INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

Production of bacterial cellulose from kombucha SCOBY: optimization of the bioprocess and industrial application

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Dissertation submitted to Escola Superior Agrária de Bragança to obtain the Degree of Master in Biotechnological Engineering under the scope of the double diploma with Saint-Petersburg State Institute of Technology

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Bragança
2021

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Acknowledgements

First and foremost, I would like to express my deepest gratitude to my supervisors Professor Paula Rodrigues and Professor António Peres for reading my numerous revisions and helping to make some sense out of the confusion. Without their help and support, this thesis would not be possible. Paula Rodrigues, thanks for your continuous support, trust, patience and persistent help. António Peres, thanks for the guidance, advice and help throughout this year.

Thanks to my home university, especially to vice-head of International Dpt. Elena Aleksandrova, head of tecnology of microbiological synthesis department Mark Shancyan and my supervisor Associate Professor Tatiana Lisitskaia for the opportunity to pursue master studies and gain valuable skills and experience in Portugal.

My sincere gratitude to Nuno Azoia, from the Centre for Nanotechnology and Smart Materials (CeNTI), for the new knowledge and experince in my one-month internship. I am really grateful for my time working with you. Thanks to CeNTI for the chance to undertake this internship.

I also wish to express my thanks to engineer Jorge Sá Morais for all the help in HPLC analysis and Stephany Rezende for the help with FTIR analysis.

To my friends, Dima, Marek, Pavol and Timea for the amazing time together.

To my parents and brother, for all the love, support and encouragement for all my pursuits.

Abstract

Bacterial cellulose (BC) is a natural polymer that has many relevant characteristics such as high water affinity, viscosity and porosity. These properties allow its use in several fields of human life and also its possible use for novel applications. However, the production of BC is limited by usage of expensive culture media, the need to provide purity of the culture and low yields. The use of a symbiotic culture of bacteria and yeasts (SCOBY) has been pointed out as a way to overcome these constrains.

Thus, in this study the most suitable bioprocess conditions that allowed achieving a high yield of BC were evaluated (e.g., temperature and carbon source concentration). Two production approaches were considered namely the cultivating of SCOBY in tea broth or the use of two isolated strains from genus *Komagataeibacter* (i.e., *Komagataeibacter* sp. SB6A and *Komagataeibacter intermedius* SB14), grown in Hestrin-Schramm (HS) medium. The fermentation broths were characterized by high performance liquid chromatography (HPLC) to better understand the fermentation process. The BC obtained in all bioprocesses was characterized by Fourier-Transformed Infra-Red (FTIR) spectroscopy. Finally, BC from SCOBY was functionalized and its surface resistivity was measured to determine its application potential for electronics.

Overall, it was found that SCOBY promoted higher yields of BC compared to the two isolated bacteria strains. Moreover, the strain SB6A was not able to produce BC in satisfactory amounts. However, no significant differences were found in the structure of BC produced by SCOBY and by the strain SB14. It was also found the functionalized BC, produced by SCOBY, could be used in electronics, although the surface resistivity needed to be improved.

Keywords: microbial symbiosis; fermentation; functionalization; biopolimers; fermented tea broth

Resumo

A celulose bacteriana (CB) é um polímero natural que possui características interessantes, tais como afinidade para a água, viscosidade e porosidade. Estas propriedades permitem que seja usada em diversas áreas de interesse, e revelam potencial para novas aplicações. No entanto, a produção de CB encontra algumas limitações devido ao uso de substratos de fermentação dispendiosos, elevada pureza e baixa produtividade. O uso de culturas simbióticas de bactérias e leveduras (SCOBY, *Symbiotic Culture Of Bacteria and Yeasts*) tem sido apontado como uma forma de ultrapassar estas limitações.

O presente trabalho teve como objetivos: i) obtenção de um processo fermentativo de elevado rendimento na produção de CB a partir de SCOBY em chá preto e de bactérias do género *Komagataeibacter* isoladas de SCOBY – *Komagataeibacter* sp. SB6A e *Komagataeibacter intermedius* SB14 – em caldo Hestrin-Schramm (HS); ii) funcionalizar a CB obtida dependendo das suas características. Os caldos de fermentação foram caracterizados por cromatografia líquida de alta precisão (HPLC) para melhor compreensão do processo fermentativo. A CB obtida foi caracterizada por espectroscopia de infravermelhos (FTIR). Por fim, a CB de SCOBY foi funcionalizada e a sua resistividade superficial foi quantificada para determinação do potencial de aplicação eletrónica.

Obteve-se maior rendimento em CB através da fermentação com SCOBY do que com as duas bactérias isoladas, sendo que a estirpe SB6A produziu quantidades muito pequenas de CB. No entanto, verificou-se semelhança estrutural entre as CB produzidas por SCOBY e pela estirpe SB14. Verificou-se também que a CB de SCOBY funcionalizada tem potencial de aplicação eletrónica, apesar de ser necessário otimizar a resistividade superficial.

Palavras-chave: simbiose microbiana; fermentação, funcionalização; biopolímeros; chá fermentado

Chapter 1: Introduction

1.1. Framework

Cellulose is considered as one of the most abundant biomaterials in the world, being possible to find it in plants, as an essential component of cellular wall. Although cellulose is present in almost all plants, cotton and wood pulp remain the most important sources, due to their richness in cellulose. During the last two decades the production of this polymer gradually increased, exceeding, in 2016, one hundred billion tons per year [1]. Such high-scale production is justified by the broad variety of applications. Cellulose is widely used in different industrial fields such as paper [2] and textile [3] production. Moreover, some of cellulose derivatives are applied in pharmaceutical applications as stabilizers and thickening agents [4], in physical chemistry to separate enantiomers [5] and in biotechnology for immobilization of proteins [6].

Another way to produce cellulose is by using bacteria and fungi [7]. Bacterial cellulose (BC) has the same structure as plant cellulose but its physical properties differ. Moreover, cellulose synthesized by bacteria is regarded as more valuable because its characteristics are more suitable for most applications. It has higher water holding capacity [8], hydrophilicity [9] and porosity [10]. These properties allow cellulose to be used as a drug delivery system, wound dress, tissue engineering agent and stabilizer. Also, cotton and wood contain lignin and other substances which make the purification process much harder for plant cellulose comparing to that of cellulose produced by microorganisms. Another advantage of BC is that, depending on the type of fermentation (static or agitated), the culture media and additives used (e.g., ethanol, water soluble polymers and polysaccharides), the produced BC can have different properties. It is also possible to functionalize BC according to the intended application. For instance, addition of water soluble polymers to the culture media could result in increasing or decreasing water holding capacity, depending on the envisaged later use of BC [11]. Moreover, the production of BC is more environmentally friendly compared to the chemical method used for obtaining plant cellulose, which could lead to some ecological problems due to the utilization of carbon disulfide (CS_2) and heavy metals [2].

In spite of all the advantages and commercial demand, BC is rarely produced due to the high cost, complexity of standard media and required additives [12]. In addition, to achieve high productivity it is required to optimize several fermentation conditions: temperature, time, media volume and superficial area. High productivity is important

because, without high yields, the usage of expensive media and additional equipment becomes impractical from an economic point of view. Thus, different methods have been sought in order to reduce the production cost. One way to cut the expenses and achieve high yields of BC is to use *kombucha*. *Kombucha* culture is a mixture of symbiotic microorganisms (called SCOBY, Symbiotic Culture of Bacteria & Yeasts), namely bacteria, mainly *Komagataeibacter xylinus*, and several yeast genera including *Zygosaccharomyces*, *Schizosaccharomyces* and *Saccharomyces* [13]. Several benefits related to the use of *kombucha* for BC production have been reported, such as yields of up to 9.5 mg/mL [7], fewer contaminations and low cost of cellulose production [13]. These features make the use of this culture a very promising approach to produce cellulose.

1.2. Objectives

The aim of this work was to develop and optimize a high yield, low-cost bioprocess to produce BC using *kombucha* culture, and to find potential industrial applications for the obtained BC.

In order to achieve this goal, the following steps were carried out:

- SCOBY and two acetic acid bacteria of the genus *Komagataeibacter* were grown under variable fermentation conditions to compare the yields of the obtained BC and their properties;
- The fermentation conditions were tentatively optimized aiming at increasing the production yield;
- Based on the yields obtained and the cellulose characteristics, the most promising growing conditions of BC were established;
- The obtained BC was characterized, namely by Fourier-transform infra-red (FTIR) spectroscopy;
- The obtained BC was functionalized envisaging a possible application in electronics

Chapter 2: Literature review

2.1. Cellulose – general aspects

Cellulose, which chemical formula is $(C_6H_{10}O_5)_n$, is a natural unbranched polymer (polysaccharide) with a molecular repeat unit of β-D-glucopyranose residues linked by β-1,4 glycosidic oxygen bonds (Figure 1).

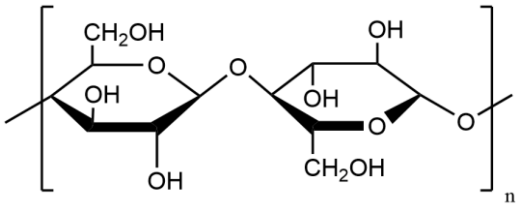

Figure 1 – Structural formula of cellulose [2].

The first mention to cellulose was made in 1839 by the French chemist Anselme Payen, who discovered it in plant matter [14]. Cellulose is in fact the main part and structural material of plant cell walls. For example, in the fibers of cotton seeds, cellulose content ranges from 95 to 99.5 %; in flax and in other fibers it represents around 60-85 %; and in wood tissues approximately 30-55 %, depending on the type of tree, its age and growing conditions [15]. A distinctive feature of cellulose is its morphological structure. Depending on the formation of fibers in the plant, the structure of these fibers, their compositions and characteristics can vary [16]. Therefore, cellulose from different sources has different properties. The cellulose characteristics determines the mechanical strength of the cell wall and plant tissue. A comparison of the properties of cellulose obtained from cotton, wood and flax is presented in Table 1. The distribution and orientation of cellulose fibers in wood cell are shown in Figure 2 [17].

Table 1 – Physio-chemical characteristics of cellulose from different plant sources [16].

	Source of cellulose		
	Cotton	**Wood**	**Flax**
Water affinity, g	140-150	125-135	95.7
Viscosity, MPa. s	15-70	30-70	21
Molecular weight, thousand units	400-500	150-220	645
Polymerization degree	2500-3500	1000-1400	3300

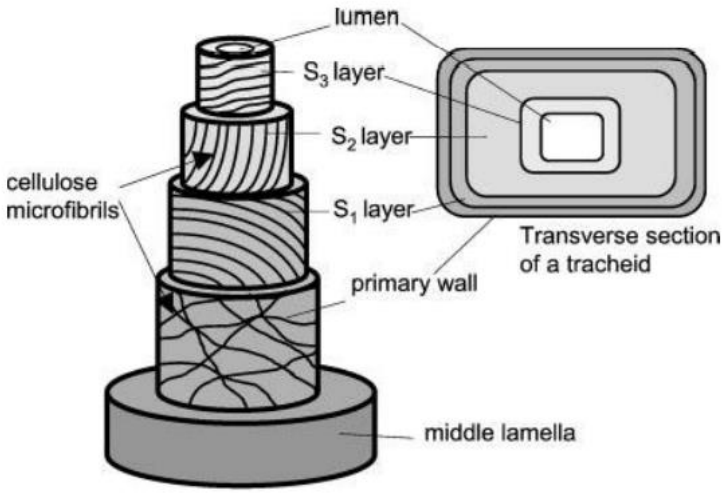

Figure 2 – Scheme of the structure of the cell wall [17].

The wall of a wood cell usually includes a primary and a secondary layer [17]. The latter contains three sublayers – outer (S1), middle (S2) and inner (S3). In the primary layer, cellulose fibers are randomly arranged and form a network structure. The cellulose fibers in the secondary casing are oriented mainly parallel to each other, which results in a high tensile strength of the plant material [17]. The degree of polymerization of cellulose in the secondary layer is higher than in the primary one. For the primary layer, the polymerization degree is 1000-3000 and for secondary layers around 15000-25000.

2.2. Bacterial cellulose

The first mention to BC goes back to 1886 in a paper published by Brown, who discovered a gelatinous film covering the entire surface of a glucose-containing medium [18]. After fifty years, Barsha and Hibbert showed that its chemical structure was similar to plant cellulose [19]. Properties of BC are considered more favorable than those of the plant counterpart. It has the capacity to retain moisture in the ratio of 309 g of water per gram of dry weight [20], withstands temperature up to 150 ℃ when it is untreated, and after chemical treatment it can withstand around 275 ℃ [21], which allows to dry the polymer without damaging its structure. At the same time, BC has good resilience, elasticity and plasticity. Its biocompatibility, high purity and absence of impurities like lignin, pectin and hemicelluloses make BC non-toxic and, therefore, suitable for medical

purposes [7]. A comparison of the properties for bacterial and plant cellulose is shown in Table 2.

Table 2 – Comparison of the average values for bacterial and plant cellulose properties [15].

Properties	Bacterial cellulose	Plant cellulose
Tensile strength, MPa	20-300	25-200
Water holding capacity, %	>95	25-35
Crystallinity, %	74-96	40-85
Purity, %	>99	<80
Degree of polymerization	14000-16000	300-10000
Porosity, %	>85	<75

2.3. Microorganisms used for cellulose synthesis

There are several gram-negative and gram-positive bacteria that are able to synthesize cellulose [22]. Among them, species of the genera *Acetobacter, Aerobacter, Achromabacter, Alcaligenes, Komagataeibacter, Pseudomonas, Rhizobium* and *Sarcina*, among others, are usually reported. Due to the highest yield, *Komagataeibacter* xylinus (syn. *Gluconoacetobacter xylinus*), *Komagataeibacter hansenii* (formerly *Acetobacter xylinum* and *Acetobacter hansenii*, respectively) and *Acetobacter pasteurianus* are the most frequently used. Depending on the bacterium that is used, the characteristics and structure of cellulose will vary [15]. Different bacteria and types of cellulose synthesized by them are shown in Table 3.

Table 3 – Cellulose-producing bacteria and structure of the obtained cellulose [15].

Bacterial genus	Structure of cellulose
Acetobacter/ Komagataeibacter	Fibril structure/ Ribbon structure
Achromabacter	Ribbon structure
Agrobacterium	Fibril structure
Aerobacter	Fibril structure
Alcaligenes	Fibril structure
Pseudomonas	Amorphous
Rhizobium	Fibril structure
Sarcina	Not defined

Another way to produce cellulose is to use microbial consortia, where several different bacteria, or even other microorganisms will interact to create a different product. One such case is the use of a SCOBY. The composition of SCOBY can vary but it always contains acetic acid bacteria like *Acetobacter* (currently syn. *Gluconoacetobacter*/*Komagataeibacter)* and *Gluconobacter*, and a wide diversity of yeasts such as *Zygosaccharomyces, Schizosaccharomyces, Saccharomyces, Dekkera/Brettanomyces, Pichia* and others [23]. Sometimes SCOBY also contains lactic acid bacteria but its presence is not essential for the growth of the other microorganisms. The main advantage of SCOBY is that the symbiotic relationship between bacteria and yeasts can promote not only the growth of bacteria but also the cellulose production. For instance, yeasts oxidize glucose to ethanol which is used by acetic acid bacteria to produce acetic acid. This production of ethanol and acetic acid enhances the production of BC [24]. These substances also inhibit the growth of other bacteria that could contaminate the culture. Another advantage of SCOBY is that it is able to grow at pH under 3 which makes the contamination less probable and, therefore, makes the production easier and cheaper. Also, SCOBY is capable to grow in the presence of polyphenolic substances [12]. Hence, this allows the use of less expensive methods to ensure the purity of the culture.

2.4. Culture methods and substrates for BC production

Depending on the application of BC, there are various methods of cultivation of cellulose-synthesizing bacteria, on which the properties of BC depend. There are two main methods of cultivation that are used: static and agitated.

The static method is the simplest and the most used one [15]. This method does not require any expensive or complex equipment. The incubation of the culture occurs in flasks for one or two weeks with pH from 2.5 to 4.5 and temperatures varying from 25 to 30 °C. The BC is obtained in the form of a film that is formed at the surface of the culture medium, as shown in Figure 3a. The physical characteristics of the film depend on many factors including disturbance of culture media, superficial area of the liquid medium (i.e., of the flask), formation of bubbles and time of incubation. After harvest, the BC assumes a yellowish color (Figure 3b) which becomes white after purification. The most frequent way to purify BC (Figure 3c) is to boil the harvested film in a 0.5 N NaOH solution and

then to wash it with distilled water until neutral pH and white color of film is obtained [13].

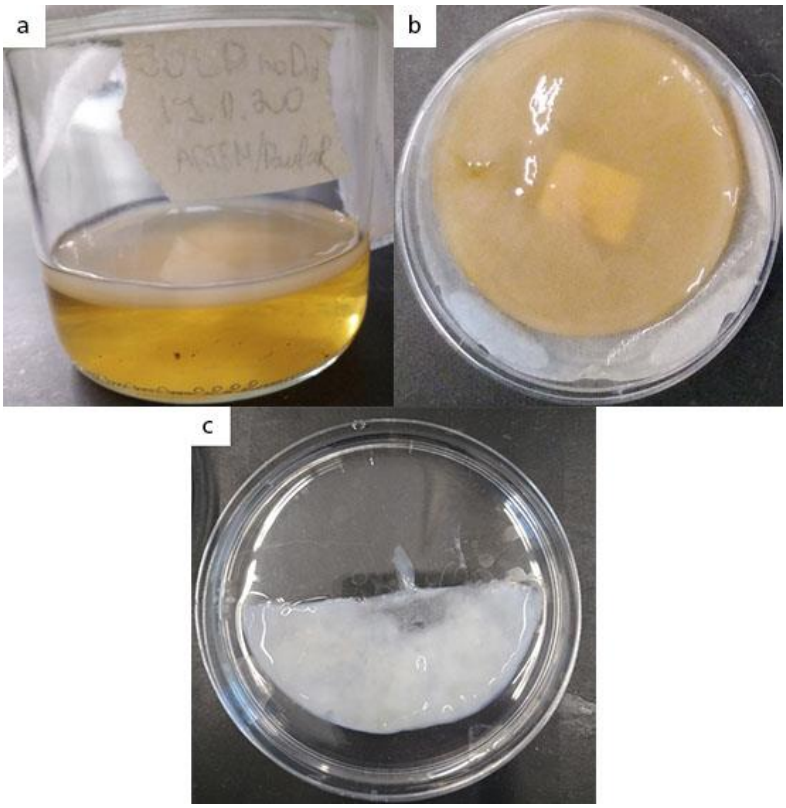

Figure 3 – (a) Bacterial cellulose production by static method, (b) bacterial cellulose film before purification, (c) purified bacterial cellulose.

The static method is easy-to-use and very undemanding and thus, it is widely used at laboratory scale [16]. However, it has several drawbacks. Firstly, cellulose-producing bacteria are aerobic microorganisms. Hence, the production of cellulose is highly dependent on the oxygen uptake to the cells, which is promoted only at the gas-liquid interface. Two other limitations of this method are related to the high cost of usual culture media used to grow bacteria as well as the moderate yields of cellulose that are achieved.

The second method of cultivation of BC is the agitated method. It has been suggested to use stirring to increase the delivery of oxygen [16]. However, it turned out that agitation did not increase the yield of BC. Moreover, it has been reported that the production of cellulose is lower in this case in comparison to the static method [25]. One of the main reasons for the decreased yield is that agitation promotes the mutation of cellulose-producing bacteria to a non-cellulose variant. When ethanol is added to the

culture media, the occurrence rate of such mutants is lowered. However, by doing that the cost of cellulose production increases [26]. Even though this method does not allow achieving high yields and the cellulose obtained in agitated conditions has different forms/structures comparing to the cellulose synthesized when the static method is applied. The form of cellulose is influenced by parameters such as time of cultivation, rotation speed, and additives used. Due to stirring and shaking, BC is synthesized in the form of spheres with different diameters (Figure 4), or in the form of fiber [27]. In fact, the agitation deeply affects some characteristics of BC, as it will have lower degree of polymerization and crystallinity index compared to the cellulose obtained in static culture. Moreover, such BC has a lower modulus of elasticity and higher water holding capacity [28]. Despite these disadvantages, this method is suggested to be the most suitable for industrial production, from an economic point of view [29].

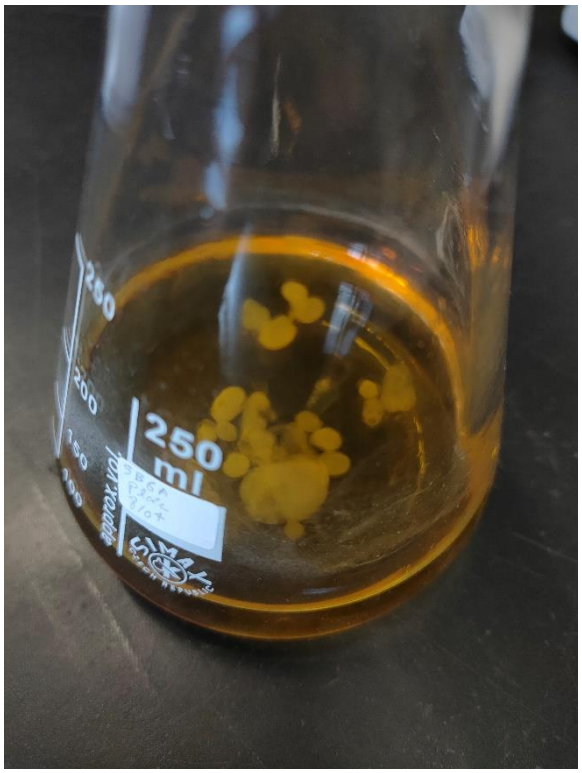

Figure 4 – Bacterial cellulose production by agitated method.

2.5. Pathway of BC synthesis

Biosynthesis of BC is a multi-step bioprocess that includes two main mechanisms: synthesis of uridine diphosphoglucose followed by polymerization of glucose. BC is considered as a secondary metabolite. Its synthesis starts from substances used as

nutrients by bacteria and can be catalyzed by different enzymes [7]. Supposed biosynthetic pathway is shown in Figure 5. When using the static method to cultivate cellulose-producing bacteria, BC fibers intertwine and form a film. The film stays in liquid-gas surface which allows bacteria to get the oxygen needed for growing and cellulose synthesis [7].

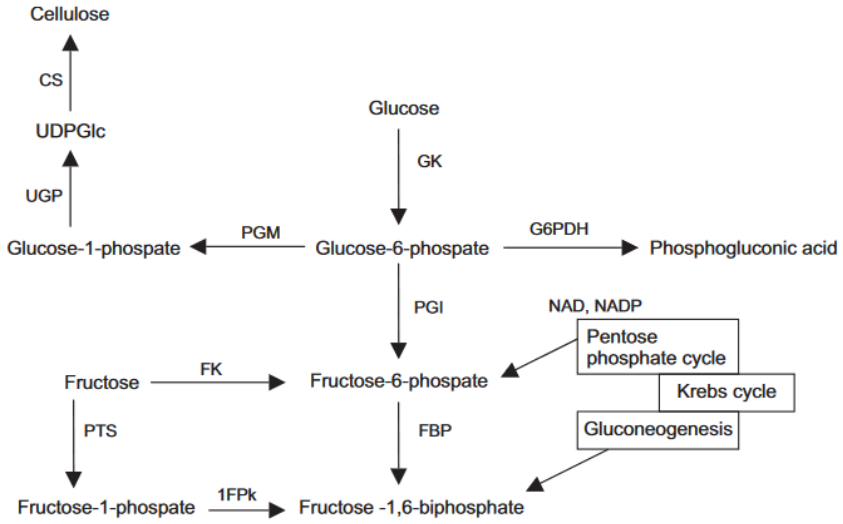

Figure 5 – Pathway of BC synthesis [7]. CS cellulose synthase, GK glucokinase, FBP fructose 1,6-bi-phosphate phosphatase, FK fructokinase, 1FPk fructose-1-phosphate kinase, PGI phosphoglucoisomerase, PMG phosphoglucomutase, PTS system of phosphotransferases, UGP pyrophosphorylase uridine diphosphoglucose, UDPGlc uridine diphosphoglucose, G6PDH glucose-6-phosphate dehydrogenase, NAD nicotinamide adenine dinucleotide, NADP nicotinamide adenine dinucleotide phosphate.

However, it's still unknown why bacteria produce cellulose. One of the theories is that BC is used by bacteria to stay on the surface near the highest oxygen concentration. Also, it is possible that BC is synthesized for the protection against environmental conditions such as ultraviolet emission and heavy-metal ions along with promote nutrient diffusion. Moreover, BC helps to prevent the drying of substrate while bacterial growth [7].

2.6. Factors influencing BC production

It is well known that BC production is dependent on several factors such as growth medium composition, type and amounts of additives, temperature and pH. Depending on the initial pH, cell growth and cellulose production is affected. Therefore, the relation between pH and production yield of cellulose should be investigated. The most

commonly used pH varies from 4 to 6-7 [7]. In this range the highest BC production achieved when pH equals 5. When pH is lower than 4 or higher 7 the production of BC drops significantly and almost ceases.

When pH drops below 4 the cellulose production yield decreases. The decrease of pH is related to the formation of different acids like acetic acid or gluconic acid [7], being crucial to be able to control it. One possible solution was suggested by Noro et al. [31] that proposed the use of corn-steep liquor as a buffer solution. Another way is to use SCOBY. Microorganisms of SCOBY are able to grow in low pH in range of 2-4 [32]. Moreover, the maximum concentration of BC hit at pH 3.

Temperature is also an important factor, affecting the cellulose production and the cell growth. The bacteria are usually cultivated at 25-30 ℃. A study conducted in India [33] evaluated the influence of temperature (20 to 45 ℃) on cellulose yield. It was found that the optimum temperature was within the range of 28-30 ℃, which corresponded to the highest production of cellulose (1.99-2.31 g/L). The lowest yield was observed at 37 ℃. When temperature exceeded 40 ℃ the cellulose production ceased.

Another important factor to note is the usage of different carbon sources. Glucose and sucrose are the most common sources of carbon used in BC production [7, 12, 13]. When they compared by BC production, for first two days BC production using glucose is higher, however, from the third day the production using glucose and sucrose makes equal. After that production of BC using sucrose exceeds production using glucose [34, 35].

The concentration of carbon source can affect the cellulose production. Thus, excess of sugar could lead to decreased BC yield [13]. This can be related to formation of byproducts such as glyconic acid, that are able to decrease pH of media and, therefore, decreasing yield of BC. However, other carbon sources have also been evaluated aiming to obtain higher yield. Some of them are shown in Table 4.

Table 4 – Different carbon sources for BC production [34].

Carbon source	BC yield after 48 h (g/L)	BC yield after 96 h (g/L)
Mannitol	2.04	3.37
Glycerol	0.82	3.75
Fructose	1.79	2.81
Galactose	0.1	0.09

It has also been reported that the cellulose production could be enhanced using different carbon and nitrogen sources as well as different additives (e.g., ethanol, organic acids, amino acids and polysaccharides). For example, it was reported that the addition of 1.4% of ethanol can quadruplicate the cellulose production [36]. At the same time introducing ethanol into the medium can be beneficial by reducing the probability of appearing non-producing mutants when the agitated production method is used.

Despite all advantages, the addition of high amounts of ethanol could influence cell growth and decrease it as well as increase the cost of production. In another work, glycerol was used as the carbon source providing the highest yield of cellulose [37] in comparison with 24 other sources, including glucose, fructose and mannitol all of which were used at 2 w/v %. The closest yield by volume when glycerol was used as carbon source (13 mg/mL of cellulose) was obtained with glucose (6.54 mg/mL) and tryptose (6.17 mg/mL). Nitrogen is also important to cell metabolism and so, it is usually added as substrate. According to several studies [37, 38, 39], one of the best compounds to use as a nitrogen source, is the yeast extract (0.5 w/v %), for which BC yields by volume ranged from 1.98 to 4.49 mg/mL have been reported. Other commonly used nitrogen sources are peptone and corn-steep liquor at 20 g/L, allowing to achieve yields by volume of 3.00 and 2.78 mg/mL of cellulose, respectively [39]. Compounds that contain nitrogen like urea, ammonium nitrate, calcium nitrate and some other inorganic substances do not affect the production of cellulose [37, 39]. Some sources of nitrogen are shown in Table 5.

Table 5 – Different nitrogen sources for BC production [40].

Carbon source	Concentration of carbon source (g/L)	BC yield (g/L)
Corn-steep liquor	10	1.07
Peptone	10	0.85
Yeast extract	10	0.85
Beef extract	10	0.75
Malt extract	10	0.26

2.7. Fermentation substrates

Two types of growth media are usually used in bioprocesses: natural, with undefined chemical composition, and chemical media, with a fully defined composition. The main advantage of chemical media is that the composition and content of the media are known. Therefore, the results can be easily reproduced [41]. However, this type of media is quite expensive due to the costly components and additives that usually contain and that are added to increase cellulose yields. On the other hand, the chemical composition of natural media is hardly known and is rarely constant. Hence, it is almost impossible to know which specific component benefits the bacteria growth. The culture media influence the growth of bacteria and, therefore it affects the production of cellulose, its yield and cost of the process.

One of the culture media commonly used for the production of BC is Hestrin-Schramm (HS) medium. It is a synthetic chemically defined medium containing 20 g/L of glucose, 5 g/L peptone, 5 g/L yeast extract, 1.15 g/L citric acid and 2.7 g/L Na_2HPO_4 [42]. The use of this medium allows to achieve high BC production yields (8.79 mg/mL) [43]. Additionally, since the medium is defined and all conditions can be easily followed and controlled, the production yield of cellulose is reproducible. This is an essential factor for a large-scale automatized production for which a small change can result in huge extra costs. Names and compositions of some media are shown in Table 6.

Table 6 – Composition of some standard media for BC production [44].

Composition (% w/v)		**Standard media**			
		HS	**Yamanaka**	**Zhou**	**Son**
Carbon source	Glucose	2	5	4	1.5
Nitrogen source	Yeast extract	0.5			
	Peptone	0.5	0.5		
	Corn-steep liquor			2	2
Phosphates	KH_2PO_4		0.3	0.2	0.3
	Na_2HPO_4	0.27			
	$Na_2HPO_4 \times 12H_2O$		0.05	0.04	0.3
Sulphates	$MgSO_4 \times 7H_2O$				
	$FeSO_4 \times 7H_2O$				0.0005
	$(NH_4)_2SO_4$		0.5	0.4	0.2
Organic acids	Citric acid	0.115			
Other substances	H_3BO_3				0.003
	Nicotinamide				0.00005

Another substrate which is often used in conjunction with SCOBY culture is *kombucha* – a fermented tea broth. It is a natural undefined medium that contains different organic acids like acetic acid, gluconic acid and lactic acid. In addition, components as ethanol, amino acids, phenolic compounds, proteins, amines, purines, some minerals (e.g., Cu, Zn, Fe) and some vitamins (e.g., B_1, B_2, B_6, B_{12}) are present in the tea broth as a result of a complex fermentation process [45].

Kombucha has several advantages compared to the HS medium. Firstly, fermented tea medium is enriched with different compounds that are sources of nitrogen, carbon and other vital elements for the bacterial growth [45]. Therefore, using *kombucha* as a growth medium decreases the amount of additives that must be added, namely of peptone, yeast extract, ethanol and other growth and cellulose promoting substances or even to not use them at all. This allows not only to increase the production yield of BC without using additional substances but also to downsize the costs.

Another advantage of fermented tea is that it contains polyphenolic compounds [45], which does not affect the SCOBY growth but inhibits growth of other microorganisms. Therefore, the use of *kombucha* along with SCOBY makes it easier to preserve the purity of the culture. Moreover, the growth of SCOBY occurs at pH of 2–4, which additionally protects the culture from contaminating microorganisms. All this makes *kombucha* a low-cost and effective medium to produce cellulose. Moreover, it also can be considered as low-contaminated medium which makes it useful in big scale production.

2.8. Applications of BC

Due to the several natural properties of BC it can be used in many different areas such as biomedicine, biotechnology, food production, textile and machine industry [46]. Some possible applications of BC are shown in Figure 6 – Different BC properties and applications.

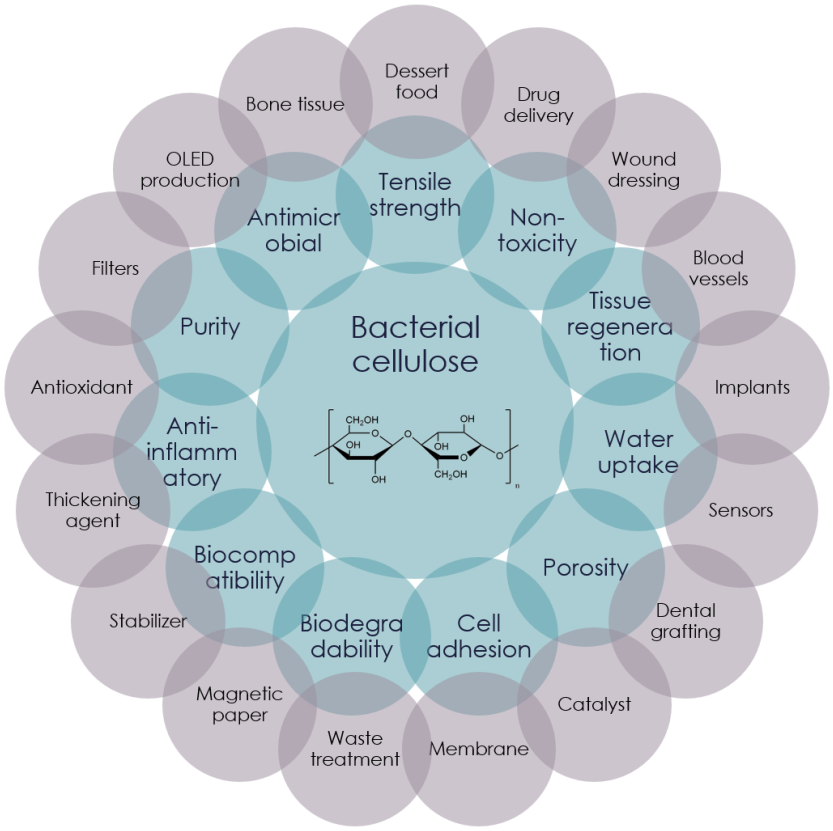

Figure 6 – Different BC properties and applications.

BC has a good water holding capacity, and it is also biodegradable and biocompatible. Therefore, one of the main applications of BC is regenerative medicine and wound healing. BC is a porous material and so, it can at the same time protect wound from infections and allow the delivery of antibiotics or other biologically active substances to the wound [47]. It is already applied as wound dressing agent created by a Brazilian company [48].

Another important area where BC can be applied is in cardio-vascular disease related problems [49], where it has been suggested that BC can replace damaged or clogged blood vessels. BC derivatives are commonly used in pharmaceutical fields. Thus, derivatives of cellulose, especially ethers and esters, can be used as a scaffold for targeted drug delivery [50]. Moreover, BC with imparted inorganic particles or organic molecules has high antibacterial effects and can be used for pharmaceutical applications [51]. Chemically pure BC can be used in food industry as a stabilizer or additive. It has been used as a dainty in some countries for a long time [52]. First, it was a traditional dessert in Philippines called "nata de coco" which is coconut milk treated with bacteria that produce BC. Also, cellulose-producing bacteria was brewed with sugar in tea broth in Asia. This beverage is known as *kombucha* and it is associated with many beneficial properties to human health like antioxidant, antimicrobial and anti-inflammatory activity [53].

Since BC is edible and non-toxic to humans it can also be utilized in food manufacturing as food wrap for perishable products [54]. It is known that cellulose is used in paper production. By using BC, it is possible to achieve high durability and flexibility of paper, which is crucial for paper banknotes [7]. Also, it was shown that these properties allow paper produced from BC to be used as filters for chemistry applications and as membranes for water purification [55]. Another field where BC can be utilized is electronics. Thus, BC membranes having indium oxide on their surface to enhance their conducting properties are promising material for organic light-emitting diodes production [56].

2.9. Functionalization of BC

Depending on the desired application, it is necessary to enhance some properties of BC, which can be done by functionalizing the BC. Functionalization can be physical, chemical or by creating composites of BC.

Two main physical modifications involve ultrasonic and gamma irradiation treatment. By using ultrasound (200 W, 20 kHz) for 15-75 minutes, the structure of BC changes, allowing to increase the temperature resistance from 208 ℃ to 250-268 ℃ [57]. It also can affect water-holding capacity and viscosity. The duration of the treatment plays a key role in the modification of properties. It was reported that continued exposure of cellulose to ultrasound (82 W) increases its crystallinity. However, if cellulose is exposed to ultrasound only for 1 minute or a short period the crystallinity is reduced approximately by half [58]. Exposing cellulose to gamma irradiation can result in an increased porosity that can play an important role when a drug delivery application is envisaged [59].

For the chemical modification of cellulose, the following processes are often used: use of NaOH, oxidation, hydrophobization and graft polymerization. The use of NaOH in small amounts allows (1.5 – 6 wt.%) the purification of BC and the destruction of the remaining bacteria [60]. However, if it is used in higher concentrations (above 6 wt.%) it can promote the conversion of the less thermal stable form of cellulose to the more stable one. Different concentrations of NaOH have been reported in the literature being the most common value around 4 - 6 wt.% [60].

Esterification and etherification of cellulose have also been pointed out as important ways to obtain derivatives of cellulose. These compounds can be used in many areas like materials for cell seeding, tissue regeneration and wound dressing [61], as well as stabilizers for cosmetics [62]. The most commercially important esters and ethers of cellulose are methyl and carboxymethyl cellulose derivatives [63].

Oxidation of BC is commonly carried out using $KMnO_4$, $NaClO_4$, H_2O_2, or HIO_4. This process leads to the formation of derivatives that have in its structure carbonyl or carboxyl groups. These derivatives have better solubility and biodegradability, making them suitable for applying as composites with metal nanoparticles [64], materials for bone tissues [65] and as artificial muscles [66]. Cellulose that contains carbonyl groups in its structure, especially aldehydes, can react with many different substances. This allows further functionalization of cellulose leading to derivatives with new properties. 2,3-

dihydrazone cellulose obtained by reaction of dialdehyde cellulose with hydrazine can be applied in tissue engineering [61]. Derivatives of cellulose having carboxyl groups are known for their use in surgery. Oxycellulose has a good capability to staunch bleeding and, therefore, is used as a hemostatic agent [67]. In addition, these substances have antimicrobial and antibacterial properties, which also makes them ideal for using in surgery and medicine [67].

Hydrophobization of BC allows further reactions with nanocomposites and organic polymers [68]. One way to achieve an adequate hydrophobization of cellulose is by reacting it with silanes like Me_3SiCl. These substances only affect the solubility in water without changing important properties such as crystallinity or morphology. It was reported that cellulose modified with silane groups have hydrophobic and oleophilic properties and, thus, could solubilize organic compounds [69]. Another way of hydrophobization is acetylation [70]. As in the first case, OH-groups of cellulose are substituted with ester groups, which are more hydrophobic. This method also does not change other properties of cellulose. A subcase of acetylation is crosslinking where polymers are used. This also can be a way to hydrophobize the surface of BC through the use of crosslinking agents to bond -COOH-groups of BC which easily can react with H_2O molecules [71]. By crosslinking hydrogen atom in carboxylic group is substituted by hydrophobic part of another molecule which have a stronger bond (Figure 7). Therefore, this prevents the reaction of BC with water.

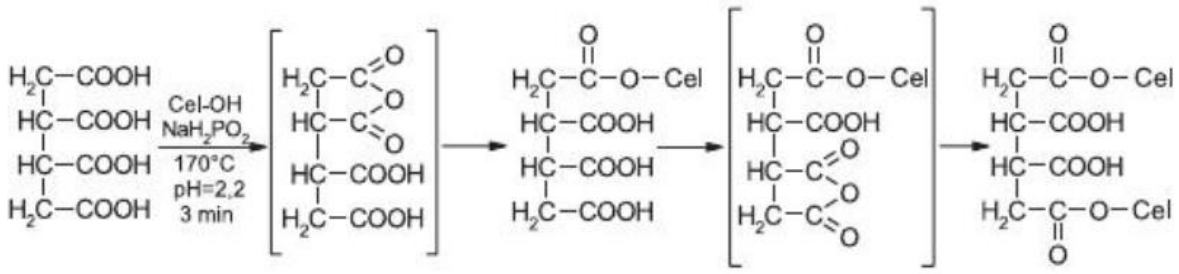

Figure 7 – The mechanism of crosslinking reaction of cellulose with carbon acid [72].

The graft polymerization is another way to modify properties of cellulose. It allows to combine properties of several polymers by adding them as branches to the backbone polymer [73]. The characteristics of the modified cellulose depend on the properties of the copolymers that are branched to it. Thus, by using this method, cellulose

can attain envisaged characteristics such as better elasticity, thermal stability and hydrophobicity [73].

BC can also be modified by creating composites of BC. This modification includes alteration of structure by adding different chemical substances and nanocomposites to the culture during incubation (*in situ*) or after incubation (*ex situ*). In the first case additives are added in culture medium during BC formation; therefore, they are gradually becoming part of the BC's fiber structure.

Moreover, depending on the additives not only the yield but also the morphology, different properties of cellulose and bacterial cells can be affected. Thus, it was reported that the addition of two antibiotics, chloramphenicol and nalidixic acid, caused the change in fiber width and elasticity modulus [74]. Addition of these substances increased the average width from 117 nm for untreated cellulose to 147 nm for cellulose treated with chloramphenicol and to 228 nm with nalidixic acid. Also, elasticity module was increased by 34-42 %. The suggested mechanism is that in the presence of such substances, ribbon of cellulose merge with each other allowing the formation of aggregates. However, some substances can decrease fiber width. In the same study, dithiothreitol was used. The width of the cellulose fibers treated with this substance were reduced almost by a half. Dithiothreitol shortens the cells and, therefore, reduces the number of sites where cellulose is synthesized. Incorporating different composites into growing BC can not only enhance its natural properties but also provide wide range of new characteristics. Polymers, inorganic and organic substances can be used as composites. Introducing water soluble polymers to the culture medium can affect water holding capacity and porosity [11].

However, the *in situ* method has a major drawback [75]. Liquid-born particles gradually start to gravitate. In case of static method BC is synthesized on the air-liquid surface and stays there until it is harvested. In result, the particles are not able to fixate in BC's structure and gravitate. In case of agitated method particles are prevented from sedimentation and stay in the suspension. However, BC obtained this way has spherical shape and, therefore, it cannot be used in some cases in which other form of BC such as film or gel is necessary.

Another way this problem can be solved is to functionalize fully synthesized and harvested BC [76]. The interaction between BC and additive can be either through

sorption or hydrogen bonding. In case of sorption additive's particles delve into and stay in BC's structure, whereas for hydrogen bonding it's necessary for additive to have such groups that can have bonds with OH-groups of BC. The way of obtaining BC composites is shown in Figure 8. This way is considered easier than in situ method and in this case BC's structure does not undergo any changes.

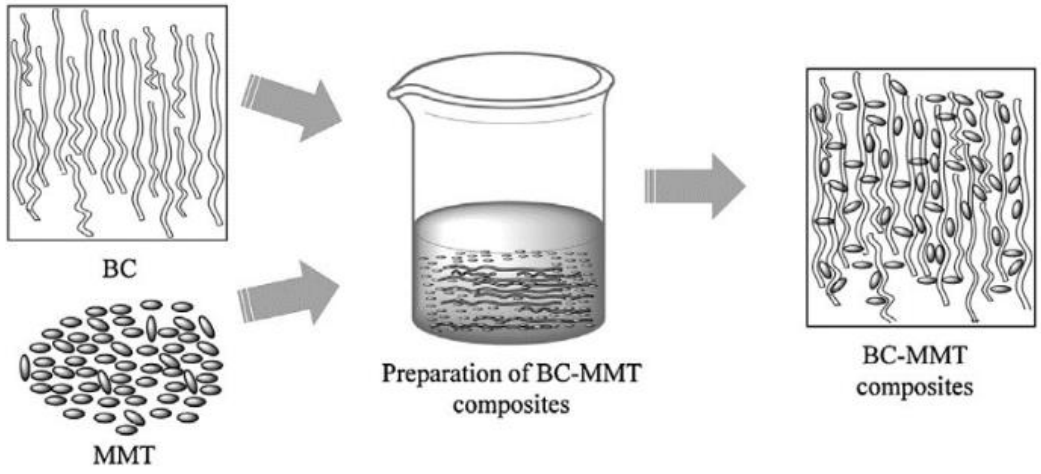

Figure 8 – *Ex situ* method of obtaining BC composites [76].

Thus, it was possible to obtain BC composite with chitosan through hydrogen bonding between OH-groups of BC and NH-groups of chitosan [77]. The same way composites with collagen [78], hydroxylapatite [79] and hyaluronic acid [80] were obtained to create a functionalized BC with enhanced mechanical for biomedical applications. Scientists from China functionalized BC with silver nanoparticles to enhance antimicrobial properties. BC fibers were place into solution of silver which was gradually heated to obtain nanoparticles. These nanoparticles delved into BC structure through sorption which lead to the antimicrobial activity [81]. The same way in BC structure were incorporated metal oxides [82], carbon nanotubes (CNT) [83] and montmorillonite [76].

Although this method is easy for use and can be applied to functionalize BC for different applications, it also has some drawbacks. The main problem of this method is that it's highly dependent on the size and nature of additive. The particles which are too big are not able to penetrate BC pores, therefore, only nanoparticles and smaller can delve

into BC structure. Moreover, hydrophobic materials are not able to interact with highly hydrophilic BC.

Chapter 3. Materials and Methods

3.1. Preparation of the fermentation broth and maintenance of the inoculum

3.1.1. Microbial characterization of the SCOBY

The SCOBY used in the present work was obtained from a homemade *kombucha* using a commercial SCOBY (no brand identified). The pellicle and broth of this *kombucha* were screened for the composition of the SCOBY by culture-dependent methods in a parallel work [84]. In that study, the pellicle and the broth were cultivated in three culture media: De Man, Rogosa, Sharpe agar (MRS, Biolife, Italy) with 0.1 % cycloheximide, for the growth of lactic acid bacteria; Glucose, Yeast Extract and Calcium Carbonate agar (GYC: 50 g/L glucose; 10 g/L yeast extract; 15 g/L de $CaCO_3$; 9 g/L bacteriological agar) with 0.1 % cycloheximide, for acetic acid bacteria; and Sabouraud Dextrose Agar (SDA; Liofilchem, Italy), for yeasts. From this, two acetic acid bacteria – *Komagataeibacter* sp. (belonging to the species complex *Komagataeibacter rhaeticus/xylinus/hansenii*; nine isolates) and *Komagataeibacter intermedius* (two isolates) – and four yeast species - *Brettanomyces bruxellensis* (eight isolates), *Brettanomyces anomalus* (one isolate), *Zygosaccharomyces bailii* (one isolate) and *Saccharomyces cerevisiae* (one isolate) were isolated and molecularly identified. Representative strains of bacteria and yeasts were deposited in the *Centro de Investigação de Montanha* (CIMO-IPB) culture collection (CC-CIMO; codes CIMO 21LE007-CIMO 21LE010 for bacteria; CIMO 21LE001-CIMO 21LE006 for yeasts), and the DNA sequences were deposited in GenBank (Accession numbers OK274314- OK274317 for bacteria; OK274330-OK274335 for yeasts).

3.1.2. Preparation of the fermentation broth for SCOBY

A homemade *Kombucha* was used as inoculum. The SCOBY culture was cultivated and maintained in black tea broth. Black leaf tea (brand Salutem) was purchased from a local supermarket. The method proposed by Sharma and Bhardwaj [13], with some modifications, was used for obtaining the mother culture and its further maintenance. First, distilled water (1 L) was poured into a two-liters glass flask and heated in microwave until boiling. After that, 3.5 g of black tea leaves were added to the boiled

water, stirred and left to infuse for 10 minutes. The leaves were then removed by filtration, and the tea was left to cool for 20 minutes. After cooling, 200 mL of tea were poured into 500 mL glass flasks and 10 % (w/v) sucrose was added to the flasks and stirred until dissolved. Then 10 % (v/v) SCOBY fermentation broth (*Kombucha*) and 10 % (w/v) of BC pellicle were added. The flasks were covered with cheese cloth and then incubated at 30 ℃ for 14 days. This procedure was repeated every two weeks, to keep the inoculum fresh, or whenever necessary for the trials.

3.1.3. Preparation of the fermentation broth for *Komagataeibacter* strains

Two *Komagataeibacter* strains previously isolated (*cf.* section 3.1.1) were used as inoculum: *Komagataeibacter* sp. SB6A (= CIMO21LE007; GenBank Acc. No. OK274314; belonging to the species complex *Komataeibacter rhaeticus/xylinus/hansenii*) and *Komagataeibacter intermedius* SB14 (= CIMO21LE010; GenBank Acc. No. OK274317). Standard HS medium (20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 2.7 g/L Na_2HPO_4 and 1.26 g/L citric acid) was chosen for cultivating the bacteria. The bacteria were incubated in an incubator (Pol-Eko, ST2 Premium Top+) at 30 ℃ for 14 days under static conditions. The produced BC was separated from the medium, dried, purified and used for the following experiments.

3.2. Preliminary experiment

3.2.1. Experimental setting

A preliminary experiment was set to obtain the information on the duration of the fermentation by monitoring the pH change. Also, to check the reports for optimal pH, compare it with the obtained results and adjust by adding buffers if there is a big difference between two values.

To carry out this experiment, fermentation broth for SCOBY was prepared as described in 3.1.2. Six flasks with 100 mL of tea were used as replicas. The tea broth contained 3.5 g/L of black tea leaves and 10 w/v % sucrose, and incubated (Pol-Eko, ST2 Premium Top+) for 8 days at 30 ℃. Every day the pH was measured at the same hour.

3.2.2. Measurement of pH

To measure the pH a volume of 5 mL was taken from fermentation broth by pipette trying not to disturb the cellulose layer. A pH meter (inoLab, pH 7310) was used to measure the pH of the fermentation broth.

3.2.3. Statistical analysis

Statistical analysis was performed using the Software Statistical Program Social Sciences (SPSS) version 27.0 (SPSS Inc., Chicago, IL). Shapiro-Wilk test was used for normality testing. The results are presented as mean ± SD (standard deviation) ($n = 6$). The Kruskal-Wallis non-parametric test was used to analyze the statistical difference of pH between days ($p < 0.05$).

3.3. Optimization of the BC production process

3.3.1. Influence of tea concentration and incubation temperature on bacterial cellulose production using SCOBY

The effects of the amount of tea and incubation temperature have a crucial impact on the yield of cellulose and, therefore, were decided to be studied. Three concentrations of tea (2.5, 3.5 and 4.5 g/L) and three temperatures (25, 27.5 and 30 ℃) were investigated. The flasks with the extreme tea concentrations (2.5 and 4.5 g/L) were tested for 25 and 30 ℃. The flasks with 3.5 g/L tea concentration were incubated only at 27.5 ℃. Flasks were incubated for 12, and 20 days. When incubated for 14 days the same range of tea concentration (2.5, 3.5 and 4.5 g/L) but different temperatures were studied (28, 30 and 32 ℃). Afterwards, a 2^K factorial design (where k is number of factors) was applied (using the Design-Expert®, version 7.1.5 – trial version from Stat-Ease Inc.), for studying two main effects (i.e., tea concentration and temperature). Seven flasks were prepared for the experiment, four of them corresponded to the combinations of minimum and maximum levels of tea concentration and temperature, the central point was done in triplicate. The study aimed at establishing the optimal temperature and tea concentration for maximizing the yield of BC on a dry weight basis.

3.3.2. Experimental design

A 2^2 factorial design was used to study the main factors: temperature and tea concentration at combination of minimum and maximum levels and 3 replicas of central point (Table 7).

Table 7 – 2^2 factorial design range and levels.

Microorganism	Variable	Symbol	Level of the variable for 14 days experiments			Level of the variable for 12 and 20 days experiments		
			-1	0	+1	-1	0	+1
SCOBY	Temperature (°C)	x_1	28	30	32	25	27.5	30
	Tea concentration (g/L)	x_2	2.5	3.5	4.5	2.5	3.5	4.5
Komagataeibacter SB14	Temperature (°C)	x_1	28	30	32	-	-	-
	Cell concentration (cells/mL)	x_2	10^3	10^5	10^7	-	-	-

The statistical treatment for 2 factors were coded using Equation (1).

$$X_i = \frac{x_i - x_0}{\Delta x_i}, i = 1, 2 \quad (1)$$

where *Xi* is the coded value of the independent factor, x_i – the real value of the independent factor, x_0 – the real value of the independent factor at the central point, Δx_i – the step change value.

The behavior of the system is expected to be explained by a first-order equation, used for predicting the optimal total factor of safety yield point (y), based on the coded values of independent factors (x_i), as expressed in Equation (2).

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \varepsilon \quad (2)$$

where y is the predicted response; the β's are parameters which are determined using multiple linear regression model (MRLM), being β_0 the intercept, β_1 and β_2 the linear coefficients and β_{12} the interaction coefficient; x_1 and x_2 are the coded independent factors and ε is a random error term of the regression model, which should be independent and follow a normal distribution, in accordance to the requirements of the MRLM.

The significance of obtained model was assessed by analysis of variance (ANOVA). The quality of the fit obtained using the regression model equation was statistically checked by the magnitude of two diagnostic residuals: the multiple (R^2) or adjusted coefficient of determination (R^2_{adj}) and the predicted coefficient of determination (R^2_{pred}). The R^2_{adj} -values describe the goodness of fit, showing how well current runs can be reproduced by the mathematical model. The R^2_{pred} -values describe the goodness of prediction, showing how well new experiments can be predicted using the mathematical model. For a model to be considered satisfactory the values of R^2_{adj} and R^2_{pred} should be higher than 0.75 and 0.60, respectively. If the values are lower than 0.25, the model cannot be used and it is considered useless [85].

3.3.3. Influence of bacteria concentration and incubation temperature on bacterial cellulose using *Komagataeibacter* strains

The effect of bacteria concentration was studied using two *Komagataeibacter* strains, previously isolated from SCOBY. The bacteria were grown for 4 days in GYC Agar medium. After that, a mass of cells was suspended in 10 mL of HS broth and cells were counted using a Neubauer counting chamber to obtain a concentration of 4×10^8 cells/mL. This suspension was diluted to obtain the test concentrations 10^7, 10^5 and 10^3 cells/mL. The flasks with extreme concentrations of bacteria were tested at 28 and 32 ℃. The flasks with 10^5 cells/mL of bacteria were incubated only at 30 ℃. The assays followed a 2^K experimental factorial design (Design-Expert software), at two levels, and for two main effects (i.e., bacteria concentration and temperature), with three replicas at the central point. Flasks were incubated for 14 days.

3.4 Characterization of the fermentation broth

3.4.1. Composition analysis of the BC fermentation broth by HPLC

After the fermentation, broth samples of, 1 mL, were withdrawn from the fermentation vessel. Then, they were filtered (0.2 μM) to an Eppendorf and frozen until analysis. When needed the filtered samples were diluted in ultra-pure water and subjected to HPLC analysis. A 10 μL sample of the filtrate was manually injected into the HPLC system.

The fermentation broth was analyzed for sugars (glucose, sucrose), organic acids (lactic acid, citric acid, acetic acid and malic acid), ethanol, and glycerol by high-performance liquid chromatography (HPLC), as described by [86]. The system was equipped with a binary pump (Varian Prostar 220), an injector (Rheodyne 7725i; Loop 20 μL), and an infrared detector (Varian RI-4) (Figure 9). Data were analyzed using the Varian Chromatography Workstation software (version 4.5). The chromatographic separation was achieved under isocratic elution with a prepacked HPLC analysis column (Aminex HPX-87H form Bio Rad: 300 × 7.8 mm, hydrogen form, 9 μm particle size, 8 % cross linkage, pH range of 1–3) operating at 30 °C (Jones Chromatography 7981 oven). The mobile phase was a solution of sulfuric acid (0.005 M), pumped at 0.6 mL/min, for a total running time of 30 min. The injection volume was 10 μL. Calibration curves, limits of detection (LOD), and limits of quantification (LOQ) were determined with seven concentration levels of HPLC-grade standards of each of the previously mentioned compounds. Sugars ranged from 0.1 to 8 g/L, organic acids ranged from 0.02 to 1.5 g/L, and the alcohols ranged from 0.4 to 3.5 %. LOD and LOQ were calculated according to [87]. The concentration was calculated as g/L for sugars, organic acids and glycerol, and in percentage for ethanol, considering the dilution factors.

Figure 9 – HPLC system.

The performance parameters of the HPLC method for each of the compounds are presented in Table 8.

Table 8 – Performance parameters of the HPLC method for the analyzed compounds (g/L).

			Equipment	
Compound	**Equation**	**R^2**	**LOD (g/L)**	**LOQ (g/L)**
Glucose	$y = 1.305 \times 10^6 x - 5.198 \times 10^3$	1.0000	0.02	0.06
Sucrose	$y = 0.992 \times 10^6 x + 3.190 \times 10^3$	1.0000	0.07	0.22
Ethanol	$y = 6.449 \times 10^6 x - 25.726 \times 10^3$	1.0000	0.01	0.04
Citric acid	$y = 0.849 \times 10^6 x - 4.054 \times 10^3$	1.0000	0.01	0.04
Acetic acid	$y = 1.126 \times 10^6 x - 4.223 \times 10^3$	0.9999	0.02	0.08

3.5. Characterization of BC

3.5.1. Purification of BC

Two different substances were used for the BC purification assays [88]. For this purpose, 3% hydrogen peroxide and 100% bleach were used. After several BCs were produced, they were placed in Petri dishes and washed with distilled water several times. After that, 50 mL of the purification substance was poured into the Petri dishes. The BC was left in the Petri dish for 4 hours. After that, the contents of the Petri dishes were poured out and the BCs were washed again 3 times with distilled water (water purification

system WIN-645UP). The main aim of purifying BC was to clean films from microorganisms and constituents of medium and to reduce color.

3.5.2. Determination of BC yield

After the fermentation, the cellulose was removed from the nutrient medium, weighed (fresh weight, FW), then dried in a drying oven (Memmert UL 60) at 60 ºC until BC got constant weight, and weighed again (dry weight, DW).

The yield of BC was calculated as g of BC per L of broth for FW and DW.

3.5.3. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analysis were performed on previously dried and purified BC on ABB MB 3000 spectrophotometer (Zurique, Suiça) at 4 cm^{-1} resolution, between 4000 - 500 cm^{-1} at 32 scans/min (Figure 10). Spectra were acquired and treated using the software Horizon MB version 3.4. The background was acquired every two consecutive assays. For each sample of BC, the spectra were acquired at several points (from 4 to 15) due to the heterogeneity of the surface, which could be seen from visual inspection. Finally, for analysis, raw FTIR spectra and its 1st and 2nd derivatives were used [89].

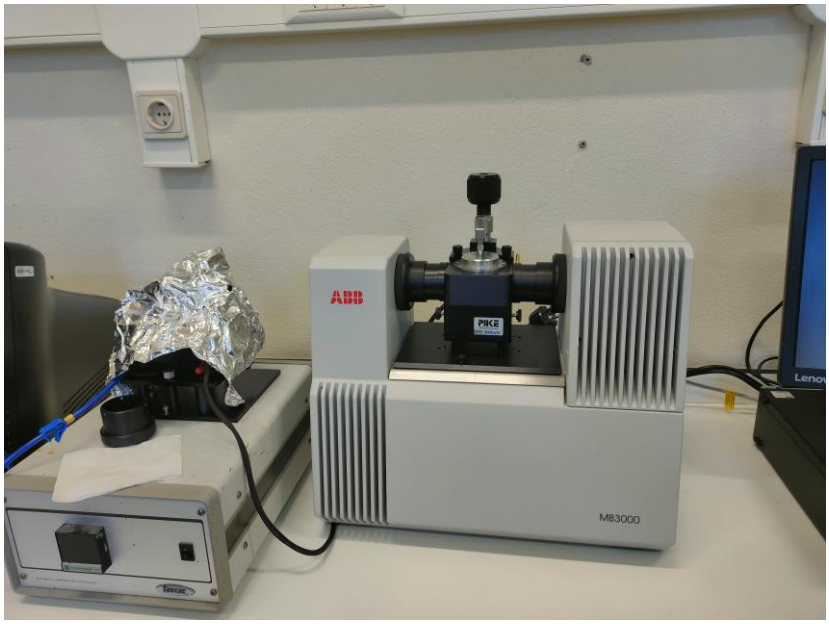

Figure 10 – FTIR equipment.

3.6. Functionalization of BC

The experiments for BC functionalization were developed in Centre for Nanotechnology and Smart Materials (CeNTI; https://www.centi.pt/), a research and development Institute, located in Famalicão, north of Portugal. One of the fields of interest for the institute is the functionalization and smartization of materials. Moreover, it is equipped with modern, cutting-edge technology. BC films were functionalized to obtain conductive BC. For these studies only BC produced by SCOBY were used.

3.6.1. Crosslinking

The crosslinking procedure followed the methodology previously described by Šauperl et al. [90]. Briefly, 100 g of 1,2,3,4-butanetetracarboxylic acid (BTCA; Merck) were put in a 1 L beaker with 100 g of $NaH_2PO_2.H_2O$ (Fluka). After that, 1 L of deionized water were added to obtain a 10% dilution. The prepared solution had yellowish color. After that, the previously purified BC was placed into 50 mL of solution, in a Petri dish and was left overnight at room temperature in dark. After 12 h the solution became transparent indicating that cellulose absorbed the $NaH_2PO_2.H_2O$ and BTCA. Then the BC was dried in an oven (ED 53 model from Binder Inc., USA) for 2.5 h at 100°C.

To assess the effectiveness of the crosslinking the water uptake capability of untreated (raw) and crosslinked BC (treated) was studied. For that, the respective BC were weighted before (W_b) and after (W_a) being placed in a Petri dish with water, for 24 hours.

The water uptake (W_u) was calculated using Equation (3).

$$W_u = \frac{W_a - W_b}{W_b} \times 100\ \%. \quad (3)$$

3.6.2. Functionalization of BC with carbon nanotubes

In order to functionalize BC with carbon nanotubes (CNT) the procedure described by Yoon et al. [91] was followed. So, 50 mg of CNT (NC 7000, Nanocyl) were put into a 200 mL beaker with 100 mL of deionized water (Kotterman 1033). Then 1.2 mL of cetyltrimethylammonium chloride (CTAC), which is used as surfactant, were added. After that the solution was directly treated with ultrasounds (Hielscher

UIP1000hd, 20 kHz, 57 µm wave amplitude, BS2d22 probe (22 mm diameter), 340 W power input) for 45 minutes. Then BC films were divided in 3 pieces, put into Petri dishes with 10, 15 and 20 mL of CNT solution. Two differently purified BCs were undergone the experiment treated with H_2O_2 and bleached.

3.6.3. Functionalization with waxy maize

The gel of waxy maize was obtained as described by Dastidar and Netravali [92]. So, 0.5 g of waxy maize (Waxylis, food grade) were put into a 200 mL beaker with 50 mL of deionized water in order to obtain a 1% (v/w) solution. After that, 0.05 mL of glycerol were added. Then the solution was stirred (200 rpm) using a magnetic stirrer and heated at 95 ℃. When the gel solution was obtained, it was poured into a Petri dish, and the BC functionalized with CNT was immersed into it and left overnight at room temperature in the dark. After that the Petri dish was washed with distilled water (WIN-645UP) and BC was left to dry at room temperature. Figure 11 shows the preparation of the waxy gel.

Figure 11– Preparation of waxy gel.

3.6.4. Measurement of surface resistivity

The surface resistivity of the treated BC was measured 3 times in different places using a multimeter (Agilent 34410A Digital Multimeter; Figure 12) for non-treated BC (NT-BC), CNT functionalized BC (CNT10-BC, CNT15-BC and CNT20-BC for BC incorporated with 10, 15 and 20 mL solution of CNT, respectively), and CNT-BC with waxy maize covered surface (CNT-WM-BC).

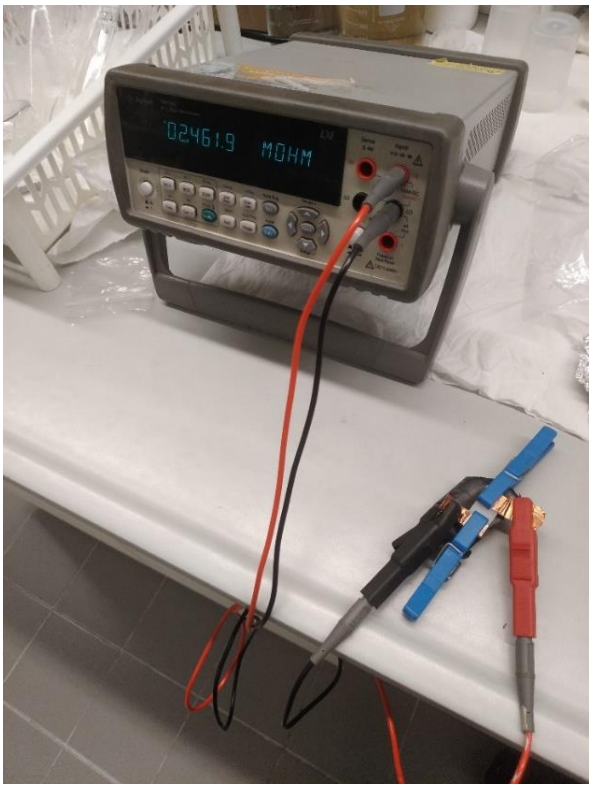

Figure 12 – Measurement of surface resistivity.

Chapter 4: Results and discussion

4.1 Preliminary experiments

4.1.1. pH measurement

First, the change in pH was measured to spot the beginning and the end of the fermentation, to determine the time of incubation of BC. For that SCOBY was incubated at 30 ℃, 3.5 g/L of black leaf tea and with 10 % w/v of sucrose (6 replicas). According to the literature the optimal pH is around 3 [32], therefore, in case it differed from the optimal, the addition of buffer or acids could be necessary. The pH and corresponding BC yields are shown in Figure 13.

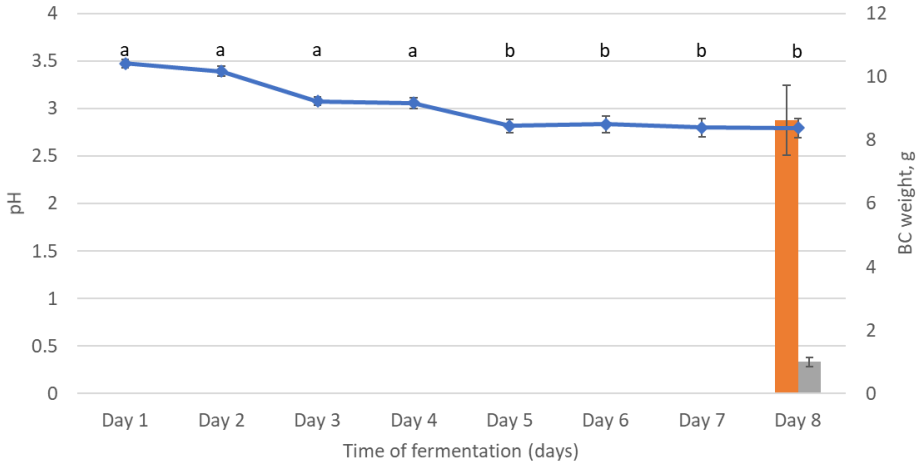

Figure 13 – pH of the fermentation broth throughout 8 days of fermentation by SCOBY, and the fresh (orange bar) and dry (gray bar) weights obtained at the end of fermentation (average ± SD, n=6).

At the fourth to fifth day of fermentation there was a significant drop in pH ($p < 0.05$), which was most likely due to the fact that the yeasts and bacteria produced acids from the beginning of fermentation. To the fifth day the amount of accumulated acid was high which led to the pH lowering [7]. In general, after the fifth day the pH stabilized at 2.8, which was consistent with the literature and should result in the highest yield of BC [32].

Throughout this assay it was found that the constant measuring of the pH disturbed the formation of BC, leading to the formation of bubbles and lowered the expected BC yield (Figure 14). Therefore, it was decided to cease this experiment and to not measure

the pH during the fermentation in the following assays. In this way, the pH measurement was conducted after BC was harvested.

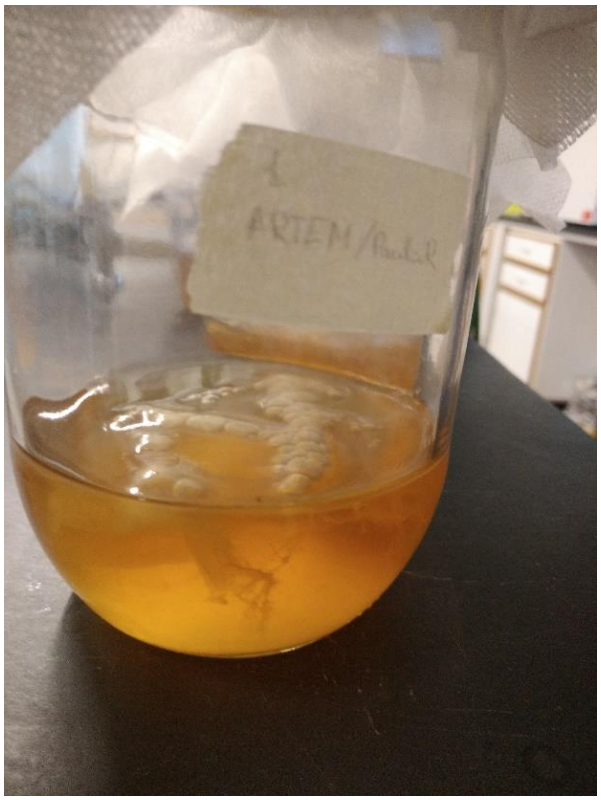

Figure 14 – Formation of bubbles during BC production.

4.2 BC production

4.2.1 SCOBY

When incubated under the specified conditions in tea broth, SCOBY produced a visible BC film on the fourth day of incubation. Starting from day 10, a fully-formed BC film was visible on the liquid-air surface and was ready to be harvested (Figure 15) However, the longer BC stayed in culture media more yield of BC was obtained. One of the main advantages of this way of producing BC is that SCOBY is growing very fast and at the same time producing acids and, therefore, reduces pH. This allowed to use laboratory equipment and carry on with the fermentation without thorough sterilization.

Figure 15 – Formation of cellulose after four days (a), and after 10 days (b) of fermentation at 27.5 ºC using 3.5 g/L of black tea.

The tea concentration and temperature are amongst the main factors which have a direct impact on BC yield. They both affect the growth of bacteria which is essential for BC biosynthesis. Regarding the initial studied fermentation conditions for SCOBY ($28 \leq T \leq 32$ ºC; $2.5 \leq$ Tea concentration ≤ 4.5 g/L), the implemented experimental design led to the data shown in Table 9, which corresponded to 14 days of fermentation.

For the fresh weight of the BC produced by SCOBY, it was not possible to establish a significant model, based on the selected 2^2 factorial design.

For the BC yield on a dry weight basis, at a 5% significant level, it was possible to establish a significant model (*P*-value = 0.0034) without lack of fit (*P*-value = 0.0821) based on only one significant factor, i.e., the temperature (*P*-value = 0.0034). The established model (in the actual factors, not using the coded ones) for the yield of BC, in g of dry weight (DW) per liter of broth, is given by Equation (4).

$$BC_{yield}\left(\frac{g_{DW}}{L}\right) = -271.73 + 10.65 \times T(ºC) \quad (4)$$

However, the determination coefficient (R^2) and the R^2_{adj} were low (0.8454 and 0.8145, respectively) being the R^2_{pred} even lower (0.6771). Even so, the R^2_{pred} was in

reasonable agreement with the R^2_{adj}. On the other hand, the adequate precision was greater than 4 (equal to 9.882) showing an acceptable signal to noise ratio. Although the model could be used to navigate the design space, the fitting and predicting performances did not allow finding the optimal conditions.

The highest yield by DW of BC (76.6 g/L) was achieved by applying the highest temperature (32 ℃) and using the highest tea concentration (4.5 g/L). This was an expected result because the favorable temperature range for SCOBY is from 30 to 32 ℃ according to the literature [33] and the media had the highest amount of nutrients from tea. Decreasing temperature and tea concentration led to a fall in the BC yield. Thus, the BC yield by DW of the middle point (3.5 g/L of tea and 30 ℃) was lower. The lowest yield by DW of BC (25.8 g/L) was observed for the lowest temperature (28 ℃) and the lowest amount of tea concentration (2.5 g/L). It was seen that the second-highest yield by DW of BC (71.6 g/L) was achieved at the lowest tea concentration (2.5 g/L) and highest temperature (32 ℃). This could be related to the fact that the temperature accelerates the bacterial growth and the amount of nutrients provided by the lowest concentration of tea was enough for bacteria to consume for 2 weeks.

This hypothesis was supported by another set of experiments where SCOBY was incubated for 12 and 20 days. For the studied fermentation conditions for SCOBY ($25 \leq T \leq 30$ ºC; $2.5 \leq$ Tea concentration ≤ 4.5 g/L), the implemented experimental design led to the data shown in Table 11, after 12 and 20 days of fermentation.

Table 9 – Fermentation conditions, fresh and dry weights, yield by volume and the composition of fermentation broth after 14 days of fermentation by SCOBY.

Sample	Tea concentration (g/L)	Temperature (°C)	FW (g)	DW (g)	Yield of DW (g/L)	pH	Concentration of substance				
							Glucose (g/L)	Sucrose (g/L)	Ethanol (%)	Citric acid (g/L)	Acetic acid (g/L)
4.5LD32	4.5	32	28.5	7.66	76.6	2.3	86.94	22.40	0.07	12.83	7.62
4.5LD28	4.5	28	23.9	3.72	37.2	2.6	21.3	50.78	0.13	3.03	3.36
3.5LD30(1)	3.5	30	27.7	4.16	41.6	2.3	37.0	33.77	0.10	4.31	3.31
3.5LD30(2)	3.5	30	25.8	4.38	43.8	2.2	48.7	35.56	0.10	6.68	3.83
3.5LD30(3)	3.5	30	21.8	3.78	37.8	2.4	33.4	46.57	0.11	3.69	1.05
2.5LD32	2.5	32	19.8	7.16	71.6	2.4	81.4	18.5	0.05	9.09	2.58
2.5LD28	2.5	28	18.9	2.58	25.8	2.2	41.6	31.84	0.13	5.76	3.62

FW- fresh weight, DW - dry weight. The initial concentration of sucrose obtained by HPLC is 64.37 g/L, other substances were not found in the broth before the fermentation; seven samples were prepared for the experimental design, four of them correspond to the combinations of minimum and maximum levels of tea concentration and temperature, the central point was done in triplicate.

Table 10 – Fermentation conditions, fresh and dry weights, yields by volume for SCOBY incubated for 12 days and 20 days.

Sample	Tea concentration (g/L)	Temperature (°C)	12 days				20 days			
			FW (g)	DW (g)	Yield DW by volume (g/L)	DW/FW (%)	FW (g)	DW (g)	Yield DW by volume (g/L)	DW/FW (%)
4.5LD30	4.5	30	20.2	2.95	29.5	14.9	36.8	7.89	78.9	21.4
4.5LD25	4.5	25	14.9	1.0	10	6.7	31.7	4.4	44.0	13.9
3.5LD27.5 (1)	3.5	27.5	11.7	1.4	14	12.0	26.2	4.88	48.8	18.6
3.5LD27.5 (2)	3.5	27.5	12.3	2.3	23	18.7	17	4.5	45.0	26.5
3.5LD27.5 (3)	3.5	27.5	9.4	1.3	13	13.8	28.3	5.5	55.0	19.4
2.5LD30	2.5	30	21.6	2.36	23.6	10.9	19.1	3.6	36.0	18.8
2.5LD25	2.5	25	11	0.75	7.5	6.8	5.8	0.65	6.5	11.2

FW- fresh weight, DW-dry weight; seven samples were prepared for the experimental design, four of them correspond to the combinations of minimum and maximum levels of tea concentration and temperature, the central point was done in triplicate.

After 12 days of fermentation, for the fresh weight it was possible to find a significant model (*P*-value = 0.0120) based on only one significant factor, i.e., the temperature (*P*-value = 0.0120), without lack of fit (*P*-value = 0.3531). However, the initial screening factorial design revealed a critical issue, i.e., the model showed a significant curvature (*P*-value = 0.0141) which could not be accounted by the first-order or interaction terms. Thus, an augment factorial design (which was not implemented, due to experimental difficulties) would be required, such as a central composite augmented design, which would allow including center points and axial runs to estimate the quadratic terms.

Nevertheless, the established model, based on the actual factors is as follows (Eq. 5):

$$BC_{fresh\ weight}(g) = -26.89 + 1.59 \times T(ºC) \quad (5)$$

Low R^2 and the R^2_{adj} were found (0.8265 and 0.7831, respectively) being the $R^2_{predicted}$ rather low (0.4131), not allowing the model to be used for prediction. Indeed, the adjusted and R^2_{pred} values are quite different probably indicating a large block effect or a possible problem with the model and/or data (e.g., existence of outliers). The adequate precision was greater than 4 (equal to 8.190) showing an acceptable signal to noise ratio and the possibility of using the model to navigate the design space.

A similar analysis was conducted for the BC yield after the 12 days of fermentation. The analysis showed that a significant model (*P*-value = 0.0168), also based on the temperature effect, the only significant effect (*P*-value = 0.0168), could be established, (Eq. 6, for the actual factors):

$$BC_{yield}\left(\frac{g_{DW}}{L}\right) = -80.25 + 3.56 \times T(ºC) \quad (6)$$

The model did not show lack of fit (*P*-value = 0.7892) neither a curvature effect (*P*-value = 0.7472). However, once again, low R^2 and the R^2_{adj} were found (0.7960 and 0.7450, respectively) being the R^2_{pred} rather low (0.4508), not allowing the model to be

used for prediction. Similarly, since the adjusted and R^2_{pred} values were quite different the model should be used with precaution. The adequate precision was greater than 4 (equal to 6.035) showing an acceptable signal to noise ratio and the possibility of using the model to navigate the design space. Contrary to the other models, the quality of the proposed model could be slightly enhanced by adding the tea concentration factor, although it was not significant.

For the 20 days of fermentation with SCOBY, the analysis was repeated. It was found that, contrary to the previous analysis, the BC fresh weight depended on the tea concentration, which was found to be a significant effect (*P*-value = 0.0297), but no more on the temperature. The established significant model (*P*-value = 0.0297), in actual factors, was (Eq. 7):

$$BC_{fresh\ weight}(g) = -14.80 + 10.90 \times Conc_{Tea}\left(\frac{g}{L}\right) \quad (7)$$

Although being significant, the model showed quite low values for the R^2, R^2_{adj} and R^2_{pred} (0.7323, 0.6654 and 0.1242, respectively), showing that the experimental design did not allow to obtain a feasible estimating or predicting model.

On the contrary, for the yield in dried BC, a significant model could be established (*P*-value = 0.0031) possessing satisfactory regression and prediction capabilities (0.9785, 0.9642 and 0.9147 for R^2, R^2_{adj} and R^2_{pred}, respectively) based on both studied factors, which were found to be significant (*P*-values of 0.0053 and 0.0028 for the temperature and tea concentration, respectively) (Eq. 8):

$$BC_{yield}\left(\frac{g_{DW}}{L}\right) = -286.08 + 10.65 \times T(ºC) + 4.10 \times Conc_{Tea}\left(\frac{g}{L}\right) \quad (8)$$

Moreover, the model did not show lack of fit (*P*-value = 0.6462) neither any curvature issue (*P*-value = 0.0916), possessing a high adequate precision (equal to 21.736). As can be seen from Eq. (8), both factors contributed positively to the increase of the BC yield. The 3D response surface on the design space is shown Figure 16 – 3D response surface described by the model established for the BC yield, after 20 days of

fermentation using SCOBY. An optimal (maximum) value of the BC yield (78 g/L, DW), within the design space could be determined, according to the model for a temperature of 30 ºC and a tea concentration of 4.5 g/L.

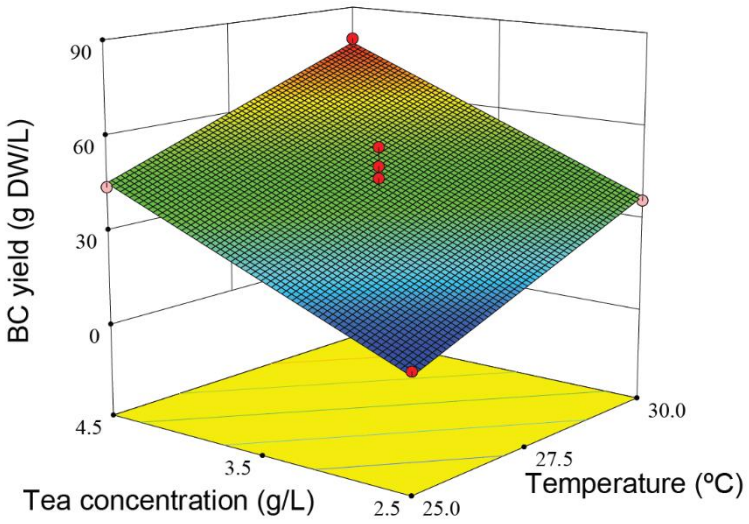

Figure 16 – 3D response surface described by the model established for the BC yield, after 20 days of fermentation using SCOBY.

It can be seen that for 12 days the trend was the same as for SCOBY incubated for 14 days. The highest yield (29.5 g/L) was for the highest temperature regardless of the amount of tea. The lowest yield (7.0 g/L) of BC corresponds to the lowest temperature. Moreover, it could be seen that the highest yield of BC fell drastically from 7.66 g to 2.95 g when the temperature was lowered by 2 from 32 to 30. When the temperature was lowered to 25 ℃ the production of BC was very slow.

When SCOBY was incubated for 20 days the pattern became different. The two highest yields were achieved by the highest tea concentration. The lowest concentration of tea corresponded to the lowest BC production. Also, it could be seen that SCOBY incubated under 30 ℃ for 20 days had almost the same yield BC as SCOBY incubated under 32 ℃ for 14 days.

The obtained BC incubated for 12, 14 and 20 days is shown in Figure 17.

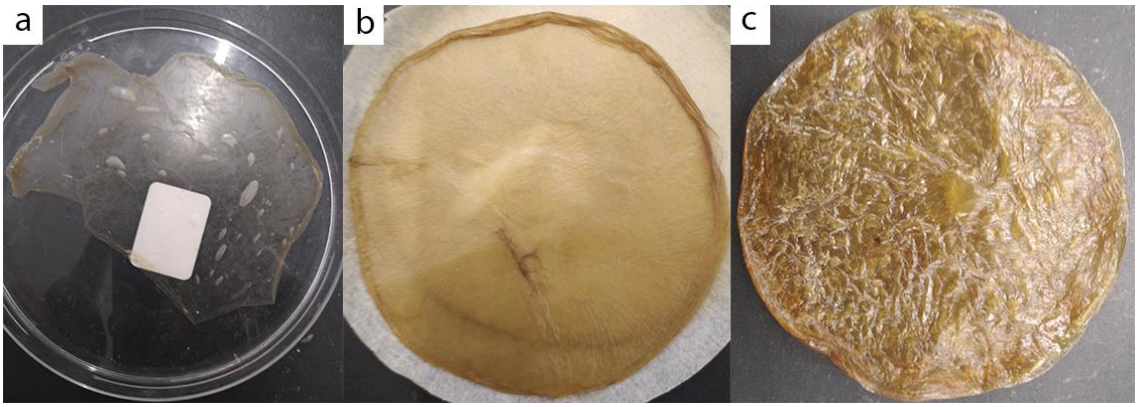

Figure 17 – BC incubated at tea concentration 3.5 g/L: (a) for 12 days at 27.5 ℃; (b) for 14 days at 30 ℃; (c) for 20 days at 27.5 ℃.

Overall, it was found that the best temperature in this study for SCOBY incubation was 32 ℃, which is in agreement with the literature [33]. Also, it was seen that in a shorter period of time (2 weeks and less) 2.5 g of tea was enough for SCOBY to grow and produce the same amount of BC incubated under the highest temperature as when 4.5 g of tea was used. However, when the period of time was longer the amount of nutrients provided by the lowest concentration was not enough. At the same time even in the case of the lowest temperature SCOBY could produce BC with good yield by DW (44.0 g/L) with the highest tea concentration. This means that if bacteria have enough time and nutrients to grow BC still can be produced. The lowest yield by DW (lower than 10 g/L) of BC in case of the lowest temperature and amount of tea regardless of time might be related to the fact that bacteria grew very slowly and at the moment when it was ready to produce BC there were not enough nutrients in the medium.

Another important thing to discuss is the composition of the fermentation broth. It was analyzed by HPLC for BC incubated for 14 days. This analysis could help understand the process of BC synthesis better. It could be through finding which substances were present in the solution and under what conditions the production of these substances was the highest.

The consumption of the initially added sucrose depended on the temperature at which bacteria were incubated. Therefore, the concentration of sucrose for samples incubated at 32 ℃ was lower than for the samples incubated at 30 or 28 ℃. Conversely, the uptake of sucrose was falling with the decrease of the temperature. It could also be noticed that for the sample with the lowest concentration of tea and temperature

(2.5LD28) the consumption of sucrose was the same as for the samples with tea concentration at 3.5 g and temperature 30 ℃. Even though the uptake of sucrose was the same as for samples at the middle point, the yield by DW for this sample was the lowest

The presence of ethanol in all samples could be explained by the yeast fermentation. The higher temperature corresponded to the lowest concentration of ethanol which increases with its lowering. This could be explained because ethanol is a volatile substance which means that increasing the temperature results in higher evaporation [93]. The presents of ethanol could affect the BC yield and the increase of the yield depends on the concentration of ethanol [36]. As for acids the highest concentration was achieved by the sample with the highest tea amount and temperature (4.5LD32), whereas, the production of acids by others samples was in almost the same range. It was also reported that presence of citric and acetic acids enhances the BC production [7].

4.2.2 *Komagataeibacter* sp.

The results obtained from the fermentation developed by the two strains of bacteria, *Komagataeibacter* sp. SB6A and *K. intermedius* SB14, are shown in **Ошибка! Источник ссылки не найден.** and Table , respectively.

Table 11 – Fermentation conditions, fresh and dry weights, yield by volume and the composition of fermentation broth after 14 days of fermentation for *Komagataeibacter* SB06A.

Sample	Number of cells (cells/mL)	Temperature (℃)	FW (g)	DW (g)	Yield of DW (g/L)	pH	Glucose (g/L)	Citric acid (g/L)
10^3Kx32	10^3	32	contaminated	-	-	-	-	-
10^3Kx28	10^3	28	contaminated	-	-	-	-	-
10^5Kx30 (1)	10^5	30	1.23	0.099	0.99	3.6	17.6	1.17
10^5Kx30 (2)	10^5	30	1.93	0.083	0.83	3.5	16.5	3.22
10^5Kx30 (3)	10^5	30	1.17	0.083	0.83	3.5	16.7	2.58
10^7Kx32	10^7	32	1.1	0.054	0.54	3.6	18.8	0.89
10^7Kx28	10^7	28	0.33	0.035	0.35	3.8	19.1	0.89

FW- fresh weight, DW-dry weight; seven samples were prepared for the experimental design, four of them correspond to the combinations of minimum and maximum levels of tea concentration and temperature, the central point was done in triplicate.

Table 12 – Fermentation conditions, fresh and dry weights, yield by volume and the composition of fermentation broth after 14 days for *Komagataeibacter* SB14.

Sample	Number of cells, cells/mL	Temperature (℃)	FW (g)	DW (g)	Yield of DW (g/L)	pH	Glucose (g/L)	Citric acid (g/L)	Acetic acid (g/L)
10^3Kx32	10^3	32	9.5	0.3	3.0	3.41	9.3	4.56	ND
10^3Kx28	10^3	28	14.3	0.44	4.4	3.53	11	3.82	ND
10^5Kx30	10^5	30	14.3	0.55	5.5	3.51	7.4	6.07	ND
10^5Kx30	10^5	30	14.6	0.56	5.6	3.49	7.7	6.55	ND
10^5Kx30	10^5	30	16.9	0.61	6.1	3.46	7.9	6.7	ND
10^7Kx32	10^7	32	15.9	0.62	6.2	3.72	4.7	4.31	1.85
10^7Kx28	10^7	28	20.5	0.67	6.7	3.79	3.9	3.73	1.59

FW- fresh weight, DW-dry weight; seven samples were prepared for the experimental design, four of them correspond to the combinations of minimum and maximum levels of tea concentration and temperature, the central point was done in triplicate. ND – substance not found in the broth.

After 14 days of incubation in HS medium, BC was produced by at different cell concentrations of *Komagataeibacter* sp. SB06A. When BC was produced with the lowest cell concentration (10^3 cells/mL) the culture media got contaminated (Figure 18). Since two flasks with the lowest cell concentrations were contaminated it was not possible to create any model for *Komagataeibacter* sp. SB06A. The contamination could be related to the fact that bacteria in that concentration were not able to grow fast enough and produce enough acids to decrease pH. The BC yields for other concentrations were all less than 0.1 g (DW). Moreover, the yield for the highest concentration (10^7 cells/mL) was lower than the middle one (10^5 cells/mL) which could mean that the optimal temperature for bacterial growth was 30 ℃. The initial concentration of glucose was 20 g/L. As could be seen from **Ошибка! Источник ссылки не найден.**, the glucose consumption for the bacteria in the central point was 3 g/L for 14 days, while the bacteria incubated at 28 and 32 ℃ consumed only 1 g/L of glucose.

In the fermentation broth of *Komagataeibacter* bacteria isolated from the SCOBY only two substances were present (glucose and citric acid). The absence of ethanol could be explained by the fact that it was produced by yeast fermentation. This was probably the reason of the low yield of BC which corresponded to other articles saying that ethanol could promote BC yield [24, 36]. Another thing was the absence of acetic acid. *Komagataeibacter* is considered acetic bacteria and, therefore, the acid should have been detected in the fermentation broth. One possible explanation for the absence of acetic acid

is that it was produced and then consumed. The consumption of acetic acid was also shown by Jayabalan et al. [94], where the concentration of acetic acid started to decrease after 15 days.

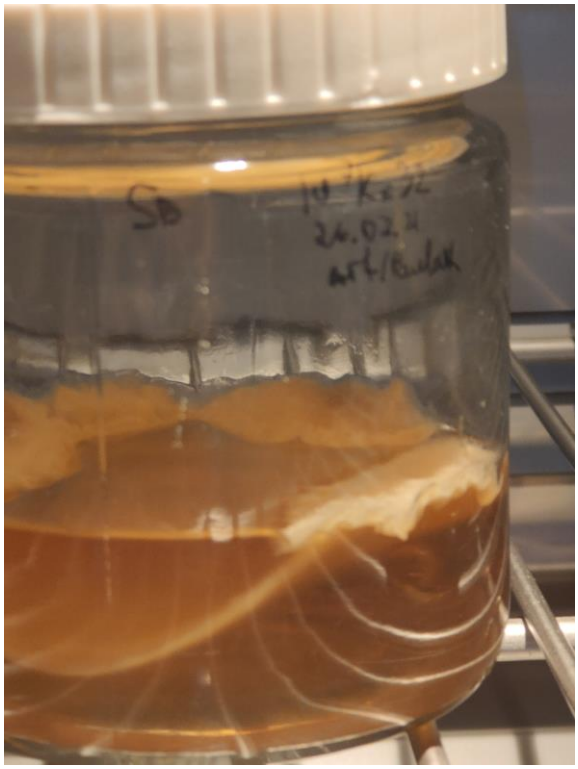

Figure 18 – Contaminated flask of *Komagataeibacter* SB06A in HS medium with cell concentration 10^3 incubated at 32 ºC.

K. intermedius SB14 suits better for BC production than the previous one because the yield by volume was higher than 1 g/L. Based on the experimental design for *K. intermedius* SB14, and under the studied experimental conditions, an analysis was performed aiming to establish a model that would allow determining the optimal initial cell concentration and temperature. The decimal logarithmic of the cell concentration was used as the factor under study.

For the BC fresh weight, a significant model (*P*-value = 0.0153) based on both factors, which revealed to be significant (*P*-value of 0.0273 and 0.0123, for the temperature and the decimal logarithm of the number of cells, respectively), could be found (Equation 6):

$$BC_{fresh\ weight}(g) = 42.42 - 1.18 \times T(ºC) + 1.58 \times log_{10}(nº\ cells) \quad (6)$$

The model did not show any lack of fit (*P*-value = 0.9504) or curvature (*P*-value = 0.8230) and had an adequate precision equal to 12.51, with satisfactory regression and prediction capabilities (0.9384, 0.8973 and 0.8593 for R^2, R^2_{adj} and R^2_{pred}, respectively). As can be seen from Eq. (6), the temperature had a negative impact on the BC fresh weight, meaning that the BC fresh weight was promoted by lower temperatures. On the other hand, the initial number of cells had a positive effect, meaning that the BC fresh weight was increase when higher initial levels of cells were used. The 3D response surface on the design space is shown in Figure 19. An optimal (maximum) value of the BC fresh weight (20.55 g), within the design space could be determined, according to the model for a temperature of 28 ºC and an initial cell number of 10^7 cells/mL.

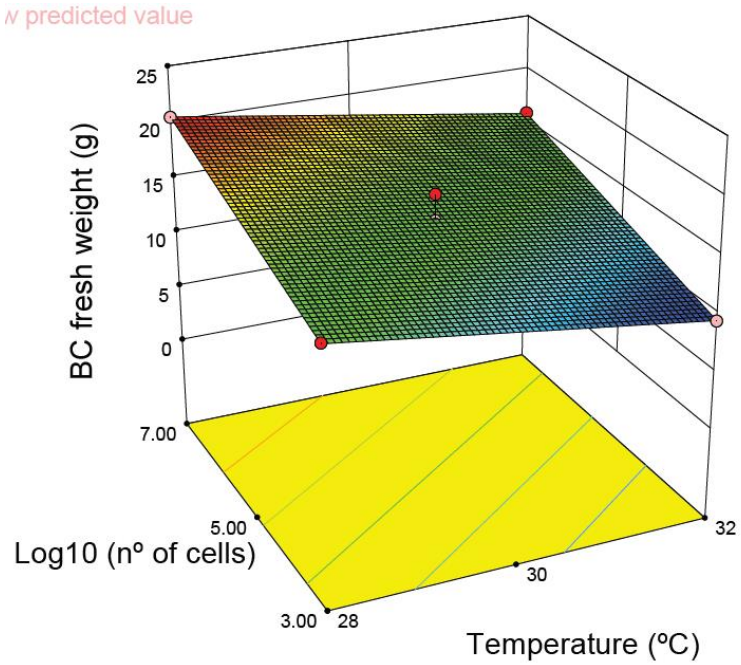

Figure 19 – 3D response surface described by the model established for the BC fresh weight, after 14 days of fermentation using *Komagataeibacter* SB14.

For the BC yield in dry weight, a significant model (*P*-value = 0.0099) based on both factors was established, although the temperature was only significant at a 10% significance level (*P*-value of 0.0823 and 0.0050, for the temperature and the decimal logarithm of the number of cells, respectively), could be found (Equation 7):

$$BC_{yield}\left(\frac{g_{DW}}{L}\right) = 8.76 - 0.24 \times T(ºC) + 0.69 \times log_{10}(nº\ cells) \qquad (7)$$

The model did not show any lack of fit (*P*-value = 0.2965) or curvature (*P*-value = 0.1018) and had an adequate precision equal to 13.25, with satisfactory regression performances (0.9539 and 0.9232 for R^2 and R^2_{adj}, respectively) but with less satisfactory predictive capability (R^2_{pred}= 0.5825). As can be seen from Eq. (7) and in-line with the results for the BC fresh weight, the temperature had a negative impact on the BC yield and the initial number of cells had a positive effect. The 3D response surface on the design space is shown in Figure 20. An optimal (maximum) value of the BC yield (6.93 g DW/L), within the design space could be determined, according to the model also for a temperature of 28 ºC and an initial cell concentration of 10^7 cells/mL.

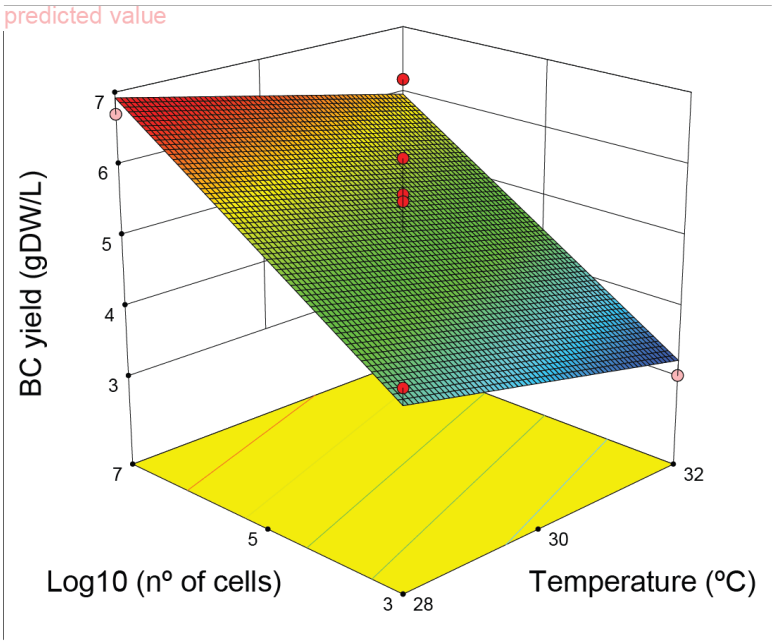

Figure 20 – 3D response surface described by the model established for the BC yield in dry weight, after 14 days of fermentation using *Komagataeibacter* SB14.

The lowest yield (3.0 g/L) corresponded to the lowest cell concentration incubated at 32 ℃. When for the same cell concentration, the temperature was decreased to 28 ℃, the yield is increased by 1.4 g/L coming to 4.4 g/L. The trend was the same for the highest concentration (6.7 g/L for 28 ℃ and 6.2 g/L for 32 ℃). The yield for the central point was very close to the values for highest concentration (5.5 – 6.1 g/L), however, the consumed glucose in this case was lower by 3 g/L. It also could be seen that for *Komagataeibacter* SB14 the same two substances (glucose and citric acid) were found by HPLC as for *Komagataeibacter* SB06A. Interestingly, for the highest cell concentration

the acetic acid was found in HPLC spectra for both detectors for *Komagataeibacter* SB14 (Figure 21).

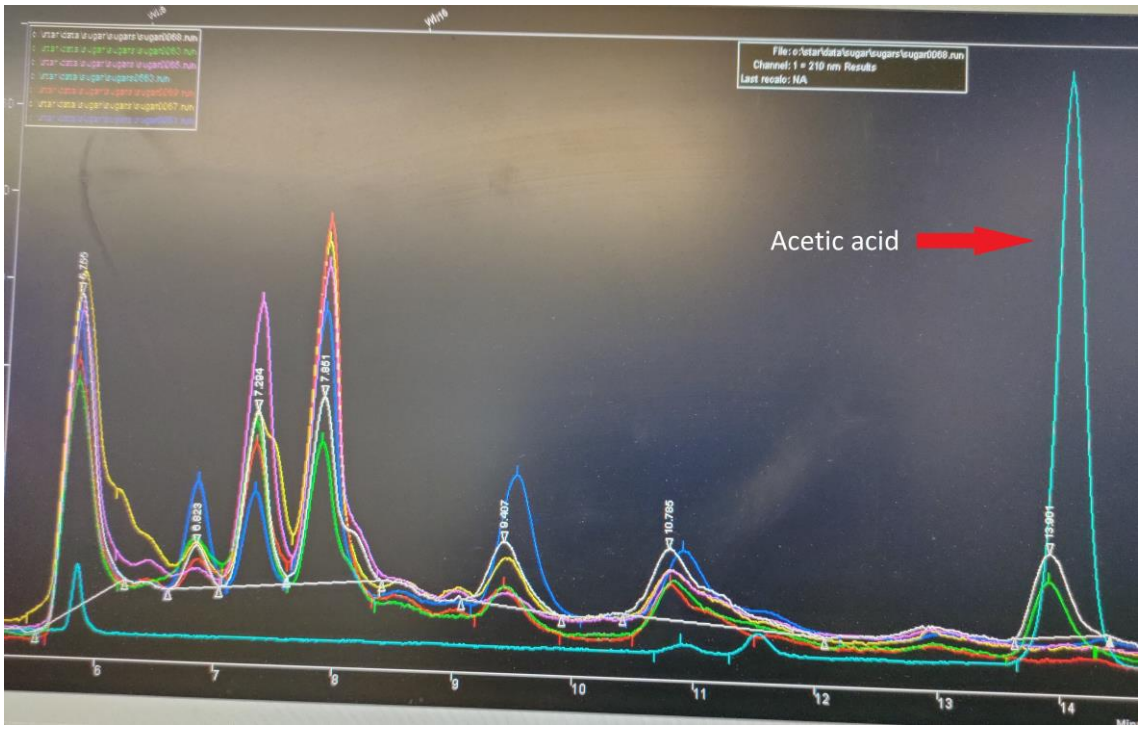

Figure 21 - UV spectrum of the composition of fermented broth from *Komagataeibacter* SB06A and SB14, and standard for acetic acid. Red – 10^5Kx30 SB06A; Blue – 10^7Kx32 SB06A; Yellow – 10^3Kx32 SB14; Pink – 10^5Kx30 SB14; Green – 10^7Kx32 SB14; White - 10^7Kx28 SB14; Turquoise – standard for acetic acid.

This means that acetic acid was produced only the strain SB14. However, even with the highest cell concentration (10^7 cells/mL) the acetic acid concentration was low (1.5-1.9 g/L). Probably, the production of acetic acid in other conditions was so low that it was instantly consumed or transformed and, therefore, cannot be found in the broth.

4.2.3. Comparison of SCOBY and *Komagataeibacter* sp.

Overall, the production of BC by *Komagataeibacter* strains was much lower comparing to SCOBY. The sugar uptake by *Komagataeibacter* SB06A was low and did not exceed 5 g/L for 14 days. The highest yield (0.099 g/L of BC) obtained by this strain was in central point (concentration 10^5 cells/mL, temperature 30 ℃). This is compliant with the reported optimal temperature being 30 ℃ for *Komagataeibacter xylinus* [95].

However, the yield reported in that study was more than 20 times higher (2.2 g/L) after 7 days of fermentation. This could mean that the strain *Komagataeibacter* SB06A does not fit for BC production in given conditions.

Komagataeibacter SB14 had a more than 30 times higher yield (starting from 3.0 g/L) than *Komagataeibacter* SB06A. Moreover, the wet weight of BC produced by *Komagataeibacter* SB14 was close to that produced by SCOBY. Although, the wet weight for the *Komagataeibacter* SB14 was almost the same as for SCOBY, the yield by volume was 10 times lower (3.0 – 6.7 g/L for *Komagataeibacter* SB14 strain and 25.8-76.6 g for SCOBY). This could be due to the fact that ethanol was present in SCOBY fermentation broth which was able to increase BC yield [24, 36]. Also, it was reported that the bacteria and yeasts contained in SCOBY could promote each other growth and, therefore, increase BC yield [7]. This made SCOBY the best culture for BC production.

The choice on the best conditions using SCOBY depends on the fermentation time of the BC. If it is necessary to achieve higher yields and continue the fermentation more than it is necessary to use higher amount of tea (4.5 g/L). However, in this case the temperature can be lowered to 30 ℃ to lower energy consumption and, therefore, the costs of production. In case when it is necessary to achieve good yields of BC (up to 70 g/L) in less time, the less amount of tea can be used (2.5 g/L), but the temperature should be 32 ℃.

When compared to BC production in previous studies, it could be observed that the yield achieved with SCOBY was the highest among all of them (76.8 g/L by SCOBY in this work and 15.3 g/L by Hwang *et.al.* [96]) (Table 13). This was considered as a good result since one of the goals of the study was to achieve the highest yield without increasing the expenses of BC production. However, the time used by [96] to produce 15.3 g/L of BC was 6 time lower than in this study. This could be due to the fact that, at first, the yeasts from SCOBY had to produce enough nutrients for bacteria to grow, which takes 4 to 5 days. Also, the yield from bacteria isolated from SCOBY was more than 10 times less than the bacteria presented in other studies which means that these bacteria grow better in symbiotic culture than alone.

Table 13 – BC production of different microorganisms

Microorganism	Supplement, (concentration)	Culture medium	Culture time	Yield (g/L)	Reference
A. xylinum BRC 5	Oxygen, (0.6 % saturation)	CSL	50 h	15.30	[96]
G. hansenii PJK (KCTC 10505 BP)	Ethanol, (1 v/v %)	Basal medium	48 h	1.72	[97]
G. hansenii PJK	Ethanol, (1 v/v %)	Basal medium	5 days	2.31	[98]
Acetobacter sp. V6	no	Synthetic medium	8 days	4.16	[99]
Acetobacter sp. A9	Ethanol, (1.4 v/v %)	Synthetic medium	7 days	5.42	[36]
A. xylinum BPR2001	no	Molasses medium	72 h	7.82	[100]
Acetobacter xylinum ssp. sucrofermentans BPR2001	agar, (0.6 w/v %)	CSL-fructose	80 h	11.60	[101]
Acetobacter xylinum ssp. sucrofermentans BPR2001	no	CSL-fructose	67 h	7.8	[102]
Acetobacter xylinum E25	Ethanol, (1 v/v %)	Yamanaka medium	14 days	5.35	[103]
G. xylinus strain (K3)	green tea, (3g/L)	CSL	7 days	3.34	[40]
Gluconacetobacter xylinus IFO 13773	lignosulphonate, (1 w/v %)	HS	7 days	16.32	[104]
Acetobacter xylinum NUST4.1	sodium alginate, (0.04 w/v %)	Seed medium	5 days	6.00	[105]
Gluconacetobacter xylinus IFO 13773	no	HS	7 days	5.76	[106]
Gluconacetobacter sp. RKY5	no	Synthetic medium	144 h	5.63	[107]
Co-culture of *Gluconacetobacter* sp. st-60–12 and *Lactobacillus mali* JCM1116	no	CSL	5 days	4.20	[108]
Komagataeibacter SB06A	no	HS	14 days	0.99	This work
Komagataeibacter SB14	no	HS	14 days	6.70	This work
SCOBY	no	Tea broth	14 days	76.80	This work

4.3. Purification of BC

Hydrogen peroxide have not completely cleaned the BC even after 12 hours. The BC had a yellowish color after cleaning (Figure 22a**)**. When bleach was used, only two hours were sufficient to clean the BC. However, after drying, BC treated with bleach

becomes crispy and brittle (Figure 22b**)**, which can affect its usage in most cases. However, after placing into water BC gained its elasticity back.

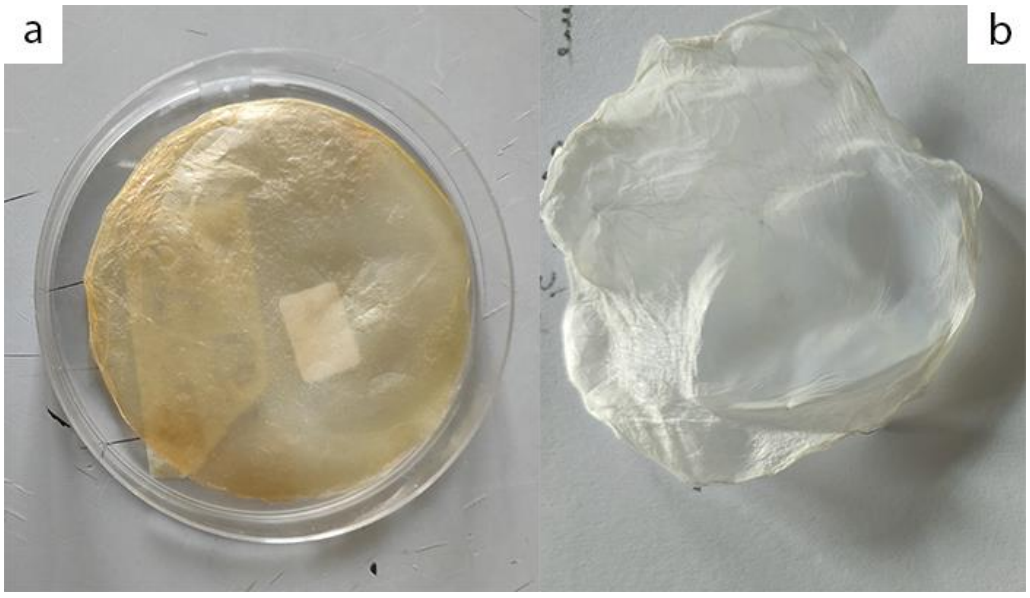

Figure 22 – Bacterial cellulose films purified by different substances: (a) - bacterial cellulose purified with peroxide; (b) - bacterial cellulose treated with bleach;

4.4. Characterization of BC

4.4.1. FTIR analysis

When examined by naked eye the surface of BC films looked heterogeneous (Figure 23).

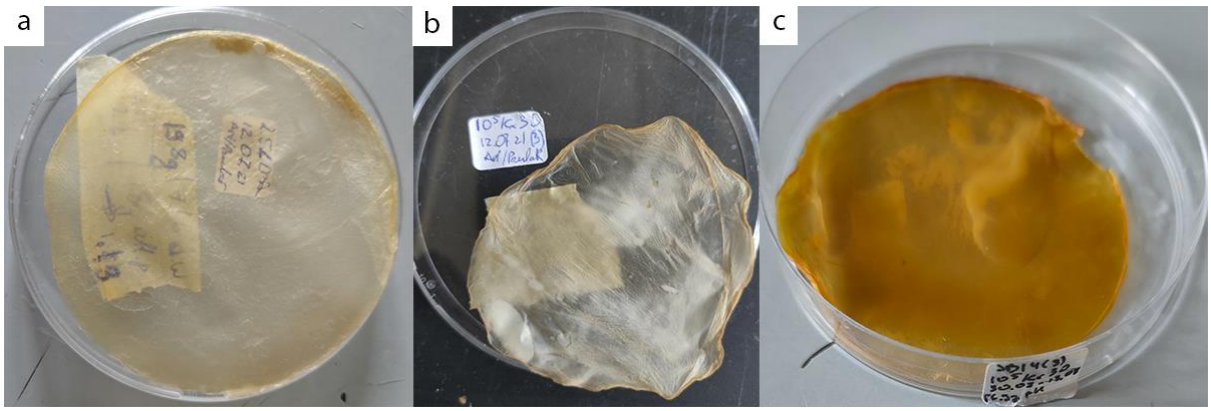

Figure 23 – BC incubated by: (a) SCOBY, (b) *Komagataeibacter* SB06A, (c) *Komagataeibacter* SB14

Therefore, it was decided to study the homogeneity of BC by acquiring FTIR spectra in 15 points (Figure 24).

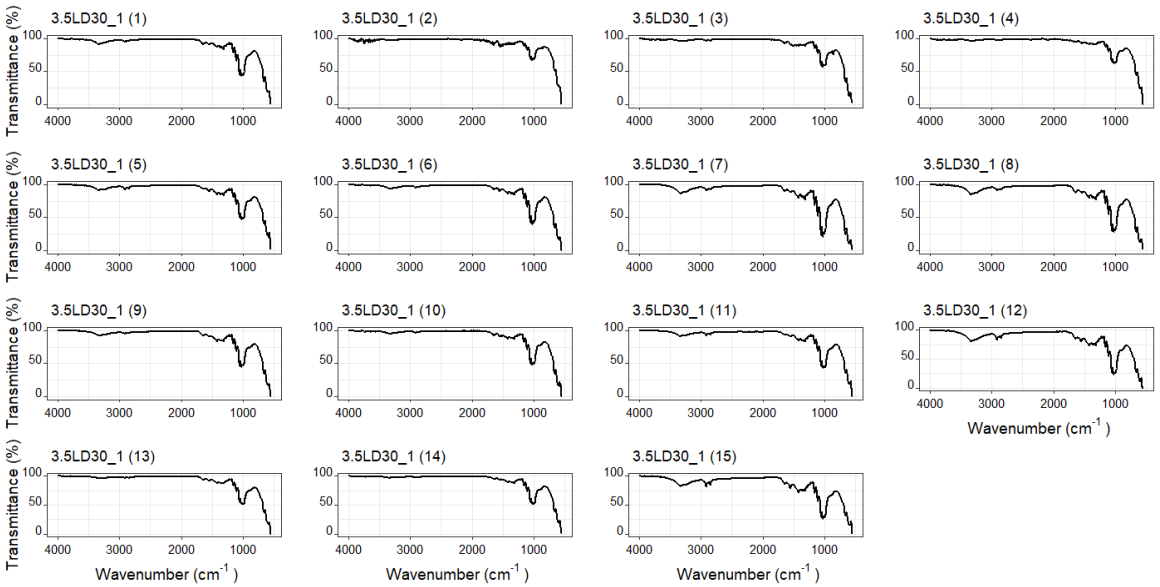

Figure 24 – FTIR spectra for 15 points of 3.5LD30(1) sample.

It was seen that the transmittance highly differs even in the same film. This means that the thickness of the film was different depending on the place of BC film and, therefore, the structure was not homogenous. However, to compare BC films produced by SCOBY, *Komagaeibacter* strains and to other BC from other studies it was decided to create a mean spectrum from obtained spectra for 4 points.

BC has many different groups and bonds which can be characterized using FTIR. The FTIR spectra for BC produced by SCOBY is shown in Figure 25.

According to the literature the typical absorption bands for BC spectrum are hydroxyl groups, methylene stretching vibration and the C-O-C and C-OH stretching vibrations of sugar ring [109]. The –OH group vibrations correspond the absorbing band around 3500 cm^{-1}. Near this band there is a C-H stretching vibration of CH_3 and CH_2 groups at 2890 cm^{-1}. The peak of 1160 cm^{-1} corresponds to the C-C stretching, while the band at 1108 cm^{-1} is the C-O-C stretching vibration. The C-OH stretching vibration of secondary and primary alcohols of BC is at 1055 cm^{-1} and 1028 cm^{-1}, respectively [109, 110]. All the groups described above were found in obtained FTIR spectra in the same area as reported in other studies. No additional signals were found. Overall, the obtained

FTIR spectra of BC produced by SCOBY and two *Komagataeibacter* bacteria are consistent with other reports [109, 111].

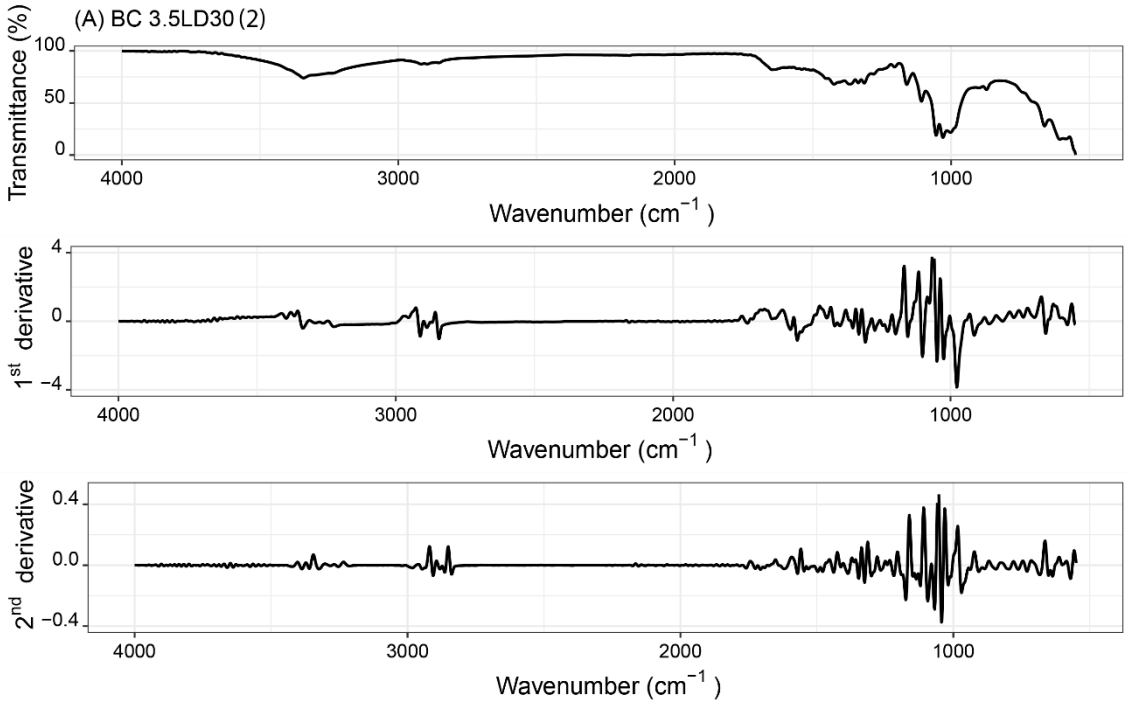

Figure 25 – FTIR for BC produced by SCOBY

When compared to the BC produced by 2 others bacteria the structure of BC was the same because the bands of absorption appeared at the same wavenumbers (Figure 26). However, the transmittance could differ a lot which could be due to differences in the thickness of the analyzed films. Also, in another study the same thing was reported that the transmittance of BC can vary depending on medium [109].

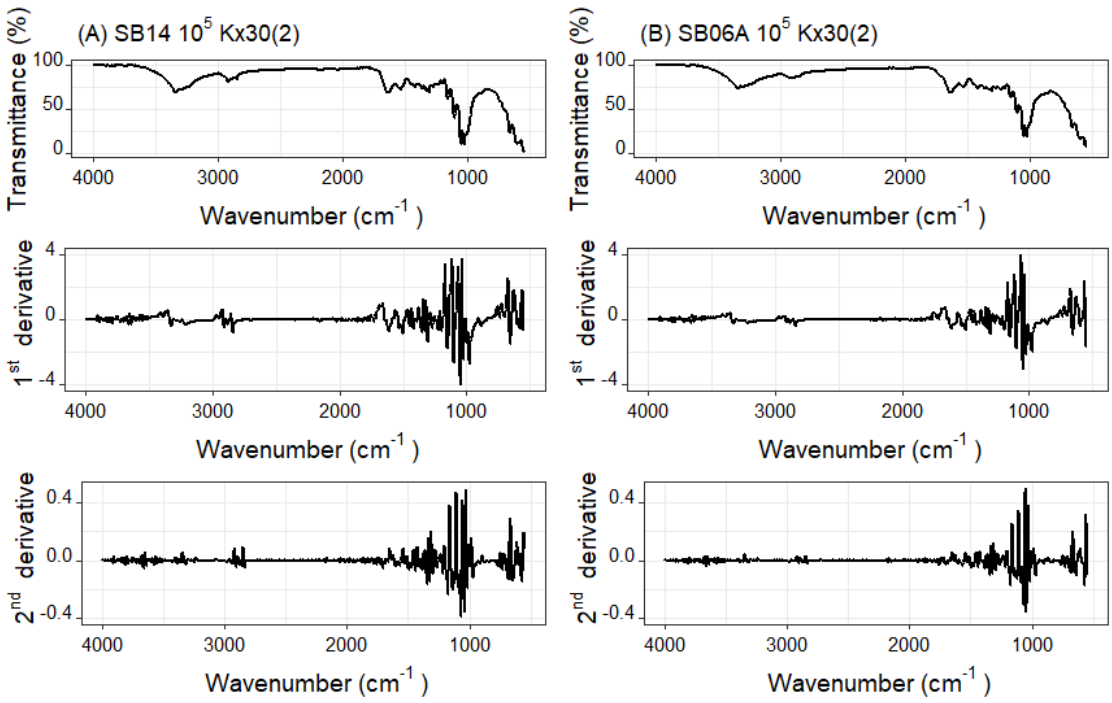

Figure 26 – FTIR for BC produced by two *Kamagataebacter* bacteria

When compared in terms of structure BC films produced by *Komagataeibacter* strains and SCOBY were structurally the same. However, the yield of BC produced by SCOBY was higher by 10 times, therefore, it was decided to functionalize only those BC.4.5. Functionalization of SCOBY BC

4.5.1. Crosslinking

To be used in electronics the water uptake ability of BC should be as low as possible. Therefore, BC could be crosslinked to increase the hydrophobicity.

The availability and reactivity of hydroxylic groups of BC making it possible to crosslink with polycarboxylic acids and formaldehyde, however, first are considered preferable, due to the fact that they are less harmful for the environment [112, 113]. Among all carboxylic acids BTCA was chosen because it is the most effective one in the crosslinking process. The concentration of BTCA was decided to be 10 % because it was shown that water uptake ability at first is lowering with the increase in concentration, but after with large concentrations it starts to rise [89].

To determine the difference in water uptake of raw and treated BC both pieces were weighed and then put in a Petri dish with water for 24 hours and then weighed again.

The initial weight of crosslinked BC was 0.14 g, whereas untreated BC was 0.06 g. After 24 h. crosslinked BC weighed 0.19 g and untreated 0.31 g, meaning that crosslinked BC absorbed 35% of its initial weight and untreated films absorbed 400%. Thus, the capacity of BC to absorb water after treatment was reduced by more than 10 times. Therefore, crosslinking with BTCA was successful in decreasing the water uptake capability and swelling of BC.

4.5.2. Functionalization of BC with CNT and coating with waxy maize

A good *ex situ* way to increase the conductive ability of BC is to incorporate carbon nanotubes (CNT) into it (**Ошибка! Источник ссылки не найден.**). The main advantage of this method is that it is easily performed. The BC film is placed in a CNT solution, CNT are absorbed and, thus, the conductivity of film increases. However, when the modified BC came into contact with other surfaces, CNT loss was observed. This could lead to the decrease in conductivity. Thus, it was decided to cover the surface of the bacterial cellulose with a polymeric substance. Amylose contained in waxy maize could be used as such a substance for the protection of BC surface.

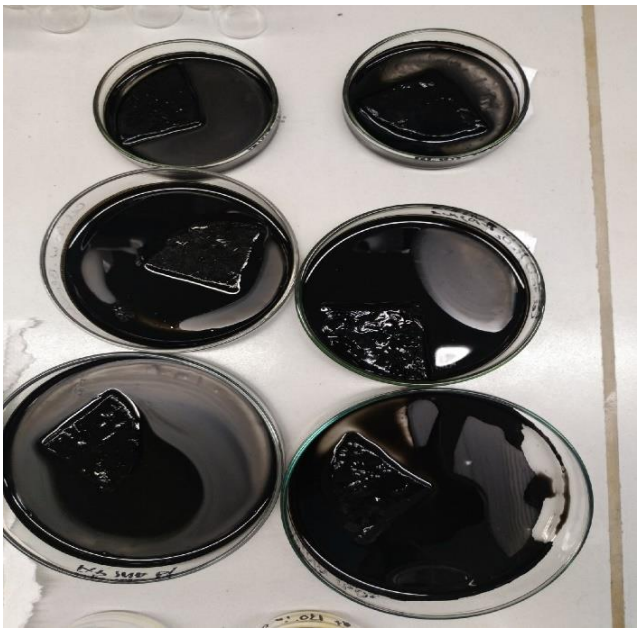

Figure 27 – Cellulose put into the CNT solution

4.5.3. Measurement of the surface resistivity

After performing these experiments, the surface resistivity was measured. The data for untreated BC, BC with different CNT concentration and BC with CNT covered with waxy maize gel are shown in Table 14.

Table 14 - Values for surface electrical resistivity of differently functionalized BC

Treatment	**Surface electrical resistivity (Ohm/cm) $\times 10^2$**				**Surface electrical conductivity (S/cm) $\times 10^{-3}$**
	1	2	3	Average	
UN - BC	2.29×10^4				4.37×10^{-4}
CNT10-BC/ H_2O_2	2.23	2.25	2.10	2.19 ± 0.07	4.56 ± 0.17
CNT10-BC/ bleach	1.10	1.69	2.55	1.78 ± 0.73	6.33 ±.2.6
CNT15-BC/ H_2O_2	1.18	1.04	1.10	1.11 ± 0.07	9.06 ± 0.58
CNT15-BC/ bleach	1.39	1.60	1.71	1.56 ± 0.16	6.30 ± 0.86
CNT20-BC/ H_2O_2	1.30	1.82	1.53	1.55 ± 0.26	6.57 ± 1.10
CNT20-BC/ bleach	1.17	1.83	1.84	1.61 ± 0.38	6.48 ± 1.79
CNT15-WM-BC/ H_2O_2	1.63	1.66	1.86	1.72 ± 0.13	5.84 ± 0.41
CNT15-WM-BC/ bleach	1.75	1.74	2.10	1.86 ± 0.21	5.40 ± 0.56

As can be seen from Table 14, no great differences were found between the 3 different volumes (10, 15, 20 mL) of carbon nanotubes solution with concentration (0.5 mg/mL). This means that even at the lowest volumes added, the amount of CNT was the highest amount that BC could absorb. However, when compared with unmodified BC this modification reduced the surface resistivity by about 10^4 times. The coating of BC with gel lead to a 1.67-fold decrease in the conductivity. However, it is expected that, when coated with waxy mazy, BC can be used for a longer period without the decrease in conductivity

Comparing to functionalized BC with increased conductivity in others studies (Table 15) the values achieved in this were average for conductivity.

Table 15 – Surface electrical conductivity for differently functionalized BC

Functionalizing substance	**Way of functionalizing**	**Surface electrical conductivity (S/cm)**	**Reference**
CNT	Modification *ex situ*	2.30×10^{-2}	[90]
polyaniline	Oxidative polymerization	8.20×10^{-4}	[114]
polyaniline	Oxidative polymerization	5.00×10^{-2}	[115]
PEDOT:PSS	Oxidative polymerization	1.50	[116]
PEDOT:PSS	Oxidative polymerization	0.26	[117]

It appeared that the best substance for increasing conductivity was poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS) which gives the highest value (1.5 S/cm). CNT and polyaniline were less effective compared to the PEDOT:PSS, however, they still increase the conductivity by $10^4 – 10^5$ times.

Chapter 5: Conclusions

This work was devoted to the biosynthesis, characterization and application of BC.

In the first stage, the preliminary experiment was carried out to obtain the information on the duration of the fermentation by monitoring the pH change. The experiment showed that the pH was consistent to that reported before for SCOBY and also that pH measuring throughout the fermentation process interfered with the production of BC.

Two different ways of producing BC were studied, namely by using SCOBY and pure *Komagataeibacter sp.* Strains, under several fermentation conditions. To establish a model and identify the optimal conditions for maximizing the BC yields, 2^2 experimental designs were used. This analysis lead to the conclusion that SCOBY performed the best among all microorganisms. *Komagataeibacter* sp. SB06A was found to be not suitable for BC production under the studied conditions, since the yield of BC did not exceed 1 g/L. The BC producing capability of *Komagataeibacter* SB14 was better than of *Komagataeibacter* SB06A and was similar to those obtained in other studies, achieving a yield of 6.7 g/L of DW after 14 days of fermentation. The use of SCOBY allowed to achieve the highest DW yield, i.e., 76.6 g/L after 14 days of fermentation. The time of incubation played a key role on the influence of temperature and tea concentration on BC yield. When SCOBY was incubated for 14 days or less, temperature was the key factor which determined the yield of BC. However, when the culture was left for more than two weeks the concentration of tea had more influence on the BC production. Thus, the best conditions should be selected regarding to the needed incubation time. In this study, the best conditions for 14 days were: a concentration of tea of 2.5 g/L and a temperature of 32 ℃.

The analysis of the fermentation broth of the SCOBY and the two bacteria was made to compare its composition. The presence of yeasts in SCOBY cause the delivery of ethanol and glucose in the broth. Unexpectedly, acetic acid was not detected in the broths of the acetic acid producing *Komagataeibacter* strains, which is probably related to its consumption or transformation into other substances.

All BC films were characterized using FTIR. The BC films had a heterogeneous structure, however, the mean spectra showed that, overall, they have a similar structure compared to films reported in the literature.

Based on the experimental design and characterization, SCOBY was chosen to proceed with a functionalization in which it was intended to enhance the electrical properties of the obtained BC. It was crosslinked with BTCA, then functionalized with different amount of CNT and covered with waxy maize for protection. The electrical resistance of the surface of the functionalized BC was then measured. It was found that the value of resistivity was in the range of functionalized BC reported earlier.

This makes it promising to conduct further studies on the possibility of applying BC in electronics. It is possible to study other ways of functionalization BC to compare and find the best conditions to acquire the BC with electrical properties. Moreover, it is possible to enhance the production of BC produced by pure bacteria, functionalize and compare it to functionalized BC produce by SCOBY.

References

[1] Nechyporchuk O, Belgacem MN & Bras J, 2016. Production of cellulose nanofibrils: A review of recent advances. Industrial Crops and Products 93: 2–25.

[2] Klemm D, Heublein B, Fink HP & Bohn A, 2005. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angewandte Chemie International Edition 44: 3358–3393.

[3] Kaminski K, Jarosz M, Grudzien J, Pawlik J, Zastawnik F, Pandyra P & Kołodziejczyk AM, 2020. Hydrogel bacterial cellulose: a path to improved materials for new eco-friendly textiles. Cellulose 27: 5353–5365.

[4] Thoorens G, Krier F, Leclercq B, Carlin B & Evrard B, 2014. Microcrystalline cellulose, a direct compression binder in a quality by design environment—A review. International Journal of Pharmaceutics 473: 64–72.

[5] Goetmar G, Zhou D, Stanley BJ & Guiochon G, 2004. Heterogeneous Adsorption of 1-Indanol on Cellulose Tribenzoate and Adsorption Energy Distribution of the Two Enantiomers. Analytical Chemistry 76: 197 – 202.

[6] Linder AP, Bergman R, Bodin A & Gatenholm P, 2003. Mechanism of Assembly of Xylan onto Cellulose Surfaces. Langmuir 19: 5072 – 5077.

[7] Chawla PR, Bajaj IB, Survase SA & Singhal RS, 2009. Fermentative Production of Microbial Cellulose, Food Technology and Biotechnology 47: 107–124.

[8] Rebelo AR, Archer AJ, Chen XL, Liu CQ, Yang G & Liu Y, 2018. Dehydration of bacterial cellulose and the water content effects on its viscoelastic and electrochemical properties. Science and Technology of Advanced Materials 19: 203-211.

[9] Bishop C, 2007. Vacuum deposition onto webs, films, and foils, Elsevier.

[10] Elham EA & Amir KA, 2013. Influence of Fermentation Condition and Alkali Treatment on the Porosity and Thickness of Bacterial Cellulose Membranes. The Online Journal of Science and Technology 3: 194-203.

[11] Seifert M, Hesse S, Kabrelian V & Klemm D, 2004. Controlling the water content of never dried and reswollen bacterial cellulose by the addition of water-soluble polymers to the culture medium. Journal of Polymer Science, Part A: Polymer Chemistry 42: 463–470.

[12] Emiljanowicz KE & Malinowska-Pańczyk E, 2019. Kombucha from alternative raw materials – The review. Food Science and Nutrition 60: 3185-3194.

[13] Sharma C & Bhardwaj NK, 2019. Biotransformation of fermented black tea into bacterial nanocellulose via symbiotic interplay of microorganisms. International Journal of Biological Macromolecules 132: 166-177.

[14] Payen A, 1838. Memoir on the composition of the tissue of plants and of woody material. Comptes Rendus 7:1052–1056.

[15] Wang J, Tavakoli J & Tang Y, 2019. Bacterial cellulose production, properties and applications with different culture methods – A review. Carbohydrate Polymers 219: 63-76.

[16] Khivetin VV & Ginzburg LN, 1993. Flax at the turn of XX and XXI, IPO "Polygran", Russia.

[17] Plomion C, Leprovost G & Stokes A, 2001. Wood formation in trees. Plant Physiology 124: 1513-1523.

[18] Brown AJ, 1886. On an acetic ferment which forms cellulose. Journal of the Chemical Society Transactions 49: 432–439.

[19] Barsha J & Hibbert H, 1934. Structure of the cellulose synthesized by the action of *Acetobacter xylinus* on fructose and glycerol. Canadian Journal of Research 10: 170-179.

[20] Schrecker S & Gostomski P, 2005. Determining the water holding capacity of microbial cellulose. Biotechnology Letters 27: 1435–1438.

[21] Yamamoto H & Horii F, 1994. In Situ crystallization of bacterial cellulose I. Influence of polymeric additives, stirring and temperature on the formation celluloses Iα and Iβ as revealed by cross polarisation/magic angle spinning (CP/MAS) 13C NMR spectroscopy. Cellulose 1: 57-66.

[22] Jonas R & Farah LF, 1998. Production and application of microbial cellulose. Polymer Degradation and Stability 59: 101–106.

[23] Laureys D, Britton SJ & Clippeleer JD, 2020. Kombucha Tea Fermentation: A Review. Journal of the American Society of Brewing Chemists 78: 165-174.

[24] Naritomi T, Kouda TH & Yoshinaga F, 1998. Effect of ethanol on bacterial cellulose production from fructose in continuous culture. Journal of Fermentation and Bioengineering 85: 598–603.

[25] Czaja W, Romanovicz D & Brown RM, 2004. Structural investigations of microbial cellulose produced in stationary and agitated culture. Cellulose 11: 403-411.

[26] Mohite BV, Salunke BK & Patil SV, 2013. Enhanced Production of Bacterial Cellulose by Using *Gluconacetobacter hansenii* NCIM 2529 Strain Under Shaking Conditions. Applied Biochemistry and Biotechnology 169: 1497–1511.

[27] Gu J & Catchmark JM, 2012. Impact of hemicelluloses and pectin on sphere-like bacterial cellulose assembly. Carbohydrate Polymers 88: 547-557.

[28] Watanabe K, Tabuchi M & Morinaga Y, 1998. Structural features and properties of bacterial cellulose produced in agitated culture. Cellulose 5: 187.

[29] Hu Y, Catchmark JM & Vogler EA, 2013. Factors Impacting the Formation of Sphere-Like Bacterial Cellulose Particles and Their Biocompatibility for Human Osteoblast Growth. Biomacromolecules 14: 3444-3452.

[30] Masaoka S, Ohe T & Sakota N, 1993. Production of cellulose from glucose by *Acetobacter xylinum.* Journal of Fermentation and Bioengineering 75: 18–22.

[31] Noro N, Sugano Y & Shoda M, 2004. Utilization of the buffering capacity of corn steep liquor in bacterial cellulose production by *Acetobacter xylinum*. Applied Microbiology and Biotechnology 64: 199–205.
[32] Goh WN, Rosma A, Kaur B, et al., 2012. Fermentation of black tea broth (Kombucha): I. Effects of sucrose concentration and fermentation time on the yield of microbial cellulose. International Food Research Journal 19: 109-117.

[33] Rangaswamy BE, Vanitha KP & Hungund BS, 2015. Microbial cellulose production from bacteria isolated from rotten fruit. International journal of polymer science 2015: 280784.

[34] Mikkelsen D, Flanagan BM, Dykes GA & Gidley MJ, 2009. Influence of different carbon sources on bacterial cellulose production by *Gluconacetobacter xylinus* strain ATCC 53524. Journal of Applied Microbiology 107: 576-583.

[35] Pourramezan, GZ, Roayaei AM & Qezelbash QR, 2009 Optimization of culture conditions for bacterial cellulose production by *Acetobacter* sp. 4B-2. Biotechnology 8: 150-154.

[36] Son HJ, Heo MS, Kim YG & Lee SJ, 2001. Optimization of fermentation conditions for the production of bacterial cellulose by a newly isolated *Acetobacter* sp. A9 in shaking cultures. Biotechnology and Applied Biochemistry 33: 1–5.

[37] Aswini K, Gopal NO & Uthandi S, 2020. Optimized culture conditions for bacterial cellulose production by *Acetobacter senegalensis* MA1. BMC Biotechnology 20: 46.

[38] Yang YK, Park SH, Hwang JW, Pyun YR & Kim YS, 1998. Cellulose production by *Acetobacter xylinum* BRC5 under agitated condition. Journal of Fermentation and Bioengineering 85: 312–317.

[39] Jia S, Ou H, Chen G, Choi D, Cho K, Okabe M & Cha WS, 2004. Cellulose production from *Gluconobacter oxydans* TQ-B2. Biotechnology and Bioprocess Engineering 9: 166–170.

[40] Nguyen VY, Flanagan B, Gidley MJ & Dykes GA, 2008. Characterization of cellulose production by a G*luconacetobacter xylinus* strain from kombucha. Current Microbiology 57: 449–453.

[41] Shuler M & Kargı F, 1992. Bioprocess Engineering: Basic Concepts. Prentice Hall. New Jersey.

[42] Hestrin S & Schramm M, 1954. Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. Biochemical Journal 58: 345-352.

[43] Silva R, Santa Brígida AI, Rosa MF, Silva Neto RM, Spinosa WA, Filho E & Figueirêdo MC, 2020. An approach for implementing ecodesign at early research stage: a case study of bacterial cellulose production. Journal of Cleaner Production 269: 122-245.

[44] Mohammadkazemi F, Doosthoseini K & Azin M, 2015. Effect of ethanol and medium on bacterial cellulose (BC) production by *Gluconacetobacter xylinus* (PTCC 1734). Cellulose Chemistry and Technology 49: 455–462.

[45] Jayabalan R, Malbaša RV, Lončar ES, Vitas JS & Sathishkumar M, 2014. A Review on Kombucha Tea—Microbiology, Composition, Fermentation, Beneficial Effects, Toxicity, and Tea Fungus. Food Science and Food Safety 13: 538-550.

[46] Jozala AF, Lencastre-Novaes LC, Lopes AM, de Carvalho Santos-Ebinuma V, Mazzola PG, Pessoa-Jr A & Chaud MV, 2016. Bacterial nanocellulose production and application: a 10-year overview. Applied Microbiology and Biotechnology 100: 2063–2072.

[47] Czaja W, Krystynowicz A, Bielecki S & Brown Jr. RM, 2006. Microbial cellulose – The natural power to heal wounds. Biomaterials 27:145–151.

[48] Johnson DC & Neogi AN, 1989. Sheeted products formed from reticulated microbial cellulose. US patent 4863565.

[49] Bäckdahl H, Helenius G, Bodin A, Nannmark U, Johansson BR, Risberg B & Gatenholm P, 2006. Mechanical properties of bacterial cellulose and interactions with smooth muscle cells. Biomaterials 27: 2141–2149.

[50] Gong K, Rehman IU & Darr JA, 2008. Characterization and drug release investigation of amorphous drug–hydroxypropyl methylcellulose composites made via supercritical carbon dioxide assisted impregnation. Journal of Pharmaceutical and Biomedical Analysis 48: 1112–1119.

[51] Hu W, Chen S, Yang J, Li Z & Wang H, 2014. Functionalized bacterial cellulose derivatives and nanocomposites. Carbohydrate Polymers 101:1043–1060

[52] Budhiono A, Rosidi B, Taher H & Iguchi M, 1999. Kinetic aspects of bacterial cellulose formation in nata-de-coco culture system. Carbohydrate Polymers 40: 137–143.

[53] Stagg, GV & Millin DJ, 1975. The nutritional and therapeutic value of tea - a review. Journal of the Science of Food and Agriculture 26: 1439–1459.

[54] Padrao J., Gonçalves S, Silva JP, Sencadas V, Lanceros-Méndez S, Pinheiro AC, Vicente AA, Rodrigues LR & Dourado F, 2016. Bacterial cellulose-lactoferrin as an antimicrobial edible packaging. Food Hydrocolloids 58: 126-140.

[55] Mautner A, Lee KY, Tammelin T, Mathew AP, Nedoma AJ, Li K & Bismarck A, 2015. Cellulose nanopapers as tight aqueous ultrafiltration membranes. Reactive and Functional Polymers 86: 209–214.

[56] Karakawa M, Chikamatsu M, Nakamoto C, Maeda Y, Kubota S, & Yase K, 2007. Organic Light-Emitting Diode Application of Fluorescent Cellulose as a Natural Polymer. Macromolecular Chemistry and Physics 208: 2000–2006.

[57] Tischer PC, Sierakowski MR, Westfahl H & Tischer CA, 2010. Nanostructural reorganization of bacterial cellulose by ultrasonic treatment. Biomacromolecules 11: 1217–1224.

[58] Paximada P, Dimitrakopoulou EA, Tsouko E, Koutinas AA, Fasseas C & Mandala IG, 2016. Structural modification of bacterial cellulose fibrils under ultrasonic irradiation. Carbohydrate Polymers 150: 5–12.

[59] Molina de Olyveira G, Maria Manzine Costa L & Basmaji P, 2013. Physically modified bacterial cellulose as alternative routes for transdermal drug delivery. Journal of Biomaterials and Tissue Engineering 3: 227–232.

[60] Gea S, Reynolds CT, Roohpour N, Wirjosentono B, Soykeabkaew N & Bilotti E, 2011. Investigation into the structural, morphological, mechanical and thermal behaviour of bacterial cellulose after a two-step purification process. Bioresource Technology 102: 9105–9110.

[61] Pillay V, Danckwerts MP, Bhatt R, Setshedi R & Patel R, 2005. Preparation of Hydroxypropylcellulose Membranes using Slow Ionotropic Reactions Configured in an Experimental Design. Journal of Bioactive and Compatible Polymers 20: 395–414.

[62] Shukla S, Brinley E, Cho HJ & Seal S, 2005. Electrospinning of hydroxypropyl cellulose fibers and their application in synthesis of nano and submicron tin oxide fibers. Polymer 46: 1213–1214.

[63] Chang C & Zhang L, 2011. Cellulose-based hydrogels: Present status and application prospects. Carbohydrate Polymers 84: 40-53.

[64] Feng J, Shi Q, Li W, Shu X, Chen A & Xie X, 2014. Antimicrobial activity of silver nanoparticles in situ growth on TEMPO-mediated oxidized bacterial cellulose. Cellulose 21: 4557–4567.

[65] Lai F, Miao YE, Huang Y, Zhang Y & Liu T, 2016. Nitrogen-doped carbon nanofiber/molybdenum disulfide nanocomposites derived from bacterial cellulose for high-efficiency electrocatalytic hydrogen evolution reaction. ACS Applied Materials & Interfaces 8: 3558–3566.

[66] Kim SS, Jeon JH, Kim HI, Kee CD & Oh IK, 2015. High-fidelity bioelectronic muscular actuator based on graphene-mediated and TEMPO-oxidized bacterial cellulose. Advanced Functional Materials 25: 3560–3570.

[67] Pavliňák D, Švachová V, Vojtek L, Zarzycká J, Hyršl P, Alberti M & Vojtová L, 2014. Plasma-chemical modifications of cellulose for biomedical applications. Open Chemistry 13: 229–235.

[68] Foresti ML, Vázquez A & Boury B, 2017. Applications of bacterial cellulose as precursor of carbon and composites with metal oxide, metal sulfide and metal nanoparticles: A review of recent advances. Carbohydrate Polymers 157: 447–467.

[69] Sai H, Fu R, Xing L, Xiang J, Li Z & Li F, 2015. Surface modification of bacterial cellulose aerogels: Web-like skeleton for oil/water separation. Applied Materials & Interfaces 7: 7373–7381.

[70] Lee KY, Quero F, Blaker J, Hill CS, Eichhorn S & Bismarck A, 2011. Surface only modification of bacterial cellulose nanofibres with organic acids. Cellulose 18: 595–605.

[71] Jiang F & Hsieh YL, 2017. Cellulose Nanofibril Aerogels: Synergistic Improvement of Hydrophobicity, Strength, and Thermal Stability via Cross-Linking with Diisocyanate. ACS Applied Materials & Interfaces 9: 2825–2834.

[72] Yang CQ & Qilie W, 1996. Formation of Cyclic Anhydride Intermediates and Esterification of Cotton Cellulose by Multifunctional Carboxyl Acids, An IR Study. Textile Research Journal 66: 595–603.

[73] Roy D, Semsarilar M, Guthrie JT & Perrier S, 2009. Cellulose modification by polymer grafting: a review. Chemical Society Reviews 38: 2046-2064.

[74] Yamanaka S & Sugiyama J, 2000. Structural modification of bacterial cellulose. Cellulose 7: 213–225.

[75] Hu W, Chen S, Yang J, *et al*, 2014. Functionalized bacterial cellulose derivatives and nanocomposites. Carbohydrate Polymers 101: 1043-1060.

[76] Shah N, Ul-Islam M & Parka JK, 2013. Overview of Bacterial Cellulose Composites: A Multipurpose Advanced Material. Carbohydrate Polymers 98: 1585– 1598.

[77] Ul-Islam M, Shah MN, Ha JH & Park JK, 2013. Effect of chitosan penetration on physico-chemical and mechanical properties of bacterial cellulose. Korean Journal of Chemical Engineering 28: 1736-1743.

[78] Cai ZJ & Yang G, 2011. Bacterial Cellulose/Collagen Composite: Characterization and First Evaluation of Cytocompatibility. Journal of Applied Polymer Science 120: 1736-1743.

[79] Hong L, 2006. Hydroxyapatite/bacterial cellulose composites synthesized via a biomimetic route. Materials Letters 60: 1710-1713.

[80] Lopes TD, Riegel-Vidotti IC, Grein A, *et al*, 2014. Bacterial cellulose and hyaluronic acid hybrid membranes: Production and characterization. International Journal of Biological Macromolecules 67: 401-408.

[81] Yang G, Xie J, Deng Y, *et al*, 2012. Hydrothermal synthesis of bacterial cellulose/AgNPs composite: A "green" route for antibacterial application. Carbohydrate Polymers 87: 2482-2487.

[82] Barud HS, 2015. Biocellulose-based flexible magnetic paper. Journal of Applied Physics 117: 17-34.

[83] Wang W, 2011. Amperometric hydrogen peroxide biosensor based on the immobilization of heme proteins on gold nanoparticles-bacteria cellulose nanofibers nanocomposite. Talanta 84: 71-77.

[84] Leite LAS, 2021. Isolamento e identificação molecular de bactérias e leveduras de SCOBY e sua aplicação na produção de celulose bacteriana. Degree report, Licenciatura em Biologia e Biotecnologia, Escola Superior Agrária do Instituto Politécnico de Bragança. 25 pp.

[85] Mandenius CF, & Brundin A, 2008. Bioprocess optimization using design-of-experiments methodology. Biotechnology Progress 24: 91–1203.

[86] Silva LC, de Souza Lago H, Rocha MOT, *et al*, 2021. Craft Beers Fermented by Potential Probiotic Yeast or Lacticaseibacilli Strains Promote Antidepressant-Like Behavior in Swiss Webster Mice. Probiotics and Antimicrobial Proteins 13: 698–708.

[87] Amarasekara AS, Wang D & Grady TL, 2020. A comparison of kombucha SCOBY bacterial cellulose purification methods. SN Applied Sciences 2: 240.

[88] Taverniers I, De Loose M & Van Bockstaele E, 2004. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. Trends in Analytical Chemistry 23: 535–552

[89] Uncu O, Ozen B & Tokatli F, 2019 Use of FTIR and UV–visible spectroscopy in determination of chemical characteristics of olive oils. Talanta 201:65-73.

[90] Šauperl O, Stana-Kleinschek K, & Ribitsch V, 2009. Cotton Cellulose 1, 2, 3, 4 Buthanetetracarboxylic Acid (BTCA) Crosslinking Monitored by some Physical—chemical Methods. Textile Research Journal 79: 780–791.

[91] Yoon SH, Jin HJ, Kook MC, & Pyun YR, 2006. Electrically Conductive Bacterial Cellulose by Incorporation of Carbon Nanotubes. Biomacromolecules 7: 1280–1284.

[92] Dastidar TG & Netravali A, 2013. Cross-Linked Waxy Maize Starch-Based "Green" Composites. ACS Sustainable Chemistry & Engineering 1: 1537–1544.

[93] Abdel-Banat BMA, Hoshida H, Ano A, Nonklang S & Akada, R, 2009. High-temperature fermentation: how can processes for ethanol production at high temperatures become superior to the traditional process using mesophilic yeast? Applied Microbiology and Biotechnology 85: 861–867.

[94] Jayabalan R, Marimuthu S & Swaminathan K, 2007. Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. Food Chemistry 102: 392–398

[95] Volova TG, Prudnikova, SV, Sukovatyi AG & Shishatskaya EI, 2018. Production and properties of bacterial cellulose by the strain *Komagataeibacter xylinus* B-12068. Applied Microbiology and Biotechnology 102: 7417–7428.

[96] Hwang JW, Yang YK, Hwang JK, Pyun YR & Kim YS, 1999. Effects of pH and dissolved oxygen on cellulose production by *Acetobacter xylinum* BRC5 in agitated culture. Journal of Bioscience and Bioengineering 88: 183–188.

[97] Jung JK & Park HN, 2005. Bacterial cellulose production by *Gluconoacetobacter hansenii* in an agitated culture without living non-cellulose producing cells. Enzyme and Microbial Technology 37: 347–354

[98] Park JK, Jung JY & Park YH, 2003. Cellulose production by *Gluconacetobacter hansenii* in a medium containing ethanol. Biotechnology Letters 25: 2055–2059.

[99] Son HJ, Kim HG, Kim KK, Kim HS, Kim YG & Lee SJ, 2003. Increased production of bacterial cellulose by *Acetobacter* sp. V6 in synthetic media under shaking culture conditions. Bioresource Technology 86: 215–219.

[100] Bae S & Shoda M, 2004. Bacterial cellulose production by fed-batch fermentation in molasses medium. Biotechnology Progress 20: 1366–1371.

[101] Bae Y & Sugano M, 2004. Improvement of bacterial cellulose production by addition of agar in a jar fermentor. Journal of Bioscience and Bioengineering 97: 33–38.

[102] Chao Y, Ishida T, Sugano Y & Shoda M, 2000. Bacterial cellulose production by *Acetobacter xylinum* in a 50L internal-loop airlift reactor. Biotechnology and Bioengineering 68: 345–352.

[103] Krystynowicz A, Czaja W, Wiktorowska-Jezierska A, Gonçalves-Miœkiewicz M, Turkiewicz M & Bielecki S, 2002. Factors affecting the yield and properties of bacterial cellulose. Journal of Industrial Microbiology and Biotechnology 29: 189–195.

[104] Keshk S & Sameshima K, 2006. Influence of lignosulfonate on crystal structure and productivity of bacterial cellulose in a static culture. Enzyme and Microbial Technology 40: 4–8.

[105] Zhou LL, Sun DP, Hu LY, Li YW & Yang JZ, 2007. Effect of addition of sodium alginate on bacterial cellulose production by *Acetobacter xylinum*. Journal of Industrial Microbiology and Biotechnology 34: 483–489

[106] Keshk & Sameshima K, 2006. The utilization of sugar cane molasses with/without the presence of lignosulfonate for the production of bacterial cellulose. Applied Microbiology and Biotechnology 72: 291–296.

[107] Kim SY, Kim JN, Wee YJ, Park DH & Ryu HW, 2006. Production of bacterial cellulose by *Gluconacetobacter* sp. RKY5 isolated from persimmon vinegar. Biotechnology and Applied Biochemistry 131: 705–715.

[108] Seto A, Saito Y, Matsushige M, Kobayashi H, Sasaki Y, Tonouchi N, Tsuchida T, Yoshinaga F, Ueda K & Beppu T, 2006. Effective cellulose production by a coculture of *Gluconacetobacter xylinus* and *Lactobacillus mali*. Applied Microbiology and Biotechnology 73: 915–921.

[109] Güzel M. & Akpınar Ö, 2018. Production and Characterization of Bacterial Cellulose from Citrus Peels. Waste and Biomass Valorization 10: 2165-2175

[110] Park JK, Park YH & Jung JY, 2003. Production of bacterial cellulose by *Gluconacetobacter hansenii* PJK isolated from rotten apple. Biotechnology and Bioprocess Engineering 8: 83–88.

[111] Wan YZ, Hong L, Jia SR, Huang Y, Zhu Y, Wang YL & Jiang HJ, 2006. Synthesis and characterization of hydroxyapatite–bacterial cellulose nanocomposites. Composites Science and Technology 66: 1825–1832.

[112] Yang CQ, Lan X, Shiqi L & Yanqiu J, 1998. Nonformaldehyde Durable Press Finishing of Cotton Fabrics by Combining Citric Acid with Polymers of Maleic Acid. Textile Research Journal 68: 457–464.

[113] Choi HM, Welch CM & Morris NM, 1994. Nonphosphorus Catalysts for Formaldehyde-Free DP Finishing of Cotton with 1,2,3,4-Butanetetracarboxylic Acid. Textile Research Journal 64: 501–507.

[114] Müller D, 2012. Electrically conducting nanocomposites: preparation and properties of polyaniline (PAni)-coated bacterial cellulose nanofibers (BC). Cellulose 19: 1645-1654.

[115] Hu W, Chen S & Yang Z, 2011. Flexible electrically conductive nanocomposite membrane based on bacterial cellulose and polyaniline. Journal of Physical Chemistry B 115: 8453-8457.

[116] Müller D, 2016. Flexible PEDOT-nanocellulose composites produced by in situ oxidative polymerization for passive components in frequency filters. Journal of Materials Science: Materials in Electronics 27: 8062-8067.

[117] Khan S, Ul-Islam M & Khattak WA, 2015. Bacterial cellulose-poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) composites for optoelectronic applications. Carbohydrate Polymers 127: 86-9