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PRODUCTION OF CELLULOSE BY *GLUCONACETOBACTER SP.*

S.U. Pokalwar[1]*, M. K. Mishra[1] and A.V. Manwar[2]

[1]*Gennova Biopharmaceuticals Ltd. Plot P-1, Hinjewadi, Pune (MS) India*
[2]*Department of Microbiology, Dnyanopasak College, PO Box 54, Parbhani (MS) India*

Abstract

Bacterial cellulose (BC) displays unique physical, chemical and mechanical properties including high crystallinity, high water holding capacity, large surface area, elasticity, mechanical strength and biocompatibility. It has found to be very beneficial in the treatment of second and third degree burn cases. BC appears to be one of the best materials to promote wound healing from burns. Another important aspect of BC is preservation of natural resources (plants). The use of trees for the production of paper and construction materials leads to deforestation. Bacterial Cellulose seems to be the only alternative for plant cellulose because bacterial cellulose has better physicochemical properties. Also the production rate of bacterial cellulose is faster than plant cellulose production.

Keywords: Biopolymer, Bacterial cellulose, Biocompatibility, Physicochemical properties

Introduction

Bacterial cellulose displays unique physical, chemical and mechanical properties that include high crystallinity, high water holding capacity, large surface area, elasticity, mechanical strength and biocompatibility [1] thus bacterial cellulose has potential applications in food preparations such as desserts, thickeners and sausage, in drug delivery agents, capsule shells, Oil spill cleanup sponge; mineral and oil recovery; leather products, sports items; ultra filters for water purification; audio speaker diaphragm; plywood laminates; specialty papers and polyesters; automotive and aircraft bodies are some of the areas where BC also have potential applications [2] Bacterial cellulose appears to be one of the best materials to promote wound healing from second and third degree burns [1] Bacterial cellulose as a substitute of natural resources (plants) could help in protecting global climate by preventing our fast disappearing forest (the use of trees for the production of paper and construction materials).

Plant kingdom is a major source of cellulose. Biosynthesis of plant cellulose involves polymerization of glucose residues using UDP-glucose as a substrate with enzyme cellulose synthase, which together with several accessory enzymes form the homopolymers of β-I, 4 -glucan into a cellulose microfibril of 36 glucan chains [3, 4, 5]. Cellulose exists in two different allomorphs, called Iα and Iβ, that can be distinguished by '3C-NMR [6]. Microfibril size can also vary among organisms, in general ranging from elementary fibril of 36 chains to the very large fibrils of the cellulosic algae, which can contain more than 200 chains. These fibrils are highly organized that they can diffract as a single pure crystal [5].

Several genera that have shown the ability to synthesize cellulose include *Sarcina*, *Agrobacterium*, *Rhizobium*, and *Acetobacter also known as Gluconacetobacter* [7, 8]. The most efficient producer of BC, a Gram negative and acetic acid bacterium *Gluconacetobacter xylinus* is the model microorganism for basic and applied studies on cellulose. *G. xylinus* produces an extra-cellular gel-like material or pellicle, which comprises of a random assembly of cellulose ribbons, composed of a number of micro-fibrils [9]. This organism and its product were first identified and characterized over a century ago [10].

It is assumed that bacteria produces cellulose by defense mechanism against sunlight to protect from UV radiation but exact mechanism of cellulose production is not known till date [11].

Cellulose biosynthesis

The use of bacteria to produce cellulose has many advantages in addition to their rapid growth and ability to be maintained under controlled conditions. Bacteria offer unique possibilities for the isolation of mutants that could help in obtaining over producing strain. A single *G. xylinus* cell may polymerize up to 200000 glucose molecules [12] into β-1,4-glucan chains which, extruded into the surrounding medium, typically achieve the form of a single, twisted, ribbon like bundle of microfibrils. The ribbon elongates in direct association with the cell envelope and remains associated during cell division; this is reported by a statistical analysis of cellulose production in growing cultures [13]. The model of cellulose biogenesis in which the stages of glucose polymerization and microfibril assembly are tightly coupled processes was

* Corresponding Author, *Email*: santosh.pokalwar@gennova.co.in

first proposed for *G. xylinus* on the basis that substances which bind to nascent polyglucan aggregates and prevent crystallization into microfibrils accelerate the rate of chain synthesis. Static cultures of *G. xylinus* are characterized by a thick cellulosic surface mat, called a pellicle, in which the embedded cells of this obligate aerobe have direct contact with the liquid/air interface [12]. The bacterium grows and produces cellulose from a wide range of substrates [14] and is devoid of cellulase activity [12]. In contrast, plant cellulose fibrils make up an integral part of the complex polysaccharide cell wall matrix. A distinct advantage in studying *G. xylinus* is that its cellulose fibril product is a metabolically inert, highly pure extracellular deposit. For many years, the study of cellulose biogenesis in *G. xylinus* was confined to physiological and morphological approaches concerned with identifying the intracellular precursors of polyglucose chains or characterizing the rise of the sugar nucleotide precursor. The rate of cellulose production in *G. xylinus* is roughly proportional to the rate of cell growth and is independent of the source of carbon. This feature, together with the fact that cellulose represents a dead end with respect to glucose metabolism, led to a convenient method for the description of metabolic pathways in this bacterium in which the rate and pattern of incorporation of labeled carbon atoms from various substrates into the insoluble glucan polymer were determined [15]. On the basis of such studies, the suitability of a particular substrate may be understood in terms of the two amphibolic pathways operative in this bacterium: (i) The pentose cycle for the oxidation of carbohydrates and (ii) The citrate cycle for the oxidation of organic acids and related compounds [16]. These pathways of carbon metabolism are unique to *G. xylinus*. The inability to metabolize glucose anaerobically in *G. xylinus* lies in the fact that it lacks phosphofructose kinase, which is a key enzyme for glycolysis [17]. Gluconeogenesis occurs in *G. xylinus* from oxaloacetate via pyruvate, because of the unusual regulation of the enzyme oxaloacetate decarboxylase and pyruvate phosphate dikinase. Thus, cellulose arises in this organism from a metabolic pool of hexose phosphate that is sustained directly by the phosphorylation of exogenous hexoses and indirectly via the pentose cycle and the gluconeogenic pathway. The conversion of hexose phosphate to cellulose is direct in the sense that it does not necessarily include intermediary cleavage of the carbon skeleton of the hexose moiety [18]. The flow of hexose phosphate carbon toward cellulose or through the pentose cycle appears to be regulated by an energy-linked control mechanism in which the crossover point could be at the ATP-sensitive NAD-linked glucose-6-phosphate dehydrogenase.

Synthesis of bacterial cellulose is regulated multi-step process, involving a large number of both individual enzymes and complexes of catalytic and regulatory proteins, whose supramolecular structure has not yet been well defined. The process results the synthesis of uridine diphosphoglucose (UDPGlc) followed by glucose polymerization into β1, 4-glucan chain, and nascent chain association in a characteristic ribbon-like structure, that may contain hundreds or even thousands of individual cellulose chains.

Pathways and mechanisms of anabolic process

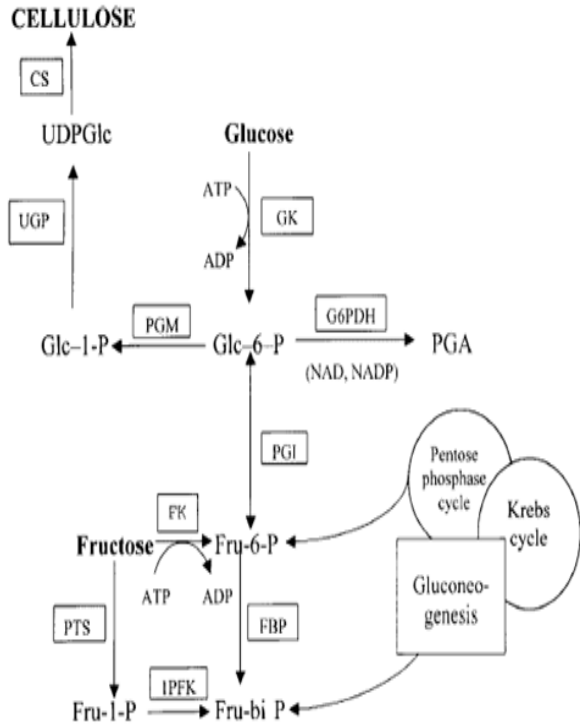
Cellulose synthesis in *G. xylinus* (Fig.1) is depends on the physiological state of the cell and involves either the pentose phosphate cycle or the Krebs cycle, coupled with gluconeogenesis [11]. Glycolysis does not operate in acetic acid bacteria since they do not synthesize the crucial enzyme of this pathway - phosphofructose kinase [11]. In *G. xylinus*, cellulose synthesis is tightly associated with catabolic processes of oxidation and consumes as much as 10% of energy derived from catabolic reactions. Bacterial cellulose production does not interfere with other anabolic processes, including protein synthesis [11]. *G. xylinus* converts various carbon compounds, such as hexoses, glycerol, dihydroxyacetone, pyruvate, and dicarboxylic acids, into cellulose, usually with about 50% efficiency. The latter compounds enter the Krebs cycle and due to oxalacetate decarboxylation to pyruvate undergo conversion to hexoses via gluconeogenesis, similarly to glycerol, dihydroxyacetone, and intermediates of the pentose phosphate cycle [11]. The direct cellulose precursor is UDPGlc, which is a product of a conventional pathway, common of many organisms, including plants, and involving glucose phosphorylation to glucose-6-phosphate (Glc-6-P), catalyzed by glucokinase, followed by isomerization of this intermediate to Glc-1-P, catalyzed by phosphoglucomutase, and conversion of the latter metabolite to UDPGlc by UDPGlc pyrophosphorylase. This last enzyme seems to be the crucial one involved in cellulose synthesis.

Fermentation

A wide range of carbon sources maybe used for cellulose production including lactate, ethanol, glycerol, molasses, sucrose and other sugars such as fructose and particularly glucose [19]. Suitable initial carbon source concentration is within the range of 2-100 g/L. Sugar can be added to the growing culture to maintain a certain desirable concentration for optimum cellulose production. Various feedstocks for nitrogen sources can be utilized including corn steep liquor. The pH for cultivation of cellulose producing organism is 5.0. The pH can be controlled by means of buffers such as citrate, or by the addition of base or acid to the medium to maintain the pH in the desired range. Three main methods for bacterial cellulose production are (i) Static

culture (ii) Submerged culture and (iii) The rotating disk system.

Figure 1 : Pathways of carbon metabolism in *G. xylinus [11]*



CS- cellulose synthase ; FBP- fructose-1, 6-biphosphate phosphatase ; FK- glucokinase ; G6PDH-glucose -6-phosphate dehydrogenase; 1PFK- fructose-1-phosphate kinase; PGI-phospho glucoisomerase; PMG- phosphogluco mutase; PTS - system of phosphotransferases; UGP-pyrophosphorylase UDPGlc ; Fru-bi-P,fructose -1,6-bi-phosphatase; Fru-6-P,fructose-6-phosphate; Glc-6(1)-P - glucose- 6(1)-phosphate; PGA- phosphogluconic acid; UDPGlc - uridine diphosphoglucose.

Static cultures

The traditional method of production has been on the surface or static cultures. This method is very simple and low-tech, and is the only widely used method for cellulose production. *G. xylinus* produces cellulose at the air/liquid interface, and cellulose production is inhibited by mixing and aeration. In static production, an inoculated medium is poured into shallow trays. It is covered and allowed to incubate for 5 to 20 days until the pellicle nearly fills the tray. The pellicle is removed and washed to eliminate the cells, and can be processed as desired. During growth, the cellulose propagates from the surface of the culture, once the pellicle has formed. Several researchers have shown that the uppermost layer of the pellicle is the only one growing and the cells that are left further into the pellicle become inactive or die from lack of oxygen (Figure 2). The static production is very simple but has some drawbacks. The diffusion of oxygen limitation, especially when the pellicle is large, and prevents growth from proceeding at its maximum rate and even leads to cell death. The trays, once inoculated, cannot be disturbed until harvest time. This makes measurements nearly impossible, since probes cannot be stuck through the cellulose or removed for cleaning, and thus pH cannot be measured or controlled. This constantly changing environment does not encourage rapid growth, and pH cannot be maintained at optimum levels. Another disadvantage of this system is the inability to access the media to make changes, such as maintaining a certain substrate concentration or adding a modifying reagent.

Fig. 2: Bacterial cellulose produced in static culture.

Submerged / Agitated cultures

The limitations of static cultures (diffusion, controllability, and scale-up) can be overcome through switching to submerged production. One of the problems that hindered the industrial application of bacterial cellulose is its low yield from static culture system. Cellulose can be economical mass produced utilizing agitated culture system. Corn steep liquor was found to be the most suitable organic nitrogen source for bacterial cellulose production. Lactate in corn steep liquor stimulated cell growth and bacterial cellulose production [20].

The modes of operating the biological reactor can be done by batch fermentation, fed batch fermentation and continuous fermentation, each having its own advantages. Batch fermentation may be sufficient for producing microbial cellulose in the form of pellicle for bulk applications. Fed-batch fermentation or continuous processing may be desirable especially in the production of modified or composite microbial cellulose pellicles. A major hurdle in the successful commercialization of the technology is the lack of

available biochemical engineering knowledge on the large-scale production of bacterial cellulose. The product of this system is not in pellicle form, but rather is formed as reticulated cellulose granules (Figure 3). The snow- like, fibrous and rice- like cellulose assemblies are synthesized in agitated culture [21]. Many of the benefits of bacterial cellulose are lost if the fibers are not organized into a defined pellicle, and applications for this product have been limited.

Figure 3: Bacterial cellulose produced in agitated culture

Modified airlift reactor

Bacterial cellulose in pellicles was formed on the air-liquid interface of a static culture, while bacterial cellulose in fibrous form was obtained by using a stirred-tank reactor. The crystallinity index, Young's modulus, and the degree of polymerization of bacterial cellulose in fibrous form were lower than for those in pellicle form. The high shear stress during agitation led to morphological and structural abnormalities in the bacterial cellulose. Moreover, the accumulation of bacterial cellulose in fibrous form during cultivation increased the viscosity of the broth, causing difficulty in mixing and reducing the oxygen-transfer rate. *G. xylinus* for bacterial cellulose production was cultivated in a modified airlift reactor; better results were obtained than from a conventional bubble column. After 72 h of cultivation, the final concentration of bacterial cellulose was 7.72 g/l and the productivity was 0.107 g/l per h in the modified airlift reactor. The concentration of bacterial cellulose was about three times higher than that produced in the conventional bubble column. Moreover, the bacterial cellulose produced using the modified reactor formed a unique elliptical pellet (the average diameter was 10 mm), which is different from the fibrous form produced using the stirred-tank reactor [22].

Rotating disk reactor

Bacterial cellulose can also be produced in traditional stirred or agitated bioreactors. However, this type of culturing typically produces a non-pellicle form of bacterial cellulose. This production method is highly susceptible to strain instability which is demonstrated by the cell's loss of ability to produce cellulose and gradual cell overgrowth [23].

Recently, bacterial cellulose has been produced in a rotating disk reactor (RDR). This system consists of a cylindrical trough half-filled with inoculated media. Flat, circular disks are mounted on a centered shaft and rotated through the trough with a motor. The cells adsorb onto the surface of the disks, most likely as a result of the extruding fibers, and form a pellicle on the disk. After 8-12 h nearly all of the cells are entrained on the disks leaving the broth clear. Solid disks are inferior to perforated or meshed disks, as the holes allow significantly more medium hold-up on the disks, and therefore faster and stronger film formation. Wet cellulose formation per unit area is higher in this system, but the pellicle has twice the water holding capacity of a typical static pellicle. There are several advantages to operating in an RDR rather than with static surface cultures. Although production rates per area are equal, volumetric rates are higher. As the disks rotate, they are in contact with liquid medium half the time and air the other half. This reduces or eliminates the diffusion limitations of static cultures. Also, since the cellulose is on the disks and not in solution, the medium can be sampled and controlled during operation without disturbing the pellicle. Scale-up in this system could be easier than in static production because the troughs can be enlarged (United States Patent 5955326).

Application of bacterial cellulose

Relatively few industries using microbial cellulose are organized; however, as the product becomes better known and its properties are further exploited, this undoubtedly will change. A few representative product areas and potential products are described below:

Cotton is comprised of a purer form of cellulose, but generally only a portion of the fibers is long enough for textile production. The cellulose produced by bacteria is markedly different than cellulose obtained from plant with many advantages that can be commercially useful. Plant cellulose mixed with hemicellulose, lignin and plant chemical products. To obtain pure cellulose chemical treatment like sulphuric acid, NaOH and other chemicals have to be used that leads to increase the product cost, biological oxygen demand and chemical oxygen demand. Bacterial cellulose is devoid of lignin and hemicellulose, is extremely hydrophilic, and has excellent shape and strength retention. It can be produced from many different substrates, its properties can be altered as it

forms rather than afterward, and it can be made to just about any shape or size in the reactor vessel.

Cellulose made from trees must endure a many stages of pulping process to remove lignin and other compounds. This step is especially costly but is necessary for paper manufacturing. Bacterial cellulose is pure and does not have to endure harsh processing. Because of the fewer steps required to process bacterial cellulose, less money is spent and less waste is generated. But a greater advantage is that the cellulose fibers remain intact because of the simpler processing, and thus the cellulose remains stronger and retains is attractive properties.

The bacterial cellulose pellicle is extremely hydrophilic, absorbing 60 to 700 times its weight in water. By adding certain substrates or by manipulating the operating conditions it is possible to change the properties of the cellulose. For instance, the addition of carboxymethyl cellulose during cellulose formation increases the water holding capacity to 1000 times its dry weight.

Sony Corporation, in conjunction with Ajinomoto developed the first audio speaker diaphragms using microbial cellulose (US Patent 4,742,164). The unique dimensional stability of microbial cellulose gives rise to a sound transducing membrane which maintains high sonic velocity over a wide frequency ranges, thus being the best material to meet the rigid requirements for optimal sound transduction. In time, it is expected that larger speaker diaphragms will be made of microbial cellulose.

In the early 1980's Johnson and Johnson pioneered in exploratory investigations on the use of microbial cellulose as a liquid loaded pad for wound care (US Patent 4,655,758, 4,588,400). Microbial cellulose has been investigated as a binder in papers, and because it consists of extremely small clusters of cellulose microfibrils, this property greatly adds to strength and durability of pulp when integrated into paper. The unique gel-like properties of microbial cellulose, combined with its complete indigestibility in the human intestinal tract, make this an attractive food base.

BC can be used as biomaterials for blood-contacting devices as it does not induce plasma coagulation to any great extent. In comparison with PET and PTFE, the BC material performed very well and was found to induce the least and slowest activation of the coagulation cascade [24].

Conclusions

In the era of declining forests, global climate changes, continuing expansion of industrialization, it is reasonable to consider the consequences of an alternative source of cellulose. This is an excellent opportunity to take advantage of new development since the intense interest in bacterial cellulose products. The demand for bacterial cellulose outpaces the supply largely because of lack of investment in research and development to optimize production on a large scale.

Can bacterial cellulose compete with traditional cellulose sources? This question remains unanswered until commercial scale up and fermentation development become mature. In the meantime, specialty products could be economically produced from bacterial cellulose. Perhaps this brief excursion into bacterial cellulose will provide a stimulus for the renewal of interest in combining traditional cellulose technology with biotechnology to create growth in a new and exciting field.

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