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Produção de celulose bacteriana usando meios alternativos para aplicação na indústria de alimentos

Bacterial cellulose production using alternatives media for application in food industry

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição, da Universidade Federal do Estado do Rio de Janeiro como requisito parcial para obtenção do título de Doutorado em Alimentos e Nutrição.

Orientador: Dr. Otniel Freitas Silva
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Dedico este trabalho aos meus pais, avôs, avós e padrinhos

que sempre se orgulharam das minhas conquistas

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“...é preciso amor pra poder pulsar,
é preciso paz pra poder sorrir, é
preciso a chuva para florir...”

Almir Sater

RESUMO

Celulose é um polímero natural biodegradável que tem sido usado em diversas áreas para substituir polímeros sintéticos. A partir da celulose é possível obter a nanocelulose. Materiais na escala nano vêm despontando na área de embalagens. A nanocelulose têm sido usada satisfatoriamente para melhorar as características de outros compósitos e se apresenta como uma alternativa sustentável e promissora. A nanocelulose pode ser de origem bacteriana ou de vegetal apresentando-se na forma de fibrilas (CNF) ou de nanocristais (CNC). A celulose bacteriana (CB) apresenta a vantagem por ser mais pura, sem lignina e hemicelulose. No entanto, apresenta alto-custo de produção e por isso ainda não é produzida em larga escala. No presente trabalho, meios de cultivo formulados a partir de fontes alternativas e agroindustriais tais como suco de caju e melaço de soja foram avaliadas para a produção de membranas e de esferas de CB por *Acetobacter xylinus* ATCC 53582. Além disso, foi apresentado um projeto de *startup*, incluindo fluxograma de produção e de custo de instalação, para a produção de membranas de CB. Membranas de CB foram produzidas por *Gluconacetobacter hansenii* ATCC 23769, incorporadas com antimicrobiano nisina e usadas para embalar queijo Minas frescal. Utilizando suco de caju com melaço de soja (meio CSM), foi possível obter membrana CB com alto rendimento (4.50 g L-1), com as mesmas características físico-químicas da CB obtida com o meio padrão HS (4.03 g L-1) e custo de produção 7 vezes menor. O custo de produção de CSM foi estimado em U$ 60 kg-1 de membrana de CB e o custo dos equipamentos para instalação da startup estimados em U$ 21.000. *Acetobacter xylinus* ATCC 53582 foi capaz de formar esferas em meio HS quando submetido a agitação orbital 150 rpm (0,72 g L-1) e quando submetido a agitação em meio HS com álcool (0,83 g L-1). Nos meios alternativos avaliados, houve formação de CB na forma fibrosa, irregular e em forma de asterisco. Estudos adicionais devem ser realizados para otimizar a formação de esferas de celulose. A CB incorporada com nisina 2500 UI mL-1 reduziu o crescimento de *Listeria monocytogenes* ATCC 19117 em 1 log CFU g-1 no queijo Minas Frescal após armazenamento por 7 dias.

Palavras chaves: Bactéria. Celulose. Biopolímero. Nanocelulose. Meios alternativos

ABSTRACT

Cellulose is a natural biodegradable polymer that has been used in many areas to replace synthetic polymers. From cellulose it is possible to obtain nanocellulose. Materials on the nanoscale are emerging in the packaging area. Nanocellulose has been used satisfactorily to improve characteristics of other composites already used and presents itself as a sustainable and promising alternative. Nanocellulose is extracted from bacterial or vegetable source, and presents forms as fibrils (CNF) or nanocrystals (CNC), Bacterial Cellulose (BC) has the advantage of being pure, without lignin and hemicellulose. However, it has a high cost of production becoming a limiting factor for large-scale BC production. In the present work, culture media formulated from alternative and agroindustrial sources, such as cashew apple and soybean molasses were evaluated for membranes and spheres of BC production using *Acetobacter xylinus* ATCC 53582. In addition, a startup project was designated, including production flowchart and installation cost, for BC membrane production. BC membranes were produced by *Gluconacetobacter hansenii* ATCC 23769, incorporated with nisin antimicrobial and used to pack *Minas frescal* cheese. Using cashew juice with soybean molasses (CSM medium), it was possible to obtain a high yield BC membrane (4.50 g L^{-1}), with the same BC physicochemical characteristics produced with HS standard (4.03 g L^{-1}) and production cost was 7 times lower. The cost of producing CSM was estimated at U$ 60 kg^{-1} of cellulose membrane and the equipment used for startup installation estimated at U$ 21.000. *Acetobacter xylinus* ATCC 53582 was able to form spheres in HS medium when subjected to 150 rpm orbital agitation (0.72 g L^{-1}) and under HS agitation with alcohol (0.83 g L^{-1}). Alternative media used, produced fibrous, irregular and asterisk BC. Further studies should be necessary to optimize the formation of cellulose spheres. BC incorporated with 2500 IU mL^{-1} reduced *Listeria monocytogenes* ATCC 19117 growth up to1 log UFC UFC g^{-1} in *Minas Frescal* cheese after storage for 7 days.

Keywords: Bacteria. Cellulose. Biopolymer. Nanocellulose. Alternatives media.

SUMMARY

CHAPTER 2
Production and characterization of *Acetobacter xylinus* bacterial cellulose using cashew apple juice and soybean molasses

INTRODUÇÃO

Nas últimas décadas a preocupação com os impactos causados ao meio ambiente tem sido crescente. A busca por polímeros biodegradáveis visando substituir os materiais plásticos e consequentemente diminuir a exploração dos combustíveis fósseis, tem levado ao desenvolvimento de novas tecnologias e compósitos. Assim os biopolímeros, tais como o polissacarídeo celulose, surgem como uma versátil e promissora alternativa para busca e desenvolvimento de novos produtos com alto valor agregado. A celulose pode ser de origem vegetal ou bacteriana. Nos vegetais se apresenta associada a hemicelulose e a lignina, portanto, para ser obtida, precisa ser submetida a processos de extração. Já a celulose de origem bacteriana, é produzida principalmente pelas bactérias do gênero *Acetobacter* de forma pura.

Tanto a celulose vegetal como a bacteriana podem ser usadas para obtenção de celulose na escala nano, a nanocelulose. Nos últimos anos a nanocelulose vem possibilitando o desenvolvimento de novos compósitos associados com quitosana, pectina e amido principalmente na área de embalagens. No entanto, assim como para todos os demais nanomateriais, mais estudos ainda precisam ser realizados para garantir que o uso da nanocelulose em alimentos não oferece riscos à saúde. Enquanto isso, o uso de antimicrobianos, como a nisina por exemplo, vêm sendo satisfatoriamente testados em embalagens de celulose vegetal, bacteriana e demais compósitos com celulose para controle de crescimento de *Listeria monocytogenes* em queijos e carnes.

A celulose bacteriana apresenta características superiores a celulose vegetal, porém a sua produção em larga escala ainda não está bem estabelecida. O meio de cultivo tradicionalmente usado requer insumos como fontes de carbono e nitrogênio onerosos, o que inviabiliza o custo de produção em escala comercial. Muitos estudos têm buscado reaproveitar subprodutos agroindustriais como melaços, sucos, cascas e bagaço de frutas como alternativa para reduzir o custo de produção. Alguns desses estudos vêm mostrando até mesmo rendimento superior ao obtido com o meio padrão, no entanto, os autores não apresentam uma avaliação econômica de quanto representa

a redução esse custo. Além disso, para ser implementado, é preciso assegurar que o subproduto apresente vasta disponibilidade durante o ano, a fim de que o processo de produção de celulose bacteriana não seja comprometido devido a oscilação da disponibilidade da matéria prima levando a uma alteração do custo de produção. Nesse trabalho, a celulose bacteriana foi produzida pelas espécies *Gluconacetobacter hansenii* ATCC 23769 e *Acetobacter xilinus* ATCC 53582.

Dependendo da forma de cultivo, a celulose bacteriana pode ser produzida como biofilmes (cultivo estático) ou pequenas esferas (cultivo agitado). Alguns autores afirmam que as esferas de celulose apesar de apresentarem características como cristalinidade inferior aos biofilmes de celulose, elas apresentam melhor aplicabilidade na área biomédica como carreadores de medicamentos, uso em partículas magnéticas para remoção de metais pesados e imobilização de enzimas. No entanto a produção de celulose bacteriana usando cultivo agitado ainda apresenta muitos desafios tais como definir velocidade de agitação, condições do inóculo (volume e tempo), pH do meio, bactéria produtora entre outros.

Assim esta tese dividida em 5 capítulos apresenta i) uma revisão do uso de nanocelulose em embalagens ii) a produção e caracterização da celulose bacteriana obtida por cultivo estático usando melaço de soja e suco de caju iii) a avaliação econômica do custo de implementação de uma planta de produção de celulose bacteriana usando meio alternativo iv) a produção de celulose e parâmetros de produção de esferas de celulose e por último v) um estudo de caso com avaliação do crescimento microbiano em queijos minas frescal.

INTRODUCTION

In the last decades the concern about the impacts caused to the environment has been increasing. The search for biodegradable polymers to replace plastic materials and consequently reduce the exploitation of fossil fuels has led to the development of new technologies and composites. Thus biopolymers, such as polysaccharide cellulose, appear as a versatile and promising alternative for the search and development of new products with high added value. Cellulose may be of plant or bacterial origin. In vegetables it is associated with hemicellulose and lignin, so to be obtained, it must be submitted to extraction processes. Cellulose of bacterial origin is mainly produced by bacteria of the genus *Acetobacter* in pure form.

Both plant and bacterial cellulose can be used to obtain nano-scale cellulose, nanocellulose. In recent years nanocellulose has enabled the development of new composites associated with chitosan, pectin and starch mainly in the area of packaging. However, as with all other nanomaterials, more studies still need to be done to ensure that the use of nanocellulose in food does not pose any health risks. Meanwhile, the use of antimicrobials, such as nisin for example, has been satisfactorily tested in vegetable, bacterial and other cellulose composites packaging for growth control of *Listeria monocytogenes* in cheese and meat.

Bacterial cellulose has characteristics superior to vegetable cellulose, but its large scale production is not yet well established. The traditionally used cultivation medium requires inputs such as expensive carbon and nitrogen sources, which makes the cost of production on a commercial scale unfeasible. Many studies have sought to reuse agroindustrial by-products such as molasses, juices, peels and fruit cake as an alternative to lower the cost of production. Some of these studies have even shown higher productivity than the standard medium, however, the authors do not present an economic assessment of how much this reduction represents. In addition, to be implemented, it must be ensured that the by-product is widely available throughout the year, so that the bacterial cellulose production process is not compromised due to the fluctuation of the availability of the raw material leading to a change in the production cost. In this work, the

bacterial cellulose was produced by the species *Gluconacetobacter hansenii* ATCC 23769 and *Acetobacter xilinus* ATCC 53582.

Depending on the form of cultivation, bacterial cellulose can be produced as biofilms (static cultivation) or small spheres (agitated cultivation). Some authors claim that cellulose spheres, despite having characteristics such as lower crystallinity than cellulose biofilms, have better applicability in the biomedical area as drug carriers, use in magnetic particles for removal of heavy metals and immobilization of enzymes. However, the production of bacterial cellulose using agitated cultivation still presents many challenges such as defining agitation speed, inoculum conditions (volume and time), pH of the medium, producing bacteria among others.

Thus this thesis divided into 5 chapters presents i) a review of the use of nanocellulose in packaging ii) the production and characterization of bacterial cellulose obtained by static cultivation using soybean molasses and cashew juice iii) the economic evaluation of the cost of implementing a bacterial cellulose production plant using alternative means iv) the cellulose production and production parameters of cellulose spheres and finally v) a case study with the evaluation of microbial growth in minas frescal cheese.

Chapter 1

Overview of nanocellulose in food packaging

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Abstract: ***Background*****:** The rising concern with environmental preservation has led to increasing interest in biodegradable polymer composites from renewable sources. These composites, usually referred to as “green”, have many industrial applications. Nanocellulose has enormous potential for use in innovate packaging.

Discussion**:** Before marketing, new products must be approved: they must be safe and not pose undue risks to consumers or the environment. Many countries have been adjusting their regulatory frameworks to deal with nanotechnologies, including nanocellulose packaging. This paper presents an overview and discusses the state of the art of different nanocellulose materials used for packaging, and the regulatory measures required, including laws and guidelines for safety assessment.

Conclusion**:** Some current packaging materials already include nano-encapsulated agrochemicals or antimicrobial nanoparticles, making them active and intelligent materials for food packaging. Also, other packages made with nanocellulose products are under investigation and may enter the market in the near future.

Keywords: Cellulose nanofibrils, Bacterial cellulose, Biopolymers, Active packaging, Nanomaterials, Nanotechnology Regulation

1. INTRODUCTION

Nowadays there is a trend to replace synthetic polymers with biodegradable and/or bio-based polymers. Plastic waste is a growing environmental problem and the continuous shortage of fossil resources also contributes to increase the interest in biopolymers. Natural polymers such as collagen, elastin, alginate, chitosan, starch, and cellulose have all been investigated for various uses [1]. Among these polymers, cellulose is the most abundant in nature and is a renewable, biocompatible and non-toxic material. It can be derived from a variety of plant sources, such as wood (hardwood and softwood), seed fibers (cotton, coir), bast fibers (flax, hemp, jute, ramie), grasses (bagasse, bamboo) [2] and also alternative sources such as marine animals (tunicate), algae and bacteria. At present, wood pulp and cotton fibers are the most important industrial sources of cellulose [3]. However, non-wood plants have received attention as a source of cellulose in recent years [4].

Besides cellulose, lignocellulosic biomass contains hemicellulose, lignin and a small amount of extractives. Wood species can be distinguished as hard and softwoods based on their anatomical features [5]. Hardwoods have a more complex, heterogeneous and rigid structure than softwoods due to their high Runkel ratio (cell wall thickness divided by lumen radius) [6]. Since softwood generally contains less lignin, the fiber delignification and purification processes for cellulose are easier, less harmful to cellulose and consume less energy [7].

In plants, cellulose has a well-organized architecture of microfibril elements composing cells. Cellulose is the primary structural component responsible for the mechanical strength of the plant. Each cell represents a fiber, with a width of 10–50 μm (depending on the source), consisting of cell wall layers, which have a total thickness of 1–5 μm [8].

Cellulose polymer chains are formed by glucose molecules linked together by β-1,4 glucosidic bonds, forming anhydroglucose units. Two anhydroglucose units compose anhydrocellobiose. The cellulose degree of polymerization is usually expressed as a number of anhydroglucose units, which varies depending on the cellulose source and the isolation/ purification method [9].

Cellulose particles with at least one dimension in nanoscale (1–100 nm) are considered nanocellulose. Plant nanocellulose can be divided into two main categories: (i) cellulose nanocrystals (CNC) or cellulose whiskers, and (ii) cellulose nanofibrils (CNF), also known as nanofibrillated cellulose (NFC), microfibrillated cellulose (MFC) or cellulose nanofibers [10]. Bacterial cellulose (BC) is also considered a nanocellulose. CNC and CNF are produced by the disintegration of cellulose fibers into nanoscale particles (top–down process), while BC is generated by a buildup of nanofibers (bottom–up process) from molecular sugars by bacteria [11].

The study of nanocellulose began in 1880 with the description of bacterial cellulose. Fig. 1 represents the principal milestones of the research involving cellulose and nanocellulose in the food industry to date.

Turbak and collaborators in the late 1970s first used the term nanocellulose, when the hierarchical structure of fibers was deconstructed and considerable quantities of nanocellulose were obtained [12]. Nanocellulose has received considerable attention in recent years, evidenced by the significant rise in the number of scientific articles published, as can be observed in Fig. 2.

Cellulose and cellulose derivates have several applications. It has been successfully used in wound dressings, burn treatments, medical devices, tissue regeneration [13], biosensing materials, electronic paper, the food industry [14] and in various devices.

This paper reviews the impact, benefits and challenges of the use of plant and bacterial nanocellulose in the food packaging materials, including laws and guidelines for safety assessment.

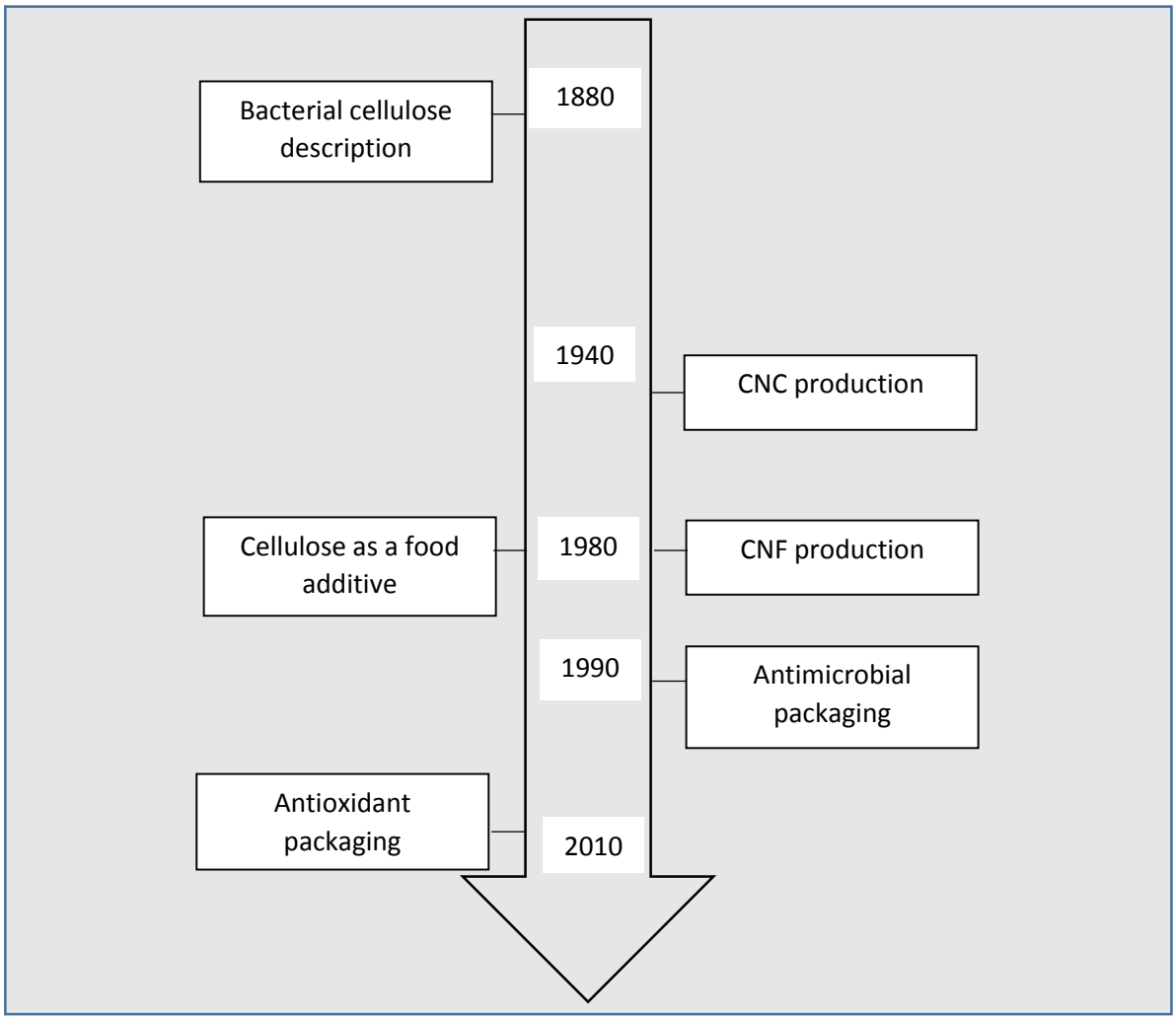

Figure 1. The principal milestones in cellulose and nanocellulose research since 1880

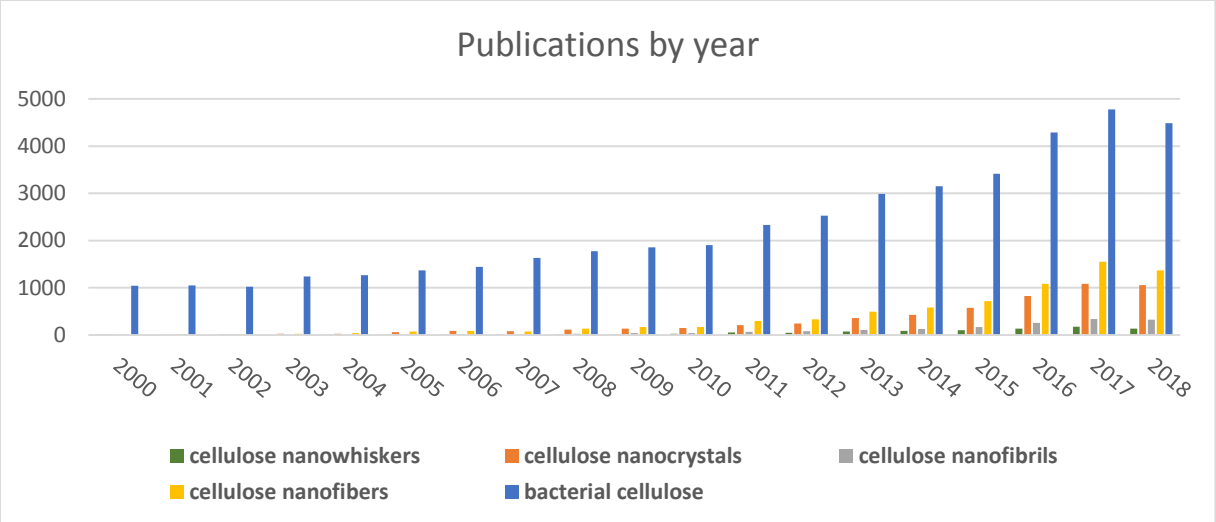

Figure 2. Number of publications per topic of interest related to nanocellulose over the last 18 years. Search criteria: Topic key words were *cellulose nanowhiskers*, *cellulose nanofibers*, *cellulose nanocrystals*, *bacterial cellulose*, *cellulose nanofibrils* Source: Science direct, September 4, 2018.

2. CELLULOSE NANOCRYSTALS

Cellulose consists of crystalline (ordered) regions along with some amorphous (disordered) regions in varying proportions. When cellulose microfibrils are subjected to mechanical and chemical extraction methods, the highly crystalline regions can be extracted and cellulose nanocrystals (CNCs) are obtained [15].

In the early 2000s, the rod-like nanosized cellulose particles were referred to as “cellulose whiskers”. Although this term is still used in patent vocabulary, in more recent years the preferred term in patents has been “cellulose nanocrystals” or “nanocrystalline cellulose” [15].

CNCs were first produced by Ranby in 1949 using acid hydrolysis of cellulose fibers dispersed in water [16]. CNCs have been produced using various types of acid treatments, such as hydrochloric, sulfuric, and phosphoric acids. Concentrated sulfuric acid is commonly used. However, each treatment leads to specific functional groups on the nanoparticle surface, so this factor affects the colloidal stability. For example, hydrochloric acid causes poor colloidal stability, whereas sulfuric acid leads to high colloidal stability. Additionally, the processing conditions used during hydrolysis, such as the reaction time and temperature, are critical in controlling the yield and quality of CNCs [17].

CNC morphology generally depends on the source of cellulose. CNCs obtained from wood were reported to have diameter and length in the range of 3–5 nm and 100–300 nm [18], respectively, while sea animals such as tunicates produce nanocrystals with a diameter of 13 –20 nm and length of 500–2,000 nm [19].

3. CELLULOSE NANOFIBRILS

CNFs are aggregations of elementary fibrils containing crystalline and amorphous parts, with a few micrometers in length and 10-100 nm in diameter. Contrary to straight cellulose nanocrystals, CNFs are long and flexible nanoparticles [20]. Turbak et al. [21] and Herrick et al. [22] discovered CNFs by passing a softwood pulp aqueous suspension through a high-pressure homogenizer several times. After repeated homogenization, they obtained a diluted dispersion of CNFs, with a gel-like appearance.

The isolation of CNFs can also be performed by a wide variety of mechanical techniques, such as refining [23], grinding [24], cryocrushing [25], extrusion [26] and blending [27].

Moreover, intensive research has been performed to enhance fibrillation and reduce the high energy costs of the disintegration process [28]. Biological/chemical pretreatment methods such as enzymatic hydrolysis [29], carboxylation [30] and sulphonation [31] appear to be promising methods to produce CNFs economically and efficiently.

4. BACTERIAL CELLULOSE

In 1886 Brown, while working with acetic acid bacteria, reported for the first time the synthesis of an extracellular gelatinous material whose chemical composition was equivalent to cell-wall cellulose [32].

Certain algae and some bacterial genera such as *Acetobacter, Rhizobium, Agrobacterium, Aerobacter, Achromobacter, Azotobacter, Salmonella, Escherichia*, and *Sarcina* produce cellulose as part of their normal metabolic processes. BC is aerobically generated in aqueous culture media containing a sugar source. It is produced in the form of a pellicle under shaking cultivation or a sheet on the surface of the culture medium

under static cultivation [33]. The time of the process ranges from a few days to two weeks [34].

BC has the same chemical composition as plant cellulose. However, it is free of other polymers, such as lignin, hemicelluloses and pectin. This high purity and organized structure gives BC higher crystallinity, thermal stability, and mechanical strength than plant cellulose. BC also presents high water-absorbing capabilities (in hydrogel form), moderate biocompatibility, and partial degradability [35].

The possibility of obtaining highly pure cellulose nanofibrils without large energy input, as required for the traditional MFC preparation, has attracted interest in this type of nanocellulose over the last two decades [15].

Despite various commercial uses, BC is still expensive to produce, limiting its use as an alternative to plant cellulose. The synthetic media used for BC production contribute to its high production cost. For this reason, numerous efforts have been made to develop new and cheaper methods to obtain BC, which include the design of new bioreactors [36], the study of alternative carbon sources such as waste materials, and the discovery of new bacterial strains [37].

5. NANOCELLULOSE IN FOOD PRODUCTS

Turbak and co-workers, in a series of scientific publications and patents, first proposed a variety of applications for microfibrillated nanocellulose as a food additive [12, 21, 38, 39]. They demonstrated that nanocellulose is an excellent suspending medium for other solids and an emulsifying base for organic liquids. During the same period, Mizuguchi and co-workers also published studies of nanocellulose as a food additive in bean jam [40], sauce and soy soup [41].

There was a gap in scientific publications and patents on nanocellulose as a food additive between the 1980s and 2000, but motivated by the recent global interest in nanotechnology, research has resumed [15].

For instance, in 2002 Cantini et al. [42] patented the “Use of cellulose microfibrils in a dry form in food formulations.” The invention focuses on use the dry form of a combination of nanocellulose with at least one polyhydroxylated compound as an

additive, which acts as a stabilizer and thickener in the formulation. Another patented process involving nanocellulose as a stabilizer was developed by Yano and co-workers in 2014 [43]. They used nanocellulose to increase the period in which a frozen dessert can retain its shape.

Nanocellulose, besides being a thickener, stabilizer and texture modifier, is also a low-calorie additive that can replace emulsifiers [14]. Lin and Lin concluded that adding 10% bacterial cellulose to a typical emulsified meat product (Chinese-style meatballs) improved the sensorial properties [44]. Bacterial nanocellulose was also tested in low-lipid meat sausages with successful results [45].

It seems that nanocellulose, like other polysaccharides, can act as a cryoprotectant in freeze-dried probiotic bacteria. Nanocellulose is adsorbed on the surface of microorganisms and forms a viscous layer that prevents the growth of ice crystals by increasing the solution's viscosity [46]. The large-scale production of fermented foods has become an important area in the food industry, so the challenges to produce large quantities of probiotic cultures must be overcome [47].

Khorasani and Shojaosadati [48], aiming to increase *Bacillus coagulans* probiotic survivability, tested a prebiotic nanocomposite using bacterial nanocellulose (BNC), pectin and an *S. commune* aqueous extract. This study demonstrated that BNC could be used as a nanoscale prebiotic biopolymer to improve probiotic encapsulation. Also, the prebiotic nanocomposite made using BNC enhanced the stability of *B. coagulans* during long-term storage at different temperatures.

Another nanocellulose application in the food industry is dietary fiber, defined as the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine but undergo complete or partial fermentation in the large intestine. Dietary fiber promotes a range of health benefits. It can reduce the risk of chronic diseases such as diabetes, obesity, cardiovascular disease and diverticulitis. In addition, it can prevent constipation and lower the blood lipid and glucose levels [49]. Consequently, nanocellulose can be a potential functional food ingredient because it provides human health benefits. [50].

Besides nanocellulose applications in food products, bacterial cellulose gel, called Nata, is a traditional dessert in Southeast Asia and is now spreading worldwide. Coco

Nata and Pina Nata are now available, with their respective flavors (coconut and pineapple) [14].

6. NANOCELLULOSE IN FOOD PACKAGING

6.1. ADVANTAGES OF ITS USE

The most important function of food packaging is to maintain the quality and safety of food products during storage and transportation. Therefore, it is important to extend the shelf life of food products by preventing unfavorable factors such as spoilage by microorganisms and chemical contaminants; permeation of water vapor, oxygen, carbon dioxide, volatile compounds and moisture; exposure to light and external physical forces. Consequently, the packaging materials should provide physical protection and create adequate physicochemical conditions to guarantee food quality [51].

The packaging industry traditionally uses materials based on glass, aluminum, tin, and fossil derived synthetic plastics. These materials have high strength and good barrier properties, but they also have some disadvantages from the economic and environmental points of view [52]. Considering the biodegradability aspect, nanocellulose is a biopolymer that is extensively used nowadays for packaging applications in the food industry [53]. Nanocellulose can work synergistically with other materials such as metals [54], minerals [55] and lignin [56] to improve the mechanical, rheological, and barrier properties of many polymeric systems.

Cellulosic nanomaterials have the capacity to form hydrogen bonds that allows the material to create a dense network, which hinders various molecules from passing through [52]. This is an important property for barrier applications, an important factor in the packaging industry. Nanocellulose promotes the development of new materials and improvement of the properties of conventional materials. Nanoscale cellulose can be used as a filler in the manufacture of composites, providing interesting features. These properties, besides others such as renewability, absence of competition with food crops, biodegradability and/or biocompatibility, are in line with the tenets of a sustainable economy, less dependent on fossil sources [57].

The diffusion of molecules between two adjacent volumes separated by a thin film of solid polymer or membrane happens in three basic steps: the sample surface adsorbs

the diffusing molecule; then the diffusing molecule passes through the film or membrane; and finally the diffusing molecule is desorbed from the film or membrane surface on the other side. So the gas permeability mainly depends on the dissolution of gas and its diffusion rate in the film [52].

Nanocellulose contains a high proportion of crystalline regions that are essentially impermeable to gas molecules [58]. Consequently, nanocellulose presents good barrier properties, especially related to oxygen transfer. In comparison to cellulose fibers, nanocellulose can form more complex and smaller pores due to its significantly higher surface area and high aspect ratio. This type of nanocellulose network can decrease the permeability by increasing the density within the film [59].

Syverud and Stenius [60] showed that NFC can act as a strong gas barrier. They produced NFC films with an oxygen transmission rate (OTR) of around 17 ± 1 mL $m^{-2}day^{-1}$ [59]. Such values are in accord with the recommended rate, which is below 10-20 mL $m^{-2}day^{-1}$ and are comparable to the best synthetic polymers, like polyvinylidene chloride-coated oriented polyester (9-15 mL $m^{-2}day^{-1}$), of approximately the same thickness.

The gas solubility is similar for NFC and CNCs but in NFC films, the oxygen molecules penetrate more slowly because more entanglements are present in the NFC structure. Consequently, NFC films usually have less oxygen permeability than CNC films [61].

6.2. LIMITATIONS OF USE

The barrier properties to oxygen of nanocellulose are good, but the same trait is not observed for water vapor. The reason is the high affinity between water and nanocellulose, so the water barrier tends to be weak for unmodified nanocellulose materials [52]. In terms of water vapor barrier properties, a comparison between nanocellulose and cellulose demonstrated a decrease in water adsorption and water vapor transmission rate in nanocellulose, due to the rigid network within the films [62]. The lowest water vapor transmission rate obtained by Rodionova et al. [63] in NFC films (both pure and partially acetylated) was 173 g m^{-2} day^{-1}, a very high value compared to 16.8 g m^{-2} day^{-1} obtained for polyethylene [64].

Some researchers have been working on improving these barrier properties. Sharma and co- workers [65] reduced by 50%, the water vapor permeability of CNF films by heating them at 175 °C for 3 h compared to untreated CNF films. The explanation for this result is that the reduction in porosity of the material hinders diffusion, increasing the crystallinity and hydrophobicity of the material. Researchers have shown improvements in water vapor and gas barrier properties by developing methods such as coating CNFs with polymers, grafting other polymers onto CNFs or using a high aspect ratio filler material to obtain a composite membrane. The inclusion of high aspect ratio filler materials avoids chemical modification of the fibers, but its disadvantage is that the filler materials usually limit recyclability and biodegradability of the resulting composite material [66].

6.3. CHITOSAN NANOCELLULOSE FILMS

An area of growing interest is the preparation of antimicrobial edible films to control foodborne microbial outbreaks, besides maintaining quality, freshness, and safety [67]. Chitosan (CHT), a cationic (1-4)-2-amino-2-deoxy-d-glucan, is industrially produced from chitin, the second most abundant polysaccharide in nature [68]. Chitin and chitosan are natural antimicrobial compounds against an extensive variety of microorganisms, including bacteria, yeasts and molds [69]. Chitosan is a non-toxic and biodegradable compound [70].

Chitosan films have poor mechanical properties, limiting their applications. Functional properties of chitosan-based composites can be improved by reinforcement with nanocellulose [71]. Nanocomposites (NCPs) are novel polymer matrices that have nanoparticles incorporated with at least one dimension in nanoscale [72]. Chitosan cellulose compounds have been studied by several groups. In these studies, the researchers incorporated nanocellulose particles in chitosan and analyzed the mechanical and barrier properties of the films obtained [73-75]. Li et al. [76] reported that by increasing the cellulose nanowhisker (CNW) concentration from 0 to 20 wt%, the dry tensile strength of chitosan nanocomposite films increased from 85 to 120 MPa, while the wet tensile strength increased from 9.9 to 17.3 MPa. They also observed that incorporation of CNW enhanced water resistance and thermal stability of chitosan films.

In another work, Wu et al. [77] found that 32% loading of CNF in chitosan films produced by the solution casting method caused 12- and 30-fold improvements in the tensile strength and Young's modulus, respectively. Since different concentrations of nanocellulose have been applied in various works, it is necessary to obtain the optimum range in order to know the best properties for chitosan- nanocellulose nanocomposites.

Dehnad et al. [78] tested the antimicrobial properties of chitosan-nanocellulose films in meat. They reported that this nanocomposite showed inhibitory effects against gram-positive and gram-negative bacteria and decreased the lactic acid bacteria population. El Samahy et al. [79] obtained similar results. The authors used a mixture containing dry nanocellulose and chitosan in different concentrations, coated on bagasse paper sheets. The paper sheet containing 0.4 g of nanocellulose and 0.6 g of chitosan showed very good antimicrobial activity against food poisoning bacteria, *Salmonella* and *Staphylococcus aureus*.

The technique used to prepare chitosan-nanocellulose films is important because it affects the dispersion of nanocellulose in chitosan and consequently controls the interaction between these two constituents. Therefore, different processes have been employed to produce nanocomposite films. The most common processes used are freeze drying [80], the layer-by-layer procedure [81], and electrospinning [82].

6.4. STARCH NANOCELLULOSE FILMS

Among natural polymers, starch has received attention due to its various advantages, such as low cost, wide availability from many plants and total compostability without the formation of toxic residues [83]. In their raw form, starches are organized into semicrystalline granules, with poor mechanical properties and high water affinity. The addition of nanofibers aims to improve some of these properties.

Lendvai et al. [84] investigated the influence of microfibrillated cellulose (MFC) in thermoplastic starch (TPS). The raw starch/glycerol and the plasticized starch/water ratios were set at 4/1 and 6/1, respectively. Up to 20 wt % of two different MFC types (of varying mean length and diameter) were incorporated in the plasticizer. The mechanical properties of the TPS biocomposites were improved with MFC. The yield strength was improved by 50% and the stiffness by 250% upon adding 20 wt% of MFC compared to

TPS. The reinforcing effect of the MFC was more prominent in the starch than in the glycerol (plasticizer)-rich phase of the TPS.

Slavutski and Bertuzzi [85] determined the effect of variations of assay parameters such as the water vapor gradient (driving force of the permeation process) and water vapor pressure values on each side of the starch/CNC nanocomposite films. The incorporation of CNC in the starch film matrix improved its water resistance and water barrier properties. The decrease in surface hydrophilicity and the improvement in the water vapor barrier properties with the addition of CNC showed that these nanocomposites have excellent potential as new biomaterials for applications in food packaging and conservation.

Montero et al. [86] found similar improvements in starch/CNC nanocomposite films. In this study, plasticized starches from different plant sources (tubers, cereals and legumes) were tested with glycerol content and reinforced with cellulose nanocrystals by the solution casting method. The incorporation of cellulose nanoparticles gives more homogeneous surfaces, increased the rigidity of films, the thermal stability and moisture resistance.

On the other hand, González et al. [87] tested starch/CNC together and concluded that the barrier to water vapor remained almost insensitive to nanoreinforcement in spite of the improved mechanical properties and higher oxygen permeability values compared to those of the unfilled matrix.

6.5. PECTIN NANOCELLULOSE FILMS

Pectin is a methylated ester of d-galacturonic acid that contributes to tissue integrity and rigidity in plant cell walls [88]. The main industrial sources of pectin extraction are apple pomace and citrus peels [89].

Edible films produced from pure pectin have poor barrier, and thermomechanical properties and weak water resistance due to their hydrophilic nature, and they have weak water barrier properties at high relative humidity [90]. Thus, many strategies have been investigated to overcome these disadvantages, including blending with plasticizers such as glycerol, acetylated monoglycerides, polyethylene glycol, and sucrose [91]; combination with hydrophobic compounds [92]; crosslinking [93]; and nanoreinforcement

of biopolymers to produce nanocomposites [94]. Recent studies have evaluated the use of silver nanoparticles [95], chitosan [96] and montmorillonite [97] as potential pectin-based nanocomposite packaging materials for foods.

Edible pectin film reinforced with 5% cellulose nanocrystals (CNC) presented good dispersion of CNC in the pectin matrix, suggesting appropriate interaction between the filler and matrix, in agreement with the mechanical results. The tensile strength increased by up to 84% and water vapor permeability decreased by 40% [98].

7. ACTIVE PACKAGING

Postharvest losses of agricultural products are significant worldwide. Nowadays, consumers demand the development of active materials with properties to enhance the shelf life and safety of packaged food. This demand poses one of the most challenging research issues [99]. Research is being focused specifically on the use of renewable resources, the improvement of barrier properties, and the introduction of new functionalities for packaging [100].

Active packaging is one of the innovative concepts in food packaging. This technology is based on the concept of incorporating certain components into the packaging systems that release or absorb substances into or from the packed food or the surrounding environment to prolong shelf life and sustain the quality, safety and sensory characteristics of the food. The most important active packaging concepts include moisture absorbers, antimicrobial packaging, carbon dioxide emitters, oxygen scavengers and antioxidant packaging [101].

Natural polymers exhibit several advantages as coatings and films, such as edibility, biodegradability, biocompatibility and barrier properties. The use of nanocellulose as an insoluble matrix for a controlled release system is, nevertheless recent. It was first developed by Kolakovic et al. [102] in a study emphasizing the diffusion-control role of nanocellulose in drug release.

In another controlled release study, Lavoine et al. [103] investigated the effect of nanocellulose coatings on caffeine release. The influence of the nanocellulose coating was represented by the cumulative amount of caffeine released as a function of the number of washing steps. In comparison with the paper impregnated in the caffeine

solution, the samples coated with the nanocellulose released the caffeine more progressively, and the proportion of caffeine released between each washing step was smaller than the proportion released by the samples without nanocellulose. The slowest release of caffeine was observed for samples coated with the mixture of nanocellulose and caffeine.

Cost of implementation should be considered when evaluating smart packaging inventions. This universal challenge for smart packaging can be reduced through improved economies of scale and the reduction of waste. It is necessary to guarantee enough functional activity without unnecessary excess, for example, using sufficient active packaging coating to prevent deterioration without overcompensating and therefore wasting these often expensive agents. The use of nanocomposites in smart packaging is an option to reduce implementation cost [104].

7.1. ANTIMICROBIAL FILMS

Traditionally, antimicrobial agents are added directly into food formulations, but this practice can result in excessive amounts of the antimicrobial agents, which may change the taste of the food [105]. Dipping, spraying or brushing techniques are used to deposit antimicrobial substances on the food surface to prevent colonization by undesirable microorganisms. However, direct application of antimicrobial substances can cause the inactivation or evaporation of active agents and rapid migration into the bulk of the foods [106]. Therefore, inactivation of the antimicrobials by food components or dilution below active concentration can occur.

The reason for incorporating antimicrobials in packaging is to prevent the growth of microorganisms on the surface of foods, where a large portion of spoilage and contamination occurs. This concept can reduce the addition of larger quantities of antimicrobials into the bulk of the food. The gradual release of an antimicrobial from a packaging film to the food surface can be an advantage over dipping and spraying [107].

Edible films have been incorporated with several antimicrobial substances (bacteriocins, essential oils and polyphenols) in order to obtain antimicrobial active packaging. Bacteriocins are antimicrobial peptides synthesized by bacteria that can kill

closely related bacteria [108]. Nisin is the most studied bacteriocin and is currently considered the only bacteriocin licensed as a GRAS food additive. It is commercially used as a natural preservative [109].

In order to obtain an antimicrobial effect against *Listeria monocytogenes*, bacterial cellulose films were exposed to a dilution series of nisin and then used to pack frankfurters. Bacterial cellulose films exposed to the nisin solution at a high concentration (2500 IU mL−1) for 6 h was found to decrease sharply the number of *L. monocytogenes* by ∼2 log CFU g^{-1} after 2 days of storage in samples covered with these films, and then remained constant until the end of experiment. The use of nisin in bacterial cellulose films extended the microbiological shelf life of frankfurters [110].

In a similar study using nanocomposite films made of polylactic acid-cellulose nanocrystals (PLA-CNC), Salmieri et al. [111] tested the effect of nisin release on the inactivation of *L. monocytogenes* in ham. Bioactive PLA-CNC-nisin films significantly decreased *L. monocytogenes* in ham during storage from day 1 and caused total inhibition from day 3. The percentage of nisin release increased continuously from day 0 to day 14, up to 21%. These results showed the potential application of PLA-CNC-nisin films for controlling the growth of pathogens in meat products.

The same research group in another work tested the nanocomposite film PLA-CNC containing oregano essential oil for vegetable packaging. Essential oils rich in phenolic compounds have been reported to have a wide spectrum of antimicrobial activity. Oregano is one of the most effective antibacterial agents [112]. Carvacrol, thymol, p-cymene and γ-terpinene are the principal constituents of oregano essential oil [113]. Microbiological analysis of mixed vegetables indicated that PLA–CNC–oregano films induced a quasi-total inhibition of *L. monocytogenes* in vegetables until day 14. These results demonstrated the strong antimicrobial capacity of PLA–CNC– oregano films for packaging of vegetables [114].

7.2. ANTIOXIDANT FILMS

After microbial growth, lipid oxidation is the main cause of food spoilage. In particular, foods with high lipid content are susceptible to deterioration. This is the case of nuts, vegetables and fish oils, as well as other fishery products and meat. The oxidation of lipids in foodstuffs results in the development of off-flavors, typical of rancidity, rendering the product unacceptable for human consumption [115]. Other negative effects are the formation of toxic aldehydes [116] and the loss of nutritional quality because of polyunsaturated fatty acid (PUFA) degradation [117].

Consumers are increasingly demanding healthier and safer food products, prompting research into novel preservation techniques. To reduce lipid oxidation, strategies such as the direct addition of antioxidants to foods or the design of suitable packaging technology have been applied. The direct addition of antioxidant compounds on the food surface has the potential limitation that once the active compounds have been consumed, the protection ends and the food quality can degrade [118]. Currently, antioxidant active packaging systems are novel alternatives for packaging based on the incorporation of antioxidant agents into packaging materials to improve the stability of oxidation-sensitive food products.

Antioxidant active films prepared with polyphenols such as gallic acid, grape seed extract, thyme extract, murta leaf extract, tea polyphenols, tea catechins and others have been widely used to protect food from oxidation [119-123].

Polypyrrole is a conjugated polymer synthesized by chemical oxidation or electrochemical polymerization of pyrrole in an aqueous solution, producing antioxidant properties [124]. Bideau et al. [125] reported the effectiveness of a polypyrrole/nanocellulose composite for food preservation. The values of oxygen permeability obtained in the nanocomposite (16.5 cm^3/m^2/day) were competitive with synthetic polymers traditionally used in food packaging, such as polyvinyl alcohol (14 cm^3/m^2/day) or polyethylene terephthalate (19 cm^3/m^2/day), with film thickness of 12 μm [126]. Besides this, the polypyrrole/nanocellulose composite has the ability to preserve bananas for five days. No brown color was visible on the bananas, so there was no trace of oxidation.

In another work using a nanocomposite composed of chitosan-xylan/cellulose nanocrystals, Bao et al. [127] observed that the nanocomposite films possessed good antibacterial activity against *S. aureus* and *E. coli* and good antioxidant activity. The antibacterial property was due to the chitosan and the antioxidant property was due to the xylan. In addition, when increasing the mass fraction of CNC to 12 wt%, the tensile strength and elongation at break of the nanocomposite films were increased significantly, whereas the swelling percentage of the films decreased with the increment of CNC content.

Wang et al. [128] prepared films with antioxidant activity by combining chitosan and epigallocatechin-3-gallate, with nano-bacterial cellulose (BC) as a reinforcement agent. They concluded that the addition of nano BC in a concentration of 5% improved the mechanical properties, thermal stability and solubility, and sustained the release of epigallocatechin-3-gallate from the film into the food.

Table 1 summarizes the main papers focusing on nanocellulose films reported in this review.

Table 1. Summary of the studies on the application of nanocellulose in food packaging

Films	Publication year	Reference
Chitosan nanocellulose	2012	[73]
	2014	[77]
	2014	[78]
	2017	[79]
Starch nanocellulose	2016	[84]
	2014	[85]
	2017	[86]
	2015	[87]
Pectin nanocellulose	2017	[98]

Antimicrobial	2008	[110]
	2014	[111]
	2001	[113]
	2014	[114]
Antioxidant	2017	[125]
	2012	[126]
	2018	[127]
	2018	[128]

8. NANOCELLULOSE PATENTS

The pioneers in describing the hydrolysis of cellulose was Ranby in the early fifties [16]. In the same period, Battista and co-workers [129] were also working on the acidic hydrolysis of cellulosic substrates. They obtained smaller particles with micro diameters, which they called cellulose crystallite aggregates, now better known as microcrystalline cellulose (MCC). This product has found many applications and resulted in a significant number of patents published in the sixties [15]. Although nanocellulose was discovered by Ranby in the fifties, it did not receive much attention until Turbak and coworkers [21, 38, 39], in the eighties, published a series of studies on food products using nanocellulose. Since then, many patents have been filed in this area.

Cellulose nanocrystals, cellulose nanofibrils and bacterial cellulose have all been the subject matter of patents. We analyzed the patent trends (number of patents granted over the years by the USPTO). In this survey, bacterial cellulose was involved in the largest number of patents (Fig. 3). The summary of the principal patents related to nanocellulose in food and food packaging films are presented in **Table 2.**

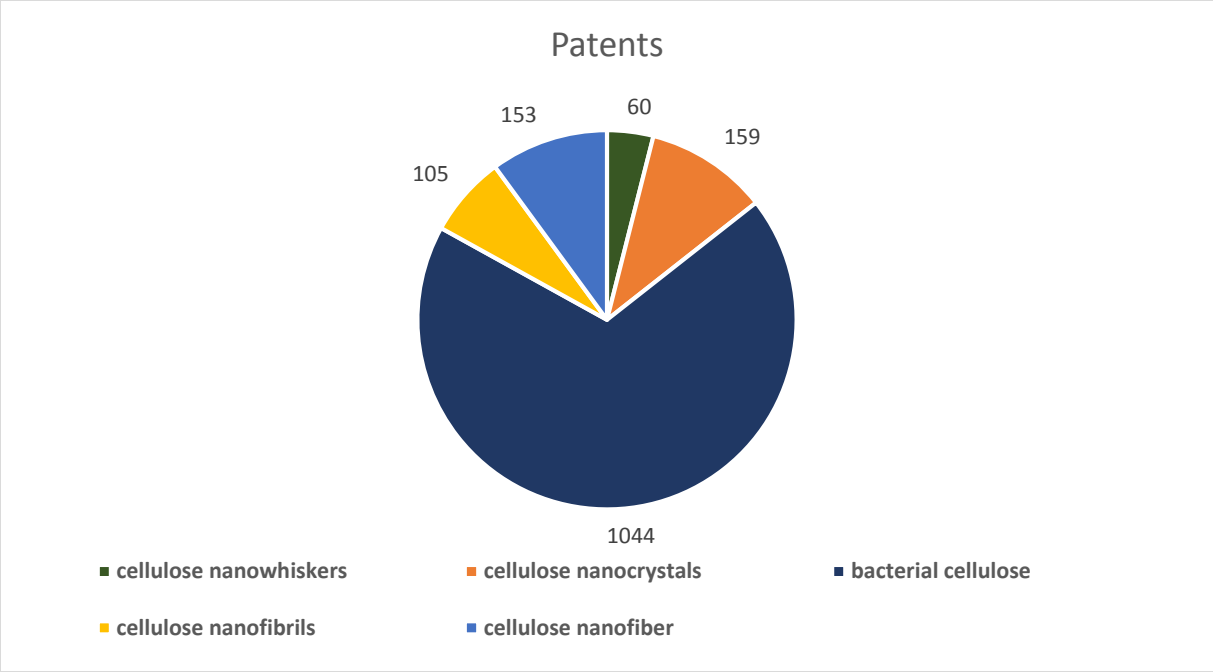

Figure. 3. Total number of patents per subject matter related to nanocellulose granted by the USPTO Search criteria: Topic key words were *cellulose nanowhiskers*, *cellulose nanofibers*, *cellulose nanocrystals*, *bacterial cellulose*, *cellulose nanofibrils* Source: USPTO, January 3rd 2019.

Table 2. Principal patents involving nanocellulose in food and food packaging films

Description	Patent number	Ref.
Suspensions containing microfibrillated cellulose	US 4378381 (1983)	[21]
Food products containing microfibrillated cellulose	US 4341807 (1982)	[38]
Suspensions containing microfibrillated cellulose.	US 4487634 (1984)	[39]
Bean jam or food composition prepared by using the same	JP58190352 (1983)	[40]
Liquid or pasty seasoning composition	JP58190369 (1983)	[41]
Use of cellulose microfibrils in dry form in food formulations	US 6485767 (2002)	[42].
Use of essentially amorphous cellulose nanofibrils as emulsifying and/or stabilizing agent	CN1438918A (2003)	[130]
Edible food packaging film	CN102145779A (2011)	[131]
Frozen dessert and frozen dessert material	US 20140342075 A1 (2014)	[43]

Nano-cellulose coatings to prevent damage in foodstuffs	US20140272013 A1 (2014)	[132]
Degradable food packaging film with antibacterial function	CN108276598A (2018)	[133]

9. NANOTOXICOLOGICAL AND REGULATORY ASPECTS

Although the use of nanomaterials for packaging has several advantages, their social and commercial acceptance is still not clear. This is related to doubts of manufacturers and consumers regarding nanotechnology. Those doubts are associated to the health and safety of workers, due to possibly harmful environmental effects, and to potential human health effects such as oxidative damage, inflammation of the gastrointestinal tract, cancers and lesions of the liver and kidneys due to acute toxic responses [134]. The possible migration of nanomaterials to foods is one of the main concerns, so before using nanomaterials in packaging that will be in contact with food products, migration tests should be performed to ensure the safety of those products.

Aspects of nanotoxicology and safety of nanomaterials (NMs) must be considered to ascertain the risks to health and the environment, based on the precautionary principle as the basis for nanotechnology regulation [135]. Amenta and collaborators [136] provided an overview of differing approaches to regulatory solutions worldwide for the use of nanotechnology in food and feed production. At that time, according the authors, only the European Union (EU) and Switzerland had nano-specific provisions incorporated in existing legislation, whereas other countries counted on non-legally binding guidance and standards for industry.

Considering that NMs can have properties that are very different from those of the non-nanoform of the same material, safety data generated about the latter form are not necessarily adequate for the nanoform [137]. Therefore, tests that were developed for the safety assessment of chemicals might not be (directly) applicable to NMs, or completely new tests might be required.

Despite the large number of research projects on NMs in recent years, investigation specifically addressing regulatory needs is still rare. At the present, certain key needs can be identified from the regulatory standpoint, such as: a) implementation of regulatory definitions for NMs (to reliably measure the particle size); b) implementation of nanoingredient labeling of products; and c) safety testing including (eco)toxicity and *in vitro* testing methods. The safety of NMs should be maintained throughout their life cycle, because they can change at each of these steps, in terms of size, agglomeration/aggregation state or surface properties [138].

Several countries have been active in examining regulatory frameworks to deal with nanotechnologies. The EU and Switzerland incorporated nano-specific provisions into their legislation for food industries [136].

Mandatory labeling of the content of NMs is already part of EU legislation on food, cosmetics and biocides. All nanoingredients have had to be clearly indicated in the list of ingredients, with the names of followed by the word “nano” in brackets, since December 2014 [139] and September 2013 [140], respectively.

In the Americas, the US Food and Drug Administration (FDA) does not have any specification for nanotechnology-based products and has not yet adopted a regulatory definition of NMs. Brazil is one of the leading countries in nanotechnology research and development in Latin America, but no specific regulation exists in the country [141]. In May 2010, a proposal to introduce labeling of food, drugs and cosmetics containing NMs was presented to the Brazilian Senate but was rejected, and no other proposal for regulation has been approved by Congress.

However, some Asian countries are active in the production and regulation of NMs. Many of these countries have established standards and certification systems for nano-enabled products. In Thailand, for example, the National Nanotechnology Centre (NANOTEC) has identified 10 flagship programs of national priority, including industrial standards for nano-products, called NANO-MARKS, and “Food Quality” aimed at improving and monitoring the quality of Thai food prepared using nanotechnology [136].

CURRENT & FUTURE DEVELOPMENTS

The incorporation of nanomaterials in agri/feed/food products is growing. New products are in development and expected to enter the market in the near future. However, due to their specific properties and based on the precautionary principle, these prospective new products must be tested to ensure their safety to human health and the environment. Research specifically addressing regulatory needs is necessary. For this reason, the challenge for governments is still to stimulate research on NMs and continue to find an optimal level of regulation that considers the potential benefits and risks of nanotechnology.

The use of a multi-method approach is recommended to classify NMs correctly, because until now no single method has been able to cover the whole size range and all the different types of NMs. There are challenges in the choice of equipment (techniques), metrics for defining properties, protocols for sample preparation, measurements and possibly protocols for conversions of the test results into a parameters of definition. For these reasons, existing methods need to be further developed and improved, especially if they are to be applied in complex matrices such as food.

CONCLUSION

Nanocellulose is a natural polymer that has enormous potential for use in the food industry as ingredient or innovate packaging. Some current packaging materials already include nano-encapsulated agrochemicals or antimicrobial nanoparticles, making them active and intelligent materials. Nanotechnological applications will trigger new market opportunities, with new products and consequently profits. However, new products must be approved. Collaboration among countries on NM regulatory aspects is required to share information and ensure protection for people and the environment.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Chapter 2

Production and characterization of *Acetobacter xylinus* bacterial cellulose using cashew apple juice and soybean molasses

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ABSTRACT: Bacterial cellulose (BC) has been largely used in biomedical and technological fields. The use of agro-industrial byproducts as alternative source of carbon and nitrogen in culture media reduces the BC cost production, adds value to the byproducts and minimizes the environmental impact. In this study, the use of cashew apple juice and soybean molasses were evaluated to produce BC by *Acetobacter xylinus* in comparison to the usual Hestrin and Schramm medium (HS). BC produced in static cultivation was characterized by X-ray diffraction, Fourier transform infrared spectroscopy and thermogravimetric analysis. The BC production (4.50 g L^{-1}) obtained from the medium using cashew apple juice as carbon source (20 g L^{-1}) with soybean molasses as nitrogen source (10 g L^{-1}) was superior than HS medium (4.03 g L^{-1}). Morphological analysis showed that bacterial celluloses produced with agro-industrial byproducts combined were similar to those found for the pellicle obtained from HS medium.

KEYWORDS: biopolymer; carbon sources; cost production; bacteria; agro-industrial waste; nanotechnology

1. INTRODUCTION

Cellulose is the most abundant polysaccharide available in the nature. The use of cellulose in paper production is one of the most known application [1]. However, considering the exploitation of fossil resources and the environmental problems related to it, the biopolymers such as cellulose, is replacing traditional synthetic polymers at different applications, since they are biodegradable [2].

Cellulose is usually obtained from wood and natural fibers, but it is also produced by others organisms, especially by bacteria belonging to the genera *Acetobacter*. *Acetobacter xylinus* is an aerobic gram-negative bacteria that has been used as a model for bacterial cellulose investigations, due to its relatively high capability to synthesize it [3]. The mechanism of transforming the glucose to cellulose requires four principal steps: (1) phosphorylation of glucose by glucokinase to glucose-6-phosphate; (2) isomerization of glucose-6-phosphate to glucose-1-phosphate by phosphoglucomutase; (3) conversion of glucose-1-phosphate to uridine diphosphate glucose (UDP-glucose) by UDP-glucose pyrophosphorylase; (4) and the synthesis of cellulose from UDP-glucose by cellulose synthase [4].

Bacterial cellulose (BC) is traditionally used as a dessert called “nata-de-coco” in South-east Asia [5]. However, BC has attracted attention because of its unique characteristics such as being naturally free of lignin and hemicellulose [6]. Besides it, BC presents high porosity, high water retention capacity, high mechanical strength, high crystallinity, low density, biodegradability, biocompatibility and non-toxicity [7]. These features make BC suitable for technological applications conducting materials and electrical devices [7], biomedicine, pharmacology [8], and food packaging [9]. Additionally, BC has been used to develop new materials and composites including the nanostructured ones [10].

Despite the potential for a wide range of commercial applications, BC is expensive to be manufactured [11]. The synthetic media commonly used for BC production is composed by glucose, yeast extract, peptone and mineral salts [12]. The use of various waste materials such as orange and pineapple juices [13], sisal juice [14], molasses [15],

grape skins aqueous extract, cheese whey, crude glycerol and sulfite pulping liquor [2] were evaluated as alternative and economic nutrient sources to reduce cost production of BC.

The use of agroindustrial residues from widely spread crops worldwide improves the cost effectiveness of BC production. Cashew apple tree *(Anacardium occidentale)* is an important tropical plant grown mainly in West Africa, India, Nigeria, Vietnam, Brazil and Indonesia [16]. The cashew apple is underused, as the main interest of this production system is its nut, due to the interest in exporting cashew nut [17]. The cashew apple juice is rich in sugars, vitamins and minerals, because of this it can be also used to produce high added value products such as dextran, lactic acid, mannitol and oligosaccharides [18]. In addition to cashew apple, soybean (*Glycine max*) is another important crop cultivated in many countries around the world [16]. Soybean molasses is generated from the production of soy protein concentrate. It is a byproduct mainly composed of soluble carbohydrates, proteins, lipids and ashes. Soybean molasses has been used in fermentations process to produce ethanol, butanol, poly-hydroxyalkanoate and lactic acid [19].

In the present work cashew apple juice and soybean molasses were evaluated as carbon and nutrient sources for BC production in static condition. The BC productivity using cashew apple juice and soybean molasses media were compared to the synthetic media HS [12]. BC samples obtained were characterized by FT-IR, XRD and TGA in order to verify if their intrinsic properties were maintained.

2. MATERIALS AND METHODS

2.1 SUBSTRATES

The cashew apple juice was collected in Pacajus (Ceará, Brazil). The soybean molasses was kindly donated by the Selecta® soybean (Araguarí, Minas Gerais, Brazil). The substrates were stored in plastic containers at 4 ºC until their uses.

2.2 CHARACTERIZATION OF SUBSTRATES

Dry extract and ashes were determined by Thermogravimetric Analyzer TGA-2000 (Las Navas Instruments, USA) after drying at 105 ºC and calcination at 550 ºC, respectively, until constant weight. Proteins were determined by Lowry method [20]. Lipids were determined with ether-extraction using an automatic extraction system (Ankom XT15, USA). Total reducing sugars (glucose and fructose) were determined by DNS method [21]. Soybean molasses was hydrolyzed with the addition of H_2SO_4 to pH 1.5 and heating at 80 °C for 10 min before sugar determination [15]. Results are presented as average of three analyses.

2.3 MICROORGANISM AND CULTURE MEDIA

Acetobacter xylinus strain ATCC 53582 was used in the present study for BC production. For the preculture, the bacteria was inoculated from HS agar medium to four tubes containing 5 mL HS broth medium. The tubes were incubated at 30 °C for 48 h. After this period, the pre cultures were mixed and used to inoculate the media.

Medium HS: The standard medium used in this study comprised the following (g L^{-1}): glucose - 20; peptone - 5; yeast extract -5; disodium phosphate (anhydrous) - 2,7; citric acid (monohydrate) - 1,15 [11].

Medium Soybean Molasses (SM): The molasses was treated before used as medium. The molasses was diluted 2-fold (w/v) with H_2SO_4 2M and heated at 80 °C for 10 minutes to hydrolyze sucrose [15]. Hydrolyzed molasses was centrifuged at 2000 g 20 min^{-1} to separate suspended solid material. Reducing sugars in supernatant were determined by DNS method and molasses was diluted with distilled water to 20 g L^{-1} reducing sugars to final concentration. The solution was adjusted to pH 5.55 with NaOH 1 M.

Medium Cashew apple juice with yeast extract (CYE): Cashew apple juice was filtered through a qualitative filter paper (Unifil, Germany) to separate suspended solid material. Reducing sugars were determined by DNS method and juice was diluted with distilled water to 20 g L^{-1} reducing sugars final concentration. Separately a solution yeast extract (10 g L^{-1}) was prepared and added during inoculation.

Medium Cashew apple juice with Soybean molasses (CSM): Cashew apple juice filtered was diluted with distilled water to 20 g L^{-1} reducing sugars. Crude soybean molasses was added as nitrogen source in 10 g L^{-1} of protein before sterilization.

2.4 BC PRODUCTION CONDITIONS

After prepared, in triplicate, 50 mL of each medium were distributed in a 250 ml Erlenmeyer flask and autoclaved at 121°C 15 min^{-1}. The culture was started by inoculating 3% (Density optical at 600 nm: 0.04 -0.07) of the preculture. Cultivations were performed at 30 °C for 7 days under static conditions.

2.5 BC RECOVERY AND PURIFICATION

After fermentation, the media was centrifuged (Thermo Fisher Scientific, Waltham, MA, USA) 2000 g 15 min^{-1} and the pH of each supernatant was measured (pH meter Ionlab PH-500B-I, Araucária, PR, Brazil). The supernatant was submitted to sugars analysis (DNS method). The BC membranes were harvested and purified by alkali treatment. The membranes from HS medium (CB HS), Soybean Molasses (CB SM), Cashew apple juice with yeast extract (CB CYE) and Cashew apple juice with Soybean molasses (CB CSM) were immersed in NaOH 1 M at 80 ºC for 1 h. Finally, BC membranes were rinsed with distilled water until pH 7.0. BC membranes were lyophilized at −53 °C for 30 h in a Liotop® Model L101 (Liotop®, São Carlos, SP, Brazil).

2.6 FT- IR SPECTROSCOPY

BC membranes were examined using a Nicolet Nexus 470 (Thermo Fisher Scientific, Waltham, MA, USA). All the spectra were acquired from 4000 to 400 cm^{-1} with a scan frequency of 32 s^{-1} and a resolution of 4 cm^{-1}.

2.7 THERMOGRAVIMETRIC ANALYSIS (TGA)

The analyses were performed using Thermogravimetric Analyzer TGA-2000 (Las Navas Instruments, South Carolina, USA). About 0.2 g of membranes were heated from 20 to 750 °C at a heating rate of 20 °C min^{-1} under nitrogen atmosphere.

2.8 X-RAY DIFFRACTION (XRD)

XRD patterns of BC membranes produced were acquired using a diffractometer D2 Phaser (Bruker, Karlsruhe, Germany), 2θ in the range between 10° - 30° and a scan rate of 0.5° min^{-1}, with a step size of 0.02° and step time of 4 s. The crystallinity index of the BC membranes was calculated according to the Segal method [22] (Eq. 1).

$$CrI = [(I002 - Iam)/I002] \times 100 \quad [Eq. (1)].$$

where I_{002} is the intensity of maximum diffraction of crystalline region at about $2\theta = 22.5°$, and I_{am} is the intensity of diffraction attributed to the amorphous region at about $2\theta = 18°$.

2.9 STATISTICAL ANALYSIS

The statistical analysis was carried out using the GraphPad Prism version 5.01 software. The statistical significance of the evaluated data was analyzed by one-way analysis of variance (ANOVA) and the Tukey's test with significance level $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

3.1 CHARACTERIZATION OF SUBSTRATES

Cashew apple juice presents 105 g L^{-1} reducing sugars, 0.32 g 100 g $^{-1}$ ashes and 0.05 g 100 g $^{-1}$ total nitrogen. Molasses contains 153 g L^{-1} reducing sugars, 67 g L^{-1} proteins and 11.62 g 100 g $^{-1}$ ashes which represent, respectively, nitrogen and minerals sources, these components are important for bacteria cell growth [23]. The obtained results are similar to ones presented by Das & Arora [24] and Caldeirao et al. [25].

Considering that cashew apple juice are poor in nitrogen, two different possibilities of N_2 supplementation were evaluated. In the medium CYE, the cashew apple juice medium was supplemented with yeast extract that is a common nitrogen source used in culture media [12]. In the medium CSM, the supplementation was done with soybean molasses.

3.2 BACTERIAL CELLULOSE PRODUCTION

Table 1 presents the comparison of pH, sugar consumption and productivity among the four media evaluated in the beginning and at the end of fermentation.

Table 1: *A. xylinus* bacterial cellulose *yield* in different carbon source culture *medium*

Medium	pH initial	pH final	Delta pH	Sugar initial (g L^{-1})	Sugar final (g L^{-1})	Delta Sugar	Cellulose production (g L^{-1})	Cellulose production/Sugar consumption
HS	5.95	4.05	-1.9	20.85	5.41	15.44^{a}	4.03^{a}	0.26^{b}
CSM	5.32	5.06	-0.26	31.69	18.59	13.09^{b}	4.50^{a}	0.34^{a}
SM	5.55	5.70	+0.15	20.95	13.16	7.79^{c}	2.23^{b}	0.28^{b}
CYE	5.42	5.94	+0.52	20.99	9.24	11.75^{d}	4.54^{a}	0.39^{a}

Means in the same column with different superscript letters are significantly different (P<0.05)

The initial pH varied between 5.32 until 5.95 in the experiments. Considering the media formulated with cashew apple juice and soybean molasses, pH slightly decreased in CSM medium and it increased in SM and CYE media. The pH decreased more significant in the medium HS (delta= 1.9).

The initial sugar concentration was determined by DNS method in approximately 20 g L^{-1} as pre-determined, except in CSM. The CSM showed the highest sugar content (31.69 g L^{-1}) because beyond cashew apple juice, the soybean molasses contributed not only as a nitrogen source but also as a sugar source. Reducing sugars in soybean molasses before hydrolysis were 45 g L^{-1}.

The sugar consumption was maximum in HS (15.44 g L^{-1}) and minimum in SM (7.79 g L^{-1}). All the media demonstrated that cellulose was produced. The minimum production was found in SM (2.23 g L^{-1}) and the maximum occurred in both CYE (4.54 g L^{-1}) and CSM (4.50 g L^{-1}). CYE and CSM showed the highest cellulose production yield over total initial sugars, demonstrating an efficient conversion.

Considering the cellulose production over the total initial sugar content, it is possible to conclude that the use of cashew apple juice added with other nitrogen source, such yeast extract or soybean molasses is a promising mainly ingredient in culture media. The obtained cellulose productivity was higher than HS medium. The estimated cost of HS media and CSM was 1.76 USD L^{-1} and 0.27 USD L^{-1}, respectively (data not published yet).

3.3 FT-IR SPECTROSCOPY

FT-IR analyses of BC was developed in order to study the structural and chemical effects of the evaluated culture media. All films presented some common bands in FT-IR spectra (Figure 1), indicating that the celluloses produced had similar chemical structure despite being produced with alternative substrates. The functional groups characteristic of BC with the main bands and respective assignments are shown in Table 2.

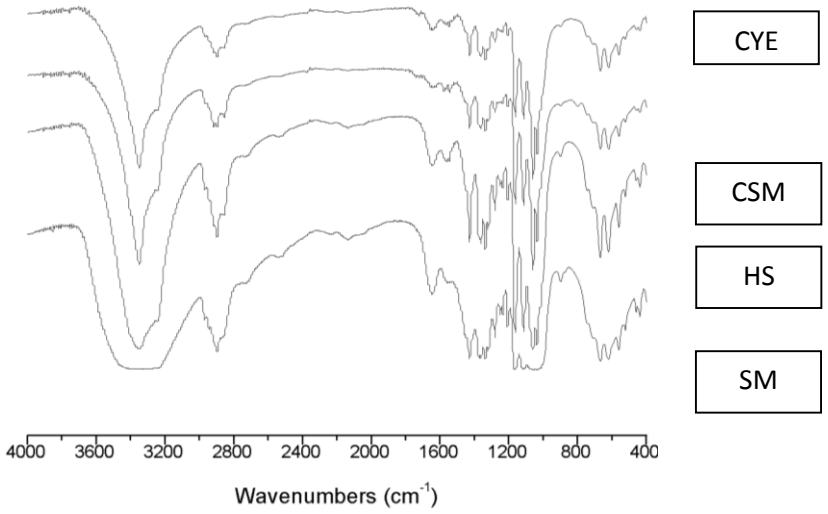

Figure 1. FT-IR spectra of the *A. xylinus* BC samples from CYE, CSM, HS and SM medium.

Table 2. FT-IR spectra of bacterial cellulose (BC) from *A. xylinus* obtained in HS, SM, CYE and CSM medium

	Wave number (cm^{-1})				
HS	**SM**	**CYE**	**CSM**	**Assignment**	**Reference**
3349	3334	3348	3348	OH (Celulose I)	[26]
2895	2895	2895	2916	CH2	[27]
1427	1428	1425	1426	HCH,OCH	[28]
1359	1360	1367	1359	CH	[28]
1163	1163	1162	1162	C-O-C	[27]
1060	Not identified	1059	1059	C-O	[29]
667	666	669	666	C-OH	[28]

Only BC produced with soybean molasses medium presented some broad bands, probably related to the amount of amorphous fractions in cellulose [30]. Peaks at 1735 cm^{-1} assigned for C=O groups in proteins and lipids were not identified [31] showing that the purification was efficient. No evidence of change in cellulose type (I to II) due to the purification treatment was observed in the FT-IR spectrum. Cellulose II is represented by the peak at 3488 cm^{-1} and –OH stretching assigned at 3447 cm^{-1}[32].

3.4 CRYSTALLINITY ANALYSIS

The crystallinity influences specifically the mechanical properties of the materials [33]. The X-Ray diffraction patterns (Figure 2) of the BC samples from HS, CYE and CSM medium showed three main peaks at 2θ =14.5°, 16.4°, and 22.5°, which are usually attributed to the crystallographic planes of 101 (amorphous region), 10 (amorphous region), and 200 (crystalline region), which characterize cellulose I [34]. No peaks are found in correspondence of 2θ =12.1º and 20.8º, which are characteristic of cellulose II [35]. This

again demonstrates that the two-step purification process did not change the structure of BC obtained from cellulose I to cellulose II.

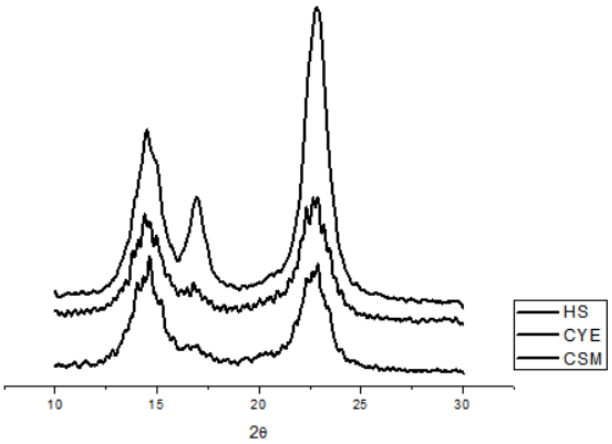

Figure 2. XRD patterns of the BC from *A. xylinus* from HS, CYE and CSM medium.

The HS media showed the highest crystallinity (87%), followed by CSM (80%) and CYE (79%). These values are similar to others reported in the literature [36, 37, 38].

Cellulose produced using SM showed a lower crystallinity than cellulose produced in the others media with values of 35% (data not shown). In a similar study, Vasquez et al. [37] also observed that cane molasses cultivating medium produced BC with slightly lower crystallinity than others carbon sources. Considering the crystallinity is related to the aggregation of microfibrils, molasses should present some constituents that may have interfered with the crystallization process [39].

3.5. THERMOGRAVIMETRIC (TGA ANALYSIS)

TGA curves are shown in Figure 3. The DTG peaks found for different media tested ranged from 384 to 412 °C (Table 3).

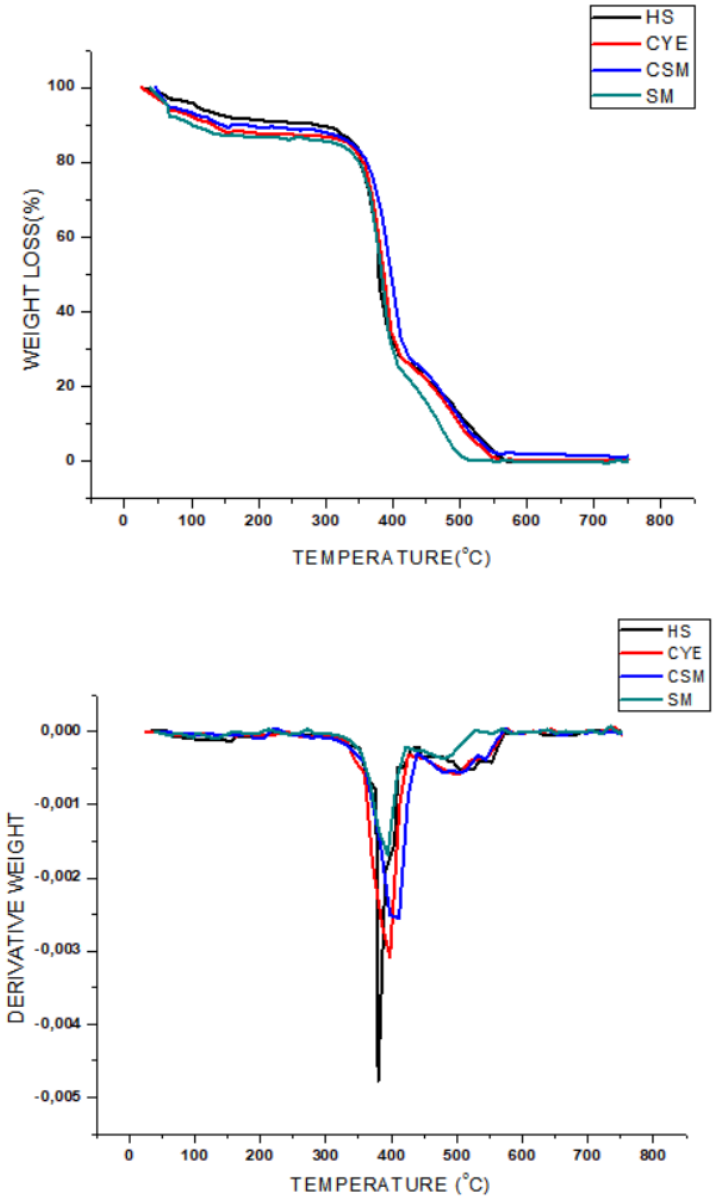

Figure 3. TGA and DTG curves from *A. xylinus* bacterial cellulose yield in different carbon source culture medium

Table 3. TGA from *A. xylinus* bacterial cellulose yield in different carbon source culture medium

medium	T onset (°C)	T endset (°C)	Peak temperature (°C)	Residual mass (%)
SM	325	422	394	0,45
CSM	330	452	412	1,40
CYE	332	439	398	0,62
HS	335	406	384	0,73

According to the literature, the degradation process of cellulose constituted by depolymerization, dehydration and decomposition reactions of the glycosidic units. This can occur in the temperature range of 250 – 400 °C [40]. The values of the DTG peaks obtained for the membranes after purification, in the different types of media tested, are in accordance with the values found in the literature [41, 31]. These observations allow us to conclude that the obtained membranes had good thermal stability and can be applied in fields where this feature is important to be considered, i.e. polymer composites for electronic devices [42].

CONCLUSION

Several agroindustry wastes have been evaluated as nutrient source for bacterial cellulose production and the search for an alternative low-cost source of carbon is still a constant challenge for scientific research. In this work, the bacterial cellulose obtained from *A. xylinus* was produced in alternatives media. The culture medium formulated with cashew apple juice and soybean molasses (CSM) was the most productive medium. This bacterial cellulose produced presented similar physical and chemical properties than cellulose produced with HS medium. Considering the results obtained, the CSM is a promising and effective low-cost medium that can be used to produce bacterial cellulose.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 3

Startup Model- **Medium Scale** Production **Plant** of Bacterial Cellulose by static cultivation using an alternative culture medium

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ABSTRACT

Bacterial cellulose (BC), due to its unique characteristics, has stood out over the years in the area of biopolymers. However, large-scale production is still expensive and it is restricted to laboratory scale. In this work, it is presented a system production by static cultivation medium scale plant for BC using cashew apple juice and soybean molasses as an alternative cultivation medium (CSM) for *Gluconacetobacter xylinus* ATCC 53582 strain. Previous study concluded that BC produced with this medium presented higher productivity and similar physicochemical characteristics than the synthetic medium HS (Hestrin & Schiram). The present study evaluated that CSM presented lower cost of production, considers aspects of productivity, plant localization, costs with equipments and raw materials, professionals required, sectors of production, flowchart production and potential buyers. Commercial production of bacterial cellulose will allow the development of new products in the biomedical, packaging and electronic area.

Keywords: Polymer; *Gluconacetobacter xylinus* ATCC 53582; cashew apple juice; soybean molasses; nanocellulose.

INTRODUCTION

Currently the commercial production of bacterial cellulose is restricted to use in beverages and dessert in Asian countries [1]. However, considering that it is a biopolymer, it can and should be extended to other areas. Besides having the advantage of being free of hemicellulose and lignin, in comparison with plant cellulose, bacterial cellulose can be produced during all seasons of the year, in any region of the world and does not require large area of cultivation [2]. However, the cost of large-scale production is still a challenge because it is costly. Researches have been studying strategies to improve productivity and reduce cost production such as designing advanced reactors, using additives in culture medium (carboxymethylcellulose, organic acids, ethanol) and agroindustrial waste as nutrient source [3].

The use of synthetic media is advantageous only on a laboratory scale. On a larger scale it is necessary to establish and optimize the use of alternative nutrient sources for the growth of *Gluconacetobacter* bacteria and consequently cellulose formation [4]. Some cultivation media using agroindustrial by-products have already been reported however, there are few cost reports of using these by-products, as well as, the required infrastructure for the commercial production of bacterial cellulose.

In this work, we present a medium scale bacterial cellulose production plant by static cultivation using cashew apple juice and soy molasses as a cultivation medium. These substrates when tested separately did not present satisfactory bacterial cellulose yield. However, when used in combination (CSM media), they have been shown to be a source of nitrogen and sugars for bacterial cellulose formation by *Gluconacetobacter xylinus* ATCC 53582 [5]. The BC produced presented higher productivity than the synthetic medium HS (Hestrin & Schramm) and without compromising chemical and physical characteristics of the membrane.

The choice of these substrates is due to the fact that Brazil has a prominent position in cashew and soybean production, but considering that many other countries are also major producers of these crops, this study can be used in other countries to

formulate this medium (CSM). Brazil is the second largest soybean producer in the world, behind only the United States [6].

Cashew cultivation is focused on cashew nut production than on fruit and juice consumption. Cashew nuts are widely used in confectionery, have high added value and are exported from Brazil to other countries. Even though cashew fruit is used in the production of juices and sweets, it is still underused [7]. Soybean molasses is a byproduct resulting from the processing of soy to obtain the protein concentrate. It has been reused in animal nutrition and more recently in the production of alcohol [8].

MATERIAL & METHODS

CSM MEDIUM AND HS MEDIUM COST PRODUCTION

The standard medium HS comprised the following (g L^{-1}): glucose - 20; peptone - 5; yeast extract -5; disodium phosphate (anhydrous) - 2,7; citric acid (monohydrate) - 1,15 [9].

The media Cashew apple juice with Soybean molasses (CSM) is prepared considering the physicochemical characteristics from soybean molasses and cashew apple juice substrates (Table 1). Cashew apple juice is filtered and diluted with distilled water to 20 g L^{-1} reducing sugars. Crude soybean molasses is added as nitrogen source in 10 g L^{-1} of protein before sterilization.

Comparing the cost production of HS medium (Table 2) and the cost production of CSM medium (Table 3) it is possible to conclude that the cost production BC of CSM medium is approximately six times cheaper than HS medium.

Table 1. Physicochemical characteristics from soybean molasses and cashew apple juice substrates

Substrate	**Total reducing sugars** ($g\ L^{-1}$)	**Protein** ($g\ L^{-1}$)	**Moisture** ($g\ 100g^{-1}$)	**Ashes** ($g\ 100\ g^{-1}$)	**Total Nitrogen** ($g\ 100\ g^{-1}$)	**Lipids** ($g\ 100\ g^{-1}$)
Soybean molasses	153 ±3.05	67±1.41	27.99±0.08	11.62±0.25	0.92±0.007	7.50±0.31
Cashew apple juice	105±2.86	NQ	87.48±0.02	0.32±0.01	0.05±0.001	0.72±0.09

NQ: Not quantified

Table 2. Average production cost of HS culture medium

HS Medium	**U$ kg^{-1} reagent**	**$g\ L^{-1}$**	**Average cost**
Glucose	8.27	20	0.17
Yeast Extract	207.06	5	1.04
Peptone	104.62	5	0.52
Citric acid	8.52	1.5	0.01
Disodium phosphate (anhydrous)	19.22	2.7	0.05
U$ L^{-1}			1.76
Productivity (g) cellulose L^{-1} (7 days)		4.03	
Production cost (U$ kg^{-1} cellulose)			**437**

Table 3. Average production cost of CSM culture medium

CSM Medium	U$ L^{-1} reagent	mL L^{-1}	g L^{-1}	Average cost
Cashew apple juice	1.29	200	NA	0.25
Soybean molasses	0.12	150	NA	0.02
U$ L^{-1}				0.27
Productivity (g) cellulose L^{-1} (7 days)			4.50	
Production cost (U$ kg^{-1} cellulose)				**60**

The amount of substrate used may vary slightly between batches due to physicochemical characteristics of the product.

COMPARISON BACTERIAL CELLULOSE PRODUCTIVITY BETWEEN OTHERS ALTERNATIVES SOURCES

All over the years different agro industrial wastes have been evaluated to produce BC. In comparison with others alternative medium, the medium CSM presented superior productivity in many cases (Table 4). None of the studies presented in table 4 considered the cost of production in publications.

Table 4. Comparison among different agroindustrial wastes medium for the BC production

Agroindustrial wastes	Additional nutrients	Maximum BC productivity	References
Cashew apple juice and soybean molasses (CSM)	No	4.50 g L^{-1}	[5]
Citrus peels	No	3.92 g 100 g^{-1} peel	[10]
Sugar cane juice and pineapple residues	No	3.24 g L^{-1}	[11]
Cashew tree exudates	yes	6.0 g L^{-1}	[7]
Citrus peel and pomace	yes	5.7 g L^{-1}	[12]
Cane molasses	No	1.0 g L^{-1}	[13]
Waste beer yeast	yes	7.0 g L^{-1}	[14]
Litchi extract	yes	2.5 g L^{-1}	[15]

PLANT LOCATION

In choosing the location of the BC production plant, preference should be given to proximity to the raw material supplying region. In Brazil, the state of Ceará, is the largest cashew producing state in the country and the major soy processing companies are located in the south center. In this case, the plant should preferably be located near the cashew producing region since the fruit has higher perishability than molasses.

GENERAL COST ASPECTS

Besides the culture media cost, it is necessary to consider the implementation cost with equipments and materials used during the process. The plant production of BC should comprise areas as: Raw material storage room, Raw material analysis and media preparation room, Cleaning membrane and sterilization room, Inoculation and cultivation room, Expedition room, Locker room, Office and Bathroom. In some of these areas, it is necessary to equip properly. Consulting three different suppliers it was possible to estimate the average cost of the main appliances required in each room (Table 5). The total estimated cost with equipment and material was calculated in U$ 21.000. In this study, a cost analysis did not consider the price of freight raw material transportation, nor electricity and water.

Table 5. Main equipments required for BC production

Raw material storage room		
Equipment	**Specification/volume**	**Average cost**
Horizontal Freezer	534 L	487
RAW MATERIAL ANALYSIS AND MEDIA PREPARATION ROOM		
Equipment	**specification/volume**	**Average cost**
Autoclavable trays	L=20 x P=10 x A=2	300
Glassware	-	1500
Filter paper	Qualitative	300
Reagents	-	1000
Balance	Precision 5 kg	1500
pH meter	-	500
Vacuum pump	-	500
Furniture	-	2000
CLEANING MEMBRANE AND STERILIZATION ROOM		
Equipment	**Specification/volume**	**Average cost**
Vertical Autoclave	30 L L^{-1}=540 x P= 530 x A=1300 mm	1700
INOCULATION AND CULTIVATION ROOM		
Equipment	**specification/volume**	**Average cost**
Bacteriological incubator	480 L - L=800 x P=600 x A=1000 mm	5.000
Laminar Flow	Vertical type100	4.000
Drying oven	180 L=820 x P=710x A=1,140 mm	1460
Air curtain	4.5 m	150
Germicide lamp	11 watts	75
EXPEDITION ROOM		
Equipment	**Specification/volume**	**Average cost**
Vacuum sealer	Sealing bar 28 cm	375

SUPPORT TEAM

The initially support team would be formed by microbiologist, chemist, logistics operator, administrator and marketing specialist. Spends with personal remuneration were not considered in this study.

SYSTEM OF PRODUCTION

BC production can be done in trays and the membranes produced must be marketed dried in vacuum sealed packaging or wet after sterilization (Figure 1). Cultivation time directly influences membrane thickness and therefore may vary from 3 to 10 days depending on the applicability of the membrane.

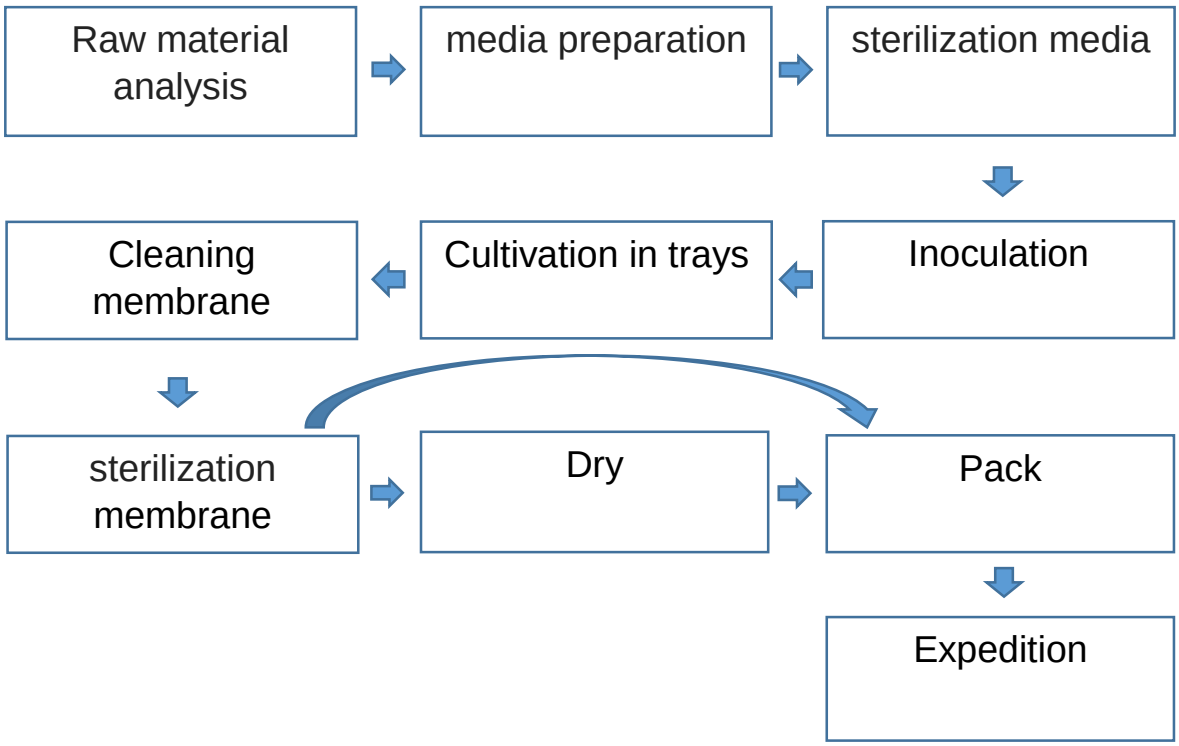

Figure 1. Flowchart of bacterial cellulose production

CONSUMER MARKET

Bacterial cellulose can be commercialized for use in native form or for products development in several areas such as: polymers (new composites), electrical sensors, biomedical (dressings and surgical materials), food industry (emulsifiers) and packaging, for studies involving nanocellulose among others.

CONCLUSIONS

The marketing of Bacterial cellulose (BC) must be expanded considering its suitable uses in several areas. There is a need to reduce the cost of culture medium for BC production. The cashew apple juice with soybean molasses medium (CSM) represents a productivity and economic alternative. The cost production BC of CSM medium is approximately six times cheaper than HS medium and the estimated cost with equipment and material was calculated in U$ 21,000. Considering that these agroindustry wastes are available in several countries, medium scale production plant of BC startup proposed in this project can be implemented in all over the world.

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Chapter 4

Bacterial cellulose production by *Acetobacter xylinus* ATCC 53582 under agitated culture medium

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ABSTRACT

Cellulose is a versatile, biodegradable and nontoxic biopolymer that has been used in many different materials. Cellulose can be extracted from plants or be produced by bacteria using biotechnological processes. Bacterial cellulose (BC) can be synthesized extracellularly mainly by aerobic gram-negative bacteria of the genus *Acetobacter.* Depending on culture conditions, BC is produced as a biofilm (static culture) or sphere (agitated culture). However, the parameters for production cellulose in spherical shape have not yet established precisely. In this work, BC production by *Acetobacter xylinus* ATCC 53582 under agitated culture in HS medium standard was studied. Parameters such as inoculum volume, inoculum age, type of agitation (orbital and stirrer), cultivation days and the use of additives (celluclast enzyme 0,1% and ethanol 1%) were evaluated. Considering that medium cost is an important aspect and that our group successfully produced BC membranes using alternative media, the best conditions determined under agitated culture for HS medium were repeated with media containing cashew apple juice and soybean molasses. The BC productivity in HS medium was superior than obtained in alternative media.

Keywords: Bacterial cellulose, nanocomposites, sphere cellulose, agitated culture

INTRODUCTION

Bacterial Cellulose (BC) was first reported in 1886 by Brown, while he was studying acetic fermentation and observed a white gelatinous pellicle on the surface of a liquid medium [1]. In the last decades BC has received increasing attention because of its exceptional features as a polysaccharide. Such features make BC suitable for technological applications in different areas such as biomaterials, nanotechnology, pharmaceuticals, medicine, food, chemistry, packaging and paper [2,3,4]. *Acetobacter xylinus* is the most extensively used microorganism in the basic and applied studies for BC production because of its higher cellulose productivity and capability to consume different sources of carbon [5,6].

There are two methods to produce bacterial cellulose: static culture, which results in a gelatin cellulose film on the surface of the medium and agitated culture, where cellulose is synthesized in the form of fibrous suspensions, pellets (sphere) or asterisk [7]. The choice of cultivation technique depends on further cellulose application. Static culture has been applied for production of *nata de coco* [8], transducer diaphragms [9], wound care dressing materials and skin substitutes [10]. Agitated culture can generate spheres of cellulose for drug delivery [11], stabilizer in food [12], adsorption of heavy metallic ions in wastewater treatment [13] and immobilization of enzymes [14]. In static culture, BC production depends on the surface area of the culture more than the volume of medium. This means that the wider the surface area, the higher the BC production, which is inappropriate for large-scale cultures. Because of this, some authors consider agitated culture more suitable for the commercial production of BC [15].

Many studies related to cellulose production by agitated culture are being carried out. However, the parameters and conditions for production cellulose in spherical shape have not yet established precisely. Culture medium, stirring speed, flask volume, inoculum volume and the bacteria are important parameters to consider [16]. Cellulose production in fermenters with continuous agitation and aeration can cause spontaneous appearance of cell- mutants (cellulose non producers), which contributes to a decline in cellulose synthesis [17].

Considering the impressive potential for a wide range of commercial applications, investigation has been conducted to produce large-scale and cost-effective commercial BC. Many studies focus on screening and genetically modifying high yield BC strains, optimizing media using agroindustrial wastes to reduce production costs, as well as using additives to improve culture media formulation, fermentation parameters and bioreactors models to improve productivity [18].

In this work, it was studied optimal parameters conditions for BC production by *Acetobacter xylinus* ATCC 53582 under agitation in standard medium. Later, *A. xylinus* was cultivated in alternative media formulated with cashew apple juice and molasses [19] to evaluate BC yield.

MATERIALS AND METHODS

MICROORGANISM AND CULTURE CONDITIONS

Acetobacter xylinus strain ATCC 53582 was acquired from the collection of tropical culture (CCT), (André Tosello Foundation), Campinas - SP, Brazil.

To stablish the best conditions of BC production, *A. xylinus* was cultivated in the standard medium HS under agitation. The parameters evaluated were inoculum volume (1%, 5%, 10%), inoculum age (48 and 72 h), orbital agitation in shaker (100 and 150 rpm) and using stirrer (360 rpm), cultivation days (3 to 7 days) and the use of additives (celluclast enzyme 0,5% and ethanol 1%) w. For the pre-culture, *A. xylinus* was inoculated from HS agar medium to tubes containing 5 mL HS broth medium. The tubes were incubated at 30 °C. After this period, the pre-cultures were mixed and used to inoculate culture medium (50 mL) in a 250 mL Erlenmeyer. Celluclast (Novozymes®) is a cellulase enzyme and it was added to liberate enwrapped cells [13]. Cellulase was added to the culture after 24hours of incubation during 1 hour under agitation and then the medium was centrifuged at 12.000g for 15 minutes (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was discarded; *A. xylinus* cells were centrifugally washed with deionized water to remove residual cellulase and then added in culture medium sterilized. Ethanol

(Sigma, America) was added to autoclaved medium during the inoculation to avoid formation of mutants' cells [20].

CULTURE MEDIA

BC production was evaluated in HS medium and alternative media formulated with Soybean Molasses, cashew apple juice with yeast extract, cashew apple juice with Soybean molasses as previously described [19]. All media were adjusted to pH 5.55 with NaOH 1 M.

Medium HS: The standard medium used in this study comprised the following (g L^{-1}): glucose 20 g; peptone – 5 g; yeast extract 5 g; disodium phosphate (anhydrous) - 2.7 g; citric acid (monohydrate) - 1.15 g [21].

Medium Soybean Molasses (SM): The molasses was hydrolyzed before used as medium. The molasses was diluted 2-fold (w/v) with H_2SO_4 2M, heated at 80 °C for 10 minutes and centrifuged at 2000 g 20 min^{-1} to separate suspended solid material. Reducing sugars in supernatant were determined by DNS method and molasses was diluted with distilled water to 20 g L^{-1} reducing sugars to final concentration.

Medium Cashew apple juice with yeast extract (CYE): Cashew apple juice was filtered through a qualitative filter paper (Unifil, Germany) to separate suspended solid material. Reducing sugars were determined by DNS method and juice was diluted with distilled water to 20 g L^{-1} reducing sugars to final concentration. Separately a solution yeast extract (10 g L^{-1}) was prepared and added during inoculation.

Medium Cashew apple juice with Soybean molasses (CSM): Cashew apple juice filtered was diluted with distilled water to 20 g L^{-1} reducing sugars. Crude soybean molasses was added as nitrogen source in 10 g L^{-1} of protein before sterilization.

BC PURIFICATION

After fermentation, the media were centrifuged (Thermo Fisher Scientific, Waltham, MA, USA) at 2000 g for 15 min^{-1}. The pH of each supernatant was measured (pH meter Ionlab PH-500B-I, Araucária, PR, Brazil) and submitted to sugars analysis by DNS method. BC produced was harvested and immersed in NaOH 0.1 M at 80 ºC for 1 h. Finally, BC were rinsed with distilled water until pH 7.0, lyophilized at −53 °C for 30 h in a freeze dryer (Liotop® Model L101 (Liotop®, São Carlos, SP, Brazil) and weighted.

STATISTICAL ANALYSIS

The statistical analysis was carried out using the GraphPad Prism version 5.01 software. The statistical significance of the evaluated data was analyzed by one-way analysis of variance (ANOVA) and the Tukey's test with significance level $\alpha = 0.05$.

RESULTS AND DISCUSSION

PARAMETER DEFINITION USING HS MEDIUM

Firstly, the parameters definition considered BC qualitative analysis because in many cases the bacteria grew, caused medium turbidity, but did not produce bacterial cellulose. The experiments were done in triplicate and repeated. Considering this, the inoculum volume was defined as 5% (v/v), inoculum age as 72 hours and cultivation days until third day because BC was disintegrated during prolonged cultivation and caused turbidity in medium.

BC PRODUCTIVITY

In the conditions tested, the use of Celluclast did not contribute to BC formation and the turbidity in medium increased. Czaja et al. (2004) used Celluclast in agitated culture and successfully obtained sphere bacterial cellulose synthetized by *Acetobacter xylinum* NQ5 (ATCC 53582) [22].

In the present study it was possible to obtain spheres of cellulose in HS medium and asteristics/irregular forms of cellulose in CSM, CYE and SM alternatives media under orbital agitation (Figure 1). The addition of ethanol in cultures submitted to orbital shaker showed no difference in cellulose shape but improved BC productivity in HS and CSM medium (Table 1). Under agitated culture *Gluconacetobacter hansenii* PJK (KCTC 10505BP) improved BC productivity to 2,31 g/L in 5 days when the etanol was added in medium [23].

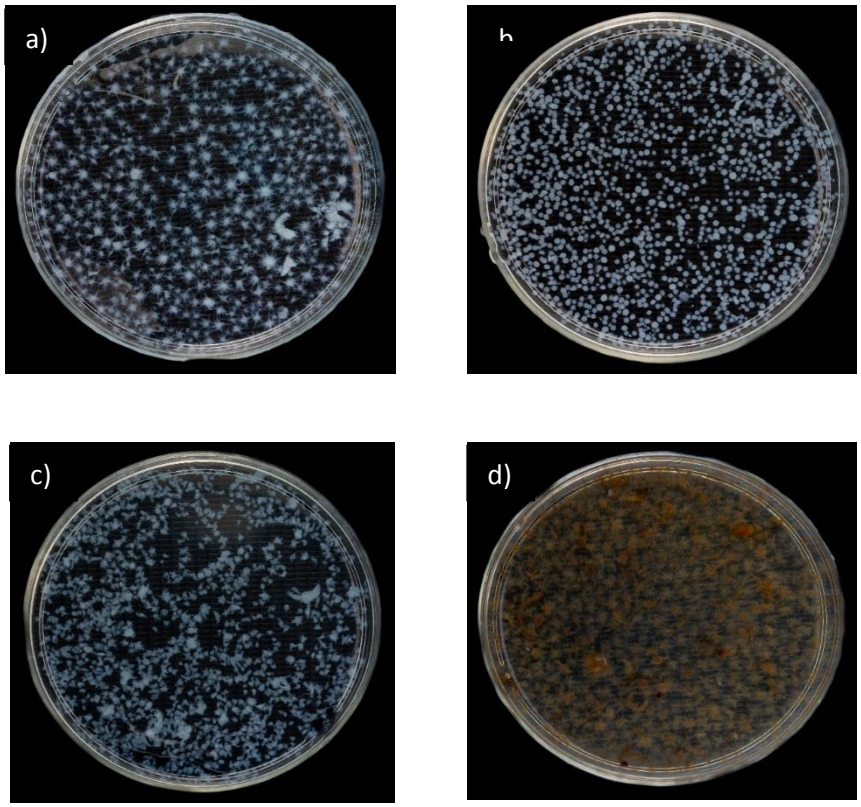

Figure 1: BC formed in different media under orbital agitation and without additives a) CYE medium b) HS medium c) SM medium d) CSM medium

Table 1. BC productivity and cellulose shape in media evaluated under orbital agitation

Media	cellulose productivity ($g\ L^{-1}$)	Cellulose shape
HS	0.52^{c}	sphere
HS + ethanol 1%	0.72^{a}	sphere
CSM	0.34^{d}	asteristic
CSM + ethanol 1%	0.52^{c}	asteristic
SM	$0,32^{d}$	irregular
SM + ethanol 1%	0.30^{d}	irregular
CYE	0.61^{b}	asteristic
CYE + ethanol 1%	$0.,54^{c}$	asteristic

In agitation using stirrer, after 24 hours, it was already possible to see spheres of cellulose synthetized only in HS medium with ethanol. In HS medium without ethanol, it was formed large BC irregular forms, while in alternatives media were formed fibrous cellulose (Figure 2). The addition of ethanol improved BC productivity only in CSM medium (Table 2).

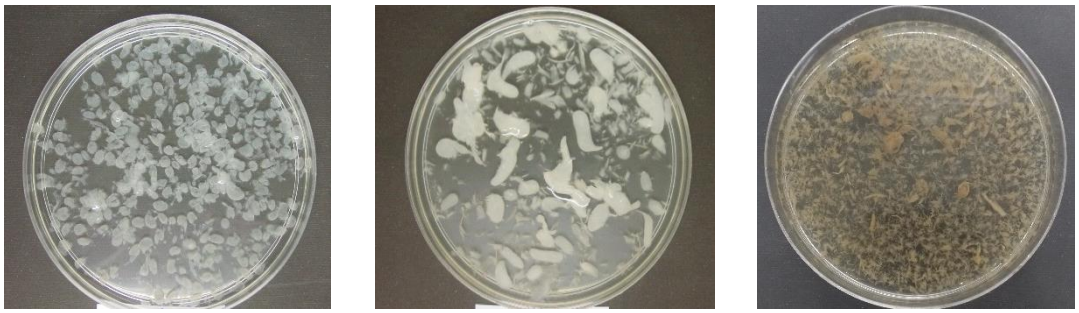

Figure 2: BC formed using stirrer a) HS medium with ethanol b) HS medium without ethanol c) CSM medium without ethanol

Table 2. BC productivity and cellulose form in media evaluated under agitation with stirrer

Media	cellulose productivity ($g\ L^{-1}$)	Cellulose form
HS	0.83^{a}	irregular
HS + ethanol 1%	0.78^{a}	spheres
CSM	0.55^{c}	fibrous
CSM + ethanol 1%	0.65^{b}	fibrous
SM	0.48^{c}	fibrous
SM + ethanol 1%	0.54^{c}	fibrous
CYE	0.51^{c}	fibrous
CYE + ethanol 1%	0.44^{c}	fibrous

BC productivity by *A. xylinus* ATCC 53582 obtained under agitated culture was lower compared with static culture [19]. More studies involving different agitation velocity and use of other additives such as carboximethilcelulose and pectin should be done.

Mohite et al. (2013) investigated the optimal fermentation conditions for BC production by *Gluconacetobacter hansenii* NCIM 2529 under shaking conditions. BC production achieved 5 g/L with 6 days of fermentation, agitation speed 170 rpm and sucrose as carbon source. Further increase in agitation speed decreased the BC production. The authors attributed this to excessive oxygen supply and changes in fluid dynamics [24].

Hu et al. (2013) studied the factors impacting the formation of sphere bacterial cellulose by *Gluconacetobacter xylinus* JCM 9730. The agitation speed was fixed in 125 rpm but the inoculum volume used to inoculate 100 mL of médium varied (1, 2, 4, 8 and 16 mL). Inoculum volume superior than 2 mL reduced the number of spheres, but formed larger spheres. The spheres diameters ranging from 5 to 10 mm in 2 mL of inoculum and 22 mm in 16 mL inoculum. Maximum BC concentration was 1,4 g/L [11].

CONCLUSIONS

In this work *Acetobacter xylinus* ATCC 53582 produced bacterial cellulose (BC) under orbital agitated culture and using stirrer. The optimal productivity was obtained

using 5% of inoculum pre cultured for 72 hours. In general, bacterial cellulose must be recovered from media until the third day of cultivation to avoid fragmentation and turbidity. The use of Celluclast did not favor BC formation. The use of ethanol stimulated the formation of sphere BC in agitation using stirrer for medium HS, but it didn't work for alternatives culture media. In both agitation modes, productivity in HS medium was superior (0,72 g/L with ethanol in orbital shaker and 0,83 g/L without ethanol in agitation with stirrer) than obtained in alternative media. More studies should be performed aiming to improve the BC productivity by *Acetobacter xylinus* ATCC 53582 under agitation.

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Chapter 5

Use of Bacterial Cellulose Incorporated with the Antimicrobial Nisin for Cheese Packaging

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Abstract

Listeriosis is a disease caused by *Listeria monocytogenes* bacteria that can cause miscarriage. It is important to control this bacterium's growth on foods, mainly dairy products and meats. Nisin is a bacteriocin widely used for this control, and it can be used in active food packaging. In this study, bacterial cellulose (BC) was produced and impregnated with nisin at four concentrations (10000 IU mL^{-1}, 5000 IU mL^{-1}, 2500 IU mL^{-1} and 1000 IU mL^{-1}) during 0, 2, 4 and 6 hours. The most efficient nisin concentration was determined on Tryptone Soy Agar (TSA). BC films with and without incorporation of nisin at 2500 IU mL^{-1} after 4 hours of exposure were used to pack Minas Frescal cheese. After 7 days of storage, the use of nisin in BC films reduced the bacterial load of *Listeria monocytogenes* by 1 log CFU g^{-1}. Bacterial cellulose demonstrated potential applicability in antimicrobial packaging films.

Keywords: Biofilm, active packaging, nisin, *Listeria*, listeriosis, food disease

1. INTRODUCTION

Cheese is widely consumed around the world. However, during storage, contamination with bacteria, mold and yeast can lead to the development of unpleasant flavors and aromas, as well as pose health threats. Therefore, cheese makers pursue ways to increase shelf life as well as the quality and safety of cheese products (Costa et al. 2018).

Antimicrobial agents can be incorporated in packaging films to extend food storage periods. Controlled release of these agents inhibits the growth of microorganisms and consequently prolongs the shelf life of packaged products (Quintavalla and Vicini 2002). The main antimicrobials tested in edible films are the bacteriocins nisin (Cleveland et al. 2001) and natamycin (Ture et al. 2011), the enzyme lysozyme (Duan et al. 2007) and various essential oils (Artiga-Artigas et al. 2017).

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria (Cleveland et al. 2001). Nisin is a bacteriocin produced by *Lactococcus lactis* (Gross and Morell 1971) and has been considered generally recognized as safe (GRAS) by the Food and Agriculture Organization (FAO) since 1988. It is widely used in several countries in products such as milk, cheese, other dairy products, canned tomatoes and other vegetables, canned soups, mayonnaise and baby foods (Müller-Auffermann et al. 2015). In Brazil, nisin is approved for use as a biopreservative in all types of cheese up to a maximum of 12.5 mg kg^{-1} (ANVISA 1996). Moreover, it has the advantage of not changing the taste of food while inhibiting *Listeria monocytogenes*, a bacterium that contaminates milk and dairy products (Santos et al. 2018).

The gram-positive bacterium *Listeria monocytogenes* causes listeriosis, disease whose clinical manifestations include miscarriage, sepsis, meningoencephalitis, gastroenteritis and fatal foodborne infection. Pregnant women, neonates, aged and debilitated patients are predominantly affected. *Listeria monocytogenes* is the only species of the genus *Listeria* that is a human pathogen (Vázquez-Boland et al. 2001).

Some studies have examined the use of nisin in milk and cheese, but nisin can also inhibit the multiplication of lactic acid bacteria, depending on the dose used in cheese manufacture, and can consequently affect the desired sensory characteristics. The use of nisin in cheese packaging is effective because it does not interfere in cheese production (Kykkidou et al. 2007).

Conventional packaging materials are mainly petroleum based, but due to environmental and sustainability issues, the use of edible films and coatings has been increasingly investigated (Fajardo et al. 2010). Among the packaging materials available, cellulose products have attracted growing interest due to their edibility, biodegradability and potential as good carriers of a wide range of antimicrobial agents (Cagri et al. 2004).

In the early 2000s, the incorporation of nisin in cellulose-based packaging films was reported (Scannell et al. 2000; Luchansky and Call, 2004). Besides its plant source, cellulose can also be produced by bacteria *Gluconacetobacter xylinus* (Brown 1886). In static culture, bacterial cellulose is synthesized as a film on the surface of the growth medium. The utility of bacterial cellulose to the producing microorganism is not clear. There are several theories, such as: retaining moisture to prevent bacteria from dehydrating; helping bacteria to become floatable in an aerobic environment; reducing the opportunity for organisms other than cellulose-producing bacteria; and protecting bacteria from the hazardous effects of UV radiation because of its opaque nature (Rajwade et al. 2015).

Bacterial cellulose membranes have unique characteristics compared to other cellulose sources, such as high purity, crystallinity, tensile strength and water retention capacity, so they have good potential for a variety of applications (Iguchi et al. 2000).

The use of adsorbed nisin in bacterial cellulose films has potential applicability in smart packaging for control of *Listeria monocytogenes* in cheese. In this work, bacterial cellulose films were produced and nisin was incorporated as antimicrobial. The film was evaluated as primary packaging for Minas Frescal cheese (uncured cheese made in the Brazilian state of Minas Gerais) aiming at controlling *Listeria monocytogenes*. To the best of our knowledge, this is the first study using bacterial cellulose incorporated with nisin for cheese packaging.

2. MATERIALS AND METHODS

2.1 PRODUCTION OF BACTERIAL CELLULOSE

HS medium (Hestrin and Schramm 1954) was inoculated (3% v/v) with *Gluconacetobacter hansenii* ATCC 23769 strain (pre-cultivated in HS broth at 28 ºC for 48 hours) and incubated (Infors HT Ecotron shaker, Bottmingen, Switzerland) statically at 28 ºC for 72 hours. Then the films were washed with distilled water, subjected to alkaline treatment with 0.5 mol L^{-1} of NaOH for 1 h at 90 ºC and then washed with distilled water to neutral pH.

Bacterial cellulose films for nisin absorption assays were produced in sterile centrifuge tubes with 5 cm diameter containing 5 mL of HS medium. Bacterial cellulose (BC) for packaging cheeses was produced in Petri dishes (15 cm) containing 35 mL of HS medium.

2.2 INCORPORATION OF NISIN BY ADSORPTION METHOD

A nisin solution of 50000 IU/mL was prepared by dissolving 0.5 g of nisin (Sigma-Aldrich - Gillingham, Dorset, UK) in 10 mL of 0.01M HCl. The solution was centrifuged at 3000 g for 15 min in a sterile centrifuge tube and the supernatant was filtered through a 0.45 µm membrane (Komitopoulou et al. 1999). This solution was diluted with 0.01M HCl to obtain other nisin concentrations: 10000 IU mL^{-1}, 5000 IU mL^{-1}, 2500 IU mL^{-1} and 1000 IU mL^{-1}. In a laminar flow cabinet (Nuaire Class 2, Plymouth, Minnesota, United States), the produced BC was immersed in nisin solutions during 0, 2, 4 and 6 hours, followed by immersion in sterile 15% glycerol solution. All BC samples were oven dried at 60 °C for 1 hour (Bunker NI1705, Piracicaba, São Paulo, Brazil).

2.3 ANTIMICROBIAL ACTIVITY ASSAY

Listeria monocytogenes ATCC 19117 strain was inoculated in brain-heart infusion broth (BHI) (Oxoid - Basingstoke, Hampishire, England), incubated for 18 hours at 30 °C. Then the culture was diluted in 0.9% NaCl at a concentration of 10^{-2} CFU mL^{-1} and 1 mL of the diluted culture was used to inoculate Tryptone Soy Agar (TSA) (Difco – Detroit, Michigan,

United States) by the pour plate method. After the medium solidified, BC membranes with different nisin concentrations were positioned over the agar surface, in the middle of the plate. The agar plates were then incubated at 30 °C for 48 h (Bunker NI1705, Piracicaba, São Paulo, Brazil) and the antimicrobial activity of the cellulose films was observed.

2.4 INOCULATION OF CHEESES AND PACKAGING

The bacterial suspensions used in this procedure were prepared from cultures of *Listeria monocytogenes* ATCC 19117. To obtain the suspensions, the microorganism was activated in BHI broth at 37 °C for 24 h. Subsequently, they were subjected to decimal dilutions in 0.1% peptone water and the inoculum concentration was adjusted with a Densimat densometer (Biomerieux, Craponne, France) to 10^{-2} and 10^{-4} CFU mL^{-1}. The number of inoculum colonies was determined by plating on TSA agar plates after incubation at 37 °C for 24 h (Bunker NI1705, Piracicaba, São Paulo, Brazil).

Minas Frescal cheese (25 g) was produced in the laboratory of Embrapa Agroindústria de Alimentos. Samples of cheese without *Listeria* inoculum were considered as controls. Inoculated cheese samples were submerged in 0.85% saline containing 10^{2} and 10^{4} CFU mL^{-1} *Listeria monocytogenes* for 20 minutes. After this, they were packaged in bacterial cellulose with or without nisin. Figure 1 shows cheese with and without bacterial cellulose. The samples were stored at 10 ºC for 1 day or 7 days, as presented in Table 1. After the incubation period, all the cheese samples were submitted to *Listeria* microbiological analysis.

A B

Figure 1: Minas Frescal cheese without bacterial cellulose (A) and with bacterial cellulose packaging (B).

Table 1: Storage conditions of cheese with or without nisin incorporated in bacterial cellulose

Cheese	Bacterial Cellulose	Inoculum	Storage days
Control	NA*	NA*	24 h
	with nisin		
	without nisin		
Inoculated	with nisin	10^2	7 days
	without nisin		
Inoculated	with nisin	10^4	7 days
	without nisin		

*NA: Not applicable. Chesse samples were not packaged

2.5 MICROBIOLOGICAL ANALYSIS

BCs of incubated cheeses were removed and transferred to 225 mL of sterile 0.1% peptone water in sterile Stomacher® bags. The cheese was manually compressed and homogenized in the solution followed by successive dilutions. An aliquot of 0.1 mL of each dilution was spread on the surface of Oxford agar (Oxoid - Basingstoke, Hampishire, England) in Petri dishes. The dishes were then incubated at 30 °C for 48 hours (Bunker NI1705, Piracicaba, São Paulo, Brazil).

2.6 STATISTICAL ANALYSIS

The statistical analysis was carried out using the GraphPad Prism version 5.01 software. The statistical significance of the evaluated data was determined by one-way analysis of variance (ANOVA) and the Tukey test with significance level $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

3.1. ANTIMICROBIAL ACTIVITY ASSAY

The antibacterial activity of the bacterial cellulose films incorporated with nisin against *L. monocytogenes* on TSA plates can be seen in Figure 2. The antimicrobial activity of bacterial cellulose films was proportional to the concentration of nisin solution. These concentrations were studied considering that below 625 IU mL^{-1}, nisin, despite presenting antimicrobial activity in synthetic media (Franklin et al. 2004; Grower et al. 2004; Singh et al. 2001), was found to be inefficient in food (Nguyen et al. 2008), and above 10000 IU mL^{-1} becomes economically prohibitive. The diffusion assay showed that the lowest dilution combined with the shortest period caused inhibition of *L. monocytogenes* of 2500 IU mL^{-1} of nisin after 4 hours. There was no inhibition zone on cellulose films containing nisin at 1000 IU mL^{-1}. Based on these results, active bacterial cellulose films for cheese packaging were prepared with exposure to 2500 IU mL^{-1} nisin for 4 h.

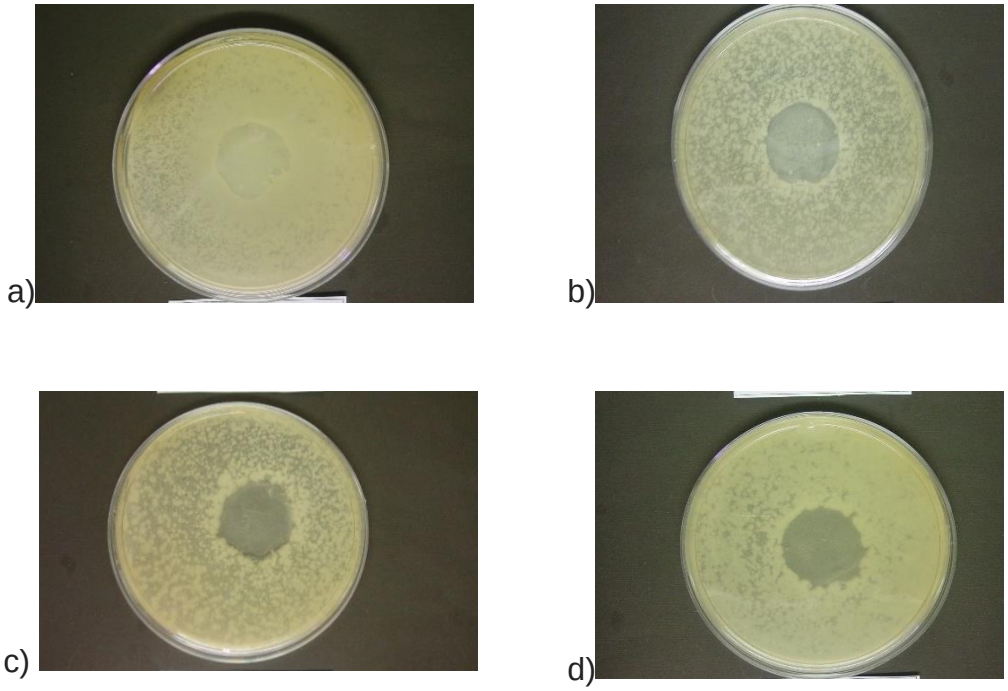

Figure 2: Antimicrobial activity of cellulose films against *L. monocytogenes* on TSA plates. Cellulose film exposed to different nisin solutions for 4 hours a) 1000 IU mL^{-1} b) 2500 IU mL^{-1} c) 5000 IU mL^{-1} d) 10000 IU mL^{-1}

3.2 MICROBIOLOGICAL ANALYSIS

In this study, the antimicrobial activity in synthetic media was enough to control *Listeria* growth in cheese, even though some previous studies have shown that nisin has stronger antimicrobial activity against *L. monocytogenes* in synthetic media than in foods (Delves-Broughton et al. 1996; Rose et al. 1999).

In all conditions, the use of BC with nisin at 2500 IU mL^{-1} as primary packaging of Minas Frescal cheese reduced the presence of *Listeria monocytogenes* when the cheese was stored under refrigeration (10 ºC). After 24 hours, the control sample with nisin presented the lowest count among the controls (5.4 log CFU g^{-1}). The control with cellulose film not containing nisin presented a higher count compared to the control without cellulose film. A similar result was reported by Nguyen et al. (2008) and the authors attributed this to the effect of the hydrated cellulose film, providing a better microenvironment for bacterial growth than the surface without cellulose.

After 7 days, cheese inoculated with 10^2 presented 6.1 – 7.1 log CFU g^{-1} and cheese inoculated with 10^4 presented 7.1 – 8.0 log CFU g^{-1}. In both cases, samples packaged in BC with nisin presented a reduction of 1 log CFU g^{-1} in comparison with BC without nisin.

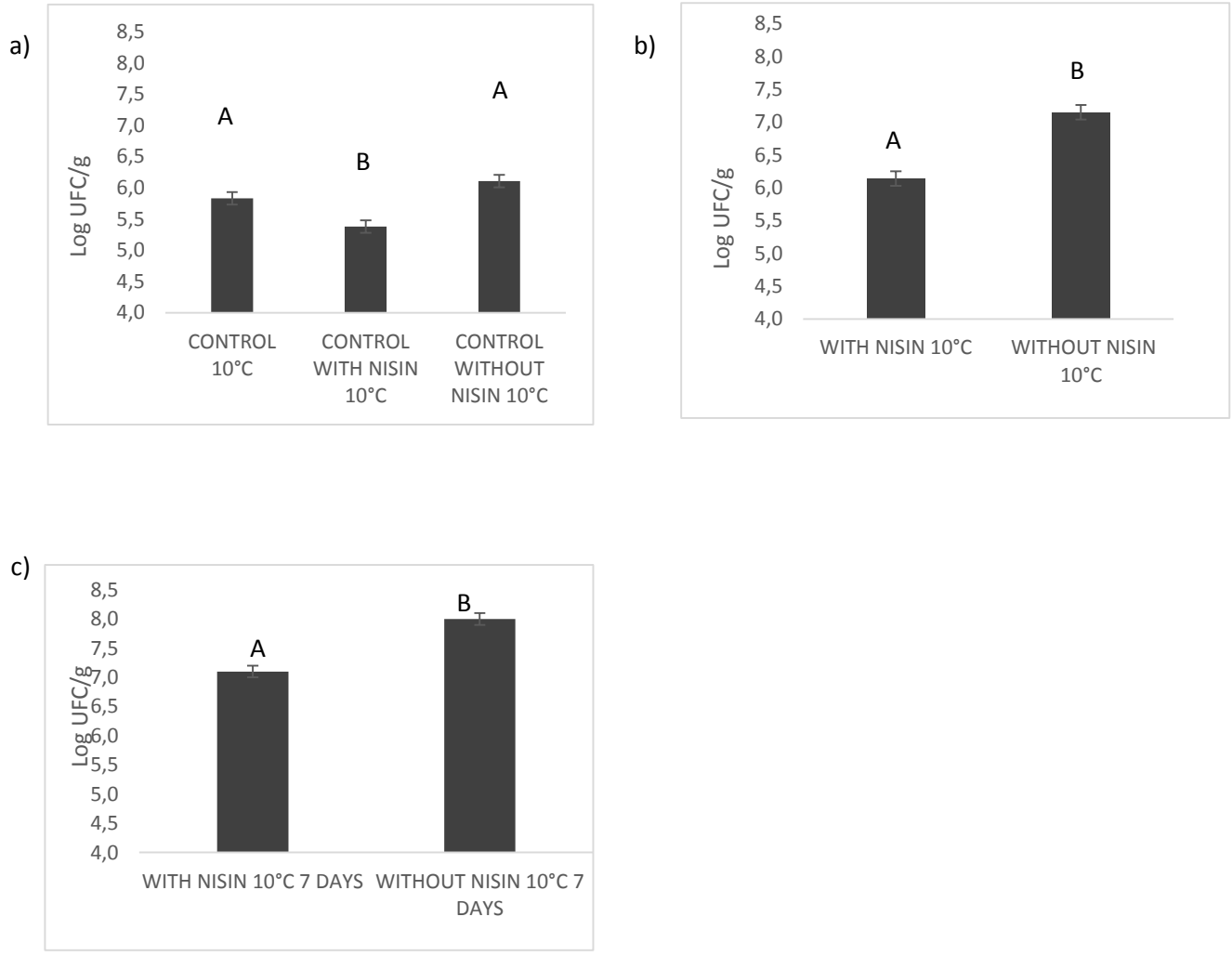

Figure 3: Numbers of *L. monocytogenes* in cheese (a) without packaging, packaging with nisin and without nisin for 24 hours (b) inoculated cheese (10^2) with nisin and without nisin after 7 days (c) inoculated cheese (10^4) with nisin and without nisin after 7 days.

Few studies have reported the use of BC as active packaging, but all of them have demonstrated satisfactory results. These studies have focused on control of

contamination in sausage. Using BC films, Zhu et al. (2010) tested ε–polylysine, Padrão et al. (2016) investigated lactoferrin, and Nguyen et al. (2008) evaluated nisin. Cellulose food packaging incorporated with nisin has been investigated with cellulose from plants (Luchansky and Call, 2004; Scannell et al. 2000) and more recently with nanocellulose composites (Salmieri et al. 2014; Weishaupt et al. 2018). The use of nanocellulose, including nanocellulose from BC, is a trend in food packaging (Souza et al. 2019; Vasconcelos et al. 2017).

CONCLUSIONS

Bacterial cellulose (BC) films were produced and incorporated with nisin at 2500 IU ml $^{-1}$ after 4 hours exposure. These films were efficient to control *Listeria monocytogenes* growth on TSA agar and cheese. The use of these films reduced *Listeria* growth by 1 log CFU g^{-1} in Minas Frescal cheese after storage for 7 days. The use of BC in active food packaging is a promising and sustainable alternative.

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CONCLUSÕES GERAIS E DESENVOLVIMENTO ADICIONAIS

A nanocelulose pode ser obtida a partir de celulose vegetal ou bacteriana (CB). Embora a celulose bacteriana seja mais pura, ainda não é produzida em larga escala. Utilizando suco de caju com melaço de soja (meio CSM), foi possível obter membrana de CB pela *Acetobacter xylinus* ATCC 53582 com alta produtividade (4.50 g L^{-1}), mesmas características físico-químicas da celulose obtidas com o meio padrão HS (4.03 g L^{-1}) e com menor custo de produção. O custo de produção de CSM foi estimado em US $ 60 kg^{-1} de celulose, enquanto o custo de produção do meio HS foi de US $ 437 kg^{-1} de celulose (7 vezes superior).

O custo da produção de celulose por litro de meio de cultura foi avaliado comparando os custos dos subprodutos agroindustriais utilizados no meio alternativo com os custos dos reagentes utilizados no meio padrão HS. Além disso, foi apresentado um projeto de *startup* para a produção estática de CB, incluindo custos de equipamentos (estimados em US $ 21.000) e fluxograma de produção.

A CB na forma de esferas apresenta várias possibilidades de aplicação. No entanto, a produção dessas esferas depende da cepa e dos parâmetros de cultivo. A cepa *Acetobacter xylinus* ATCC 53582 foi capaz de formar esferas em meio HS quando submetido a agitação orbital 150 rpm (0,72 g L^{-1}) e quando submetido a agitação em meio HS com álcool (0,83 g L^{-1}). Nos meios alternativos avaliados, houve formação de celulose na forma fibrosa, irregular e em forma de asterisco. O uso de Celluclast não favoreceu a formação de CB.

Em um estudo de caso, na CB produzida por *Gluconacetobacter hansenii* ATCC 23769 foi incorporada nisina antimicrobiana 2500 UI mL-1 e usada para embalar queijo Minas Frescal. O uso desses filmes reduziu o crescimento de *Listeria monocytogenes* em 1 log CFU g^{-1} no queijo Minas Frescal após armazenamento por 7 dias.

As embalagens de nanocelulose representam uma barreira eficiente contra o oxigênio. No entanto, embora a capacidade de absorção de água seja menor que a da celulose, essa característica ainda limita o seu uso. Assim, estudos adicionais precisam

ser direcionados a fim de melhorar esta característica. Além disso, o uso de nanomateriais ainda requer mais estudos sobre toxicidade. A colaboração entre governos se torna necessária para regulamentar os nanomateriais, incluindo nanocelulose.

GENERAL CONCLUSIONS AND FURTHER DEVELOPMETS

Nanocellulose can be obtained from plant or bacterial celulose (BC). Although bacterial cellulose is purer, it is not yet produced on large scale. Using cashew apple juice with soybean molasses (CSM medium) it was possible to obtain bacterial cellulose membrane by *Acetobacter xylinus* ATCC 53582 with high productivity (4.50 g L^{-1}), same physicochemical characteristics of cellulose obtained with the standard medium HS (4.03 g L^{-1}) and with lower production cost. The cost production of CSM was estimated in U$ 60 kg^{-1} cellulose while cost production of HS medium was U$ 437 kg^{-1} cellulose (7 times higher).

The cost of cellulose production per liter of culture medium was evaluated by comparing the costs of agro-industrial by-products used in the alternative medium with the costs of reagents used in the HS standard medium. In addition, a startup project for BC static production was presented, including equipment costs (estimated in U$ 21.000) and production flowchart.

The BC in the form of spheres presents several application possibilities. However, the production of these spheres depends on the strain and the cultivation parameters. *Acetobacter xylinus* ATCC 53582 was able to form beads in HS medium when subjected to orbital agitation 150 rpm (0.72 g L^{-1}) and when subjected to stirring in HS medium with alcohol (0.83 g L^{-1}). In the alternatives media tested, there was formation of cellulose in fibrous, irregular and asterisk shape. The use of Celluclast did not favor BC formation.

In a case study, BC produced by *Gluconacetobacter hansenii* ATCC 23769 was incorporated with the antimicrobial nisin 2500 IU mL^{-1} and used to pack Minas Frescal cheese. The use of these films reduced *Listeria monocytogenes* growth by 1 log CFU g^{-1} in Minas Frescal cheese after storage for 7 days.

Nanocellulose packaging represents an efficient barrier against oxygen. However, although the water absorption capacity is lower than that of cellulose, this feature still limits its use. Thus, further studies need to be directed in order to improve this feature. In

addition, the use of nanomaterials still requires further toxicity studies. Collaboration between governments is needed to regulate nanomaterials, including nanocellulose.