by adding 2 volumes of a polar organic solvent – 96% alcohol. Then it was centrifuged 6000 rpm for 15 min in order to separate the precipitated exopolysaccharide.

2.6. Characterization of bacterial cellulose and pullulan by scanning electron microscopy

EPS samples were pre-coated with a thin layer of a platinum-palladium alloy (Pt/Pd 80/20) and examined on a JSM-7800F scanning electron microscope (Jeol, Japan). During the study, the diameter of microfibers was measured. In the calculation used a sample of data, including 100 measurements.

2.7. Viscosity measurement

P samples produced from *A.pullulans* were dissolved in deionized water (5 mg/ml). Viscosity was measured using a viscometer (Model SV-100) at 25 °C. Pure pullulan from Sigma was used as control.

2.8. Pullulan content

Polysaccharides produced from *A.pullulans* were assayed for sensitivity to pullulanse to determine the P content [30]. Each polysaccharide sample was first ethanol-precipitated, dried and subsequently resuspended at a final concentration of (0.1%, w/v) in 50 mM sodium acetate buffer (pH 5.0). Pullulanase from *Bacillus acidopullulyticus* (Sigma) was added to a concentration of 0.5 U/ml. After mixing, the mixture was incubated for 24 h at 25 °C.

2.9. The study of mechanical properties of bacterial cellulose films

The mechanical properties of the films were determined on an Instron tensile testing machine (USA) in the uniaxial mode by indicators: tensile strength (MPa) and relative elongation at disruption (%). The measurements were carried out at a temperature of (25±2) °C and a relative humidity of (55±5)%, with the specimen deformation rate set at 100 mm/min.

2.10. Nutrient media (NM)

a) for the growth of bacterial cellulose producer strain:

Classical Hestrin-Schramm (HS) medium recommended for industrial synthesis of BC (g/ L): glucose – 20, sodium hydrogen phosphate – 2.7, peptone – 5, yeast extract – 5, citric acid – 1.15;

Modified Hestrin-Schramm (HS) medium (g/L): glucose – 20, sodium hydrogen phosphate – 2.7, peptone – 5, yeast extract – 5, citric acid – 1.15, ethanol – 10;

NM with mannitol: (g/L): mannitol – 25, peptone – 3, yeast extract – 5;

NM-1 (g/L): whey – 20, sodium hydrogen phosphate – 2.7, peptone – 5, yeast extract – 5, citric acid – 1.15, ethanol – 10;

NM-2 (g/L): molasses – 20, sodium hydrogen phosphate – 2.7, peptone – 5, yeast extract – 5, citric acid – 1.15, ethanol – 10;

NM-3 (g/L): waste hydrolysates from cereal crops processing – 20, sodium hydrogen phosphate – 2.7, peptone – 5, yeast extract – 5, citric acid – 1.15, ethanol – 10;

NM-4 (g/L): glycerin – 20, peptone – 5, yeast extract – 5, citric acid – 1.15, ethanol – 10.

Industrial waste served as a source of carbon and nitrogen in the medium. Peptone was added to the medium as a nitrogen deficiency agent, and yeast extract was used as a source of vitamins.

The media were adjusted to pH 6.5–7.0 with icy acetic acid and 6HNaOH. Sterilization was performed by autoclaving at 110 °C for 20 min.

b) for the growth of pullulan producer strain:

Malt medium (g/L): malt extract – 30.0; peptone from soy flour – 3.0;

Glucose-peptone medium (g/L): glucose – 10, peptone – 5, KH_2PO_4 – 1, $MgSO_4$ – 0.5;

Sabouraud medium (g/L): dextrose – 20.0; peptone – 5.0; casein – 5.0;

Capek-Dox medium (g/L): sucrose – 30, $NaNO_3$ – 2, KH_2PO_4 – 1, $MgSO_4x7H_2O$ – 0.5, KCl – 0.5, $FeSO_4$ – 0.01.

Modified carbon source Capek-Dox medium (g/L): sucrose (glucose, molasses, treacle) – 30; 40; 50, $NaNO_3$ – 2, KH_2PO_4 – 1, $MgSO_4x7H_2O$ –0.5, KCl – 0.5, $FeSO_4$ – 0.01.

Modified nitrogen source Capek-Dox medium (g/L): sucrose 30, $(NH_4)_2SO_4$ – 45; 60; 75 or $NaNO_3$ – 15; 20; 25 or peptone – 5; 7.5; 10; KH_2PO_4 – 1, $MgSO_4x7H_2O$ – 0.5, KCl – 0.5, $FeSO_4$ – 0.01.

Modified molasses Capek-Dox medium (g/L): molasses 5 and 10%, KH_2PO_4 – 1, $MgSO_4x7H_2O$ –0.5, KCl – 0.5, $FeSO_4$ – 0.01.

2.11. Statistical processing of experimental results

All assays were conducted in parallel triplicates and repeated three times. Data are presented as means per group ± standard errors of the means. The statistical significance of differences between means was calculated using analysis of variance (ANOVA) ($P < 0.05$).

3. Results and discussion

3.1. Development of a nutrient medium from industrial wastes for bacterial cellulose producer

Stage 1. Selection of a basic media for optimization

A new strain of *Gluconoacetobacter xylinus* C-3 was used in the work, which in terms of productivity exceeds the *Gluconoacetobacter xylinus* B-11240 and *Gluconoacetobacter hansenii* B-6756 collection strains, recommended for industrial production of cellulose. The optimal choice of culture media and conditions for cultivation is important for the growth of cellulose-forming bacteria, since the growth of bacteria influences the stimulation of BC production.

The preparation of bacterial cellulose was carried out on several media: medium with mannitol, HS medium, and modified HS medium. The strain was grown at 30 °C for 7 days. It was shown that a modified HS medium with 0.1% ethanol provided the maximum polymer yield (4.56 g/L), so it was decided to use it as a basis for further optimization studies (Fig. 1).

Probably the presence of citric acid in classical and modified HS medium affected the increase in the productivity of the strain. It is believed that citric acid is utilized by producers as an energy source when the glucose is deficient in a nutrient medium.

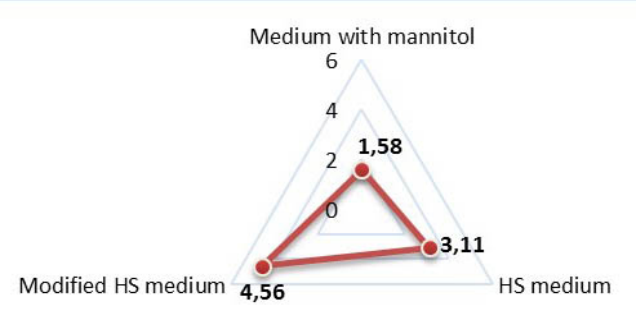


Fig. 1. Production efficiency of *Gluconoacetobacter xylinus* C-3 strain on various media.

Table 1
Parameters of bacterial cellulose biotechnological production efficiency

Nutrient medium	Dry weight of cells, g/L (X)	Dry weight of film, g/L (P)	Utilized glucose, g/L (S)	P/X, %	P/S, %	Economical coefficient, X/S, %
Medium with mannitol	0.7±0.05	1.58±0.02	10±0.65	225.71	15.8	7.00
HS medium	1.47±0.11	3.11±0.03	14±1.01	211.56	22.21	10.50
Modified HS medium	2.94±0.08	4.56±0.02	18±0.07	155.10	0.25	16.33

Ethanol in the composition of the modified HS medium induces the production efficiency of cellulose strains, since it creates a NADH reduced form, decreasing the redox potential of the medium, which favorably influence the synthesis of bacterial cellulose [29]. In addition, ethanol suppresses spontaneous mutations of cellulose-synthesizing bacteria, which reduce their productivity, and can be used as an additional carbon source.

To control the process and compare the BC production efficiency on different media, economical coefficient in relation to carbon substrate, characterizing the degree of energy transfer in the substrate to the product was calculated (Table 1).

The maximum economical coefficient was observed when cultivating a producer strain of bacterial cellulose on a modified HS medium. A decrease of this parameter when growing a strain on an HS medium and a medium with mannitol is associated with a slow growth rate to the log–phase due to the lack of any component of the nutrient medium (limiting) or due to the accumulation of metabolites unfavorable for growth in the medium (inhibition). The maximum polymer yield (4.56 g/L), and the economic efficiency indicator of BC production (16.33%), provided on a modified HS medium with 0.1% ethanol, made it possible to use it as a basis for further optimization studies.

Stage 2. Selection of the optimal nutrient medium on the basis of industrial wastes for bacterial cellulose producer

As a result of the analysis of the industrial sector of Republic of Kazakhstan, it was decided to use whey, molasses, hydrolyzed shells of cereals and glycerin as a source of carbon and nitrogen in the medium. Peptone was added to the medium as a nitrogen deficiency agent, and yeast extract was used as a source of vitamins. Table 2 shows the data reflecting the results of determining the mass of BC films formed on a HS modified medium, Nutrient medium-1 (NM-1), NM-2, NM-3, NM-4.

According to the data obtained, the amount of BC formed by this strain on a medium with whey is comparable to that synthesized on a standard HS medium. The producer synthesizes cellulose from glucose. Therefore, any sugar contained in the fermentable substrate should be converted to glucose. Lactose present in whey is a disaccharide that breaks down into glucose and galactose. Galactose is considered as the least suitable carbon source for cellulose-forming bacteria [31]. This is due to the fact that the membrane transport of galactose is inefficient, therefore, its conversion into cellulose takes place with a low yield [5]. It is necessary to take into account the fact that whey is a perishable product, this is a significant drawback of the wide use of a nutrient medium based on it.

Table 2
Efficiency of *Gluconoacetobacter xylinus* C-3 strain on classical HS medium and waste-based media

Nutrient medium (NM)	BC film weight (g/L)	Significance of difference with control
Modified HS Medium	4.56±0.02 (control	
NM-1 with whey	4.73±0.03	$p > 0.01$
NM-2 with molasses	8.21±0.02	$p < 0.01$
NM-3 with cereal shell hydrolyzates	6.89±0.01	$p < 0.01$
NM-4 with glycerin	3.05±0.12	$p > 0.01$

On the other hand, a medium with hydrolyzed seem to be quite promising subject to availability of cheap technologies for hydrolysates production, since it ensures the high yield of BC on it. This technology was developed by Siberian scientists, where the enzymatic hydrolyzate of oat, miscanthus and flax fruit shells is used as a nutrient medium for obtaining a BC [32]. It was shown that enzymatic hydrolysates are biologically benign, suitable for obtaining products of microbiological synthesis and do not need additional processing to release them from harmful impurities [28]. Since such hydrolysates can be obtained from any renewable cellulose-containing plant resources, and the media based on them can be used in different microbial synthesis productions, such developments look quite promising from a technological and ecological point of view.

A lower productivity of the strain was noted on a glycerol medium. Although the weight of film synthesized by the strain on NM-4 medium is 33.11% higher than on HS medium. Similar data were obtained by other authors, who found that during static cultivation, the yield of BC on glycerol media is lower than on glucose media [33].

NM-2 medium based on molasses is more favorable for bacterial cellulose synthesis in comparison with standard HS medium and media containing dairy and agro-industry wastes.

Since molasses is a by-product of the final crystallization stage in sugar production process, it is one of the most economical carbon sources in microbiological industry. Molasses contains about 80% dry matter, about 57% of which is represented by sugars. It is also rich in proteins and organic nitrogen. In addition, molasses contains significant amounts of sulfur in the form of sulfides and dioxides [34].

The mass yield of a polymer on NM-2 medium is 1.8 times higher than on the classical HS medium. This may be due to the fact that a mixture of carbohydrates (sucrose, glucose and fructose) is present in molasses. Initially, the producer consumes glucose, and then gradually other sugars. By analogy with the metabolism in human body, these sugars have a «low glycemic index».

It is well known that acetic acid bacteria producing cellulose oxidize glucose to gluconic acid [31]. The transformation of glucose into gluconic acid leads to a significant decrease in the pH of the culture broth and blocks the synthesis of BC. When a producer grows on molasses, intensive formation of gluconic acid does not occur, the pH level remains almost at the same level (Fig. 2).

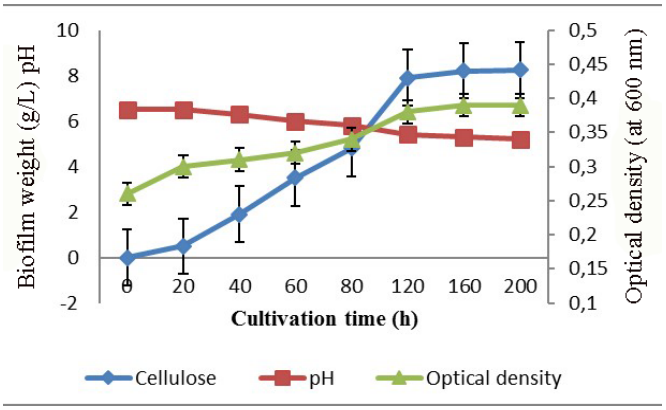


Fig. 2. Dynamics of bacterial cellulose synthesis of by *Gluconoacetobacter xylinus* C-3 on NM-2.

In addition, molasses contains phenolic compounds similar to lignin [11], which are also slowly consumed, and therefore the pH slightly changed, which was accompanied by an increase in the rate of cell growth and the formation of BC in them. It was established that the synthesis of BC is associated with an increase in acetic acid bacteria and the conditions corresponding to the maximum yield of bacteria correspond to the maximum BC output.

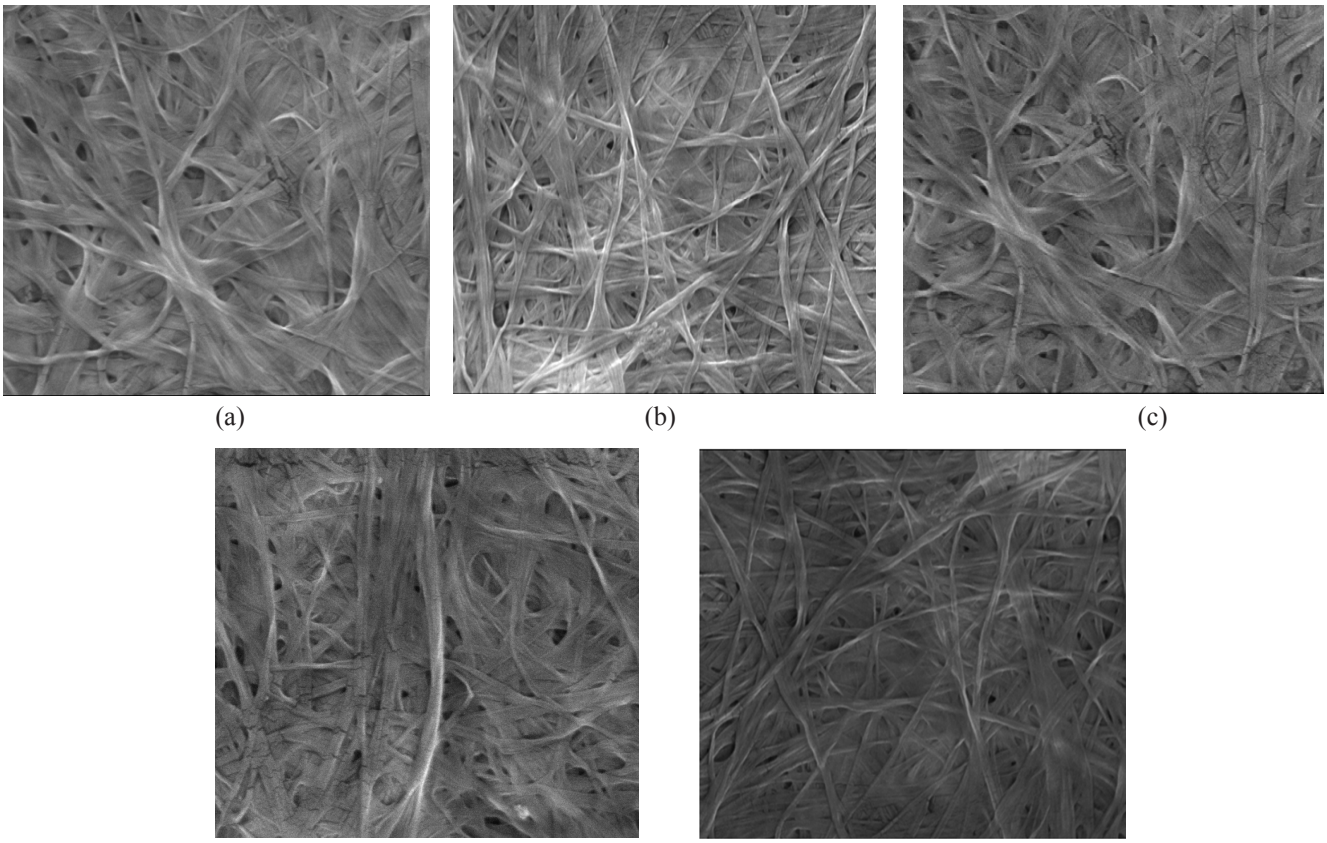
In addition to carbohydrates, nitrogen compounds are also present in molasses: some amino acids, nucleic acids, vitamins [34]. Their presence frees from the need to use additional sources of nitrogen nutrition, such as peptone and yeast extract.

However, it should be noted that in molasses there are minerals and heavy metals that have a toxic effect on the growth of microorganisms and the synthesis of the product [36]. Suspended impurities and heavy metals of molasses can be removed by treatment with H_2SO_4, and $Ca_3(PO_4)_2$, which was used in the work to increase the enzymatic yield of the polysaccharide. In addition, such pretreatment leads to a decrease in the content of carbohydrates, the content of which in native molasses is quite high (up to 23.6% by volume) [29], and a relatively low concentration of sugar in molasses (up to 5% by volume) is a necessary condition for effective BC production.

The results indicate that molasses – a product of the final stage of crystallization of sugar production process, is an ideal carbon source for BC producer cultivation.

Stage 3. Study of the structural and mechanical properties of bacterial cellulose obtained by cultivating a producer on an optimized media with industrial waste

The composition of the nutrient medium can



(a) (b) (c)

(d) (e)

Fig. 3. SEM images of BC films synthesized by strain*:* *Gluconoacetobacter xylinus* C-3 on different medium: (a) – HS mod.; (b) – NM-1; (c) – NM-2; (d) – NM-3; (e) – NM-4 (increase x25.000).

affect the BC structure [5, 34, 36]. Determining the quality of this polymer properties are its structural features, which determine the mechanical strength. The structural properties of the obtained gel films of BCs were examined on a JSM-7800F scanning electron microscope to detect possible differences in the morphology of film surface, as well as comparing the diameter and location of microfibrils of polymers relative to each other (Fig. 3).

Microfibrillary ribbons that form a nano-gel film of bacterial cellulose grown on waste media do not significantly differ from each other. Statistical processing of the results was done to determine the average and most common microfibril diameter. In the calculation used a sample of data, including 1000 measurements.

The thickness of a single fiber is in the range of 15–150 nm, which is 100 times thinner than microfibrils of plant cellulose. To determine the average diameter of microfibrils, statistical processing was performed according to a special program. In the calculation a sample of data, including 100 measurements was used. According to the calculations, the average diameter of BC nanofibrils was about 30±5 nm.

Gel films obtained on media with different types of wastes have an interconnected porous matrix structure with a large surface area. All BC films are flat and smooth. The microfibrils of the BCs are connected into ribbon-like fibers with a thickness of one millionth of a centimeter.

The presence of a uniform distribution of the fibers of BC matrix provides high mechanical strength of the films, which is an important indicator of the biomaterials quality. The strength of the films was determined on a universal “Instron” tensile tester machine in an uniaxial mode by two parameters: tensile strength (MPa) and elongation at break (%) (Table 3).

The BC films synthesized on the medium with molasses showed a high strength index compared with the “standard” ones. This value is rather high compared with the mechanical parameters of many flat oriented layers of organic polymers [37]. The strength of the material synthesized on the medium with molasses may be related to the appearance of hydrogen bonds between OH-groups of cellulose and OH-groups of substances in the composition of molasses. The increase in hydrogen bonds correlates with a high tensile strength in the material.

Table 3
Mechanical properties of bacterial cellulose formed on various nutrient media

Mechanical indicators	Modified HS medium	NM-1 with milk whey	NM-2 with molasses	MN-3 with cereal shell hydrolyzates	NM-4 with glycerin
Tensile strength (MPa)	17.01±0.5	26.16±0.6	37.12±0.2	20.03±0.3	24.01±0.5
Elongation at break (%)	8.01±0.7	4.56±0.1	3.28±0.2	6.23±0.1	5.01±0.1

The cost of 1 liter of standard HS medium is 0.52$, the NM-2 media on the basis of molasses is 0.05$. So, the cost price of 1 g of BC on HS medium is 0.11$, and on NM-2 medium – 0.0053$. Thus, the new media with molasses, providing a high level of BC biosynthesis by a producer, is cost-effective. In general, the use of media based on food and agro-industry wastes can significantly reduce the cost of BC and other products of microbiological synthesis technology, and opens up broad prospects for the development of new waste disposal technologies.

3.2. Growth and production of A. pullulans exopolysaccharides depending on composition of nutrient medium

Stage 1. Selection of a basic media for optimization

Aureobasidium fungi are able to consume a variety of substrates, because they produce a wide range of enzymes and have a high metabolic activity [38, 9]. P has a different composition and structure depending on the carbon and nitrogen sources [39, 40]. According that, producer strain C8 strain was studied for the ability to accumulate exopolysaccharide on traditional nutrient media for cultivation of yeast: malt, glucose-peptone, Sabouraud and Czapek-Dox.

It was shown that the Czapek-Dox medium with sucrose as a carbon source was characterized by the highest yield efficiency of EPS. The yield of the polysaccharide was 14.09±0.46 g/l. Utilization of the substrate in this media was 25.03±1.08 g/l which also exceeded the amount of consumed substrate in other variants (Fig. 4).

Found on the results the optimization of a nutrient media, ensuring the highest yield of EPS, was based on varying of carbon and nitrogen sources in a Czapek-Dox media.

It is known that the selection of carbon sources is an important factor affecting the yield of EPS. Some authors, in particular Chi Z. with co-authors [38], consider glucose as an optimal carbon source in culture medium. Others believe that the maximum yield of polysaccharide is possible on a medium containing other sources [9]. Such substrates can be industrial waste containing a large amount of simple sugars. In this regard, a by-product of beet sugar production – beet molasses (forage molasses) and starch syrup (treacle) – a product of incomplete acid or enzymatic hydrolysis of corn starch were used as carbon sources. The biological value of these compounds is the presence of carbohydrates, biologically active substances, amino acids, vitamins and trace elements [23, 24]. A significant advantage of the proposed raw materials is their low cost, as well as availability and ease of use.

Stage 2. Selection of the optimal source of carbon and nitrogen in the nutrient medium for pullulan producer

Previous studies have demonstrated that *A. pullulans* has the capability to grow on a variety of substrates, and even with agricultural waste without chemical or enzymatic pretreatment due to the multiple enzyme systems available for saccharification of complex sugars [30]. At the next stage, the source of carbon and its concentration were optimized to increase the yield of exopolysaccharide. Sucrose, glucose, molasses and treacle in the amount of 3.0; 4.0; 5.0% were added in the Czapek-Dox nutrient medium.

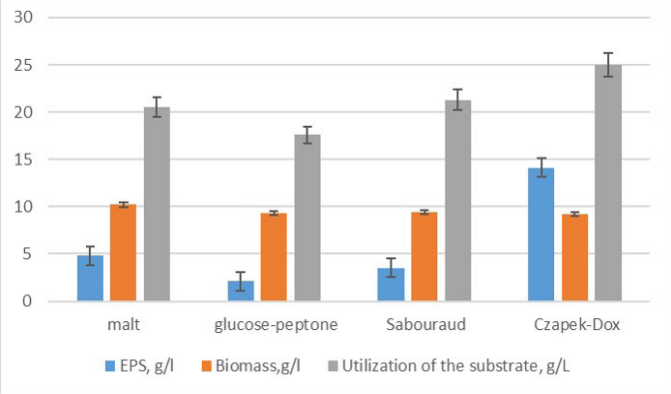


Fig. 4. The influence of nutrient medium composition on the yield of EPS (g/L) by *A.pullulans* C8 strain.

Table 4
Efficiency of EPS production by *A. pullulans* C8 strain on a medium with various carbon sources

Carbon source (g/l)	Dry cell weight, g/l (X)	Dry EPS weight, g/l (P)	Utilized glucose, g/l (S)	P/X, %	P/S, %	Economical coefficient X/S, %
Sucrose 3%	0.208±0.01	1.22±0.06	25.21±1.26	586.54	4.84	0.83
Sucrose 4%	0.396±0.02	3.25±0.16	34.12±1.71	820.71	9.53	1.16
Sucrose 5%	0.532±0.03	7.14±0.36	40.25±2.01	1342.11	17.74	1.32
Glucose 3%	2.932±0.15	5.52±0.28	26.32±1.32	188.27	20.97	11.14
Glucose 4%	5.123±0.26	6.58±0.33	35.12±1.76	128.44	18.74	14.59
Glucose 5%	6.987±0.35	9.34±0.47	40.55±2.03	133.68	23.03	17.23
Molasses 3%	3.964±0.20	4.25±0.21	25.22±1.26	107.21	16.85	15.72
Molasses 4%	7.044±0.35	8.93±0.45	36.12±1.81	126.77	24.72	19.50
Molasses 5%	8.312±0.42	10.08±0.50	38.34±1.92	121.27	26.29	21.68
Treacle 3%	1.272±0.06	3.02±0.15	22.11±1.11	237.42	13.66	5.75
Treacle 4%	1.312±0.07	2.88±0.14	31.13±1.56	219.51	9.25	4.21
Treacle 5%	1.4±0.07	5.03±0.25	36.51±1.83	359.29	13.78	3.83

The choice of these concentrations was justified by excess carbon source (i.e. above 5%) was reported exhibiting inhibition effect on P production [41]. The reason could be due to the suppression effect of sugars on enzymes related to P production, such as α-phospoglucose mutase, UDPG-pyrophosphrylase and glucotransferase [42].

The results showed the dependence of biomass growth and P production on increasing concentration of carbon in the medium (Table 4).

It was shown, that the most favorable carbon sources are glucose and molasses at a concentration of 5% leading to 9.34±0.47 and 10.08±0.50 g/l EPS yield respectively. This indicates the possibility of using sugars contained in molasses for the accumulation of P.

However, the main component of the media along with carbon for the development of microorganisms is nitrogen, since it is a part of amino acids and is involved in formation of peptide bonds of a protein. We are suggest that nitrogen source also plays an important role on P production. In our case for similar rates of carbon utilization, the diversion of glucose from incorportation into cellular material to the elaboration of polysaccharide is dependent on ammonium ion concentration. Depletion of nitrogen from the medium could trigger the synthesis of pullulan. A possible reason is that the presence of nitrogen will stimulate glucolysis through its activating effect on phosphofructokinase [2].

Traditionally, ammonium sulphate, sodium nitrate and peptone are used in EPS accumulation media [9]. When studying the effect of nitrogen sources, it was found that the strain is more preferable to assimilate ammonium sulfate and peptone (Table 5).

Table 5
Efficiency of EPS production by *A. pullulans* strain on a medium with various nitrogen sources

Nitrogen source (g/l)	Dry cell weight, g/l (X)	Dry EPS weight, g/l (P)	Utilized glucose, g/l (S)	P/X, %	P/S, %	Economical coefficient X/S, %
$(NH_4)_2SO_4$ 4.5%	0.596±0.03	6.74±0.34	14.25±0.71	1130.87	47.30	4.18
$(NH_4)_2SO_4$ 6.0%	0.968±0,05	7.21±0,36	15.86±0.79	744.83	45.46	6.10
$(NH_4)_2SO_4$ 7.5%	0.352±0.02	9.09±0.45	19.54±0.98	2582.39	46.52	1.80
$NaNO_3$ 1.5%	3.772±0.19	5.66±0.28	10.54±0.53	150.05	53.70	35.79
$NaNO_3$ 2.0%	3.408±0.17	6.02±0.30	12.91±0.65	176.64	46.63	26.40
$NaNO_3$ 2.5%	4.028±0.20	6.32±0.32	14.55±0.73	156.90	43.44	27.68
peptone 0.5	2.424±0.12	6.51±0.33	15.11±0.76	268.56	43.08	16.04
peptone 0.75	3.14±0.16	7.3±0.37	17.24±0.86	232.48	42.34	18.21
peptone 1.0	3.54±0.18	12.79±0.64	18.34±0.92	361.30	69.74	19.30

Table 6
The efficiency of EPS production by *A. pullulans* C8 strain on the medium with molasses as a source of carbon and nitrogen

Carbon source (g/l)	Dry cell weight, g/l (X)	Dry EPS weight, g/l (P)	Utilized glucose, g/l (S)	P/X, %	P/S, %	Economical coefficient X/S, %
Molasses 5%	7.752±0.39	11.49±0.57	3.25±0.16	148.22	353.54	238.52
Molasses 10%	11.260±0.56	34.66±1.73	8.86±0.44	307.82	391.20	127.09

The maximum accumulation of exopolysaccharide 12.79±0.64 g/l was achieved when using a medium with peptone 1%, while the economic coefficient was 19.30%. Thus, research results of media optimization showed, that the modified Czapek-Dox medium with 5% molasses as carbon source and 1% peptone as nitrogen source is preferable for the exopolysaccharide accumulation.

Stage 3. Polysaccharide production on molasses medium

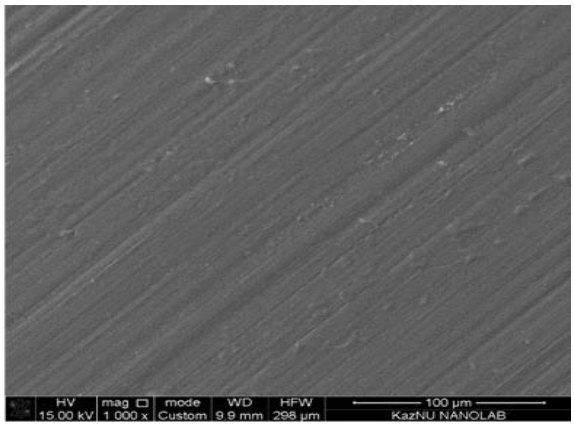
Molasses contains not only 57.0% total sugar, but also 11–13% protein and amino acid (organic nitrogen) and 0.09% nitrate [23, 24]. In previous experiments, it was found that for a successful synthesis of pullulan, a rather 1% concentration of nitrogen sources. We believed that molasses contains the necessary amount of nitrogen for the producer. This was the basis for the next series of experiments in which the producer was grown without an additional source of nitrogen – peptone, which is the most expensive component of this medium. The results of this study are summarized in Table 6.

Based on Table 6, the modified Capek-Dox media with molasses as a source of carbon and nitrogen, contributed to a good growth of the producer: the number of cells in culture medium significantly exceeded this indicator on sucrose and glucose (Table 5). The yield of exopolysaccharide on Capek-Dox media with 10% molasses was higher than in a standard Czapek-Dox medium and amounted to a maximum – 34.66±1.73 g/l. However, a change in the appearance of the polysaccharide was observed: it acquired a flocculent structure, and also colored into light brown color for molasses characteristics (Fig. 5).

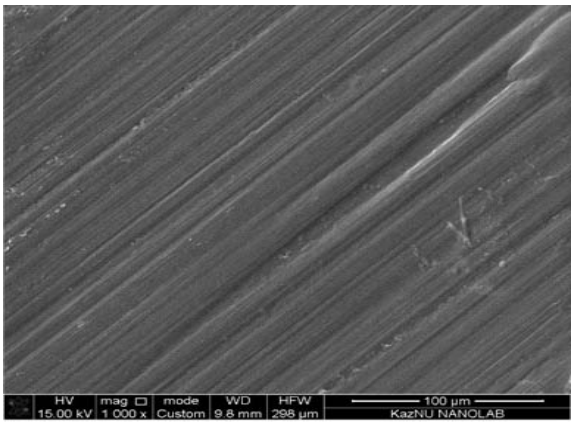
The surface morphology microstructure characterization of pullulan obtained on different media was evaluated by SEM. As shown in Fig. 6, Pullulan obtained on the medium with molasses and standard Czapek-Dox with sucrose looks the same.

(a) (b) (c)

Fig. 5. Precipitation of polysaccharide from medium with molasse (a) and glucose (b, c).



(a)



(b)

Fig. 6. SEM images of pullulan films obtained on basic (a) and modified Czapek-dox media with 10% molasses (b).

Table 7
Comparison of pullulan content and viscosity obtained from pH profiles

Sample	Pullulan (%)	Viscosity (cP)
Pullulan Czapek-Dox media	94.5±2.3	2.3±0.1
Pullulan 10% Molasses media	91.2±1.9	2.1±0.2
Control (Sigma pullulan)	95.1±2.1	2.4±0.1

Since the carbon source can affect the properties of P, in addition to the external structure, purity and viscosity were determined – key physicochemical indicators of the quality of this polymer [43, 44]. Quality analysis of produced pullulan demonstrated 91.2–94.5% purity (Table 7). The viscosity results also indicated high similarity of the studied samples.

Therefore, the replacement of carbon source for molasses in a Czapek-dox media for pullulan production did not alter the pullulan content and viscosity of the polymer. The pullulan samples showed superior values for all parameters.

The cost of 1 l of standard Czapek-dox media is 0.60 $, the modificated media on the basis of molasses is 0.05 $. So, the cost price of 1 g of EPS on Czapek-dox medium is 0.30 $, and on molasses medium – 0.013 $. Thus, the new media with molasses, providing a high level of EPS biosynthesis by a producer, is cost-effective. Thus, the cost of P is reduced by at least 20 times (taking into account the cost of alcohol for the extraction of P).

In summary, both to improve the production of BC and P and to reduce production cost are very important and are the main concern of this research. The main goal of this research is to improve the production of both BC and P by exploring new fermentation methods.

4. Conclusions

An optimal nutrient medium has been selected on the basis of the sugar production waste – molasses for the producer of BC. The nutrient medium contains 2% molasses as a carbon source, 1% yeast extract and peptone in a 1: 1 ratio as a nitrogen source, 0.3% sodium hydrogen phosphate, 0.1% citric acid and 1% ethanol. Cultivation of the *Gluconoacetobacter xylinus* C-3 strain on this medium for 7 days at 25–30 °C ensures its high productivity ensuring 8.21±0.02 g/L BC yield. The composition of the optimized nutrient medium with molasses provides high mechanical properties of bacterial cellulose (tensile strength – 37.12±0.2 MPa and elongation at break – 3.28±0.2%) and does not affect the microfibrillar structure of the polymer.

A modified Czapek-Dox medium with 10% molasses as carbon and nitrogen sources is preferable for the exopolysaccharide accumulation by *A. pullulans* C8 strain. The optimized media has an advantage over the traditionally used media in terms of the efficiency of exopolysaccharide accumulation and cost reduction as a result of inclusion of industrial waste. The yield of pullulan exopolysaccharide in media was 34.66±1.73 g/l, that is higher than in a standard Czapek-Dox medium. The surface morphology and microstructure of the pullulan films obtained on different media showed minor changes in morphology. Furthermore, pullulan did not differ in terms of the analyzed parameters in the purity and viscosity. The replacement of carbon and nitrogen source for molasses in a Czapek-dox media for pullulan production did not alter the content characteristics of the polymer.

The advantages of molasses: low cost, availability and ease of use. This approach will reduce the cost of these polysaccharides and submit a way to remove these wastes. The development of ecologically clean methods of cellulose synthesis is of great interest to solve the important problems of biosphere ecology and deforestation.

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