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Review

A survey of cellulose biosynthesis in higher plants

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Abstract Cellulose plays a central role in plant development and its biosynthesis represents one of the most important biochemical processes in plant biology. However, the corresponding molecular mechanisms are not well understood, despite the progress made in the past years in the identification of genes that code for the catalytic subunits of the cellulose synthases and other proteins potentially involved in cellulose formation. A major bottleneck is the high instability of the cellulose synthase complexes and their location in the plasma membrane. Additional efforts are currently being made to unravel the mechanisms of cellulose biosynthesis. Indeed, understanding how cellulose is formed and how its crystallinity is achieved is relevant not only for studying plant development, but also for improving the digestibility of the plant biomass, which is foreseen as an alternative to fossil fuels for the production of energy. This review summarizes the major unanswered questions related to the process of cellulose biosynthesis, and describes the recent progress that has been made in the area through the combination of biochemical approaches and molecular genetics.

Key words: Callose, carbohydrate biosynthesis, cellulose synthase, cell wall.

Cellulose, an abundant polymer with many applications

Cellulose is a major component of the plant biomass and as such it is often described as the most abundant macromolecule on earth. Cellulose plays a central role in the regulation of the cell volume, the determination of the cell shape and size, the mechanical protection of the plant and its defence against pathogens. It is synthesized at the surface of the cell and organized into crystalline microfibrils that govern the mechanical properties of the cell wall by their strength and stiffness. Cellulose microfibrils are embedded in a complex matrix of carbohydrates, phenolic polymers and structural proteins. The resulting biocomposite can accommodate a variety of mechanical requirements during plant life. Owing to its abundance and unique properties, cellulose is exploited in a wide number of applications. Traditionally, paper and cardboard are produced from wood pulps while regenerated cellulose is extensively used in the textile industry. Numerous other products are based on cellulose derivatives such as cellulose esters and ethers, which are valuable for instance for the preparation of coatings, films, plastics, adhesives, paints, pharmaceuticals, cosmetics, etc (Engelhardt 1995; Klemm et al. 2005). But in addition to these traditional sectors, novel areas are emerging with the necessity to find alternatives to our current but unsustainable use of fossil fuels to supply energy, chemicals and materials. Of particular importance is the production of bioethanol from cellulose, which has the potential to significantly contribute to the replacement of petroleum-derived fuels for transportation (Ragauskas et al. 2006). Numerous initiatives are being taken to overcome the recalcitrance of cellulosic plant cell walls to digestibility, and facilitate the cost-effective production of liquid biofuels from plant biomass by fermentation (see for instance Frederick et al. 2008). In addition, plant cell walls represent one of the most elegant natural composite materials, and the physical and mechanical properties of cellulose nanocrystals are comparable to those of the best synthetic materials. Thus, another field of great potential is the biomimetic engineering of cellulose-based materials that combine environmental friendliness and/or biocompatibility with high performance and increased functionality (Teeri et al. 2007). However, the major bottleneck towards such applications is our present poor understanding of the mechanisms of cellulose formation, deposition and self-assembly with other cell wall components.

Cellulose structure and biosynthesis: the unanswered questions

In addition to higher plants, numerous organisms belonging for instance to the algal or oomycete families synthesize cellulose as a cell wall component (Brown 1996). Some bacterial species, for example the Gram negative bacterium *Gluconacetobacter xylinus*, extrude cellulose in their surrounding environment as a virtually pure carbohydrate. Cellulose is a homopolymer of glucose residues that are linked to each other by β-(1→4) linkages. The degree of polymerization of cellulose chains varies greatly with the origin of the polymer (800 to up to 10000 for plant celluloses; Klemm et al. 2005). This apparently simple carbohydrate forms crystalline structures that have required several decades of research to be solved. Native cellulose – or cellulose I –occurs in two different crystalline forms designated I_α and I_β, which coexist in variable proportions depending on the origin of the cellulose (Atalla and VanderHart 1984; VanderHart and Atalla 1984). Cellulose I_α consists of triclinic unit cells while the I_β allomorph exhibits a monoclinic type of unit cells (Sugiyama et al. 1991; Finkenstadt and Millane 1998). These two forms of cellulose differ by the orientation of some hydrogen bonds, which influences the fine conformation of individual chains within the crystals (Nishiyama et al. 2002). Typically, the cellulose produced by *G. xylinus* is enriched in the I_α allomorph whereas higher plants contain a higher proportion of the I_β allomorph (VanderHart and Atalla 1984). Both allomorphs of cellulose I consist of parallel chains of β-(1→4) glucan (see for instance Chanzy and Henrissat 1985; Kuga and Brown 1988), but the molecular events that determine the preferential formation of either of the crystalline forms are not known. Cellulose II, another form of cellulose, has been reported to occur in nature in very few instances such as in the marine alga *Halicystis*, the bacterium *Sarcina* or mutants of the bacterium *G. xylinum* (Delmer 1999, and references therein). Thus, cellulose I is by far the most abundant natural form of the polymer. However, cellulose II can be artificially produced from cellulose I by two processes known as regeneration and mercerization (Franz and Blaschek 1990; Klemm et al. 2005). The latter involves the swelling of the polymer in aqueous NaOH solutions as a first step, whereas cellulose is first dissolved in specific solvents such as *N*-methylmorpholine-*N*-oxide during the regeneration process (Franz and Blaschek 1990; Klemm et al. 2005). In both cases, a final recrystallization step leads to the formation of cellulose II which is thermodynamically more stable than cellulose I and, as opposed to the latter, consists of antiparallel chains (Langan et al. 2001). Mercerization and regeneration are used to improve the properties of natural yarns and fabrics. Viscose and Lyocell are typical examples of products of these processes (Klemm et al. 2005). Several models have been proposed to explain the mode of formation of cellulose II from cellulose I, but the actual molecular events accompanying this transition are unclear.

Enzymatic complexes located in the plasma membrane are responsible for the biosynthesis of cellulose. They were first identified in the alga *Oocystis apiculata* as globular structures present at the tip of elongating cellulose microfibrils (reviewed in Brown 1996). For this reason, they were designated as terminal complexes (TCs). TCs have been visualized in several organisms and they are currently known as occurring in two main forms (Brown 1996). Linear TCs correspond to arrays of synthesizing enzymes. Generally observed in algae and in the bacterium *G. xylinus*, their organization differs from one organism to another by the number of synthesizing units in each row (Brown 1996). Rosettes represent the other form of TCs. They are characteristic of higher plants (Brown 1996) and consist of six globular structures which are each possibly composed of six cellulose synthase catalytic subunits (CesA) (Figure 1). According to this hypothesis, a rosette would be responsible for the simultaneous elongation of 36 β-(1→4)-glucan chains that co-crystallize in a microfibrillar form (Delmer 1999). This number is compatible with the observed lateral size of elementary cellulose microfibrils commonly visualized in higher plants (Delmer 1999). However, other results indicate that the size of a microfibril is rather consistent with the packing of 18 chains per microfibril or even less (Ha et al. 1998). In fact, the actual number of active catalytic subunits in individual TCs, which defines the number of chains per elementary microfibril has never been experimentally demonstrated. It is only in 1999 that the presence of the catalytic subunits responsible for cellulose polymerization was firmly demonstrated in rosettes (Kimura et al. 1999). Apart from the evidence that CesA proteins are physically associated to the enzyme complex, the identity of the other possible protein components of the cellulose synthase machinery and their stoichiometry are unknown. This is essentially due to the difficulty to purify to homogeneity the cellulose synthase as an intact complex. Thus, molecular genetic approaches have been undertaken as an alternative to the challenging biochemical characterization of the enzyme, using particularly the powerful model *Arabidopsis thaliana*. This has allowed the identification of a number of mutants that are affected in cellulose biosynthesis. Some of the proteins coded by the corresponding genes have been proposed to be associated to the cellulose synthase machinery, but their physical occurrence within the complexes remains to be determined. Typical examples of candidates possibly associated to the enzyme are a membrane-bound

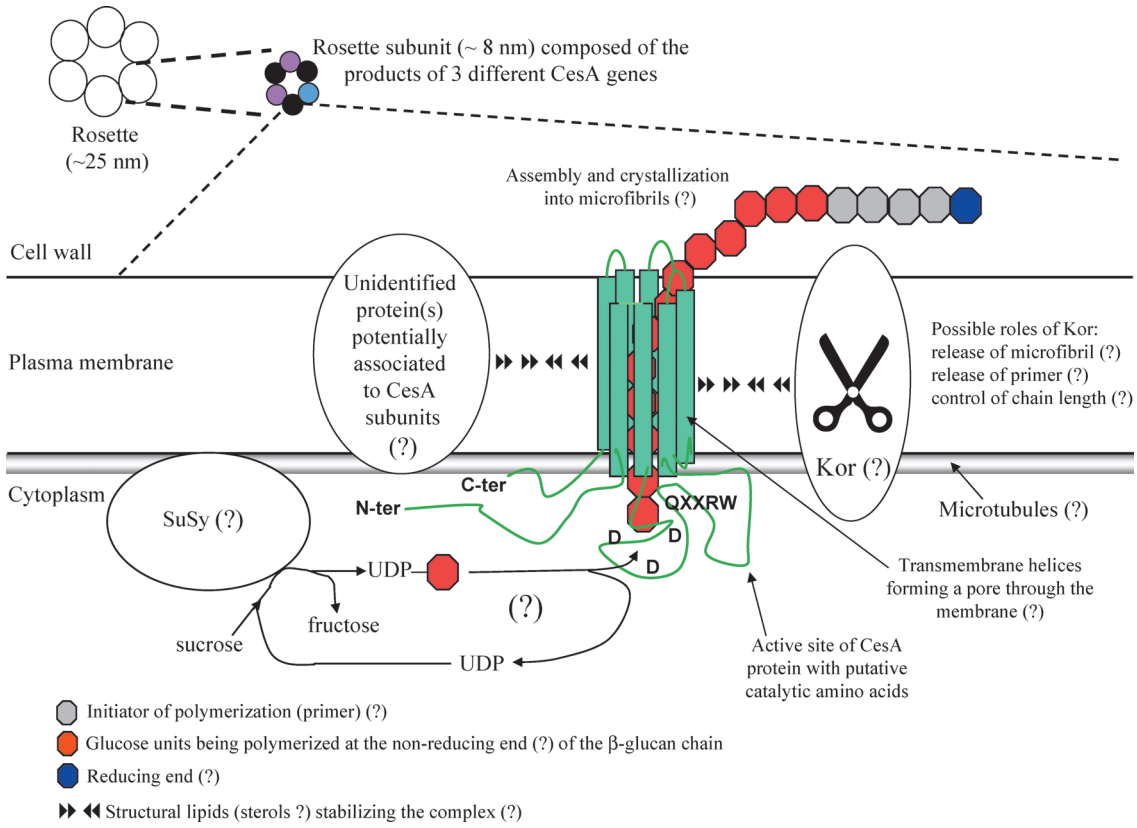

Figure 1. Hypothetical model for cellulose biosynthesis in higher plants. (?) refers to aspects that remain to be clarified including (a) involvement of a primer to initiate polymerization; (b) orientation of the glucan chain being extruded; (c) mechanism of translocation of the cellulose chains across the plasma membrane (after Delmer 1999); (d) involvement of crystallization subunits for microfibril formation; (e) association of membrane-bound proteins to the CesA subunits; (f) the mode of interaction of the cellulose synthase complex with microtubules; and the roles of (g) Korrigan (Kor); (h) sucrose synthase (SuSy); (i) structural lipids. Other proteins such as regulation subunits are not represented. The stoichiometry of the different subunits in the complex is not known. (Adapted from Bulone 2006).

form of sucrose synthase (Amor et al. 1995), a membrane-bound endo-(1→4)-β-glucanase designated as Korrigan (Nicol et al. 1998), annexins (Hofmann et al. 2003), actin, tubulin, a putative lipid transfer protein, a metallothionein, etc (for reviews on the possible role of these proteins in cellulose biosynthesis see Haigler et al. 2001; Doblin et al. 2002; Mølhøj et al. 2002). The identification of cytoskeleton-related proteins is consistent with the multiple reports that suggest an involvement of cortical microtubules in the deposition of cellulose microfibrils (see for instance Baskin 2001; Somerville 2006). In addition, the movement of CesA proteins in the plasma membrane has been shown to follow linear tracks that coincide with the orientation of cortical microtubules (Paredez et al. 2006), while drugs that interfere with cellulose biosynthesis decouple the ordered deposition of cellulose microfibrils from microtubule orientation (Himmelspach et al. 2003).

CesA proteins present 8 putative transmembrane helices that are involved in anchoring the enzyme in the plasma membrane (Delmer 1999). A large soluble part of CesAs is predicted to be located on the cytoplasmic side of the membrane where UDP-glucose, the substrate of CesAs, is synthesized by UDP-glucose pyrophosphorylase. Sucrose synthase may also contribute directly to the synthesis of the cellulose synthase substrate (Amor et al. 1995) (Figure 1). Experimental data obtained *in vitro* support the involvement of sitosterol-β-glucoside as a primer for cellulose synthesis (Peng et al. 2002), but this remains to be firmly demonstrated *in vivo*. The cytoplasmic soluble part of CesA proteins contains the putative catalytic amino acids that form β linkages in the cellulose chains, from a UDP-glucose molecule in which the glucose moiety is in the α configuration (Figure 1). This so-called inverting mechanism is most likely coupled to the addition of glucose units to the non-reducing end of the growing glucan chains (Figure 1), as suggested by data obtained with the blackberry cellulose synthase (Lai Kee Him et al. 2002). This is further supported by the situation in the bacterium *G. xylinus* where cellulose chains have also been described to be elongated from their non-reducing ends (Koyama et al. 1997). As implied by the putative topology of CesA proteins and the cytoplasmic localization of UDP-glucose, the catalytic event occurs on the cytoplasmic side of the plasma membrane (Figure 1). Thus, the growing glucan chains need

to be extruded across the plasma membrane to reach the cell wall and this occurs by a so far unknown mechanism. In an attractive speculative model, it was suggested that the 8 putative transmembrane helices born by the CesA protein delimit a pore through the membrane to allow the extrusion of the glucan chains (Delmer 1999) (Figure 1). Several other hypothetical models involving for instance porin-like proteins or flippases have been proposed to describe the translocation of the cellulose chains across the plasma membrane (Brown and Saxena 2000; Lai Kee Him et al. 2003; Bulone 2006). After synthesis, the β-(1→4)-glucan chains must be released in the cell wall. This might require a hydrolytic activity such as that of the membrane-bound endoglucanase Korrigan (Figure 1). Another function of the latter protein might be the control of the degree of polymerization of the cellulose chains or the release of a primer (Mølhøj et al. 2002), if a primer is indeed required to initiate cellulose polymerization (Figure 1). But the actual role of Korrigan in cellulose biosynthesis remains to be demonstrated. The assembly of the cellulose chains as crystalline microfibrils is a poorly understood process that could be spontaneous or assisted by proteins (Figure 1). In addition to the protein candidates mentioned above, subunits involved in the regulation of the cellulose synthase machinery could be part of the complex.

Molecular biology and biochemistry of cellulose biosynthesis

Numerous homologues of the *CesA* genes that were first identified in the cotton fiber (Pear et al. 1996) have been isolated in several plants. These functional homologues were typically identified by sequence similarity and/or through the production and characterization of mutants that exhibit a lower cellulose content or that are resistant to herbicides targeted to cellulose biosynthesis. Examples are the *CesA* genes from *A. thaliana* (Arioli et al. 1998; Taylor et al. 1999; Fagard et al. 2000; Taylor et al. 2000; Scheible et al. 2001), maize (Holland et al. 2000), tobacco (Doblin et al. 2001), barley (Burton et al. 2004), hybrid aspen (Djerbi et al. 2004) and poplar (Djerbi et al. 2005). Genome analyses and EST sequencing of the most studied plant species like *A. thaliana*, rice, barley and poplar have allowed the identification of up to 18 different *CesA* genes in each species. This raises the question of the actual function of these multiple genes. Immunoprecipitation experiments have revealed that specific sets of 3 gene products are associated in the same complex. In *A. thaliana*, where 10 different *CesA* genes have been identified, immunoprecipitation experiments have recently shown that the products of *CesA1*, *3* and *6* form a complex (Desprez et al. 2007). In addition, mutations in any of these 3 genes affect cellulose biosynthesis in primary walls (Arioli et al. 1998; Fagard et al. 2000; Cano-Delgado et al. 2003). Similarly, a set of 3 different *Arabidopsis* genes, namely *CesA4*, *7* and *8*, is specifically involved in cellulose biosynthesis in secondary walls (Taylor et al. 1999, 2000 and 2003). Microarray data have shown that the products of these latter 3 genes are co-regulated (Brown et al. 2005; Persson et al. 2005) and immunoprecipitation experiments revealed that they interact in a complex (Taylor et al. 2003). Thus, in *A. thaliana* it seems that active cellulose synthase complexes consist of the products of 3 different *CesA* genes, and that complexes with different CesA compositions are required for primary and secondary cell wall formation. However, some of the 10 *Arabidopsis CesA* genes are at least partially redundant. For instance, *CesA2* and *CesA5* are partially redundant with *CesA6*, and there is experimental evidence supporting that the corresponding proteins compete for the same position in the cellulose synthase complex (Desprez et al. 2007; Persson et al. 2007). The association of the CesA proteins in a given complex possibly occurs through direct interactions between putative N-terminal zinc finger domains rich in cysteine (Kurek et al. 2002). The formation of these interactions may represent an early step of the rosette assembly (Doblin et al. 2002). Interestingly, expression analyses of *CesA* genes in the hybrid aspen *Populus tremula* × *tremuloides* show that 4 genes are more specifically expressed during xylogenesis (Djerbi et al. 2004). However, there are no experimental data available on the number of different *CesA* gene products required to form a cellulose synthase complex in this species. In addition to the involvement of specific sets of *CesA* genes in the polymerization of the glucan chains, some other *CesA* genes could be specifically required at a different step of the cellulose biosynthesis process. For instance, some *CesA* genes may be specifically involved in the synthesis of cellodextrins that could act as primers to initiate cellulose polymerization (see for instance Taylor et al. 1999, 2000 and 2003; Peng et al. 2002; Read and Bacic 2002).

The purification of plant cellulose synthase complexes has been hindered by the inherent instability of the enzyme and, consequently, the difficulty to extract it from the plasma membrane in an intact and active form. Detergent extracts of plasma membranes typically synthesize no or very little cellulose *in vitro* in the presence of UDP-glucose. Instead, the preparations readily catalyze the formation of a linear β-(1→3)-glucan (Delmer 1987; Okuda et al. 1993). The latter is synthesized by callose synthase, an enzyme that uses UDP-glucose as a sugar donor and is bound to the plasma membrane by transmembrane domains. It has been proposed that callose and cellulose synthases correspond to a single enzyme that synthesizes either polysaccharide depending on its

conformation and/or the presence of regulation factors (Delmer 1999). This hypothesis has however been challenged in the last years by the identification of plant genes that code for putative catalytic subunits of callose synthases. The products of these genes designated as *Gsl* for "Glucan Synthase Like" do not show any significant similarity with the plant *CesA* proteins (Cui et al. 2001; Doblin et al. 2001; Hong et al. 2001; Li et al. 2003). In particular, they do not contain the D,D,D,QXXRW motif common to all CesAs and to a large majority of processive glycosyltransferases (Saxena et al. 1995; Campbell et al. 1997). Based on sequence analyses and reaction mechanism, CesAs are grouped in glycosyltransferase family 2 together with numerous enzymes from a large range of organisms, while Gsl proteins form glycosyltransferase family 48 (Coutinho and Henrissat 1999; http://www.cazy.org). In addition, the predicted molecular weight of the *Gsl* gene products is in the range 190–220 kDa compared to 110–120 kDa for CesAs (see for instance Pear et al. 1996, and Li et al. 2003). However, the evidence that the catalytic subunits of the cellulose and callose synthases correspond to different proteins does not rule out the possibility that the enzyme complexes may contain some identical subunits.

Even though it is still not possible to assay cellulose synthases routinely, significant progress has been made over the years on the development of methods that allow *in vitro* synthesis of cellulose using plant enzymes, thereby opening opportunities for the direct characterization of the cellulose synthase machinery using biochemical approaches. The first successful *in vitro* synthesis of cellulose was achieved using detergent extracts from mung bean and cotton plasma membranes (Kudlicka et al. 1995 and 1996; Kudlicka and Brown 1997). Later, mg amounts of cellulose were synthesized *in vitro* using an enzyme preparation from cell suspension cultures of blackberry (Lai Kee Him et al. 2002). This allowed the full characterization of the cellulose synthesized *in vitro*, which was found to be of a significantly higher crystallinity than the cellulose extracted from primary walls of the same cells. More recently, the *in vitro* synthesis of cellulose was achieved using digitonin extracts of plasma membranes from the hybrid aspen *P. tremula* × *tremuloides* (Colombani et al. 2004) (Figure 2). In particular, conditions for assaying callose and cellulose synthase activities were determined. While typically only 30% of cellulose relative to 70% of callose can be synthesized *in vitro*, up to 50% of cellulose was obtained by using detergent extracts of plasma membranes from suspension cultures of hybrid aspen cells harvested in their stationary growth phase (Colombani et al. 2004). Interestingly, the higher cellulose synthase activity in the stationary phase coincided with the level of expression of the *CesA* genes associated to secondary cell wall biosynthesis (Ohlsson et al. 2006).

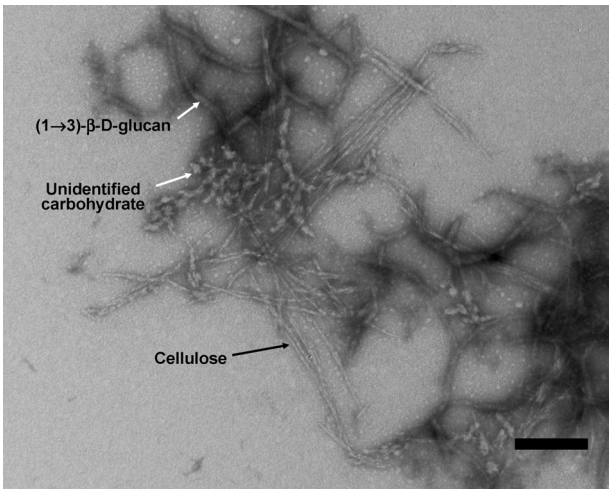

Figure 2. Micrograph showing the morphology of the polysaccharides synthesized *in vitro* by a digitonin-extract of plasma membranes from hybrid aspen (*Populus tremula* × *tremuloides*) cells. Two of the structures were unequivocally identified as cellulose and (1→3)-β-D-glucan, while the third one was not characterized. However, its morphology points towards short aggregates of (1→3)-β-D-glucan chains, or possibly cellulose II. Bar = 200 nm

Previous results obtained in our group suggested that the detergents used for protein extraction influence the general organization and structure of the glucan synthase complexes, and indirectly the morphology and structure of *in vitro* synthesized β-(1→3)-glucans (Lai Kee Him et al. 2001; Pelosi et al. 2003). In addition, during the optimization of the conditions for *in vitro* synthesis of cellulose from different plant species, the choice of the detergent to extract cellulose synthase in an active form was found to be species specific (Lai Kee Him et al. 2002; Colombani et al. 2004). From these observations it was hypothesized that the callose and cellulose synthases are located in detergent-resistant microdomains (DRM) similar to lipid rafts in animal cells, but with lipid compositions that vary from one plant species to another (Lai Kee Him et al. 2002). Our latest results on the isolation of DRM from purified plasma membranes of plant cells confirmed this hypothesis (Bessueille et al., submitted for publication). In particular, the isolated DRM were able to synthesize *in vitro* polysaccharides that were unequivocally identified as callose and cellulose. Many different cellular tasks have been ascribed to DRM (see for instance Bhat and Panstruga 2005), but our data represent the first link between DRM and callose and cellulose biosynthesis in higher plants. It is noteworthy that sterols, which are enriched in DRM, have been shown to be crucial for cellulose synthesis as well as for elongation and cell wall expansion in *A. thaliana* (Schrick et al. 2004). Thus, it is tempting to speculate that some lipids from the sterol family are required *in vivo* for the stability of the cellulose synthase complexes in the plasma membrane (Figure 1). If it is the case, a successful *in*

vitro synthesis of cellulose may be achieved only with relatively mild detergents that allow the extraction of the intact machinery together with its stabilizing sterols. The full characterization of plant DRM is a promising approach for the identification of the proteins required for cellulose and callose synthesis. It opens new opportunities for understanding in more details the molecular mechanisms of the synthase complexes, which is of great relevance in developmental biology and for a number of applications, such as for instance the manipulation of cellulose crystallinity to improve the production processes of cellulosic ethanol.

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