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Selection and optimization of cultivation conditions for bacterial cellulose producer

Abstract. The aim of this study was to increase productivity of *Komagataeibacter xylinus* C3 strain on nutrient media using industrial wastes as carbon source. By-product of sugar production, molasses, was selected for the cultivation of bacterial cellulose (BC) producer. It has been shown that the significant accumulation of BC (11.9 g/L) occurs on molasses medium with 7 days of cultivation in static conditions, which is 1.6 times higher than on modified Hestrin and Schramm (MHS) medium. BC synthesized on molasses medium is of high mechanical properties (tensile strength – 38.34 MPa and relative elongation at break – 3.34%). The replacement of carbon source for molasses in MHS medium for BC production did not alter the polymer structure and its microfibrillar nature was not affected by the composition of the medium. The introduction of molasses as nutrient source promoted a significant cost decrease of culture media by almost 2.8 times relating to MHS medium cost.

Key words: bacterial cellulose, industrial waste, morphological properties, mechanical parameters, cost-effective production.

Introduction

The main object of study and practical use for the production of bacterial cellulose (BC) are acetic acid bacteria belonging to the family of *Acetobacteriaceae* [1-3]. Currently, 14 genera are present in this family: *Acetobacter*, *Acidiphilium*, *Acidocella*, *Acidomonas*, *Craurococcus*, *Asaia*, *Gluconobacter*, *Paracraurococcus*, *Rhodopila*, *Roseomonas*, *Stella*, *Kozakia*. The species differ in their ability to develop on media with high concentrations of acetate and glucose. The names of the genera *Acetobacter* and *Gluconobacter* in this family have been known for a long time, but the taxons of the remaining genera were isolated and published only after 1989. Until 1998, the species *Acetobacter xylinum* was considered in the scientific literature as a separate species, but belonged to the subspecies *Acetobacter aceti*. In 1998, this species was again reclassified as *Gluconacetobacter xylinus*. The species *Acetobacter hansenii* has been reclassified as *Gluconacetobacter hansenii*. Later, the genus *Gluconacetobacter* was again reclassified into the genus *Komagataeibacter* [4].

It is believed that the cells of bacteria synthesizing cellulose are immobilized in the polymer net in order to maintain the entire population in the space between air and liquid. I.e., cellulose biosynthesis is physiologically expedient for producers, and is an important evolutionary mechanism for the survival of producers of this polymer [5].

Bacterial cells created during the synthesis process of glucose chains exit through tiny pores that are present on its cell membrane [6]. Cellulose-synthesizing enzymes involved in the glucose polymerization reaction produce several glucan chains that form 1.5-nanometer subelement fibrils. These fibrils (glucose chains) then combine to form microfibrils, which later assemble and form cellulose ribbons [7]. Typically, 10 to 100 microfibrils form a ribbon approximately 7 nm thick and 70-145 nm wide. These ribbons (nanofibers) subsequently create a web-like net structure with a large number of empty spaces between the fibers [8, 9].

The main factors affecting the productivity of bacteria are the composition of the nutrient medium and the conditions of their cultivation. The optimal choice of nutrient medium and conditions for cultivation is also important for cellulose-forming bacteria, whose productivity depends primarily on the carbon source. They synthesize it from glucose, which is used in the classical Hestrin and Schramm

(HS) medium [10]. However, a number of papers provide information on the influence of other carbon sources on BC biosynthesis [11-13]. Most often their authors used sucrose, fructose, galactose, mannitol and glycerin. It has been shown that sucrose and fructose provide the highest BC yield, followed in descending order by manitol, glycerin and galactose [13]. The authors explained the results obtained by the ability of bacteria to form glucose from different carbon sources, since any substrate must initially be converted into glucose and only after that it polymerizes into cellulose [13]. When using mannitol, fructose or glucose constant rates of cellulose formation are observed as a result of efficient transport through the cell membrane (initially mannitol is converted into fructose). Galactose has been identified as the least suitable carbon source because its transport through the cell membrane is inefficient.

One of the problems limiting the BC production is a high cost. The price of the fermentation medium is 30-50% of the total cost of the target product, which plays a crucial role in microbial fermentation.

The use of waste and by-products of some industries in fermentation media can increase the profitability of BC production. In this regard, many studies have focused on the development of media using industrial waste: cognac extract [14], fruit juices [15], maple syrup [16], sugar cane juice, wastewater from the production of rice wine [17], grape oilcake [18], wastewater from confectionery factories, corn liquor, dairy and soy whey [19]. In a number of works, agro-industrial wastes were tested for these purposes: wheat straw [20], activated sludge [21], spruce hydrolysate [22], hydrolysates of technical cellulose, as well as waste from biodiesel production, such as crude glycerin and by-products of acetone-butanol-ethanol fermentation [23]. Thus, the range of agricultural and industrial waste that could be used in fermentation media for the growth of producers and the formation of BC by them is quite wide.

In this regard, the purpose of this work is to select and optimize the cultivation conditions that ensure the maximum yield of BC.

Materials and methods

Obtaining BC under surface cultivation conditions. The inoculate was obtained by transferring a colony from an agar culture with *Komagataeibacter xylinus* C-3 strain into 100 ml of modified HS (MHS) medium, and then incubated at 30°C for 48 hours. In MHS medium, in contrast to the classical HS medium, the glucose content is reduced to 1% and ethanol is added at a concentration of 0.5% [24]. Composition of MHS medium (g/L): glucose – 10, sodium hydrogen phosphate – 2.7, peptone – 5, yeast extract – 5, citric acid – 1.15, ethanol – 5.

The resulting culture was vigorously shaken to release the immobilized cells from the synthesized cellulose film, followed by filtration of the suspension through sterile nets. The cells were then precipitated by centrifugation at 10,000 rpm. The titer of cells in the inoculum was determined by optical density and adjusted to a cell density of 5×10^8 CFU/ml using a UV-1601 PC spectrophotometer (Shimadzu, Japan).

Liquid-phase stationary cultivation of the BC producer was carried out in MHS medium with glucose, as well as in a condition where production wastes were used instead of glucose. Nutrient media based on whey, molasses and glycerin of the following compositions:

- medium with whey (g/L): whey – 20, sodium hydrogen phosphate – 2.7, peptone – 5, yeast extract – 5, citric acid – 1.15, ethanol – 5;
- medium with molasses (g/L): molasses – 20, sodium hydrogen phosphate – 2.7, peptone – 5, yeast extract – 5, citric acid – 1.15, ethanol – 5;
- medium with glycerin (g/L): glycerin – 20, peptone – 5, yeast extract – 5, citric acid – 1.15, ethanol – 5.

The media were poured into 100 ml flasks, then 1 ml of inoculate was added. Cultivation was carried out at 30°C for 7 days.

BC films were separated from the culture fluid and periodically washed with 0.5-1% NaOH solution at 80°C heating until the cells were removed. Then, the cellulose samples were washed from the NaOH solution with distilled water, 0.5% acetic acid solution and once more with distilled water until a neutral reaction. The obtained cellulose samples were stored in distilled water at 5°C.

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

Characterization of bacterial cellulose samples by scanning electron microscopy. Samples of cellulose were precoated with a thin layer of a platinum-palladium alloy (Pt/Pd 80/20) and examined using a JSM-7800F scanning electron microscope (Jeol, Japan). The average diameter of the BC nanofiber was calculated according to the obtained (at least 100) values, calculations were carried out using Origin Pro 9.1 program (Originlab Corporation, USA).

Determination of the strength of films. The mechanical data of BC was measured using the Instron bursting machine (USA) in uniaxial mode according to the parameters: tensile strength (MPa)

and elongation at disruption (%). Tensile tests were conducted with a set sample deformation at constant speed of 100 mm/min.

Statistical analysis. Statistical comparison was performed using an unpaired test, followed by one-way analysis of variance (ANOVA) using Dunnett's multiple comparison test. All statistical data of the analysis were carried out using the SPSS 16.0 software package (SPSS Inc., USA).

Results and discussion

Despite the fact that there are many publications using cheap sources of raw materials for the production of BC [14-23], it is necessary to clarify the technological parameters for each producer and a specific strain. 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, at the next stage, studies were conducted to determine the possibility of using cheap carbon and energy sources as part of the nutrient medium for the *K. xylinus* C-3 strain.

Dairy and sugar production are well developed in the Republic of Kazakhstan, the waste of which are whey and molasses. One of the ways to use them is to develop simple and cheap nutrient media based on them for production BC.

In addition, the number of distilleries, including those producing bioethanol, is increasing every year. One of the main components of organic waste in the production of biofuels is glycerin. A significant amount of glycerin is formed during the industrial distillation of alcohol in distillation columns and the production of bioethanol from vegetable raw materials. Waste disposal occupies one of the key positions in the organization of environmentally safe and cost-effective production.

In this regard, it was decided to use whey, molasses and glycerin, which were introduced into MHS medium instead of glucose as carbon source. Figure 1 shows the results of determining the mass of BC films formed on MHS and waste media.

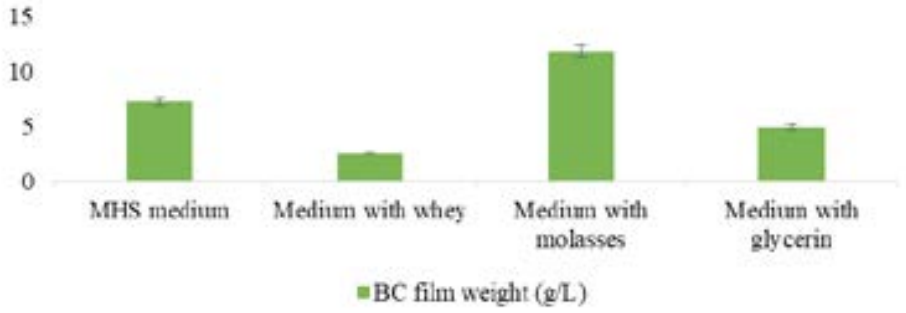


Figure 1 – Productivity of *K. xylinus* C-3 strain on MHS medium and waste-based media

Judging by obtained data, the lowest BC yield was observed on the medium with whey (2.5 g/L). The producer synthesizes cellulose from glucose. Therefore, any sugars contained in the fermented substrate must be converted to glucose. Lactose presented in whey is a disaccharide that breaks down into glucose and galactose. Galactose is considered the least suitable carbon source for cellulose-forming bacteria [13]. Besides, the proportion of mutants that unable to synthesize cellulose increases during cultivation on media with galactose or lactose. The increase in the number of cellulose-negative cells leads to competition for a substrate with a population of cellulose-positive cells. It was found that not a dense, but a gel-like film was formed on these media. The fact is that the presence in the population of a significant proportion of cellulose-negative mutants not involved in the synthesis of cellulose leads to a noticeable decrease in the overall rate of polymer accumulation, which in turn prevents the formation of a dense film [24]. A similar pattern was observed in our experiments.

It should also be taken into account that whey is a perishable product, which is a significant disadvantage of the widespread use of a nutrient medium based on it.

Low productivity of the strain was also noted on the glycerin medium. The weight of the film synthesized by the strain is 4.87 g/L, which is 32.6% less than on MHS medium. Similar data were

obtained by other authors, who found that with static cultivation, the yield of BC on glycerin medium is lower than on glucose medium [25, 26].

The most favorable medium for BC synthesis is a medium based on molasses [27-29]. It is one of the most economical carbon sources in the microbiological industry. Molasses is a by-product of the final stage of crystallization in the sugar production process. Due to the high content of sucrose in molasses, it is actively used as a raw material for the preparation of nutrient media. Molasses contains about 80% of dry substances that approximately 57% is represented by sugars. However, at such concentration, microorganisms will not be able to grow, so molasses was diluted with distilled water 10 times. The relatively low sugar concentration in molasses is a prerequisite for efficient cellulose production.

It should be noted that molasses contains minerals and heavy metals that have a toxic effect on the growth of microorganisms and the synthesis of the product [30]. Suspended impurities and heavy metals of molasses were removed by treatment with hydrochloric acid at 70-80°C. In addition, this treatment will also allow sucrose to be hydrolyzed to its glucose and fructose monomer.

The polymer mass yield on the molasses medium is 11.9 g/L, which is 1.6 times more than on MHS medium (7.23 g/L). The reason may be that molasses contains a mixture of carbohydrates (sucrose, glucose and fructose). Primarily, the producer consumes glucose, and then gradually other sugars.

There is noted a pH decline cause of glucose oxidation to a gluconic acid that could impact on formation of BC [15]. However, synthesis of gluconic acid is suppressed in a medium based on molasses, and pH stays steady (Figure 2).

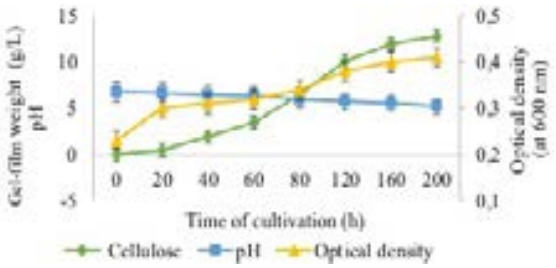


Figure 2 – Dynamics of BC biosynthesis on the medium with molasses

Furthermore, molasses includes phenolic compounds with guaiacyl and syringyl linkages comparable to lignin [29], which are likewise slowly consumed, resulting in a modest pH shift, which was accompanied by an increase in cell proliferation and the synthesis of BC. It was discovered that BC synthesis was linked to the growth of acetic acid bacteria, and that the conditions applied to the highest number of bacteria also correspond to the highest BC production.

Molasses includes nitrogen molecules such as amino acids, nucleic acids, and vitamins apart from carbohydrates. In MHS medium, peptone and yeast extract serve as sources of nitrogen nutrition. Peptone is an expensive component (48,775 tenge/kg). On the one hand, yeast extract also contains nitrogen compounds necessary for microorganisms (peptides, free amino acids and nucleotides). On the other hand, it also contains all the vitamins of group B. And, finally, some researchers point out that the absence of peptone in the nutrient medium does not affect the productivity of *K. xylinus* strain [31]. In this regard, the producer strain was grown under the same conditions, but on a medium without peptone (Table 1).

Table 1 – The mass of the BC film (g/L) formed on media with different concentrations of peptone

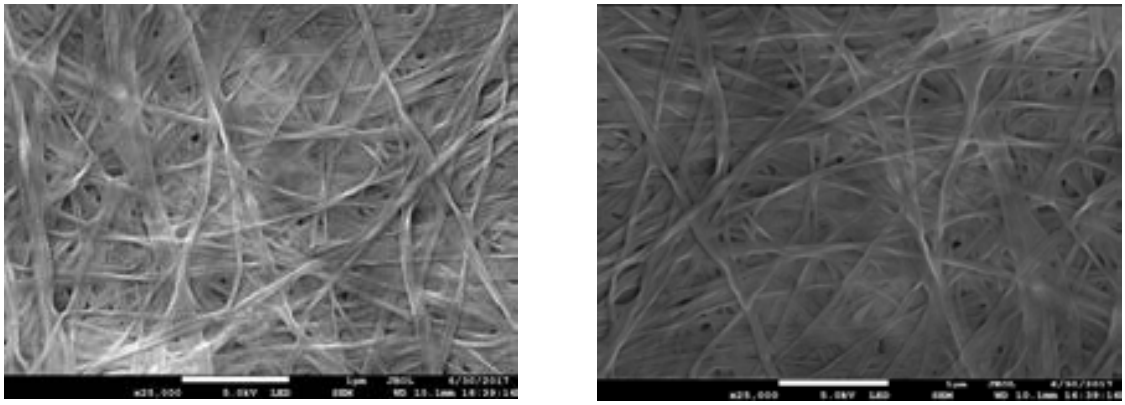
Medium	Content of peptone (%)		
	0	3	5
MHS with glucose	7.12±0.3	7.19±0.4	7.23±0.4
Medium with molasses	11.65±0.4	11.79±0.2	11.9±0.4
Note: * in all cases, there were no significant differences between the indicators (p≥0,05)			

Under the conditions of this experiment, no significant difference in the level of productivity of the strain was found, the discrepancy in the mass of the BC films was within the confidence interval, at least in the conditions of the experiment. Probably, organic nitrogen compounds contained in molasses and yeast extract fill the needs of the strain in this element. This eliminates the need to use an additional source of nitrogen nutrition, such as pepton. The removal of peptone from the medium based on

molasses will reduce the cost of the BC production technology.

Thus, the proposed composition of the medium with molasses (g/L): molasses – 20; sodium hydrophosphate – 2.7; yeast extract – 5; citric acid – 1.15; ethanol – 5. A patent for utility model No. 5756 was obtained for it under the name "Nutrient medium for the cultivation of the bacterial cellulose producer *Komagateibacter xylinus*" dated 08.01.2021 [32].

The production nutrient medium not only significantly affects the cost of the obtained products, but also determines their quality [16, 21, 33]. The BC was examined on a scanning electron microscope to detect possible differences in the gel film (Figure 3).



A B

Figure 3 – SEM images of BC films synthesized by the *K. xylinus* C-3 strain on various media.
Note: A – MHS; B – medium with molasses (magnification x25 000)

Microfibrillar ribbons forming a nano-gel film of BC grown on MHS and media based on molasses do not differ from each other. All BC films had a flat and smooth surface. The thickness of BC microfibrils averaged 15–150 nm. The average diameter of BC fibrils was approximately 30±5 nm. The interconnected porous matrix structure of films formed on media had a considerable surface area. BC microfibrils are combined together into one million of a centimeter thick ribbon-like fibers. It is feasible to provide not only the needed vapor and gas permeability, but also to retain different biologically active components in the structure of films.

The presence of a BC framework with uniform fiber distribution density assures that films have a high mechanical strength, which is an important measure of biomaterial quality [34]. The strength of the films was determined on the universal breaking machine Instron (USA) in uniaxial mode by two indicators: tensile strength (MPa) and elongation value (%) (Table 2).

Judging by the results presented in Table 2, the tensile strength of the BC formed on MHS medium was 28.54±0.4 MPa, and the elongation value was 4.89±0.2. The elongation value (%) of BC gel film formed on the media based on molasses was 3.34±0.2. BC films synthesized on molasses medium had the highest strength value (38.34±0.2) compared to those obtained on MHS medium. This value is sufficiently high compared to the mechanical parameters of many flat oriented layers of organic polymers. It is known that high tensile strength correlates with an increase in the number of hydrogen bonds in the material [30]. This suggests that an increase in the strength of BC synthesized on the medium with molasses may be due to the occurrence of hydrogen bonds between OH-groups of cellulose and OH-groups in composition of molasses. The cost of 1 liter of MHS medium is 743 tenge, molasses-based media is 435 tenge (Table 3).

Table 2 – Mechanical properties of BC formed on various nutrient media

Mechanical strength indicators	MHS medium	Medium with molasses
Tensile Strength (MPa)	28.54±0.4	38.34±0.2
Elongation at break (%)	4.89±0.2	3.34±0.2

Table 3 – Calculation of the cost of nutrient media for cultivation of *K. xylinus* C3

Medium	Components of medium	Price (tg per 1 kg)	Number of ingredients (g/L)	Price (tg for 1 liter of medium)
MHS medium	glucose	7,000	10	70
	sodium hydrogen phosphate	103, 800	2.7	280
	peptone	48, 775	5	244
	yeast extract	26, 400	5	132
	citric acid	5,700	1.15	7
	ethanol	2,000	5	10
Total	743			
Medium with molasses	molasses	300	20	6
	sodium hydrophosphate	103, 800	2.7	280
	yeast extract	26, 400	5	132
	citric acid	5,700	1.15	7
	ethanol	2,000	5	10
Total	435			

The cost of 1 g of BC on MHS medium – 103 tenge and on molasses-based medium – 37 tenge, which reduces the cost of BC production technology by almost 2.8 times (Table 4).

Thus, the new molasses medium is cost-effective for the cultivation of the BC producer as well as provides a high level of gel-film biosynthesis.

Table 4 – Calculation of the cost of BC on different media

Medium	BC output on media (g/L)	The cost of the medium (tg per 1L)	Cost of BC (tg for 1 g/L)
MHS medium	7.23	743	103 tg
Medium with molasses	11.9	435	37 tg

Conclusion

Production of BC is costly with defined chemical medium, i.e., HS medium. Therefore, most researchers are searching alternative from the available wastes in order to reduce the cost. To overcome these difficulties, there has been a research on the enhanced production of BC, by selection and optimization of the producer static cultivation using whey, glycerin and molasses, as substrate.

An optimal nutrient medium based on the waste of sugar production – molasses was selected for the BC producer, which provides a 2.8-fold reduction in the cost of the synthesized biopolymer. Surface cultivation of *K. xylinus* C3 strain on a medium with molasses for 7 days increases the productivity of the film formation to 11.9 g/L.

BC, obtained on the medium with molasses forms a network of micro-(15-35 nm) and macrofibrils (50-150 nm), providing high mechanical properties (tensile strength – 38.34±0.2 MPa; elongation at break – 3.34±0.2%).

Thus, the composition of optimal nutrient medium for BC production was developed using molasses as a substrate. By-product of sugar production promoted significant gains of BC in mass and yield compared to MHS medium, without any difference in microfibrillar structure of polymer.

An optimized nutrient medium based on sugar production waste – molasses can be used to scale the production of BC. The use of media based on food and agro-industrial waste can significantly reduce the cost of technology for obtaining not only BC, but also other products of microbiological synthesis and

opens up broad prospects for the development of new technologies for the disposal of these wastes.

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