Bacterial Cellulose and Method of Fabrication Thereof

Technical Field

The present invention relates, in general terms, to bacterial cellulose and their methods of fabrication thereof.

Background

Biopolymers are natural polymers produced by the cells of living organisms. Like other polymers, biopolymers consist of monomeric units that are covalently bonded in chains to form larger molecules. There are three main classes of biopolymers, classified according to the monomers used and the structure of the biopolymer formed: polynucleotides, polypeptides, and polysaccharides. Polynucleotides, such as RNA and DNA, are long polymers of nucleotides. Polypeptides include proteins and shorter polymers of amino acids; some major examples include collagen, actin, and fibrin. Polysaccharides are linear or branched chains of sugar carbohydrates; examples include starch, cellulose, and alginate. Other examples of biopolymers include natural rubbers (polymers of isoprene), suberin and lignin (complex polyphenolic polymers), cutin and cutan (complex polymers of long-chain fatty acids), melanin, and polyhydroxyalkanoates (PHAs).

Bacterial cellulose (BC) is a biopolymer with a wide range of applications across various industries due to its unique properties, including its high purity, mechanical strength, biocompatibility, and ability to retain large amounts of water. Their main applications are in the food industry, medical field, and materials science. For instance, BC can be used to create edible films and coatings for fruits, vegetables, and other food products, extending their shelf life and preserving freshness by providing desired oxygen and moisture barrier properties. Regarding medical applications, BC can be used in wound care products, including dressings and bandages, due to its excellent moisture retention, biocompatibility, and transparency, which allows for easy monitoring of wounds. As material, BC fibers can be used to produce sustainable textiles and clothing items with unique properties such as moisture-wicking, breathability, and biodegradability. Additionally, BC can be combined with other biomaterials to create nanocomposites with improved properties for various applications.

BC can be produced by certain strains of bacteria, including gram-negative bacteria of the genera Komagataeibacter (Gluconacetobacter), Agrobacterium, Achromobacter, Aerobacter, Alcaligenes, Rhizobium, Pseudomonas, and Salmonella, as well as gram-positive bacteria Sarcina and Leifsonia. Commercial production of BC involves cultivating specific strains of bacteria in a controlled fermentation process. The specific details of BC production can vary depending on the bacterial strain used, the intended application of the BC, and the equipment and facilities available.

Despite of all advantages of bacterial cellulose in many applications, its manufacture remains relatively expensive, mainly due to high cost of culture media and low productivity of the known strains. Therefore, low-cost feedstocks and biowastes are in demand to bring down the production cost of bacterial cellulose. Those feedstocks can be agricultural residues (molasses and pre-treated wheat straw), food industry by-products (fruit peels) as well as municipal organic waste and paper mill sludge. Furthermore, there is awareness that brewery and bioethanol production result in substantial amounts of spent grain and spent yeast (approximately 36.4 million tons annually), posing considerable challenges in waste management. This situation can be both costly and environmentally troublesome. These by-products could be a suitable carbon source for certain BC-producing bacteria. For instance, it was reported the production of bacterial cellulose by Gluconacetobacter hansenii CGMCC 3917 using only waste beer yeast as nutrient source. However, in the lab study above, waste beer yeast had to go through both harsh chemical and thermal treatments, thus, bringing up the operational cost and limiting the commercialization of the process.

It would be desirable to overcome or ameliorate at least one of the above-described problems.

Summary

The present disclosure concerns a method of producing bacterial cellulose, comprising:
incubating a starter culture of about 20L to about 50L with a production medium of at least about 200L in order to form a culture for forming the bacterial cellulose;
wherein the starter culture comprises at least one Komagataeibacter strain selected from Komagataeibacter nataicola, Komagataeibacter rhaeticus, Komagataeibacter

xylinus, and Komagataeibacter intermedius; and
wherein the production medium comprises a spent yeast liquor which is not treated thermally or chemically.

In some embodiments, the starter culture further comprises a Komagataeibacter (Gluconacetobacter) strain selected from G. sucrofermentans, G. hansenii, G. europaeus, G. entanii, G. saccharivorans, G. swingsii, G. oboediens, G. liquefaciens, G. sacchari, G. diazotrophicus, G. johannae, G. azotocaptans, G. medellinensis, or a combination thereof.

In some embodiments, the spent yeast liquor is obtained from a brewery and/or bioethanol fermentation process.

In some embodiments, the production medium comprises:

a) sugar at about 1 % w/v to about 18 % w/v relative to the production medium;

b) spent yeast liquor at about 2 % w/v to about 12 % w/v relative to the production medium; and

c) disodium phosphate (Na_2HPO_4) at about 0.1 % w/v to about 1 % w/v relative to the production medium.

In some embodiments, the production medium further comprises spent grain filtrate at about 5 % w/v to about 50 % w/v relative to the production medium.

In some embodiments, the spent grain filtrate is formed by suspending a spent grain in an aqueous medium at a ratio of about 1:3 to form a sludge and filtering the sludge.

In some embodiments, the spent grain is obtained from a brewery and/or a bioethanol fermentation process.

In some embodiments, the production medium further comprises citric acid at about 0.05 % w/v to about 1 % w/v relative to the production medium.

In some embodiments, the production medium is characterised by a pH of about 4 to about 6, or a Brix value of about 3 to about 6.

In some embodiments, the step of incubating the culture is performed at about 20 °C to about 40 °C. In some embodiments, the step of incubating the culture is performed at about 30 °C.

In some embodiments, the step of incubating the culture is performed under static conditions. In some embodiments, the step of incubating the culture is performed without agitation of the starter culture.

In some embodiments, the step of incubating the culture is performed for about 2 days to about 20 days. In some embodiments, the step of incubating the culture is performed for about 4 days to about 7 days.

In some embodiments, the step of incubating the culture is performed in a fermentation tray with a volume of about 1 L to about 2 L.

In some embodiments, the starter culture is added to the production medium about 5 % v/v to about 40 % v/v relative to the culture medium.

In some embodiments, the method further comprises a step of culturing a seed inoculum in a propagation medium and/or an activation medium in order to form the starter culture. In some embodiments, the seed inoculum comprises a pellicle formed from at least one Komagataeibacter strain.

In some embodiments, the seed inoculum is added to the propagation medium and/or activation medium at about 10 % v/v to about 30 % v/v relative to the starter culture. In some embodiments, the seed inoculum is added to the propagation medium and/or activation medium at about 20 % v/v relative to the starter culture.

In some embodiments, the step of forming the starter culture is performed at about 20 °C to about 40 °C. In some embodiments, the step of forming the starter culture is performed at about 30 °C.

In some embodiments, the step of forming the starter culture is performed under static conditions. In some embodiments, the step of forming the starter culture is performed without agitation of the starter culture.

In some embodiments, the step of forming the starter culture is performed for about 2 days to about 20 days. In some embodiments, the step of forming the starter culture is performed for about 4 days to about 7 days.

In some embodiments, the propagation medium comprises:

a) sugar at about 3 % w/v to about 20 % w/v relative to the propagation medium; and

b) spent yeast liquor at about 2 % w/v to about 12 % w/v relative to the propagation medium.

In some embodiments, the propagation medium further comprises citric acid at about 0.05 % w/v to about 1 % w/v relative to the propagation medium.

In some embodiments, the propagation medium is characterised by a pH of about 4 to about 6.

In some embodiments, the step of forming the seed inoculum is performed at about 20 °C to about 40 °C. In some embodiments, the step of forming the seed inoculum is performed at about 30 °C.

In some embodiments, the step of forming the seed inoculum is performed under static conditions.

In some embodiments, the step of forming the seed inoculum is performed for about 2 days to about 10 days. In some embodiments, the step of forming the seed inoculum is performed for about 4 days to about 7 days.

In some embodiments, the method further comprises a step of activating the at least one Komagataeibacter strain in an activation medium in order to form the seed inoculum.

In some embodiments, the activation medium comprises:

a) sugar at about 1 % w/v to about 20 % w/v relative to the activation medium; and

b) yeast extract powder at about 0.1 % w/v to about 2 % w/v relative to the activation medium.

In some embodiments, the activation medium further comprises citric acid at about 0.05 % w/v to about 1 % w/v relative to the activation medium.

In some embodiments, the activation medium is characterised by a pH of about 4 to about 6.

In some embodiments, the step of incubating the culture comprises forming a bacterial cellulose membrane in order to form the bacterial cellulose.

In some embodiments, the method further comprises a step of harvesting the bacterial cellulose membrane when a pH of the culture is about pH 2 to about pH 3.

In some embodiments, the method further comprises a step of washing the bacterial cellulose membrane and compressing the bacterial cellulose membrane.

In some embodiments, after the bacterial cellulose is harvested, residual media from the method are recycled in order to produce another batch of bacterial cellulose.

In some embodiments, the method is characterised by a bacterial cellulose yield of more than about 80%.

The present disclosure concerns a bacterial cellulose formed using the method as disclosed herein.

In some embodiments, the bacterial cellulose is characterised by a thickness of about 1 cm to about 3 cm.

In some embodiments, the bacterial cellulose is characterised by a tensile strength of about 100 MPa to about 200 MPa.

In some embodiments, the bacterial cellulose is characterised by an elongation at break value of about 20 % to about 50%.

In some embodiments, the bacterial cellulose is formable as a foam, hydrogel, aerogel, cardboard sheet, biofilm, film, sheet, or membrane.

Brief description of the drawings

Embodiments of the present invention will now be described, by way of non-limiting example, with reference to the drawings in which:

Figure 1 shows a flow-process diagram for the production of bacterial cellulose using spent grain and spent yeast.
Figure 2 shows bioreactor system for continuous operation of BC production.
Figure 3 shows stacked trays for static fermentation.
Figure 4 shows the production of bacterial cellulose (opaque membrane) when growing in production medium Buyo2, containing both spent grain filtrate and spent yeast liquor.

Detailed description

Large quantities of spent grain and spent yeast are generated during brewery and bioethanol production, leading to significant waste management challenges, which can expensive and environmentally problematic. It is estimated that, regarding brewer's spent grain alone, approximately 36.4 million tons are generated annually. Without effective strategies for reusing these by-products, valuable resources including nutrients, carbon sources, and potential energy sources encapsulated in spent grain and spent yeast may go to waste. For instance, brewer's spent grain is rich in hemicellulose, cellulose, proteins, lignin, oil and phenolic compounds while brewer's spent yeast is a source of protein, minerals, vitamins and saccharides. Traditional disposal methods, such as using by-products as animal feed, have limitations. Exploring more diverse and sustainable applications for these by-products requires innovative solutions and technologies.

Further, bacterial cellulose is a high-value biomaterial with a wide range of applications, yet it is challenging to mass-produce on an industrial scale due to the need for expensive carbon-rich virgin feedstock and the absence of an optimized and efficient process for large-scale production. By providing a more effective and sustainable approach to the

utilization of brewery and bioethanol by-products on an industrial scale using specialized bacterial species and their combinations, at least one of these challenges may be addressed.

The present disclosure predicated on the understanding that when tested at laboratory scales, to form bacterial cellulose, the brewery wastes as mentioned above have to be chemically or thermally pre-treated, either via a two-step process or a one-step process. Furthermore, the production of bacterial cellulose was conducted only in small volumes (50 mL to 110 mL), only one strain was used in each study, and no wastewater recycling was reported. This demonstrates that these lab-scale processes are not linearly scalable, and optimising the processes for industrial scale production is challenging.

The present disclosure concerns a process of using a specific bacterium or bacterial mixtures for the efficient utilization of by-products including spent grain and spent yeast from brewery or bioethanol production. The present disclosure further concerns applying these specialized microorganisms to transform the aforementioned by-products into bacterial cellulose at a large scale. For example, the method comprises steps such as starter culture preparation, by-product loading, medium concentration and composition, as well as other fermentation parameters and techniques. This comprehensive approach is designed to enhance the yield of bacterial cellulose production at an industrial scale, contributing to a more sustainable and resource-efficient utilization of brewery and bioethanol by-products.

Biopolymers comprises monomeric units made from monomers. Biopolymers often have a well-defined structure, though this is not a defining characteristic (for example lignocellulose). The exact chemical composition and the sequence in which these units are arranged is called the primary structure, in the case of proteins. Many biopolymers spontaneously fold into characteristic compact shapes (secondary structure and tertiary structure), which determine their biological functions and depend in a complicated way on their primary structures. In contrast, synthetic polymers have much simpler and more random (or stochastic) structures. This leads to a molecular mass distribution that is missing in biopolymers. In fact, as their synthesis is controlled by a template-directed process in most in vivo systems, biopolymers of a type (say one specific protein) are all alike: they all contain similar sequences and numbers of monomers and thus all have

the same mass. This monodispersity is in contrast to the polydispersity encountered in synthetic polymers. As a result, biopolymers often have a dispersity of 1.

"Biopolymers" is also contrasted from "biobased polymers". Biobased polymers are polymers chemically or biologically synthesized (fully or partially) from biomass monomers, such as polyesters (e.g., polyhydroxyalkanoates (PHAs) and polylactic acid (PLA)). In this regard, the only polymers that can be regarded as both biopolymers and biobased polymers are those that are biologically produced (by microbes) from biomass carbon sources (e.g., sugars and lipids), and examples of these include PHAs, bacterial cellulose, gellan gum, xanthan gum, and curdlan.

Polysaccharides (sugar polymers) can be linear or branched and are typically joined with glycosidic bonds. The exact placement of the linkage can vary, and the orientation of the linking functional groups is also important, resulting in α- and β-glycosidic bonds with numbering definitive of the linking carbons' location in the ring. In addition, many saccharide units can undergo various chemical modifications, such as amination, and can even form parts of other molecules, such as glycoproteins.

Cellulose is structured with stacked chains that result in stability and strength. The strength and stability comes from the straighter shape of cellulose caused by glucose monomers joined together by glycosydic bonds. The straight shape allows the molecules to pack closely. Cellulose is used vastly in the form of nano-fibrils called nano-cellulose. Nano-cellulose presented at low concentrations produces a transparent gel material. This material can be used for biodegradable, homogeneous, dense films that are very useful in the biomedical field.

Accordingly, the present disclosure concerns a method of producing bacterial cellulose, comprising:

incubating a starter culture with a production medium in order to form a culture for forming the bacterial cellulose ;

wherein the starter culture comprises at least one Komagataeibacter strain selected from Komagataeibacter nataicola, Komagataeibacter rhaeticus, Komagataeibacter xylinus, and Komagataeibacter intermedius; and

wherein the production medium comprises a spent yeast liquor which is not treated thermally or chemically.

In some embodiments, the method of producing bacterial cellulose comprises:
incubating a starter culture of about 20L to about 50L with a production medium of at least about 200L in order to form a culture for forming the bacterial cellulose ;
wherein the starter culture comprises at least one Komagataeibacter strain selected from Komagataeibacter nataicola, Komagataeibacter rhaeticus, Komagataeibacter xylinus, and Komagataeibacter intermedius; and
wherein the production medium comprises a spent yeast liquor which is not treated thermally or chemically.

Pretreatment of spent grain and spent yeast are used in order to maximise the nutritional value obtainable. For example, pretreatment using sulphuric acid or phosphoric acid is recommended in order to break down proteins and lignocellulosic material so that microorganism may efficiently utilise them. The inventors have found that such a pretreatment step is not necessary when fermenting with specialized bacteria, and may further be advantageous. As there is no need to conduct pre-treatment, either chemically or thermally, cost-effectiveness is enhanced, environmental impact reduced, and the process simplified. In particular, eliminating the need for these chemical and thermal pre-treatments reduces operational costs, making the overall utilization process more economically viable.

It was found that Komagataeibacter nataicola, Komagataeibacter rhaeticus, Komagataeibacter xylinus, and Komagataeibacter intermedius show high adaptation with the media containing spent yeast liquor and spent grain filtrate. In some embodiments, the starter culture comprises *Komagataeibacter rhaeticus* and *Komagataeibacter xylinus*. In some embodiments, the starter culture comprises Komagataeibacter nataicola, Komagataeibacter rhaeticus, and Komagataeibacter xylinus. In some embodiments, the starter culture comprises Komagataeibacter rhaeticus, Komagataeibacter xylinus, and Komagataeibacter intermedius. In some embodiments, the starter culture comprises Komagataeibacter nataicola, Komagataeibacter rhaeticus, Komagataeibacter xylinus and Komagataeibacter intermedius.

Additionally, the presence of other Komagataeibacter strains in combination with at least one of the above mentioned 4 strains does not negatively impact the method. In

some embodiments, the starter culture comprises a bacterium selected from *Komagataeibacter nataicola*, *Komagataeibacter rhaeticus*, *Komagataeibacter xylinus*, *Komagataeibacter intermedius*, Komagataeibacter (Gluconacetobacter) strains such as G. sucrofermentans, G. hansenii, G. europaeus, G. entanii, G. saccharivorans, G. swingsii, G. oboediens, G. liquefaciens, G. sacchari, G. diazotrophicus, G. johannae, and G. azotocaptans, G. medellinensis, or a combination thereof. In some embodiments, the starter culture further comprises a Komagataeibacter (Gluconacetobacter) strain selected from G. sucrofermentans, G. hansenii, G. europaeus, G. entanii, G. saccharivorans, G. swingsii, G. oboediens, G. liquefaciens, G. sacchari, G. diazotrophicus, G. johannae, G. azotocaptans, G. medellinensis, or a combination thereof.

A combination of bacterial strains allows the bacterial consortium to efficiently utilize a broader range of nutrients, particularly from complex substrates as spent grain and spent yeast. The usage of bacterial mixtures also increases robustness and resilience to variations in fermentation conditions.

In some embodiments, the production medium comprises:

a) sugar at about 1 % w/v to about 18 % w/v relative to the production medium;

b) spent yeast liquor at about 2 % w/v to about 12 % w/v relative to the production medium; and

c) disodium phosphate (Na_2HPO_4) at about 0.1 % w/v to about 1 % w/v relative to the production medium.

"Saccharide" or 'sugar" includes within its scope monosaccharide, disaccharide, oligosaccharide, polysaccharide, and also includes their isomers thereof. Monosaccharides, also called simple sugars, are the simplest forms of sugar and the most basic units (monomers) from which all carbohydrates are built. Examples of monosaccharides include glucose (dextrose), fructose (levulose), and galactose. Monosaccharides are the building blocks of disaccharides (such as sucrose and lactose) and polysaccharides (such as cellulose and starch). A disaccharide is the sugar formed when two monosaccharides are joined by a glycosidic linkage. Like monosaccharides, disaccharides are simple sugars soluble in water. Three common examples are sucrose, lactose, and maltose. An oligosaccharide is a saccharide polymer containing a small number (typically three to ten) of monosaccharides. Polysaccharides are long-chain

polymeric carbohydrates composed of monosaccharide units bound together by glycosidic linkages. This carbohydrate can react with water (hydrolysis) using enzymes as catalysts (such as amylase, cellulase, chitinase, etc.), which produces constituent sugars (monosaccharides, or oligosaccharides). They range in structure from linear to highly branched. Examples include storage polysaccharides such as starch, glycogen and galactogen and structural polysaccharides such as cellulose and chitin.

In some embodiments, the sugar is selected from glucose, galactose, fructose, xylose, sucrose, lactose, maltose, trehalose, sorbitol, mannitol, maltodextrin, raffinose, stachyose, fructo-oligosaccharide, amylose, amylopectin, modified starch, glycogen, dextran, chitosan, glycosaminoglycans, alginate, ulvan, gum Arabic, gellan gum, cellulose, hemicellulose, ethylcellulose, methylcellulose, pectin, hydrocolloid and a combination thereof. In some embodiments, the sugar is sucrose.

In some embodiments, the sugar is about 1 % w/v to about 18 % w/v relative to the production medium, about 1 % w/v to about 16 % w/v, about 1 % w/v to about 14 % w/v, about 1 % w/v to about 12 % w/v, about 1 % w/v to about 10 % w/v, about 1 % w/v to about 9 % w/v, about 1 % w/v to about 8 % w/v, about 1 % w/v to about 7 % w/v, about 1 % w/v to about 6 % w/v, about 1 % w/v to about 5 % w/v, about 1 % w/v to about 4 % w/v, about 2 % w/v to about 4 % w/v, or about 3 % w/v to about 4 % w/v.

Spent yeast liquor refers to a supernatant of a spent yeast mixture after sedimentation of the yeast. In this regard, while the liquor may also be obtained by centrifuging spent yeast, this is not necessary. In some embodiments, the spent yeast liquor is obtained from a brewery and/or bioethanol fermentation process. The spent yeast liquor may be utilised directly from a brewery and/or bioethanol fermentation process; i.e. without alterations. Spent yeast liquor may comprise proteins, minerals, vitamins, nitrogen, and enzymes.

In some embodiments, the spent yeast liquor has the following characters: total organic carbon is about 8 % w/v to about 15 % w/v, total nitrogen content is about 0.5 % w/v to about 1.5 % w/v, total protein content is about 4 % w/v to about 10 % w/v, carbohydrate content is about 3 % w/v to about 5 % w/v, total fat content is about 0.1 % w/v to about 0.5 % w/v, and ash content is about 1 % w/v to about 2 % w/v. In

some embodiments, the spent yeast liquor has the following characters: total organic carbon is about 13.8 % w/v, total nitrogen is about 1.09 % w/v, total protein content is about 6.8 % w/v, carbohydrate content is about 4.35 % w/v, total fat content is about 0.39 % w/v, and ash content is about 1.26 % w/v.

In some embodiments, the Brix value of the spent yeast liquor is about 7 to about 14, potential alcohol is about 4 to about 7.0, humidity is about 65 % to about 90 % and pH is about 4.5 to 6.5. In some embodiments, the Brix value of the spent yeast liquor is 8.7, the potential alcohol is about 4.8, humidity is about 86.6 %, and pH value is pH 5.9.

In some embodiments, the spent yeast liquor is not thermally or chemically pre-treated. This means that that the spent yeast liquor is not subjected to heat treatment nor chemicals are added.

In some embodiments, the spent yeast liquor is about 2 % w/v to about 12 % w/v relative to the production medium, about 2 % w/v to about 10 % w/v, about 2 % w/v to about 9.5 % w/v, about 2 % w/v to about 9 % w/v, about 2 % w/v to about 8.5 % w/v, about 2 % w/v to about 8 % w/v, about 2 % w/v to about 7.5 % w/v, about 2 % w/v to about 7 % w/v, about 2 % w/v to about 6.5 % w/v, about 2 % w/v to about 6 % w/v, about 2 % w/v to about 5.5 % w/v, about 2 % w/v to about 5 % w/v, about 2.5 % w/v to about 5 % w/v, about 3 % w/v to about 5 % w/v, about 3.5 % w/v to about 5 % w/v, or about 4 % w/v to about 5 % w/v.

In some embodiments, the disodium phosphate (Na_2HPO_4) is about 0.1 % w/v to about 1 % w/v relative to the production medium. In other embodiments, the concentration is about 0.1 % w/v to about 0.9 % w/v, about 0.1 % w/v to about 0.8 % w/v, about 0.1 % w/v to about 0.7 % w/v, about 0.1 % w/v to about 0.6 % w/v, about 0.1 % w/v to about 0.5 % w/v, about 0.2 % w/v to about 0.5 % w/v, about 0.3 % w/v to about 0.5 % w/v, or about 0.4 % w/v to about 0.5 % w/v.

Spent grain filtrate refers to a filtrate obtained from spent grain. Spent grain filtrate may comprise organic carbon, nitrogen, proteins, carbohydrates and other components.

In some embodiments, the spent grain filtrate has the following characters: total organic

carbon is about 10 % w/v to about 30 % w/v, total nitrogen content is about 0.5 % w/v to about 1.5 % w/v, total protein content is about 4 % w/v to about 10 % w/v, carbohydrate content is about 5 % w/v to about 15 % w/v, total fat content is about 0.1 % w/v to about 1 % w/v, and ash content is about 0.5 % w/v to about 2 % w/v. In some embodiments, the spent grain has the following characters: total organic carbon is about 20.6 % w/v, total nitrogen is about 1.1 % w/v, total protein content is about 6.7 % w/v, carbohydrate content is about 10 % w/v, total fat content is about 0.6 % w/v, and ash content is about 0.7 % w/v.

In some embodiments, the production medium further comprises spent grain filtrate at about 5 % w/v to about 50 % w/v relative to the production medium. In some embodiments, the spent grain filtrate is about 5 % w/v to about 45 % w/v, about 5 % w/v to about 40 % w/v, about 5 % w/v to about 35 % w/v, about 5 % w/v to about 30 % w/v, about 10 % w/v to about 30 % w/v, about 10 % w/v to about 28 % w/v, about 10 % w/v to about 26 % w/v, about 10 % w/v to about 24 % w/v, about 10 % w/v to about 22 % w/v, about 10 % w/v to about 20 % w/v, about 10 % w/v to about 18 % w/v, about 10 % w/v to about 16 % w/v, about 12 % w/v to about 16 % w/v, or about 14 % w/v to about 16 % w/v.

In some embodiments, the spent grain filtrate is not thermally or chemically pre-treated. This means that that the spent grain filtrate is not subjected to heat treatment nor other chemicals are added.

In some embodiments, the spent grain filtrate is formed by suspending a spent grain in an aqueous medium at a ratio of about 1:3 to form a sludge and filtering the sludge. The resulting filtrate is the spent grain filtrate. In some embodiments, the spent grain is suspended in an aqueous medium such that the average moisture content of the spent grain is about 70% to about 90%, about 70% to about 85%, about 70% to about 80%, or about 70% to about 75%.

The term 'aqueous medium' used herein refers to a water based solvent or solvent system, and which comprises of mainly water. Such solvents can be either polar or non-polar, and/or either protic or aprotic. Solvent systems refer to combinations of solvents

which resulting in a final single phase. Both 'solvents' and 'solvent systems' can include, and is not limited to, pentane, cyclopentane, hexane, cyclohexane, benzene, toluene, dioxane, chloroform, diethylether, dichloromethane, tetrahydrofuran, ethyl acetate, acetone, dimethylformamide, acetonitrile, dimethyl sulfoxide, nitromethane, propylene carbonate, formic acid, butanol, isopropanol, propanol, ethanol, methanol, acetic acid, ethylene glycol, diethylene glycol or water. Water based solvent or solvent systems can also include dissolved ions, salts and molecules such as amino acids, proteins, sugars and phospholipids. Such salts may be, but not limited to, sodium chloride, potassium chloride, ammonium acetate, magnesium acetate, magnesium chloride, magnesium sulfate, potassium acetate, potassium chloride, sodium acetate, sodium citrate, zinc chloride, HEPES sodium, calcium chloride, ferric nitrate, sodium bicarbonate, potassium phosphate and sodium phosphate. As such, biological fluids, physiological solutions and culture medium also falls within this definition.

In some embodiments, the spent grain is obtained from a brewery and/or a bioethanol fermentation process.

In some embodiments, the production medium further comprises citric acid at about 0.05 % w/v to about 1 % w/v relative to the production medium. In other embodiments, the concentration is about 0.05 % w/v to about 0.9 % w/v, about 0.05 % w/v to about 0.8 % w/v, about 0.05 % w/v to about 0.7 % w/v, about 0.05 % w/v to about 0.6 % w/v, about 0.05 % w/v to about 0.5 % w/v, about 0.05 % w/v to about 0.4 % w/v, about 0.05 % w/v to about 0.3 % w/v, about 0.05 % w/v to about 0.2 % w/v, or about 0.05 % w/v to about 0.1 % w/v.

In some embodiments, the production medium is characterised by a pH of about 4 to about 6.

In some embodiments, the production medium is characterised by a Brix value of about 3 to about 6. In some embodiments, the production medium is characterised by a Brix value of 4.5.

When tested with at least 3 different spent grain and spent yeast samples from different brewers, and different spent gain and spent yeast batches from the same brewer, the method consistently produced bacterial cellulose.

In some embodiments, the step of incubating the culture comprises incubating the starter culture with a first production medium to form an intermediate culture and incubating the intermediate culture with a second production medium in order to form a production culture. This allows the cells to adapt to the new medium, and thus provide for a higher yield. Preferably, the first production medium and the second production medium used are the same. For example, if the first production medium is Buyo2, the second production medium is preferably Buyo2.

In some embodiments, the step of incubating the culture is performed at about 20 °C to about 40 °C. In some embodiments, the temperature is about 20 °C to about 38 °C, about 20 °C to about 36 °C, about 20 °C to about 34 °C, about 20 °C to about 32 °C, about 22 °C to about 32 °C, about 24 °C to about 32 °C, about 26 °C to about 32 °C, or about 28 °C to about 32 °C. In some embodiments, the step of incubating the culture is performed at about 30 °C.

In some embodiments, the step of incubating the culture is performed under static conditions. This means that the starter culture is allowed to incubate without external disturbance. In some embodiments, the step of incubating the culture is performed without agitation of the starter culture.

In some embodiments, the step of incubating the culture is performed for about 2 days to about 20 days. In some embodiments, the duration is about 2 days to about 18 days, about 2 days to about 16 days, about 2 days to about 14 days, about 2 days to about 12 days, about 2 days to about 10 days, or about 2 days to about 8 days. In some embodiments, the step of incubating the culture is performed for about 4 days to about 7 days.

In some embodiments, the starter culture is mixed with the production medium prior to its transfer or incubation in a fermentation tray. Alternatively, in some embodiments, the starter culture is added to a fermentation tray comprising the production medium. In this method, instead of mixing the media and cultures before pouring to the fermentation trays, the freshly sterilized and hot media is poured to the fermentation trays first (to sterilize the trays as well), and when the media are cooled to less than about 30 °C, the starter culture may be added directly to the tray and mixed.

In some embodiments, about 10L to about 50L of starter culture is mixed with at least about 200L of production medium. In some embodiments, about 20L to about 50L of starter culture is mixed with about 400L to about 1600L of production medium. In some embodiments, about 40L of starter culture is mixed with at least about 1500L of production medium.

In some embodiments, about 20L to about 50L of starter culture is mixed with at least about 200L of a first production medium to create the intermediate culture. In some embodiments, about 100L to about 300L of grown intermediate culture is mixed with about 800L to about 1000L of second production medium. In some embodiments, about 40L of starter culture is mixed with at least about 200L of the first production medium. In some embodiments, about 200L of intermediate culture is mixed with at least about 800L of the second production medium.

In some embodiments, the step of incubating the culture is performed in a fermentation tray with a volume of about 1 L to about 2 L. Preferably, the volume may be about 1 L to about 1.5 L. It was found that if the volume is too large, it is difficult for cells at the bottom to produce cellulose, as the cellulose formation occurs at the air/cellulose pellicle interface and not at the medium/cellulose interface. Large fermentation volumes maximize the utilization of production facilities, leading to lower production costs per unit of product. The media and starter cultures were prepared and mixed in autoclavable bioreactors before transferring them to stackable fermentation trays, facilitating the scalability of the entire production process.

In some embodiments, the starter culture is added to the production medium about 5 % v/v to about 40 % v/v relative to the culture medium. In some embodiments, the concentration is about 5 % v/v to about 35 % v/v, about 5 % v/v to about 30 % v/v, about 5 % v/v to about 28 % v/v, about 5 % v/v to about 26 % v/v, about 5 % v/v to about 24 % v/v, about 5 % v/v to about 22% v/v, about 5 % v/v to about 20 % v/v, about 6 % v/v to about 20 % v/v, about 8 % v/v to about 20 % v/v, about 10 % v/v to about 20 % v/v, about 12 % v/v to about 20 % v/v, about 14 % v/v to about 20 % v/v, or about 18 % v/v to about 20 % v/v. In some embodiments, the starter culture is added to the production medium about 20 % v/v relative to the culture medium.

In some embodiments, the method further comprises a step of culturing a seed inoculum in a propagation medium and/or activation medium in order to form the starter culture. In some embodiments, the method further comprises a step of culturing a seed inoculum in a propagation medium in order to form the starter culture. In some embodiments, the seed inoculum comprises a pellicle formed from at least one Komagataeibacter strain.

Accordingly, the method of producing bacterial cellulose comprises:

a) culturing a seed inoculum in a propagation medium and/or activation medium in order to form a starter culture, the seed inoculum comprises a pellicle formed from at least one Komagataeibacter strain; and

b) incubating the starter culture with a production medium in order to form a culture for forming the bacterial cellulose;

wherein the production medium comprises a spent yeast liquor which is not treated thermally or chemically;

wherein the at least one Komagataeibacter strain is selected from Komagataeibacter nataicola, Komagataeibacter rhaeticus, Komagataeibacter xylinus, Komagataeibacter intermedius or a combination thereof.

Seed inoculum refers to the material which is used for inoculation, i.e., the introduction of microorganisms into a culture. Inoculation is carried out to grow microorganisms in a culture under specific growth conditions.

In some embodiments, the seed inoculum is added to the propagation medium and/or activation medium at about 10 % v/v to about 30 % v/v relative to the starter culture. In some embodiments, the concentration is about 10 % v/v to about 28 % v/v, about 10 % v/v to about 26 % v/v, about 10 % v/v to about 24 % v/v, about 10 % v/v to about 22% v/v, about 10 % v/v to about 20 % v/v, about 12 % v/v to about 20 % v/v, about 14 % v/v to about 20 % v/v, or about 16 % v/v to about 20 % v/v. In some embodiments, the seed inoculum is added to the propagation medium and/or activation medium at about 20 % v/v relative to the starter culture.

It was found that when a pellicle is cultured, the reactivated strain is more stable and may establish the starter culture after a few cycles. Pellicles contain very active bacteria, so they can grow faster, shortening the culture time. It was found that pellicles may be

used to create the starter culture or to re-activate the strain(s), as it can be challenging to transfer pellicles during the production stage.

In some embodiments, the step of forming the starter culture is performed at about 20 °C to about 40 °C. In some embodiments, the temperature is about 20 °C to about 38 °C, about 20 °C to about 36 °C, about 20 °C to about 34 °C, about 20 °C to about 32 °C, about 22 °C to about 32 °C, about 24 °C to about 32 °C, about 26 °C to about 32 °C, or about 28 °C to about 32 °C. In some embodiments, the step of forming the starter culture is performed at about 30 °C.

In some embodiments, the step of forming the starter culture is performed under static conditions. This means that the starter culture is allowed to incubate without external disturbance. In some embodiments, the step of forming the starter culture is performed without agitation of the starter culture.

In some embodiments, the step of forming the starter culture is performed for about 2 days to about 20 days. In some embodiments, the duration is about 2 days to about 18 days, about 2 days to about 16 days, about 2 days to about 14 days, about 2 days to about 12 days, about 2 days to about 10 days, or about 2 days to about 8 days. In some embodiments, the step of forming the starter culture is performed for about 4 days to about 7 days.

In some embodiments, the step of forming the starter culture comprises:

i) conditioning the seed inoculum in a first propagation medium and/or activation medium in order to form a conditioned seed inoculum; and

ii) culturing the conditioned seed inoculum in a second propagation medium in order to form the starter culture.

Conditioning the seed inoculum prevents the cells from being "shocked", and in the process, may cause cell death. It was found that this may increase the bacterial cellulose yield.

The first propagation medium may be the same as the second propagation medium. Alternatively, the first propagation medium may comprise the same components as the second propagation medium but with at least one component at a lesser concentration.

In some embodiments, the propagation medium comprises:

a) sugar at about 3 % w/v to about 20 % w/v relative to the propagation medium; and

b) spent yeast liquor at about 2 % w/v to about 10 % w/v relative to the propagation medium.

In some embodiments, the propagation medium comprises a spent yeast liquor which is not treated thermally or chemically.

In some embodiments, the sugar is about 3 % w/v to about 20 % w/v relative to the propagation medium, about 3 % w/v to about 18 % w/v, about 3 % w/v to about 16 % w/v, about 3 % w/v to about 14 % w/v, about 3 % w/v to about 12 % w/v, about 6 % w/v to about 12 % w/v, or about 8 % w/v to about 12 % w/v. In some embodiments, the sugar is about 10 % w/v relative to the propagation medium.

In some embodiments, the spent yeast liquor is about 2 % w/v to about 10 % w/v relative to the propagation medium, about 2 % w/v to about 9.5 % w/v, about 2 % w/v to about 9 % w/v, about 2 % w/v to about 8.5 % w/v, about 2 % w/v to about 8 % w/v, about 2 % w/v to about 7.5 % w/v, about 2 % w/v to about 7 % w/v, about 2 % w/v to about 6.5 % w/v, about 2 % w/v to about 6 % w/v, about 2 % w/v to about 5.5 % w/v, about 2 % w/v to about 5 % w/v, about 2.5 % w/v to about 5 % w/v, about 3 % w/v to about 5 % w/v, about 3.5 % w/v to about 5 % w/v, or about 4 % w/v to about 5 % w/v.

In some embodiments, the propagation medium further comprises citric acid at about 0.05 % w/v to about 1 % w/v relative to the propagation medium. In other embodiments, the concentration is about 0.05 % w/v to about 0.9 % w/v, about 0.05 % w/v to about 0.8 % w/v, about 0.05 % w/v to about 0.7 % w/v, about 0.05 % w/v to about 0.6 % w/v, about 0.05 % w/v to about 0.5 % w/v, about 0.05 % w/v to about 0.4 % w/v, about 0.05 % w/v to about 0.3 % w/v, about 0.05 % w/v to about 0.2 % w/v, or about 0.05 % w/v to about 0.1 % w/v.

In some embodiments, the propagation medium is characterised by a pH of about 4 to about 6.

In some embodiments, the method further comprises a step of activating the at least one Komagataeibacter strain in an activation medium in order to form the seed inoculum.

In some embodiments, the step of forming the seed inoculum is performed at about 20 °C to about 40 °C. In some embodiments, the temperature is about 20 °C to about 38 °C, about 20 °C to about 36 °C, about 20 °C to about 34 °C, about 20 °C to about 32 °C, about 22 °C to about 32 °C, about 24 °C to about 32 °C, about 26 °C to about 32 °C, or about 28 °C to about 32 °C. In some embodiments, the step of forming the seed inoculum is performed at about 30 °C.

In some embodiments, the step of forming the seed inoculum is performed under static conditions. This means that the seed inoculum is allowed to incubate without external disturbance or without agitation.

In some embodiments, the step of forming the seed inoculum is performed for about 2 days to about 10 days. In some embodiments, the duration is about 2 days to about 18 days, about 2 days to about 16 days, about 2 days to about 14 days, about 2 days to about 12 days, about 2 days to about 10 days, or about 2 days to about 8 days. In some embodiments, the step of forming the seed inoculum is performed for about 4 days to about 7 days.

In some embodiments, the activation medium comprises:

a) sugar at about 1 % w/v to about 20 % w/v relative to the activation medium; and

b) yeast extract powder at about 0.1 % w/v to about 2 % w/v relative to the activation medium.

In some embodiments, the sugar is about 1 % w/v to about 20 % w/v relative to the activation medium, about 1 % w/v to about 18 % w/v, about 1 % w/v to about 16 % w/v, about 1 % w/v to about 14 % w/v, about 1 % w/v to about 12 % w/v, about 1 % w/v to about 10 % w/v, about 1 % w/v to about 8 % w/v, or about 1 % w/v to about 6 % w/v. In some embodiments, the sugar is about 5 % w/v relative to the activation medium.

Yeast extract powder is a mixture of amino acids, peptides, vitamins and carbohydrates. In some embodiments, the yeast extract powder is about 0.1 % w/v to about 2 % w/v relative to the activation medium, about 0.1 % w/v to about 1.8 % w/v, about 0.1 % w/v to about 1.6 % w/v, about 0.1 % w/v to about 1.4 % w/v, about 0.1 % w/v to about 1.2 % w/v, about 0.1 % w/v to about 1 % w/v, about 0.1 % w/v to about 0.8 % w/v, about 0.1 % w/v to about 0.6 % w/v, about 0.2 % w/v to about 0.6 % w/v, or about 0.4 % w/v to about 0.6 % w/v. In some embodiments, the yeast extract is about 0.5 % w/v relative to the activation medium.

In some embodiments, the activation medium further comprises citric acid at about 0.05 % w/v to about 1 % w/v relative to the activation medium. In other embodiments, the concentration is about 0.05 % w/v to about 0.9 % w/v, about 0.05 % w/v to about 0.8 % w/v, about 0.05 % w/v to about 0.7 % w/v, about 0.05 % w/v to about 0.6 % w/v, about 0.05 % w/v to about 0.5 % w/v, about 0.05 % w/v to about 0.4 % w/v, about 0.05 % w/v to about 0.3 % w/v, about 0.05 % w/v to about 0.2 % w/v, or about 0.05 % w/v to about 0.1 % w/v.

In some embodiments, the activation medium is characterised by a pH of about 4 to about 6.

In some embodiments, the activation medium is characterised by a Brix value of about 3 to about 6. In some embodiments, the activation medium is characterised by a Brix value of 4.5.

In some embodiments, the step of incubating the culture medium comprises forming a bacterial cellulose membrane in order to form the bacterial cellulose. The bacterial cellulose membrane may be a pellicle. The bacterial cellulose membrane may be formed on a surface of the culture medium.

In some embodiments, the method further comprises a step of harvesting the bacterial cellulose membrane when a pH of the culture medium is about pH 2 to about pH 3.

In some embodiments, the method further comprises a step of wiping the bacterial cellulose membrane. In some embodiments, the method further comprises a step of

washing the bacterial cellulose membrane and compressing the bacterial cellulose membrane. The bacterial cellulose membrane may be hydraulic pressed to remove the solvent or water.

After the cleaning step, the pellicle mainly contains water and cellulose, and some insignificant amount of bacteria. If needed, bacteria can be removed by NaOH and/or H_2O_2 treatment.

In some embodiments, the residual media are recycled. In some embodiments, after a first batch of bacterial cellulose is harvested, a residual medium from the first method is recycled into a second production or method in order to form a second batch of bacterial cellulose. The residual media may be recycled back into the activation medium, propagation medium and/or production medium. As the production yield is more than 80 %, less than 20 % residual media left after production and the residual media may be reused. This lowers the cost for wastewater treatment, reducing the environmental impact of the production process, thus it aligns with sustainability goals.

In all the media, such as activation medium, propagation medium, and production medium, ethanol may be added. The concentration may be about 0.5 % v/v to about 2.5 % v/v relative to the medium. In other embodiments, the concentration is about 0.5 % v/v to about 2 % v/v, about 0.5 % v/v to about 1.5 % v/v, or about 0.5 % v/v to about 1 % v/v.

In some embodiments, the method is characterised by a bacterial cellulose yield of more than about 80%. In other embodiments, the bacterial cellulose yield is more than about 82%, about 84%, about 86%, about 88%, or about 90%.

The present disclosure concerns a bacterial cellulose formed using the method as disclosed herein.

The present disclosure concerns a bacterial cellulose, comprising a Komagataeibacter strain residue at less than about 1 % w/w. In other embodiments, the Komagataeibacter strain residue is less than about 0.9 % w/w, about 0.8 % w/w, about 0.7% w/w, about 0.6 % w/w, about 0.5 % w/w, about 0.4 % w/w, about 0.3 % w/w, about 0.2 % w/w, or about 0.1 % w/w.

In some embodiments, the bacterial cellulose is characterised by a thickness of about 1 cm to about 3 cm. In other embodiments, the thickness is about 1 cm to about 2.8 cm, about 1 cm to about 2.6 cm, about 1 cm to about 2.4 cm, about 1 cm to about 2.2 cm, about 1 cm to about 2 cm, or about 1 cm to about 1.8 cm.

In some embodiments, the bacterial cellulose is characterised by a tensile strength of about 100 MPa to about 200 MPa. In other embodiments, the tensile strength is about 110 MPa to about 200 MPa, about 110 MPa to about 190 MPa, about 110 MPa to about 180 MPa, about 110 MPa to about 170 MPa, about 110 MPa to about 160 MPa, about 110 MPa to about 150 MPa, or about 110 MPa to about 140 MPa.

In some embodiments, the bacterial cellulose is characterised by an elongation at break value of about 20 % to about 50%. In other embodiments, the elongation at break value is about 25 % to about 50%, about 30 % to about 50%, about 30 % to about 45%, or about 30 % to about 40%.

In some embodiments, the bacterial cellulose is formable as a foam, hydrogel, aerogel, cardboard sheet, biofilm, film, sheet, or membrane.

Examples

The overall process for the production of bacterial cellulose using spent grain and spent yeast was shown in Figure 1.

Medium preparations

Four media were prepared including activation medium GY (Table 1), propagation medium BuyoF1 (Table 2), production medium Buyo2 (Table 3), and production medium Buyo4 (Table 4). Medium GY contains 50 g/L sucrose and 5 g/L yeast extract powder. Medium BuyoF1 contains 100 g/L sucrose and 45 mL/L spent yeast liquor. Medium Buyo2 contains 150 mL/L spent grain filtrate, 40 mL/L spent yeast liquor, 30 g/L sucrose, and 5 g/L disodium phosphate (Na_2HPO_4). Medium Buyo4 contains 40 mL/L spent yeast liquor, 30 g/L sucrose, and 5 g/L Na_2HPO_4. All media were added with 1 g/L citric acid and 5 mL/L ethanol, and the pH was adjusted to pH 4.0 using acetic acid.

The media were sterilized either by autoclaving at 121°C for 15 minutes or by pumping through a continuous sterilization system at the same temperature with a flow rate of 1,000 L/h. Subsequently, the culture media were cooled down to 28-30°C before usage. For a 1,000-L fermentation scale, with a 20 % culture volume, 800 L of media were prepared.

Table 1. Composition of activation medium GY

No	Component	Concentration	Percentage (w/v, v/v)
1	Sucrose	50 g/L	5
2	Yeast extract powder	5 g/L	0.5
3	Ethanol	5 mL/L	0.5
4	Citric acid	1 g/L	0.1
5	Acetic acid	To adjust pH to pH 4.0	

Table 2. Composition of propagation medium BuyoF1 for starter culture

No.	Component	Concentration	Percentage (w/v or v/v)
1	Sucrose	100 g/L	10
2	Spent yeast liquor	45 mL/L	4.5
3	Ethanol	5 mL/L	0.5
4	Citric acid	1 g/L	0.1
5	Acetic acid	To adjust pH to pH 4.0	

Table 3. Composition of medium Buyo2 for bacterial cellulose production

No.	Component	Concentration	Percentage (w/v or v/v)
1	Spent grain filtrate	150 mL/L	15
2	Spent yeast liquor	40 mL/L	4
3	Sucrose	30 g/L	3
4	Na_2HPO_4	5 g/L	0.5
5	Ethanol	5 ml/L	0.5
6	Citric acid	1 g/L	0.1
7	Acetic acid	To adjust pH to pH 4.0	

Table 4. Composition of medium Buyo4 for bacterial cellulose production

No.	Component	Concentration	Percentage (w/v or v/v)

1	Spent yeast liquor	40 mL/L	4
2	Sucrose	30 g/L	3
3	Na_2HPO_4	5 g/L	0.5
4	Ethanol	5 mL/L	0.5
5	Citric acid	1 g/L	0.1
6	Acetic acid	To adjust pH to pH 4.0	

Preparation of spent grain filtrate

There was no thermal or chemical pre-treatment needed for the usage of spent grain. Wet spent grain from brewery or ethanol manufacturers was directly re-suspended in water at the ratio of 1:3, i.e. 50 kg wet spent grain in 150 L water; the average moisture content of wet spent gain was approximately 75%. The sludge was then filtered using a centrifugal dehydrator (1,000 rpm) with 200-mesh cloth filter, and the filtrate was collected to add to medium Buyo2.

Preparation of spent yeast liquor

No thermal or chemical pre-treatment was required for the utilization of spent yeast liquor collected from brewery or bioethanol fermentation processes. The spent yeast liquor was used as-is or filtered using a centrifugal dehydrator (1,000 rpm) with 200-mesh cloth filter, and then added to the media BuyoF1, Buyo2 and Buyo4.

Preparation of seed inocula and starter cultures at an industrial scale

Komagataeibacter strains including *Komagataeibacter nataicola*, *Komagataeibacter rhaeticus*, *Komagataeibacter xylinus*, and *Komagataeibacter intermedius* that were found to be adapted well with media containing spent grain as well as spent yeast were stored at -80 °C as individual strains or a mixture of strains for long-term storage. Strain mixtures can consist of all four strains (*K. nataicola*, *K. rhaeticus*, *K. xylinus* and *K. intermedius*), combinations of three strains (e.g. *K. nataicola*, *K. rhaeticus* and *K. xylinus*), or combinations of two strains (e.g. *K. rhaeticus* and *K. xylinus*).

To produce the seed inoculum, individual strains or a mixture of strains from the frozen stocks, were activated in a propagation medium or activation medium GY, which had already been sterilized and cooled to 28-30°C. The seed inoculum cultures were grown in glass Petri dishes with 30 mL of medium GY. For a 1000-L fermentation scale, cells were grown in 40 glass Petri dishes at 28-30 °C under static conditions for 4 days until

pellicles formed. These pellicles were then transferred into medium BuyoF1, and cultured for 4-7 days at 28-30 °C in a static condition to adapt the cells to the presence of spent yeast liquor, forming 40L of starter cultures. The transfer of pellicles is preferred for reactivating strains and establishing starter cultures after several cycles of solution transfer.

The starter culture may then be transferred to a production medium (Buyo2 or Buyo4) for producing bacterial cellulose.

Production of bacterial cellulose in static conditions at an industrial scale

Medium Buyo2 that contains both spent grain filtrate and spent yeast liquor, or medium Buyo4 that contains spent yeast liquor, but not spent grain filtrate, were used for BC production. The media (up to 800 L) were sterilized in bioreactors at 121°C for 15 minutes, or via a continuous sterilization system with a flow rate of 1,000-1200 L/h at 121°C, the media were then cooled down to 28-30 °C using a heat-exchange method with cold water. Exchanged water is collected to prepare media including activation, propagation and production media. A series of bioreactors is used (Figure 2), allowing BC production to operate on a continuous basis.

The starter culture (40 L) was added to the media (Buyo2 or Buyo4) at 20 % (v/v) in 500-L bioreactors, and the cells were allowed to grow at 28-30°C under static conditions for 4-7 days to adapt the cells with the production media. When the pellicles were established, the culture (20 %, v/v) was transferred to the corresponding media (Buyo2 or Buyo4) in 1500-L bioreactors, and the solutions were mixed, and 1L was pumped into each plastic fermentation tray (the inner dimension of 32 cm x 26 cm x 4.5 cm, with the maximum volume of 3,744 cm^3). Fermentation trays were then stacked (Figure 3), each stack contains up to 40 trays, of which the top tray was empty, served as the cover for underneath trays.

Fermentation trays and fermentation tray stacks were kept static without shaking at 28-30 °C for the production of BC for up 7 days. Bacterial cellulose formed pellicles floating above the media (Figure 4). When the media were depleted, or after up to 7 days of culturing, pellicles (also known as BC membranes) were harvested. The pH value of the media at harvest was around pH 2.0-3.0. The harvested BC membranes were briefly wiped to remove media, and washed with water; the adsorbed water was finally

removed from the BC membranes by hydraulic press machines. The residual media as well as water from the washing step and pressing step are collected for recycling.

The BC production yield from *K. nataicola* ranged from 85 to 89 % (wet weight) (Table 5), the strain grew and consumed almost all liquid in the fermentation trays. The produced bacterial cellulose formed firm, flexible and opaque membranes, and these BC membranes can be easily handled without breakage or tear. It found that no cellulose pellicles were formed in the BC-producing media in the absence of spent grain filtrate and/or spent yeast liquor. Furthermore, cellulose membranes were prone to break and tear in the absence of Na_2HPO_4.

Table 5. The BC production yield by K. nataicola from 1 L of media Buyo2 or Buyo4

Medium	**Strain**	**Wet-weighted BC (mg)**	**BC thickness (mm)**	**% BC yield (wet weight)**	**% BC yield (dried weight)**
Buyo2	*K. nataicola*	890 ± 6	0.86 ± 0.09	89	5.3
Buyo4	*K. nataicola*	850 ± 2	0.89 ± 0.07	85	5.1

Recycling residual media and wastewater

The residual media were filtered using centrifugal dehydrators (1,000 rpm) equipped with 200-mesh cloth filters to eliminate any potential pellets. Subsequently, the filtrate, along with the water used for washing BC and the water from pressing BC during harvesting, were combined. If necessary, the pH of collected water was adjusted to 4.0 using NaOH. The collected solution was then employed to dissolve chemical components for the BC production media, i.e. media Buyo2 and Buyo4.

Conclusion

Utilizing waste materials or low-cost feedstocks for bacterial cellulose (BC) production not only reduces production costs but also contributes to sustainability by repurposing materials that would otherwise be discarded as waste. Herein, we selected specialized bacteria and their mixtures to create a fermentation process for BC production directly on spent grain or spent yeast without any chemical or thermal pre-treatment. The application of bioreactors for inoculum and culture preparation, along with the use of static fermentation, allows for BC production at an industrial scale. Specifically, seed inocula and starter cultures were prepared in 500-L and 1500-L bioreactors, while

fermentation was carried out using stackable fermentation trays. Optimization of fermentation conditions is essential to maximize BC yield and quality when using by-products from alcoholic fermentation. We optimized by-products loading, medium composition, and fermentation conditions to achieve high yields of bacterial cellulose, with maximum wet yields of BC exceeding 85%. Additionally, we recycled residual media and wastewater to minimize production costs and environmental impacts.

It will be appreciated that many further modifications and permutations of various aspects of the described embodiments are possible. Accordingly, the described aspects are intended to embrace all such alterations, modifications, and variations that fall within the spirit and scope of the appended claims.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Throughout this specification and the claims which follow, unless the context requires otherwise, the phrase "consisting essentially of", and variations such as "consists essentially of" will be understood to indicate that the recited element(s) is/are essential i.e. necessary elements of the invention. The phrase allows for the presence of other non-recited elements which do not materially affect the characteristics of the invention but excludes additional unspecified elements which would affect the basic and novel characteristics of the method defined.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Claims

1. A method of producing bacterial cellulose, comprising:
incubating a starter culture of 20L to 50L with a production medium of at least 200L in order to form a culture for forming the bacterial cellulose;
wherein the starter culture comprises at least one Komagataeibacter strain selected from Komagataeibacter nataicola, Komagataeibacter rhaeticus, Komagataeibacter xylinus, and Komagataeibacter intermedius;
wherein the production medium comprises a spent yeast liquor which is not treated thermally or chemically;
wherein the step of incubating the culture is performed under static conditions in a fermentation tray with a volume of 1 L to 2 L.

2. The method according to claim 1, wherein the starter culture further comprises a Komagataeibacter (Gluconacetobacter) strain selected from G. sucrofermentans, G. hansenii, G. europaeus, G. entanii, G. saccharivorans, G. swingsii, G. oboediens, G. liquefaciens, G. sacchari, G. diazotrophicus, G. johannae, G. azotocaptans, G. medellinensis, or a combination thereof.

3. The method according to claim 1 or 2, wherein the production medium comprises:
a) sugar at 1 % w/v to 18 % w/v relative to the production medium;
b) spent yeast liquor at 2 % w/v to 12 % w/v relative to the production medium;
c) disodium phosphate (Na_2HPO_4) at 0.1 % w/v to 1 % w/v relative to the production medium; and
d) citric acid at 0.05 % w/v to 1 % w/v relative to the production medium;
e) ethanol at 0.5 % w/w to 2.5 % w/w relative to the production medium;
wherein the spent yeast liquor is obtained from a brewery and/or bioethanol fermentation process;
wherein the production medium is characterised by a pH of 4 to 6 and a Brix value of 3 to 6.

4. The method according to any one of claims 1 to 3, wherein the production medium further comprises spent grain filtrate at 5 % w/v to 50 % w/v relative to the production medium;
wherein the spent grain filtrate is formed by suspending a spent grain in an aqueous

medium at a ratio of 1:3 to form a sludge and filtering the sludge; and
wherein the spent grain is obtained from a brewery and/or a bioethanol fermentation process.

5. The method according to any one of claims 1 to 4, wherein the step of incubating the culture is performed at 20 °C to 40 °C, and for 2 days to 20 days.

6. The method according to any one of claims 1 to 5, wherein the starter culture is added to the production medium 5 % v/v to 40 % v/v relative to the production culture.

7. The method according to any one of claims 1 to 6, wherein the method further comprises a step of culturing a seed inoculum in a propagation medium and/or activation medium in order to form the starter culture, wherein the seed inoculum comprises a pellicle formed from at least one Komagataeibacter strain;
wherein the seed inoculum is added to the propagation medium and/or activation medium at 10 % v/v to 30 % v/v relative to the starter culture.

8. The method according to claim 7, wherein the step of forming the starter culture is performed at 20 °C to 40 °C, under static conditions, and for 2 days to 20 days.

9. The method according to claim 7 or 8, wherein the propagation medium comprises:
a) sugar at 3 % w/v to 20 % w/v relative to the propagation medium;
b) spent yeast liquor at 2 % w/v to 12 % w/v relative to the propagation medium; and
c) citric acid at 0.05 % w/v to 1 % w/v relative to the propagation medium;
e) ethanol at 0.5 % w/w to 2.5 % w/w relative to the propagation medium;
wherein the propagation medium is characterised by a pH of 4 to 6.

10. The method according to any one of claims 7 to 9, wherein the method further comprises a step of activating the at least one Komagataeibacter strain in an activation medium in order to form the seed inoculum.

11. The method according to claim 10, wherein the step of forming the seed inoculum is performed at 20 °C to 40 °C, under static conditions, and for 2 days to 20 days.

12. The method according to any one of claims 7 to 11, wherein the activation medium comprises:

a) sugar at 1 % w/v to 20 % w/v relative to the activation medium;

b) yeast extract powder at 0.1 % w/v to 2 % w/v relative to the activation medium; and

c) citric acid at 0.05 % w/v to 1 % w/v relative to the activation medium;

e) ethanol at 0.5 % w/w to 2.5 % w/w relative to the activation medium;

wherein the activation medium is characterised by a pH of 4 to 6.

13. The method according to any one of claims 1 to 12, wherein the step of incubating the culture comprises forming a bacterial cellulose membrane and harvesting the bacterial cellulose membrane when a pH of the culture is pH 2 to pH 3.

14. The method according to any one of claims 1 to 13, wherein the method further comprises a step of washing the bacterial cellulose membrane and compressing the bacterial cellulose membrane.

15. The method according to any one of claims 1 to 14, wherein after the bacterial cellulose is harvested, a residual medium from the method is recycled in order to form another batch of bacterial cellulose.

16. The method according to any one of claims 1 to 15, wherein the method is characterised by a bacterial cellulose yield of more than 80 %.

17. The method according to any one of claims 1 to 16, wherein the bacterial cellulose is formable as a foam, hydrogel, aerogel, cardboard sheet, biofilm, film, sheet, or membrane.