Direct quantitative determination of adsorbed cellulase on lignocellulosic biomass with its application to study cellulase desorption for potential recycling

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Effective hydrolysis of pretreated lignocellulose mediated by cellulase requires an in-depth understanding of cellulase adsorption and desorption. Here we developed a simple method for determining the adsorbed cellulase on cellulosic materials or pretreated lignocellulose, which involves (i) hydrolysis of adsorbed cellulase in the presence of 10 M of NaOH at 121 °C for 20 min, and (ii) the ninhydrin assay for the amino acids released from the hydrolyzed cellulase. The major lignocellulosic components (*i.e.*, cellulose, hemicellulose, and lignin) did not interfere with the ninhydrin assay. A number of cellulase desorption methods were investigated: pH change as well as the use of detergents, high salt solution, and polyhydric alcohols. The pH adjustment to 13.0 and the elution by 72% ethylene glycol at neutral pH were among the most efficient approaches for desorbing the adsorbed cellulase. For the recycling of active cellulase, a modest pH adjustment to 10.0 may be a low-cost viable method to desorb active cellulase. It was found that more than 90% of cellulase for hydrolysis of the pretreated corn stover could be recycled by washing at pH 10.0.

Introduction

Cost-effective liberation of fermentable soluble sugars from non-food lignocellulosic biomass is still the largest obstacle to large-scale implementation of biorefineries.[1,2] Biomass saccharification usually involves two steps—biomass pretreatment/fractionation followed by hydrolysis mediated by cellulase[3–5] or chemical catalysts.[6,7] Enzymatic hydrolysis features high selectivity and mild reactions but suffers from its poor stability and high prices. Significant advances in a 27 fold cost reduction of cellulase have been made through production process optimization and cellulase engineering.[4,8] Cellulase cost, which could range from 30 to more than 100 (US) cents per gallon of cellulosic ethanol, is still far more expensive than that of starch-hydrolyzing enzymes for corn kernel based ethanol biorefineries (*e.g.*, 2–5 cents per gallon of starchy ethanol). A cost reduction in cellulase utilization is one of the central tasks for production of low-cost cellulosic ethanol. Several approaches can be conducted for decreasing cellulase costs: (i) decreasing cellulase loading (*e.g.*, gram of cellulase used per gram of glucan) by increasing substrate reactivity of pretreated biomass[9–11] and/or recycling costly cellulase,[12–15] (ii) increasing cellulase performance (unit per gram of cellulase) by using cellulase engineering,[4,16,17] and (iii) decreasing cellulase production costs (dollar per gram of cellulase).[4,18] Intensive efforts have been made to improve performance of cellulase by several enzyme companies and a number of enzyme laboratories because cellulase would be the largest industrial enzyme market, but limited advances have been reported so far.

The study of cellulase adsorption and desorption is of great importance for understanding the cellulose hydrolysis mechanism and evaluating the potential of cellulase recycling. Different from common enzyme recycling in aqueous homogeneous reactions *via* enzyme immobilization, cellulase in heterogeneous hydrolysis can be easily recycled by re-adsorbing free cellulase in the aqueous phase onto newly-added insoluble substrates.[12–15] Also, desorption of cellulase can be conducted through the addition of reagents, such as Tween, urea, alkali, glycerol, and Triton X-100.[19]

Adsorption of cellulase on the surface of cellulose is a prerequisite of cellulose hydrolysis. After hydrolysis, significant amounts of adsorbed cellulase are released to the aqueous phase.[20–22] Adsorption of cellulase on the surface of cellulose and pretreated biomass is often described by the Langmuir equation based on the reversible adsorption assumption.[3,5,22,23] In fact, adsorption of cellulase components is not strictly reversible. For example, binding of all *Thermobifida fusca* cellulase components to bacterial microcrystalline cellulose is irreversible.[24] On bacterial microcrystalline cellulose, approximately 10% of the bound *Trichoderma* cellobiohydrolase I and ~30–40% of the bound *Trichoderma* cellobiohydrolase II are irreversible, respectively.[25] For pretreated biomass, competitive cellulase adsorption by lignin along with cellulose makes cellulase adsorption/desorption more complicated.[26–28]

The adsorbed cellulase is often calculated based on the mass difference of initial cellulase and free cellulase[22,27,29] since most protein assays, such as UV, Bradford, Lowry, and bicinchoninic acid (BCA), cannot be applied directly for determining adsorbed cellulase on the surface of cellulose or lignocellulosic materials.

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Desorbed or free cellulase has been measured based on activity,[19,21,30] but cellulase activity assays are subject to changes in cellulase composition.[4,31] When reversibly adsorbed cellulase is washed by a large volume of solution, very low concentrations of free cellulase result in a challenge for accurate assays of protein mass concentration and/or enzyme activity. Therefore, radio-labeled cellulases have been used to study reversibility of their adsorption/desorption.[25,26] However, this technology requires protein purification, protein labeling, and a costly radioactivity detection instrument. Recently, a direct method for measuring adsorbed cellulase has been developed based on nitrogen element analysis.[29] However, this method is only a rough measurement because it detects all nitrogen-containing compounds, such as alkaloids, ammonia, protein in plant samples, and a relatively costly analytical instrument is required.

In this study, desorption of cellulase under different elution conditions, such as pH, buffer, salt, and detergents, was studied. The adsorbed cellulase was measured by complete hydrolysis to amino acids followed by the ninhydrin assay.

Experimental section

Chemicals and materials

All chemicals were reagent grade and purchased from Sigma Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Microcrystalline cellulose (Avicel PH105) was purchased from FMC (Philadelphia, PA). Regenerated amorphous cellulose (RAC) was prepared through Avicel dissolution in concentrated phosphoric acid followed by regeneration in water.[32] Birchwood xylan containing more than 90% xylose units was purchased from Sigma Aldrich. Kraft lignin[33] was isolated from bagasse through the Kraft pulping and NaOH treatment at 170 °C, gifted from Dr Scott Renneckar at Virginia Tech (Blacksburg, VA). The fungal cellulase Spezyme CP was a gift from Genencor (Palo Alto, CA). Corn was grown from Biomass AgriProducts (Harlan, IA). The tub-ground materials for corn stover were approximately nine months old. Dilute sulfuric acid pretreated corn stover was produced in a pilot-scale continuous vertical reactor at 190 °C, 0.048 g acid/g dry biomass, 1 min residence time, and a 30% (w/w) total solid loading.[27] Cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) was conducted by using 85% H_3PO_4 at 50 °C, 1 atm, and 45 min, as described elsewhere.[9,27] Lyophilized cellulase powder, as a standard protein for the adsorbed cellulase assay, was prepared through total protein precipitation by trichloroacetic acid (TCA) for soluble sugar removal,[34] and then washing by ice-cold acetone for TCA removal.

Protein assays

Protein mass concentration was measured by using the Bradford,[35] ninhydrin, or UV280 assays.[36] Dry bovine serum albumin (BSA) and lyophilized cellulase powder were used as reference standards. The ninhydrin assay can be described as follows: 100 μL of the protein solutions containing up to 100 μg of protein (protein concentrations of up to 1 mg/mL) were mixed with 300 μL of 13.5 M NaOH and autoclaved at 121 °C for 20 min for complete protein hydrolysis. After cooling down to room temperature, the solutions were neutralized by adding 500 μL of 100% acetic acid, followed by adding 500 μL of 2% ninhydrin reagent while mixing well. After boiling for 10 min and cooling down to room temperature, the samples were diluted by three volumes of 95% ethanol. After centrifugation for removing solids, 200 μL of the colored supernatant was added into a 96-well microplate. The absorbance of the supernatants was read by the BioTek multi-detection microplate reader at a 570 nm wavelength. The readings from the microplate reader were normalized to 1 cm length of light path. The inferences from Avicel, RAC, xylan, lignin or pretreated corn stover samples on the protein assay were determined according to the ninhydrin assay.

Cellulase adsorption and desorption

Adsorption of cellulase was conducted at a total volume of 1000 μL of a 50 mM sodium citrate buffer (pH 4.8) for Fig. 3 (see later), containing various amounts of Avicel or dilute acid (DA)-pretreated corn stover at 4 °C. Final cellulase concentrations used were 0.35 mg/mL for Avicel and 0.15 mg/mL for DA corn stover. After 10 min adsorption followed by a centrifugation at 13 000 g for 5 min, samples were washed by an excessive amount of water (200 μL each, 4 times). The adsorbed cellulase was suspended in 100 μL of distilled water, transferred to glass tubes, and then measured by the ninhydrin assay.

The experiments in Table 1 were conducted by using 200 μL of washing solvent, such as 1 M sodium chloride, 80% ethylene glycol, 50% glycerol, 0.01% Tween 80, 0.01% Triton X-100, 1.1% sodium dodecyl sulfate, 50 mM citrate solution with pH adjusted to 8, 9 and 10, and 0.135 M sodium hydroxide (pH 14), followed by 200 μL of water washing. The non-washable cellulase on 5 mg of Avicel or 1 mg of DA corn stover was measured by the ninhydrin assay.

Total cellulase (P_{total}, mg/mL) was measured by the Bradford method.[35] The adsorbed protein (P_{ads}, mg/mL) was calculated based on a difference between P_{total} and P_{free}, or measured by the ninhydrin assay directly.

Enzymatic hydrolysis of pretreated biomass

The pretreated corn stover samples were hydrolyzed at the substrate concentration of 10 g of glucan per L in a 50 mM sodium citrate buffer (pH 4.8) supplemented with 0.5 g/L sodium azide. The hydrolysis was conducted with an enzyme loading of 15 filter paper units of cellulase and 30 units of Novozyme 188 β-glucosidase per gram of glucan (12.3 mg cellulase and 9.4 mg β-glucosidase per gram of glucan) at 50 °C with a shaking rate of 250 rpm. The samples were taken for product and protein assays during the hydrolysis. Soluble sugar was measured by HPLC and free protein was measured by the Bradford assay.[27] After 72 h hydrolysis followed by centrifugation, biomass residues were washed in an excessive amount of the washing solvent (pH 10.0 citrate solution). The non-washable (irreversibly-bound) cellulase in the biomass residuals was measured by the ninhydrin assay.

Results and discussion

Although mass concentration of soluble proteins can be measured by a number of assays,[37] most of them cannot be

applied to measure adsorbed proteins on the surface of solid materials or in the presence of solid particles. Previously, we measured the adsorbed cellulase on pure cellulose, by using the Lowry assay after SDS desorption.[21,30] But this method is not applicable to pretreated biomass, containing hemicellulose and lignin, due to the interference from these lignocellulose components.

The ninhydrin assay has been used for quantifying the total amount of amino acids and analyzing amino acid components.[37,38] Protein contents in plant samples have been measured by the ninhydrin assay in the presence of tannin.[39–42] Both tannin and lignin are polyphenols with similar structures and chemical properties.[39] In addition, prior to the ninhydrin assay, protein samples must be hydrolyzed to amino acids in the presence of alkali. This information suggested that the bound cellulase on the surface of lignocellulosic materials could be measured by the ninhydrin assay because bound cellulase was hydrolyzed to free amino acids and the presence of lignin could not interfere with the ninhydrin assay.

Figure 1 shows that two free proteins (BSA and cellulase) have different slopes in terms of protein mass concentration by the ninhydrin (A), Bradford (B), and UV280 (C) assays due to differences in their amino acid compositions. Since assays of mass protein concentrations are protein-composition dependent, it is important to choose the right protein as a reference for these assays. For example, the cellulase assay was conducted based on cellulase as a reference or BSA as a reference with an adjustment coefficient (*i.e.*, 1 g BSA = 0.8 g cellulase for the ninhydrin method).

The effects of lignocellulosic biomass components were investigated on the ninhydrin-based adsorbed cellulase assay (Fig. 2B). They included pure cellulosic samples (Avicel and RAC), hemicellulose (birchwood xylan), lignin, monosaccharides (glucose and xylose), as well as DA-pretreated corn stover, and COSLIF-pretreated corn stover. Figure 2B shows nearly no readings for all tested lignocellulosic components, suggesting that they did not interfere with the ninhydrin assay. Similar slopes without any significant difference were obtained for the cellulase samples in the absence and presence of DA-pretreated corn stover from 100 μg to 1 mg per sample (Fig. 2A). These results validated the feasibility of determining the adsorbed cellulase by using ninhydrin in the presence of cellulose, lignin, hemicellulose, and lignocellulose, when their masses are less than 1 mg per sample.

The total cellulase (P_{total}) during enzymatic cellulose hydrolysis includes—free (unbound) cellulase (P_{free}), reversibly bound cellulase (P_{rev}), and irreversibly bound cellulase (P_{irr}) as below,

$$P_{total} = P_{free} + P_{rev} + P_{irr}$$

Determination of P_{irr} is important for knowing the potential of cellulase recycling, and the desorption efficiency depends on the conditions of the washing solvents. Fig. 3 shows that the amounts of adsorbed cellulase ($P_{rev} + P_{irr}$), measured by a direct ninhydrin assay, are close to those measured by the indirect method ($P_{total} - P_{free}$) in the presence of Avicel and DA-pretreated corn stover. These results suggest that the direct ninhydrin assay can measure the adsorbed cellulase on pure cellulose and pretreated lignocellulose. A fraction of adsorbed cellulase on Avicel and pretreated corn stover may be washed away by water (Fig. 3). After excessive de-ionized water washing, more bound cellulase was removed from Avicel than from pretreated corn stover, suggesting that pretreated biomass can bind cellulase more tightly. The results also indicate that pH-neutral water washing was not efficient to remove adsorbed cellulase.

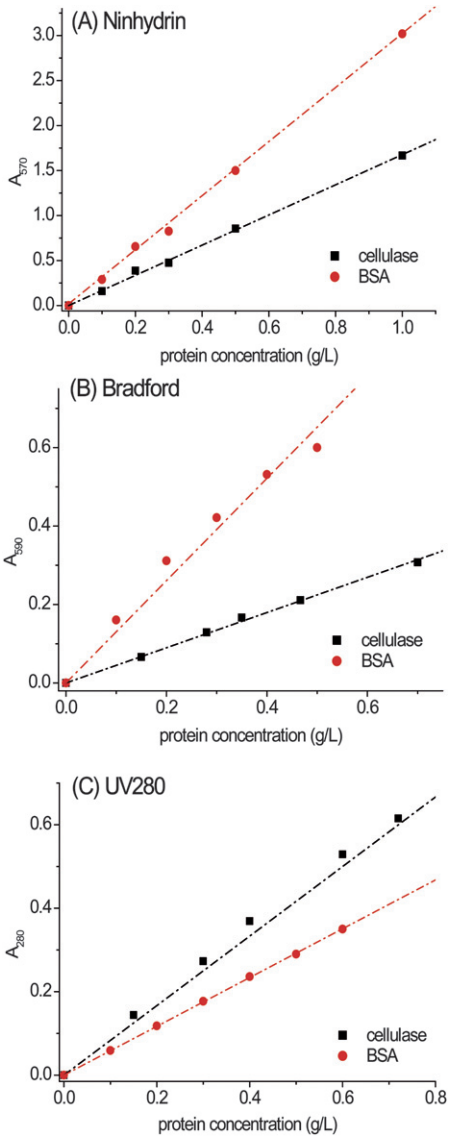

Fig. 1 Standard curves of different free protein assays with BSA and cellulase as references.

Table 1 shows the effects of desorption conditions on desorption efficiency of cellulase for Avicel and DA-pretreated corn stover. Desorption efficiency was associated with several experimental factors (*e.g.*, solvent type, ratio of solvent to adsorbent, adsorbent type, *etc.*). Since the ultimate goal was to economically recycle active desorbed cellulase, the use of a large volume of solvent was not practical. Polyhydric alcohols, such as ethylene glycol and glycerol, are more efficient for removing adsorbed cellulase than mild detergents (*e.g.*, Tween 80, and Triton X-100) or strong detergents (*e.g.*, SDS). Similar results

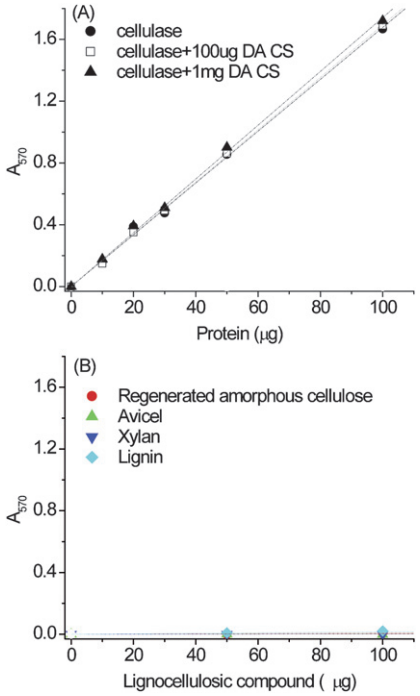

Fig. 2 Effects of two different concentrations of DA-pretreated corn stover (DA CS) on cellulase assays (A) and effects of various concentrations of lignocellulose components (regenerated amorphous cellulose (RAC), microcrystalline cellulose (Avicel), birchwood xylan, and kraft lignin) on the zero cellulase assays (B). One hundred μL of the sample solution with cellulase concentrations from 0.1 to 1.0 g/L (final concentration) or no cellulase added was mixed with 5 μL or 50 μL 20 g/L biomass slurry or other lignocellulose components. The mixtures were reacted with the ninhydrin reagent as described in the Experimental section.

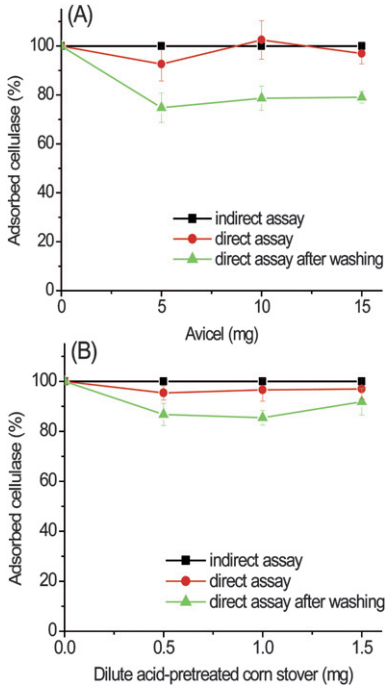

Fig. 3 Comparison of Ninhydrin assay (direct assay), with Bradford (indirect assay, difference = total – free) for Avicel (A) and DA corn stover (B). Substrate amounts at different mass concentration had the same binding capability.[27]

Table 1 Cellulase removal efficiency from Avicel and DA-pretreated corn stover after following various washing steps

	Cellulase desorption efficiency (%)	
Washing condition	Avicel	DA corn stover
Polyhydric alcohol		
72% Ethylene glycol	81 ± 5.5	76 ± 4.5
45% Glycerol	77 ± 3.3	74 ± 2.9
Detergent		
0.01% Tween80	38 ± 1.1	28 ± 0.8
0.01% Triton X-100	42 ± 1.5	39 ± 4.7
1% SDS	46 ± 4.0	42 ± 3.5
pH adjustment		
pH = 8	55 ± 4.6	35 ± 1.1
pH = 9	57 ± 1.5	48 ± 4.8
pH = 10	61 ± 3.9	56 ± 1.6
pH = 13	85 ± 3.7	94 ± 4.1
Salt		
1M NaCl	10 ± 0.5	6 ± 0.4
Deionized water (pH ~5.0)	8.9 ± 0.8	5.2 ± 0.3

have been reported by Otter and co-workers.[19] It was found that 72% ethylene glycol (EG) was the most effective, removing 81 ± 5.5% and 76 ± 4.5% of adsorbed cellulase from pure cellulose and pretreated biomass, respectively. EG was also previously used for desorbing the cellulose-binding module tagged protein for protein purification.[43] It was found that a pH increase, from 5 to 8, 9, and 10, increased desorption efficiency by 61 ± 3.9% compared to those observed prior to cellulase deactivation at a higher pH range. At pH 13, desorption efficiencies were 85% and 94% for Avicel and DA-pretreated corn stover, respectively. But cellulase was deactivated under these conditions. Although high concentration salt (1 M NaCl) was used to desorb cellulase,[44,45] it was found to be inefficient at the tested condition. From a cost-effective point of view, adjustment of the solution pH was more operative for desorbing the bound cellulase compared to the addition of other chemicals.

Fig. 4 shows profiles of glucan digestibility and free protein concentration for corn stover pretreated by DA and COSLIF, at a typical enzyme loading of 15 filter paper units of cellulase and 30 units of β-glucosidase per gram of glucan. Glucan digestibility of the COSLIF-pretreated corn stover reached 97% at hour 24, while the DA-pretreated corn stover exhibited considerably slower enzymatic hydrolysis rates with a final glucan digestibility of 84% at hour 72 (Fig. 4A). The free protein concentrations in both cases were decreased after the first 2 hours, indicating a rapid cellulase adsorption required for cellulose hydrolysis. At the beginning of hydrolysis, more cellulase was adsorbed by the COSLIF-pretreated corn stover than the DA-pretreated corn stover, consistent with faster hydrolysis rates and larger substrate accessibility to cellulase.[27] Later, the concentration of free cellulase rose, mainly due to bound cellulase release accompanying substrate consumption.[22] Similar dynamic trends of free cellulase were reported previously.[13,15] Notably, the protein concentration in the supernatant decreased slightly after ~20 h, which might be because some newly-exposed lignin after

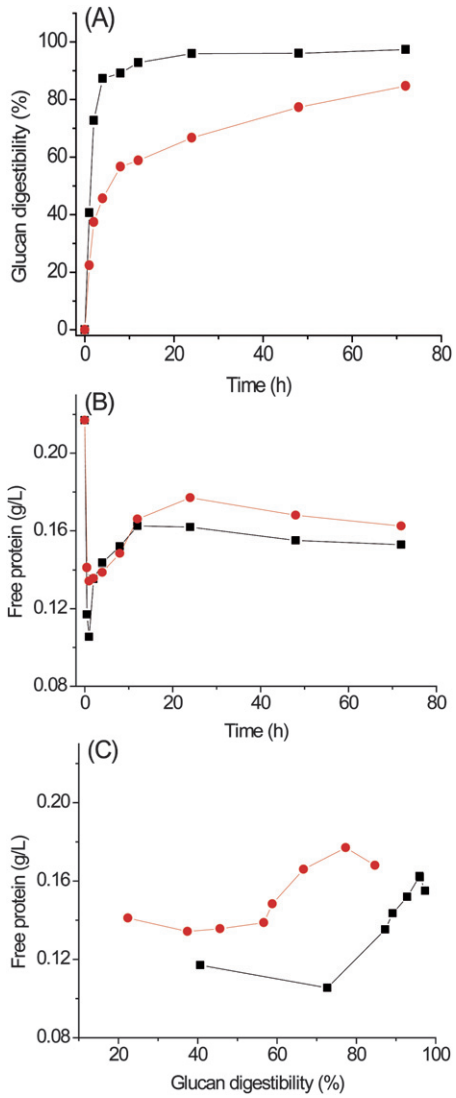

Fig. 4 Hydrolysis profiles (A) and protein concentration changes in the supernatant (B and C) for pretreated corn stover at 50 °C, 250 rpm, 10 g glucan/L with the enzyme loading of 15 filter paper units of cellulase and 30 units of β-glucosidase per gram of glucan (0.216 g/L of total protein concentration). ■ COSLIF, ● DA pretreatment.

cellulose hydrolysis adsorbed free cellulase at the end of hydrolysis (Fig. 4B). Moreover, it was found that more cellulase was adsorbed by COSLIF-pretreated corn stover than DA-pretreated corn stover at the same glucan digestibility (Fig. 4C), because the former had more total substrate accessibility.[27] At the end of hydrolysis, more cellulase was adsorbed on the COSLIF-pretreated corn stover due to its higher non-cellulose accessibility to cellulase than DA-pretreated corn stover.[27]

After 72-hour enzymatic hydrolysis and sequential washing at pH 10.0, the remaining adsorbed cellulase was quantified by the ninhydrin assay (Table 2). Although COSLIF-pretreated biomass adsorbed more cellulase than DA-pretreated biomass, the bound cellulase on the former was washed away more easily than the latter. The removal efficiencies were 81.2% and 71.8% on COSLIF- and DA-pretreated biomass, respectively. The irreversibly bound cellulase (*i.e.*, net cellulase loss) for the

Table 2 Cellulase desorption from the enzymatic hydrolysis residues of the corn stover pretreated by DA and COSLIF by using water washing at pH = 10[a]

	DA	COSLIF
Adsorbed enzymes on residue (% of initial protein)	28.0 ± 3.2	31.9 ± 2.1
Washable enzyme (% of adsorbed protein)	71.8 ± 3.0	81.2 ± 2.3
Overall recovery potential (% of initial protein)	92.1 ± 3.9	94.0 ± 2.7

[a] Adsorbed cellulase on hydrolysis residue and remaining cellulase after desorption were quantified by a direct ninhydrin assay. The hydrolysis was conducted at 10 g per L of glucan in a 50 mM sodium citrate buffer (pH 4.8) with an enzyme loading of 15 filter paper units of cellulase and 30 units of Novozyme 188 glucosidase per g of glucan (12.3 mg cellulase and 9.4 mg glucosidase per g of glucan).

COSLIF-pretreated biomass was ~6% of initially added total cellulase, suggesting a great potential for cellulase recycling. If cellulase stability can be enhanced greatly,[16,17] cellulase recycling will greatly decrease enzyme costs.

Two major factors preventing practical cellulase recycling are (i) inefficient cellulase release from pretreated biomass, particularly the irreversible adsorption on lignin, which can be addressed by a pH switch (shown here), addition of surfactant, or a more efficient lignin removal during biomass pretreatment, and (ii) cellulase denaturation over time, which can be overcome by cellulase engineering for better thermostability.[4,16,17]

Conclusion

The present work shows that the ninhydrin assay is a simple, fast, and low-cost assay for determining the adsorbed cellulase on cellulosic materials and pretreated lignocellulosic biomass. Protein hydrolysis releases the adsorbed cellulase, and lignocellulose components (cellulose, lignin, and hemicellulose) did not interfere with the protein assay. This assay would be useful for evaluating the feasibility of cellulase recycling for lignocellulosic biomass pretreated by different pretreatment approaches. A modest pH switch from 5.0 to 10.0 would be a cost-effective way to desorb active cellulase for its recycling. Therefore, it is important to improve cellulase thermostability by protein engineering so that recycling costly cellulase could be conducted through high-pH water washing.

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