UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS EXATAS E DE TECNOLOGIA
DEPARTAMENTO DE ENGENHARIA QUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

VANESSA MOLINA DE VASCONCELLOS

"Síntese de celulose bacteriana a partir de resíduos lignocelulósicos via evolução adaptativa"

São Carlos, SP – Maio de 2019

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“Synthesis of bacterial cellulose from lignocellulosic residues via adaptive laboratory evolution”

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Profa. Dra. Cristiane Sanchez Farinas

“One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don't throw it away”.

(Stephen Hawking)

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RESUMO

A celulose bacteriana (CB) é produzida, principalmente, pelo gênero de bactérias *Komagataeibacter*. Trata-se de um biopolímero livre de impurezas, construído a partir do entrelaçamento de nanofibras de celulose, que apresenta propriedades notáveis como: elevada resistência mecânica, alta capacidade de retenção de água, susceptibilidade a modificações químicas e biocompatibilidade. Tais singularidades tornam a CB um produto de grande interesse em diversas aplicações, que variam de aplicações nobres, como na área médica e farmacêutica, a menos sofisticadas, como material de reforço em polímeros. No entanto, para a síntese de CB é necessário que o meio de cultura seja rico em glicose, um dos pontos chave que encarecem o produto final. Neste contexto, a presente tese visa contribuir na redução dos custos envolvidos no processo de síntese de CB utilizando como substrato uma fonte de C alternativa: a fração líquida obtida a partir do pré-tratamento hidrotérmico de resíduos lignocelulósicos de palha de milho e bagaço de cana-de-açúcar gerado nas biorefinarias. Um desafio a ser superado para tal aplicação está relacionado à presença de inibidores no meio, e contornar esse entrave com a adição de uma etapa de detoxificação com carvão ativado, por exemplo, implica em um aumento no custo do processo. Assim, a alternativa proposta foi a obtenção de uma cepa produtora de celulose resistente aos inibidores, obtida através da técnica de evolução laboratorial adaptativa, utilizando a fração líquida de palha de milho como meio de cultura. O desenvolvimento da cepa de *K. hansenii* adaptada tornou possível converter diretamente uma forma diluída de glicose em CB, um nanomaterial de alto valor agregado. Enquanto a estabilidade genética, sua natureza e mecanismos por trás da síntese ainda precisam ser elucidados, a adaptação evolutiva desta bactéria produtora de nanocelulose não apenas tornou a cepa adaptada resistente aos inibidores, mas também capaz de produzir significativamente mais CB em comparação com a cepa original. Além disso, a CB da cepa adaptada manteve as mesmas propriedades das CB produzidas no meio de cultura padrão, indicando o potencial da estratégia proposta.

Palavras chave: celulose bacteriana, *Komagataeibacter hansenii*, resíduos lignocelulósicos, evolução laboratorial adaptativa.

ABSTRACT

Bacterial cellulose (BC) is produced mainly by the *Komagataeibacter* genus. BC is a biopolymer free of impurities, built from the interlacing of cellulose nanofibers, which presents remarkable properties, such as: high mechanical strength, high water retention capacity, susceptibility to chemical modifications and biocompatibility. The BC is a product of great interest in several biotechnological applications, ranging from noble applications, e.g. medical and pharmaceutical fields, to less sophisticated ones, e.g. reinforcement material in polymers. For the BC synthesis is necessary that the culture medium be rich in glucose, one of the key points that increase the final product. In this context, the present thesis aims to contribute to the reduction of costs involved in the process of CB synthesis using as substrate an alternative source of C: the liquid hot water (LHW) pretreated liquid fraction of lignocellulosic wastes (corn stover and sugarcane bagasse) produced in biorefineries. A challenge to be overcome is related to the presence of inhibitors in the medium, and bypassing this barrier with the addition of a detoxification step with activated charcoal, for example, implies an increase in process cost. The alternative presented in this work was to obtain an inhibitor resistant cellulose producing strain through the adaptive laboratory evolution technique, using the HLW pretreated liquid fraction from corn stover as culture medium. The development of the adapted *K. hansenii* strain makes possible to directly convert, and thereby concentrate, a dilute form of glucose into an insoluble, readily recovered and value-added cellulose product. While genetic stability, its nature and mechanisms behind the synthesis still need to be revealed, the evolutionary adaptation of this nanocellulose-producing bacteria not only made the bacterium resistant to inhibitors but also capable of producing significantly more nanocellulose compared to the original strain. In addition, the nanocellulose of the adapted strain maintained the same properties as those produced in the standard culture medium.

Keywords: bacterial cellulose, *Komagataeibacter hansenii*, lignocellulosic waste, adaptive laboratory evolution.

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AZEREDO, H., BARUD, H. S., FARINAS, C. S., VASCONCELLOS, V. M., & CLARO, A. M. (2019). Bacterial Cellulose as a Raw Material for Food and Food Materials Packaging Applications. **Frontiers in Sustainable Food Systems***, 3*, 7.

General Considerations

Goals and Overview

The goal of this thesis was to evaluate the viability of bacterial cellulose (BC) production by *Komagataeibacter hansenii* (ATCC 23769) using the residual sugars present in the liquid fraction of lignocellulosic materials pretreatment process.

Specific Goals

i. Develop a standard protocol for the synthesis, purification, drying and characterization of bacterial cellulose films;

ii. Evaluate the potential use of glucose and xylose for bacterial cellulose production and growth by the microorganism;

iii. Evaluate the toxicity level of the liquid fraction from hot liquid water pretreatment using two lignocellulosic substrates (sugar cane bagasse and corn stover);

iv. Investigate the toxicity of different individual phenolic compounds in the culture medium;

v. Apply the adaptive laboratory evolution technique to obtain a cellulose-producing bacterium resistant to lignocellulosic inhibitors and capable to use the sugars in the liquid fraction from hot liquid water pretreatment for the production of bacterial cellulose.

Chapters Overview

This thesis was organized in four chapters. The chapter 1 provides a brief introduction about the bacterial cellulose (BC) polymer, the producing microorganisms, how the biopolymer is synthesized, its characteristics and field of application, the bioprocesses that can

be applied for its synthesis, the alternative culture media that is being used, and it also describes the adaptive laboratory evolution technique as a tool to obtain adapted strains.

The chapter 2 reports on the development of a standard protocol for the whole BC production process. That includes a description of the experimental procedures and results regarding the bacterial cellulose synthesis, purification and drying steps as well as the physical-chemical characterization techniques, such as field emission scanning electron microscopy (FESEM) for morphological analysis, thermogravimetric analysis (TGA) for the evaluation of thermal degradation behavior, crystallinity characterization and chemical composition of the film by X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR), respectively.

The chapter 3 describes the experimental procedures and results related to the use of different carbon sources for the synthesis of BC. The main results of this chapter are about the use of the LHW pretreatment liquid fraction from sugarcane bagasse and corn stover. For this purpose, both liquid fractions were used without and with a detoxification step with activated charcoal. This chapter also presents the results of the use of glucose and xylose in different proportions as a source of carbon in the standard culture medium, analyzing the bacterial behavior and the production of the cellulose film in medium with different concentrations of C5 and C6 sugars. And also, the results of the addition of inhibitors in the standard culture medium using phenolic compounds such as: vanillin, tannic acid, ρ-coumaric acid and ferulic acid. The physical-chemical characterization of the all produced films is also presented in this chapter.

The chapter 4 reports on the adaptation of the bacteria to the inhibitors present in culture medium containing the liquid fraction from the liquid hot water pretreatment of corn stover. *K. hansenii* was successfully adapted to the medium containing phenolics inhibitors after 12 rounds of culturing experiments in their presence. The adapted strain was tested in different

culture media and the evolutionary adaptation of this nanocellulose-producing bacteria not only made the bacterium resistant to inhibitors, but also able to produce significantly more nanocellulose compared to the original strain. The physical-chemical characterization of the produced films is also presented in this chapter. Preliminary results about the bacteria adaptation in culture medium contain LHW liquid fraction from sugarcane bagasse and in a culture medium containing synthetic compounds based on the same liquid fraction are also presented here.

1. Bacterial Cellulose: a brief review

1.1 Origin of Bacterial Cellulose: A Source of Pure Cellulose

The cellulose is considered the most abundant biomacromolecule in the earth, it is a renewable source produced by almost all groups of living organisms. In nature, the plants are the main producers of cellulose, but it can be also synthesized by algae, tunicate (marine animal) and bacteria (Donini *et al.*, 2010). Vegetable cellulose is an intracellular polysaccharide that constitutes the cell wall of plants in a complex matrix intertwined with hemicellulose and lignin, its extraction involves chemical or enzymatic processes (Mosier *et al.*, 2005). Whereas cellulose produced by bacteria is a pure extracellular polysaccharide easy to obtain (Donini *et al.*, 2010).

The bacterial cellulose (BC) was initially observed in a fermented beverage, known as kombucha tea, and as dessert from Philippines called nata-de-coco. Kombucha is a popular beverage originating (perhaps) from northeast China and brought to Europe by Portuguese and Dutch explorers. The kombucha tea or “tea fungus” is a fermented beverage produced by a symbiotic relationship of acetic acid bacteria and yeast through the fermentation, using as substrate hot aqueous green or black tea extracts and sucrose. During the kombucha fabrication a cellulosic mat is produced at the air-liquid interface of the culture media, where a cell mass of bacteria and yeasts becomes attached. The dominant bacteria in kombucha culture include *Komagataeibacter xylinus*, and yeasts from the genera *Zygosaccharomyces*, *Schizosaccharomyces*, *Saccharomyces* (Gama *et al.*, 2016).

Nata-de-coco is a BC used as raw material for a traditional dessert from Philippines. Many similarities exist between kombucha and nata de coco fermentation system, regarding the microbial strains involved, culture conditions, fermentation kinetics, and product formation. The nata-de-coco fermentation BC is also formed using as substrate coconut water enriched with several carbohydrates and amino acids. The BC formed is cut in cubes and immersed in a

sugar syrup. Nata-de-coco flavor may have changed altering the source of culture medium, producing similar products e.g. nata-de-pina (from pineapple juice). Nowadays, a variety of nata-like products are being produced added as texture-rich cubes to beverages, yogurts, and jellies (Azeredo *et al.*, 2019).

1.2 Species of Cellulose-producing Bacteria

A variety of wild-type nata-de-coco from several regions of the Philippines was used as samples for the isolation of cellulose-producing microorganisms. The starter cultures in the fermentation process were composed of an *Acetobacter* strains mixed population. The 38 isolates obtained were characterized and differentiated based on different aspects, such as the pellicle type and colony morphology, carbon-utilization pattern (BiologTM assay), amount of cellulose production and 16S rRNA sequence (Bernardo *et al.*, 1998). The synthesis of a particular type of BC sheet by the interaction between the different types of *Acetobacter* strains is still uncertain. Different strains or their combinations may affect the BC production, not only its properties, e.g. a thick or a thin film or a soft or tough pellicle, but also its yields (Gama *et al.*, 2016).

Several species of bacteria and cyanobacteria have been reported to produce extracellular cellulose. These include strains from the genera *Acetobacter, Komagataeibacter, Agrobacterium, Aerobacter, Rhizobium and Gluconobacter* (Kaushal e Walker, 1951; Napoli et al., 1975; Matthysse et al., 1995; Römling, 2002; Hungund e Gupta, 2010). The production of cellulose by *Komagataeibacter xylinus* (former *Gluconacetobacter xylinus*) was discovered in 1886 by A. J. Brown, who observed that the microorganism produced cellulose in the presence of glucose and oxygen (Brown, 1886). The *Komagataeibacter xylinus* and *K. hansenii*, are known to be the highest cellulose producer, they are a wide group of strictly aerobic, Gram negative and acetic acid organisms endowed with the ability to oxidize a wide

variety of carbohydrates, alcohols, and sugar alcohol into acetic acid and other organic acids (such as gluconic, fumaric, citric, oxoacids, and ketones) and even amino acids. The *Komagataeibacter* genus has been the target of significant interest in academic and commercial exploitation. (Gama *et al.*, 2016).

Nowadays, the genetic manipulation of wild strains for development of a genetic engineered bacteria is a potential strategy that has been studied for enhancing BC production and yield, as well improve its properties (Islam *et al.*, 2017).

1.3 Bacterial Cellulose Biosynthesis

The *Komagataeibacter xylinum* is the bacterium model used to describe the synthesis of cellulose, due to its ability to produce cellulose from a wide range of carbon/nitrogen source. The BC is synthesized in two intermediary steps: i) the formation of 1,4-b-glucan chains and ii) the assembly and crystallization of cellulose chains (Lee *et al.*, 2014).

Cellulose-producing bacteria operate in the pentose-phosphate cycle or the Krebs cycle, depending on the physiological state of the cell coupled with gluconeogenesis (Ross *et al.*, 1991). The pentose-phosphate cycle involves the oxidation of carbohydrates and the Krebs cycle involves the oxidation of acetate-derived carbohydrates, fat, and proteins, such as oxalosuccinate and a-ketoglutarate (L. Nelson e M. Cox, 2012).

The BC biosynthesis is a strictly regulated process with multi-step reactions, involving several individual enzymes, catalytic complexes, and regulatory proteins. It contains four key enzymatic steps when glucose is used as carbon source; they are: i) phosphorylation of glucose by glucokinase ii) isomerization of glucose-6-phosphate (Glc-6-P) to glucose-1-phosphate (Glc-1-P) by phosphoglucomutase, iii) synthesis of UDPglucose (UDPGlc) by UDPG-pyrophosphorylase (UGPase), and iv) cellulose synthase reaction (Figure 1.1). UDPGlc, which is common in many organisms, is the direct cellulose precursor. UGPase is thought to play an

important role in cellulose synthesis since it is approximately 100 times more active in cellulose producers than that of noncellulose producing bacteria (Valla *et al.*, 1989).

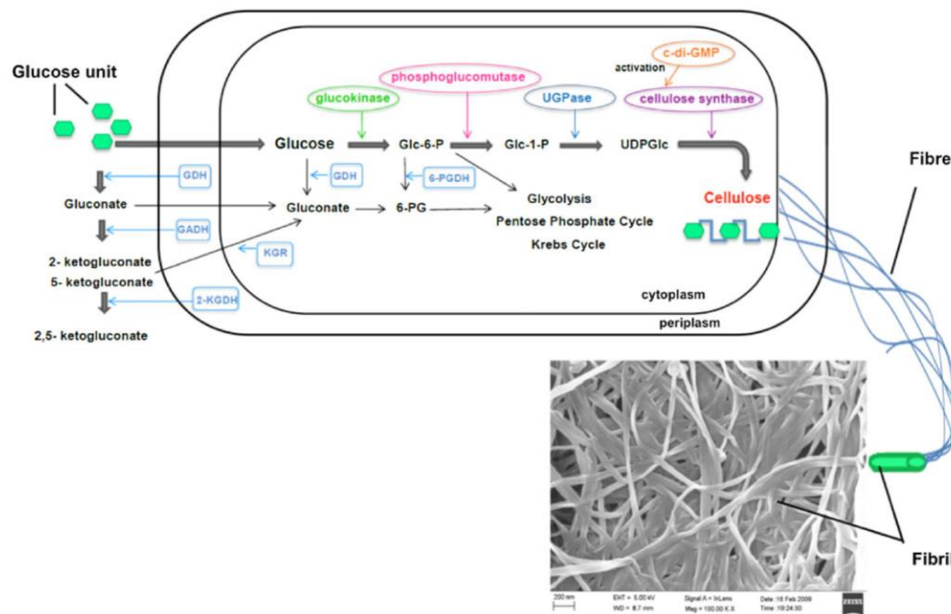


FIGURE 1.1A schematic showing the major metabolic pathways of *A. xylinum* and the assembly of cellulose molecules into nanofibrils. Obtained from (Lee et al., 2014) with permission provided by John Wiley and Soons and Copyright Clearance Center.

When disaccharides, such as sucrose and maltose, are used as carbon source for cellulose-producing bacteria, the biosynthesis of BC starts with the hydrolysis of disaccharides into monosaccharides, such as glucose and fructose. The cellulose chains are first synthesized inside the bacteria. The biosynthesis of cellulose is catalyzed by cellulose synthase, it is tightly bound to cytoplasmatic membrane and appears to be very unstable. Two hypotheses for this mechanism in *K. xylinum* have been reported, involving or not a lipid intermediate. Although pathways of UDPGlc are relatively well known, the molecular mechanisms of glucose polymerization into long and unbranched cellulose chains are still elusive to scientists (Valla *et al.*, 1989; Reiniati *et al.*, 2017). The assembly and crystallization of cellulose chains is the rate-limiting step (Chawla, Prashant R *et al.*, 2009).The unique structure and properties of

cellulose result from a course of extrusion of chains and their assembly outside the cell. These molecules are then spun through cellulose export components to form protofibrils, which are approximately 2–4 nm in diameter. A ribbon shaped microfibril of approximately 80 nm is assembled from these protofibrils(Chawla, P. R. *et al.*, 2009; Reiniati *et al.*, 2017)

1.4 The Bacterial Cellulose Polymer

Cellulose is composed of D-glucose units linked by (1→4) glycosidic bonds (L. Nelson e M. Cox, 2012). The polymorph of cellulose and its derivatives have been well documented. Six polymorphs of cellulose (I, II, III_1, III_2, IV_1 and IV_2) can be interconverted. Cellulose I, or native cellulose, is the form found in nature. Cellulose II, the second most extensively studied form, may be obtained from cellulose I by either of two processes: a) regeneration, which is the solubilization of cellulose I in a solvent followed by reprecipitation by dilution in water to give cellulose II, or b) mercerization, which is the process of swelling native fibers in concentrated sodium hydroxide. Celluloses III_1 and III_2 are formed, in a reversible process, from celluloses I and II, respectively, by treatment with liquid ammonia or some amines, and the subsequent evaporation of excess ammonia. Polymorphs IV_1 and may be prepared by heating celluloses III_1 and III_2 respectively (Osullivan, 1997). It is generally known that the cellulose I is a mix of two crystalline modifications I_α (triclinic) and I_β (monoclinic), the monoclinic form is thermodynamically stable, thus I_α can be transformed into cellulose I_β (Nelson e O'connor, 1964). Bacterial cellulose is a semi-crystalline material, its structure is composed of cellulose I_α and I_β. The I_α polymorph predominates in cellulose from bacterial origin, although after alkaline treatment I_α transforms, at least partially, to I_β (Tasker *et al.*, 1994; Wada *et al.*, 2001; Ford *et al.*, 2010). The difference in the α/β fraction in the polymer will affect the strength and solubility of the material (Wada *et al.*, 2001).

The X-ray diffraction technique has been used to evaluate the fine structure of BC, changes in microstructures, such as crystallinity (%), index cristalynite (IC), crystal size and identification of polymorphs. The peaks that indicate the presence of cellulose I are peak 1 (~15°) corresponds to the 100 plane of cellulose I_α or to the 110 plane of cellulose I_β, peak 2 (~17°) corresponds to the 010 plane of cellulose I_α or to the 110 plane of cellulose I_β and peak 3 (~23°) corresponds to the 110 plane of cellulose I_α or to the 200 plane of cellulose I_β (Wada *et al.*, 2001). Each diffracted peak presents only one contribution of the corresponding diffractions of the phase I_α and I_β, which is difficult to estimate due to overlapping of the planes reflections. The BC diffractograms also have the presence of the amorphous region (I_{am}~20°).

Bacterial NanoCellulose (BNC) has received substantial interest owing to its unique structural features and impressive physicomechanical properties. Using the full potential of BC requires knowledge of characteristic and properties of cellulose. The properties and application of bacterial cellulose as a membrane largely depend on the properties of the surface area. One of the basic methods of surface analysis are microscopic methods. The quality of the information obtained with microscopy is mainly dependent on resolution and contrast. The most common methods of surface characterization of bacterial cellulose are SEM (scanning electron microscopy), TEM (transmission electron microscopy), and AFM (atomic force microscopy). Each BNC nanofiber is a bundle of cellulose nanofibrils which are aggregates of extended cellulose chains (Gama *et al.*, 2016).

The contact angle, parameter crucial for the surface characterization, is connected with membrane surface chemistry (hydrophilicity, membrane surface charge) and with membrane–solute(s)–solvent interaction. By measuring the contact angle we can define useful polymer properties such as the ability of coating or adhesion properties of the surface (Gama *et al.*, 2016).

One of the most important analytical techniques for polymer characterization is infrared spectroscopy (IR), which is a technique based on the vibrations of the atoms of a molecule. Typical FTIR spectra of BNC present some specific bands, such as: the C─O─C chemical bonding appears at the wavenumber near 1160 and 900 cm^{-1}; strong peak that appears at 1060 and 1030 cm^{-1} are the indicative of C─O stretching at C3, C─C stretching, and C─O stretching at C6; the absorption peak of carbonyl groups (C═O) with intramolecular hydrogen bonds is also found at around 1650 cm^{-1}, the peaks of 3350 cm^{-1} and shouldering around 3400–3500 cm^{-1} indicates O─H stretching, 2800–2900 cm^{-1} indicates C─H stretching; A small peak that appears at 672 and 711 cm^{-1} corresponds to the out-of-plane bending of C─O─H. Besides that, there is also chemical bonding between carbon and hydrogen (C─H bonding). This bonding appears between 1430 and 1290 cm^{-1} wavenumber. The band that appears at 1635–1640 cm^{-1} has been attributed to the absorbed water bending vibrations present in cellulose. From the FTIR spectrum, it is possible to determine the crystallinity index of bacterial cellulose, its composites and estimate the polymorph fraction Iα/Iβ (Kataoka e Kondo, 1999; Bi *et al.*, 2014; Gama *et al.*, 2016).

The high purity and organized structure of bacterial cellulose (BC) provides remarkable properties for this biomaterial such as high thermal stability, crystallinity, degree of polymerization, mechanical strength, capacity for water retention and capacity of chemical modifications, being also biocompatible and biodegradable (Esa *et al.*, 2014; Islam *et al.*, 2017). These excellent properties make BC attractive for various biotechnological applications including biomedical and pharmaceutical applications, such as tissue regeneration, wound dressing, artificial skin, controlled drug realize (Ullah *et al.*, 2016), proteins immobilization (Kim *et al.*, 2017), as matrix and reinforcement material for nanocomposites with biotechnological applications (Cacicedo *et al.*, 2016) and optical, electronic and magnetic devices (Sabo *et al.*, 2016). Others attractive including cosmetic application (emulsion

stabilizer), food applications (Nata de Coco as a desert, food additive), sewage purification, pulp and paper applications (Iguchi *et al.*, 2000; Shah *et al.*, 2010; Ang *et al.*, 2012; Esa *et al.*, 2014).

1.5 Bioprocess Conditions for Bacterial Cellulose Production

The development of cost-effective production of BC involves the selection of bioprocess conditions. BC biosynthesis occurs in submerged cultures under static and dynamic conditions, in flasks or in bioreactors, the form of culture directly affects the structure of the cellulose formed. The cellulose is synthesized as a film in cultures under static condition, diverse shapes and sizes vessels can be used, so the BC membrane takes the shape of the object where it was grown. In static culture a film is formed at the liquid-air interface, as a set of crosslinked crystalline strands, which increases in thickness with increasing culture time. (Borzani e Desouza, 1995; Shah *et al.*, 2013; Esa *et al.*, 2014). The cultivation usually requires 5–20 days, until the BC sheet nearly fills the tray, since the BC production depends on the area of the air/liquid interface (Lin *et al.*, 2013). The cellulose membrane produced in the medium tends to entrap the bacteria, which limits their oxygen supply, and the nutrients are constantly consumed, so that their concentration decreases over time, which limits the BC production (Esa *et al.*, 2014). Possible explanations of the cellulose formation at the interface are that BC is formed as a self-defense mechanism to protect bacteria from the damaging effects of UV light or to help bacteria float in order to secure sufficient oxygen supply (Reiniati *et al.*, 2017). The traditional static culture is time demanding, with low productivity, which may hinder its industrial application (Lin *et al.*, 2013). Fed-batch cultivation is an interesting strategy to overcome this problem. A study conducted by Shezad *et al.* (2009) showed that the addition of new alternative medium culture during cultivation in a continuous process regime increased two to three times the yield process in fed-batch cultivation compared to batch cultivation.

Recently, bioreactors that produce BC in a higher yield under nearly static conditions have been developed to produce BC sheets–such as Horizontal Lift Reactor (Kralisch (Kralisch *et al.*, 2010), rotary biofilm contactor (Kim *et al.*, 2007), and aerosol bioreactor (Hornung *et al.*, 2007).

In agitated cultivation, oxygen is continuously mixed into the medium, so the BC is produced with enhanced yield compared with static culture, which contributes to cost reduction (Ul-Islam *et al.*, 2015). The agitated fermentation process may lead to several forms of cellulose, from fibrous suspension to spheres and pellets (Esa *et al.*, 2014). Although the BC yield in agitated culture is usually considered to be higher than in static culture, a major drawback of agitation is the increased probability of mutation of cellulose producing cells into cellulose-negative mutants under the high turbulence and shear stress (Park *et al.*, 2003; Kim *et al.*, 2007). New reactor designs have been proposed and supplementing the culture medium with ethanol has been reported to prevent the accumulation of those mutants, increasing BC production (Son *et al.*, 2001; Ul-Islam *et al.*, 2015).

For commercial production, large scale, semi-continuous, or continuous fermentation methods are more suitable to meet the demand. The maximum production of BC is always the objective, however the properties for the intended application needs to be followed (Lin *et al.*, 2013). The effects of cultivation type (static or agitated; batch, or fed batch process), the bacterial strain and the culture medium need to be carefully evaluated in order to obtain BC with yields high enough to improve its feasibility for the selected application.

The control of process variables is considered as key factor toward optimizing BC syntheses, in order to improve BC yield, the most important process variables that should be optimized and controlled during the cultivation process includes pH, oxygen supply, and temperature (Lee *et al.*, 2014; Islam *et al.*, 2017). Optimum pH for cell growth and BC production depends on the bacterial strain, being usually between 4.0 and 7.0 (Reiniati *et al.*,

2017). The pH of the culture medium could vary during cultivation due to the accumulation of secondary metabolites from the consumption of sugars and nitrogen sources (e.g. gluconic, acetic, or lactic acids) (Lee *et al.*, 2014). The control of the temperature during cultivation for BC production is also important, since it can have an effect on BC yield and properties as well. The effect of temperature on BC production by *Komagataeibacter xylinus* showed that the optimal temperature range for cell growth and BC production is around 28–30◦C, while the physiological temperature range is wide (20–37◦C) (Volova *et al.*, 2018) Aeration is another important process variable to control, because the BC-producing bacteria are highly aerobic, so a suitable oxygen supply is crucial (Wu e Li, 2015). However, while low dissolved oxygen content hinders bacteria growth and BC production, a high oxygenation can favor the production of gluconic acid (Lee *et al.*, 2014; Islam *et al.*, 2017)

To improve the yield of CB production, many researches are being carried out focusing on the isolation of overproductive strains, optimization of cultivation conditions (pH control, by-product control, variations in medium supplementation conditions, varied carbon sources and transport of oxygen) and also in the development of modified bioreactors (Donini *et al.*, 2010; Shah *et al.*, 2013; Esa *et al.*, 2014; Lee *et al.*, 2014)

The process to obtaining BC after culture involves two steps: the physical separation of the BC produced from the culture medium and the chemical purification of the biopolymer. Nevertheless, the downstream processing for BC is usually easier and cheaper when compared to the procedures required to purify plant derived cellulose.

The BC can be removed from the culture medium applying simple procedures: for static cultures the BC film produced can be simply harvested, and for the agitated cultivation the BC can be removed by filtration or centrifugation. The recovered BC from the broth contains some impurities like remaining cells and nutrients, thus it needs to be purified before application. The most widely used procedure for BC purification is the alkali treatment, some care should

be taken with the alkali treatment severity because concentrated solutions may transform the cellulose polymorph and, consequently, modify its mechanical properties (Chawla, P. R. *et al.*, 2009; Reiniati *et al.*, 2017).

The downstream processing of BC can also include a drying step. There are different methods of drying the BC including drying at room temperature, oven drying, freeze drying, and supercritical drying. The drying process of BC changes its characteristics and properties; thus, it should be chosen according to the final application of the material (Zeng, Laromaine e Roig, 2014; Vasconcellos e Farinas, 2018). It is important to note that choice of BC downstream processing can impact the final characteristics and price of the material, and therefore should be selected based on its desired application.

1.6 Biosynthesis of cellulose from various carbon source

The optimal design of the medium is very important for the growth of microorganism and thus stimulating the formation of products. Macro- and micronutrients required for the growth of a microorganism are carbon, nitrogen, phosphorus, sulphur, potassium and magnesium salts. Secretion of cellulose is usually most noticeable when the bacteria are supplied with an abundant carbon source and a minimal nitrogen source (Chawla, P. R. *et al.*, 2009).

BC production requires a glucose-rich culture medium. Although other carbohydrates such as fructose, maltose, xylose, starch and glycerol also been tried, usually, glucose and sucrose are used as carbon sources for cellulose production, resulting in high production costs, which limits BC potential applications. (Chawla, Prashant R *et al.*, 2009; Hungund *et al.*, 2013). The conventional culture medium used for BC production is the Hestrin and Schramm (HS) medium, which contains glucose, peptone, yeast extract, citric acid and sodium phosphate dibasic as carbon, and nitrogen sources (Hestrin e Schramm, 1954; Cacicedo *et al.*, 2016).

The *Komagataeibacter* genus are widely used for BC production, due to its capacity to use a wide range of carbon/nitrogen sources (Lee *et al.*, 2014; Islam *et al.*, 2017). In order to address this high cost limitation, the use of alternative culture media for BC production has been investigated, including the use of fruit juices (Kurosumi *et al.*, 2009; Hungund *et al.*, 2013), sugar cane molasse (Keshk, S. e Sameshima, K., 2006; Tyagi e Suresh, 2016), brewery waste(Shezad *et al.*, 2010), among others (Islam *et al.*, 2017). The evaluation of fruit juices including orange, pineapple, apple, Japanese pear, and grape for BC production by *Acetobacter xylinum* NBRC 13693 showed that orange and Japanese pear juices were suitable medium for BC production, resulting in increased yields of BC (Kurosumi *et al.*, 2009). Other examples of fruit juice as culture medium, including pineapple, pomegranate, muskmelon, water melon, tomato, orange, and also molasses, sugarcane juice, coconut water, coconut milk have been also used as alternative carbon sources for bacterial cellulose production (Hungund *et al.*, 2013).

Carbohydrates present in lignocellulose-derived liquid process streams as well as hydrolysates have the potential to be used as feedstock for bacterial cellulose production (Castro, C. *et al.*, 2011; Kiziltas *et al.*, 2015). Formation of bacterial nanocellulose has been reported from konjac power (Hong e Qiu, 2008), waste fibers from pulp and paper mill (Cavka *et al.*, 2013), spruce wood (Guo *et al.*, 2013), sugarcane bagasse (Cheng *et al.*, 2016) and corn stalk (Cheng *et al.*, 2017). However, the pretreatment process of lignocellulosic biomass generates compounds which are inhibitors of biochemical reactions, including furan aldehydes (e.g. furfural and 5-hydroxymethyl-furfural), aliphatic acids (e.g. acetic and formic acid), phenolics and other aromatic compound derived from lignin degradation (Cao *et al.*, 2013; Cao *et al.*, 2015; Kim *et al.*, 2016; Michelin *et al.*, 2016); (Ximenes *et al.*, 2010; 2011; Ximenes *et al.*, 2017). Thus, the addition of a detoxification step for the removal of the inhibitors derived from the pretreatment of lignocellulosic is required.

1.7 Adaptative laboratorial evolution: a natural mutation selection methodology

Adaptive laboratory evolution (ALE) is a methodology of natural selection that has been used with great success for different biotechnological applications, improving yields and reducing production costs (Brooks *et al.*, 2011). In contrast to rational engineering strategies and directed modification of specific enzymes, ALE has the advantage of letting nonintuitive beneficial mutations occur in many different genes and regulatory regions in parallel. The most prominent organisms that have been studied for ALE towards nutrient sources and environmental stresses are *Escherichia coli* and *Saccharomyces cerevisiae* (Dragosits e Mattanovich, 2013).

ALE can be a tool for achieve latent pathway activation, for phenotype optimization and for environmental adaptation. ALE used as tool for environmental adaptation is usually done by gradually reducing the amount of supplements from passage to passage while cells remain in the exponential phase. The growth environment is changing with each passage, forcing cells to adapt and to reduce their dependency on the supplement, and subsequently reroute their metabolic and regulatory network (Portnoy *et al.*, 2011).

Adaptive laboratory evolution may be designed to enhance bacterial tolerance to inhibitors. Examples include improvement of fitness of *Klebsiella pneumoniae* with respect to 2,3 butanediol accumulation (Li *et al.*, 2016), evolution of cytochrome P450 BM-3 monoxygenase to organic cosolvents used in hydroxylation and epoxidation reactions (Seng Wong *et al.*, 2004), and improvement of xylose utilization by *Corynebacterium glutamicum* (Radek *et al.*, 2017). Other applications include the development of antibiotic overproduction by Streptomyces clavuligerus (Charusanti *et al.*, 2012), and enhancement of tolerance of the ethanologenic yeasts *Scheffersomyces stipites* (Slininger *et al.*, 2015) and *Saccharomyces cerevisiae* to lignocellulosic hydrolysates for ethanol biofuel production (Almario *et al.*, 2013).

1.8 Lignocellulosic biomass and Liquid Hot Water Pretreatment

Lignocellulosic biomass from agriculture and forestry, which includes agro-industrial residues, forest-industrial residues, energy crops, and other materials, is the most abundant bioresource to consider as feedstock for biorefineries (Jönsson e Martín, 2016; Dos Santos *et al.*, 2018).

Plant cell walls consist primarily of cellulose (20–50% on a dry weight basis), hemicellulose (15–35%), and lignin (10–30%). In order to effectively process this complex lignocellulosic structure through biochemical conversion in biorefineries, a pretreatment step for increasing the accessibility of enzymes to cellulose and hemicellulose is usually required, due to recalcitrance of the material (Mosier *et al.*, 2005; Ximenes *et al.*, 2017).

The amount and nature of the formed degradation products, many of which are inhibitory to downstream biocatalytic processes, is directly related to the pretreatment method and conditions. In the Liquid Hot Water (LHW) pretreatment water penetrates into the biomass under high pressure, hydrates cellulose, and removes most of the hemicelluloses and a minor part of lignin. The solubilization of hemicelluloses is catalyzed by hydronium ions at an elevated temperature, while acetic acid and other organic acids generated from hemicellulose also facilitate this process. Although lignin is partially depolymerized and solubilized during hot water pretreatment, complete delignification is not possible using hot water alone due to the recondensation of soluble components originating from lignin (Jönsson e Martín, 2016; Ximenes *et al.*, 2017).

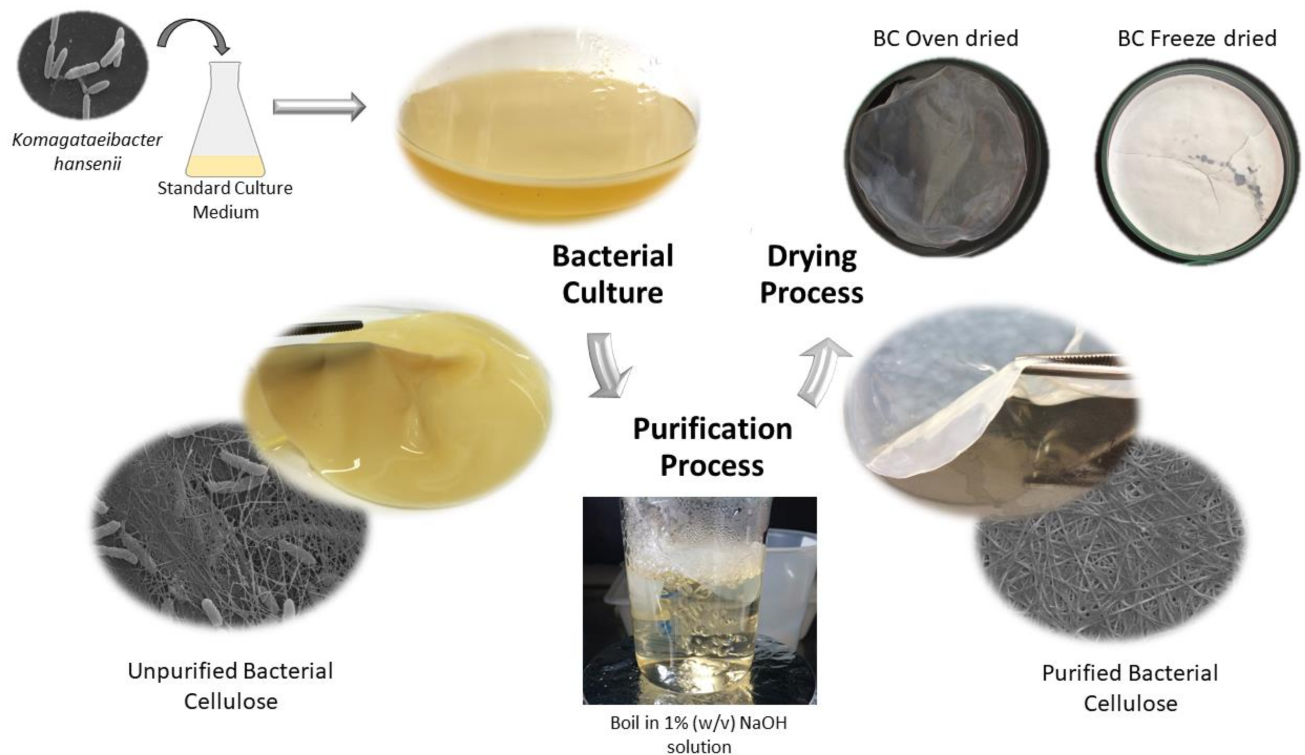
The chemical differences between feedstocks have a major impact on the formation of inhibitors during pretreatment. Liquid hot water pretreatment of lignocellulosic materials including corn stover, corn fiber, switchgrass, sugarcane and hardwoods containing low concentrations of glucose results in an aqueous stream and a solids fraction that is separately

utilized for enzyme hydrolysis and fermentation to ethanol (Zeng et al., 2007, 2011a, 2011b; Ladisch et al., 2014; Ko et al., 2015). This liquid fraction derived from liquid hot water besides containing a low amount of glucose, also contains glucans, acetic acid, aldehydes, and xylose and xylo-oligosaccharides in addition to phenolic compounds. This aqueous stream also contains other components that inhibit enzymes and microorganisms (Ximenes *et al.*, 2017).

2. Bacterial cellulose films produced by *Komagataeibacter hansenii* under different drying processes

* The content of this chapter is an adaptation of the scientific article entitled: “The effect of drying process on the properties of bacterial cellulose films from *Gluconacetobacter hansenii*” by Vasconcellos, V. M.; Farinas, C. S., published in Chemical Engineering Transactions.

Reference: CEt. 2018, 64, 145-149.



2.1 Abstract

Bacterial cellulose (BC) is a polymeric material that presents unique structural and mechanical properties, high crystallinity, biocompatibility, and biodegradability. All these excellent properties make BC attractive for various biotechnological applications, ranging from biomedical to electronic devices. However, the cultivation conditions to produce BC as well as the downstream processing steps can affect the properties of BC films by modifying the microstructure of the material. This paper reports on the effect of drying method on BC films produced by *Komagataeibacter hansenii* using two different procedures (oven drying at 50°C and freeze-drying). Structural changes in the BC films were evaluated using scanning electron microscopy (SEM), thermogravimetric analyses (TGA), X-ray diffraction (XRD), and Fourier-Transform Infrared (FT-IR). The two samples were visually different, as the oven dried BC was transparent while the freeze-dried BC was whitish. SEM micrographs showed that the samples had similar interweaving, but the freeze-dried material presented a higher porosity while oven dried presented collapsed fibers, leading to a volume reduction of the film. The analysis of the thermal stability showed that the films have a similar degradation profile, starting the process of degradation at 319°C for the oven and at 325°C for the freeze-dried samples. The BC films showed also similar crystallinities (85%), although their diffratograms exhibit different peaks suggesting that the drying process changed the percentage of I_α/I_β polymorphs of the films, which was corroborated by the FT-IR results. These differences in the BC films characteristics submitted to different drying procedures can have an impact on their mechanical properties and water absorption capability, thus potentially influencing the type of possible applications.

2.2 Introduction

The bacterial cellulose (BC) is a polymeric material that presents remarkable properties as nanostructure, high crystallinity, capacity of chemical modifications, biocompatibility,

biodegradability, high mechanical strength and capacity for water retention, slow capacity for water evaporation, non-toxicity, and ability to be molded 3-dimensional and with chemical modifications structures during biosynthesis. In face of all these excellent properties, the BC becomes attractive for various biotechnological applications including biomedical, cosmetic, food, pulp, paper, optical, electrical, magnetic, BC membranes and sewage purification (Iguchi *et al.*, 2000; Shah *et al.*, 2010; Ang *et al.*, 2012; Esa *et al.*, 2014).

BC is produced mainly by the acid bacteria *Komagataeibacter*, such as *K. xylinus* and *K. hansenii*, by cultivation in a liquid medium rich in carbohydrates, chemically defined or complex, under stirring or static condition (Iguchi *et al.*, 2000; Shah *et al.*, 2013; Esa *et al.*, 2014). The current challenges limiting the application of BC in a broader and larger scale is related to bioprocess developments to increase its production.

The process used to produce BC affects directly its properties and consequently its application. The effects of cultivation type (static or agitated; batch or fed batch process), the different bacterial strains and the culture medium (complex or industrial waste) on the BC properties has been widely studied. Besides, the downstream processing treatment of the BC can also impact the final characteristics of the material. However, how the drying process affects the properties of BC still needs to be further investigated, since most of the studies compare the properties of the film produced under different cultivations conditions using a unique drying process or during the preparation of BC composites (Hu *et al.*, 2014; Zeng, Laromaine, Feng*, et al.*, 2014). The aim of this work was to evaluate how the drying process of BC affects its properties. For this purpose BC films were produced *by K. hansenii* under static cultivation, followed by purification step and then dried under two different procedures, oven dried or freeze-dried. Both resulting BC films were then fully characterized by microscopic, thermogravimetric, X-ray and FT-IR analysis.

2.3 Materials and Methods

2.3.1 Microorganism

K. hansenii ATCC 23769 was purchased from Fundação André Tosello de Pesquisa e Tecnologia—FAT (Campinas, Brazil). The bacterium was maintained in slants of mannitol agar medium at 4°C. The stock was renewed monthly *K. hansenii* is an acetobacter, gram negative and strictly aerobic bacterium.

2.3.2 Preparation of initial inoculum and cultivation

For all assays were used the Hestrin and Schramm (H&S) culture medium (Hestrin e Schramm, 1954), contained (%, w/v): 2.0% glucose, 0.5 bacto peptone, 0.5 yeast extract, 0.27 disodium phosphate (anhydrous), 0.115 citric acid, pH adjusted to 5.0 with a citric acid solution. The culture media was sterilized at 121°C in an autoclave for 15 min and transferred to Erlenmeyer flasks. To activate the bacterial the inoculum (preculture) was prepared adding 1 mL of stock culture into 100 mL of the H&S medium in a 500 mL Erlenmeyer flask incubated at 28 °C for 72 h under static conditions. Samples were aliquoted every 24 hours to measure absorbance, pH and glucose concentration. The quantification of the glucose concentration was performed with a enzymatic kit for glucose measurement (Labtest, Brazil) and the absorbance of the aliquots was measured in a spectrophotometer with a wavelength of 640 nm. The main culture was started by inoculating 4, 8 or 16 v/v% of the inoculum into 100 mL of the HS medium in a 500 mL Erlenmeyer flask, the culture was incubated at 28 °C for 7 days under static conditions.

2.3.3 Purification and drying process

The purification was carried out by separating the BC from the medium and boiling (~95°C) in a 1% (w/v) sodium hydroxide solution for 60 min for removal of cells and medium

embedded in the cellulose film. After purification, the film was rinsed with distilled water until neutral pH. The BC films were either oven dried (Oven FANEM 502, Brazil) at 50 °C on a silicon surface for 48 hours, until constant weight (Oven-dried BC) or frozen at -30 °C and then freeze dried (Freeze Drier LIOBRAS 101, Brazil) on petri dishes (Freeze-dried BC).

2.3.4 Characterization Analyzes

Scanning Electron Microscopy SEM analysis was performed on a microscope JEOL (Model JSM-6510) equipped with a secondary electron detector (SEI) and operated at 10 kV. For these analyses, all samples were attached to aluminum stubs using adhesive carbon tape and then coated with gold (Leica Sputter Coater - SCD050 Coating system). The BC films were fixed directly onto the carbon tape. Bacterial sample was prepared on a slice of glass, using for fixing a Karnovsky solution. For dehydration, the samples were washed with acetone solution at different concentrations and freeze dried. The samples used for all analyses were randomly picked from the BC films.

Thermogravimetric analysis (TGA) of BC samples was carried out using a TA instruments (TGA Q500 V6.3 build 189) using sample of about 7 mg in a platinum sample holder. Each sample was scanned over a temperature range from ambient temperature to 600°C at a heating rate of 10 °C/min under nitrogen atmosphere with a sample flow rate 50 mL/min and balance flow rate 50 mL/min to avoid sample oxidation. Thermogravimetric derivative (DTG) was also obtained from the data.

The X-ray diffraction (XDR) was performed with a high-resolution X-ray diffractometer (Shimadzu 6000) with a Ni-filtered Cu Kα (1,540562 Å) radiation source operated at voltage 30 Kv and 30 mA electric current. The dried BC samples were scanned from 5° to 85° 2θ range with a step of 0,02°, a step time of 4,0 sec and scan speed 2°/min. The % Crystallinity was calculated by two methods, the Height peak method (%C_H) Eq(1) (Segal *et al.*, 1959) and the

Area peak method Eq(2) (%C_A) (Park *et al.*, 2010). A peak fitting program (OringiPro) was used, assuming pVoigt functions for each crystalline peak and a broad peak around 22° assigned to the amorphous phase. The equations used are below:

$$\%C_H = \frac{I_{002}-I_{AM}}{I_{002}} x100 \quad (1) \qquad\qquad \%C_A = (1-\frac{A_A}{A_T}) * 100 \quad (2)$$

Where I_{002} is the maximum intensity of the lattice diffraction (2Θ of 22° to 23°) and I_{AM} is that of the amorphous material where the intensity is minimum (2Θ of 18° to 19°), A_A is the area relative to the amorphous fraction and A_T is the total area of the original diffractogram.

Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR) spectroscopy analyses was carried out on the BC films on a Vertex 70 FTIR spectrometer (Brunker), equipped with a Universal ATR accessory, using 32 scans and a resolution of 4 cm^{-1}, over the range 4000-400 com^{-1} and their background was recorded with an empty cell for each sample. The spectra's were normalized and cellulose I_α fraction (Kataoka e Kondo, 1999) and I_α, I_β content (Bi *et al.*, 2014) were calculated using the peak heights at 750 and 710 cm^{-1} as showed the following equations:

$$I_\alpha \text{fraction} = \frac{A_{750}}{A_{750} + A_{710}} \quad (3) \qquad\qquad I_\alpha = 2.55 * I_\alpha \text{fraction} - 0.32 \quad (4)$$

2.4 Results and Discussion

2.4.1 Time and size of inoculum

A high concentration of cells (Hu *et al.*, 2013) and exponential growth phase may lead in rapid aggregation of cellulose fibers followed by a fast production of BC film in a short period of time in an appropriate environment. For a good production of bacterial cellulose (BC), by cultivation of *K. hansenii*, an ideal inoculum is needed, therefore an evaluation of growth phase and the cell concentration of the inoculum was performed. The analyze of the growth phase of bacteria was realized monitoring the absorbance (considering the turbidity of

the medium as indicative of increase in biomass), pH and glucose concentration of the medium for 72 hours under static conditions using the H&S culture medium at 28 °C. The results of this monitoring are shown in Figure 2.1. It's possible observe that between 24 and 48 hours there is a significant change in the parameters analyzed, the absorbance increases more than 4 times accompanied by a large drop in pH and glucose concentration. These results indicate the end of the lag phase and beginning of the exponential growth phase, in this phase the cell reproduction is extremely active and the growth rate of the microbial population becomes constant, it is the period of high metabolic activity of the cell. It was also possible to observe the beginning of the formation of BC film in 24 hours process, it appeared to be very thin and transparent. Subsequently, the CB-layer film thicken until the end of the process (data not shown). It was selected 72 hours as the incubation time for the inoculum, ensuring that the cells were in their better growth phase.

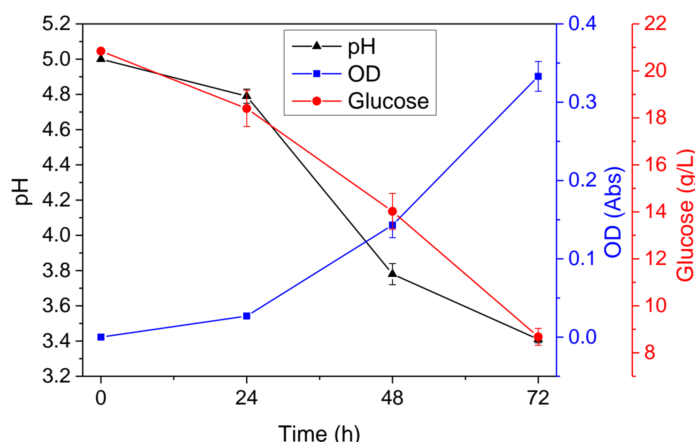


FIGURE 2.1 Growth profile of *K. hansenii* versus time: glucose consumption, pH and optical density (OD) of inoculum under 72 hours of static cultivation at 28 °C using the HS culture medium

To evaluate possible changes in the production of BC at the end of cultivation, it was tested different initial cell concentrations. They were evaluated three inoculum sizes of 4, 8

and 16% (v/v), the amount of BC obtained after 7 days of static cultivation at 28 °C using the H&S culture medium and oven dried is shown in Figure 2.2.

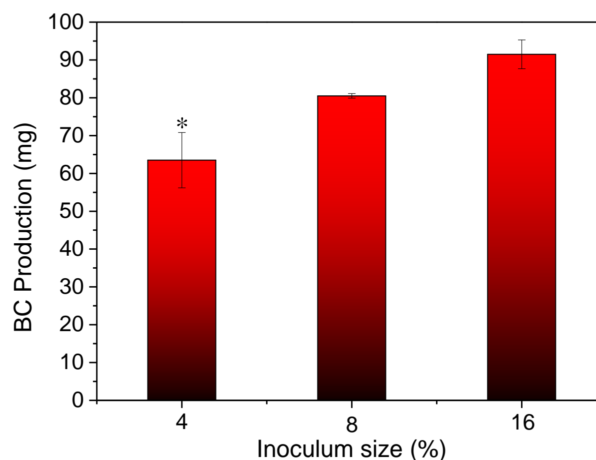


FIGURE 2.2 BC production after 7 days of static cultivation at 28 °C using the HS culture medium under different inoculums sizes 4%, 8% and 16% (v/v). The column labeled by asterisk (*) indicates that inoculum size has significant difference ($p < 0.05$).

This result indicate that initial concentration of cell affects the production of CB, low cell concentrations (4%) decreases the production of the film, while higher concentrations increase BC. However there is no significant difference in the production of BC for higher cell concentration (8 and 16%), this may be related to the decrease of the substrate or a larger initial concentration of cells has left the inappropriate environment by the release of secondary metabolites, limiting bacterial growth. Similar result was found by (Hu *et al.*, 2013) who tested inoculum size of 1, 2, 4, 8 and 16%. Thus, the selected inoculum size was 8% (v / v) to study the drying process.

2.4.2 Purification and Drying Process of BC

Morphological characterization of *K. hansenii,* the crude BC film and the purified BC oven dried was carried out using SEM. From Figure 2.3, it is possible to show that the

purification process was effective in removing bacteria intertwined in cellulose film and traces of culture medium. The purification process also increased the BC films transparency.

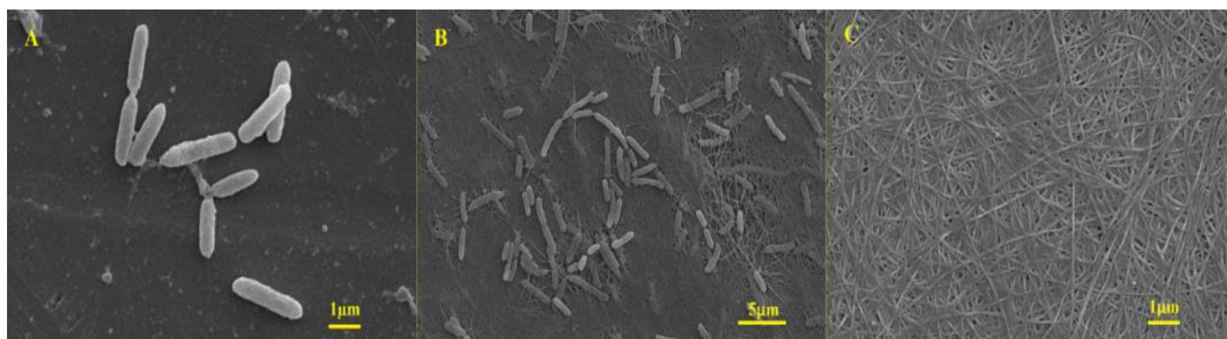


FIGURE 2.3 SEM micrographs of *K. hansenii* (ATCC- 23769) (A), BC films after 7 days of static cultivation at 30 °C before (B) and after (C) purification with NaOH 1 % (w/v) for 60 min at 100°C.

The BC samples produced and purified were subjected to two different drying processes: oven dried or freeze-dried. Figure 2.4 shows the appearance of the samples at the end of the different drying processes. It can be seen that the over dried BC (Figure 2.4 (A)) has transparency while the freeze-dried BC (Figure 2.4 (B)) does not, looking like a “Styrofoam”. The structure of the cellulose membranes was investigated with higher resolution using SEM (Figure 2.4 (C) and (D)), in which the BC fibers dispersion and the interfacial adhesion are shown. Similarly, to previous reports, BC films show a hierarchical structure with pores of different sizes from macro to micro scale. It is not able that both samples show similarity between the interweaving of the fibers. However, the freeze-dried BC has a higher porosity while the oven dried sample the fibers are more collapsed, presenting a lower porosity.

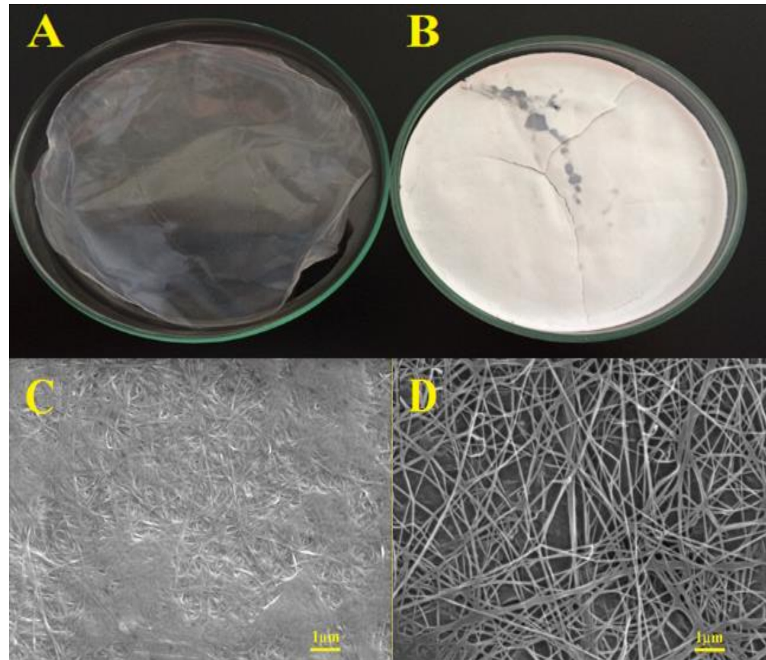


FIGURE 2.4 BC films oven dried (A) and freeze dried (B) and SEM micrographs of BC films oven dried (C) and freeze dried (D).

The thickness of the oven-dried membrane was much lower than the freeze-dried, 12 ± 2 and 627 ± 84 μm, respectively. The greater thickness of the freeze-dried film is directly related to the loss of transparency of the film, because the intertwining of the fibers interferes with the angle of the light that is incident and the angle of the light that is reflected. A simple visual observation does not provide information about the crosslink forming the film, but probably the reduced volume of oven dried BC is related to the collapse of the polymer chains that form the structural network of the membrane. The diversity of applications and biological functions of BC membranes are based on their distinct morphology, and the intertwining of the fibers in films is responsible for its considerable mechanical strength and water absorption capacity. Zeng et al. (2014b) confirmed these differences evaluating three different drying processes: room temperature drying, freeze drying and supercritical CO_2 drying, and found out that mechanical properties, such as penetration depth, hardness, and also water absorption capacity were modified by the drying process, and each process favors a specific characteristic.

2.4.3 BC Characterization

The TGA and DTG curves of bacterial cellulose are presented in Figure 2.5. It is possible to observe that oven-dried BC and freeze-dried BC have similar behavior in relation to heat degradation, showing the typical single step thermal degradation profile. Three stages of mass loss were associated with the TGA curves. A first stage below 150 °C, with less than 10% weight loss, is associated with the release of water and other volatile compounds present in the film. A second stage, at range 250 - 450 °C, shows a severe weight loss. This event is associated with the process of cellulose degradation, involving depolymerization, dehydration and decomposition of glycosyl units followed by the formation of a charred residue (Mohammadkazemi *et al.*, 2015). The third stage extends to the usual ending test temperature at 600°C. The single step confirms that the adopted purification process promoted the removal of all contaminants.

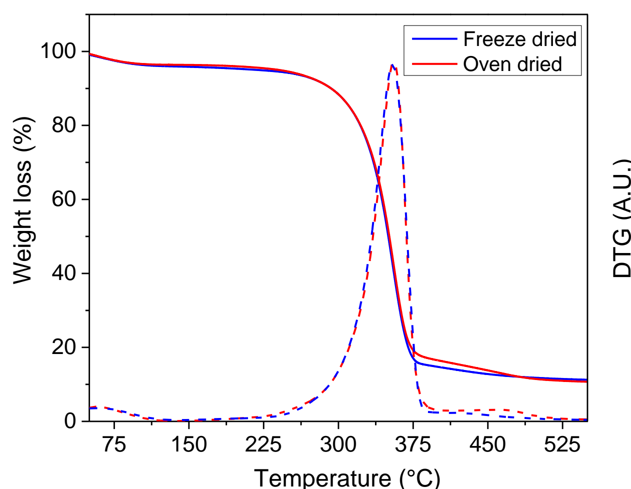


FIGURE 2.5 TGA (---) and DTG (- - -) of cellulose produced by *K. hansenii* in HS medium under different drying processes.

The results of temperature of T_{onset} and main DTG peak are listed in Table 2.1. It was observed no significant differences between BC samples under the drying processes studied. This indicates that the drying process did not affect the thermal degradation. The high thermal

resistance of BC with a decomposition temperature around 355°C is in agreement with previous studies uisng alkaline treatment and similar drying processes, freeze dried (Mohite e Patil, 2014) and oven dried (Zeng, Laromaine e Roig, 2014; Kiziltas *et al.*, 2015; Mohammadkazemi *et al.*, 2015).

TABLE 2-1 Details of TGA, DTG, DRX and FT-IR characterization of BC films under different drying processes

BC Sample	T_{onset} (°C)	Main DTG peak temp. (°C)	%C_H	%C_A	I_α fraction	I_α	I_β
Oven dried	319.6	355.4	84	68	0.35	57.2	42.8
Freeze dried	325.2	354.5	85	64	0.37	62.3	37.7

The BC is a semi-crystalline material and its structure is composed of cellulose I, predominantly by polymorph I_α (Zeng, Laromaine e Roig, 2014). To compare the microstructure changes in the BC oven dried and freeze-dried, X-ray diffraction was used. The diffractograms of the samples are presented in Figure 2.6 (A). The peaks found for the two samples only suggest the presence of cellulose I, peak 1 (~15°) corresponds to the 100 plane of cellulose I_α or to the $1\bar{1}0$ plane of cellulose I_β, peak 2 (~17°) corresponds to the 010 plane of cellulose I_α or to the 110 plane of cellulose I_β and peak 3 (~23°) corresponds to the 110 plane of cellulose I_α or to the 200 plane of cellulose I_β (Wada *et al.*, 2001). The samples have different peaks and intensity, each diffracted peak presents only one contribution of the corresponding diffractions of the phase I_α and I_β, which is difficult to estimate due to overlapping of the planes reflections. The diffractograms also reveals the presence of the amorphous region in BC (I_{am}~20°). The % crystallinity (%C) was calculated using two different

methods and the results are presented in Table 2.1. The $\%C_H$ showed no difference between the samples dried by different processes and both have high crystallinity ($\%C_H$~85%), when the peak area was used there was also no significant difference in crystallinity between the drying processes, but the $\%C_A$ value was reduced in almost 20% ($\%C_A$~65%). The difference of the values between the methods was expected, since the height method is an empirical measurement to allow relative rapid comparison between samples. However, there is some reasons that it should not be used as a method for estimating the amount of crystalline and amorphous material in a cellulose sample, as the height used for amorphous fraction is not aligned with the maximum height of the amorphous peak, only the highest crystalline peak is used in the calculation and only the height is taken into account being that width also plays a very important role (Park *et al.*, 2010).

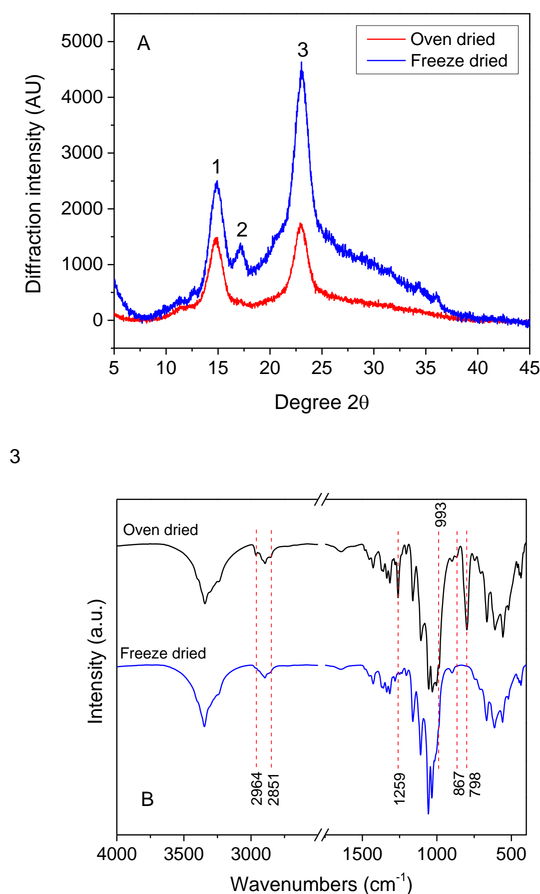


FIGURE 2.6 X-ray diffractogram (A) and FTIR vibrational spectra (B) of BC produced by *K. hansenii* in HS medium under different drying processes.

The results suggest that different drying methods are not influencing the crystallinity of BC, but the methods are changing the proportion of polymorphs I_{α}/I_{β}. Zeng and co-work (2014) found similar crystallinity results and peaks for films to dried at room temperature and freeze-dried.

The FT-IR spectra of oven- and freeze-dried cellulose are presented in Figure 2.6 (B). It is possible to observe that the films have similar profile, the absorption bands at 798, 867, 1035, 1637, 2851, 2919, 2964 and 3350 cm^{-1} in the spectra characterize the material as bacterial cellulose. The region between the bands 3600-3000 cm^{-1} corresponds to hydrogen bond O-H stretching, hydrogen bonds for cellulose I include two intramolecular bonding namely, O(2)H-O(6) bonding (3405-340 cm^{-1}) and O(3)H-O(5) bonding (3340-3375 cm^{-1}) and one

intramolecular bonding O(6)H-O(3) (3230-3310 cm^{-1}) (Fan *et al.*, 2012). The presence of the bands 1429, 1163, 1111, 897 cm^{-1} also characterizes the formation of cellulose I (Nelson e O'connor, 1964). These results indicate that the drying process used was not able to transform cellulose I into II, but the increased intensity of the band around 993 cm^{-1} in the oven-dried film suggests the formation of a new crystal structure with a cellulose II characteristic (Yue *et al.*, 2015). The appearance of cellulose II may be related to the process of film purification with sodium hydroxide, as the appearance of cellulose II after the alkaline purification process has been reported by Zeng (2014).

It is possible to observe some changes around 800-950 cm^{-1} band. This region is sensitive to the amount of amorphous cellulose, i.e. broadening of this band indicates higher amount of disordered structure (Proniewicz *et al.*, 2002; Ang *et al.*, 2012) and the increase in the intensity of the 2900 cm^{-1} band in the oven-dried cellulose spectrum are indications that this film is more crystalline (Ciolacu *et al.*, 2011). The efficiency of the purification process also can be confirmed through the FT-IR spectro, due to the absence at 1642 cm^{-1} band which relate to amide presence (Zeng, Laromaine e Roig, 2014).

Table 2.1 shows the I_α fraction and the amount of α and β polyforms of cellulose I. The results indicate that freeze dried process slightly increased the cellulose I_α amount in the BC film, suggesting that the dried process would affect the formation of two distinct crystalline variation in the crystalline structure of cellulose I: meta stable state cellulose I_α and stable state cellulose I_β (Nelson e O'connor, 1964). Bi and co-workers (2014) reported that different strains can produce cellulose film with different concentrations of cellulose I_α in agitation culture. The drying technique can modify the chemical bonds, as the disorder of cellulosic structure may be caused by the different arrangements in the angles around β-glycoside linkages and hydrogen bond rearrangement (Proniewicz *et al.*, 2002; Ang *et al.*, 2012).

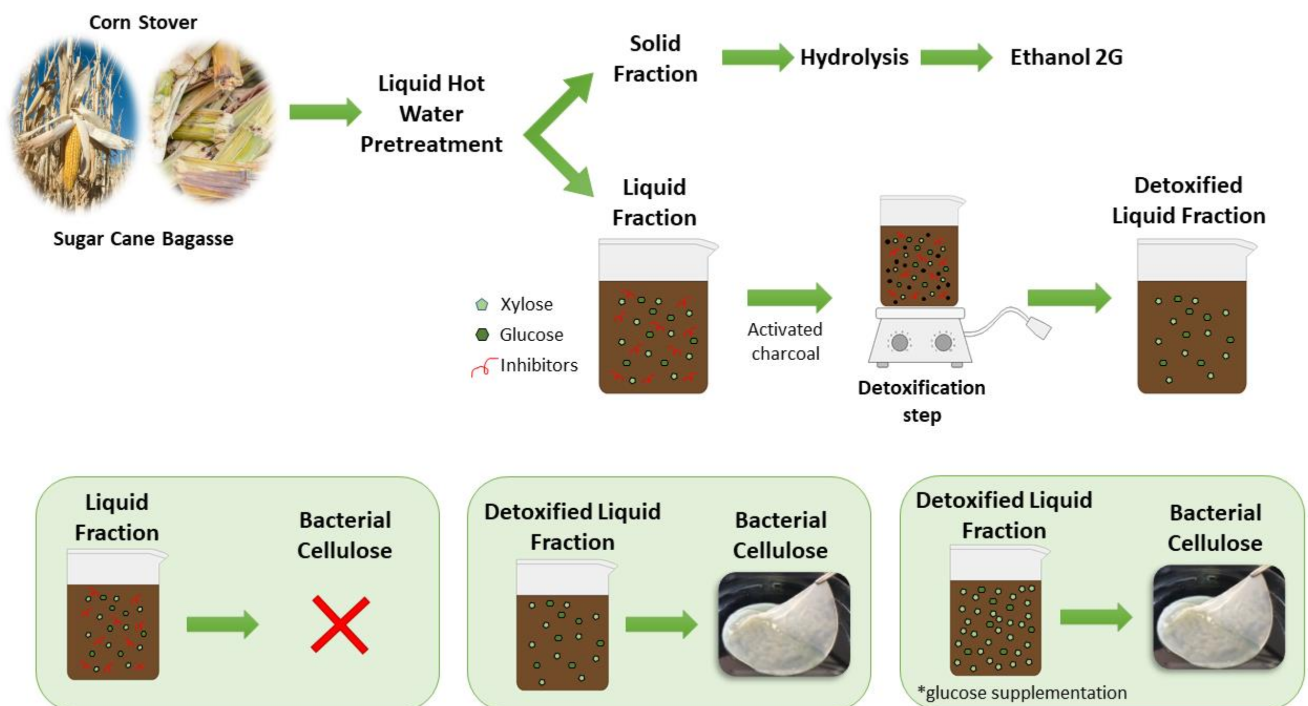
2.5 Conclusions

The results of this work using two different drying processes allowed the production of different BC films with different structural properties. Such materials could be used to create novel complex nanocomposites with different applications. The oven drying and freeze-drying methods did not affect the thermal stability and crystallinity of the film, but modified the transparency, porosity and polymorphs composition of BC films, what can interfere directly in the potential applications. A better understanding of how the drying process affects the properties of the BC films may contribute for the development of novel biotechnological materials for different applications.

3. Bacterial cellulose production using as C source the LHW pretreatment liquid fraction from lignocellulosic biomass

* The content of this chapter is an adaptation of the scientific article entitled: "Adaptive laboratory evolution of nanocellulose-producing bacterium" by Vanessa M. Vasconcellos, Cristiane S. Farinas, Eduardo Ximenes, Patricia Slininger, Michael Ladisch, published in Biotechnology and Bioengineering.

Reference: doi: 10.1002/bit.26997



3.1 Abstract

Microbial inhibitors generated after liquid hot water (LHW) pretreatment of lignocellulosic biomass can negatively affect cellulose production by bacteria. Bacterial cellulose (BC) production by *Komagataeibacter hansenii* ATCC 23769 was initially investigated here using the liquid fraction from hydrothermal pretreatment of sugarcane bagasse (SB) and corn stover (CS) as culture media. The production of cellulose in both liquid fractions was not feasible without the detoxification of the medium using activated charcoal due to the toxic character of the lignin-derived compounds. BC production using the detoxified CS fraction was 49% higher than that using standard medium, but 28% lower in the presence of detoxified SB fraction as culture medium. *K. hansenii* cultured in pretreatment liquid treated with activated charcoal to remove inhibitors also converted glucose to bacterial nanocellulose and used xylose as carbon source for growth. The cellulose-producing bacteria was inhibited in the presence of vanillin, ferulic and ρ-coumaric acids compounds at the concentration of 1.8 mg/mL, with the vanillin being the most toxic compound and inhibiting the growth of the microorganism. The properties of BC produced using liquid fractions were the same as nanocellulose generated from media specifically formulated for bacterial cellulose formation.

3.2 Introduction

The high purity and organized structure of cellulose produced by bacteria provides remarkable properties for this biomaterial such as high thermal stability, crystallinity, degree of polymerization, mechanical strength, capacity for water retention and capacity of chemical modifications, while being also biocompatible and biodegradable (Esa *et al.*, 2014; Islam *et al.*, 2017). These excellent properties make Bacterial Cellulose (BC) attractive for various

biotechnological applications (Ullah *et al.*, 2016; Kim *et al.*, 2017); (Cacicedo *et al.*, 2016); (Sabo *et al.*, 2016).

The *Gluconacetobacter* genus can metabolize a wide range of carbon/nitrogen sources for BC production (Lee *et al.*, 2014; Islam *et al.*, 2017)**,** but requires a glucose-rich culture medium with other nutrients sources, resulting in high production costs, which limits BC potential applications (Hungund *et al.*, 2013). Therefore, the use of alternative culture media for BC production has been investigated, such as fruit juices (Kurosumi *et al.*, 2009; Hungund *et al.*, 2013), sugar cane molasse (Keshk, S. e Sameshima, K., 2006; Tyagi e Suresh, 2016) and brewery waste (Shezad *et al.*, 2010).

Carbohydrates present in lignocellulose-derived media has the potential to be used as an alternative cost-effective feedstock for BC production (Castro, C. *et al.*, 2011; Kiziltas *et al.*, 2015), however, the pretreatment process of lignocellulosic biomass generates enzyme and microbial inhibitors (Ximenes *et al.*, 2010; 2011; Kim *et al.*, 2016).

Liquid hot water pretreatment of lignocellulosic materials including corn stover, corn fiber, switchgrass, sugarcane and hardwoods, generates an aqueous stream which could prove to be a valuable resource for production of bacterial nanocellulose. However, the pretreatment liquid is a biomass processing stream that also contains acetic acid and phenolic inhibitors that are found together with sugars at dilute concentrations for a range of lignocellulosic feedstocks (Ximenes *et al.*, 2010; 2011; Cao *et al.*, 2013; Cao *et al.*, 2015; Kim *et al.*, 2016; Michelin *et al.*, 2016; Ximenes *et al.*, 2017).

The inhibition effect of such compounds has been reported for the production of BC using culture medium based on the hydrolysates from konjac power (Hong e Qiu, 2008), waste fibers from pulp and paper mill (Cavka *et al.*, 2013), spruce wood (Guo *et al.*, 2013), sugarcane bagasse (Cheng *et al.*, 2016) and corn stalk (Cheng *et al.*, 2017), indicating the need for the

addition of detoxification procedures, which can add significant costs to the BC production process.

We evaluated here the feasibility of applying the liquid fraction from the hydrothermal pretreatment of sugarcane bagasse and corn stover as culture media to BC production by *K. hansenii*. The role of the xylose, the effects of adding a detoxification step with activated charcoal as well as supplementation with glucose and salts were evaluated. All BC films were characterized by microscopic, thermogravimetric, X-ray and FT-IR analysis.

3.3 Materials and Methods

3.3.1 Microorganism

K. hansenii ATCC 23769 was purchased from Fundação André Tosello de Pesquisa e Tecnologia—FAT (Campinas, Brazil). The bacterium was maintained in slants of mannitol agar medium (3 g/L bacto peptone, 5 g/L yeast extract, 25 g/L D-mannitol, and 15 g/L agar) at 4°C. The stock was renewed monthly.

3.3.2 Lignocellulosic biomass

Sugarcane bagasse, originated in the State of São Paulo (Brazil), was dried at room temperature until moisture content was lower than 10%. The dried material was ground in a knife mill and the particle diameter <2 mm selected for the pretreatment. Corn stover was harvested from central Illinois, dried at room temperature to 5–8% moisture, and ground (Model 4 Wiley, Thomas Scientific, Swedesboro, NJ) to pass through a 2 mm screen (Kim *et al.*, 2016).

Pretreatments were based on using LHW conditions as described by Kohlmann *et al.*, (1994); Kim *et al.*, (2009); Kim *et al.*, (2013); Kim *et al.*, (2015); Kim *et al.*, (2016). Pretreatment liquid (800 ml) was obtained from sugarcane bagasse after LHW pretreatment in

a 5 L reactor (Model 4580, Parr Instruments) using a 10% (wt/vol) solid loading and carried out at 195°C for 10 min. Heat up to 195°C required 75 min, and was followed by a 10 min hold at 195°C, and 30 min cooling (Vasconcellos *et al.*, 2015). Corn stover pretreatment liquid was obtained for 20% (wt/vol) CS solids loading in deionized (DI) water in 37 ml reaction tubes. The tubes were preheated to 140°C in a sandbath for 45 min, heated and held at 190°C for 15 min, and cooled by quenching in room temperature water. Heating and cooling times were 45 and 30 min, respectively.

3.3.3 Pretreatment liquids

The pH of liquids from both pretreated sugarcane bagasse and corn stover were adjusted to 5.0 with 1% (wt/vol) citric acid. The liquids from pretreating both types of lignocellulose were clarified by vacuum filtering solids through Whatman filter paper number 1 in a 70 mm Buchner funnel. Filtration required 5 min at room temperature.

Pretreatment liquid used for control runs was detoxified by addition of activated charcoal 5% [wt/vol] with agitation for 1 hr at room temperature. The suspensions were vacuum filtered using a Whatman filter paper number 1 and the pH was adjusted to 5.0 with a 1% (wt/vol) citric acid solution. The activated charcoal treatment removed 95% and 70% of the phenolic compounds from sugarcane bagasse and corn stover pretreatment liquids, respectively (Table 3-1).

The untreated and activated charcoal treated pretreatment liquids were analyzed before and after autoclaving. The compositions of pretreatment liquids themselves were initially analyzed for carbohydrates by liquid chromatography (Sluiter *et al.*, 2008) as well as for phenolics using Folin–Ciocalteu reagent (Singleton Vernon *et al.*, 1999). Reducing sugars, glucose and phenolic compounds were measured by the dinitrosalicylic acid (DNS) method (Miller, 1959), an enzymatic kit (Megazyme) and Folin–Ciocalteu reagent (Sigma-Aldrich),

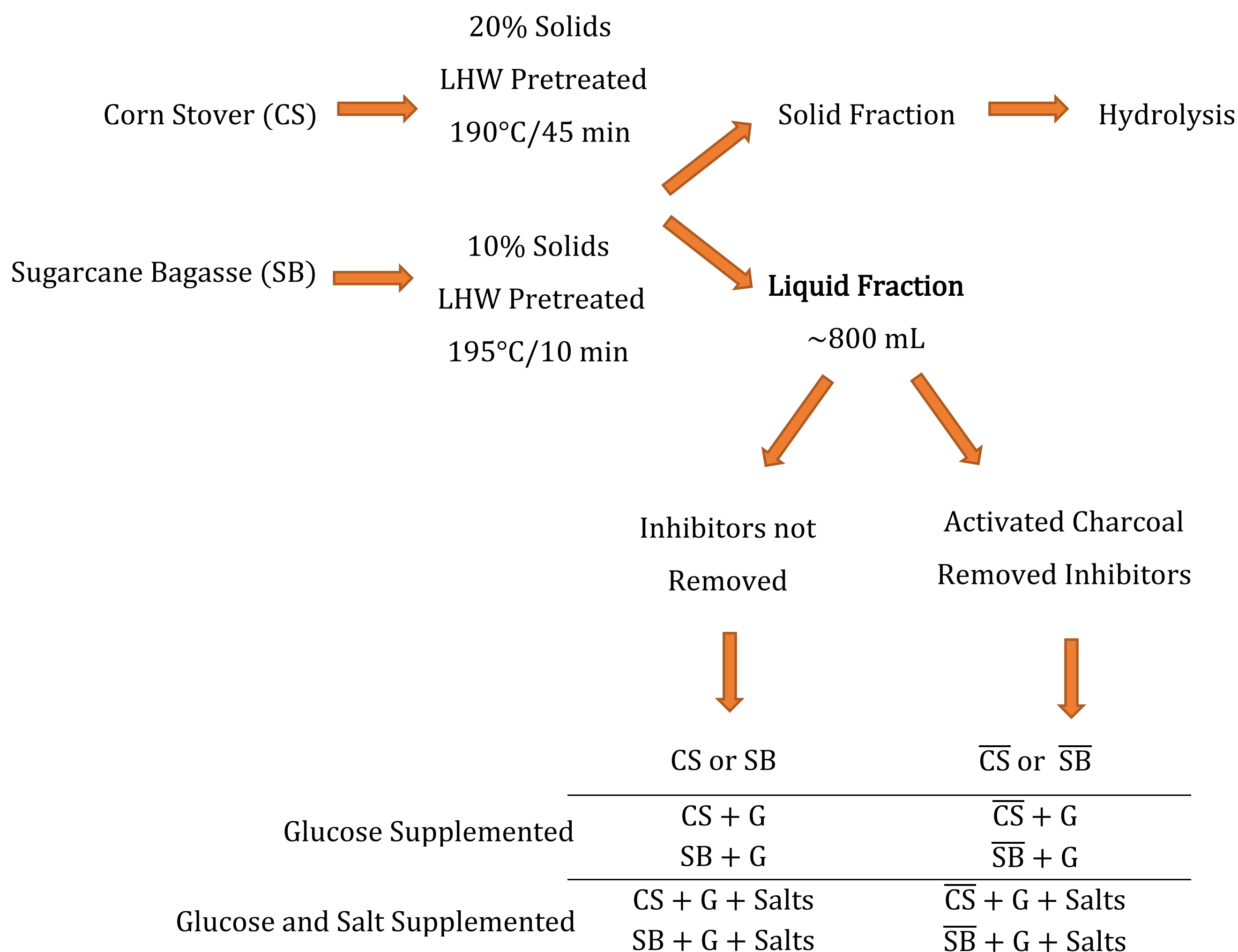
respectively (Singleton Vernon *et al.*, 1999). The total phenolic compound concentrations were expressed as gallic acid equivalents (GAE; mg/ml). During adaptive laboratory evolution protocols, total reducing sugars (includes hexoses and pentoses), glucose, and phenolics were measured calorimetrically rather than running the more time-consuming liquid chromatographic analysis for the large number of samples generated.

TABLE 3-1 Concentration of total phenolics and glucose in pretreatment liquids from sugarcane bagasse (ScB) and corn stover (CS) used as culture medium for BC production.

Liquid fraction	Total phenolics	Glucose
	(mg/L)	
ScB	1.87 ± 0.06	0.39 ± 0.02
Detoxified ScB	0.26 ± 0.01	0. 51 ± 0.01
CS	3.05 ± 0.07	1.49 ± 0.02
Detoxified CS	0.89 ± 0.06	1.54 ± 0.01

3.3.4 Culture media

Standard Hestrin and Schramm (HS) medium was used for bacterial growth (Hestrin e Schramm, 1954). This medium contains (%, wt/vol basis): 2.0 glucose, 0.5 bacto peptone, 0.5 yeast extract, 0.27 disodium phosphate (anhydrous), 0.115 citric acid, with the pH adjusted to 5.0 using a 1% (wt/vol) citric acid solution. The standard culture medium was also tested with the gradual replacement of glucose by xylose as carbon source following the ratios of 20:0, 10:10, 5:15, and 0:20 (g/L). The untreated and detoxified liquors from sugarcane bagasse and corn stover were used as culture media, under as is or nutritionally supplemented conditions. The sequence of steps in preparing these liquids is outlined in Figure 3.1.



	CS or SB	$\overline{CS}$ or $\overline{SB}$
Glucose Supplemented	CS + G SB + G	$\overline{CS}$ + G $\overline{SB}$ + G
Glucose and Salt Supplemented	CS + G + Salts SB + G + Salts	$\overline{CS}$ + G + Salts $\overline{SB}$ + G + Salts

FIGURE 3.1 Summary of preparation of culture media using liquid fraction (i.e., pretreatment liquid) from liquid hot water pretreated corn stover (CS) and sugarcane bagasse (SB). Pretreatment liquid that was clarified with activated charcoal is denoted as $\overline{CS}$ or $\overline{SB}$ respectively, with 90% of phenolics removed from sugarcane bagasse and 70% from corn stover pretreatment liquids.

The untreated and detoxified liquors supplemented with yeast extract and peptone were used as culture medium for bacterial nanocellulose production in three different ways: as is, nutritionally supplemented with glucose (+Glu), or supplemented with glucose and salts (+Glu + Salts). Final concentrations of supplemented nutrients were at the same concentrations as in the HS medium.

3.3.5 Microbial cultivation and bacterial cellulose formation

There are four steps in the protocol for the formation of bacterial cellulose: activation in agar, preparation of pre-inoculum filtrate, inoculation of HS medium with the filtrate and finally cultivation at 30°C under static conditions. Bacteria were first inoculated into HS agar medium at 30°C resulting in the formation of colonies that grow as a cloudy film. The cells were then removed from the agar and transferred to 20 ml of fresh HS medium in a shake flask.

After 24 h, a pre-inoculum was produced, of which 10 ml was added into 40 ml of fresh medium and incubated for an additional 3 days. The cultivation of bacterial nanocellulose was started by inoculating a volume corresponding to 4% (vol/vol) of filtered inoculum into fresh culture medium. Cultures were incubated for 7 days in a 50 ml Erlenmeyer using 20% of the flask volume. The amount of nanocellulose formed by the bacteria was measured after harvesting, boiling to remove cells and medium embedded in the cellulose film, filtering, drying, and weighing.

3.3.6 Purification and drying process to assess nanocellulose accumulation

The synthesized bacterial cellulose (BC) was separated from the medium using tweezers and boiled (~95°C) in a 1% (wt/vol) sodium hydroxide solution for 60 min to, as mentioned in previous section, remove cells and medium embedded in the cellulose film. The film was then rinsed with distilled water at ambient temperature until neutral pH was achieved followed by oven drying at 50°C for 48 h to constant weight. The resulting sheet of about 5 cm diameter was stored at 50% humidity for further analysis, to have standard conditions for the nanocellulose characterization work, minimizing interference in the analyses by humidity.

3.3.7 BC characterization

BC samples (after purification and drying) were subjected to analyses to characterize the resulting material. BC samples were attached onto aluminum stubs using adhesive carbon tape,

coated with carbon (Leica Sputter Coater—SCD050 coating system), and examined by field emission scanning electron microscopy using a JEOL microscope (model JSM-607 1F) operated at 2 kV and with a resolution of 1 μm.

Thermogravimetric analysis (TGA) analyses of BC samples were carried out in a TA instrument (TGA Q500 V6.3 build 189) using a platinum sample holder. Each sample (~7 mg) was heated from ambient temperature to 600°C range, at a heating rate of 10°C/min under nitrogen atmosphere, to avoid sample oxidation, and with a sample flow rate 50 ml/min.

X-ray diffractogram (DRX) was performed with a high-resolution X-ray diffractometer (Shimadzu 6000) with a Ni-filtered Cu Kα (1,540,562 Å) radiation source operated at voltage 30 kV and 30 mA electric current. The BC samples were scanned from 5° to 45° 2θ range and scan speed 0.5°/min. The crystallinity index (CrI) was calculated by Segal method (Segal *et al.*, 1959), where CrI expresses the relative degree of crystallinity, I_{002} is the maximum intensity of the lattice diffraction (2θ of 22–23°) and I_{AM} represents the amorphous phase of the material (the minimum intensity 2θ between 18° and 19°).

$$CrI\ (\%) = \frac{(I_{002}-I_{AM})}{I_{002}} x100 \qquad (1)$$

Fourier-transform infrared (FTIR) spectroscopy analyses were carried out on the BC samples in a Vertex 70 FTIR spectrometer (Brunker), equipped with a Universal ATR accessory, using 32 scans and a resolution of 4 cm^{-1}, over the range 4000-400 cm^{-1} and their background was recorded with an empty cell for each sample. The spectra were normalized at band 1,030 cm^{-1}. The apparent I_α mass fraction (f_α^{IR}) was calculated using the peak heights at 750 cm^{-1} (contribution from cellulose I_α) and 710 cm^{-1} (contribution from cellulose I_β) as showed in Equation (2). The mass fraction of cellulose I_α (f_α) was calculated using the relationship between FTIR and CP-MAS 13C NMR according to Equation (3) (Yamamoto *et al.*, 1996).

$$f_\alpha^{IR} = \frac{A_{750}}{A_{750}+A_{710}} \qquad (2)$$

$$f_\alpha = 2.55 * f_\alpha^{IR} - 0.32 \qquad (3)$$

3.4 Results and Discussion

3.4.1 Xylose as a carbon source for bacterial growth

The liquid fractions from LHW-pretreated lignocellulosic biomass are rich in pentoses from the degradation of hemicellulose, thus the possible consumption of these sugars by the bacteria as an energy source or bacterial cellulose production was evaluated. For the initial evaluation of the ability of *K. hansenii* to metabolize xylose, the standard culture medium (HS) was tested for the bacterial cultivation varying the amount of glucose and xylose (Table 3-2). An increase of xylose in the culture medium proportionately reduced the weight of the nanocellulose matrices with the absence of nanocellulose production when xylose was the sole C source (0 G:20 X in Table 3-2), possibly due to xylose being consumed by *K. hansenii* to support its growth. These results are in agreement with previous reports (Cavka *et al.*, 2013; Kiziltas *et al.*, 2015). The xylose and acetic acid present in the pretreatment liquids, while utilized as growth substrates by *K. hansenii*, are insufficient to form bacterial films, being required glucose for the synthesis of bacterial nanocellulose (Kiziltas *et al.*, 2015).

TABLE 3-2 Characterization of BC films synthesized in the presence of different ratios of glucose (G) and xylose (X) as carbon source in inhibitor-free Hestrin and Schramm (HS) media

Samples	Weight of nanocellulose formed (dry basis, mg)	Glucose (g/L)		Reducing sugar (g/L)		Estimated xylose consumption	
		Initial[1]	Final[2,3]	Initial[1]	Final[2,3]	(g/L)	%
Standard	5.67 ± 0.34	19.70	4.51 ± 0.74	20	6.56 ± 0.23	N/A	N/A
10G:10X	2.76 ± 0.14	10.70	0	22.3	6.96 ± 0.24	4.64	40
5G:15X	1.50 ± 0.23	5.58	0	23.4	9.60 ± 0.35	8.22	46
0G:20X	0	0	0	21.8	18.15 ± 0.83	3.64	16

[1]Initial = before cultivation; [2]Final = after 168 hrs cultivation; [3]based on 3 replicates

3.4.2 Effect of xylose on structure of nanocellulose

Nanocellulose films produced using different proportions of glucose/xylose showed a hierarchical structure with pores of different sizes from macro to microscale and similarity between the interweaving of the fibers (Figure 3.2). While the replacement of glucose by xylose did not cause modification in the interweaving of the fibers, the matrix produced using 5:15 (g glucose:g xylose) appears to consist of collapsed fibers.

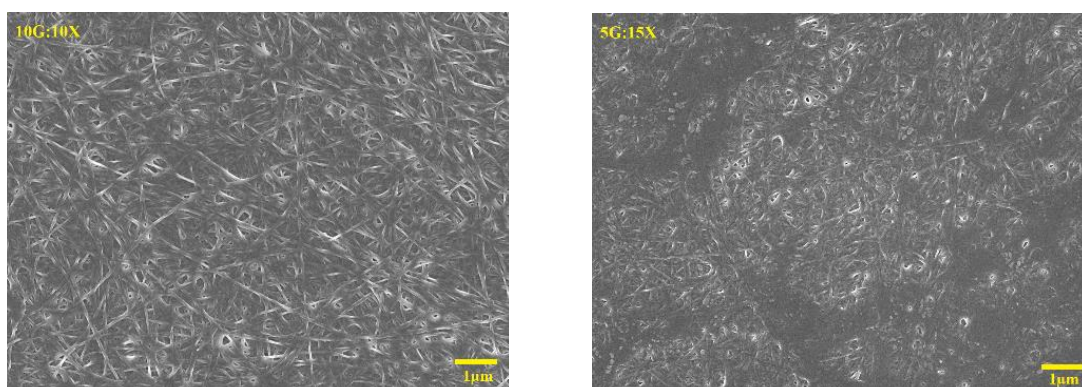


FIGURE 3.2 FESEM micrographs of BC films produced by unadapted Komagataeibacter hansenii using standard culture medium (HS) and standard culture medium with the gradual replacement of glucose (G) by xylose (X).

The change in the carbon source did not affect the thermal degradation behavior of all nanocellulose produced with xylose in the culture medium (T_{onset}~320°C, Figure 3.3, Table 3-3) compared to nanocellulose obtained from HS medium. Both forms gave a typical single step profile (Mohammadkazemi *et al.*, 2015).

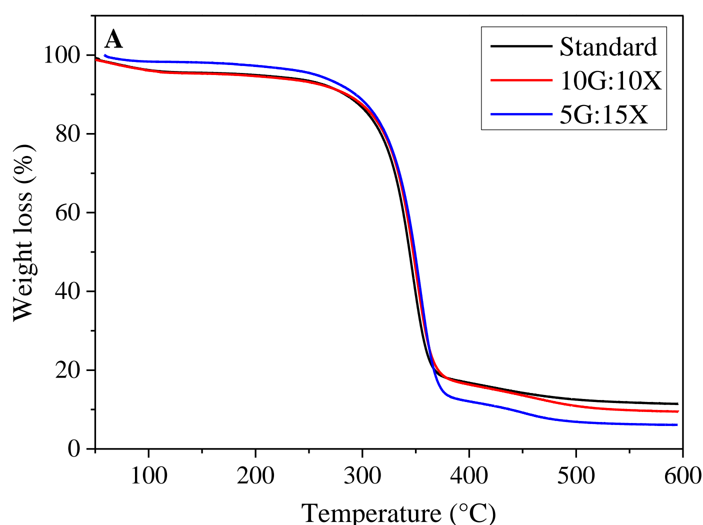


FIGURE 3.3 Weight loss curves from TGA analyses of BC films produced by *Komagataeibacter hansenii* using standard culture medium (HS) and standard culture medium with the gradual replacement of glucose (G) by xylose (X).

TABLE 3-3 Characterization details of BC films synthesized under different amounts of glucose (G) and xylose (X).

Samples	T_{onset} (°C)	CrI (%)	f_{α}^{IR}	f_{α}
Standard	319.7	86.8	0.31	0.48
10G:10X	323.0	84.1	0.35	0.57
5G:15X	325.7	79.1	0.35	0.59
0G:20X	-	-	-	-

To compare the microstructure changes in the bacterial nanocellulose films obtained using different proportions of glucose/xylose, X-ray diffraction was used (Figure 3.4). Peak 1 (~15), peak 2 (~17°), and peak 3 (~23°) found in the diffractograms for all bacterial nanocellulose samples suggest the presence of cellulose I only. The diffractograms also reveal the presence of an amorphous region consistent with observations by (Wada *et al.*, 2001).

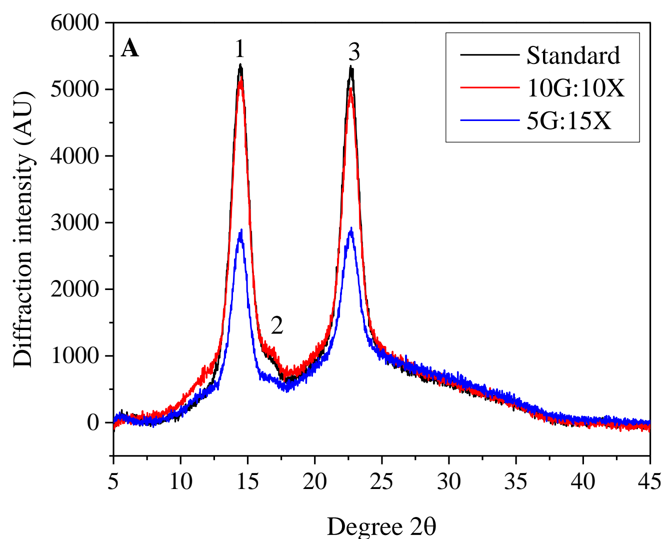


FIGURE 3.4 X-ray diffractogram of BC films produced by *Komagataeibacter hansenii* using standard culture medium (HS) and standard culture medium with the gradual replacement of glucose (G) by xylose (X).

The crystallinity index (CrI) shows a slight decrease with increasing xylose proportion in the culture medium (86–79%), with all the films retaining a high crystallinity (Table 3-3). Profile of the FT-IR spectra (Figure 3.5) also characterize all of the films as bacterial cellulose and cellulose type I (Table 3-4) with a band at 867 cm^{-1}. This is consistent with C–H deformation (Proniewicz *et al.*, 2002; Ang *et al.*, 2012). The fraction of polymorphs I_α (Table 3-3) further showed the cellulose I_α-rich type was not affected by xylose in the culture medium.

TABLE 3-4 FT-IR group frequency of absorption bands of bacterial cellulose

Group frequency (cm^{-1})	Origin and Assignment	Ref.
2995-4000	Free and hydrogen-bonded OH stretching	Yue *et al.*(2015)
2892	C-H streching	Yue *et al.*, (2015)
2851	CH_2 symmetrical stretching	Nelson e O'connor (1964)
~1637	O-H bending vibration of adsorbed water molecules	Nelson and O'Connor (1964)

1467	Methyl group deformations	Yue et al. (2015)
1429	CH2 scissoring motion	Fan *et al.*(2012); Nelson & O'Connor (1964)
1368	C-H bending	Yue *et al*. (2015)
1316	CH_2 rocking vibration at C6	Fan *et al*. (2012); Yue *et al*. (2015)
1259	G ring streching	Fan *et al*. (2012)
1232	COH bending at C6	Fan *et al*. (2012)
1204	C-O-C symmetric stretching, OH plane deformation	Fan *et al*. (2012)
1163	C-O-C asymetric bridge streching	L. Nelson & M. Cox (2012); Yue *et al*. (2015)
1105	Anti-symmetric ring stretch	Yue *et al*. (2015)
1046~1018	C-C, C-OH, C-H ring and side group vibrations	Yue *et al*. (2015); Fan *et al*. (2012)
993	C-O valence vibration	Yue *et al*. (2015)
800-950	C-H deformation	Ang *et al*. (2012); Proniewicz et al. (2002)
897	C-O-C stretching at the the β(1-4) glycosidic linkages	Yue et al. (2015)
662	C-OH out-of-plane bending	Fan et al. (2012)

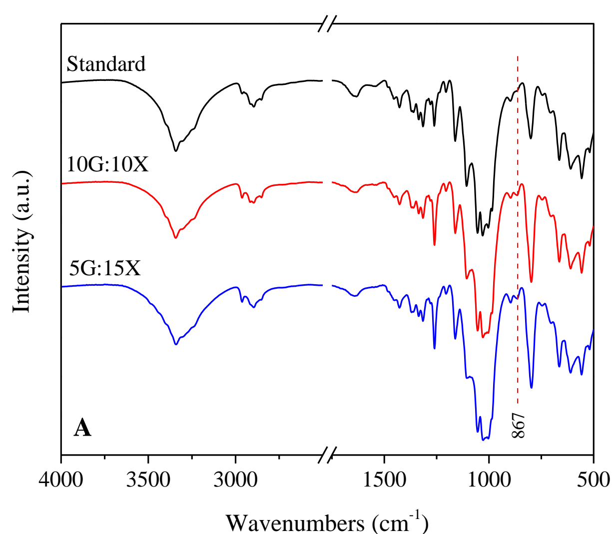


FIGURE 3.5 FTIR vibrational spectra of BC films produced by *Komagataeibacter hansenii* using standard culture medium (HS) and standard culture medium with the gradual replacement of glucose (G) by xylose (X). The spectrums were normalized using the band intensity at 1030 cm^{-}.

3.4.3 Use of sugarcane bagasse and corn stover liquid fraction as culture medium for BC production

Nutritional supplementation of full-strength (100%) pretreatment liquid from corn stover and sugarcane bagasse that had not been treated with activated charcoal was insufficient to achieve growth of *K. hansenii* (data are not shown). Most likely *K. hansenii* was not able to overcome the inhibitory effect of the phenolics in the pretreatment-derived liquid. In comparison, pretreatment liquid from corn stover and sugarcane bagasse treated with activated charcoal facilitated the growth of the bacterium and the production of the cellulose film (Table 3-5).

TABLE 3-5 Comparison of Nanocellulose Productivities in Detoxified Sugarcane Bagasse and Corn Stover

Fermentation Media	Pretreatment Liquid from Bagasse		Pretreatment Liquid from Corn Stover	
	Weight of nanocellulose formed (dry basis, mg)	Yield $\frac{\text{mg nanocellulose}}{\text{mg/glucose}}$	Weight nanocellulose formed, mg (dry basis, mg)	Yield $\frac{\text{mg nanocellulose}}{\text{mg/glucose}}$
Hestrin-Schramm	5.1 ± 0.3	0.026[a]	4.5 ± 0.4	0.023[a]
Detoxified	3.7 ± 0.2	0.740[b]	6.7 ± 0.7	0.440[b]
Detoxified + Glucose	5.5 ± 0.2	0.028[a]	10.2 ± 0.6	0.051[a]
Detoxified + Glucose + Salts	9.4 ± 0.2	0.047[a]	13.1 ± 0.8	0.066[a]

[a]Final culture media volume of 10 mL with 20 mg glucose/mL or total weight of 200 mg glucose.

[b]Final culture media volume of 10 mL with 0.5 mg glucose/mL of glucose derived from biomass after liquid hot water pretreatment, or total weight of 5 mg glucose, for bagasse; and 1.54 mg glucose/mL derived from biomass after liquid hot water pretreatment or total weight of 15.4 mg of glucose for corn stover (Table 3-1).

When detoxified pretreatment liquid was supplemented to the same glucose concentration as HS standard medium, the detoxified liquid gave, respectively, similar or even higher yields for sugarcane bagasse (i.e., 0.028 vs. 0.026 g nanocellulose/g glucose for HS medium) and corn stover (i.e., 0.051 vs. 0.023 g nanocellulose/g glucose for HS medium; Table 3-5). When glucose and salts were combined at the same concentration as in HS medium, production of bacterial cellulose increased about two tothree times (Table 3-5). Different dilutions of the glucose through addition of xylose (cf. control to 10:10 to 5:15 [glucose:xylose] in Table 3-3) also reduced the yield of nanocellulose by about the same amount.

Bacterial nanocellulose synthesis occurs in two steps: (a) the intracellular formation of 1,4-β-glucan chains, involving four key enzymes and (b) the assembly and crystallization of cellulose chains, with cellulose chains being extruded outside the cell where the chains selfassemble into fibrils. Although these processes occur in an extracellular space, they are considered to be cell directed because the formed nanocellulose film structures are governed by the pattern of extrusion sites associated with the cell (Chawla, Prashant R *et al.*, 2009; Lee *et al.*, 2014; Reiniati *et al.*, 2017).

3.4.4 Characterization of the BC film produced using detoxified liquid fraction from sugarcane bagasse and corn stover

The BC films produced using the detoxified bagasse pretreatment liquid (Figure 3.6) showed a hierarchical structure with different pore sizes and similarity between the interweaving of the fibers, but the thickness of the ribbons seemed to have increased in the presence of detoxified bagasse liquor in relation to the standard HS culture medium.

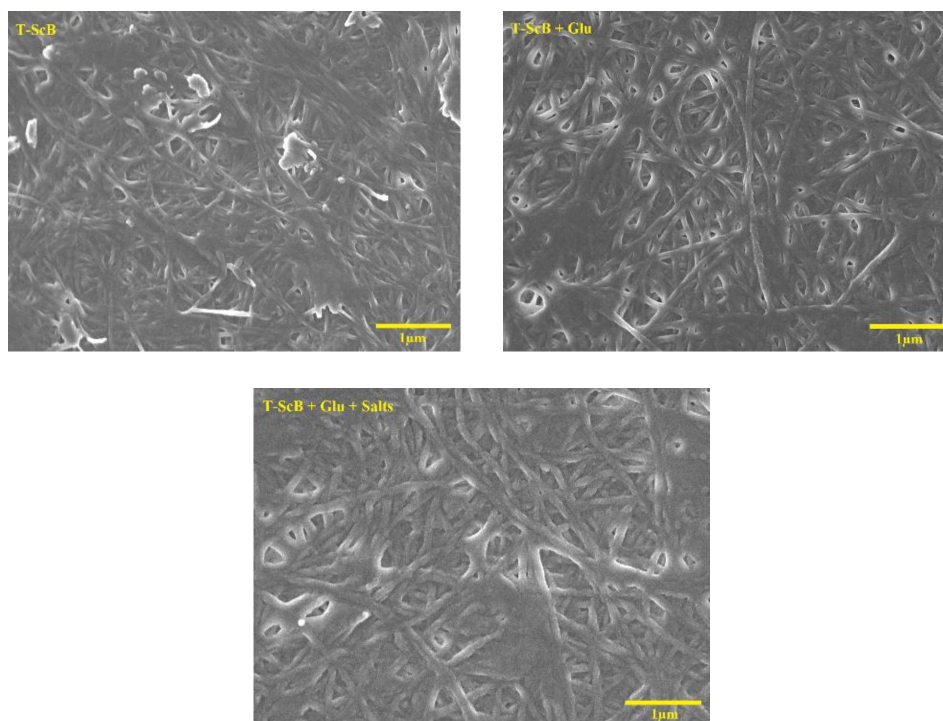


FIGURE 3.6 FESEM micrographs of BC films produced by *K hansenii* using detoxified liquid fraction (i.e., pretreatment liquid) from liquid hot water pretreated sugarcane bagasse.

The use of detoxified bagasse liquid fraction as culture medium, with nutritional supplementation or not, did not affect the behavior in relation to heat degradation compared to BC produced using standard HS culture medium, showing the typical single step profile (Figure 3.7). Also it was observed no differences in the T_{onset} between these samples (Table 3-6).

TABLE 3-6 BC films synthesized using detoxified sugarcane bagasse liquid fraction (D-SB) and detoxified cron stover liquid fraction (D-CS), raw and nutritionally supplemented as the standard culture medium: weight and characterization details.

Samples	T_{onset} (°C)	CrI (%)	f_α^{IR}	f_α
Standard (SB)	313.5	73.2	0.31	0.48
D-SB	314.6	73.1	0.34	0.55
D-SB + Glu	307.5	66.5	0.35	0.56
D-SB + Glu + Salts	310.3	89.1	0.34	0.55
Standard (CS)	295.5	79.0	0.31	0.49
D-CS	319.0	*	0.22	0.25
D-CS + Glu	319.3	75.4	*	*
D-CS + Glu + Salts	329.3	*	*	*

* the data could not be calculated

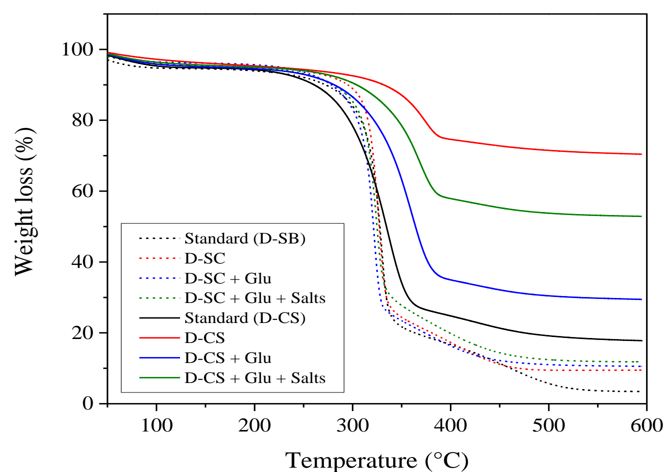


FIGURE 3.7 Weight loss curves from TGA analyses of BC films produced by *K. hansenii* using standard culture medium (HS) and detoxified sugarcane bagasse liquid fraction (D-SB) and treated corn stover liquor (D-CS), raw and nutritionally supplemented as the standard culture medium.

The peaks found in the diffractogram for the three samples using detoxified bagasse liquid suggest the presence of cellulose I only, and the diffractograms also reveals the presence of the amorphous region (Figure 3.8).

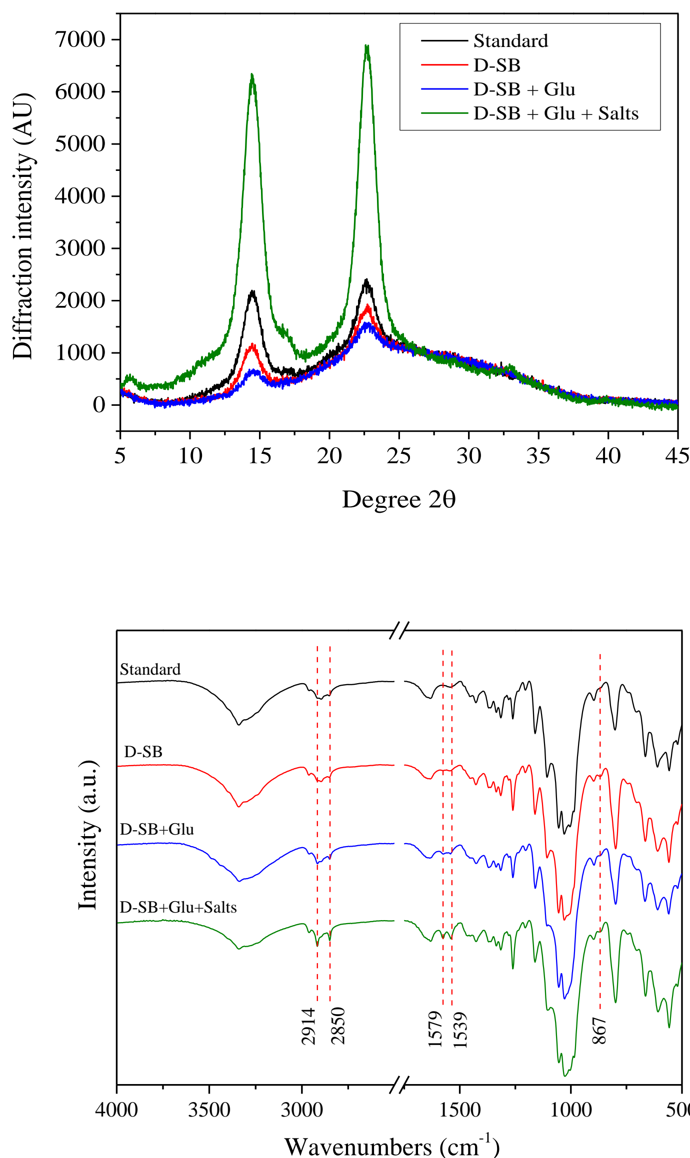


FIGURE 3.8 X-ray diffractogram and FT-IR vibrational spectra of BC films produced by *K. hansenii* using standard culture medium (HS) and detoxified sugarcane bagasse liquid fraction (D-SB), raw and nutritionally supplemented as the standard culture medium. The FT-IR spectrums were normalized using the band intensity at 1030 cm^{-1}.

The CrI of the films produced with detoxified bagasse liquid increased as the culture medium was being supplemented (Table 3-6), probably due to the better availability of substrate and salts, which can act as cofactors in the reactions of BC synthesis, allowing a better polymerization process (Lee *et al.*, 2014). The use of treated bagasse liquor as culture medium increase I_{α} fraction (Table 3-6).

The BC film produced with charcoal-treated corn stover liquid fraction showed a different structure interweaving of the fibers, and agglomerates appear to be adhered to the ribbons surface. However, the glucose and salts supplementation produced cellulose films with an interweaving and pore size similar to the BC films using the standard HS medium (Figure 3.9).

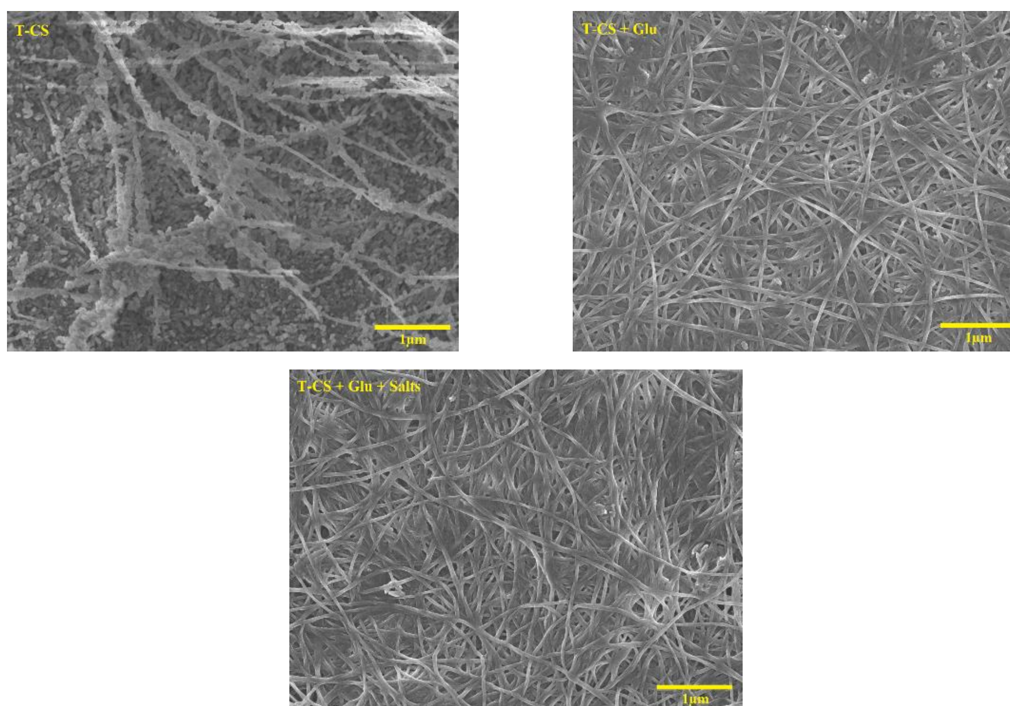


FIGURE 3.9 FESEM micrographs of BC films produced by *K. hansenii* using detoxified liquid fraction (i.e., pretreatment liquid) from liquid hot water pretreated corn stover.

The T_{onset} remained high for all films produced using corn stover liquid fraction (Table 3-3). Although these films exhibited the same behavior in relation to thermal degradation, the difference in the mass loss found suggests that the treatment with activated charcoal or the corn stover liquor medium composition interfered in the production of BC (Figure 3.7). X-ray diffractograms (Figure 3.10 A) and FT-IR spectra (Figure 3.10 B) also suggest that the crystallinity and chemical structure of the films produced using corn stover liquid fraction were altered in relation to BC produced using standard HS culture medium. The CrI (%) of the BC film produced with D-CS + Glu was similar to the BC film produced in the standard HS medium (Table 3-6). For the other conditions using corn stover liquor, the CrI (%) value could

not be determined due to contaminated spectrum. The changes in the FT-IR spectra (Figure 3.10 A) indicates that BC films produced in the presence of corn stover liquor are cellulose I_β-dominant type (Kataoka e Kondo, 1999), the disappearance of the band at 750 cm^{-1} prevents the estimation of fraction I alpha.

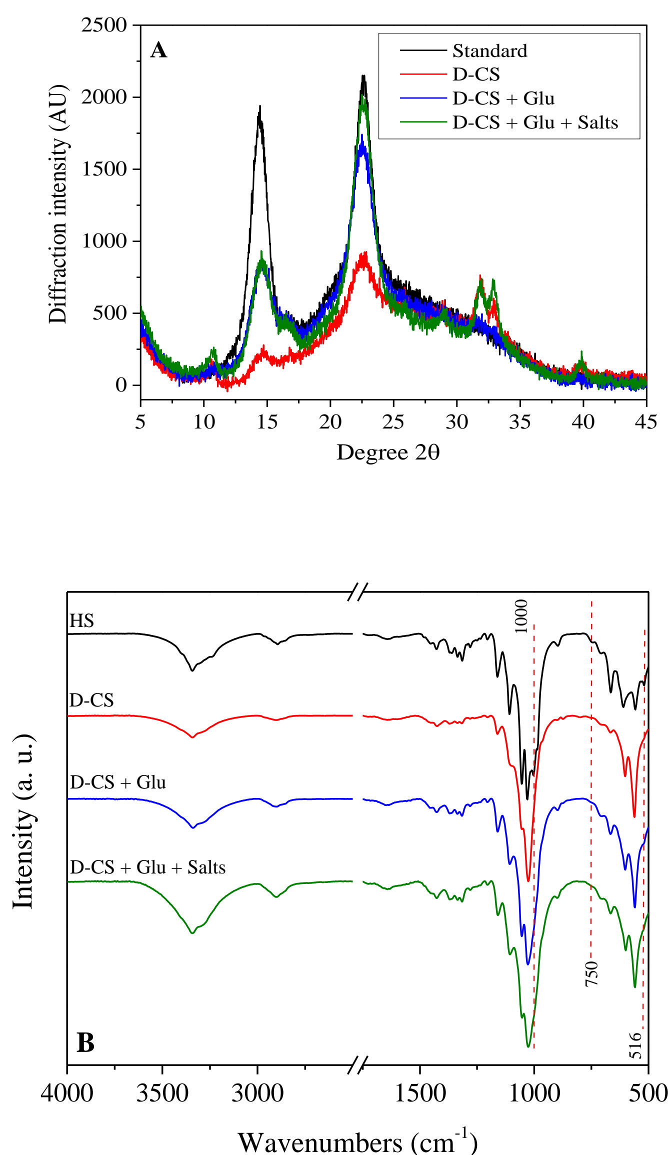


FIGURE 3.10 X-ray diffractogram (A) and FT-IR vibrational spectra (B) of BC films produced by *K. hansenii* using standard culture medium (HS) and detoxified corn stover liquid fraction (D-SB), raw and nutritionally supplemented as the standard culture medium. The FT-IR spectrums were normalized using the band intensity at 1030 cm^{-1}.

3.4.5 Inhibition of nanocellulose production by phenolic compounds

Phenolic compounds inhibit cellulose-hydrolyzing enzymes (Ximenes *et al.*, 2010; 2011; Kim *et al.*, 2013; Kim *et al.*, 2015; Dos Santos *et al.*, 2018) and cellulose producing bacteria (Guo *et al.*, 2013; Kiziltas *et al.*, 2015). We confirmed that individual addition of 1.8 g/L of ferulic, tannic, ρ-coumaric acids or vanillin to the HS medium inhibited the synthesis of bacterial cellulose by the original strain when their concentrations were similar to those in pretreatment liquid from sugarcane bagasse (Table 3-7).

TABLE 3-7 Effect of addition of phenolic compounds on weight of nanocellulose produced by *K. hansenii*.

Media (with 20 mg/mL glucose)	Weight of nanocellulose formed (dry basis, mg)	Yield $\frac{\text{mg nanocellulose}}{\text{mg glucose}}$
Hestrin and Schramm Media	5.1 ± 0.3	0.025
Phenolic added (at 1.8 mg/mL)		
Vanillin	0	0.0
Ferulic acid	2.4 ± 0.3	0.012
ρ-coumaric acid	4.5 ± 0.7	0.022
Tannic acid	5.4 ± 0.4	0.027

Vanillin prevented the production of nanocellulose film altogether (Table 3-7). Ferulic and ρ-coumaric acids decreased nanocellulose production by 50% and 12%, respectively. Tannic acid, which completely deactivates β-glucosidase and inhibits cellulolytic enzymes, (Ximenes *et al.,* 2010, 2011), had little effect on the amount of nanocellulose produced. However, nanocellulose film produced in the presence of tannic acid had collapsed fibers (Figure 3.11).

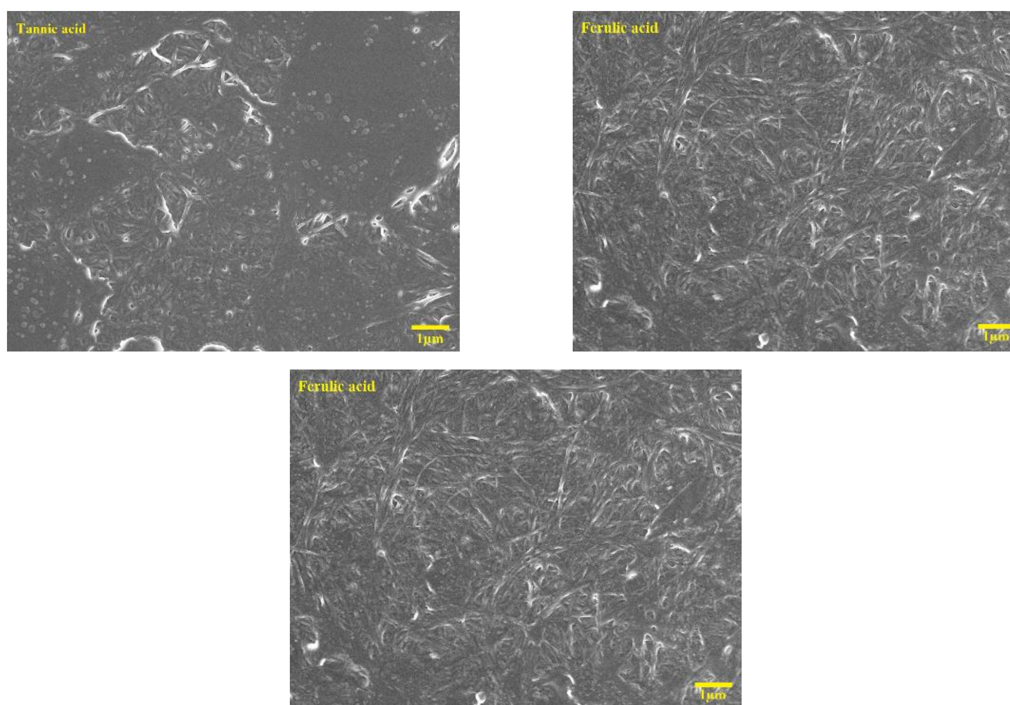


FIGURE 3.11 FESEM micrographs of BC films produced by *Komagataeibacter hansenii* using standard culture medium (HS) with the addition of phenolic compounds (1.8 mg/mL).

These results indicate that the amount and type of phenolic compound inhibits differently the microorganism for BC production. Phenolic compounds have been found to have a considerable inhibitory effect in the fermentation of lignocellulosic hydrolysates, and low molecular weight phenolic compounds are considered to be most toxic (Guo *et al.*, 2013).

The phenolic compounds added did not affect the behavior in relation to heat degradation, as all the samples presented the typical single step profile (Figure 3-12 A), and also no differences in T_{onset} were observed (Table 3-8).

TABLE 3-8 Characterization details of BC films BC films produced by *K. hansenii* using standard culture medium (HS) with the addition of phenolic compounds (1.8 mg/mL)

Samples	T_{onset} (°C)	CrI (%)	f_α^{IR}	f_α
Standard	313.5	73.2	0.31	0.48
Ferulic acid	308.9	81.4	0.31	0.47
Tannic acid	301.0	83.0	0.32	0.49
P-coumaric acid	316.4	80.5	0.36	0.60

The peaks found in X-ray diffractograms (Figure 3-12 B) and the FT-IR spectra (Figure 3-12 C) for all samples suggest the presence of cellulose type I only and the presence of the amorphous region. The presence of phenolics did not affect the CrI of the film, neither the I_{α} fraction (Table 3-8).

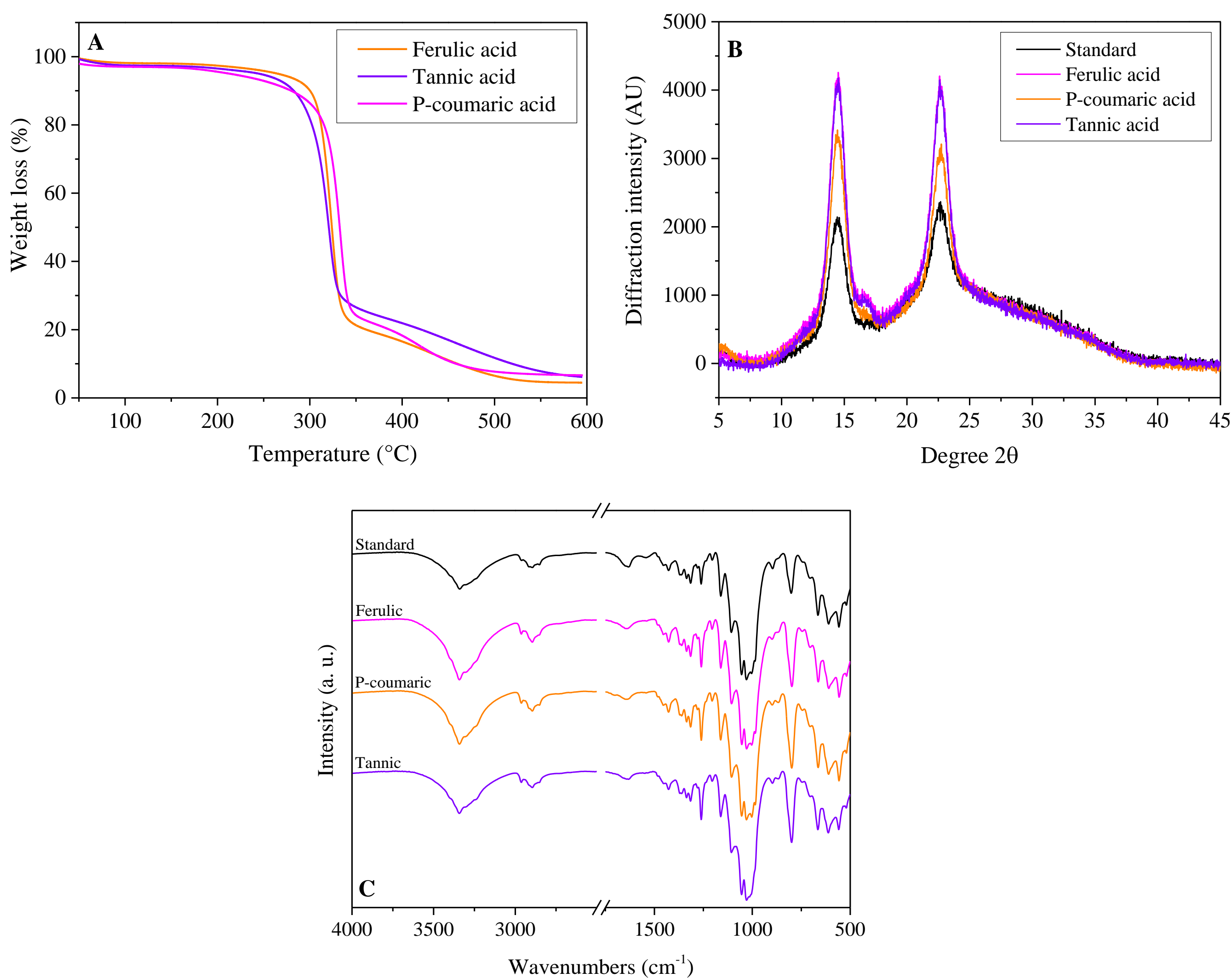


FIGURE 3.12 Weight loss curves from TGA analyses (A), X-ray diffractogram (B) and FT-IR vibrational spectra (C) of BC films produced by *K. hansenii* using standard culture medium (HS) with the addition of phenolic compounds (1.8 mg/mL). The FT-IR spectrums were normalized using the band intensity at 1030 cm^{-1}.

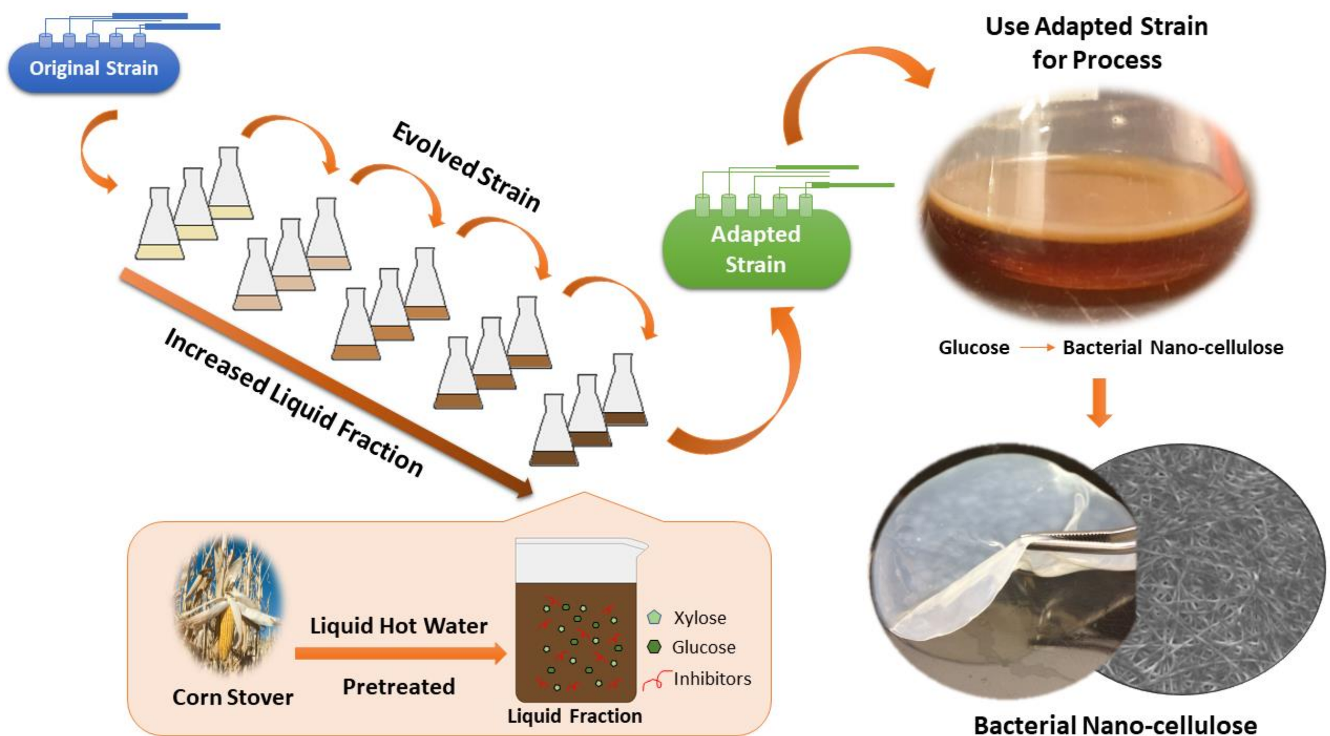
3.5 Conclusions

The liquid fractions of the LHW pretreatment from bagasse and corn stover have potential to be used as a source of C for bacterial cellulose production. However, the inhibitors in these media are toxic to the bacteria and an additional detoxification step is required for BC production. BC production using the detoxified CS fraction was 49% higher than that using standard medium, but 28% lower in the presence of detoxified SB fraction as culture medium. The bacterium cultured in pretreatment liquid detoxified with activated charcoal to remove inhibitors was capable to convert glucose to bacterial nanocellulose and used xylose as carbon source for growth. The cellulose-producing bacteria was inhibited in the presence of vanillin, ferulic and ρ-coumaric acids compounds at the concentration of 1.8 mg/mL. The vanillin was the most toxic compound, inhibiting the growth of the microorganism, while tannic acid, a known enzyme inhibitor, did not affect cellulose production. The physicochemical characteristics of all produced cellulose films were similar to the nanocellulose generated in a nutritional media specifically formulated for bacterial cellulose formation.

4 Adaptive laboratory evolution of nanocellulose-producing bacterium

* The content of this chapter is an adaptation of the scientific article entitled: "Adaptive laboratory evolution of nanocellulose-producing bacterium" by Vanessa M. Vasconcellos, Cristiane S. Farinas, Eduardo Ximenes, Patricia Slininger, Michael Ladisch, published in Biotechnology and Bioengineering.

Reference: doi: 10.1002/bit.26997



4.1 Abstract

Adaptive laboratory evolution through 12 rounds of culturing experiments of the nanocellulose-producing bacterium *Komagataeibacter hansenii* ATCC 23769 in a liquid fraction from hydrothermal pretreatment of corn stover resulted in a strain that resists inhibition by phenolics. The original strain generated nanocellulose from glucose in standard Hestrin and Schramm (HS) medium, but not from the glucose in pretreatment liquid. The properties of this cellulose were the same as nanocellulose generated from media specifically formulated for bacterial cellulose formation. However, attempts to directly utilize glucose proved unsuccessful due to the toxic character of the lignin-derived phenolics, and in particular, vanillin and ferulic acid. Adaptive laboratory evolution at increasing concentrations of pretreatment liquid from corn stover in HS medium resulted in a strain of *K. hansenii* that generated bacterial nanocellulose directly from pretreatment liquids of corn stover. The development of this adapted strain positions pretreatment liquid as a valuable resource since *K. hansenii* is able to convert and thereby concentrate a dilute form of glucose into an insoluble, readily recovered and value-added product-bacterial nanocellulose.

4.2 Introduction

Nanocellulose produced by *Komagataeibacter* species is characterized by thermal stability, mechanical strength, and water retention in a biocompatible and biodegradable material (Esa *et al.*, 2014; Islam *et al.*, 2017). Fermentation derived nanocellulose has a translucent optical character (Figure 4.1 a,b) due to the nanometer diameters of fibers that make up the matrix (Figure 4.1c) and give it strength despite a void fraction of 90% or more. Potential applications include hydrocolloid additives for food, and medical uses for wound dressings, tissue replacement, and drug delivery (Ullah *et al.*, 2016; Da Gama e Dourado, 2018).

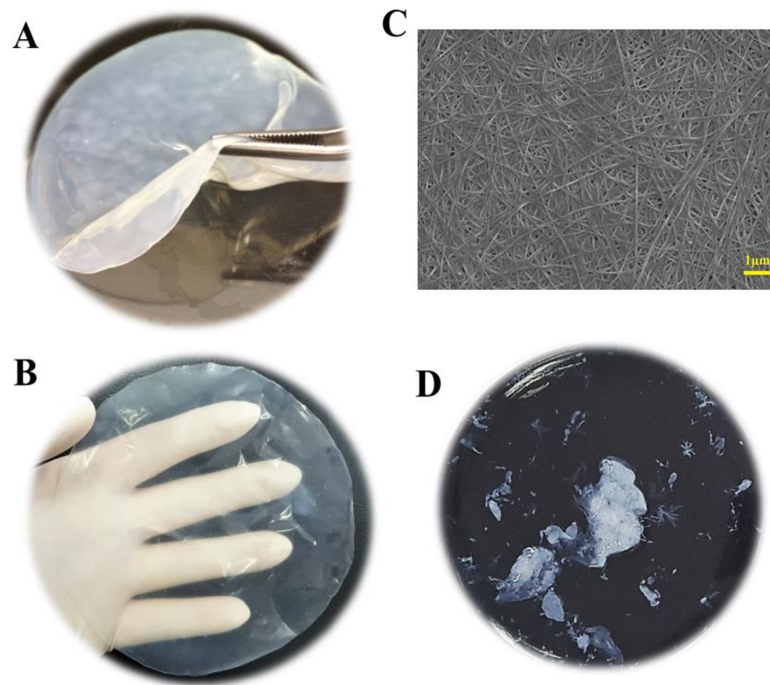


FIGURE 4.1 (a) Bacterial nanocellulose film generated in a culture dish being harvested using tweezers; (b) transparent property illustrated by fingers that are visible behind the film after harvesting, purification, and drying; (c) scanning electron micrograph image of dried sheet of bacterial cellulose nanofibers showing matrix structure; (d) nanocellulose formed in shake flask (images courtesy of EMBRAPA Instrumentação).

Bacterial nanocellulose was discovered in pineapple peels in 1819 and evolved into a traditional fermented food business in the Pacific region. Numerous food and other uses have been identified and tested since then. Biomedical application as a wound dressing for second- and third-degree burns has evolved since 2006 and films of bacterial nanocellulose formed by Komagataeibacter species have recently been shown to be suitable for food packaging (Azeredo *et al.*, 2017).

Carbohydrates present in lignocellulose-derived liquid process streams as well as hydrolysates have the potential to be used as feedstock for bacterial cellulose production (Castro, Cristina *et al.*, 2011; Kiziltas *et al.*, 2015). Formation of bacterial nanocellulose has been reported from konjac powder (Hong e Qiu, 2008), waste fibers from pulp and paper mill (Cavka *et al.*, 2013), spruce wood (Guo *et al.*, 2013), sugarcane bagasse, and corn stalks

hydrolysates (Cheng *et al.*, 2016; Cheng *et al.*, 2017). Production of bacterial nanocellulose in agitated culture has potential for scale-up, although the product is formed as aggregates rather than sheets (Figure 4.1 d) (Chen *et al.*, 2017; Chen *et al.*, 2018).

Liquid hot water pretreatment of lignocellulosic materials including corn stover, corn fiber, switchgrass, sugarcane, and hardwoods containing low concentrations of glucose results in an aqueous stream and a solids fraction that is separately utilized for enzyme hydrolysis and fermentation to ethanol (Figure 4.2) (Zeng *et al.*, 2007; Zeng *et al.*, 2012a; b; Ladisch *et al.*, 2014; Ko *et al.*, 2015). Pretreatment liquids from a range of lignocellulose biomass materials contain inhibitory phenolic constituents (Table 4.1). Inhibitor-tolerant *Komagataeibacter* species could position the pretreatment liquid as a valuable resource for production of bacterial nanocellulose by directly converting, and thereby concentrating the glucose present at low concentration levels into an insoluble, readily recovered value-added product (Zhang, Winestrand, Chen, *et al.*, 2014; Zhang, Winestrand, Guo, *et al.*, 2014; Zou *et al.*, 2017). However, pretreatment liquid contains, in addition to dilute sugars (such as glucose and xylose), acetic acid and phenolic inhibitors in a range of lignocellulosic feedstocks (Table 4.1) (Cao *et al.*, 2013; Cao *et al.*, 2015; Kim *et al.*, 2016; Michelin *et al.*, 2016; Ximenes *et al.*, 2010; 2011; Ximenes *et al.*, 2017). Hence, inhibitor tolerant bacteria are required to avoid costs of removing inhibitors (Guo *et al.*, 2013), particularly phenolics, and would enable the generation of bacterial nanocellulose in the presence of inhibitors generated by liquid hot water (LHW) pretreatment.

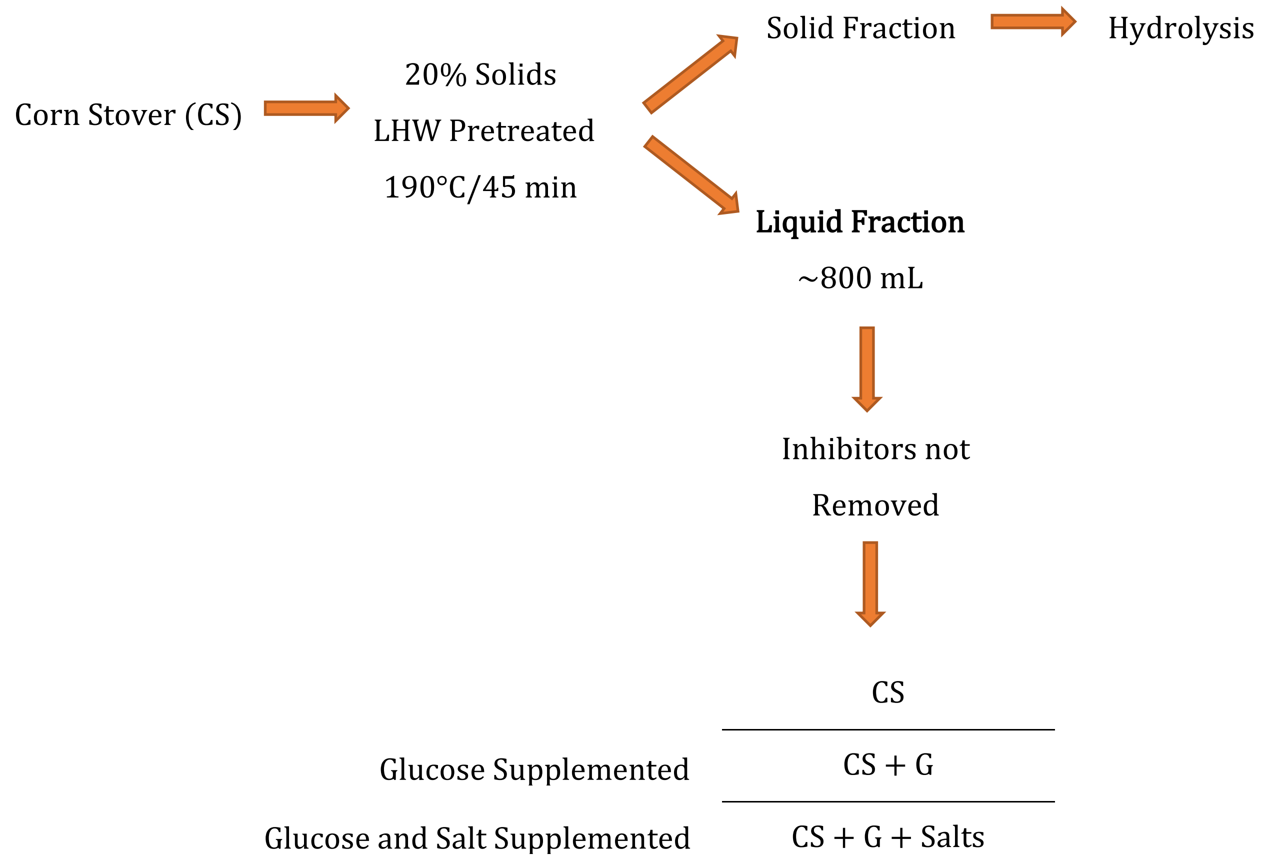


FIGURE 4.2 Summary of preparation of culture media using liquid fraction (i.e., pretreatment liquid) from liquid hot water (LHW) pretreated corn stover (CS).

TABLE 4-1 Compositions of liquid hot water pretreatment

Biomass Material	Corn Stover [1]	Corn Fiber [2]	Sugarcane Bagasse [3]	Switch Grass[4-6]	Hardwood (Maple)[7]
Pretreatment Temperature (°C)	190	160	200	200	200
Time (min.)	45	20	30	10	20
Severity[8]	4.88	3.45	5.07	4.594	4.89
Solids Loading (%)	20	20	10	15	23
Composition (g/L) Glucose	0.25	0.96	0.4	0.2	0.1
Xylose	1.99	2.82	7.6	1.3	1.1
Arabinose	na[9]	7.03	1.2	0.4	0.3
cellobiose	0.34	na	na	na	na
Gluco-oligosaccharides	na	na	0.8	na	na
Xylo-oligomers	21.7	na	6.5	na	na
Acetic Acid	2.8	0.56	3.4	1.2	2.7
Phenolics	3.09	0.84	1.4	na	1.3
HMF	0.141	0.01	0.3	0.04	nd[10]
Furfural	0.7	0.27	4.8	0.52	0.5

(Kim *et al.*, 2016), this work [1]; (Kurambhatti *et al.*, 2018)[2]; (Michelin *et al.*, 2016)[3]; (Kim, Youngmi *et al.*, 2011)[4]; (Ximenes *et al.*, 2013)[5], (Ximenes *et al.*, 2017)[6]; (Kim, Y. *et al.*, 2011)[7]; $\varpi = 12.1$ was fitted to data for xylan dissolution[8] na = not available[9]; nd: not detected[10].

Adaptive laboratory evolution may be designed to enhance bacterial tolerance to inhibitors. Examples include improvement of fitness of *Klebsiella pneumoniae* with respect to 2,3 butanediol accumulation (Li *et al.*, 2016), evolution of cytochrome P450 BM-3 monoxygenase to organic cosolvents used in hydroxylation and epoxidation reactions (Seng Wong *et al.*, 2004), and improvement of xylose utilization by *Corynebacterium glutamicum* (Radek *et al.*, 2017). Other applications include the development of antibiotic overproduction

by Streptomyces clavuligerus (Charusanti *et al.*, 2012), and enhancement of tolerance of the ethanologenic yeasts *Scheffersomyces stipites* (Slininger *et al.*, 2015) and *Saccharomyces cerevisiae* to lignocellulosic hydrolysates for ethanol biofuel production (Almario *et al.*, 2013).

We report the adaptive laboratory evolution of *Komagataeibacter hansenii* ATCC 23769 to enhance its ability to generate bacterial nanocellulose from liquid fraction of LHW pretreated corn stover. Adaptive laboratory evolution, as recently discussed by (Lacroix *et al.*, 2017) is particularly relevant for pretreatment liquid from sugarcane bagasse, corn stover, and hardwood, since these represent current major candidates for cellulosic biorefineries (Ladisch *et al.*, 2014; Dos Santos *et al.*, 2018). Our work resulted in an adapted strain of *K. hansenii*, that unlike the wild-type, is capable of directly converting glucose in pretreatment liquid to bacterial nanocellulose.

4.3 Materials and Methods

4.3.1 Microorganism

K. hansenii ATCC 23769 was purchased from Fundação André Tosello de Pesquisa e Tecnologia—FAT (Campinas, Brazil). The bacterium was maintained in slants of mannitol agar medium (3 g/L bacto peptone, 5 g/L yeast extract, 25 g/L D-mannitol, and 15 g/L agar) at 4°C. The stock was renewed monthly.

4.3.2 Lignocellulosic biomass

Corn stover was harvested from central Illinois, dried at room temperature to 5–8% moisture, and ground (Model 4 Wiley, Thomas Scientific, Swedesboro, NJ) to pass through a 2 mm screen (Kim *et al.*, 2016).

Pretreatments were based on using LHW conditions as described by Kohlmann *et al.*, (1994); Kim *et al.*, (2009); Kim *et al.*, (2013); Kim *et al.*, (2015); Kim *et al.*, (2016). Corn

stover pretreatment liquid was obtained for 20% (wt/vol) CS solids loading in deionized (DI) water in 37 ml reaction tubes. The tubes were preheated to 140°C in a sandbath for 45 min, heated and held at 190°C for 15 min, and cooled by quenching in room temperature water. Heating and cooling times were 45 and 30 min, respectively.

4.3.3 Pretreatment liquids

The pH of liquids from corn stover were adjusted to 5.0 with 1% (wt/vol) citric acid. The liquid from pretreating lignocellulose were clarified by vacuum filtering solids through Whatman filter paper number 1 in a 70-mm Buchner funnel. Filtration required 5 min at room temperature. The corn stover filtrate was used to formulate the media used for adaptive evolution of *K. hansenii*. Compositions are summarized in Table 4-2. Hydroxymethylfurfural was not detected.

The pretreatment liquids were analyzed before and after autoclaving. The compositions of pretreatment liquids themselves were initially analyzed for carbohydrates by liquid chromatography (Sluiter *et al.*, 2008) as well as for phenolics using Folin–Ciocalteu reagent (Singleton Vernon *et al.*, 1999). Reducing sugars, glucose and phenolic compounds were measured by the dinitrosalicylic acid (DNS) method (Miller, 1959), an enzymatic kit (Megazyme) and Folin–Ciocalteu reagent (Sigma-Aldrich), respectively ((Freitas *et al.*, 2019)Singleton et al., 1999). The total phenolic compound concentrations were expressed as gallic acid equivalents (GAE; mg/ml). During adaptive laboratory evolution protocols, total reducing sugars (includes hexoses and pentoses), glucose, and phenolics were measured calorimetrically rather than running the more time-consuming liquid chromatographic analysis for the large number of samples generated.

TABLE 4-2 Compositions of pretreatment liquids and culture media used for adaptive evolution of *Komagataeibacter hansenii*

Culture Media	Concentrations of Key Components, mg/mL				mg/mL
	Glucose	Xylose	Arabinose	Acetic Acid	Total phenol
Hestrin and Schrumm	20	0	0	0	0
Corn Stover Pretreatment Liquids					
100%	1.54	2.75	4.02	3.16	3.05
80% supplemented	23.6	nd	1.50	1.93	2.44
60% supplemented	23.8	nd	1.14	1.46	1.83

nd = not detected.

4.3.4 Culture medium

Standard Hestrin and Schramm (HS) medium was used for bacterial growth (Hestrin e Schramm, 1954). This medium contains (%, wt/vol basis): 2.0 glucose, 0.5 bacto peptone, 0.5 yeast extract, 0.27 disodium phosphate (anhydrous), 0.115 citric acid, with the pH adjusted to 5.0 using a 1% (wt/vol) citric acid solution.

The liquid fraction supplemented with yeast extract and peptone were used as culture medium for bacterial nanocellulose production in three different ways: as is, nutritionally supplemented with glucose (+Glu), or supplemented with glucose and salts (+Glu + Salts). Final concentrations of supplemented nutrients were at the same concentrations as in the HS medium. The sequence of steps in preparing these liquids is outlined in Figure 4.2.

4.3.5 Microbial cultivation and bacterial cellulose formation

There are four steps in the protocol for the formation of bacterial cellulose: activation in agar, preparation of pre-inoculum filtrate, inoculation of HS medium with the filtrate and finally cultivation at 30°C under static conditions. Bacteria were first inoculated into HS agar

medium at 30°C resulting in the formation of colonies that grow as a cloudy film. The cells were then removed from the agar and transferred to 20 ml of fresh HS medium in a shake flask.

After 24 hr, a pre-inoculum was produced, of which 10 ml was added into 40 ml of fresh medium and incubated for an additional 3 days. The cultivation of bacterial nanocellulose was started by inoculating a volume corresponding to 4% (vol/vol) of filtered inoculum into fresh culture medium. Cultures were incubated for 7 days in a 50 ml Erlenmeyer using 20% of the flask volume. The amount of nanocellulose formed by the bacteria was measured after harvesting, boiling to remove cells and medium embedded in the cellulose film, filtering, drying, and weighing.

4.3.6 Purification and drying process to asses nanocellulose accumulation

The synthesized bacterial cellulose (BC) was separated from the medium using tweezers (as illustrated in Figure 4.1a) and boiled (~95°C) in a 1% (wt/vol) sodium hydroxide solution for 60 min to, as mentioned in previous section, remove cells and medium embedded in the cellulose film. The film was then rinsed with distilled water at ambient temperature until neutral pH was achieved followed by oven drying at 50°C for 48 hr to constant weight. The resulting sheet (Figure 4.1b) of about 5 cm diameter was stored at 50% humidity for further analysis, to have standard conditions for the nanocellulose characterization work, minimizing interference in the analyses by humidity.

4.3.7 Adaptative evolution of bacteria to LHW biomass pretreatment derived inhibitors

Pretreatment liquid separated by vacuum filtration from 20% corn stover solids (Figure 4.2) after LHW pretreatment was used for adaptive evolution of the *K. hansenii* to obtain an inhibitor-tolerant strain. For purposes of adaptive evolution, the pretreatment liquid was supplemented with glucose and salts resulting in a phenol-containing standard culture medium,

(20 g/L glucose, 0.27 g/L disodium phosphate [anhydrous], 0.115 g/L citric acid, with the pH adjusted to 5.0 using a 1% [wt/vol] citric acid solution; Table 4-2). The corn stover pretreatment liquid was added to HS media at volume ratios of 8:2 to 1:9, thus proportionately decreasing inhibitor levels. Six rounds of culturing experiments were carried out in 96-well plates (Figure 4.3) followed by another six rounds in 48-well plates. Once microbial growth in the presence of lignin-derived phenolic inhibitors was achieved (first 6 rounds of culturing experiments), rounds 7-9 were cultured in 48-well plates with each well containing 400 µl medium, followed by cultivation of rounds 10–12 in 800 µl medium, since the larger volumes made it easier to observe membrane formation.

Incubation was static at 30°C. Each condition was performed in triplicate and a serial transfer after culture of 5% (vol/vol) of the total volume into the fresh medium was performed every 3–4 days. The standard HS culture medium (no inhibitors) was used as the control. Samples with bacterial cellulose films with a 1–2 mm thickness similar to those from standard HS medium were visually selected and used to inoculate the next round of media.

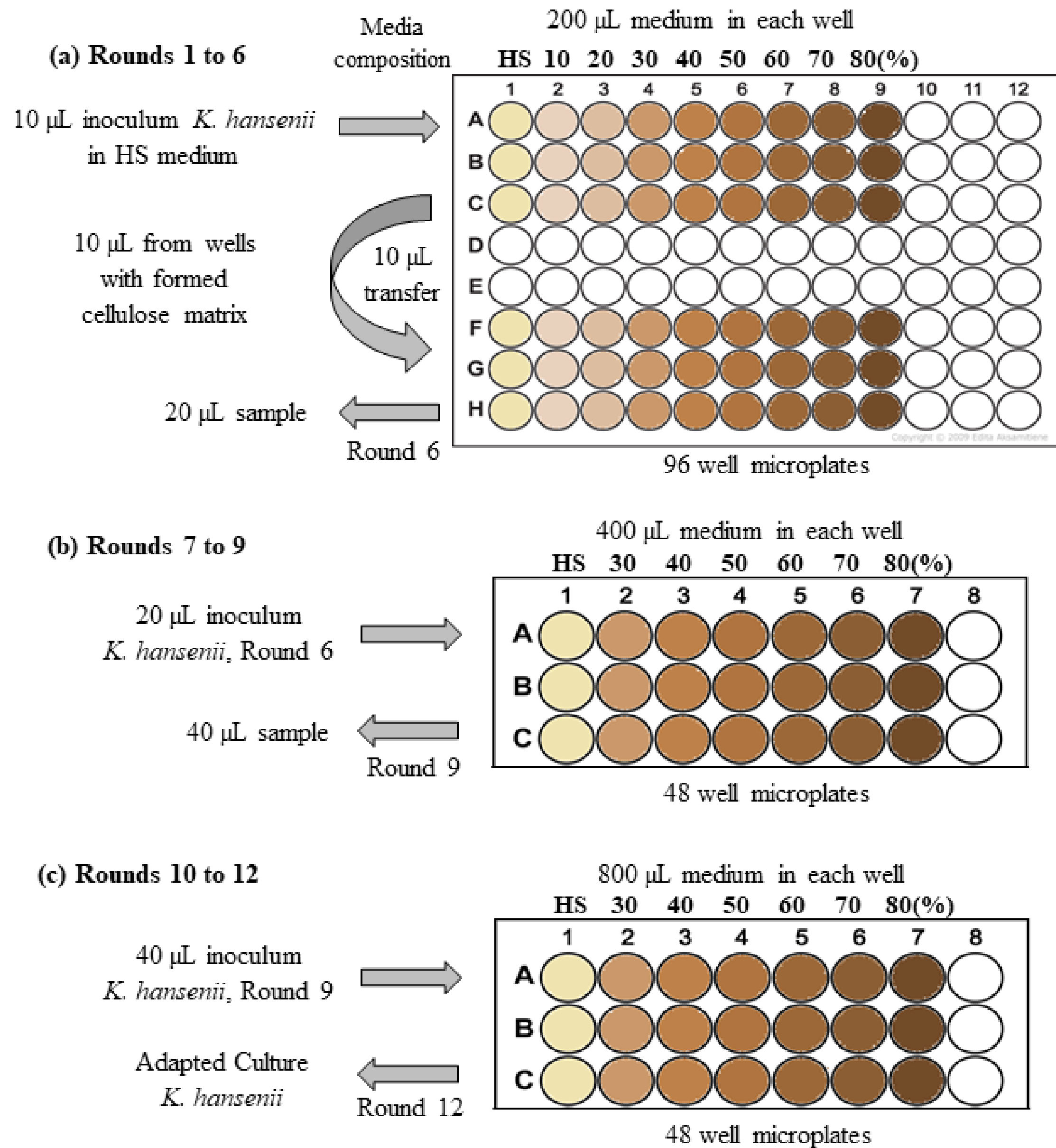


FIGURE 4.3 Experimental procedure for the evolutionary adaptation of Komagataeibacter hansenii in pretreated corn stover liquid fraction (CS) nutritionally supplemented with glucose and salts. Rounds of culturing experiments 1–6 and 7–10 were performed in 96- and 48-well plates, respectively with Hestrin and Schrumm (HS) media supplemented with 10–80% CS pretreatment

liquid as described in Section 4.3. Media color darkens with increasing pretreatment.

4.3.8 BC characterization

BC samples (after purification and drying) were subjected to analyses to characterize the resulting material. BC samples were attached onto aluminum stubs using adhesive carbon tape, coated with carbon (Leica Sputter Coater—SCD050 coating system), and examined by field emission scanning electron microscopy using a JEOL microscope (model JSM-607 1F) operated at 2 kV and with a resolution of 1 µm.

Thermogravimetric analysis (TGA) analyses of BC samples were carried out in a TA instrument (TGA Q500 V6.3 build 189) using a platinum sample holder. Each sample (~7 mg) was heated from ambient temperature to 600°C range, at a heating rate of 10°C/min under nitrogen atmosphere, to avoid sample oxidation, and with a sample flow rate 50 ml/min.

X-ray diffractogram (DRX) was performed with a high-resolution X-ray diffractometer (Shimadzu 6000) with a Ni-filtered Cu Kα (1,540,562 Å) radiation source operated at voltage 30 kV and 30 mA electric current. The BC samples were scanned from 5° to 45° 2θ range and scan speed 0.5°/min. The crystallinity index (CrI) was calculated by Segal method ((Segal *et al.*, 1959), where CrI expresses the relative degree of crystallinity, I_{002} is the maximum intensity of the lattice diffraction (2θ of 22–23°) and I_{AM} represents the amorphous phase of the material (the minimum intensity 2θ between 18° and 19°).

$$\text{CrI}\ (\%) = \frac{(I_{002}-I_{AM})}{I_{002}} \text{x}100 \qquad (1)$$

Fourier-transform infrared (FTIR) spectroscopy analyses were carried out on the BC samples in a Vertex 70 FTIR spectrometer (Brunker), equipped with a Universal ATR accessory, using 32 scans and a resolution of 4 cm^{-1}, over the range 4000-400 cm^{-1} and their background was recorded with an empty cell for each sample. The spectra were normalized at band 1,030 cm^{-1}. The apparent I_α mass fraction (f_α^{IR}) was calculated using the peak heights at 750 cm^{-1} (contribution from cellulose I_α) and 710 cm^{-1} (contribution from cellulose I_β) as showed in Equation (2). The mass fraction of cellulose I_α (f_α) was calculated using the

relationship between FTIR and CP-MAS 13C NMR according to Equation (3) (Yamamoto *et al.*, 1996).

$$f_\alpha^{IR} = \frac{A_{750}}{A_{750}+A_{710}} \qquad (2)$$

$$f_\alpha = 2.55 * f_\alpha^{IR} - 0.32 \qquad (3)$$

4.4 Results and Discussion

The original strain of *K. hansenii* (ATCC 23769) showed an initial tolerance to inhibitors when pretreatment liquid from corn stover was diluted to 30% of its original concentration. Adapted laboratory evolution followed the sequence summarized in Table 4-3. After 12 rounds of culturing experiments, the bacterium became adapted to inhibitors and was able to form bacterial nanocellulose film in culture medium containing 80% (vol/vol) of liquor with added components resulting in a HS standard similar medium containing inhibitors from corn stover pretreatment liquid.

The fitness of the adapted bacteria was subsequently tested in the different culture medium. Results were compared against the unadapted strain (Table 4-4). While the original strain only produced nanocellulose after a detoxification step, the adapted strain produced it in the untreated liquor medium, and actually achieved 49% more nanocellulose with less supplementation of glucose compared to standard medium (1.5 g/L of glucose vs. 20 g/L in standard medium). However, when glucose was added to the pretreatment liquids to achieve same concentration as used in the standard medium (20 g/L), a lower yield of mg nanocellulose/mg glucose was observed compared to that with lower glucose concentration (1.5 g/L glucose).

TABLE 4-3 Evolutionary adaptation of *K. hansenii* in corn stover liquor (CS) nutritionally supplemented with glucose and salts at the same concentration of the standard culture medium.

Rounds	HS	CS concentration (%)							
		10	20	30	40	50	60	70	80
1	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	-	-
2	+ + +	0	0	+ + +	+ +	+ +	+ +	+	+
3	+ + +	0	0	+ + +	+ +	+ +	+	-	-
4	+ + +	0	0	+ + +	+ + +	+ +	+ +	+	+
5	+ + +	0	0	+ + +	+ + +	+ +	+ +	+	-
6	+ + +	0	0	+ + +	+ + +	+ + +	+ +	+ +	+
7	+ + +	0	0	+ + +	+ + +	+ + +	+ +	+ +	-
8	+ + +	0	0	+ + +	+ + +	+ + +	+ +	+ +	+ +
9	+ + +	0	0	+ + +	+ + +	+ + +	+ + +	+ +	+
10	+ + +	0	0	0	+ + +	+ + +	+ + +	+ + +	+ +
11	+ + +	0	0	0	0	+ + +	+ + +	+ + +	+ +
12	+ + +	0	0	0	0	0	+ + +	+ + +	+ + +

Note: The cross symbols represent the thickness of the BC film (+++ same as the standard, ++ between standard and 1 mm, + >1 mm and − no BC synthesized), the grey spaces are the samples selected to start the next round of culturing experiment and 0 indicates the point at which testing in the indicated medium was terminated. Abbreviation: HS: Hestrin and Schramm medium.

Kurosumi *et al.*, (2009) also observed better yields of bacterial cellulose production in different types of fruit juices compared to synthetic media, but a glucose inhibitory effect at higher concentrations may not apply here, since others have seen effective bacterial cellulose production at concentrations even higher than 20 g/L (Cakar *et al.*, 2014). The results show that the adapted strain is capable of both improved glucose utilization and nanocellulose productivity; however, further investigation is needed to understand what mechanism and compounds are involved in triggering the membrane formation with higher yield here at lower concentration of glucose.

TABLE 4-4 Characterization of BC films synthesized by adapted *K. hansenii*

Strain	Culture medium	Weight of nanocellulose formed (dry basis, mg)	Yield $\frac{\text{mg nanocellulose}}{\text{mg/glucose}}$	T_{onset} (°C)	CrI (%)	f_{α}^{IR}	f_{α}
Original	Standard[a]	5.2 ± 0.6	0.026	284.3	79.5	0.34	0.57
	CS + Glu + Salts[a]	0	0	-	-	-	-
12th Round	Standard[a]	4.8 ± 0.2	0.024	284.2	70.8	0.33	0.53
	CS[b]	7.1 ± 0.3	0.460	292.5	77.8	[c]	[c]
	CS + Glu[b]	16.4 ± 1.0	0.082	305.8	87.1	[c]	[c]
	CS + Glu + Salts[b]	13.8 ± 0.4	0.069	315.8	85.0	[c]	[c]
	$CS_{60\%}$ + Glu + Salts[a]	16.5 ± 0.4	0.083	314.9	86.4	0.17	0.13

[a]Final culture media volume of 10 ml with 20 mg glucose/ml or total weight of 200 mg glucose.
[b]Final culture media volume of 10 ml with 1.54 mg glucose/ml or total weight of 15.4 mg of glucose.
[c]The data could not be calculated due to the disappearance of the 750 cm^{-1} band.

The phenolic compounds present in corn stover pretreatment liquid, while inhibitory to microbial growth, contribute to increased nanocellulose production by minimizing the decrease in medium pH and by decreasing the gluconic acid concentration in the medium. Previous reports showed that lignosulfonate addition in the standard HS culture medium or in sugarcane molasses improves nanocellulose production by decreasing gluconic acid concentration through chemical reactions with phenolic compounds from the lignosulfonate lignin (Keshk, Smas e Sameshima, K, 2006; Keshk, S. e Sameshima, K., 2006). The decrease in bacterial nanocellulose production in the case of HS medium has been attributed to the formation of D-gluconic and D-2-ketogluconic acids, with the pH of the medium subsequently decreasing to pH 3. When phenolic compounds are present, the pH remains at 4.

The structure of the cellulose films produced by the original and adapted strains grown in pretreatment liquid from corn stover was investigated in comparison to the BC films generated by *K. hansenii* in Hestrin and Schramm medium using higher resolution images (Figure 4.4). The BC films produced showed the same hierarchical structure with different pores sizes and similarity between the interweaving of the fibers, indicating that the adaptive evolution process did not influence the morphological structure of the film. The use of pretreatment liquid from corn stover did not change the morphological structure of fiber interweaving.

All of the nanocellulose films formed in the presence and absence of pretreatment liquid had the same heat degradation behavior and showed the typical single step profile (Mohammadkazemi *et al.*, 2015), with the Tonset about 284°C (Table 7). Thermal degradation behavior was also similar (Figure 4.5).

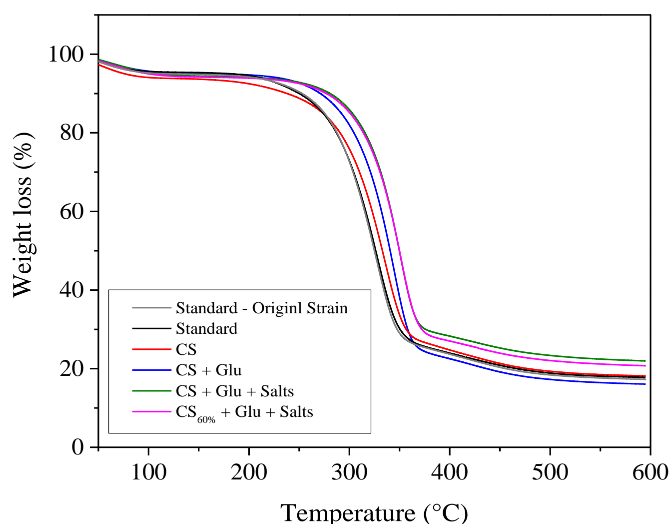


FIGURE 4.4 Weight loss curves from TGA analyzes of BC films produced by original and adapted *K. hansenii* strains in standard medium and liquid fraction of LHW pre-treated corn stover under raw and nutritionally supplemented conditions.

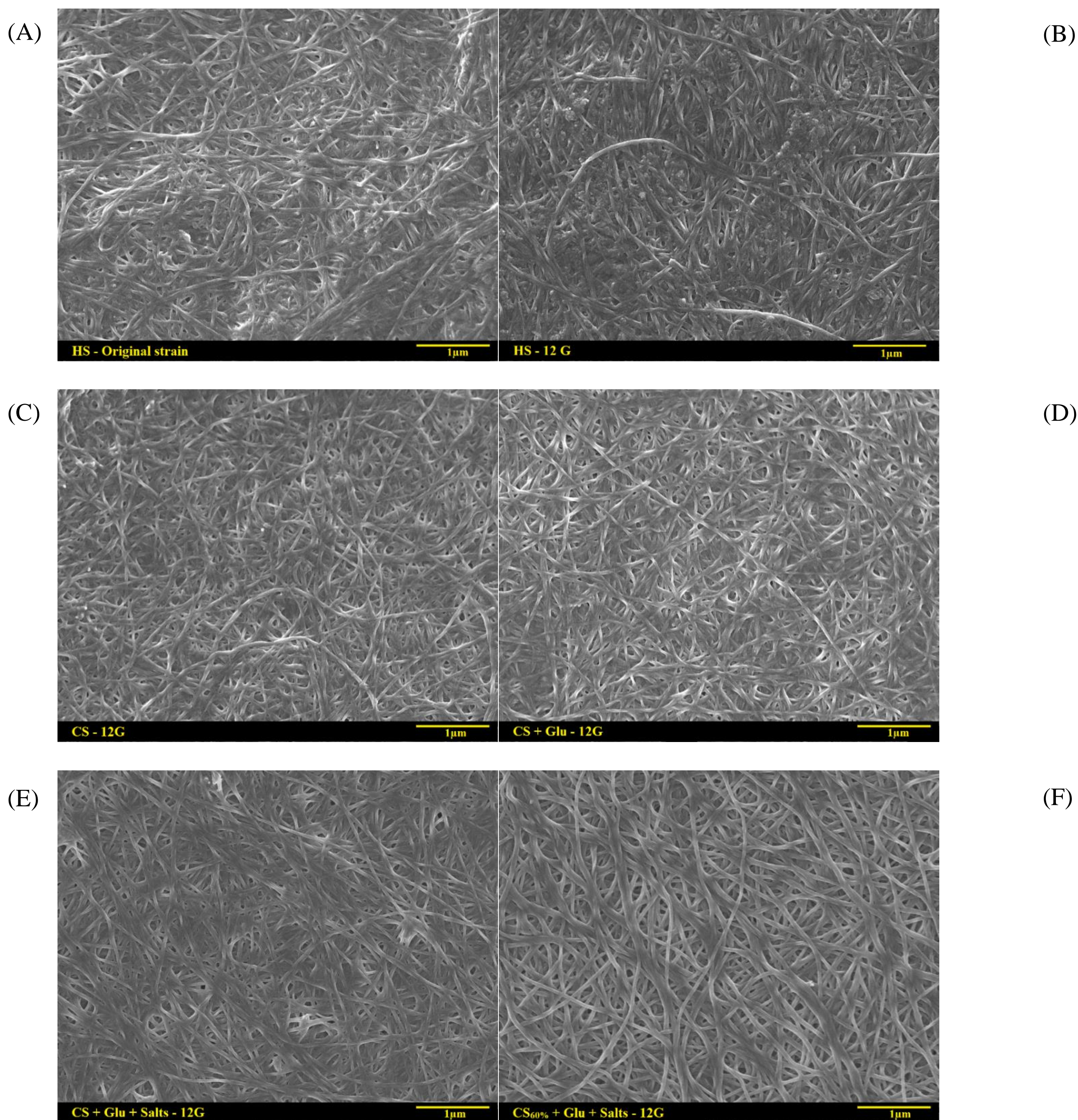


FIGURE 4.5 FESEM micrographs of BC films produced by original and adapted *Komagataeibacter hansenii* strains in standard medium and in liquid fraction of LHW pre-treated corn stover under raw and nutritionally supplemented conditions. A-F refer to: BC films produced using Hestrin and Schrumm (HS) medium by original (A) and adapted (B) strains; BC films produced by the adapted bacteria using corn stover liquid fraction (C), corn stover nutritionally supplemented with glucose (D), corn stover supplemented with glucose and salts (E), Corn stover$_{60\%}$ nutritionally supplemented with glucose and salts (F).

X-ray diffraction peaks for all analyzed samples suggest the presence of cellulose I only (Figure 4.6) and the diffractograms also revealed the presence of an amorphous region in BC (I_{am}~20°) (Wada *et al.*, 2001). The BC produced in standard HS medium by the original and adapted strains showed different CrI (79.5% and 70.8%, respectively), indicating the process of evolution contributed to the production of a less crystalline BC film in standard HS medium compared to the original strain (Table 4-4). However, when grown in the presence of CS liquor medium supplemented with glucose, the adapted bacteria produced a BC film with higher CrI (87.1%).

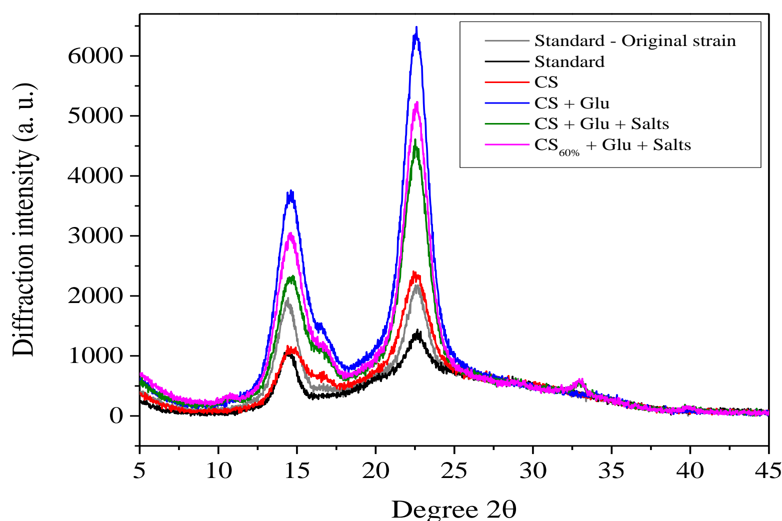


FIGURE 4.6 X-ray diffractogram of BC films produced by original and adapted *K. hansenii* strains in standard medium and liquid fraction of LHW pre-treated corn stover under raw and nutritionally supplemented conditions.

All FT-IR spectra have similar profile (Figure 4.7), with absorption bands in the spectra characterizing the material as bacterial cellulose and cellulose type I. Both original and adapted strains had the same absorption bands, indicating that the ALE process did not affect the chemicals structure of the BC film. However, the use of CS liquor modified the bands profile around 3240 and 750 cm^{-1}. The disappearance of 3240 cm^{-1} peak, which is related to a specific intramolecular bonding for cellulose I (Fan *et al.*, 2012), is correlating to the difference in

crystallinity found in the X-ray data. The disappearance of the band at 750 cm^{-1} indicates that BC films produced in the presence of CS liquor are cellulose I$_\beta$-dominant type and made not possible to estimate the fraction of cellulose (Table 4-4).

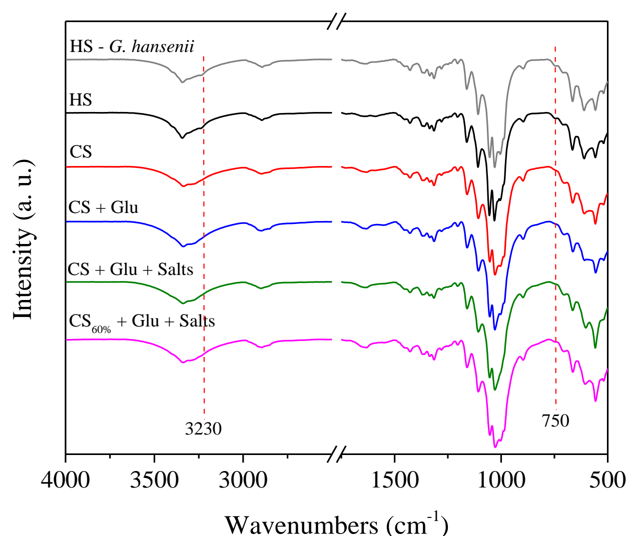


FIGURE 4.7 FTIR vibrational spectra of BC films produced by original and adapted K. hansenii strains in standard medium and liquid fraction of LHW pre-treated corn stover under raw and nutritionally supplemented conditions. The spectrums were normalized using the 1030 cm^{-1}

4.5 Conclusions

K. hansenii was successfully adapted to phenolics inhibitors after 12 round of culturing experiments in their presence. The development of this adapted strain makes possible to directly convert, and thereby concentrate, a dilute form of glucose into an insoluble, readily recovered, value-added nanocellulose product. While genetic stability, its nature and mechanisms behind it still need to be unveiled, the evolutionary adaptation of this nanocellulose-producing bacteria not only made the bacterium resistant to inhibitors, but also able to produce significantly more nanocellulose compared to the original strain. Nanocellulose from the adapted strain retained the same properties of that produced in the standard culture medium. Therefore, improved glucose utilization for cellulose synthesis was obtained, while

allowing the BC synthesis process occurring without a detoxification step and nutritional supplementation. In summary, our results indicate that new evolved strain can successfully valorize pretreatment liquid as a resource for nanocellulose production.

4.6 Supplementary data on additional attempts to adapt *K. hansenii* to inhibitors

The sugarcane bagasse hydrothermal pretreatment liquid fraction (SB, Figure 3.1) was also used as culture medium to obtain a new adapted strain, following the methodology in 48-well plates (Figure 4.3 (c)). The original strain of *K. hansenii* (ATCC 23769) showed an initial tolerance to inhibitors when pretreatment liquid from bagasse was diluted to 40% of its original concentration. Adapted laboratory evolution followed the sequence summarized in Table 4-5. After 28 rounds of culturing experiments, the bacterium became adapted to inhibitors and was able to form bacterial nanocellulose film in culture medium containing 80% (vol/vol) of liquor with added components to result in a HS standard similar medium. It was necessary to double the number of rounds of culturing to obtain an adapted strain using bagasse liquid fraction, when compared to process using corn stover.

The fitness of the adapted bacteria was subsequently tested in the different culture medium (Table 4-6). The culture media tested included: the standard HS culture medium, culture media using the liquid fraction of the sugarcane bagasse, standard culture media with substitutions of the sugars and standard culture medium with additions of phenolic compounds and furaldehydes. The substitutions and additions were based on previous experiments, e.g. xylose (Table 3-3), and on the composition of sugars and inhibitors present in the liquid fraction used (Table 4-7).

TABLE 4-5 Evolutionary adaptation of *K. hansenii* in sugar cane bagasse liquid fraction (SB) nutritionally supplemented with glucose and salts at the same concentration of the standard culture medium.

Rounds	HS	SB concentration (%)							
		10	20	30	40	50	60	70	80
1	+ + +	+ + +	+ + +	+ + +	+ +	+ +	-	-	-
2	+ + +	+ + +	+ + +	+ + +	+ +	-	-	-	-
3	+ + +	+ + +	+ + +	+ + +	+ +	+	-	-	-
4	+ + +	0	+ + +	+ + +	+ + +	+ +	-	-	-
5	+ + +	0	+ + +	+ + +	+ + +	+ +	-	-	-
6	+ + +	0	+ + +	+ + +	+ + +	+ +	-	-	-
7	+ + +	0	+ + +	+ + +	+ + +	+ + +	+	-	-
8	+ + +	0	+ + +	+ + +	+ + +	+ + +	-	-	-
9	+ + +	0	+ + +	+ + +	+ + +	+ + +	-	-	-
10	+ + +	0	0	0	+ + +	+ + +	-	-	-
11	+ + +	0	0	0	+ + +	+ + +	+ + +	+	+
12	+ + +	0	0	0	+ + +	+ + +	+	-	-
13	+ + +	0	0	0	+ + +	+ + +	+	-	-
14	+ + +	0	0	0	+ + +	+ + +	+	-	-
15	+ + +	0	0	0	+ + +	+ + +	+ + +	+	-
16	+ + +	0	0	0	+ + +	+ + +	+ +	-	-
17	+ + +	0	0	0	0	+ + +	+ + +	+ +	-
18	+ + +	0	0	0	0	+ + +	+ + +	+ + +	-
19	+ + +	0	0	0	0	+ + +	+ +	-	-
20	+ + +	0	0	0	0	+ + +	+ + +	+ +	+ +
21	+ + +	0	0	0	0	+ + +	+ + +	+ +	+ +
22	+ + +	0	0	0	0	0	+ + +	+ + +	+ +
23	+ + +	0	0	0	0	0	+ + +	+ + +	+
24	+ + +	0	0	0	0	0	+ + +	-	-
25	+ + +	0	0	0	0	0	+ + +	-	-
26	+ + +	0	0	0	0	0	+ + +	+ + +	+ +
27	+ + +	0	0	0	0	0	+ + +	+ + +	+ + +
28	+ + +	0	0	0	0	0	+ + +	+ + +	+ + +

Note: The cross symbols represent the thickness of the BC film (+++ same as the standard, ++ between standard and 1 mm, + >1 mm and − no BC synthesized), the grey spaces are the samples selected to start the next round of culturing experiment and 0 indicates the point at which testing in the indicated medium was terminated. Abbreviation: HS: Hestrin and Schramm medium.

TABLE 4-6 Weight of nanocellulose formed by *K. hansenii* adapted in bagasse liquid fraction

Culture medium composition	Weight of nanocellulose formed (dry basis, mg)
HS	10.9 ± 0.9
HS with the replacement of glucose by 10G:10X	5.8 ± 0.7
HS with the replacement of glucose by 5GX:15X	3.4 ± 0.8
HS with the replacement of glucose by 0G:20X	< 0.3
SB	0
SB + Glu	0
SB + Glu + Salts	0
HS with the replacement of glucose by sugars detected in SB	< 0.9
HS with the addition of furaldehyde detected in SB	0
HS with the addition of phenolic compounds detected in SB	12.2 ± 0.8

TABLE 4-7 Concentration of sugars and inhibitors in bagasse liquid fraction.

Sugar	mg/mL	Furaldehyde	mg/mL	Phenolic compound	mg/mL
Cellobiose	0.472 ± 0.198	HMF	0.155 ± 0.066	Vanillin	0.073 ± 0.024
Glucose	0.247 ± 0.074	Furfural	3.371 ± 1.252	Ferulic acid	0.047 ± 0.009
Xylose	8.482 ± 0.476			ρ-coumaric acid	0.173 ± 0.052
Arabinose	1.909 ± 0.120			4-hydroxybenzoic acid	0.094 ± 0.071
				4-hydroxybenzaldehyde	0.081 ± 0.008

Although the strain was adapted in the well plate experiment. The adapted strain produced was not able to synthesize BC using sugarcane bagasse liquid fraction during the test. Despite the sugars present in the liquid fraction be enough for the cellulose synthesis, not even nutritional supplementation was sufficient to induce the production of cellulose. When furaldehydes were added as inhibitors in the culture medium, the adapted strain did not produce bacterial cellulose. While the addition of 0.5 mg/mL of phenolic compounds induced the production of cellulose. The consumption of xylose by the adapted strain was similar to that

described in chapter 2. It can be concluded that the inhibitors are still responsible for the non-production of cellulose and the adaptation process was not efficient in this case.

It was used a synthetic inhibitors cocktail for the adaptation of the bacterium to inhibitors from the lignocellulosic wastes, following the methodology in 96-well plates (Figure 4.3 (a)). The synthetic cocktail was based on Freitas *et al.* (2019). The inhibitors cocktail composition is shown in table 4-8.

TABLE 4-8 Inhibitors composition of the synthetic coktail

Inhibitor	100%	50%
	mg/mL	
Hydroxymethylfurfural (HMF)	1.0	0.5
Fufural	3.0	1.5
Catechol	0.035	0.018
4-hydroxybenzaldehyde	0.22	0.11
4-hydroxybenzoic acid	0.022	0.011
Vanillin	0.1	0.05
ρ-coumaric acid	1.0	0.5
Ferulic acid	0.4	0.2
Tanic acid	1.0	0.5

The original strain of *K. hansenii* (ATCC 23769) showed an initial tolerance to inhibitors when synthetic inhibitors cocktail was diluted to 20% of its original concentration. Adapted laboratory evolution followed the sequence summarized in Table 4-9. After 27 rounds of culturing experiments, the bacterium became adapted to 50% of the inhibitors present in the synthetic cocktail. However, the synthetic cocktail proved to be more toxic than the bagasse liquid fraction. The adapted strain tolerated 2.0 mg/mL furaldehydes and 1.4 mg/mL phenolic

compounds. It is possible that furaldehydes are more toxic to cellulose-producing bacteria than phenolic compounds.

TABLE 4-9 Evolutionary adaptation of *K. hansenii* in standard culture medium (HS) with the addition of synthetic compounds simulating the sugarcane bagasse liquid fraction.

Rounds	HS	Synthetic compounds concentration (%)							
		10	20	30	40	50	60	70	80
1	+++	+++	+++	++	+	-	-	-	-
2	+++	++	+	+	-	-	-	-	-
3	+++	+++	++	+	-	-	-	-	-
4	+++	+++	+	-	-	-	-	-	-
5	+++	+++	+	-	-	-	-	-	0
6	+++	+++	++	-	-	-	-	-	0
7	+++	+++	++	-	-	-	-	-	0
8	+++	+++	+	-	-	-	-	-	0
9	+++	+++	+++	+++	++	-	-	-	0
10	+++	+++	+++	+++	-	-	-	-	0
11	+++	+++	+++	+++	+++	-	-	-	0
12	+++	+++	+++	+++	+	-	-	-	0
13	+++	+++	+++	+++	++	-	-	-	0
14	+++	+++	+++	+++	++	-	-	-	0
15	+++	+++	+++	+++	+++	-	-	-	0
16	+++	+++	+++	+++	+++	+	-	-	0
17	+++	+++	+++	+++	+++	+	-	-	0
18	+++	+++	+++	+++	+++	+++	-	-	0
19	+++	0	0	+++	-	-	-	-	0
20	+++	0	0	+++	+++	+	-	-	0
21	+++	0	0	+++	+++	-	-	-	0
22	+++	0	0	+++	+++	+	-	-	0
23	+++	0	0	+++	+++	+++	-	-	0
24	+++	0	0	+++	+++	+++	+	-	0
25	+++	0	0	+++	+++	-	-	-	0
26	+++	0	0	+++	+++	++	-	-	0
27	+++	0	0	+++	+++	++	-	-	0

Note: The cross symbols represent the thickness of the BC film (+++ same as the standard, ++ between standard and 1 mm, + >1 mm and − no BC synthesized), the grey spaces are the samples selected to start the next round of culturing experiment and 0 indicates the point at

which testing in the indicated medium was terminated. Abbreviation: HS: Hestrin and Schramm medium.

Kiziltas *et al.*, (2015) reported that the same bacterium used in this study was able to metabolize furfural (~0.08 mg/ mL), a much lower amount than that used in that cocktail. (Zou *et al.*, 2017) also related the tolerance for 4 different nanocellulose-producing strains to lignocellulose-derived inhibitors, an amount of 0.96 mg/ml e 1.90 mg/mL, of Furfural and HMF respectively, was toxic to all tested strains, completely inhibiting cellulose synthesis for two of them. Vanillin was toxic for three of the four strains estimated in the 0.30 mg/mL. Corroborating with the data presented in this study. Ferulic acid and hydroxybenzoic acid do not present toxicity to ATCC 23770 at a concentration of 0.18 mg / mL and 1.38 m / mL, respectively (Zhang et al., 2014). These concentrations are the same or greater than those used in the synthetic cocktail. This data supports the idea that furaldehydes are more toxic than phenolic compounds to cellulose-producing organisms, and that smaller phenolic compounds are more toxic than those with large molecular mass.

The results presented in this thesis indicate that substrates from lignocellulosic wastes have the potential to be applied as a culture medium for bacterial cellulose production. However, the inhibitors are toxic to the cellulose-producing bacteria, being a challenge to be overcome in this process. The development of an adapted strain to such inhibitors granted the use of that residual sugar source for the production of a value-added bacterial cellulose product. The evolutionary laboratory adaptation technique allowed the development of adapted strains, but given the different toxicity and variety of inhibitors, more studies should be developed to ensure a real fitness improvement of these microorganisms. Furthermore, the genetic stability, its nature and mechanisms behind it still need to be unveiled.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

For the BC synthesis is necessary that the culture medium be rich in glucose, one of the key points that increase the final product. In this context, the present thesis aims to contribute to the reduction of costs involved in the process of CB synthesis using as substrate an alternative source of C: the liquid hot water (LHW) pretreated liquid fraction of lignocellulosic wastes (corn stover and sugarcane bagasse) produced in biorefineries. A challenge to be overcome is related to the presence of inhibitors in the medium, and bypassing this barrier with the addition of a detoxification step with activated charcoal, for example, implies an increase in process cost. The alternative presented in this work was to obtain an inhibitor resistant cellulose producing strain through the adaptive laboratory evolution technique, using the HLW pretreated liquid fraction from corn stover as culture medium.

Through the analysis of the results presented here it can be concluded that:

- ✓ The use of fraction liquids of lignocellulosic waste for the production of bacterial cellulose is viable, however, it is necessary to add a detoxification step of the medium for the removal of the inhibitors;
- ✓ BC production using the detoxified CS fraction was 49% higher than that using standard medium, but 28% lower in the presence of detoxified SB fraction as culture medium.
- ✓ *K. hansenii* is able to metabolize xylose as an energy source, but requires glucose for cellulose synthesis;
- ✓ ELA provided an adapted strain capable to produce bacterial cellulose in a culture medium from corn stover liquid fraction without the addition of the detoxification step;
- ✓ The adapted strain obtained in the 12th round of culturing was able to use corn stover liquid fraction with and without nutritional supplementation for bacterial cellulose production;

- ✓ The adapted strain produced 4.2 mg of cellulose in standard medium, and 2.9 times more in liquor supplemented with glucose and salts (CS + Glu + Salts) and 3.4 times more in liquor supplemented with glucose (CS + Glu);
- ✓ The BC film produced under different culture mediums maintained the same excellent properties presented by the membrane produced in standard medium;
- ✓ The liquid fraction from bagasse appears to be more toxic to the microorganism, and the bacterium did not succeed in adaptation, even after 28 rounds of cultivation. Similar results were found by adding synthetic compounds to the standard culture medium simulating the sugarcane bagasse liquid fraction.
- ✓ The inhibition of bacterial cellulose production is related to the presence of phenolic compounds, but also to furaldehyde compounds, both are generated during the pretreatment of the lignocellulosic biomass.

The adaptive laboratory adaptation technique is a viable option for the development of new strains of cellulose-producing bacteria inhibitor-resistant, but it should be deeply studied. Some adjustments in the protocol may be necessary for the success of the adaptation in more toxic media. And also, further studies should be carried out to clarify issues such as: genetic stability, its nature and mechanisms behind the BC production by the adapted strain.

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