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Controlled adsorption of cellulase onto pretreated corncob by pH adjustment

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Abstract The effective recycling of cellulase requires an in-depth understanding of cellulase adsorption and desorption. In the present study, we examined the adsorption behaviors and stabilities of cellulase at different pH values. Acidic pH (<4.8) was found to favor adsorption, whereas neutral and alkaline pH (especially pH 7 and 10) favored desorption. The influence of pH on cellulase activity was temperature dependent. Under mild conditions (e.g., pH 7 and 25 °C), the effect of pH on cellulase activity was reversible, and the cellulase activity can return to almost 100% by adjusting the pH value to 4.8. However, under severe conditions (e.g. pH 10 and 50 °C), irreversible inactivation may take place. We also explored the roles of pH and temperature in cellulase adsorption kinetics and isotherms. At pH 4.8, temperature had no remarkable effect on the adsorption capacity of the cellulases onto substrate. However, at pH 7 and 10, high temperatures lead to more cellulase desorption. Only at pH 4.8 does cellulase adsorption well fit ($R^2 > 0.96$) the pseudo-first-order kinetic and Langmuir adsorption isotherm ($R^2 > 0.99$) models.

Keywords Cellulase adsorption · Cellulase desorption · Enzyme recycling · pH adjustment · Langmuir adsorption isotherm

Abbreviations

CBD	Cellulose-binding domain
SAA	Soaking in aqueous ammonia
SAA-CC	SAA-corncob
FPU	Filter paper unit
NREL	National renewable energy laboratory
HPLC	High-performance liquid chromatography
CEL-SELP	Cellulolytic enzyme lignin from steam-exploded Lodgepole pine
CEL-EPLP	Cellulolytic enzyme lignin from ethanol (organosolv)-pretreated Lodgepole pine

Introduction

The cost of cellulase has been the greatest obstacle to the large-scale implementation of cellulosic biofuel

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and related chemicals converted from lignocellulose (Margeot et al. 2009). Thus, cellulase cost reduction is one of the central tasks for producing low-cost cellulosic ethanol (Zhu et al. 2009b). Conventional approaches to reduce enzyme costs include the following: (1) decreasing cellulase production costs by various engineering means (Banerjee et al. 2010; Huang et al. 2011; Lynd et al. 2008; Zhang et al. 2006), (2) reducing cellulase loading (e.g., gram of cellulase used per gram of glucan) by breaking the recalcitrant structure of pretreated biomass (Gupta and Lee 2010; Kumar and Wyman 2009a, b; Kumar et al. 2010; Lamsal et al. 2011; Mosier et al. 2005), and (3) cutting down cellulase utilization costs by recycling cellulase (Gregg and Saddler 1996; Lu et al. 2002). Although intensive efforts have been exerted by several enzyme companies and a number of enzyme laboratories, cellulase production cost reduction remains a challenge (Tu et al. 2007a). As another approach to decrease the enzyme cost, cellulase recycling become increasingly important (Lee et al. 1995; Ramos et al. 1993; Tu et al. 2007b).

The adsorption of cellulase on the surface of lignocellulose is a prerequisite of lignocellulose hydrolysis (Jäger et al. 2010; Zhu et al. 2009b). Cellulase adsorption onto cellulosic substrates is reportedly reversible and often described by the Langmuir isotherm (Bansal et al. 2009; Dourado et al. 1999; Gerber et al. 1997). However, according to various authors, cellulase adsorption onto the pretreated lignin-rich substrates is not strictly reversible (Palonen et al. 2004; Zhu et al. 2009a). After hydrolysis, cellulases can either be freed into the supernatant (liquid phase) or remain bound to the residual substrate (solid phase). Free cellulases can readsorb onto fresh substrates (Lee et al. 1995; Ramos et al. 1993) or be collected by ultrafiltration (Lu et al. 2002; Zhang et al. 2011), but neither approach can recycle the binding enzyme. With the growth of lignin content in substrates, cellulase adsorption and desorption become more irreversible (Zhu et al. 2009a). Cellulase activity is reduced because it is irreversibly bound (Girard and Converse 1993). Thus, an effective approach for desorbing cellulases from solid substrates and recovering cellulase activity is necessary to recycle cellulase (Tu et al. 2007a).

The addition of a desorbent is a traditional approach to desorb cellulases from a lignocellulosic substrate (Otter et al. 1989). A range of chemical reagents can be used as desorbents, such as buffer (Azevedo et al. 2001; Tu et al. 2009b), Tween, urea, alkali, glycerol (Rad and Yazdanparast 1998), Triton X-100, and polyethylene glycol (Xu and Chen 2009). However, most of these desorbents are unable to desorb cellulases efficiently and cause further deactivation of desorbed cellulases. Remarkably, among these chemical reagents, the desorption of an alkaline solution is more efficient (Otter et al. 1989; Zhu et al. 2009b) due to the decreased affinity of the cellulase cellulose-binding domain with increased pH (Hu et al. 2010; Kyriacou et al. 1988). Adjusting the solution pH is more operative and cost-effective compared with adding other chemicals (Zhu et al. 2009b).

The previous desorption approach of pH adjustment is to elute cellulases using an alkaline solution (Otter et al. 1984). Unfortunately, after desorption, the eluted cellulases in the alkaline solution are difficult to collect, and the deactivation of desorbed cellulases remains unsolved. A considerable amount of work has been focused on cellulase adsorption under different physical parameters, such as temperature and ionic strength. However, there is limited information on the adsorption of cellulase enzymes at different pH values (Ding 2000; Kyriacou et al. 1988).

In the current study, we assayed the controllability of cellulase adsorption on pretreated corncob by in situ pH adjustment. We compared the adsorption of cellulase onto corncob after soaking in aqueous ammonia (SAA-CC) at different pH values. The effects of pH on cellulase stability were investigated. The adsorption kinetics and adsorption isotherm of cellulase onto SAA-CC at different pH values were then described using the pseudo-first-order kinetic model and Langmuir isotherms. Different from previous studies, we eliminated the influence of hydrolysis on adsorption and described a more realistic adsorption kinetics of cellulase onto SAA-CC.

Methods

Substrates and enzymes

Microcrystalline cellulose (Avicel PH101) and Whatman no. 1 filter paper (FP) were purchased from Sigma (St. Louis, MO, USA). Corncobs (cellulose, 37.6%; hemicellulose, 31.0%; lignin, 17.0%) used in this study were collected from a local farm (Tianjin, China). Air-

dried corncob were pre-milled and screened, and the fractions between 20 and 80 meshes were used for subsequent experiments. Cellulase preparation derived from *Trichoderma reesei* was used in this study: Spezyme CP (Genencor International, Palo Alto, CA, USA), filter paper activity = 117 FPU/mL, protein content = 35.6 mg/mL (Zhang et al. 2010b). All chemicals used were of analytical grade.

Soaking in aqueous ammonia (SAA) pretreatment

Air-dried corncob (moisture <10%, 50 g) was pretreated by soaking in aqueous ammonia (SAA) using 15% (w/w) ammonia solution (300 mL, diluted from 25% ammonia hydroxide) and a solid-to-liquid ratio (S:L) of 1:6. The pretreatment was performed in screw-capped Pyrex solution bottles (500 mL) in a water bath at 60 °C without agitation for 12 h. After pretreatment, the pretreated corncob was separated by filtering and washed with distilled water until the pH of wash water was neutral. Washed solids were oven-dried at 105 °C for 24 h and collected for subsequent experiments.

Analytical methods

Components of the pretreated corncob were determined using a standard method (NREL laboratory analytical procedure LAP-002, 2008). Soluble sugars were analyzed by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad, USA), the parameters used in the HPLC analysis were as follows: mobile phase used was 0.01 N H_2SO_4, injection volume of 20 μL, column temperature of 65 °C, and flow rate of 0.6 mL/min.

Protein content of enzyme samples was measured by the Bradford Assay (Bradford 1976) using bovine serum albumin (BSA) as the protein standard. Cellulase activities (FPU) were measured according to standard procedures (Ghose 1987).

Enzymatic hydrolysis of lignocellulosic substrates

Unless otherwise stated, all enzymatic hydrolysis experiments were performed in 50 mL of 50 mM citrate buffer (pH = 4.8) at a mass loading of 5 w/w% (based on substrate). Hydrolysis was performed by incubating at 4, 25, and 50 °C, with shaking at 120 rpm for 2 h. The cellulase loading was 30 FPU g^{-1} cellulose. Aliquots (1 mL) were removed from the reaction at different times (0, 5, 15, 30, 60, 90, and 120 min) and centrifuged to remove insoluble materials. Sugar content was measured by high-performance liquid chromatography as previously described. The hydrolysis yield of the substrate was calculated from the measured monomeric sugar content as a percentage of the theoretical sugars available in each substrate.

Effect of pH on cellulase adsorption and stability

To investigate the effect of pH on cellulase adsorption, we compared the adsorption of cellulase on different substrates at various pH values. Solutions with various pH values (pH 2–10) were pre-incubated at 25 °C for 30 min. About 1 g of SAA-CC sample (0.457 g of Avicel or 1.215 g of untreated corncob) was suspended in 20 mL of the previously prepared solutions (pH 2–10), and incubated with the same loading (30 FPU g^{-1} cellulose) of Spezyme CP at 25 °C for 2 h in a shaking water bath. After incubation, the suspensions (1 mL) were collected and centrifuged to remove insoluble materials. The protein contents of these aliquots were subsequently determined.

To determine the effect of pH on enzyme activity, a specific amount of Spezyme CP (2 FPU/mL) was first incubated in a series of solutions (pH 2–10) at 25, 37, and 50 °C for 2 h. After incubation, the activity of Spezyme CP remaining in these solutions was measured at 50 °C for 1 h (Ghose 1987). The change in protein content was determined using the Bradford assay (Bradford 1976). To study the reversible effect of pH on cellulase activity, cellulase samples were adjusted back to pH 4.8 and cellulase activities were measured again.

Effects of pH on cellulase adsorption kinetics and isotherm

To examine the adsorption kinetics, ~1 g of SAA-CC samples and 20 mL of solutions with various pH values (pH 4.8, 7, and 10) were placed in a 50 mL Erlenmeyer flask. The incubation was carried out with Spezyme CP (30 FPU g^{-1} cellulose) for 2 h at different temperatures (4, 25, and 50 °C) on a shaking water bath. Aliquots (1 mL) were collected at 0, 5, 15, 30, 60, 90, and 120 min during the incubation, and centrifuged to remove insoluble materials. The protein content of the suspensions was determined using the Bradford assay.

The adsorbed cellulase was calculated by subtracting the initial cellulase content from the non-adsorbed cellulase content in the supernatant. The cellulase adsorption kinetics of the SAA-CC samples was characterized by pseudo-first-order kinetics (Copeland 2000):

$$\Gamma(t) = \Gamma_{\max} \cdot (1 - e^{(-k_{ad} \cdot t)}) \quad (1)$$

where $\Gamma(t)$ is the amount of adsorbed cellulase (mg/g) at time t (s), $\Gamma_{\max}$ is the maximum cellulase adsorption at equilibrium (mg/g), and k_{ad} is the pseudo-first-order adsorption rate constant for approaching equilibrium (s^{-1}).

To determine the adsorption isotherm, different loadings (5 FPU g^{-1} cellulose to 30 FPU g^{-1} cellulose) of cellulase were incubated with 1 g of SAA-CC in 20 mL of 50 mM citrate buffer (pH 4.8) at 25 and 50 °C for 1 h to reach equilibrium. The protein content in the supernatant was determined for the nonadsorbed cellulase. The adsorbed cellulase was calculated from the difference between the initial cellulase dosage and the nonadsorbed cellulase. Cellulase adsorption on the SAA-CC samples was characterized by the Langmuir adsorption isotherm (Tu et al. 2009a):

$$\Gamma = \frac{\Gamma_{\max} KC}{1 + KC} \quad (2)$$

where Γ is the adsorbed protein (mg/g of SAA-CC), C is the concentration of unadsorbed protein in the bulk solution (mg/mL), and K is the Langmuir constant (mL/mg of protein).

Results and discussion

Main components of substrates

Table 1 shows the main components of corncob before and after pretreatment. After SAA, the cellulose content of corncob increased from 37.6 to 45.7%, and the hemicellulose increased from 31.0 to 35.7%. On the other hand, lignin decreased from 17.0 to 7.8%. The delignification ratio of SAA-pretreated corncob is 54.1%.

Table 1 Main chemical compositions of raw corncob, SAA-pretreated corncob

Substrate	Glucan (%)	Xylan (%)	Klason Lignin (%)	ASL[a] (%)
Raw corncob	37.6	31.0	15.0	2.0
SAA-pretreated corncob	45.7	35.7	6.4	1.4

[a] *ASL* acid soluble lignin

Effect of pH on the adsorption amount of cellulase

In previous studies, the pH showed significant effects on *Trichoderma reesei* cellulase adsorption onto Solka Floc (Kyriacou et al. 1988) and Avicel (Ding 2000). From a practical point of view, the effect of pH on cellulase adsorption onto pretreated lignocellulose is interesting to gauge. Soaking in aqueous ammonia, a promising pretreatment method, has been proven as one of the most feasible methods for lignocellulose pretreatment. It can effectively remove lignin and increase cellulose digestibility under modest conditions without high pressures and temperatures. Therefore, in the current study, we focused on the adsorption of cellulase onto SAA-CC (Huang et al. 2010; Zhang et al. 2010a, b, 2011).

To ensure cellulase adsorption onto lignocellulose, we used Avicel, untreated corncob, and SAA-CC as the substrates. The adsorption of Spezyme CP on pure cellulose and lignin-rich substrates were then compared. The same loading of cellulase (30 FPU g^{-1} cellulose) was incubated with Avicel, untreated corncob, SAA-CC samples in 20 mL solutions with different pH values (pH 2–10) for 2 h at 25 °C. Cellulase adsorption onto the different substrates were then measured (Fig. 1). Around 55, 53, and 44% of the total Spezyme CP was adsorbed onto SAA-CC at pH 4.8, 2, and 3, respectively. Increased pH from 4.8 to 10 resulted in a remarkable decrease in the adsorption of cellulases onto SAA-CC. The adsorption of cellulase at pH 7 and 10 was minimal, with only around 25% of cellulase adsorbed at pH 7 and 20% adsorbed at pH 10. Similar results were found in the adsorption of cellulase onto the pure cellulose substrate (Avicel). About 52, 43, and 34% of the total Spezyme CP was adsorbed onto Avicel at pH 4.8, 2, and 3, respectively. These data were slightly lower than the values of cellulase adsorption onto SAA-CC, which can be attributed to the more accessible surface area in SAA-CC. Increased pH from 4.8 to 10 also resulted in decreased cellulase adsorption onto Avicel. These results may indicate that Spezyme CP difficultly adsorb onto cellulose and lignin (reversible and irreversible adsorption, respectively) under

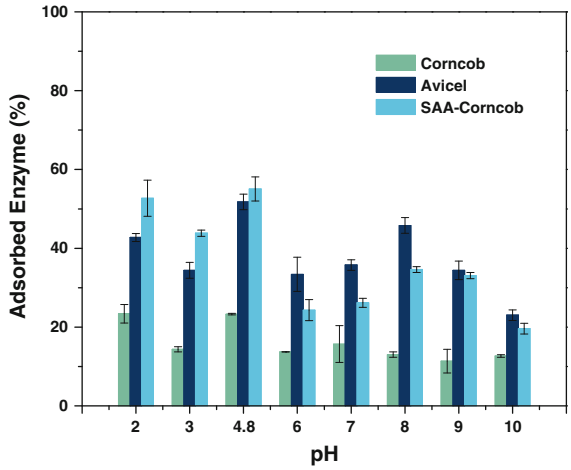


Fig. 1 The adsorption Spezyme CP onto cellulosic and lignocellulosic substrates after 2 h of equilibration at different pH values. The ~1 g SAA-CC samples (0.457 g Aivicel or 1.215 g untreated corncob) and 20 mL of solutions with various pH values (2–10) were placed into a 50 mL Erlenmeyer flask. The incubation was carried out with same loading (30 FPU g^{-1} cellulose) of Spezyme CP. The *numbers* on the *graph* represents the average of three separate measurements

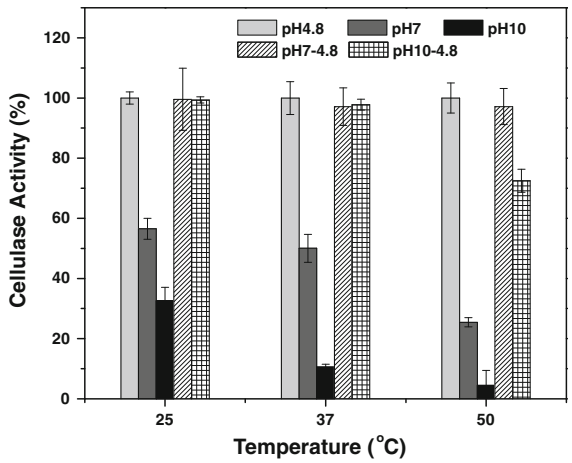


Fig. 2 The activities of Spezyme CP at different pH values and temperatures. A specific amount of Spezyme CP (2 FPU/mL) was incubated in 20 mL of solutions with different pH values (pH = 4.8, 7, 10). After incubation at 25, 37 and 50 °C for 2 h with shaking, cellulase activity remaining was measured using filter paper activity measurement, and expressed as percentage of the initially cellulase activity. To study the reversible effect of pH on cellulase activity, cellulase samples were adjusted back to pH 4.8 and then cellulase activities were measured. The *numbers* on the *graph* represents the average of three separate measurements

alkaline conditions. As shown in Fig. 1, the effect of pH on cellulase adsorption onto untreated corncob was inconspicuous because of the low accessible surface area in the original lignocellulosic substrate (Huang et al. 2010). This finding implied that the cellulase adsorption capacity increased not only with the growth of cellulose content, but also with the enhancement of accessibility in the lignocellulosic substrate after SAA treatment (Esteghlalian et al. 2001; Jeoh et al. 2007; Meunier-Goddik and Penner 1998; Palonen et al. 2004).

Ding (2000) has studied cellulase adsorption onto Avicel at pH 2–7.5, and showed that the adsorption of cellulase decreased when the pH was unequal to 4.8. Nevertheless, in the current study, pH $\leq$ 4.8 favored cellulase adsorption, whereas pH > 4.8 (especially pH 7 and 10) favored desorption, similar to the results of Zhu et al. (2009b). This finding implied that the adsorption and desorption of cellulase can be controlled by in situ pH adjustment.

Effect of pH on cellulase stabilities

The pH stability of cellulase was investigated to understand further the effect of pH on adsorption and cellulase activity. A specific amount of Spezyme CP (2 FPU/mL) was incubated in 20 mL of solutions with various pH values (pH 4.8, 7, and 10) for 2 h at different temperatures (25, 37, 50 °C). Cellulase activities at these pH values were measured following standard procedures (Ghose 1987). To study the reversible effect of pH on cellulase activity, we adjusted the pH of cellulase samples to 4.8, and then measured cellulase activities again. The pH activity of Spezyme CP is shown in Fig. 2. After incubating at 25 °C for 2 h, 100% of the FPU remained in solution at pH 4.8; around 57% FPU remained at pH 7; and only 33% FPU remained at pH 10. Interestingly, when the pH was adjusted from 7 or 10 to 4.8, the cellulase activities recovered to almost 100%. After incubating at 37 °C for 2 h, only 50% cellulase activities remained at pH 7, and 10% remained at pH 10 (Fig. 2). Nevertheless, when the pH was adjusted from 7 or 10 to 4.8, the cellulase activities recovered to more than 97%. After incubating at the saccharification temperature (50 °C) for 2 h, the cellulase activities further decreased to 25% at pH 7 and 4% at pH 10. When the pH was adjusted from 7 or 10 to 4.8, the

cellulase activities recovered to 97 and 72%, respectively. These results indicated that the effect of pH on cellulase (Spezyme CP) activity was temperature dependent. With increased temperature, the resistance of cellulase to pH adjustment decreased. The effect of pH on cellulase (Spezyme CP) activity was also reversible under low temperatures (25 °C), whereas irreversible inactivation may take place under high temperatures (e.g., 50 °C).

Considering the probable isoelectric precipitation of cellulase, we determined the loss of cellulase at different pH values under similar experimental conditions. As shown in Fig. 3, only a small amount of cellulase was lost from pH 2 to 10. The minimum free enzyme determined was 93% of the initial enzyme at pH 6. This result implied that no cellulase precipitated despite the denaturation of cellulase.

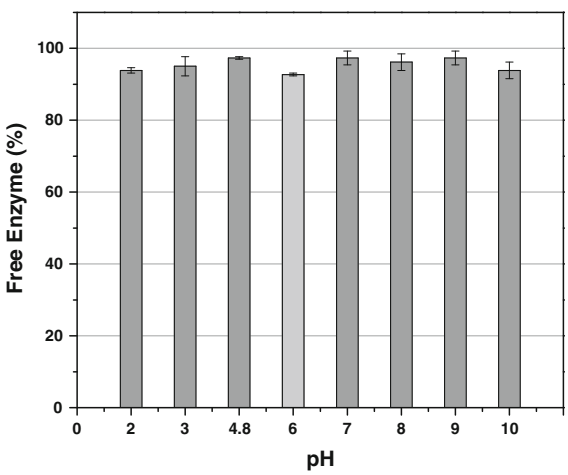


Fig. 3 Average amount of Spezyme CP remaining in solutions with various pH values after 2 h incubation at 25 °C. The numbers on the graph represents the average of three separate measurements

Kinetics of cellulase adsorption at different pH values

The process of cellulase adsorption onto pretreated lignocellulose was followed at different pH values and temperatures. Lignocellulose samples (SAA-CC) were suspended in solutions with various pH values (pH 4.8, 7, and 10). To each lignocellulose sample, the same cellulase preparations from *T. reesei* (Spezyme CP) were assessed for adsorption kinetics. The adsorption of Spezyme CP onto SAA-CC at pH 4.8 reached equilibrium within 60 min (Fig. 4a), similar to the time required for Spezyme CP adsorption onto pretreated lodgepole pine (Tu et al. 2007a). Ryu et al. (1984) have reported that increased temperature decreases the capacity of cellulase adsorption onto Avicel. Kyriacou et al. (1988) have observed reduced cellulase affinity with increased temperature. Nevertheless, under the experimental conditions described in Fig. 4a, the amount of adsorbed enzyme was around 55, 59, and 53% at 50, 25, and 4 °C, respectively. Increased temperature from 4 to 50 °C showed little effect on cellulase adsorption onto SAA-CC at pH 4.8. Similar results on cellulase adsorption onto CEL-SELP and CEL-EPLP have been reported (Tu et al. 2009a).

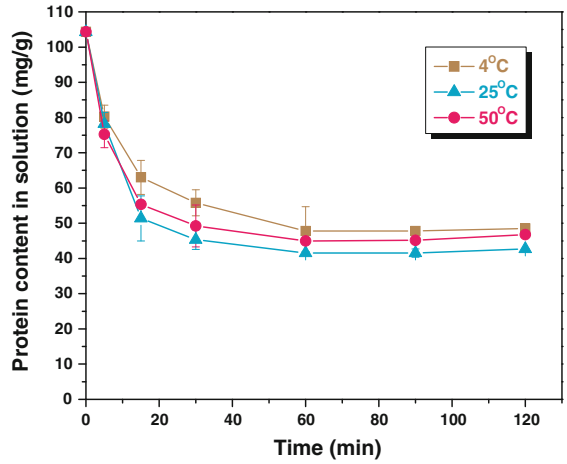
Cellulase adsorption onto SAA-CC at pH 7 and pH 10 are shown in Fig. 4b, c, respectively. After 2 h of incubation, only around 26% (25 °C) and 12% (50 °C) of the total enzyme complex were adsorbed onto SAA-CC at pH 7.0, whereas only around 20% (25 °C) and 4% (50 °C) cellulases were adsorbed at pH 10. This finding indicated that high pH values and temperatures favor cellulase desorption. The adsorption of enzymes at pH 7.0 slightly increased over time (Fig. 4b), and the adsorption of enzymes at pH 10 increased in the first 60 min before sharply decreasing until 120 min (Fig. 4c).

Cellulase adsorption kinetics onto pretreated lignocellulose at pH 4.8 was characterized by pseudo-first-order kinetics (Fig. 6). Considering the influence

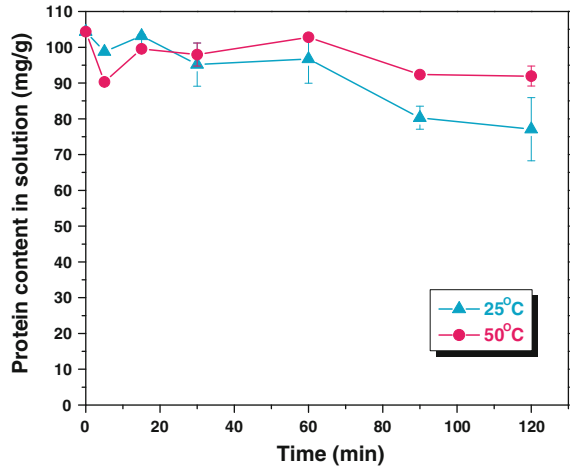
Table 2 Langmuir and kinetic adsorption parameters of cellulase

Temperature (°C)	Langmuir adsorption parameters			Kinetic adsorption parameters		
	Γ_{max} (mg/g)	K (mL/mg)	R^2	Γ_{max} (mg/g)	k_{ad} (s^{-1})	R^2
4	–	–	–	58.5 ± 2.58	0.064 ± 0.011	0.965
25	65.9 ± 2.11	0.947 ± 0.10	0.996	62.7 ± 0.67	0.103 ± 0.005	0.997
50	59.5 ± 1.32	0.858 ± 0.06	0.998	66.7 ± 1.24	0.116 ± 0.010	0.992

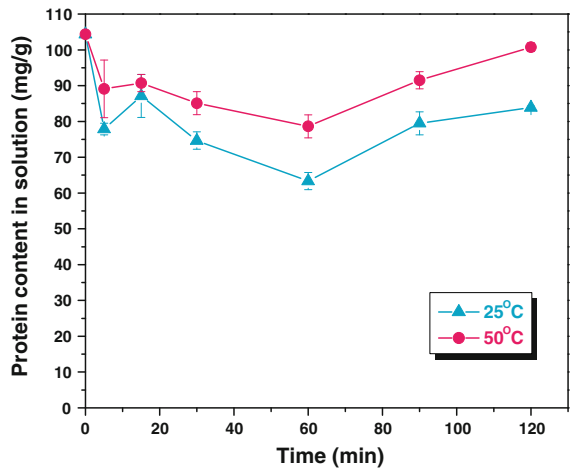
Fig. 4 Adsorption of Spezyme CP on SAA-CC at different pH and different temperatures. The ~1 g SAA-CC samples and 20 mL of solutions with various pH values (pH = 4.8, 7, 10) were placed into a 50 mL Erlenmeyer flask. The incubation was carried out with same loading (30 FPU g^{-1} cellulose) of Spezyme CP for 2 h at different temperatures on a shaking water bath. Aliquots of 1 mL were taken for each time point during the incubation. *Each point* represents the average of two separate measurements, and the *error bars* give the standard deviation



(a) Spezyme CP adsorption on SAA-CC at pH 4.8



(b) Spezyme CP adsorption on SAA-CC at pH 7



(c) Spezyme CP adsorption on SAA-CC at pH 10

of hydrolysis on adsorption, the values for Γ were calculated by the mass balance equation (Bansal et al. 2009):

$$\Gamma = \frac{C_T - C}{S} \tag{3}$$

where C_T is the initial cellulase dosage (mg/mL), and S is the substrate concentration (mg/mL). As shown in Table 2, after eliminating the loss of substrate as shown in Fig. 5, the pseudo-first-order kinetic model (Eq. 1) provided a good fit ($R^2 > 0.96$). As shown in Fig. 6, the adsorption rate of cellulases sharply dropped with decreased temperature from 50 to 4 °C. The SAA-CC substrate rapid adsorption of Spezyme CP in the first 15 min at 50 and 25 °C (52 and 47%, respectively), whereas the adsorption at 4 °C was slow within 60 min (30% at 15 min and 47% at 30 min). These results implied that low temperatures (especially 4 °C) decreased the movement abilities of cellulases along the cellulose fiber and consequently decreased the adsorption rate of cellulases. As shown in Table 2, the kinetic constant (k_{ad}) dropped with decreased temperature, which provides further proof that low temperatures decreased the adsorption rate of cellulases. The values for maximum adsorption (Γ_{max}) from 4 to 50 °C had no significant difference. Subsequently, to quantify further cellulase adsorption onto pretreated lignocellulose, an adsorption isotherm was used to characterize the cellulase adsorption capacity and adsorption affinity on SAA-CC substrates.

Adsorption isotherms of cellulase at pH 4.8

The Langmuir adsorption isotherm is extensively utilized to study cellulase adsorption onto various lignocellulosic substrates (Palonen et al. 2004; Tu et al. 2007a, 2009a). Critical assumptions that form the basis of this nonlinear model include the following: (1) the adsorption is monolayer and reversible, (2) the binding sites are uniform over the entire lignocellulose surface, and (3) the rates of adsorption and desorption are in equilibrium. The parameters of the Langmuir

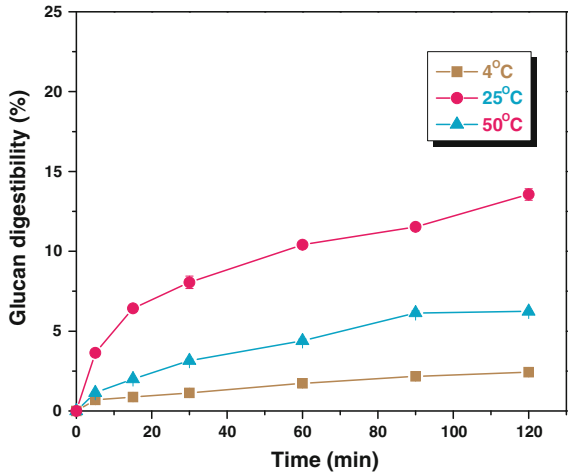


Fig. 5 Spezyme CP digestibility of SAA-corncob at pH 4.8

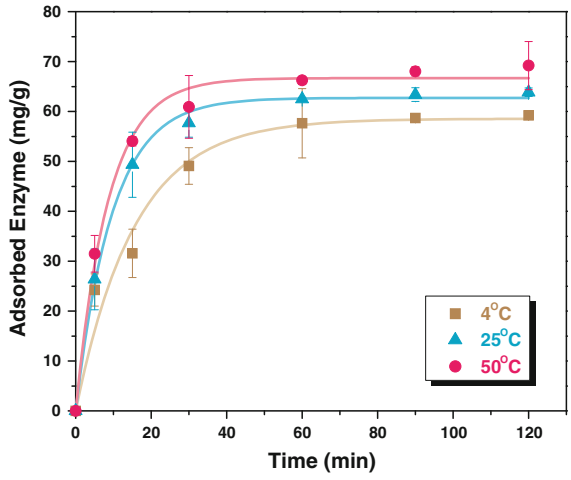


Fig. 6 Spezyme CP adsorption kinetics on SAA-CC at pH 4.8. For adsorption kinetics, ~1 g SAA-CC samples and 20 mL of 50 mM citrate buffer (pH = 4.8) were placed into a 50 mL Erlenmeyer flask. The incubation was carried out with same loading (30 FPU g^{-1} cellulose) of Spezyme CP for 2 h at different temperatures on a shaking water bath. Aliquots of 1 mL were taken for each time point during the incubation. The adsorbed cellulase was calculated from the difference between initial cellulase content and nonadsorbed cellulose content in the supernatant. *Each point* represents the average of two separate measurements, and the *error bars* give the standard deviation

adsorption isotherm show the maximum amount of enzyme that can be adsorbed onto a substrate and the affinity of adsorption.

The cellulase adsorption isotherms on SAA-CC substrates at pH 4.8 were characterized by the Langmuir adsorption isotherm (Fig. 7). To establish the adsorption isotherm, various concentrations of

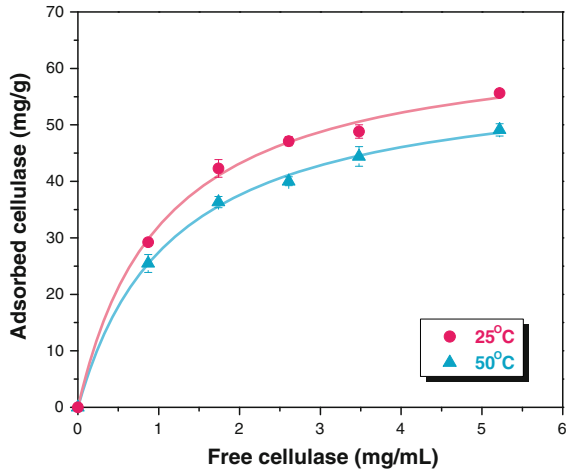


Fig. 7 Spezyme CP adsorption isotherms on SAA-CC at pH 4.8 (characterized by Langmuir adsorption isotherm). For adsorption isotherm, different loading (5–30 FPU g^{-1} cellulose) of cellulase were incubated with 5% of SAA-CC in 50 mM citrate buffer for 1 h to reach equilibrium. Protein content in the supernatant was determined as the nonadsorbed cellulase. The adsorbed cellulase was calculated from the difference between initial cellulase content and nonadsorbed cellulose content in the supernatant. *Each point* represents the average of two separate measurements, and the *error bars* give the standard deviation

cellulase were incubated with a 5% suspension of SAA-CC in 50 mM citrate buffer at 25 and 50 °C for 1 h to reach equilibrium. The protein content in the supernatant was then determined. The adsorbed cellulase was calculated from the difference between the initial cellulase content and the nonadsorbed cellulose content in the supernatant. Experimental data well fit the Langmuir adsorption isotherm models ($R^2 > 0.99$) (Table 2). The adsorption capacity of Spezyme CP onto SAA-CC at 25 °C was $\Gamma_{max} = 65.9 \pm 2.11$ mg/g substrates, which was similar to the adsorption capacity at 50 °C ($\Gamma_{max} = 59.5 \pm 1.32$ mg/g substrates). This result provided further proof that temperature had no remarkable effect on the adsorption capacity of cellulases onto SAA-CC at pH 4.8.

The Langmuir constants (K) from the adsorption isotherm represent the equilibrium affinity constants of cellulase onto the substrates. As shown in Table 2, decreased temperature from 50 to 25 °C resulted in slightly increased affinity of Spezyme CP to SAA-CC. Similarly, Tu et al. (2009a) have reported that the temperature has a much less considerable effect on cellulase adsorption onto lignin than onto cellulose. The cellulase adsorption isotherms of SAA-CC

implied that the temperature had no significant effect on cellulase adsorption onto lignin-rich substrates.

Conclusions

The present work shows that pH plays a significant role on the adsorption of cellulase onto cellulosic materials and pretreated lignocellulosic biomass. The main conclusions drawn are as follows. (1) Cellulase adsorption can be controlled by pH adjustment. Acidic pH (<4.8) favors adsorption whereas neutral and alkaline pH (especially pH 7 and 10) favors desorption. With the growth of cellulose content and accessibility in lignocellulosic substrates after pretreatment, the effect of pH on cellulase adsorption has become increasingly remarkable. (2) The effect of pH on cellulase activity is temperature dependent and partly recoverable. Cellulase is highly stable at different pH values and does not precipitate. Therefore, controlling cellulase adsorption by in situ pH adjustment is useful in facilitating cellulase recycling from pretreated lignocellulosic biomass.

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