Korean J. Chem. Eng., 34(6), 1591-1599 (2017)
DOI: 10.1007/s11814-017-0121-2
pISSN: 0256-1115
eISSN: 1975-7220

INVITED REVIEW PAPER

Recent advancements in bioreactions of cellular and cell-free systems: A study of bacterial cellulose as a model

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(*Received 2 January 2017 • accepted 25 April 2017*)

Abstract−Conventional approaches of regulating natural biochemical and biological processes are greatly hampered by the complexity of natural systems. Therefore, current biotechnological research is focused on improving biological systems and processes using advanced technologies such as genetic and metabolic engineering. These technologies, which employ principles of synthetic and systems biology, are greatly motivated by the diversity of living organisms to improve biological processes and allow the manipulation and reprogramming of target bioreactions and cellular systems. This review describes recent developments in cell biology, as well as genetic and metabolic engineering, and their role in enhancing biological processes. In particular, we illustrate recent advancements in genetic and metabolic engineering with respect to the production of bacterial cellulose (BC) using the model systems *Gluconacetobacter xylinum* and *Gluconacetobacter hansenii*. Besides, the cell-free enzyme system, representing the latest engineering strategies, has been comprehensively described. The content covered in the current review will lead readers to get an insight into developing novel metabolic pathways and engineering novel strains for enhanced production of BC and other bioproducts formation.

Keywords: Biochemical Reactions, Biological Systems, Genetic Manipulation, Metabolic Engineering, Bacterial Cellulose

INTRODUCTION

Conventional biochemical reactions and biological processes have been inspired by synthetic biology owing to the advancements in genetic and metabolic engineering. Synthetic biology refers to the application of engineering-driven approaches to accelerate the design-build-test loops for reprogramming existing and constructing novel biological systems [1] because designing a complete cellular system is often difficult and laborious. For example, the economical production of artemisinin in yeast accounts for over 150 person-years of work, and that is still ongoing [2]. Although it is still at the early stage of development, its potential advantages are quite clear. The ability to reliably and rapidly engineer biological functions will advance applications in various fields such as medicine, biotechnology, and green chemistry [3-5]. Synthetic biology mainly focuses on the construction of genetic circuits, biological modules, and synthetic pathways, which are then implemented into genetically reprogrammed organisms. This technology, however, is limited by incomplete knowledge of living processes, the complexity of living cells and cellular systems, and the intricacy of growth and adaptation of living organisms [1,2]. To overcome these discrepancies, several efforts have been made in systems biology to better understand living cells and build whole-cell models [6,7]. System biology is an emerging applied approach that uses computational and mathematical modeling to explore complex interactions within the complex biological systems. It mainly focuses on modeling and discovering emergent properties of cells, tissues, organs, and organisms functioning as a system by using metabolic engineering and cell signaling networks [8]. To date, it has presented numerous breakthroughs such as production of non-natural products, for example, 1,4-butanediol [10].

Bacterial cellulose (BC) is a biopolymer produced by specific genera of bacteria (*Acetobacter*, *Rhizobium,* and *Agrobacterium* etc.), certain algae [11-13], and cell-free system [14,15]. Although BC has the same chemical structure as that of plant cellulose, it has considerably superior physical, mechanical, and biological properties [16]. BC has received tremendous attention for its unique structural, physico-mechanical, and biological properties and a broad range of practical and potential applications in different industries [17-20]. BC synthesis by microbial cells is regulated by the cellulose synthesis operon, which is a functional unit of genomic bacterial DNA and contains multiple genes (Table 1). Two operons, the Acetobacter cellulose synthesis operon (*acsABCD*) and BC synthesis operon (*bcsABCD*), which are homologous functional units that encode the essential proteins involved in BC synthesis, have been identified in *A. xylinum* from American Type Culture Collection (ATCC) 53582 and 1306-3, respectively [21,22]. Kawano et al. reported that the downstream region of the cellulose synthase operon contains the gene *bglxA*, which encodes β-glucosidase that hydrolyzes more than three β-1,4-glucose units; therefore, any dis-

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Table 1. Illustration of function of various genes involved in BC production

Microorganism	Genes	Gene function	References
G. xylinus BPR2001	*dgc1*, *dgc2*, and *dgc3*	Activating BC synthesis	[28,29]
G. xylinus	bcs operon	Synthesis, crystallization, and extrusion of cellulose	[30]
G. xylinus	BcsA	Polymerization of uridine diphosphate glucose (UDP-Glc)	[31]
G. xylinus	BcsB	Reversibly binds large quantities of c-di-GMP which activates the AxCESB, activator of cellulose synthesis	[32,33]
G. xylinus	BcsC	Crystallization of cellulose fibrils	[22]
G. xylinus	BcsD	Extrusion of cellulose through membrane	[22]
G. xylinus	pdeA1	PdeA1 is phosphodiesterase A, and degrades cdi-GMP	[34]
G. xylinus	dgc1	Dgc1 is a di-guanilate cyclase that synthesizes c-di-GMP	[34]
A. xylinum	CMCase	BC synthesis	[35]
E. coli	*β*-Glucosidase	BC synthesis	[36]
E. coli	ORF2	Formation of highly ordered structure of BC	[37]
E. coli and *S. enterica*	*yhjQ* and *bcsZ*	Cellulose biosynthesis	[38,39]

ruption in this gene would significantly lower BC production [23]. It is well established that the yield of BC by *A. xylinum* is limited due to the formation of ketogluconate [24]. This issue was overcome through the development of ketogluconate-negative strains of *Acetobacter*, which resulted in substantial improvement in the production of BC at a much lower rate of glucose consumption [25]. Colvin was the first to utilize the cell-free approach to BC synthesis using cell-free extract obtained from *A. xylinum* and providing glucose and ATP as energy sources [26]. Recently, Ullah et al. reported on in vitro synthesis of bio-cellulose using a cell-free system developed from a single microbial strain and characterized for various structural and physico-mechanical properties [14,15]. These studies suggest that cellulose synthesis by microorganisms takes place through a series of biochemical reactions. Because a cell-free system operates in the absence of external barrier, such as a cell wall or membrane, it avoids the complexity caused by these barriers and bypasses the genetic regulation required for direct access to the inner machinery of the cell [1,27]. Compared with an in vivo system, the more controlled and simplified design has inspired rapid development of engineering foundations for cell-free systems in the last decade.

Herein, we discuss recent developments in biochemical reactions that mediate BC synthesis and examine recent progress in cell biology, systems biology, and metabolic engineering with respect to improved BC production. Besides describing BC synthesis by using model systems such as *A. xylinum* and *G. hansenii*, we portrayed the latest engineering strategy of cellulose production using a cell-free system. We compared conventional microbial production with recent advancements in BC synthesis through genetic and metabolic engineering strategies. We believe that this review will provide insights to the develop novel metabolic pathways and engineer new strains for enhanced BC production and other products formation.

BIOCHEMICAL AND BIOLOGICAL REACTIONS

Conventionally, biological systems are examined by isolating a small subset of biological components, which can then be probed individually to better understand their structure and function. This approach is based on the assumption that the interaction of biochemical components occurs in isolation, which results in discrete cause-and-effect relationships. However, understanding cellular biology at the DNA, RNA, and protein level has made it clear that biological processes do not occur in isolation, but rather within the context of complex systems of components that constitute metabolic pathways, and which are regulated by intricate networks of feedback loops [1,27]. These systems and pathways operate on a variety of levels, from RNA polymerase that interacts with a DNA strand to start DNA transcription, to a signal-transduction pathway within a cell, to complex interactions between systems of organisms [40]. With progressive development and exploration, our appreciation of the complexity of interactions within and between metabolic systems has grown. We now recognize that conventional scientific approaches severely limit our ability to understand the complex biological phenomena and interactions within and between living cells.

Consequently, the conventional reductionist approaches to understanding biological processes have recently been replaced by more advanced strategies such as synthetic biology, cell-free technology, and metabolic and genetic engineering [1,27,41]. The production of chemicals and biological molecules by living systems can be broadly classified into three categories: (1) synthesis of simple and naturally-occurring chemicals through conventional metabolic engineering strategies; (2) synthesis of naturally-occurring chemicals by complex biochemical reactions and molecules; this requires efficient production of the products precursor followed by the chemical synthesis of the product; (3) biological production of non-naturally-occurring chemicals and modification of natural biomacromolecules and bioproducts. Production of new chemicals, or improving the quality and production of existing ones by microbial cells, is a more feasible approach because of simpler genetic makeup and amenable growth rates compared with those of plant and animal systems. Therefore, the hand-in-hand development of computational technology, protein and enzyme engineering, and tools for genetic manipulation has provided the foundation for the production of green chemicals and fuels [42,43].

Metabolic engineering has proven to be a powerful tool for the sustainable production of a variety of chemicals and products. Exploration of microbial systems, biological and biochemical pathways, and metabolic mechanism has generated a wealth of valuable genetic information. Advancements in this technology and its application in cellular metabolism and biochemistry have enabled the design of novel metabolic pathways that do not exist in nature or enhance the efficacy of any particular natural biochemical pathway. Expansion or manipulation of native metabolic pathways is the first step for the establishment of a novel metabolic pathway [44]. Thus, the obstacles to enhancing the production of a desired chemical, such as microbial cellulose, by bacterial or algal species can be overcome by using the tools offered by synthetic biology and protein engineering. However, despite the establishment of a number of metabolic routes for the production of bacterial cellulose and other useful chemicals, the challenge of industrializing such products, remains. Commercialization of bacterial cellulose and its products needs to overcome several hurdles. First, the microbial cells producing cellulose need to be systematically engineered to improve titer, yield, and volumetric productivity. The manufacturing cost of this process needs to be minimized by optimization and scale-up. Lastly, the separation process needs to be economical and efficient [27,44].

Despite major milestones achieved by the use of genetic and metabolic engineering, obstacles remain in production of non-natural chemicals. These can be overcome by identifying the enzymes capable of carrying out reactions similar to those of naturally occurring chemical pathways. This is what constitutes cell-free technology.

BACTERIAL CELLULOSE

1. Advantages of Bacterial Cellulose

Bacterial cellulose (BC), an important biopolymer produced by aerobic bacteria, has received immense consideration owing to its unique features and broad spectrum applications [45]. It represents the purest form of cellulose compared to plant cellulose and has unique structural, physico-chemical, mechanical, and biological features. Its unique features such as high water holding capacity (WHC), slow water release rate (WRR), higher crystallinity and tensile features, ultrafine fiber network, and moldability into three-dimensional structures bestow BC with high potential value [17, 45,46]. Its fibril network is comprised of well-arranged three-dimensional nanofibers which bless BC with high surface area and porosity [45].

BC has been widely applied in the medical field, electrical instruments, food ingredients, and separation processes. The most promising applications of BC are in the medical field where it is extensively used as wound dressing material, burns, artificial skin, vascular grafts, scaffolds for tissue engineering, tissue regeneration, and artificial blood vessels [17,18,47]. It has been used for the preparation of several commercial products such as tires, headphone membranes, high performance speaker diaphragms, high-grade paper, makeup pads, diet-food, and textiles etc. [48]. Furthermore, it is used as carrier in drug delivery systems, enzyme immobilization, and ion exchange membrane and as biodegradable and biocompatible sensors and actuators [49-51].

Economically feasible production of BC is one of the major challenges of BC research since its first report. Production of BC

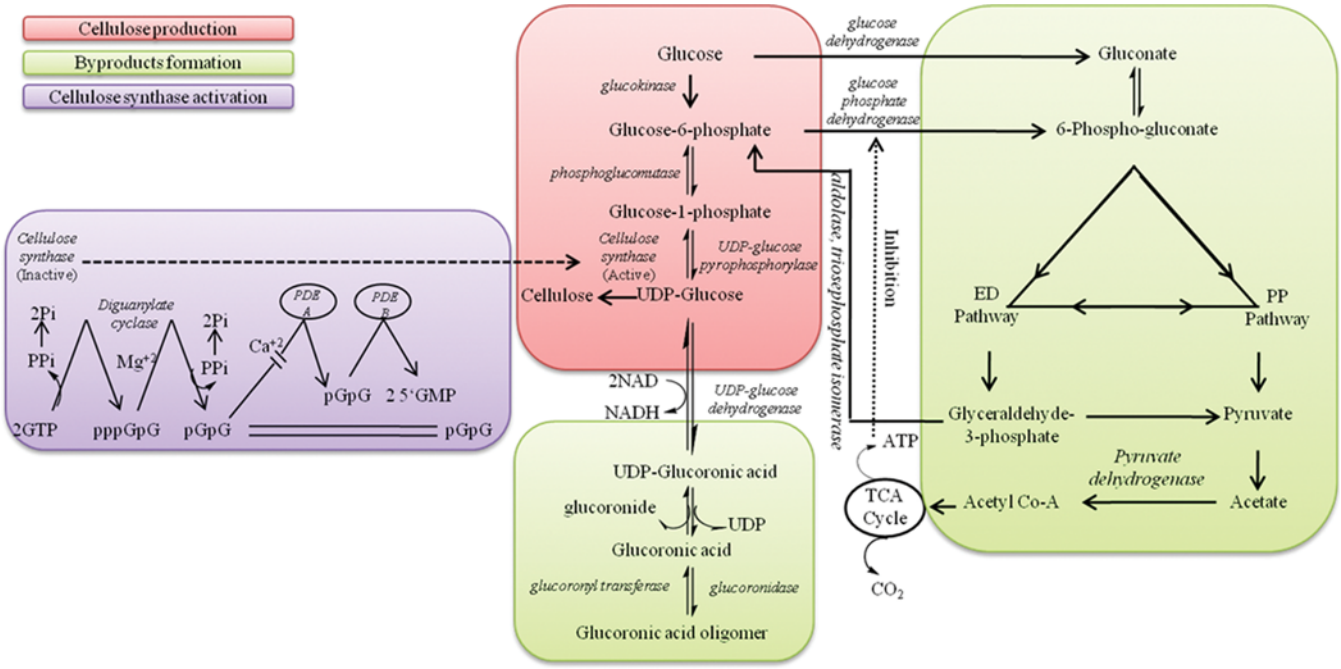

Fig. 1. Schematic representation of bio-cellulose production by the cell-free system through the principal glucose pathway, and other pathways interconnected through the activation of cellulose synthase. The scheme was developed based on literature review and results of the LC-MS/MS LTQ Orbitrap analysis. The figure has been reproduced from "Carbohydrate Polymers 132, 286 (2015)" with the permission from "Elsevier".

from a chemically defined medium is very expensive, thus limiting its pilot-scale production and value-added commercial applications. Therefore, extensive efforts have been made to improve the process efficacy, develop more advanced fermenters and cultivation strategies, develop new microbial strains, exploring and utilization of raw materials as carbon source, supplementation of medium with other components etc. with the aim to economically produce BC [52-56] (Table 2).

2. Production of Bacterial Cellulose

The structural features and synthetic mechanisms of BC are well established [14,15,57,58]; the mechanism of its biosynthesis is controlled by enzymes [14,52] (Fig. 1). During BC biosynthesis, glucose monomers unite to produce chains inside the bacterial cells. Later, these chains project to the external medium through small pores on the bacterial cell wall [14,58]. BC has been produced via oxidative fermentation using a variety of synthetic and non-synthetic media. *Gluconacetobacter xylinus* is the most studied and effective BC producer [59]. Cellulose biosynthesis occurs via biopolymerization of uridine diphosphate (UDP)-glucose nucleotide sugar precursors [60]. A series of genes involved in the synthetic process encode the catalytic subunit of cellulose synthase that binds UDP-glucose to ensure a supply of monomers for polymerization. The synthetic strategies of BC production can be classified into two categories as explained below.

2-1. Native Strategy

Since its initial discovery, BC has been synthesized through a number of synthetic routes using synthetic as well as natural media. The morphology and physiology of BC are greatly controlled by the strategy of synthesis. The overall biosynthesis is regulated by a complex set of genes and enzymes. The native strategies encompass simple production processes without the genetic alteration involved in synthetic pathways. Bacteria grow on culture media that contain either glucose or glucose precursors as the primary source of sugar. The culture is maintained under static conditions or agitation, depending upon application requirements. During static culture, the culture medium inoculated with bacterial colonies is maintained for 7 to 10 days or longer, and BC is synthesized in the form of pellicles. Conversely, agitation cultures are kept at agitation speeds ranging from 100 to 500 revolutions per minute (rpm) and produce BC in the form of small pellets [61-63].

The genetic makeup used for both synthetic strategies is similar. The gene assembly responsible for the production steps remains the same and synthesis occurs in a stepwise order. Glucose monomers combine through glycosidic linkages and produce small chains inside the cytoplasm. These small chains of cellulose protrude out of the bacterial cell wall and combine to form nano- and microfibrils. The fibrils thicken into macro-fibrils and arrange together to produce a web-shaped fibril network. Because the BC biosynthetic process occurs in aerobic bacteria, it takes place at the air-water interface. A sheet of BC is produced at the surface of the medium in static culture. In submerged agitated culture, air is supplied through continuous aeration of the medium, which enables submerged BC production. Because of continuous agitation, BC is produced in the form of small pellets. The structural features of these two methods are similar [61,62,64]. Bacterial cellulose synthesis is a four-step process that involves (a) activation of monosaccharides via formation of sugar nucleotides, (b) assembly of repeating units through polymerization, (c) concurrent addition of

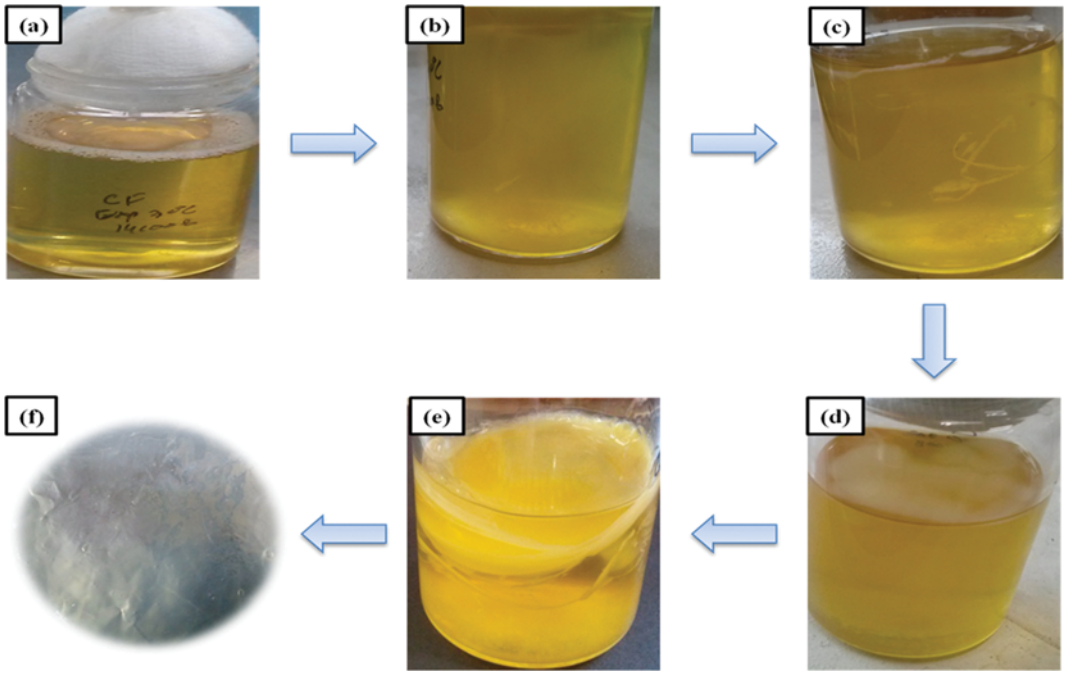

Fig. 2. Illustration of bio-cellulose production by the cell-free system. (a) Incubation of the cell-free enzyme solution with the glucose source, (b) formation of fibrils, (c) self-aggregation of fibrils, (d) formation of bio-cellulose pellicles, (e) formation of the bio-cellulose sheet through self-aggregation of pellicles, and (f) the harvested and washed bio-cellulose sheet. The figure has been reproduced from "Carbohydrate Polymers 132, 286 (2015)" with the permission from "Elsevier".

contained acyl groups (if present), and (d) excretion through the cell wall/membrane into the extracellular environment [65]. Each step of the synthesis is regulated by the availability of appropriate substrates, enzymes, and/or cofactors. A deficiency in any of the components retards or abolishes the synthesis pathway.

2-2. Engineering Strategies

Advancements in biotechnology enable researchers to manipulate the genetic makeup and synthetic mechanisms of biological processes. Altering genetic operation in cellular systems enables us to introduce additional features into a product, as well as modify its production, yield, and structural features. The most important step has been shifting production into a cell-free system, in which the entire course of BC production is performed using cell-free enzymatic processes [14]. Unlike cellular production processes, a cell-free system synthesizes glucose chains exogenously in the culture medium [14]. A cell-free system can also operate effectively under anaerobic conditions. Ullah et al. developed a cell-free system for the synthesis of bio-cellulose from *G. hansenii* PJK [14] and successfully synthesized bio-cellulose through a non-cellular enzymatic system (Fig. 2). The extract obtained from disrupted bacterial cells was confirmed for the presence of all essential enzymes and cofactors [14].

The establishment of cell-free enzyme-based bio-cellulose synthesis is an important step towards mediating the actual pathway or introducing variations to enhance production, stop the formation of byproducts, or alter the physiological and morphological behavior of the synthesized bio-product. For example, some studies show that alteration in certain enzymes can affect the structure and production of BC as well as of other bacterial polysaccharides.

Nakai et al. (2013) reported that disruption in the cellulose (carboxymethylcellulase) gene generates irregular packing of *de novo* synthesized fibrils in a cellulose-producing bacterium *Gluconacetobacter xylinus*. This alteration led to a remarkable reduction in the production of BC. In addition to reduction in productivity, structural analysis also confirmed highly twisted fibrils in the mutated cells, and particulate accumulation was observed in the culture medium [66]. Similarly, some studies have shown that the addition of endo-glucanase to culture wells causes overproduction of cellulose by *G. xylinus*, which enhances cellulose yield [67,68]; however, the production of cellulose fibrils is greatly reduced by the addition of antibodies to recombinant glucanase [69].

Some studies have observed that the cellulose biosynthetic pathway is altered by the presence of byproducts. In 2011, Ha et al. reported high BC production by a cellular system in which water-soluble oligosaccharides (WSOS) were added to the culture medium [58]. These WSOS were found to be a byproduct of BC and affected the overall BC production process [12,62]. Recently, Ullah et al. reported that the production of bio-cellulose was increased by adding cofactors to the cell-free enzyme extract of a cellulose-producing bacterial strain [14].

These studies on bio-cellulose and other exopolysaccharides suggest that biotechnology is advancing towards developing systems where pathways may be completely controlled, leading to more specific products. This approach could potentially eliminate byproduct formation and increase manageability of production, yield, and physico-mechanical properties of the product.

Table 2. Production of BC in static and agitation cultures with variety of BC producing strains, carbon sources, and supplementary materials. The table has been reproduced from "Carbohydrate Polymers 98, 1585 (2013)" with permission from "Elsevier"

Microorganism	Carbon source	Supplementary materials	Culture time (days)	Yield (g/L)	Cultivation mode	References
G. xylinus, *Trichoderma reesei*	Glucose	Fiber sludge	14	6.23	Static	[70]
G. xylinus	Glucose	Cellulosic fabrics	14	10.80	---	[71]
G. medellensis	Glucose	None	14	4.50	---	[72]
G. hansenii PJK (KCTC 10505 BP)	Glucose	Glucuronic acid oligomers	10	7.4	---	[58]
G. xylinus (PTCC, 1734)	Glucose	Date syrup	14	40.35	---	[73]
G. Persimmonis (GH-2)	Glucose	Fructose, beef extract	14	5.14	---	[74]
G. xylinus strain (ATCC 53524)	Sucrose	None	4	3.83	---	[75]
G. hansenii PJK (KCTC 10505 BP)	Waste from beer culture	None	14	8.6	---	[76]
G. xylinus strain (K3)	Mannitol	Green tea	7	3.34	---	[77]
G. xylinus (IFO 13773)	Sugar cane molasses	None	7	5.76	---	[78]
A. xylinum (ATCC 700178)	CSL-Fru	Carboxymethylcellulose	5	13.00	Agitated	[79]
A. xylinum	CSL-Fru	Sodium alginate, agar, carboxymethylcellulose	5	7.05	---	[80]
Gluconacetobacter sp. (RKY5)	Glycerol	None	6	5.63	---	[81]
A. xylinum (BPR2001)	Molasses	None	3	7.80	---	[82]
A. xylinum (BPR2001)	Fructose	Agar/Oxygen	3	14.10	---	[82]
G. hansenii PJK (KCTC 10505 BP)	Glucose	Ethanol	3	2.50	---	[83]

CELLULAR MODELS FOR BACTERIAL CELLULOSE PRODUCTION

The synthesis of BC is a complex biochemical process. During synthesis, glucose monomers are converted into cellulose through a series of biochemical reactions mediated by specific enzymes. Because cellulose pellicles reside on the surface of the culture medium, BC production does not depend on volume, but rather on the surface area of the culture. One study proposed a model in which BC would be secreted into the exterior of the cell. This model would be comprised of a row of terminal complexes (TC) using sodium dodecyl sulfate freeze-fracture labeling technique aided by transmission electron microscopy [34]. Until the 1990s, few genetic studies examined BC production; however, several trials that aimed to increase production used genetic modifications as well as medium and bacterial strain enhancement. To date, several microorganisms are known to produce BC. Below, we describe two model organisms, *Gluconacetobacter hansenii* and *Gluconacetobacter xylinus*, used for the synthesis of bacterial cellulose.

1. *Gluconacetobacter hansenii*

Gluconacetobacter hansenii is a Gram-negative bacterium that produces and secretes highly crystalline cellulose into the growth medium. It has been extensively used as a model organism for the study of cellulose synthesis. The synthesis of cellulose in *G. hansenii* involves the formation of β-1,4 glucan chains via polymerization of glucose units by a multi-enzyme cellulose synthase complex (CSC) [14]. Acetyl-coenzyme A synthetase (AcsA) is the catalytic subunit of cellulose synthase enzymes in the CSC; AcsC is required for the secretion of cellulose from a microbial cell into the culture medium. However, very little information is available about other proteins required for the assembly of crystalline cellulose. The secreted β-1,4 glucan chains further assemble into higher degrees of crystallinity and form a highly ordered structure. Cellulose synthesis occurs in four distinct reactions: sub-elementary fibrils, elementary fibrils, micro-fibrils, and ribbons. Electron microscopic analysis demonstrates that the surface of a *G. hansenii* cell has 50-80 pore-like sites located in a row along the axis of the cell; each of these sites is thought to secrete a 1.5 nm sub-elementary fibril composed of 10-15 glucan chains [84,85]. The sub-elementary fibrils produced from multiple extrusion sites aggregate to form a 3.5 nm elementary fibril [86]. The extrusion sites are grouped together. Such proximity and organization may facilitate co-crystallization of adjacent elementary fibrils to form 6-7 nm micro-fibrils, which assemble further to form bundles. The organized bundles of microfibrils form the twisting cellulose ribbon (40-60 nm), which aggregates into a cellulose pellicle produced at the surface of the culture medium [14,15].

Deng et al. used Tn5 transposon insertion mutagenesis to visually examine the formation of cellulose pellicles in the growth medium of 763 individual colonies of *G. hansenii* and found that 85 colonies formed cellulose with altered morphology [87]. Further, X-ray diffraction analysis of these altered colonies revealed that two of these colonies produced cellulose with substantially lowered crystallinity compared with that produced by wild type. Molecular analysis revealed that the gene disrupted in one of the two mutants encoded a lysine decarboxylase, whereas the other gene coded for alanine racemase. Solid-state nuclear magnetic resonance (NMR) revealed that the cellulose produced by the two mutants contained an increased amount of non-crystalline cellulose and monosaccharides associated with non-cellulosic polysaccharides compared with cellulose produced by the wild type. Analysis of monosaccharides in the cellulose produced by the two mutants demonstrated higher percentages of galactose and mannose. An analysis by field emission scanning electron microscopy (FE-SEM) revealed that the cellulose produced by these mutants was inconsistently distributed, with some regions appearing to contain deposition of non-cellulosic polysaccharides. However, the width of the cellulose ribbon produced by the mutants was comparable to that produced by wild type [87].

2. *Gluconacetobacter xylinus*

Gluconacetobacter xylinus, formerly known as *Gluconacetobacter xylinum*, is a Gram-negative bacterium that has been extensively used as a model organism to study microbial cellulose [86]. *Gluconacetobacter xylinus* abundantly produces a pure crystalline form of cellulose within the bacterial cell and secretes it into the culture medium. The secreted cellulose forms a pellicle at the air-medium interface in static culture [12,14]. Strap et al. indicate that the pellicle cellulose may be used as storage and utilized as a carbonfoodceucose a sugar source he bacteriumntence a bit; hence, I offer the following edit:ding in this sentence is a little close t source by microbial cells under starvation conditions [88]. Its utilization as food

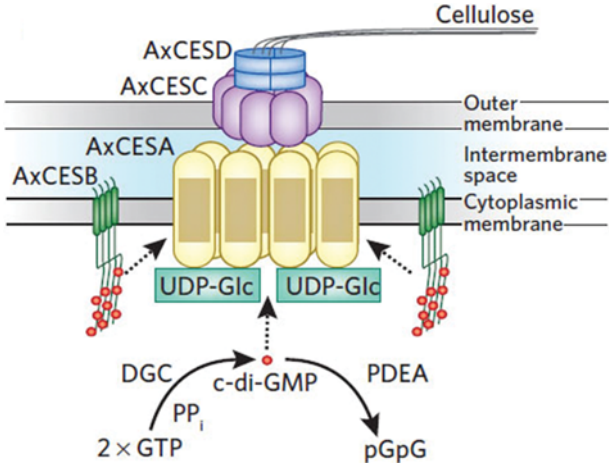

Fig. 3. Cellulose synthesis in *A. xylinum*. Schematic model of the cellulose synthesizing machinery $(A_2B_2)_4C_8D_8$ in *A. xylinum*. AxCESA, with eight to ten putative transmembrane regions (TMDs), is located in the cytoplasmic membrane and has been shown to bind the substrate UDP-Glc. Free c-di-GMP is thought to allosterically activate AxCESA activity. The majority of the cellular c-di-GMP is bound to AxCESB, which is in close proximity to AxCESA. Diguanylate cyclase (DGC) catalyzes the synthesis of c-di-GMP, whereas phosphodiesterase A (PDEA) degrades the molecule. Prediction tools suggest that AxCESD is a soluble protein and AxCESC contains one TMD. Endler et al. proposed that AxCESC is located in the outer membrane, whereas AxCESD might be located in the intermembrane space or extracellularly. The figure has been reproduced from "Nature Chemical Biology 6, 883 (2010)" with the permission from "Nature Publishing Group".

would require the presence of exo- and endo-glucanases, both of which are detectable in the culture medium [89]. Under unfavorable conditions, such as decrease in water content, variation in pH, presence of toxic substances, and UV radiation, the cellulose pellicle can provide protection to the microbial cells embedded inside [88]. *G. xylinus* produces the cellulose I allomorphic structure under static conditions, in which the linear β-1,4 glucan chains are oriented in parallel with each other with the same polarity as that of the cellulose II structure; the β-1,4 glucan chains of cellulose II are arranged randomly, making the structure of cellulose II more amorphous and easily degradable [90]. BC synthesis in *A. xylinum* is shown in Fig. 3.

Pellicin (([2E]-3-phenyl-1-[2,3,4,5-tetrahydro-1,6-benzodioxocin-8-yl]prop-2-en-1-one) is a useful tool for the study of cellulose biosynthesis in *G. xylinus*. Strap et al. conducted a chemical genetic screening of 10,000 small molecules and found that pellicin completely abolished cellulose production in *G. xylinus*. Cells were grown in the presence of pellicin and cell growth rate was found to be 1.5-times faster than that in the untreated cells; additionally, pellicin caused cell elongation [91]. However, measurement of cellulose synthesis in vitro showed that cellulose synthase activity was not directly inhibited by pellicin. In contrast, when cellulose activity was measured in pellicin-treated cells, the rate of cellulose synthesis increased eight-fold compared to untreated cells. This phenomenon was also apparent in the rapid production of cellulose when cells grown in the presence of pellicin were washed and transferred to a medium lacking the inhibitor. Thus, it was concluded that the rate of cellulose production could not be accounted for by the growth rate of microbial cells. In addition, pellicin was not found in the intracellular contents of *G. xylinus*. Moreover, pellicin was found to exert its effect extracellularly by interfering with crystallization of pre-cellulose tactoidal aggregates. This resulted in enhanced production of cellulose II (the less crystalline allomorph) as evidenced by the ratio of the acid-insoluble to acid-soluble product in *in vitro* assays and confirmed by in vivo SEM and X-ray powder diffraction (XRD) analyses.

CURRENT PROGRESS IN BACTERIAL CELLULOSE PRODUCTION

BC production by microbial cells, excretion from cells to the culture medium, structural organization of fibrils, and mechanism and regulation of BC synthesis have been studied extensively. However, BC synthesis operons have not been cloned, and no microbial cells were transformed with the BC synthesis gene produced BC [45]. Until date, the genetic modifications conducted with respect to BC production have not addressed the direct self-amplification of BC genes.

Ketogluconate is a byproduct formed during the synthesis of BC from glucose or sucrose by *G. hansenii* or *G. xylinus*. The formation of ketogluconate is undesirable because it lowers the pH of the growth medium, reduces cell growth rate, and adversely affects BC production. This has led to the development of a mutant with restricted ketogluconate synthesis using genetic engineering [92]. Generally, this can be achieved through ultraviolet (UV) mutagenesis; however, a problem with this approach is the decreased fluidity of the culture broth, which adversely affects BC production. Moreover, this mutant strain secretes acetan, which is viscous and water soluble, into the culture broth while BC production is occurring, making the medium more viscous and unfavorable for microbial cell growth. Further, acetan utilizes uridine diphosphate glucose (UDP-Glc) for self-synthesis. UDP-Glc is a starting material for cellulose synthesis and thus lowers the overall yield of BC. To prevent the synthesis of acetan, Ishida et al. developed an acetan-nonproducing mutant strain, EP1, from the parental *G. xylinus* BPR2001 [93]. However, BC production by the mutant EP1 decreased under shaking culture conditions; although under static conditions, its productivity was the same as that of the parent strain. Subsequently, the shaken culture broth of EP1 was found to contain large aggregates of cells and BC compared with that of the parent strain. These observations suggest that acetan raised the viscosity of the culture medium and prevented the coagulation of cells and BC, which ultimately resulted in an increase in BC production by *A. xylinus*. A strain with a mutation in *dgc1*, a gene known for activating BC synthesis, was developed from the parent strain BRP2001 through genetic modification and was expected to produce BC at lower rates. Unexpectedly, BC production by the mutant strain was comparable to that of the parent strain in both static and shaken cultures. Surprisingly, BC production was increased by 36% compared to that of the parent strain in a stirred-tank reactor [92]. Despite the disruption in *dgc1*, BC production increased; this can be correlated to the combinatorial effect of the two counterparts of *dgc1*, *dgc2* and *dgc3*, which are functionally similar to *dgc1*and are more stimulatory for BC production. Hence, a mutation in *dgc1* does not significantly lower BC production.

CONCLUSIONS AND BENCHMARK FUTURE PROSPECTS

Over the years, considerable focus has been on understanding microbial BC production because of its broad range of industrial applications. Several microbial and algal species have been identified for BC production. Further, several genetically engineered strains have been developed for improving BC synthesis. The enzymes involved in BC synthesis have been characterized at both protein and gene levels.

Recent advances in genetic and metabolic engineering, as well as in systems and synthetic biology, offer new avenues to further improve BC production. Systems biology is progressing towards managing complex systems more efficiently. The key to successful engineering or reengineering of biological systems is based on our understanding of their complexity. Currently, cell-free technology is being used to study the metabolism of several complex biochemical pathways. Using cell-free biology or genetic and metabolic engineering to understand BC synthesis will lead to further innovations and development of novel metabolic pathways and genetically engineered strains for enhanced BC production. The assembly of new structures, whether single genes, enzymes, cells, or complex organisms is a complicated process dependent on the interactions of its components, which is vital for the proper functioning of a new system. This can be made possible through advancements in biochemical and cellular systems and will ultimately be

based on the strategies of genetic and metabolic engineering, synthetic and systems biology, and cell-free technology.

ACKNOWLEDGEMENT

This work was supported by the Basic Science Research Program through the National Research Foundation (NRF) of Korea, funded by the Ministry of Education, Science and Technology (NRF-2014-R1A1A2055756).

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