High-Producing Strains, Cellulose Synthase and Regulation of Bacterial Cellulose

Heng Zhang, Chunlin Zhu, Yang Huang, Yinghong Lu and Dongping Sun

Chemicobiology and Functional Materials Institute, Nanjing University of Science and Technology, Nanjing 210094, China.

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Bacterial cellulose which is produced by several bacteria has played an important role in industry and medicine area based on its unique structural, functional, physical and chemical properties. However, both the scale and cost of production impede a wide range of applications of bacteria cellulose. The paper concludes the current state of knowledge of high-producing strains and discusses the mechanism of cellulose biosynthesis. Cellulose synthase, as the key enzyme, is identified from the structure, the organization of gene and the regulation. The cyclic diguanylic acid regulatory system is important in the progress of bacterial cellulose biosynthesis.

Key words: Bacterial Cellulose, Metabolic Network, High-producing Strains, Cellulose Synthase, Regulation.

Cellulose is the most abundant polysaccharide on earth, which is the main structural element of plant cell wall. In 1886, Brown first reported an extracellular gelatinous mat, named bacterial cellulose (BC), synthesized by Bacterium aceti when fermenting acetic acid. He verified this substance and determined that the chemical component of this material was same as plant-derived cellulose. Subsequently, scientists found that bacterial cellulose (BC) is a biological nanomaterial synthesized by several microorganisms belonging to the genera Acetobacter, Agrobacterium, Achromobacter, Aerobacter, Azotobacter, Pseudomonas, Salmonella, Escherichia, Rhizobium and Sarcina ventriculi with intensive studies on bacterial cellulose synthesis\(^2\). The exceptional structure of higher crystallinity and higher polymerization endows BC with superior properties, such as high purity, high water retention, high tensile strength, ultrafine reticulated structure and excellent biodegradability\(^3\). Resulting from these unique properties, BC has found a wider applications in paper, textile, food industries, and is an ideal biomaterial in cosmetics and medicine industries as a renewable natural resource\(^4\). With the increase of demand for BC, it is urgent to improve BC production at the lowest cost possible. The strategies mainly include screening of high-producing strains, optimization of metabolic process, regulatory of synthesis, and so on. Therefore, a major goal of BC research has been used to enhance the microbial conversion of glucose into cellulose. The process analysis of bacterial cellulose production provides a platform for understanding the synthesis of BC.

In the present review, we have summarized various high-producing strains and the recent
progress and regulation made to enhanced production of BC. Accordingly, this review will provide ideas for the development of novel strategies for BC production.

Screening of high-producing strains

Cellulose is the most abundant organic polymer produced by plants, some animals, algae, fungi, flagellates and bacteria [6]. In 1998, Jonas et al. [7] gave an overview about the bacteria which can secrete bacterial cellulose. It was reported that so many strains are in conditions to produce cellulose, such as the strains from the genera Gluconacetobacter (formerly Acetobacter), Agrobacterium, Pseudomonas, Rhizobium and Sarcina. Among these, Gluconacetobacter is the most efficient bacterial cellulose producing strain and used as model organism in research and commercial production, whose high BC yield reaching to around 7-8 g/L in industrial scale (Fig.1) [8].

The choice of production strains is critical for industrial production. Two strategies are proposed for the isolation of high producing strain: (1) Isolation high yield strain from environment; (2) Mutation breeding in the laboratory. The first strain producing bacterial cellulose was isolated from home made vinegar [9]. Yang et al. [8] has isolated a strain identified as Gluconacetobacter intermedius CIs26 from rotten mandarin fruit with a yield of 7.2 g/L. And this strain appeared to have potential in BC manufacture on an industrial scale. Tanskul [10] has isolated one genus namely Rhodococcus sp. MI 2 from ripe fruits and vegetables. Castro [11] isolated a new acid-resistant strain of Gluconacetobacter medellensis from the fermentation of Colombian homemade vinegar. The optimum yield of this strain was 4.5 g/L at pH 3.5, which was highly tolerant to low pH conditions. Due to bacterial cellulose producing strains can utilizet glucose effectively to form cellulose, most of the strains are separated from materials with high sugar content. Gluconacetobacter sp. was found in the production of grape wine. The BC produced by this strain has indicated a high oxygen barrier but low water barrier [9]. Besides, scientists screen efficient strains from mutants induced by physical and chemical methods. Wu [8] has got a Gluconacetobacter xilinus strain through mutagenesis induced by high hydrostatic pressure treatment. And the strain showed the highest BC yield increased from 106.03 g/L to 158.56 g/L (in the wet state).

Analysis of BC Synthesis

Bacterial cellulose is the end product of carbon metabolism. The first step of BC producing is the polymerization of several 1,4-glucan chains through the cellulose precursor, UDPGlc (uridine diphosphoglucose), at an extrusion site on the cell surface. After that, the nascent individual glucan chains are forced to form characteristic ribbons, which later secreted into the surrounding medium (Fig.2) [8]. This process is closely related to hexose monophosphate pathway (HMP), the Embden-Meyerhof-Parnas Pathway (EMP) and the Tricarboxylic Acid Cycle (TCA) [15], which involves a large number of complexes of catalytic and regulatory proteins. It is still a difficult task to find out how the nutrients metabolism in cellulose-producing bacterium, referring to carbon metabolism, nitrogenous metabolism and respiration of the microorganism. Due to the lack of the key enzyme in the 6-phosphofructokinase (PFK) or lower activity of PFK in EMP, carbohydrate catabolism of Acetobacter xilimus may be dominated by HMP, and provide energy through TCA pathway. In 1996, Tonouchi et al. [16] characterized the biosynthetic pathway of cellulose from glucose and fructose in A. xilimus BPR2001 and investigated several enzyme activities in the medium containing different carbon sources.

Simultaneously, strain BPR2001 was found to have a fructose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS). The research indicated that the carbohydrate metabolism pathway is independent with the formation of protein synthesis. De Wulf et al. [17] constructed the metabolic network of Gluconacetobacter xilimus, but the major product was keto-gluconate, which influenced pH, cell breed and BC production. In this process, they considered a parallel byproduct of acetan, a viscous water-soluble polysaccharide. In order to increase the BC production, they generated a non-keto-gluconate-producing mutant strain through UV mutagenesis. The production of BC had increased obviously. Hugo-Velasco-Bedrán and Felipe-López-Isunz [8] also made a simple analysis about the A. xilimus metabolism, but the bacterial synthesis effect on metabolic flux does not take into account. Makoto and Yasushi [8]
got a simplified scheme of bacterial cellulose production, which used glucose or fructose as the carbon source and considered the metabolism of gluconic acid (Fig.3).

Bacterial cellulose production can be improved by reducing the related byproducts, which suggests an important approach of metabolic engineering 20. Metabolic engineering is a subject trying to get a better insight into the metabolism of organisms 21. Vallino et al. 22 assumed that intracellular intermediate metabolites were in the quasi-steady state with the base of quasi-steady state assumption. They estimated the metabolic flux distribution in cells combined with biochemical reaction pathway. The steady-state of metabolic networks can be described in terms of mass balance equations. The basic metabolites within metabolic network is proportional to the energy synthetized 23. In the fermentation prophase, bacteria consumes glucose to synthesis bacterial cellulose and cells rapidly mainly through the HMP pathway. In the second stage, the metabolic flux is redistributed and a mass of carbon source flows to byproducts. It concluded that the metabolic flux of main node G6P and acetate in different stage of the biosynthesis of bacterial cellulose. Li 24 gave a further research. Their results indicated that ethanol functions as energy source for ATP generation at the early stage, which can inhibit the activity of G6PDH by the increased levels of ATP. Furthermore, by adding sodium citrate, the main byproduct citric acid in the TCA cycle is also reduced significantly. The metabolic flux in the process of cellulose production is affected by the culture conditions. Pyruvate is another key metabolite of respiration and fermentation. Through the use of metabolic networks and metabolic flux balance model, quantitative analysis of metabolic network can provide theoretical guidance for fermentation control optimization and genetic operation to generate bacteria bacterial cellulose.

**Genetics and enzyme regulation**

Cellulose biosynthesis both in plants and in prokaryotes is catalyzed by the uridine diphosphate (UDP)-forming cellulose synthase complexes (CESAs), which are membrane-embedded glycosyltransferases (GT) (Fig.3) [3]. CESAs involved the transfer of consecutive glucopyranose residues from UDP-activated glucose (UDP-Glc) to the newly formed polysaccharide chain at the anomeric carbon of the newly added sugar from ρ to υ, translocation of glucan across the plasma membrane, and coalescence of multiple glucan chains to form paracrystalline microfibrils 25,26. Research indicated that prokaryotic and eukaryotic CESAs shared a similar predicted topology including eight transmembrane helices and at least one extended intracellular glycosyltransferase loop (Fig.3) [3]. In 1958, Roelofsen first suggested that cellulose fibrils could be assembled by means of enzyme complexes located at their tips via a vitro assay and a purification protocol 27. Then in 1990, Lin and Brown 28 used Specific-labeling technique to support the opinion that cellulose synthase consisted of three polypeptide chains. The calculated molecular masses of these polypeptide chains are 90, 67 and 54 kDa, respectively. Saxonia 29 and his companions purified the cellulose synthase, and provided evidence that the CESAs is comprised two polypeptide chains with the molecular size of 83kDa and 93kDa, simultaneously corroborated contain UDPG-binding site via azine group-UDPG affinity labeling method. Inder and his colleagues 30 gave the same point, they purified the cellulose synthase from *A. xylinum* ATCC 53582 by production entrapment, and the purified preparation showed two major polypeptides of 93 and 83 kDa on lithium dodecyl sulfate(LDS)-polyacrylamide gel following electrophoresis. Further analysis corroborated that the 83kDa polypeptide is a glycoprotein which contains substrate-binding sites and active sites by lectin affinity chromatography 28. For Gram-negative bacteria, the protein complex used to product and secrete cellulose was consisted of at least three subunits (BcsA, BcsB and BcsC) 31. Detailed information about the atomic structure and biochemical mechanisms of the A and B subunits of CESAs were presented 3. Cellulose synthesis occurs within the cytosolic domain of inner membrane protein BcsA, whereas BcsB resides in the periplasm which may help to guide the chain in the extrusion process. They are fused as a single polypeptide in some species. And BcsC just required for cellulose synthesis in vivo but not in vitro 32,33.

Back to genetic basic, the cellulose synthase genes from the bacterium...
Gluconoacetobacter hansenii were described in 1990. The cellulose synthase gene of *Acetobacter xylinum* and other cellulose producing strains was all located on chromosome\(^{34}\), rather than considered the plasmid in the past. But the functional cellulose synthase is located in the cytoplasmic membrane with several predicted transmembrane domains\(^{35}\). Cellulose synthase gene and several other genes are included in the same operon in *G. Xylinus*\(^{33}\). In most species, the different subunits of the CS are encoded by different genes. As mentioned earlier, the two polypeptides of the CS are encoded by acsAB of acs operon in *G. Hansenii* ATCC 23769 and *G. xylinus* ATCC 53582\(^{36}\), while the two sigmasubunits of the CS are determined by bcsA and bcsB in *G. xylinus* 1306-3\(^{37}\). AcsA considered to be the catalytic subunit has been experimentally shown to bind the precursors UDP-glucose for the synthesis of BC. The length of the cellulose synthase acsA is between 723 to 888 amino acids. AcsB contains a regulatory domain, and binds cyclic diGMP, which is shown to be the allosteric activator of this enzyme\(^{38}\). AcsC was thought to form pores in cell walls through which synthesized cellulose can pass. Recently, Naoki Sunagawa\(^{39}\) verified that the cellulose synthase complex in *Acetobacter xylinum* contains a new member named cellulose complementing factor (Ccp). They successfully identified the coding region of the ccpAx gene, it is involved in the production and crystallization of cellulose I microfibrils. And most of CcpAx existed in the membrane fraction and contained rich structure of ±-helices. These data indicate that this protein has a direct interaction with AxCeSD through pulldown assays and ITC analysis. This work lays a foundation for further studying the mechanisms of the regulation for the cellulose synthesis. Very recently, a cellulose synthase has been identified the most conserved gene of the bcs operon among the species\(^{40}\). But the C- and N- terminal part of the protein is less well conserved. The FFCGS and RFLPL motives included in the CS could be mainly responsible for the determination of the (1\(^n\)4) specificity of the 5-glucan bond\(^{31}\). The study found that an operon encoding two ORFs was located 3.0 kb upstream of the bcs operon in *A. xylinum*, which is same as

**Fig.1.** SEM images of *Acetobacter xylinus* [45,46]

**Fig.2.** Formation of Bacterial cellulose [45,46]
Fig. 3. Architecture of the BcsA–BcsB complex. BcsA and BcsB form an elongated complex with large cytosolic and periplasmic domains. The transmembrane helices of BcsA are coloured green, the glycosyltransferase (GT) domain beige, and the C-terminal domain red. BcsB is shown in light and dark blue for its periplasmic and membrane-associated regions, respectively. The N and C termini of both subunits are indicated and the translocating glucan and UDP are shown as cyan and violet spheres. Horizontal bars indicate the membrane boundaries. IF, amphipathic interface helices of BcsA.

Fig. 4. The schematic of reactions for c-di-GMP synthesis and degradation. 2 GTP condensation reaction occurs, the first release of 1 molecules of PPI is converted into a linear dinucleotide pyrophosphate three phosphorylation of pppGpG, then release the 1 PPI molecules, and condensed to form c-di-GMP, PDE cuts a single phosphodiester from c-di-GMP, and turns c-di-GMP to be active to do not have the activity of two linear polymer pGpG.

Fig. 5. Possible c-di-GMP effector mechanisms. c-di-GMP (the small ball) binding domains act as molecular switches through interaction with protein partners.
Salmonella typhimurium and Escherichia coli. In order to define the influence of these two ORFs, Standal introduced the upstream regions into a shuttle vector pSA19. The present of the first could enhance the production of cellulose and the transposon mutation at the second ORF which caused the inability to produce BC in vivo. While in Gluconacetobacter and G. xylinus NBRC3288, three ORFs code for the CS, and only one ORF contained in the strains ATCC 23769 and closely-related ATCC 55382.

Regulation and control of the synthesis of bacterial cellulose is influenced by so many different factors. The experiment proved that approximately eight enzymes involved in the biosynthesis of bacterial cellulose, including cellulose synthase which is the characteristic and key enzyme in the process of cellulose synthesis. The last step catalyzed by the CS from glucose into polyglucan chain is the unique rate-limiting reaction. In 1987, Ross et al. found a kind of guanosine acyl oligonucleotide analogues which had evidently allosteric effect in cellulose synthase when they investigated the pathway of cellulose synthesis in A. xylinum. Two years later, they identified the active principle was c-di-GMP and put forward the concept of the cyclic diguanylic acid regulatory system (Fig.4). A. xylinum is known to produce c-di-GMP and to synthesize cellulose fibrils in response to the change of intracellular c-di-GMP concentration. The possible mechanisms of c-di-GMP have showed in Figure.3. Amikam confirmed that the C-terminal part of acsA contained the active site binding c-di-GMP, it was highly homologous to the PilZ domain. So we predict that c-di-GMP binds to acsA act as molecular switches through interaction with protein partners (Fig.5).

In order to examine the action of the activator c-di-GMP which confirmed as a bacterial second messenger, Peter and Raphael researched the kinetics of activation, the result showed that in the range of 0.3-4.0uM, the effector concentration, the activity of the CS manifested a typical Michaelis-Menten relationship via the experiments in vitro. The CS activity fell off rapidly with the concentration of the c-di-GMP below this range. And the intracellular content of cyclic diguanylic acid is dependent on a balance between the cellular levels of diguanylate cyclases (DGC) and the opposing action of the Ca²⁺-sensitive phosphodiesterases (AxPDEA)³⁴.

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