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In Situ, Rapid, and Temporally Resolved Measurements of Cellulase Adsorption onto Lignocellulosic Substrates by UV−vis Spectrophotometry

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This study demonstrated two in situ UV−vis spectrophotometric methods for rapid and temporally resolved measurements of cellulase adsorption onto cellulosic and lignocellulosic substrates during enzymatic hydrolysis. The cellulase protein absorption peak at 280 nm was used for quantification. The spectral interferences from light scattering by small fibers (fines) and particulates and from absorptions by lignin leached from lignocelluloses were corrected using a dual-wavelength technique. Wavelengths of 500 and 255 nm were used as secondary wavelengths for correcting spectral interferences from light scattering and absorption of leached lignin. Spectral interferences can also be eliminated by taking the second derivative of the measured spectra of enzymatic hydrolysate of cellulose or lignocelluloses. The in situ measured cellulase adsorptions in cellulose and lignocellulose suspensions by these two spectrophotometric methods showed general agreement with batch sampling assayed by the Bradford method. The in situ methods not only eliminated tedious batch sampling but also can resolve the kinetics of the initial adsorption process. The measured time-dependent cellulase adsorptions were found to follow pseudo-second-order kinetics.

I. Introduction

One major barrier in biochemical conversion of plant biomass to sugars for biofuel and bioproduct production is the low efficiency and high cost of enzymatic saccharification of cellulose. Adsorption of cellulase enzymes onto lignocellulosic substrates is a prerequisite step in cellulose saccharification because the substrate is insoluble and hydrolysis reaction is heterogeneous.[1] It is well known that enzymatic hydrolysis of a cellulosic substrate starts rapidly and is followed by a slow and sometimes incomplete reaction. The initial hydrolysis rate is proportional to the amount of cellulase adsorbed.[2] This suggests that enzyme adsorption kinetics in the initial hydrolysis period is very important to achieve high saccharification efficiency. It has been recognized that cellulase adsorption is significantly affected by lignin and lignin distribution (i.e., the amounts of lignin remaining on solid substrates and removed).[1,3] Furthermore, the adsorption of cellulase enzymes onto lignin causes loss of cellulase activity. The kinetics of cellulase adsorption onto lignin is different from that onto cellulose,[4] which can be used to elucidate the extent of inhibition of enzymatic cellulose hydrolysis by lignin. In view of this discussion, in situ, rapid, and temporally resolved cellulase adsorption measurements are important to produce necessary data for developing a good understanding of enzymatic hydrolysis of lignocelluloses.

Most studies reported in the literature on cellulase enzyme adsorption onto cellulose/lignocelluloses in suspension systems were based on batch sampling and then offline measurements[4−6] using solution depletion technique.[7] Cellulase in the samples was measured as protein using nitrogen analysis,[8] Lowry,[9] Bradford,[10] UV (280 nm) absorption,[11] or fluorescence[12] methods. Temporal resolution of the batch sampling and offline measurements is limited by the time to take the sample. Although the critical time period to achieve cellulase steady-state adsorption onto cellulose is often on the order of tens of minutes[13,14] and shorter than that onto lignin,[4] the batch sampling method may not be able to resolve the dynamic adsorption phenomena in the initial stage. Furthermore, it is tedious and time-consuming. Several methods have the potential for in situ and temporally resolved protein concentration measurements, including ellipsometry[15] and spectroscopic techniques.[7,11,12] Ellipsometry may not be suitable for lingocellulosic substrates because of difficulties in determining the complex refractive index of the irregular lignocellulosic surface. Most spectroscopic techniques have

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Table 1. Chemical Compositions of the Five Lignocellulosic Substrates Used in the Present Study[a]

substrate	k. lignin	a.s. lignin	arabinan	galactan	glucan	xylan	mannan	total
MDF spruce	28.0	0.8	0.5	1.7	44.8	5.6	10.3	91.8
delignified MDF	2.0	3.6	0.5	1.3	62.0	6.7	10.7	87.0
DA aspen	22.9	1.5	nd	nd	67.7	4.1	0.5	96.7
SPORL-low pH aspen	19.7	1.2	nd	nd	70.4	3.4	0.0	94.6
SPORL-high pH aspen	20.1	1.5	nd	nd	63.5	7.0	1.2	93.1

[a] "nd" stands for not detectable.

the issue of spectral interferences, such as those from light scattering by biomass particulates/fibers or from absorption by lignin, one of the major components of lignocelluloses. It is difficult to separate lignin from cellulose in lignocellulose saccharification studies. It is also difficult to eliminate light scattering from small fibers/particles in solid biomass suspensions. As a result, the accuracy of spectroscopic techniques for measurements of cellulase adsorption onto lignocellulosic substrates is compromised, even with separation steps such as centrifuge and filtration.

This study was aimed at developing effective approaches to overcome the effects of lignin and other solid particle interference on UV−vis spectrophotometric measurements of cellulase adsorption in lignocellulose suspensions. Specifically, this study demonstrated a dual-wavelength method and a spectral derivative method for correcting interferences from lignin and solid particles on the adsorption spectra of a commercial endoglucanase. By subtracting the measured cellulase concentration from the initial cellulase concentration, in situ measurements of cellulose adsorption onto lignocellulosic substrate can be achieved using UV−vis spectrophotometry.

II. Materials and Methods

Materials. Cellulose substrate was produced by disintegrating Whatman no. 1 filter paper (Whatman, England) for 12 000 revolutions in a disintegrator (TMI, Ronkonkoma, NY). Two of five lignocellulosic substrates were produced from spruce, and the other three were from aspen. The first spruce substrate was produced by directly disk milling spruce wood chips in a 30.5 cm pressurized disk refiner (Andritz Sprout-Bauer Pressurized Refiner, Andritz Sprout, Muncy, PA) using a disk plate pattern D2B-505. The milling was conducted at 7.2 bar and 166 °C with a disk plate gap of 0.06 mm, as described elsewhere.[16] This disk milling process is often used to produce wood fibers for making medium-density fiber board (MDF). The second spruce substrate was obtained by delignifying the fibers six times using a chlorite charge of 30% on oven dry (od) wood at 70 °C.[17] The three aspen substrates were produced by disk milling wood chips pretreated by dilute acid and two sulfite pretreatments to overcome recalcitrance of lignocelluloses (SPORL),[18] respectively. The pretreatments were conducted in a laboratory bomb-type pulping digester at 170 °C for 20 min. Sulfuric acid and sodium bisulfite charges on od wood were 1.1 and 0%, 1.1 and 3%, and 0 and 3% for the dilute acid (DA), SPORL-low pH, and SPORL-high pH pretreatments,[19] respectively. Disk millings were conducted under atmospheric conditions with disk plate gap of 1 mm, as described elsewhere.[20] The dilute acid and SPORL-low pH pretreatments effectively removed the recalcitrance of aspen so that the substrates have excellent enzymatic cellulose digestibility.[19] All lignocellulosic substrates were thoroughly washed with deionized water at 50 °C. The chemical compositions of the washed substrates were analyzed by the Analytical Chemistry and Microscopy Lab of the USDA Forest Products Laboratory (Table 1).

A commercial cellulase, Novozym 476 (Novozymes A/S, Denmark), a monocomponent endoglucanase (EGV), was used as received. SDS-PAGE showed a single dominant band with only traces of other bands.[21] The measured relative molecular weight was 46 kDa. The initial CMCase activity and protein concentration were 5000 ECU/mL and 6.4 mg/mL, respectively. Bovine serum albumin (BSA) (Food grade, SeraCare, Milford, MA) was used as standard to calibrate the protein content of Novozym 476. Bio-Rad (Bradford) protein assay kit was obtained from Bio-Rad Laboratories (Maryland). All other chemicals used were of analytical grade unless stated otherwise.

Apparatus for In Situ Cellulase Concentration Measurements. All experiments were conducted using a flow loop, as described elsewhere.[22] For each substrate, 1.0 g of od fibers was added to 100 mL of endoglucanase/buffer solution (0.5 mM acetate buffer, pH 4.8) in a 125 mL Erlenmeyer flask at room temperature. Novozym 476 loading was 0.1 mg protein/mL in carrying out hydrolysis experiments. Solution or suspension was first agitated using a magnetic stirring bar at 200 rpm. Solution or suspension was then pumped at a flow rate of 1.5 mg/min using a peristaltic pump (model M312, Gilson, Middleton, WI) through a Teflon tube (total volume of ∼2.0 mL) to a quartz flow cuvette (1.5 mL) and back to the flask. The flow cuvette has an optical path length of 10 mm. A stainless-steel screen tube (400 mesh, volume ∼1.0 mL) was wrapped at the inlet of the Teflon tube to filter fibers. Spectral intensities of light passing through the cuvette were recorded by a UV-vis spectrophotometer (model 8453 Agilent, Palo Alto, CA). The spectrophotometer is equipped with a kinetic operation mode for continuously recording complete UV−vis spectra every 10 s. The sampling interval of 10 s is more than sufficient to resolve the cellulose adsorption kinetics.

Measurements of Cellulase Adsorption by Batch Sampling. The endoglucanase concentrations during hydrolysis of the pure cellulose and SPORL-low pH aspen substrate were also batch sampled and then analyzed using the Bradford method with the commercial Bio-Rad (Bradford) protein assay kit.[10] About 0.8 mL of substrate hydrolysate was taken from the screen tube every 2 min. The samples were centrifuged at 13 000*g* for 1 min. The protein concentration of the supernatant of each sample was determined to calculate the amount of endoglucanase adsorption to compare with the in situ UV−vis measurements. Bovine serum albumin (BSA) was used as standard for calibration using the Bradford method. The reported data are averages of duplicates. The standard deviations were used as measurement error bars.

III. Results and Discussions

Spectral Characteristics. The protein absorption spectrum is well-characterized, with absorption mainly in the UV range.

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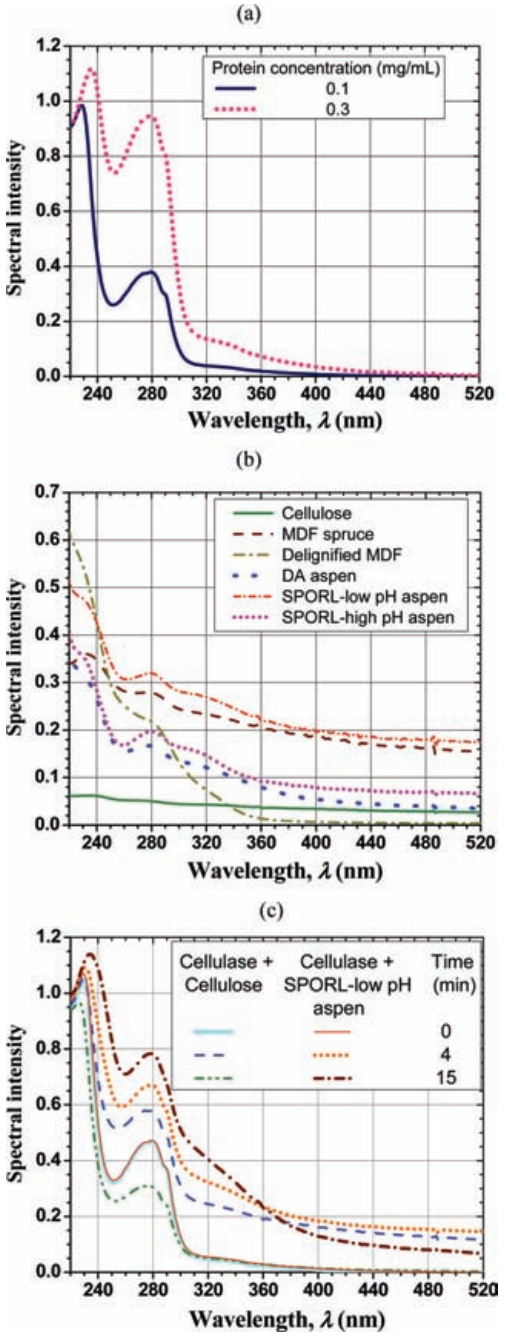

Figure 1. In situ measured UV−vis spectra of cellulase solution and lignocellulose suspensions of 1% consistency. The solution/suspensions were flowing at 1.5 mL/min and stirred at 200 rpm: (a) cellulase (endoglucanase) solutions, (b) lignocellulose suspensions, and (c) lignocellulose suspensions in the presence of cellulase.

As shown in Figure 1a, the endoglucanase has two absorption peaks, around 230 and 280 nm. The peak at 230 nm showed a shift at high concentrations (0.3 mg/mL in Figure 1a), probably due to the formation of cellulase aggregates or micelles at high concentrations. A valley at 250 nm was also observed. No spectral shift was observed at 250 and 280 nm as endoglucanase concentration increased from 0.1 to 0.3 mg/mL. The peak at wavelength 280 nm is often used for protein quantification.[11] Spectral intensity decreases rapidly beyond 280 nm. No absorption was detected beyond a wavelength of 500 nm.

Spectral interferences by small fibers (fines) and lignin of lignocelluloses are unavoidable during UV−vis measurements, even with screen filters. Light scattering from small fibers (fines) or particulates in cellulosic/lignocellulosic suspensions can be seen from the spectrum of a cellulose suspension, as shown in Figure 1b. It is featureless and covers the entire UV−vis spectral range. Lignin can be leached out from lignocellulosic substrates,[23] resulting in absorptions primarily in the UV range (Figure 1b). The lignin absorption peak at 280 nm completely overlaps the main absorption peak of protein. It is noticed that the light scattering intensities from fines are much lower than the lignin absorptions in the UV range, suggesting that lignin absorption is probably the main spectral interference. It is also noticed that the spectra for the two SPORL aspen substrates are very similar. The main difference is in the spectral intensity, which is mainly contributed by the difference in baseline shift from light scattering. The SPORL-high pH produces substrates with larger size than does the SPORL-low pH under the same disk milling conditions, as evidenced by SEM imaging.[20] More SPORL-high pH fibers were filtered out because of larger size by the stainless tubing screen in the flow loop, which resulted in lower light scattering than with the SPORL-low pH substrate.

The spectra of the cellulosic and lignocellulosic (SPORL-low pH aspen) enzymatic hydrolysates (suspensions) are a superposition of the spectral features of the cellulase absorptions and the interferences, as discussed above. The main spectral features resemble the protein absorption spectra but with a broadband feature extended beyond 300 nm to the visible range (Figure 1c). For these two adsorption experiments, the absorption spectra of endoglucanase were recorded before the addition of cellulose/lignocellulose. As expected, the two endoglucanase spectra are almost identical for each substrate at $t = 0$, suggesting excellent experimental repeatability in in situ measurements. After the addition of cellulose or pretreated aspen fibers, adsorption of endoglucanase onto the substrates proceeded. The light scattering and absorption by leached lignin were increased, as shown on the two spectra obtained at 4 min, despite the fact that endoglucanase concentrations in both suspensions were decreased by adsorption onto fibers filtered by the stainless screen. The spectral intensities of the cellulose suspension are lower than those of aspen suspensions because of the presence of lignin in the latter. Continued circulation ($t = 15$ min) caused increased lignin leaching from aspen, as evidenced by the increased lignin absorption in the UV range, despite the reduced endoglucanase adsorption in the same UV range due to adsorption. The accumulation of small fibers (fines) on the stainless tubing screen in the flow loop increased the filtration efficiency of small fibers (fines) during circulation, which reduced the light scattering, as reflected by the spectral intensities in the visible range (Figure 1c) where lignin absorption is weak (Figure 1b).

Dual-Wavelength Method. The interferences of light scattering by suspended small fibers (fines) or particulates and the absorption of lignin can be corrected for by using a dual-wavelength spectroscopic method. The method has been successfully applied to correct for the interference of lignin leached from chemical pulps on the absorption of hydrolysis products of hexeneuronic acid at 290 nm.[23] This is a very similar problem for the lignin interference on cellulase absorption at 280 nm in the present study. For any two given wavelengths, $m1$ and $m2$, the measured total spectral intensity, A, can be written as the linear contribution of the protein absorption, A_p, and the spectral intensity from interference (scattering and/or absorption), I,

$$A^{m1} = A_p^{m1} + I^{m1} \quad (1a)$$

$$A^{m2} = A_p^{m2} + I^{m2} \quad (1b)$$

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Table 2. Experimentally Determined Parameters for the Present Dual-Wavelength Method for Quantification of Cellulase (Novozyme 476) Concentration

substrate	$m1$	$m2$	σ	τ
cellulose	280	500	0	0.5430 ± 0.0020
MDF spruce		255	0.708 ± 0.001	1.0384 ± 0.0006
delignified MDF				1.2240 ± 0.0005
DA aspen				0.9702 ± 0.0012
SPORL-low pH aspen				0.9973 ± 0.0004
SPORL-high pH aspen				0.8800 ± 0.0009

When the principal ($m1$) and second ($m2$) wavelengths are properly selected, the spectral intensities of the protein absorption and the interference spectral intensity at these two wavelengths can be (linearly) correlated,[23] that is

$$A_{\mathrm{p}}^{m2} = \sigma A_{\mathrm{p}}^{m1} \quad (2a)$$

$$I^{m2} = \tau I^{m1} \quad (2b)$$

Simultaneously solving eqs 1a−2b, we have

$$A_p^{m1} = \frac{A^{m2} - \tau A^{m1}}{\sigma - \tau} = \epsilon_{\mathrm{p}}^{m1} \cdot L \cdot C_{\mathrm{p}} \quad (3)$$

where $\epsilon_{\mathrm{p}}^{m1}$ is mass absorptivity of protein at a given principal wavelength, L is the optical path length, and C_{p} is the mass concentration of the solution. Therefore, protein mass concentration can be obtained

$$C_{\mathrm{p}} = \frac{A^{m2} - \tau A^{m1}}{\epsilon_{\mathrm{p}}^{m} \cdot L(\sigma - \tau)} = \frac{A^{m2} - \tau A^{m1}}{\alpha(\sigma - \tau)} = \frac{A^{m2} - \tau A^{m1}}{\beta} \quad (4)$$

where $\alpha = (\epsilon_{\mathrm{p}}^{280} \cdot L)$. Either α or β can be calibrated using pure cellulase solutions, as will be discussed later.

A dual-wavelength method uses the measured spectral intensity at a second wavelength, $m2$, to quantify spectral interference at the principal wavelength, I^{m1}. There are many selections for these two wavelengths as long as the selection satisfies eqs 2a and 2b. Wavelength $m1$ is usually the principal absorption peak of the substance of interest; $m1 = 280$ nm for protein or cellulase measurements. At wavelength $m2$, it should have all potential interferences at the principal wavelength $m1$ (280 nm) to account for the contribution of these interferences.

For cellulase adsorption onto cellulose, I^{280} is simply from light scattering of small cellulosic fibers (fines), a featureless broadband spectrum, as shown in Figure 1b. In the visible range, that is, $m2 = 500$ nm, protein does not have absorption (Figure 1a), $A_{\mathrm{p}}^{500} = 0$ and $A^{500} = I^{500}$; therefore, eq 2a is automatically satisfied with $\sigma = 0$. Experiments were conducted using a cellulose suspension to establish eq 2b. The suspension was continuously circulated through the flow cuvette. Light scattering spectra were continuously recorded. The light scattering spectral intensities at 280 and 500 nm recorded from different times were plotted. Linear regression resulted in the τ value of 0.546 for eq 2b, as listed in Table 2.

For cellulase adsorption onto lignocelluloses, I^{280} is contributed by many factors, such as light scattering and absorptions by lignin. Because lignin absorbs primarily in the UV range, appropriate selection for the second wavelength should be in the UV range. Wavelength $m2 = 255$ nm (close to 260 nm, which has been used for correction contributions from nucleic acids[11] and lignin[23]), which gives good results to satisfy eqs 2a and 2b, was selected. It should be pointed out that spectral interference from light scattering by small fibers (fines) at 280 nm was also captured at 255 nm and corrected. Experiments were conducted using endoglucanase solutions of protein concentration ranging from 0 to 0.13 mg/mL to establish eq 2a. Linear regression of the recorded time-dependent protein absorption intensities from the endoglucanase solution at 255 nm, A_{p}^{255}, and at 280 nm, A_{p}^{280}, resulted in $\sigma = 0.708$. Spectral measurements were also conducted using the five lignocellulose suspensions in the absence of endoglucanase to establish eq 2b for each substrate. Excellent linear relations between the measured spectral intensities at 255 nm, I^{255}, and at 280 nm, I^{280}, were also obtained for each suspension. The coefficients τ obtained by linear regression for eqs 2b are listed in Table 2. A constant value of τ (= 1.2) was adopted for different wood pulps by Chai et al.[23] because the method is for applications to one type pulp, Kraft pulp, with similar lignin characteristics. The present method for cellulase measurements applies to various lignocellulosic substrates derived from different pretreatment processes, such as dilute acid SPORL with different pH values. The degree of variations among τ shown in Table 2 suggests the variations and similarities in lignin characteristics of these substrates.

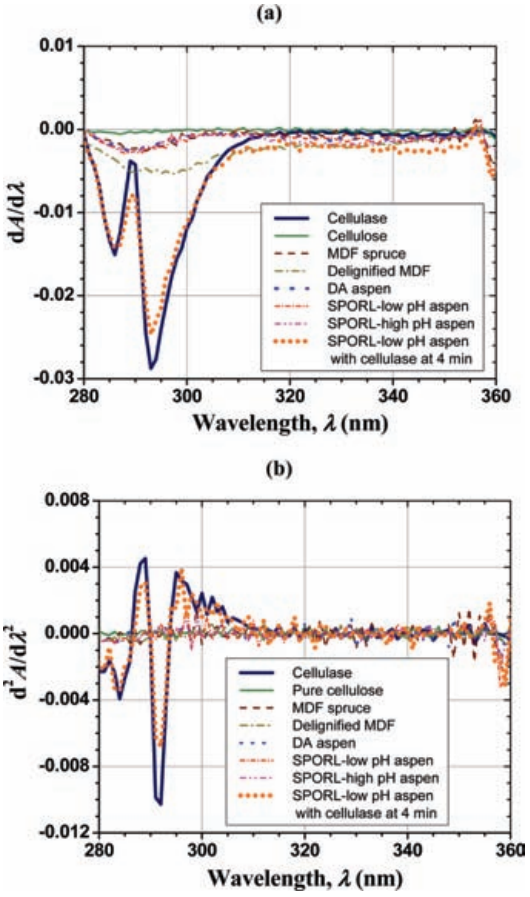

Figure 2. Derivative spectra (with respect to wavelength) derived from the spectra shown in Figure 1: (a) first-order derivative and (b) second-order derivative.

Spectral Derivative Method. The spectral derivative method uses spectral features to eliminate interference. Interferences from light scattering by small particulates or fibers (fines) are broadband and featureless (with a very small slope that almost linearly decreases with wavelength), as shown by the cellulose spectrum in Figure 1b. The first-order derivatives with respect to wavelength of this spectrum are therefore almost constant (Figure 2a). A close-to-zero value results when taking the second derivatives with respect to wavelength (Figure 2b). Absorption spectra of lignocelluloses exhibit unique features, especially the absorption peak at ~280 nm, and overlap with the absorption peak of

protein. However, lignocelluloses have no absorption peak beyond 280 nm, with absorption slowly declining in intensity (Figure 1b), similar to that of light scattering interference; that is, the second-order derivatives with respect to wavelength will be close to zero, as shown by the data from the five lignocellulose suspensions (Figure 2b). The absorption spectra of endoglucanase (Figure 1a) exhibit a very sharp decline between 280 (peak absorption) and 300 nm; furthermore, the spectra have two inflection points at wavelengths of about 285 and 295 nm (Figure 1a,c). This suggests that the first-order derivative with respect to wavelength will have two local maxima and one local minimum between 280 and 300 nm (Figure 2a). The second-order derivatives will change sign at the two inflection points and achieve one local maximum between these two points (Figure 2b). Therefore, the interferences from light scattering and absorptions from other species, mainly leached lignin, on endoglucanase (protein) absorption can be eliminated by taking the second derivative of the cellulose or lignocelluloses hydrolysate spectra with respect to wavelength. The derivative spectrum taken from the endoglucanase containing SPORL-low pH aspen suspension is very similar to the spectrum of endoglucanase between 280 and 360 nm (Figure 2b), suggesting the effectiveness of the present derivative method for eliminating spectral interferences from lignin adsorption and light scattering from particulates on cellulase absorption in enzymatic hydrolysates. Because the second-order derivative peaked around 291 nm, 291 nm was selected for cellulase concentration quantification using the expression

$$\left.\frac{d^2A}{d\lambda^2}\right|_{\lambda=291} = \chi \cdot C_p \qquad (5)$$

where χ is a calibration constant.

Method Calibration. Endoglucanase solutions were used to calibrate both methods discussed above. As shown in Figure 3a,b, excellent linear fittings of the measured spectral intensities with respect to the known endoglucanase concentrations using eqs 3 and 5 were obtained for protein concentrations below 0.25 mg/mL for eq 3 and below 0.20 mg/mL for eq 5. The slopes of the linear correlations from fitting are simply the calibration constants for eqs 3 and 5, that is, $\alpha = 3.535$ L/g and $\chi = -0.10$ L/g/nm^2, respectively.

Time-Dependent Adsorption of Cellulase onto Cellulose/Lignocellulose. Endoglucanase adsorption experiments onto a cellulosic and a SPORL-low pH aspen (lignocellulosic) substrate were conducted to demonstrate the present methods for in situ measurements. The time-dependent spectral intensities at 280 and 500 nm for the cellulosic substrate or at 280 and 255 nm for the SPORL-low pH aspen substrate represent the dynamic processes of cellulase and lignin adsorptions and light scattering caused by small fibers (fines) (Figure 4). Specifically, (1) the development of a fiber net on the stainless screen through suspension circulation provided improved filtration of fibers (fines) and decreased light scattering over time; (2) the continuous leaching of lignin increased lignin absorption; and (3) endoglucanase adsorption reduced free endoglucanase concentration in the suspension and therefore absorption over time. The spectral intensities at 500 nm for the cellulosic substrate clearly show the effects of fibers (fines) on light scattering interference. The intensities increased with time initially and then decreased because of the filtration of fibers (fines) by the development of a fiber net. This feature is also reflected on the measured spectral intensity profile at 280 nm. Endoglucanase concentration decreased monotonically with time because of adsorption onto cellulosic/lignocellulosic substrate, as shown by the calculated

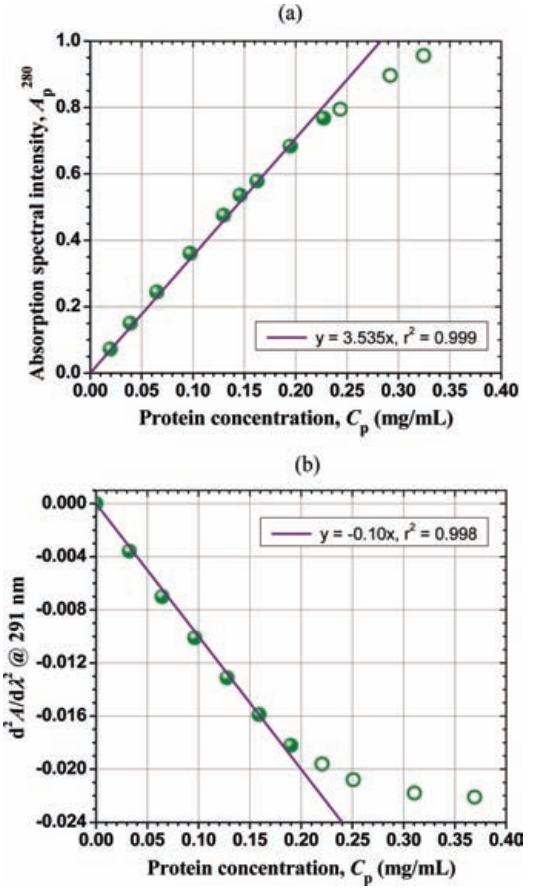

Figure 3. Calibration results using cellulase solutions conducted at flow rate 1.5 mL/min and stirred at 200 rpm: (a) dual-wavelength method and (b) spectral derivative method.

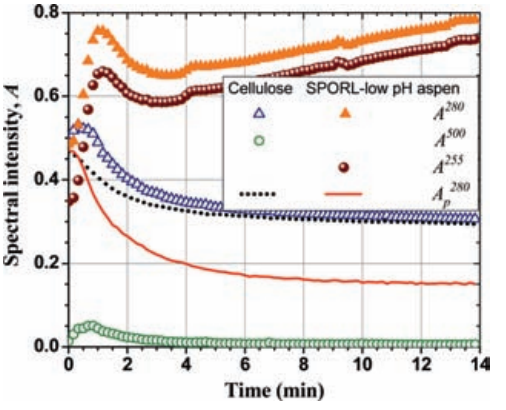

Figure 4. In situ measured time-dependent spectral intensities at two wavelengths during cellulase adsorption onto a cellulose (280 and 500 nm) and a SPORL-low pH pretreated aspen substrate (280 and 255 nm), along with calculated time-dependent protein absorption spectral intensity using the dual-wavelength method.

endoglucanase absorption intensity profile using the dual-wavelength method. The spectral intensities at 255 and 280 nm for the SPORL-low pH aspen suspension were much stronger than the intensities from light scattering at 500 nm of the cellulose spectrum due to lignin absorption. This is in addition to the fact that (1) light scattering spectral intensities at these two wavelengths are higher than that at 500 nm and (2) light scattering effect is smaller than that of lignin absorption (Figure 1b). The spectral intensities at 255 and 280 nm increased with time initially because of light scattering

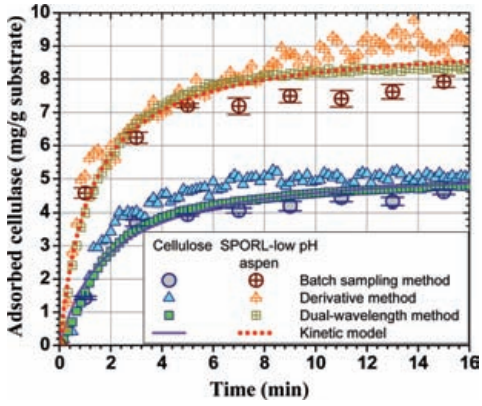

Figure 5. Comparisons of the measured time-dependent cellulase absorptions onto cellulosic and SPORL-low pH pretreated aspen substrates using the two in situ spectroscopic methods and a batch sampling method assayed by Bradford method. The data from the dual-wavelength method were used to fit to a pseudo-second-order kinetic model.

and absorption by lignin from leaching (Figure 4). The development of fiber net over time reduced the light scattering spectral intensities at these two wavelengths. Further leaching of lignin overcame the deficit produced by the reduced light scattering, which resulted in the increase in spectral intensities at these two wavelengths. The endoglucanase absorption intensities at 280 nm corrected for all interferences, A_p^{280}, can be obtained using the dual-wavelength method. The results clearly show the decline of (free) endoglucanase concentration in the suspensions with time due to endoglucanase adsorption onto the two cellulosic and lignocellulosic substrates (Figure 4). Similar results of free endoglucanase concentrations were also obtained using the spectral derivative method.

The amounts of cellulase adsorbed can be calculated by simply subtracting the measured time-dependent free cellulase in the suspensions from the initial amount of cellulase applied. The results obtained by the dual-wavelength and spectral derivative methods were compared with those obtained using batch sampling then assayed by Bradford method.[10] Good agreements among these three methods were obtained (Figure 5), suggesting the validity of the present in situ UV−vis spectrophotometric methods for rapid and temporally resolved endoglucanase adsorption measurements. The results show that the batch sampling Bradford method produced lower endoglucanase adsorption than both the dual-wavelength and spectral derivative methods in general. However, the differences are much smaller for the cellulose substrate than those of the lignocelluloses substrate. This is probably due to measurement bias in quantifying protein in samples taken from lignocellulose suspensions using the Bradford method. Lignin can bind with the dye (Coomassie Brilliant Blue) used in the Bradford method to overpredict the amount of protein in the sample. The interaction of cellulase with lignin is nonspecific, weaker than the specific binding with cellulose. As a result, more desorption of cellulase may be taken place for a lignocellulosic substrate than for a cellulosic substrate during sample preparation using centrifuge. The spectral derivative method produced highest endoglucanase adsorption among the three methods for the two experiments conducted. However, the maximum difference among the three methods (between batch sampling and spectral derivative methods) is ~20% (Figure 5). The maximum difference between the dual-wavelength and derivative methods is only ~10%. The error bars of the Bradford method in Figure 5 were the standard deviations from triplicate measurements. The average standard deviation of the dual wavelength method was ~2% based on the six replicate adsorption experiments (not shown in Figure 5 for clarity). Data scattering for the spectral derivative method is higher than that for the dual-wavelength method because of sensitivities of derivatives to experimental spectral variations from noise or errors (Figure 5). The average standard deviation of the measured endoglucanase adsorption over the initial adsorption period of 22 min (with sampling interval of 10 s) was 4.6% from duplicate measurements using the spectral derivative method (not shown in Figure 5 for clarity). Nevertheless, the simplicity of the spectral method is advantageous for real-time measurements.

Mathematical models were applied to illustrate the endoglucanase adsorption kinetics. It was found that fittings using both the first-order kinetics and diffusion models were unsatisfactory. The pseudo-second-order kinetic model

$$\frac{1}{q} = \frac{1}{k \cdot q_e^2} \cdot \frac{1}{t} + \frac{1}{q_e} \tag{6}$$

where k is the rate constant and q_e and q are the amount of cellulase adsorbed per unit mass of substrate at equilibrium and time t, respectively, provides the best fits to both sets of the endoglucanase adsorption data of the two hydrolysis experiments (Figure 5), in agreement with literature.[24] The fittings were conducted using the data determined from the dual-wavelength method. The results were k = 0.099 and 0.090 (g substrate/mg/min), q_e = 5.421 and 9.186 (mg/g substrate), for the cellulosic and pretreated aspen substrates, respectively. The difference in rate constants is very small between these two substrates. However, the amounts of endoglucanase adsorbed at equilibrium are very different. This difference can be contributed by many factors, most likely, however, by the difference in the specific surface of the two substrates. The lignocellulosic substrate is much finer than the cellulose substrate based on visual observation and SEM imaging. The initial adsorption of endoglucanase was very rapid. About 60−70% of the equilibrium adsorption amount was achieved in the first 2 min, suggesting that batch sampling may be incapable of temporally resolving the initial cellulase adsorption kinetics.

IV. Conclusions

UV−vis spectrophotometry can be applied for in situ cellulase adsorption measurements in enzymatic hydrolysis of lignocelluloses by using a dual-wavelength or a spectral derivative method. The spectral interferences from light scattering by small fibers (fines) and particulates and absorption from lignin leached from lignocelluloses can be effectively corrected using either of these two methods. The in situ measured cellulase adsorptions onto lignocellulose by these two methods are slightly higher than those by batch sampling assayed by Bradford method but are otherwise in good agreement. The derivative method produced slightly more data scattering than the dual-wavelength method. This was due to its greater sensitivities to experimental spectral variations from noise or error. However, the simplicity of the derivative method is attractive for online real-time applications.

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(24) Daoud, F. B.-O.; Kaddour, S.; Sadoun, T. Adsorption of cellulase *Aspergillus niger* on a commercial activated carbon: kinetics and equilibrium studies. *Colloids Surf., B* **2010**, *75*, 93–99.

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