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Quantitative Study of the Interfacial Adsorption of Cellullase to Cellulose

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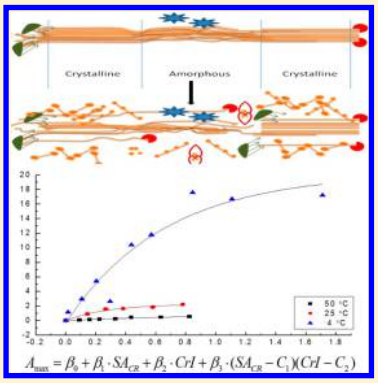
Supporting Information

ABSTRACT: The phenomenon of interfacial adsorption of cellulase to cellulose plays a significant role in its enzymatic conversion to a variety of biomaterials and biofuels. The crystallinity and surface areas are the key substrate characteristics that must be considered in its final conversion to soluble sugars. This research therefore characterized the crystallinity and surface areas of microcrystalline celluloses and hardwood pulps as a function of the interfacial activity of cellulase, and thereafter the effects of these two parameters on adsorption were modeled. The crystallinities were characterized by X-ray diffraction, while surface areas by laser scattering and Congo red adsorption. It was found that cellulase adsorption to cellulose follows a Langmuir model while statistical modeling showed that surface area, crystallinity, and their interactions were determined to be significant for cellulase adsorption over a temperature range of 4−50 °C.



1. INTRODUCTION

A green manufacturing conversion platform for the transformation of cellulose to soluble sugars by cellulase plays a critical role in utilizing cellulosic biomass for applications in a number of diverse disciplines including principally energy, materials, and medicine. This type of conversion can be economical and environmentally friendly because the raw materials and catalysts are abundant and bio-based, and the operating conditions for the conversion are biologically mild.[1,2] However, for the system to have any opportunity to achieve saccharification (hydrolysis of the sugar-based polymer chain), cellulase adsorption is considered *a priori* to be the chief prerequisite. Because of complex physiochemical surface properties of solid substrates and the inherent complexity associated with the variety of cellulase enzyme systems, these heterogeneous enzymatic reactions are extremely complicated compared to homogeneous enzymatic reactions.[3−7] Understanding their interfacial behavior is critical for an ultimate scaled-up biorefinery that encompasses the commercialization of next-generation biofuels and green materials, for the facility design and operating parameters. Cellulase adsorption to cellulose is affected by several factors. These factors can be classified into three categories: namely, the biochemical properties of the cellulase system, the physicochemical characteristics of substrates, and the operating conditions of the conversion process. The interactions among these parameters compromise the success of cellulose hydrolysis research and thus make it more challenging.[8−10] A great deal of research has studied the effects of a number of operating conditions including temperature, pH, ionic strength, surfactants, etc., in terms of the media effect.[11−14] Media effect is a function of the overall conditions of the aqueous solutions in which the biological reactions take place. A significant amount of research has also investigated how the substrate affects cellulase adsorption and subsequent cellulose hydrolysis,[15,16] but a relatively thorough and systematic study of such interfacial and heterogeneous kinetics is lacking.

The Langmuir isotherm model is the most popular model describing interfacial adsorption behavior. The model generally obeys the equation[15,17,18]

$$W_{ads} = \frac{W_{ads,max} K_p C}{1 + K_p C} \quad (1)$$

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where W_{ads} is the amount of adsorbate on a substrate (mg/g), $W_{ads,max}$ the maximum amount of adsorbate that can be adsorbed (or saturated adsorbate, mg/g), C the free concentration of adsorbate (mg/mL) at equilibrium, and K_p the adsorption constant (mL/mg). Equation 1 can be an empirical adsorption model describing cellulase adsorption but does not conform to more refined Langmuir assumptions,[19] which encompass both the homogeneity of substrate and the formation of a cellulase monolayer.

There is general agreement on how the surface and bulk composition of substrates can affect cellulose hydrolysis,[20] but it remains contentious how the physical properties of the substrates, here cellulose, affect the interfacial adsorption of heterogeneous catalytic reactions. Surface area, porosity, and crystallinity are major factors currently under investigation.[21−24] Another question is, which parameter, surface area or crystallinity, has a more pronounced effect on cellulase adsorption? The current research therefore attempted to answer these questions by fitting adsorption data with surface areas and crystallinity of selected cellulose substrates. The effect of the interaction of these two variables on adsorption is also investigated using statistical analysis.

2. EXPERIMENTAL SECTION

2.1. Materials. A commercial cellulase powder derived from *Aspergillus niger* was purchased from MP Biomedical Inc. (hereafter referred to as MPC). Microcrystalline cellulose (Avicel PH101, PH102, and PH105) was from FMC BioPolymer Corporation with nominal particle sizes of 50, 90, and 30 μm, respectively. Eucalyptus bleached kraft pulp was purchased from the National Institute of Standards and Technology (NIST, US Department of Commerce, Gaithersburg, MD 20899). The pulp was disintegrated in a disintegrator and designated as unbeaten pulp (UP hereafter). Part of UP was beaten in a valley beater[25] to 130 mL CSF freeness as compared to 560 mL for the UP and designated as beaten pulp (BP hereafter). All pulps were thoroughly washed using sodium acetate buffer (pH 4.8, ionic strength = 100 mM) three times and then stored in a cold room at 4 °C.

2.2. Crystallinity Measurement. The crystallinity indices were determined using a Philips XLF ATPS XRD 1000 (an OMNI Instruments Inc.) with a Cu target of λ = 1.54 Å. The calculation of crystallinity indices followed the procedure by Segal et al.[26] and is summarized in eq 2:

$$\mathrm{CrI} = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \tag{2}$$

where CrI is the degree of crystallinity, I_{002} the maximum intensity of the 002 lattice diffraction, and I_{am} the intensity of diffraction at the 101 lattice. Handsheets of both UP and BP were prepared by TAPPI method T 272 sp-97,[27] and MCC pellets were pressed for use in the XRD for crystallinity determination.

2.3. Surface Area Determination. Congo red, a direct dye, was used to measure the substrate surface areas. The adsorption of Congo red dye follows a Langmuir-type monolayer adsorption.[28] Adsorption measurements were conducted at room temperature with a 3.5 g to 100 mL substrate-to-SSA = liquid ratio. The saturation adsorptions (A_{max}) of Congo red on the substrates were determined by fits of Langmuir adsorption isotherms, the surface areas were calculated using eq 3

$$\mathrm{SSA} = \frac{A_{max} N_A \mathrm{SA}_{CR}}{\mathrm{MW}_{CR}} \tag{3}$$

where N_A is Avogadro's number, SA_{CR} is the surface area of a single Congo red molecule (1.73 nm^2), and MW_{CR} the molecular weight of Congo red (696.66 g/mol).

A Horiba laser scattering particle size distribution analyzer LA-300 was also used to measure the particle sizes and external surface areas of the substrates employed. The measurements were conducted at a laser wavelength of 650 nm with samples suspended in pure water. The sample amount was controlled in the range such that 70−90% of the incident light was transmitted to the detector arrays. Because of the spherical shape assumption in the LA detection system, this provides only referential information.

2.4. Measurements of Cellulase Adsorption. The adsorption of cellulase was determined using the depletion method. The cellulase concentration in solutions was determined using the absorbance method at 280 nm.[29] Adsorption incubation experiments were conducted at a 5% substrate consistency at three temperatures (4, 25, and 50 °C). The reaction mixtures were incubated in a Labline incubator shaker for temperatures at 25 and 50 °C. Adsorption experiments at 4 °C were performed in a cold room. A 60 min period was selected as the incubation time. After the incubation, the reaction mixtures were centrifugally separated in a Beckman Model TJ-6 centrifuge at 1400 rpm (an equivalent of 400g force) for 20 min. The liquid phases were then used for the determination of free protein/cellulose content (equilibrium concentration). The difference of protein/cellulase concentration in the original and equilibrium cellulase solutions gives the amount of adsorption of cellulase. Figure 1 presents the procedure used for the cellulase adsorption measurement.

2.5. Statistical Analysis of Data for Langmuir Adsorption Parameters. The Langmuir parameters were determined by fitting cellulose adsorption against the equilibrium concentration of proteins, according to eq 1; thereby, the Langmuir parameters were obtained.

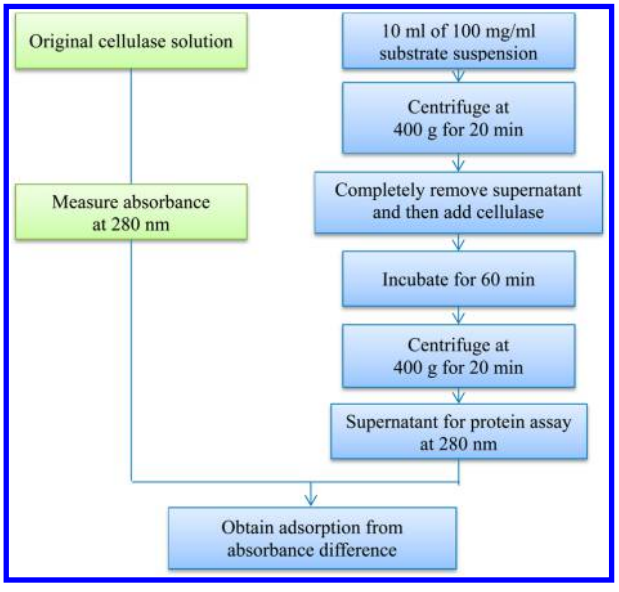

Figure 1. Flow diagram of the procedure used to determine protein adsorption by substrates in cellulase solutions.

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A statistic regression model was employed to determine the relative significance level of substrate surface area by Congo red and crystallinity indexes on cellulase adsorption. The model was

$$A_{max} = \beta_0 + \beta_1 SA_{CR} + \beta_2 CrI + \beta_3 (SA_{CR} - C_1)(CrI - C_2) \quad (4)$$

where A_{max} is the maximum adsorption determined from the Langmuir adsorption isotherm; SA_{cr} and CrI are substrate surface area by Congo red and the substrate crystallinity index by X-ray diffraction, respectively; C_1 is a constant related to the Congo red surface area and C_2 a constant related to substrate crystallinity; β_1, β_2, and β_3 are regression coefficients that describe the contributions of surface area, crystallinity indices, and their interactions to the maximum adsorption; β_0 is a regression intercept. The analysis of this model was realized by using the JMP Software developed by SAS Institute Inc.

3. RESULTS AND DISCUSSION

3.1. Crystallinity Indices of Substrates. Figure 2 is the X-ray spectrum for beaten HW kraft pulp. The shape of the diffractogram is similar to what has been reported elsewhere.[30,31] The summary of the crystallinity indices, calculated using eq 2 of the substrates, is presented in Table 1. The crystalline indices of MCCs are greater than those obtained by Ardizzone et al.[31] and Nakai et al.,[32] whose numbers were 65% and 63%, respectively, but closer to those obtained by Plonka[33] and Sottys et al.,[34] both of whom reported 75% crystallinity indices. These differences may have been caused by the instruments used for their respective research. Nevertheless, the current results for MCCs are in agreement with the crystallinity obtained by Fan et al. and Lee et al., whose numbers for MCC were 84.5% and 80.3−82.9%, respectively.[3,35] Unbeaten and beaten pulps show significantly lower crystallinities than the MCCs. The crystallinity of unbeaten pulp is very close to the crystallinity by Cheng et al., but larger than the value obtained by Park for softwood pulp.[36,37] These differences may be due to species differences. Beating defibrillates fibers, which decreases their crystallinities. Unlike MCC, most of the amorphous region of fibers remains intact which gives lower crystallinities.

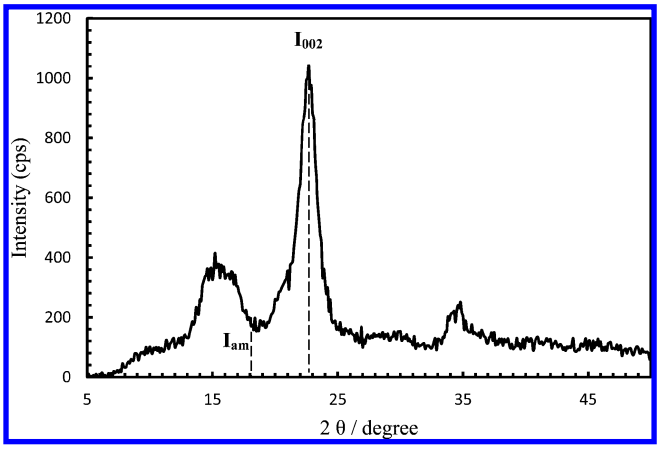

Figure 2. X-ray diffraction pattern of beaten hardwood kraft pulp.

The crystallinity values of MCCs ranged from 82.3 to 82.9, but they are not significantly different. Beating decreased the crystallinity index of the pulp, which may be due to the mechanical disruption of the crystalline regions as previously reported by Fan et al.,[3] who reported the effect of ball milling on crystallinity index. The high crystallinity indices for MCCs originate from the manufacturing process in which amorphous regions are removed by hydrolysis. The degree of cellulose crystallinity is also believed to be an important factor affecting the rate of cellulose hydrolysis.[3,39,40] However, less work has focused on its effect on adsorption. Further within the current report, a comparison will be provided to correlate the effects of crystallinity and surface area on cellulase adsorption.

3.2. Surface Areas of Substrates. The surface areas of all the substrates measured by Congo red dye are presented in Table 1. The calculated specific surface in Table 2 is based on an assumption that all the particles are spherical and are computed using eq 5:

$$SSA = \frac{A}{M} = \frac{A}{V\rho} = \frac{4\pi r^2}{(4/3)\pi r^3 \rho} = \frac{3}{r\rho} \quad (5)$$

where SSA is specific surface area in m^2/g; M is mass in g and calculated using volume V (m^3) and cellulose density ρ (assumed to be 1.5×10^6 g/m^3); and r is the radius of the cellulose particle in meters (m). The pulp fibers do not have spherical shapes, and therefore no SSA was calculated. The measured particle sizes of Avicel PH101 and PH102 by Horiba LA-300 are greater than their nominal sizes, whereas that of Avicel PH105 is smaller. This difference may be due to the internal scattering of laser light within the cellulose particles. The larger particles of Avicel PH101 and PH102 may have higher internal scattering due to more or larger pores within the particles, while Avicel PH105 has less. This could produce larger measured particle sizes for Avicel PH101 and PH102 and lower ones for Avicel PH105. A comparison of the calculated surface areas with the surface areas obtained by LA300 may imply that LA300 can only measure the external surface area of the substrates. No literature values were found for MCC particle sizes using a laser scattering technique. There are scattered data reported for the measurement of the size distributions using other techniques.[41−43] The surface areas determined by light scattering are also dependent upon the particle shape in the medium, while the surface areas measured by the Congo red methods would be less affected by such a factor as they are based on adsorption. Therefore, the surfaces determined by the Congo red sorption method would be more applicable to the adsorption of cellulase to cellulose.

Table 1. Surface Areas and Crystallinity Indices of Microcrystalline Cellulose and Hardwood Pulps

sample	species	surface area by Congo red[a] (m^2/g)	CrI[b]	CrI in the lit.[b]
pulp	beaten	49.5 ± 9.2	61.8 ± 2.1	61.0,[36] 52.8−54.8[37]
	unbeaten	19.8 ± 3 0.7	68.6 ± 0.2	70.0[36]
MCC (Avicel)	PH101	10.3 ± 1.2	82.6	65,[31] 63[32]
	PH102	9.7 ± 1.6	82.3 ± 1.2	75,[34] 84.5[3]
	PH105	12.2 ± 1.1	82.9 ± 2.0	80.3−82.9[35]

[a]Surface area by Congo red was calculated from eq 3. All results but the CrI of PH101 are presented in the form of mean value ± standard errors. [b]CrI denotes the crystallinity values calculated by eq 2. The last column of this table presents some CrI found in the literature. Their corresponding sources are denoted by superscripts.

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Table 2. Particle Sizes and Surface Areas Measured for Microcrystalline Celluloses and Unbeaten and Beaten Hardwood Pulps[a]

	particle size (μm)				
	PH101	PH102	PH105	unbeaten HW	beaten HW
P_N	50	90	30		
P_{LA}	74.2 ± 6.1	123.9 ± 9.7	25.2 ± 2.0	103.5 ± 2.6	101.0 ± 1.7
	specific surface area (m^2/mg)				
	PH101	PH102	PH105	unbeaten HW	beaten HW
S_{Cal}	0.078	0.042	0.13		
S_{LA}	0.078 ± 0.005	0.042 ± 0.004	0.216 ± 0.024	0.105 ± 0.006	0.102 ± 0.007

[a]The experimental results by laser scattering are the average of three replicates; the ranges are based on a 95% confidence interval using a *t*-distribution critical value with 3 degrees of freedom. P_N = nominal particle size, P_{LA} = particle obtained by LA-300, S_{Cal} = surface area calculated using the nominal particle size, and S_{LA} = surface area obtained by LA-300.

The surface areas of MCC are about 10 times greater than reports from the literature, such as those for Avicel PH101 reported by Badawy et al.[44] and Luukkonen et al.,[38] whose values were 1.09 and 1.14 m^2/g, respectively, using BET methods with nitrogen as the adsorbates. Gustafsson et al. reported a value of 1.36 m^2/g SSA for Avicel PH101 using krypton (Kr) as the adsorbate. One difference may be due to different saturated adsorptions at different states; namely, the aqueous states in the Congo red methods contrasted to the frozen states in the BET methods. The surface areas of unbeaten and beaten pulps are comparable to values reported previously.[45] Beating increased the surface area of pulps, which is also in agreement with previous reports.[46]

The surface areas obtained by Congo red are used for modeling cellulase adsorption hereafter.

3.3. Adsorption Isotherms. A cellulase on Avicel PH102 adsorption curve is shown in Figure 3. Avicel PH102 was incubated in a 0.75 mg/mL cellulase solution at 25 °C with a 5% solid-to-liquid ratio. Figure 3 shows that the equilibrium concentration did not change significantly after 2 min. All equilibrium cellulase concentrations were the same from 2 to 60 min, with about 90.5% of the initial cellulase concentration in solution. This demonstrates that cellulase adsorption is a fast process as confirmed by equilibrium being attained within minutes. The equilibrium time was shorter than reported elsewhere,[47,48] where estimations of 5 min using a quartz crystal microbalance technique have been reported. Jackson reported a 5−30 min period for different cellulase isozymes to reach adsorption equilibrium.[49] The difference from the results obtained by Jeong et al. may be caused by the morphology difference in substrates used, whereas the difference from Jackson's work may be due to the lack of synergism because only individual isozymes were used. Figure 3 also shows that about 9.5% of the original cellulase was adsorbed onto the Avicel PH102 upon adsorption equilibrium. All other substrates gave similar results at equilibrium time (data not shown). To obtain fully equilibrated adsorption such that substrates were not substantially changed, a 60 min incubation time was chosen. This was also the time interval used by Fan et al.[3] Ghose reported a 4% degradation of filter paper substrate in a 1 h incubation at 50 °C,[50] which would not have a statistically significant effect on substrate properties.

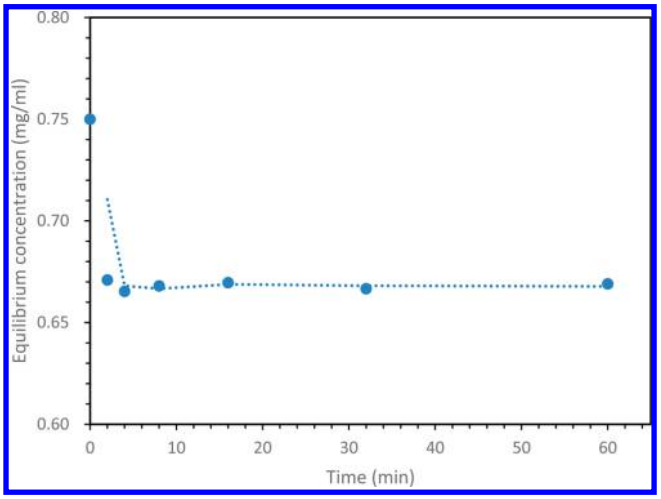

Figure 3. Adsorption kinetics of cellulase on Avicel PH102 at 25 °C. Cellulase concentration: MPC at 0.75 mg/mL.

The difference between initial (at time zero) and equilibrium concentrations gave the amount of cellulase absorbed on the substrates. A calibration curve of $C = 1.54A$ (where C is the cellulase concentration in mg/mL and A is the absorbance or optical density (OD) of solutions at 280 nm UV light (figure not shown)) was obtained to determine cellulase concentration in the solutions using BSA as the referential protein. Figure 4 shows adsorption isotherms for MPC adsorption on Avicel PH102 at 4, 25, and 50 °C. Higher temperatures produced lower cellulase adsorption, which is typical in Langmuir adsorption studies. No appreciable adsorption was observed at 50 °C. These results are in agreement with the results

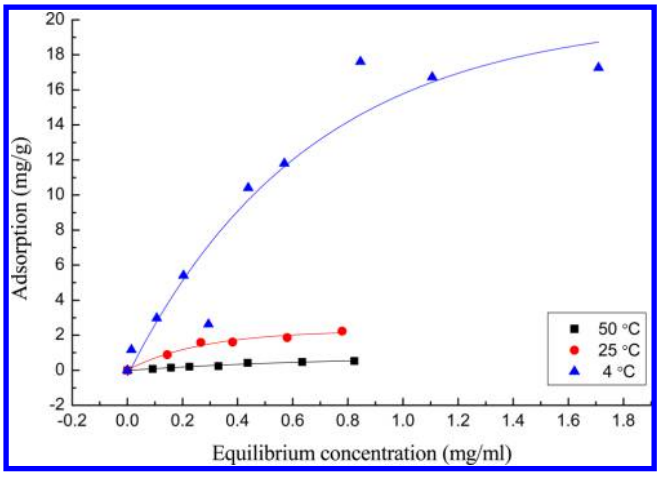

Figure 4. Adsorption isotherms for MCC PH102 at 4, 25, and 50 °C. The maximum adsorption $W_{ads,max}$ and adsorption constant K_p at 4 °C are 29.56 mg/g and 2.32 mL/mg, respectively. The constants at 25 °C are shown in Table 3. No appreciable adsorption occurred at 50 °C.

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obtained by Jackson et al. and other researchers.[49,51,52] Other substrates showed similar temperature effects (data not shown).

This adsorption behavior can be explained from E_a and typical Boltzmann distribution profiles. Higher temperature promotes a high-energy state, which generally leads to a low adsorption ratio. In terms of molecular motion, the cellulase protein molecules at lower temperature tend to cluster on the substrate surface to form a substrate−enzyme complex, which eventually leads to higher adsorption until most of the binding sites are occupied (typical enzyme binding saturation kinetics). At higher temperatures, the cellulase molecules become active and start to catalyze the hydrolysis of substrates. The release of reducing sugar may be one factor which reduces the amount adsorbed. When the temperature reaches optimum conditions for cellulase activity, the adsorption is probably low due to the movement of cellulase molecules and the constant rapid release of reducing sugars; this may explain why appreciable cellulase adsorption at 50 °C was not observed.

Adsorption isotherms for all substrates at 25 °C are presented in Figure 5. Table 3 summarizes all the relevant

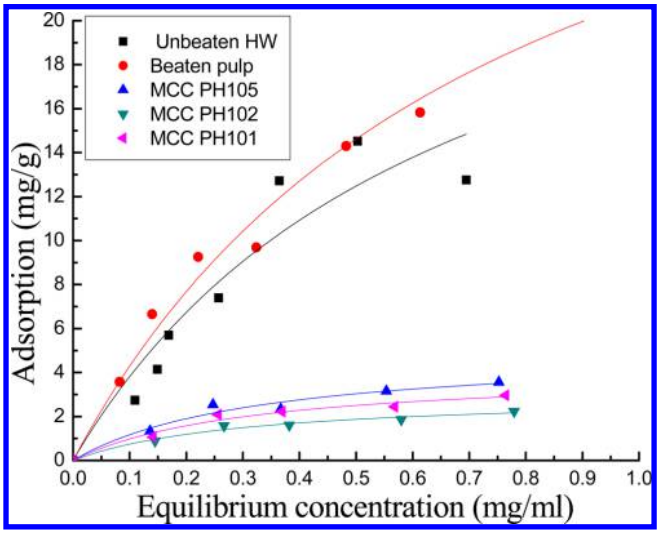

Figure 5. Adsorption isotherms for MPC on microcrystalline celluloses and unbeaten and beaten hardwood pulps at 25 °C. The plotted values were averaged from three replicates.

Table 3. Langmuir Adsorption Constants for Microcrystalline Celluloses and Pulps at 25 °C[a]

	PH101	PH102	PH105	UP	BP
K_p (mL/mg)	3.13	3.28	3.03	1.52	1.32
$W_{ads,max}$ (mg/g)	4.10	3.01	5.03	28.89	36.80
adj R-square	0.97	0.98	0.96	0.89	0.98

[a]UP = unbeaten hardwood kraft pulp, BP = beaten hardwood kraft. K_p = adsorption constant, $W_{ads,max}$ = maximum adsorption amount and adj R-squares indicate the goodness of fit. A higher value indicates a better fit.

Langmuir parameters, $W_{ads,max}$ (mg/g), K_p (mL/mg), and correlation coefficients. All the fittings but one for UP are statistically significant. The amount of adsorbed cellulase increased as the surface area of the cellulose substrate increased for microcrystalline celluloses. Avicel PH105 had the highest adsorption with its smallest particle size and largest specific surface area. Both the UP and BP show much higher adsorption than MCCs, which is supposed to be related to their greater amorphous regions. Their maximum adsorption $W_{ads,max}$ also followed this trend. $W_{ads,max}$ and K_p change in opposing directions, a finding that is in agreement with other investigations.[51−53] Numerically, the maximum adsorption is smaller and the equilibrium constants are greater than the values obtained by Peitersen et al.[52] for Avicel PH102, whose adsorption constants K_p and maximum adsorption $W_{ads,max}$ ranged from 0.53 to 1.67 mL/mg and 0.029 to 0.082 mg protein per g cellulose at the temperature range of 20−50 °C; Kim et al. investigated the adsorption of CMCase and Avicelase and three mixtures of these isozymes for Avicel PH101 in different proportions at five temperatures.[52,54] A cellulase from *Trichoderma viride* was used in both of these studies as opposed to the cellulase from *Aspergillus niger* used in the current research. The difference between this work and previous results may be partially ascribed to the different methods used. Peitersen et al. used the Folin procedure to determine protein adsorption while Kim et al. used the Lowry method to determine the enzyme (protein) concentration. No previous research has studied adsorption on Avicel PH105. A Langmuir fitting of Jackson's data[49] on dried hardwood pulp gave maximum adsorptions at 4 °C for CBH I and EG II of 16.07 mg/g and 24.02 mg/g, while the maximum adsorptions at 50 °C was 10.13 mg/g and 17.18 mg/mL. The adsorption constants obtained were 53.91 and 8.67 mL/mg for CBHI and EGII at 4 °C, and those at 50 °C were 21.61 and 9.54 mL/mg. The adsorption constants from that work are higher than those in the current report. The difference may come from the different types of cellulase enzymes used because cellulase from *Trichoderma reesei* was used in Jackson's work as opposed to *Aspergillus niger* in the current work. Indeed, Hu et al. reported that adsorption behaviors of cellulases from *Trichoderma reesei* and *Aspergillus niger* were different.[55] The basic mechanisms between these adsorption differences may be worthy of exploration at molecular levels, with emphasis on the chemical natures of catalytic cites of cellulases under development.

3.4. Statistical Model. Equation 4 was used to determine the significance levels of cellulose surface area, cellulose crystallinity, and their effect on cellulase adsorption. All surface areas and crystallinity data were used rather than their mean values to increase the degrees of freedom for model fitting. C_1 and C_2 were determined to be 74.9 and 21.3 by the JMP statistical software (SAS Institute Inc.). Table 4 summarizes the values of the constants in eq 5. It also summarizes their significance levels (p-values).

Table 4 shows that the substrate surface area, crystallinity, and their interactions all are significant at the 5% significance level ($\alpha = 0.05$, $p < \alpha$, see the table), in terms of their effects on cellulase adsorption to the substrates. It can also be argued that crystallinity and its interaction with surfaces play greater roles

Table 4. Summary of Regression Coefficients and Their Significance Levels According to the Regression Model Described in Eq 5[a]

	β_0	β_1	β_2	β_3
coeff	84.2	0.66	−1.01	0.046
p-value	0.0043	0.014	0.0035	0.0062

[a]Surface areas used for this model are those obtained by Congo red (see text). p-value indicates the significance level by which the probability that the coefficient is equal to zero is determined. Lower values mean smaller probability for the coefficient to be zero.

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in cellulase adsorption. It should be noted that this finding is different from several others. Fan et al.[3] reported that crystallinity played a more important role than surface area in a similar study, while Grethlein[24] found that surface areas were more significant. However, the results obtained in this research shows that these parameters cannot be deconvoluted and therefore must be systematically investigated simultaneously.

A direct connection of cellulase adsorption to the substrates' key characteristics, namely cellulose surface areas and crystallinities, is proposed via this model. By using this model, a tool to design the optimum cellulase dosages in research of biological conversion of cellulosic substrates for various applications is now available. The fundamental linkage of cellulase protein contents with substrate characteristics thereby provides an aid for future efforts.

4. CONCLUSIONS

The adsorption of cellulase protein to cellulosic substrates was found to follow a Langmuir adsorption isotherm where maximum adsorptions could be determined. Under the investigated situations, both the surface areas and crystallinity of chosen substrates are significant for determining the capacity of cellulase adsorption. A statistical model was also employed to explore the significance of cellulose surface area and crystallinity on cellulase adsorption. The results suggest that both the surface area and crystallinity of cellulosic substrates should be considered in any cellulase-mediated conversion platform, as all the relevant parameters are statistically significant at the significance level of $\alpha = 0.05$. The current model provides a systematic approach to determine cellulase charges based on substrates characteristics and can be an aid in designing and optimizing operational conditions.

■ ASSOCIATED CONTENT

Supporting Information

Maximum adsorptions of Congo red to the cellulose substrates that were used to determine their surface areas and their XRD spectra. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcc.5b02011.

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Notes

The authors declare no competing financial interest.

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