Biodegradation of plant biomass is a slow process in nature, and hydrolysis of cellulose is also widely considered to be a rate-limiting step in the proposed industrial process of converting lignocellulosic materials to biofuels. It is generally known that a team of enzymes including endo- and exocellulases as well as cellobiases are required to act synergistically to hydrolyze cellulose to glucose. The detailed molecular mechanisms of these enzymes have yet to be convincingly elucidated. In this report, atomic force microscopy (AFM) is used to image in real-time the structural changes in Valonia cellulose crystals acted upon by the exocellulase cellobiohydrolase I (CBH I) from Trichoderma reesei. Under AFM, single enzyme molecules could be observed binding only to one face of the cellulose crystal, apparently the hydrophobic face. The surface roughness of cellulose began increasing after adding CBH I, and the overall size of cellulose crystals decreased during an 11-h period. Interestingly, this size reduction apparently occurred only in the width of the crystal, whereas the height remained relatively constant. In addition, the measured cross-section shape of cellulose crystal changed from asymmetric to nearly symmetric. These observed changes brought about by CBH I action may constitute the first direct visualization supporting the idea that the exocellulase selec-tively hydrolyzes the hydrophobic faces of cellulose. The limited accessibility of the hydrophobic faces in native cellulose may contribute significantly to the rate-limiting slowness of cellulose hydrolysis.

Natural cellulose is a bundle of linear β-1,4-linked glucan chains held tightly in a crystalline structure by the cumulative effect of many inter- and intrachain hydrogen bonds. Cellulose produced by higher plants is the most abundant biopolymer on Earth, accounting for 40–60% by dry weight of plant cell walls. In addition to the traditional uses of cellulose in the paper, food, and textile industries, the new concept of biofuels produced by higher plants is the most abundant biopolymer on Earth, accounting for 40–60% by dry weight of plant cell walls. In addition to the traditional uses of cellulose in the paper, food, and textile industries, the new concept of biofuels produced from lignocellulosic biomass is considered a promising route to sustainable energy production. Unfortunately, lignocellulosic material is intrinsically recalcitrant to chemical and enzymatic breakdown to simple sugars that can be fermented to liquid fuels. A deeper understanding of biomass recalcitrance will be required for the potential of lignocellulosic biofuels to be real-ized (1).

In native plant cell walls, cellulose exists as nanometer scale microfibril networks embedded in matrices of other biopolymers such as hemicelluloses, pectins, and lignins. Chemical pretreatment processes are often required to remove or relo-cate these “other” matrix polymers, thereby exposing the cellulose to a follow-up enzymatic hydrolysis to produce glucose. The cellulases are a class of enzymes, produced mainly by cel-lulolytic fungi and bacteria, that catalyze hydrolysis of the β-1,4-glicosidic bonds that link the glucosyl units of cellulose. Cellulases have been classified on the basis of their modes of action on the substrate into three distinct classes that react synergistically: (i) the endo-β-(1,4)-glucanases that cleave the cellulase chain at internal positions to produce new chain ends (2), (ii) the exo-β-(1,4)-d-glucanases that cleave successive cellobysiol units from the ends of cellulose, and (iii) the β-d-glucosidas that hydrolyze cellobiose to glucose. Among these types of cellulases, the exoglucanases appear to catalyze most of the bond-cleavages in the saccharification of crystalline cellu-lose and are usually the major component of cellulase prepara-tions, especially in the case of current fungus-derived commer-cial enzymes.

Cellulose substrates isolated from different sources, though all composed of linear β-1,4-linked glucose polymers, differ sig-nificantly in structure as measured in terms of amorphous content, crystallinity, and size and shape of crystallites (3); these structural differences may substantially affect susceptibility to cellulase action. In addition, depending on variations of the patterns of inter- and intrachain hydrogen bonds, cellulose may form different crystalline allomorphs (e.g. cellulose Iα, Iβ, II, III, and IV), upon which the binding and activities of cellulases may be different. Endoglucanases acting alone may attack the non-crystalline regions effectively, but effective attack on the crys-talline portion of cellulose requires in addition the synergistic action of the exoglucanases. The fact that cellulose structure affects cellulase activities has long been documented (4), but experimental data presented in the literature have been subject to considerable uncertainty in interpretation of the relationships between cellulose structure and cellulase activities due to disagreements in identification and quantification of cellulose structure by different analytical methods (5). In the current study, cellulose crystals from the cell wall of a green alga, Valo-nia ventricosa, were intensively characterized by means of

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...atomic force microscopy (AFM). Single, continuously monitored crystals of the characterized material were used as substrates in assays visualizing and quantifying the effects of catalysis by Trichoderma reesei CBH I (Cel7A) on the structure of cellulose crystals. The ultimate objective of this study is to elucidate the reaction mechanism of cellulase on true crystalline cellulose.

The cellulase system produced by the filamentous fungus T. reesei is widely used as a commercial enzyme mixture in industry. This system contains two exoglucanases, cellobiohydrolases CBH I and CBH II, which belong to glycoside hydrolase families 7 and 6 respectively, along with five endoglucanases (I–V). It is generally believed that hydrolysis of crystalline cellulose to cellobiose is the rate-limiting step of cellulose degradation, which requires at least two type of synergistic actions, endo-exo cooperation between endoglucanases and cellobiohydrolases (CBHs), and exo-exo cooperation between CBH I and II, which remove cellobiosyl residues from, respectively, the reducing and nonreducing ends of cellulose chains (6). Among the enzymes present in the cellulase preparation from T. reesei, CBH I is the most abundant component, accounting for >60% of the total protein (7). CBH I is structurally modular, consisting of a family 7 glycoside hydrolase catalytic module and a family 1 carbohydrate-binding module (TrCBM1), connected by a proline- and threonine-rich, highly glycosylated linker peptide. In a proposed (8) mechanism for the action of CBH I on crystalline cellulose, the surface-binding family 1 CBM first binds to the planar surface of cellulose. The reducing end of one cellulose chain is then threaded into the active site tunnel of the catalytic module. The cellulose chain is advanced “processively” through the tunnel, two glucosyl residues at a time, as catalytic residues in the tunnel catalyze the hydrolysis of every second β-1,4-glucosidic bond to depolymerize the chain to free cellobiose units. This last proposed feature is based primarily on the analytical finding that CBH I produces mainly cellobiose, as well as on the structure of the catalytic module (8) and the CBM (9). A recent study employing fast AFM of CBH I on a cellulose crystal appears supportive of this proposed mechanism (10).

During the past decade, researchers have investigated cellulase activity and cellulose-cel lulase interaction through a combination of biochemical methods and wet chemistry, as well as imaging techniques such as fluorescence microscopy (11–14), transmission electron microscopy (TEM) (15, 16) and scanning electron microscopy (17). AFM has also been used to characterize the effects of different types of cellulosases upon cellulose structure (10, 13, 18–20). Previously, we have studied the interactions with cellulose of both complete cellulase enzymes (13) and their binding modules (14, 21), by employing single-molecule spectroscopy techniques, such as total internal reflection fluorescence microscopy and AFM. It has been demonstrated that AFM is a powerful analytical tool that can be used to obtain highly accurate images of the cellulose surface under physiological conditions with nanometer resolution (22, 23). The sample can be imaged without physical (freezing) or chemical (fixation) treatments; the images thus obtained therefore reflect “native” structures. However, up until now, limited information has been obtained by real-time observation of biological events in time frames long enough to capture significant effects of cellulase action. In the present study, we use AFM to image in real-time the morphological changes occurring in single crystals of Valonia cellulose as a result of hydrolysis by T. reesei CBH I. The Valonia cellulose I has mostly an I₄ structure and is a widely accepted native crystalline cellulose standard. Our objective was to learn how the enzyme affects the cellulose morphology at the nanometer scale.

**EXPERIMENTAL PROCEDURES**

*Preparation of Valonia Cell Wall Cross-section for AFM—* Frozen whole Valonia cells were fixed and embedded using the following protocol, with all steps performed in a microwave at 30 °C under vacuum except where noted. Samples were fixed in 2.5% (v/v) glutaraldehyde in PBS, washed 3 times with PBS, fixed in 1% (w/v) OsO₄ in PBS, washed 3 times with distilled H₂O, and dehydrated with increasing concentrations of ethanol (30%, 60%, 90%, 3 × 100% resin, diluted in distilled H₂O). Fixed cells were then infiltrated with EMBed 812 resin (Electron Microscopy Sciences, Hatfield, PA) in increasing concentrations of resin (7%, 15%, 30%, 60%, 90%, 3 × 100% resin, diluted in ethanol), with the last two resin exchanges lasting for 48 h each on a rotator at room temperature. The samples were then sectioned to 240 nm thickness with a Diatome diamond knife (Electron Microscopy Sciences, Hatfield, PA) on a Leica EM UTC ultramicrotome (Leica, Wetzlar, Germany). Sections were collected on freshly cleaved mica disks for AFM.

*Preparation of Valonia Cellulose Crystals for AFM Imaging in Buffer and CBH I Solution—* The cellulose crystals were isolated from Valonia cells using the previously described method (25). Purified Valonia cellulose crystals were stored at −20 °C. Freshly cleaved mica coated with an amine polymer, poly-L-lysine, provided a flat, smooth, and stationary substrate on which the cellulose crystals were firmly immobilized to overcome the forces applied by the AFM probe and to permit high resolution of the sample structure. Five μl of poly-L-lysine solution (0.1% w/v in water, Sigma-Aldrich) was added to freshly cleaved mica. After 5 min of incubation, the excess poly-L-lysine solution was removed using a spin coater (Model KW-4A, Chemat Technology, Northridge, CA) operated at 500 rpm for...
30 s followed by 4000 rpm for 30 s. After that, 5 μl of a suspension of cellulose crystals (1 mg/ml in water) was added, followed by incubation and spin coating as in the above procedure. The coated mica was then dried in an oven at gentle heat (45 °C) overnight. AFM was operated in tapping mode in buffer and in air. We found that application of a 150-μl liquid droplet (enzyme solution or buffer) to the cellulose-coated mica provided a sample in aqueous environment that was sufficient for imaging for at least 10 h without significant artifacts caused by water evaporation. CBH I (1 mg/ml) was applied to the cellulose-coated mica in acetate buffer (20 mM acetate, 0.02% NaN₃, 100 mM NaCl, pH 5.0): the same buffer, without CBH I, was used for buffer-control imaging.

AFM and Image Analysis—AFM measurements were conducted using a Multi-Mode™ scanning probe microscope with NanoScope V controller (Veeco, Santa Barbara, CA). To ensure absolute stability, the AFM was located in a specially designed laboratory with acoustic and vibration isolation. A customized Nikon optical microscope with deep focus (800× magnification) was used to aid in positioning of the AFM tip to the desired location. Silicon nitride probes and sharp nitride lever probes (Veeco) were used in tapping mode to image cellulose fibers in liquid, and DP18/HTRES/Al BS probes (MikroMasch, San Jose, CA) were used in air. Images of cross section and roughness were analyzed using Nanoscope software (version 7.30). Surface roughness was evaluated by flattening the images (second order) and calculating mean roughness (Ra) and root mean squared roughness (Rq). Ra is the arithmetic average of the absolute values of the surface height deviations measured from the mean plane, expressed as follows in Equation 1.

\[ Ra = \frac{1}{N} \sum_{j=1}^{N} |Z_j| \]  

(Eq. 1)

In contrast, Rq is root mean square average of height deviations taken from the mean image data plane, expressed as follows in Equation 2.

\[ Rq = \sqrt{\frac{\sum(Z_j)^2}{N}} \]  

(Eq. 2)

RESULTS AND DISCUSSION

Size and Shape of Valonia Cellulose Crystals—The green alga *V. ventricosa* produces large cellulose fibrils. Cross-sections of *Valonia* cellulose crystallites appear square with sizes ranging from 10 to 20 nm as observed by TEM (26, 27). The negative staining method used in those studies measured the crystallites as nonstaining objects, and it was expected that the measured size of the crystallite could be smaller than that of the actual microfibril due to staining effects and resolution limitation. In the current study, the cross-section of the *Valonia* cell wall was imaged by AFM, with height (Fig. 1A), phase (Fig. 1B), and amplitude (Fig. 1C) images being taken simultaneously. By analyzing these images, the contours of each microfibril could be seen clearly. The shape of each microfibril is hexagonal (Fig. 1D); because the microfibrils are mostly irregular with two narrow sides, they can appear pentagonal in low resolution images (Fig. 1A–C). The sizes of individual microfibrils range from 10 to 50 nm, with maximum frequency near 35 nm. The isolated cellulose crystals were also imaged (Fig. 1, E and F). Several crystals often form small bundles, and single crystals were measured in the cross-section profile of height images as being 15 to 40 nm in height with maximum frequency of 25 nm, and the length was measured as 200 nm to 2 μm with maximum frequency of 1 μm. The reason that the measured size of single isolated crystals was slightly smaller than that in native cell wall could be one of the following: 1) the measured cross-sections may not be perfectly perpendicular to the long axis of the microfibril, which would result in larger size measured than the actual one; 2) although we used a sharp tip (1 nm in apex) for the AFM imaging to minimize the tip broadening effect, it would be difficult to deconvolute such effects; or 3) the preparation process used to isolate the cellulose crystal could also be expected to cause surface damage and peeling. Nevertheless, the shape of *Valonia* cellulose observed is an irregular hexagon with two narrow sides. Incidentally, subunits corresponding to the 3–5 nm elementary fibril reported previously (28) were not observed in this study.

CBH I Binder to Cellulose and Moves—Fig. 2 shows a single cellulose crystal imaged in acetate buffer (as “zero-time control,” Fig. 2A) and at 171, 179, 188, 196, 205, 214, and 222 min, respectively, (Fig. 2, B–H) after addition of CBH I. With the same scanning size and area, we observed that cellulose fibers had well defined ridges and relatively clean surfaces in buffer. After addition of CBH I, there were some new features (right side of cellulose) showing up on the cellulose crystal. These new features could be explained as CBH I enzymes binding to the cellulose surface. These new features were observed over a period of time (222 min), as shown in Fig. 2 (B–H). The apparent size of these features is approximately a quarter of the AFM...
measured height (25 nm) of the cellulose crystal. Based on published crystal structures for the CBH I catalytic domain (8), and on small-angle x-ray scattering structures for the intact enzyme (29), projection of a bound CBH I molecule away from the cellulose surface may be inferred to be between 5 and 10 nm. A productively bound molecule (bound through both CBM and a catalytic module “threaded” upon a cellulose chain) will have a “projection height” closer to the smaller size, whereas an enzyme molecule bound to the cellulose only by the CBM might have projection heights ranging up to ~10 nm. Therefore, we propose that the new features appearing on cellulose are most likely to be CBH I enzymes. Although the AFM time-resolution (frame rate ~ 1 frame/(8 min)) used in this study is not capable of demonstrating processive motion of specific, individual CBH I molecules, the observed new features were similar to those tracked in a recent high speed AFM report (10). Fig. 2 also shows that these features change relative locations on the cellulose surface over the time imaged. Because there are apparently two different sizes of features, with their relative distances changing during the observed time frames (Fig. 2, C and D), we further speculate that the two size categories represent two different kinds of modules (i.e. catalytic and carbohydrate-binding modules) and that the observed changes in intermodule distance reflect conformational changes in the entire CBH I molecule as CBH I catalyzes bond cleavage and moves along on a cellulose chain. Rigorous statistical analysis of continuous real-time imaging using AFM will be required to confirm this speculation.

Cellulose Surface Roughness Increases with CBH I Reaction—AFM is capable of measuring the surface structure at atomic resolution. We analyzed the structural changes in the cellulose surface concomitant with the action of CBH I. One of the features obtained from AFM measurements is the degree of roughness, which can be used to analyze changes in the surface brought about by friction, adhesion, and catalytic activity. There are many mathematical approaches to calculating surface roughness from AFM images, including probability height distribution, fractal analysis, mean roughness (Ra), and root-mean-squared roughness (Rq). Ra and Rq are the most commonly reported of the measures of surface roughness that can be extracted from AFM images. Fig. 3 represents roughness measurement of the cellulose crystal shown in Fig. 2. We found that cellulose surface roughness increased (in both Ra and Rq cases) by ~0.3 nm after addition of CBH I, which further confirms that the apparent morphological changes of cellulose observed by AFM were the results of CBH I reaction. The roughness remained almost constant during a subsequent 274 min of measurement in the presence of CBH I (Fig. 3). Valonia cellulosics are known to be primarily I₆ in crystalline form (30). The distance between I₆ cellulose sheets (d₁₁₀) is 0.39 nm, which is similar to the measured surface roughness increases resulting from CBH I action, indicating that CBH I enzymes affect only the surface layer of cellulose crystals. Previously, it has been demonstrated that the TrCBM1 binds to the hydrophobic faces (1 1 0) of cellulose (21, 31, 32), and these faces are as narrow as 2–4 nm (14, 32). Presumably the hydrophobic faces consist of more than one cellulose chain, thus the roughness change may indicate that the cellulose chains are hydrolyzed individually.

Cellulose Crystal Size Decreases during CBH I Hydrolysis—Continuous images of cellulose were taken in real-time during incubation with either buffer or CBH I solution. The size and shape of the cellulose crystal were analyzed by plotting the cross-section profiles of each image frame (Fig. 4, A and B). No change was observed during an 11-h incubation in buffer (Fig. 4A). After addition of CBH I, on the other hand, Fig. 4B shows size reduction of the observed area taken from the average of two successive frames with 50 cross-section lines in each frame (based on 512 × 512 scan lines), which demonstrated that the...
average width of the cellulose crystal decreased, but the average height remained relatively constant. The apparent reduction of cellulose specifically on one side of the crystal may imply that the CBH I tends to hydrolyze cellulose from certain surfaces. It has been previously reported that the family 1 CBM from CBH I binds only to certain faces of cellulose crystal, i.e. the (1 1 0) faces (or the hydrophobic faces) in the case of Valonia cellulose (28). It could be further speculated that CBH I hydrolysis also occurs only on the hydrophobic faces as discussed below.

The cross-section area of each single cellulose crystal was further integrated based on AFM image section profiles, and a size reduction of 20% was estimated based on 11 h of measurement (Fig. 4C), whereas there was no change observed for the same period of time in buffer (Fig. 4A). The hydrolysis rate of CBH I could not be calculated, because 1) it is not known how many CBH I molecules are involved in the period of 11 h hydrolysis, and 2) it is difficult to estimate how many cellulose chains are hydrolyzed in the 20% size reduction.

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FIGURE 5. A schematic diagram of AFM measurement of a cellulose crystal, in which the cross-section of a single 25-nm cellulose crystal is represented based on cellulose I₆ structure with narrow 3-nm hydrophobic faces (1 1 0). In this schematic representation, the crystal attaches to the mica surface by its broader hydrophilic surface. If the CBH I hydrolyzes both (1 1 0) and (−1 −1 0) faces, the AFM can only measure the changes in the (1 1 0) face, resulting in cross-section profiles of AFM height imaging reduced in width from one side and unchanged in height (Fig. 4). Tip scanning profiles in red and blue lines represent cellulose before and after hydrolysis, respectively. The tip and the cellulose are not in scale.
shape provided by the manufacturer. According to this proposed orientation of the crystal on the mica surface, both hydrophobic (1 1 0) and (1 1 0) faces, are exposed and susceptible to enzymatic hydrolysis. AFM, however, can probe only the surfaces geometrically accessible to the tip, so for the situation shown in Fig. 5, only the hydrolytic removal of material from the (1 1 0) face will show up in scan profiles as a difference between scans taken before and after CBH I hydrolysis. The schematic diagram shown in Fig. 5 therefore provides a quite likely explanation for the one-sided reduction of the cellulose crystal width in Fig. 4B. In addition, Fig. 5 illustrates a reasonable explanation for the observation that no significant changes in measured crystal height are detected during enzyme hydrolysis, contrasted with substantial changes in crystal width.

It has been documented that CBH I carries out exoattack on cellulose from its reducing end generating sharpened tips (16). Fig. 6 shows a succession of real-time AFM amplitude images (9 min per scan) of the end of the same cellulose crystal, beginning (“zero time” in the figure legend) 6 h after the addition of CBH I. The amplitude mode yields clear images with high contrast. During the course of the enzyme hydrolysis, we observed various shape changes at the end of the cellulose crystal, such as from smooth (Fig. 6A, 0 min), to sawtoothed (Fig. 6B, 9 min), and then to nicked (Fig. 6C, 26 min). The fiber end appeared smooth again in Fig. 6D (249 min), but with a sharper angle than that in Fig. 6A. The observed tip sharpening is similar to that reported previously (16) where CBH I erodes bacterial cellulose and renders the reducing end pointed in TEM images. We

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extend the results of the earlier work by providing real-time images of irregularities produced in cellulose-crystal tips by CBH I-catalyzed hydrolytic erosion. For the same reason illustrated in Fig. 5, AFM only detects one side of sharpening, whereas TEM image can show sharpening from both sides. In this study, operating AFM in an aqueous environment allows us to image the process without physiological conditions without intervening chemical/physical treatments. The observed phenomenon is thus closer to that occurring in nature. A control experiment was done by imaging cellulose fiber ends exposed to acetate buffer only. A series of real-time AFM amplitude images were taken starting at 6 h after adding acetate buffer solution rather than CBH I solution. No observable changes were found in either a short time (0 to 34 min with ~9 min frame rate) or a long time scale (360 min) (Fig. 7). The ends of isolated cellulose crystals sometimes appear to be nicked branches (Fig. 7), which could have resulted from the harsh preparation conditions (strong acid/base or high temperature) of cellulose isolation.

Conclusions—Atomic force microscopy has been used to make real-time measurements, both qualitative and quantitative, of the morphological changes in single crystals of cellulose during hydrolysis by T. reesei CBH I. We demonstrated structural changes including decrease in width, increases of surface roughness, and changes in shape of fiber ends. We have observed that the cross-section of Valonia cellulose crystal is apparently an irregular hexagon with two narrow hydrophobic faces that are binding surfaces for TrCBM1, and are likely to be the surfaces from which CBH I-catalyzed reactions remove cellulose chains. To the best of our knowledge, this is the first direct measurement of CBH I hydrolysis of crystalline cellulose that supports the hypothesis that CBH I hydrolyzes only the hydrophobic faces of cellulose. We further speculate that development of pretreatment approaches aimed at modification of cellulose to increase the accessibility of the hydrophobic planes to enzyme catalyst may lead to further improvements in the efficiency of enzymatic saccharification of biomass.

REFERENCES

1. Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., and Foust, T. D. (2007) Science 315, 804–807
2. Reese, E. T., Siu, R. G., and Levinson, H. S. (1950) J. Bacteriol. 59, 485–497
3. Montanari, S., Rountani, M., Heux, L., and Vignon, M. R. (2005) Macromolecules 38, 1665–1671
4. Fan, L. T., Lee, Y. H., and Beardmore, D. H. (1980) Biotechnol. Bioeng. 22, 177–199
5. Park, S., Baker, J. O., Himmel, M. E., Parilla, P. A., and Johnson, D. K. (2010) BiofueLS 3, 10
6. Henrissat, B., Driguez, H., Viet, C., and Schülein, M. (1985) Bio/Technology 3, 722–726
7. Enari, T. M., and Niku-Paavola, M. L. (1987) Crit. Rev. Biotechnol. 5, 67–87
8. Divine, C., Stähler, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J. K., Teeri, T. T., and Jones, T. A. (1994) Science 265, 524–528
9. Kraljis, J., Clore, G. M., Nilges, M., Jones, T. A., Pettersson, G., Knowles, J., and Gronenborn, A. M. (1989) Biochemistry 28, 7241–7257
10. Igarashi, K., Koivula, A., Wada, M., Kimura, S., Penttilä, M., and Samejima, M. (2009) J. Biol. Chem. 284, 36186–36190
11. Helbert, W., Chanzy, H., Husum, T. L., Schülein, M., and Ernst, S. (2003) BiMacromolecules 4, 481–487
12. Moran-Mirabal, J. M., Santhanam, N., Corgie, S. C., Craighead, H. G., and Walker, L. P. (2008) Biotechnol. Bioeng. 101, 1129–1141
13. Liu, Y. S., Luo, Y. H., Baker, J. O., Zeng, Y. N., Himmel, M. E., Smith, S. J., and Ding, S. Y. (2010) Proc. SPIE 7571, 757103
14. Ding, S. Y., Xu, Q., Ali, M. K., Baker, J. O., Bayer, E. A., Barak, Y., Lamed, R., Sugiyama, J., Rumbles, G., and Himmel, M. E. (2006) BioTechniques 41, 435–446
15. Bayer, E. A., Chanzy, H., Lamed, R., and Shoham, Y. (1998) Curr. Opin. Struct. Biol. 8, 548–557
16. Boisset, C., Fraschini, C., Schülein, M., Henrissat, B., and Chanzy, H. (2000) Appl. Environ. Microbiol. 66, 1444–1452
17. Bohn, A., Fink, H. P., Ganster, J., and Pinnow, M. (2000) Macromol. Chem. Phys. 201, 1913–1921
18. Lee, I., Evans, B. R., and Woodward, J. (2000) Ultramicroscopy 82, 213–221
19. Liu, H., Fu, S. Y., Zhu, J. Y., Li, H., and Zhan, H. Y. (2009) Enzyme Microb. Technol. 45, 274–281
20. Quirk, A., Lipkowski, J., Vandenende, C., Cockburn, D., Clarke, A. J., Dutcher, J. R., and Roscoe, S. G. (2010) Langmuir 26, 5007–5013
21. Liu, Y. S., Zeng, Y., Luo, Y., Xu, Q., Himmel, M. E., Smith, S. J., and Ding, S. Y. (2009) Cellulose 16, 587–597
22. Baker, A. A., Helbert, W., Sugiyama, J., and Miles, M. J. (2000) Biophys. J. 79, 1139–1145
23. Ding, S. Y., and Himmel, M. E. (2006) J. Agric. Food Chem. 54, 597–606
24. Baker, J. O., Mitchell, D. J., Grohmann, K., and Himmel, M. E. (1991) in Enzymes in Biomass Conversion (Leatham, G. F., and Himmel, M. E., eds.) pp. 313–330, American Chemical Society, Washington D.C.
25. Imai, T., Poutaux, J. L., and Sugiyama, J. (2003) Polymer 44, 1871–1879
26. Chanzy, H., Henrissat, B., Vuong, R., and Revol, J. F. (1986) Holzforschung 40 (Suppl.), 25–30
27. Revol, J. F. (1982) Carb. Polymers 2, 123–134
28. Gardner, K. H., and Blackwell, J. (1971) J. Polymer Sci. Part C: Polymer Symposia 36, 327–340
29. Abuja, P. M., Schmuck, M., Pilz, I., Tomme, P., Claeyssens, M., and Esterbauer, H. (1988) Eur. Biophys. J. 15, 339–342
30. Sugiyama, J., Vuong, R., and Chanzy, H. (1991) Macromolecules 24, 4168–4175
31. Lehtiö, J., Sugiyama, J., Gustavsson, M., Fransson, L., Linder, M., and Teeri, T. T. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 484–489
32. Xu, Q., Tucker, M. P., Arenkiew, P., Ai, X., Rumbles, G., Sugiyama, J., Himmel, M. E., and Ding, S. Y. (2009) Cellulose 16, 19–26