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ISOLATION AND IDENTIFICATION OF BACTERIAL CELLULOSE PRODUCERS WITH POTENTIAL FOR MEDICINE AND BIOTECHNOLOGY

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ABSTRACT

Recent achievements in investigations of microbial polysaccharides have highlighted their importance in the development of new classes of biomaterials. Some polysaccharides of microbial origin are already widely used in various medical applications, including hyaluronic acid, dextran, alginate, and scleroglucan. Substantial attention has more recently focused on bacterial cellulose as a promising and effective biopolymer.

In this study, based on knowledge of the ecological niches of acetic acid bacteria, we isolated cellulose-producing bacteria using the fruits of grapes of different varieties and Kombucha. The most effective biocellulose producers were screened, and their biochemical properties were determined. A total of 17 acetic acid bacteria isolates were obtained, and three strains capable of producing a cellulose biofilm were selected, which were identified according to the direct nucleotide sequence of the 16S rRNA gene fragment and biochemical properties.

Key words: biocellulose, bacterial cellulose, biopolymer, nanocellulose,*Komagataeibacter,* gel-film

INTRODUCTION

Currently, extracellular bacterial cellulose has been widely used in several sectors of the economy in world practice: to create biofilters with various sizes, to immobilize microorganisms and enzymes; in the paper production and packaging industries. In the textile industry, bacterial cellulose is used to create new fabrics, in medicine for the production of artificial skin; in the high-tech industry for the production of new materials, nanocomposites, in ecology for wastewater treatment [1].

Bacterial cellulose (BC) has characteristics that are absent in plant cellulose. BC is a glucose polymer, is formed during static bacterial cultivation, is a fairly strong gel film (the ratio dry polymer/water about 1/100) with a specific architecture formed by crystalline microfibrils at the nanostructured level. Water can be replaced with organic liquid while maintaining a huge internal surface.

Bacteria produce cellulose as a means of protecting cells from adverse factors and providing competitive advantages for existence at the interface of the water-air phases. Cells of cellulose-synthesizing bacteria are immobilized in a polymer grid to maintain the entire population on the surface of the liquid phase and thus gain access to oxygen [2, 3].

Bacterial cellulose is synthesized by grams of negative bacterial species from the genera*Komagataeibacter*, *Agrobacterium*, *Aerobacter*, *Achomobacter*, *Azotobacter*, *Rhizobium*, *Escherichia*, and *Salmonella* [4]. Most often*Acetobacter xylinum* or *Gluconacetobacter xylinum* are used to obtain bacterial cellulose [5].

A single*G. xylinum* cell is capable of polymerizing 200,000 glucose molecules per second into β-1,4-glucan chains, which are then excreted into the environment, forming ribbon-like bundles of microfibrils. The fibers are synthesized on the membranes using cellulose synthase, forming a three-dimensional grid of cellulose fibers. The produced nanofibers are longer, wider and stronger than plant

cellulose fibers. Biocellulose differ from plant cellulose increased elastic and free from impurities of hemicelluloses and lignin [6].

Fibrils of bacterial cellulose are 100 times thinner than plant cellulose, they creat a highly porous material that allows to transfer antibiotics or other medicines to the wound, in doing so remaining an effective physical barrier against any external infection [7,8].

BC is not allergenic and easily sterilized without changing properties. Being similar to human skin, BC can be used as a substitute for skin in the treatment of extensive burns and non-woven coatings for chronic wounds. For this reason, cellulose is widely used to treat wounds. [9].

Compared with other biopolymers such as collagen, chitosan and gelatin, bacterial cellulose has excellent biological properties for tissue regeneration, mainly for the treatment of chronic and burn wounds [10].

In this regard, the goal of our research was the isolation and study of effective producers of bacterial cellulose.

Materials and methods

Isolation of microorganisms. Bacteria capable of producing biocellulose were isolated from rotting grapes and Kombucha. Grape berries and a suspension of Kombucha (5 ml) were inoculated into HS-broth (100 ml) composed: 2.0 % D-dextrose, 0.5% yeast extract, 0.5% peptone, 0.27% Na_2HPO_4, 0.115% citric acid, pH ć 6,0 [11]. The cultivation was carried out under static conditions at a 30 ^0C temperature for 7 days. Then, from an accumulative culture, sowing on HS-agar (1,5%) plates was performed and cultivated in an incubator at 30 ° C for 3 days.The isolation and quantification of microorganisms was carried out according to generally accepted methods, based on the identification of bacteria in nutrient solutions containing nutrition and energy components necessary for the vital activity of bacteria of each species [12].

Biochemical and physiological tests. Morphology of the cells was examined under light microscope. Gram staining was performed to select gram negative strains. Acid forming colonies were subjected to further biochemical tests. Catalase, oxidase, H_2S and indole tests were performed using discs and strips reagents (Himedia, India). Acid production from carbohydrates was investigated by using carbohydrate soaked discs with Andrede media (Himedia, India). Urea Agar Base (Christensen) (Himedia, India) was used to determine urea utilization. Hydrolysis of gelatin was investigated by inoculation of nutrient gelatin tubes (0.5% peptone, 0.3% beef extract and 12% gelatin) incubated at 37°C for 1-2 weeks. Attitude to oxygen determined by inoculating bacteria in an agar column.

Genetic identification. Genetic identification of bacteria was carried out by determining the direct nucleotide sequence of the 16S rRNA gene fragment. PCR amplification of the DNA fragment was performed using universal primers 8f 5≥-AGAGTTTGATCCTGGCTCAG-3≥ and 806R 5≥-GGACTACCAGGGTATCTAAT-3≥ The PCR products of 16S rRNA from each isolate were sequenced and the nucleotide sequences of all the studied strains were compared with the 16S rRNA nucleotide sequences available in the international NCBI database.

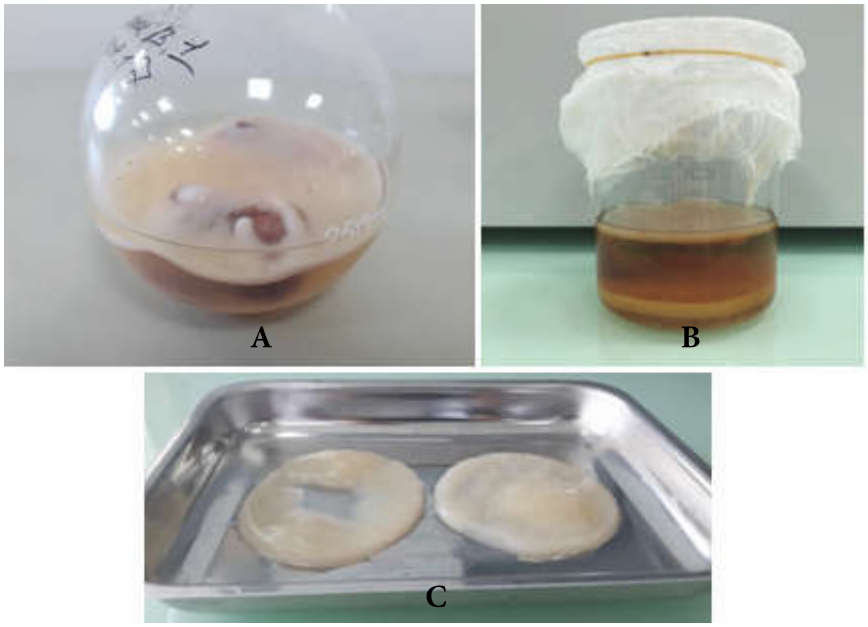
Cellulose formation and detection. Productivity was studied in 250 ml flasks with 90 ml of medium, which was inoculated with 10 ml of the starting culture and incubated at 30 °C under static conditions for 7 days. Cellulose formation was monitored by the appearance of a white film on the surface of the culture broth. Additional processing is required to confirm the structure of the pulp. Culturing liquid was centrifuged for 10 minutes at 4000 g. After washing three times with distilled water, the film were boiled for 15 minutes with 0.5 N NaOH. Cellulose is resistant to this treatment, and thus the remaining material can be considered cellulose free from microbial cells and medium components. The purified cellulose film was dried at 65 °C until a constant weight was obtained. BC productivity was expressed in dry weight g/l of culture medium [13].

RESULTS AND DISCUSSION

We have isolated 17 isolates of bacteria from Kombucha and rotting grapes. Each isolate was isolated in a pure culture and tested for biofilm formation on the HS-broth. The formed films were treated in 0.5 N NaOH to confirm the presence of cellulose.

As a result of the work, 3 isolates of acetic acid bacteria were selected that are capable of producing biocellulose. Figure 1 shows biocellulose samples obtained from rotting grapes and Kombucha.

The study of the morphological and biochemical properties of the bacteria showed that the GH1 strain forms beige shades of rounded colony when growing on solid nutrient medium, the edge is not even, the GH2 strain forms small round convex beige shades, and the GV1 strain forms round whites. The colonies of the studied cultures are glossy, with a homogeneous structure convex. According to the consistency of the colony of crops - dry. The largest colonies are formed by the GV1 strain - from 2 to 5 mm. Colonies of GH1 and GH2 strains are small - 0.5-3 mm. According to micro morphological properties, bacteria cultures are represented by gram-negative rods, located singly, in pairs or chains (figure 2, table 1).



A - film from rotting grape, B - Kombucha, C - washes biocellulose films
Fig. 1. Biocellulose formation

In relation to oxygen, the studied microorganisms were assigned to obligate erobes. All the studied microorganisms are characterized by common signs - catalase-positive, oxidase and urease are negative, acid-forming, do not form hydrogen sulfide and indole and do not dilute gelatin. The presence of gas formation is not observed (table 2).

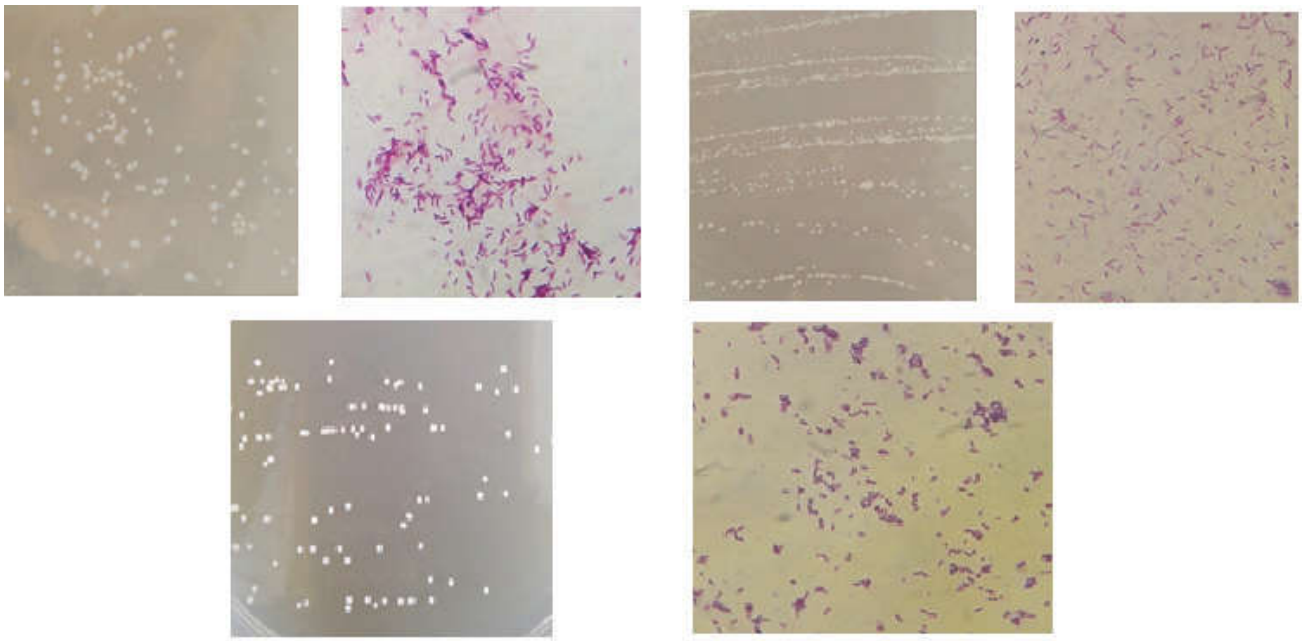


Fig. 2. Macro and micro morphological properties of cells: on the left macro properties, on the right - micro

Table 1. Morphological properties of bacteria

Strain	GH1	GH2	GV1
The form of colony	round	round	round
Optical properties of colony	glossy	glossy	glossy
Color of colony	beige	beige	white
Gram's reaction	-	-	-
The form of cells	r	r	r
Edge of colony	wavy	flat	flat
Sizes colony, mm	0,5-3	0,5-3	2-5
Side view colony	convex	convex	convex
Structure colony	homogeneous	homogeneous	homogeneous
Consistence colony	dry	dry	dry
Pigment highlighting	don't highlight	don't highlight	don't highlight
Note: r ć rods			

A study of the ability of cultures to assimilate various carbohydrates showed that the GH1 strain is able to utilize all carbohydrates except raffinose, sucrose, sorbitol, galactose and dulcitol. The GH2 strain does not metabolize sucrose, arabinose, galactose, cellobiose, xylose, and fructose. GV1 - strain does not utilize rhamnose, trehalose, xylose, adonite and fructose. The results are presented in table 3.

Table 2. Biochemical properties of bacteria isolates

Strain	Atitute towards O_2	catalase	oxidase	ureasa	Gelatin liquefaction	H_2S generation	Indole generation	Acid generation	Gas generation
GH1	Obligate aerobe	+	-	-	-	-	-	+	-
GH2	Obligate aerobe	+	-	-	-	-	-	+	-
GV1	Obligate aerobe	+	-	-	-	-	-	+	-

The dry weight of biocellulose in selected cultures during cultivation on HS medium was: GH1 - 5.05 g / l; GH2 - 5.86 g / l and GV1 - 1.16 g / l of dry weight biofilm.

According to Bergey≥s determinant, these cultures were assigned to the genus *Komogataeibacter*, which was confirmed by the genetic identification of microorganisms by the conserved locus 16S rRNA.

According to the BLAST program in National Center for Biotechnology Information (NCBI), the isolates GH1 showed 99,53%, GH2 ć 99,06% and GV1 ć 99,60% similarity with *Komogataeibacter sp.*

CONCLUSION

Bacterial cellulose is a water-insoluble extracellular polysaccharide with a simple structure and has many superior physic-chemical properties compared to plant cellulose, such as high purity, ultra-fine mesh structure, high crystallinity, high mechanical strength, high hydrophilicity, biocompatibility and ability to biodegradation. Due to these properties, biocellulose can be widely used in medicine, acoustic membranes, biomedical engineering, paper production, textile and food industries.

In this regard, studies aimed at the isolation and study of bacterial cellulose are of particular relevance.

As a result of our studies, we made the following conclusions:

We selected 3 isolates of acetic acid bacteria capable of producing cellulose on simple nutrient media with carbohydrates.

Identification of bacteria by determining the direct nucleotide sequence of the 16S rRNA fragment showed that they belong to the genus *Komagataei* -

bacter sp.

It was determined that the studied strains are represented by gram-negative rods. Colonies are rounded, dry in texture of beige and white. The size of the colonies in strains GH1 and GH2 ć 0.5-2 mm, in GV1 ć 2-5 mm. The strains have similar biochemical properties: catalase is positive, urease and oxidase are negative, have the property of acid formation, indole and hydrogen sulfide do not form, do not thin gelatin and do not have the property of gas formation.

Dry biomass of biocellulose in selected strains during cultivation on HS medium was: GH1 - 5.05 g / L; GH2 is 5.86 g / L and GV1 is 1.16 g / L of biofilm powder.

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МЕДИЦИНА ЖӘНЕ БИОТЕХНОЛОГИЯДА ПЕРСПЕКТИВАЛЫ БАКТЕРИАЛДЫ ЦЕЛЛЮЛОЗА ПРОДУЦЕНТТЕРІН БӨЛІП АЛУ ЖӘНЕ СӘЙКЕСТЕНДІРУ

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ТҮЙІН

Микробтық полисахаридтер саласындағы соңғы жетістіктер олардың биоматериалдардың жаңа кластарын дамытудағы маңыздылығын көрсетеді. Микробтан шыққан кейбір полисахаридтер қазірдің өзінде медицинаның әртүрлі салаларында кеңінен қолданылуда: гиалурон қышқылы, декстран, альгинат, склероглюкан. Қазіргі уақытта перспективалы және тиімді биополимер ретінде бактериалды целлюлозаға ерекше назар бөлінеді.

Сірке қышқылы бактерияларының экологиялық тауашалары туралы білімдерге сүйене отырып, біз целлюлоза өндірушілер бактериялардың оқшаулау көзі ретінде түрлі сортты жүзімдер мен Kombucha пайдаландық. Бұл мақалада биоцеллюлозаның тиімді өндірушілерінің биохимиялық қасиеттерін оқшаулау және зерттеу нәтижелері келтірілген. Жұмыс барысында сірке қышқылы бактерияларының 17 изоляттары оқшауланды, олардың ішінен целлюлоза биоқабыршағын шығара алатын 3 штамм таңдалды және 16S рРНҚ генінің фрагментінің бырынғай нуклеотидтер тізбегін мен биохимиялық қасиеттерін анықтау арқылы анықталды.

Негізгі сөздер: биоцеллюлоза, бактериалды целлюлоза, биополимер, наноцеллюлоза, *Komagataeibacter*, гель-қабыршақ

ВЫДЕЛЕНИЕ И ИДЕНТИФИКАЦИЯ ПРОДУЦЕНТОВ БАКТЕРИАЛЬНОЙ ЦЕЛЛЮЛОЗЫ, ПЕРСПЕКТИВНЫХ В МЕДИЦИНЕ И БИОТЕХНОЛОГИИ

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АБСТАКТ

Достижения последних лет в области микробных полисахаридов указывают на их значимость в разработке новых классов биоматериалов. Некоторые полисахариды микробного происхождения уже широко применяют в различных отраслях медицины: гиалуроновая кислота, декстран, альгинат, склероглюкан. Особое внимание в настоящее время обращено на бактериальную целлюлозу как на перспективный и эффективный биополимер.

Исходя из знания экологических ниш уксуснокислых бактерий, мы использовали плоды винограда разных сортов и чайный гриб, как источники для выделения бактерий продуцентов целлюлозы. В данной статье представлены результаты по выделению и изучению биохимических свойств эффективных продуцентов биоцеллюлозы. В ходе работы было выделено 17 изолятов уксуснокислых бактерий. Из них отобрано 3 штамма, способных продуцировать биопленку целлюлозы. Штаммы идентифицированы на основе определения прямой нуклеотидной последовательности фрагмента 16S rRNA гена и биохимических свойств.

Ключевые слова: биоцеллюлоза, бактериальная целлюлоза, биополимер, наноцеллюлоза, *Komagataeibacter*, гель-пленка