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Non-ionic Surfactants and Non-Catalytic Protein Treatment on Enzymatic Hydrolysis of Pretreated Creeping Wild Ryegrass

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Abstract Our previous research has shown that saline Creeping Wild Ryegrass (CWR), *Leymus triticoides*, has a great potential to be used for bioethanol production because of its high fermentable sugar yield, up to 85% cellulose conversion of pretreated CWR. However, the high cost of enzyme is still one of the obstacles making large-scale lignocellulosic bioethanol production economically difficult. It is desirable to use reduced enzyme loading to produce fermentable sugars with high yield and low cost. To reduce the enzyme loading, the effect of addition of non-ionic surfactants and non-catalytic protein on the enzymatic hydrolysis of pretreated CWR was investigated in this study. Tween 20, Tween 80, and bovine serum albumin (BSA) were used as additives to improve the enzymatic hydrolysis of dilute sulfuric-acid-pretreated CWR. Under the loading of 0.1 g additives/g dry solid, Tween 20 was the most effective additive, followed by Tween 80 and BSA. With the addition of Tween 20 mixed with cellulase loading of 15 FPU/g cellulose, the cellulose conversion increased 14% (from 75 to 89%), which was similar to that with cellulase loading of 30 FPU/g cellulose and without additive addition. The results of cellulase and BSA adsorption on the Avicel PH101, pretreated CWR, and lignaceous residue of pretreated CWR support the theory that the primary mechanism behind the additives is prevention of non-productive adsorption of enzymes on lignaceous material of pretreated CWR. The addition of additives could be a promising technology to improve the enzymatic hydrolysis by reducing the enzyme activity loss caused by non-productive adsorption.

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Abbreviations

CWR	Creeping Wild Ryegrass, *Leymus triticoides*
BSA	bovine serum albumin
FPU	cellulase activity
CBU	β-glucosidase activity
Tween 80	poly(oxyethylene)$_{20}$-sorbitan-monooleate
Tween 20	poly(oxyethylene)$_{20}$-sorbitan-monolaurate
SSF	simultaneous saccharification and fermentation
DI water	deionized water
HPLC	high-performance liquid chromatography

Introduction

For more than a decade, lignocellulosic biomass has been recognized as a high potential substrate for ethanol production [1]. At present, the major research interest is to develop effective processes for conversion of cellulose into ethanol through enzymatic hydrolysis with high yield and low cost. The main obstacle to the large-scale commercialization of lignocellulose-based ethanol is the significant enzyme cost contribution to the overall cost [2, 3]. To obtain high fermentable sugar yield and hydrolysis rate, high enzyme loading is normally needed along with prolonged process time [4–8]. Furthermore, enzyme recycling is difficult because of the enzyme adsorption on residual lignocellulosic material. Thus, it is necessary to develop technologies to improve the efficiency of enzymatic hydrolysis to make lignocellulose-based ethanol economically feasible.

Enzyme activity loss because of non-productive adsorption on lignin surface was identified as one of the important factors to decrease enzyme effectiveness, and the effect of surfactants and non-catalytic protein on the enzymatic hydrolysis has been extensively studied to increase the enzymatic hydrolysis of cellulose into fermentable sugars [7, 9–19]. The reported study showed that the non-ionic surfactant poly(oxyethylene)$_{20}$-sorbitan-monooleate (Tween 80) enhanced the enzymatic hydrolysis rate and extent of newspaper cellulose by 33 and 14%, respectively [20]. It was also found that 30% more FPU cellulase activity remained in solution, and about three times more recoverable FPU activity could be recycled with the presence of Tween 80. Tween 80 enhanced enzymatic hydrolysis yields for steam-exploded poplar wood by 20% in the simultaneous saccharification and fermentation (SSF) process [21]. Helle et al. [22] reported that hydrolysis yield increased by as much as a factor of 7, whereas enzyme adsorption on cellulose decreased because of the addition of Tween 80. With the presence of poly(oxyethylene)$_{20}$-sorbitan-monolaurate (Tween 20) and Tween 80, the conversions of cellulose and xylan in lime-pretreated corn stover were increased by 42 and 40%, respectively [23]. Wu and Ju [24] showed that the addition of Tween 20 or Tween 80 to waste newsprint could increase cellulose conversion by about 50% with the saving of cellulase loading of 80%. With the addition of non-ionic, anionic, and cationic surfactants to the hydrolysis of cellulose (Avicel, tissue paper, and reclaimed paper), Ooshima et al. [25] subsequently found that Tween 20 was the most effective for the enhancement of cellulose conversion, and anionic surfactants did not have any effect on cellulose hydrolysis. With the addition of Tween 20 in the SSF process for

ethanol production from softwood, Alkasrawi et al. [2] reported that the ethanol yield was increased by 8% in an even shorter time, and the amount of enzyme loading could be reduced by 50% while maintaining a constant yield. Kristensen et al. [3] reported that surfactants had a more pronounced effect on acid and steam-treated straw than ammonia and hydrogen-peroxide-treated straw. The addition of non-catalytic protein such as bovine serum albumin (BSA) was also used to enhance the enzymatic hydrolysis of cellulose in lignocellulosic biomass [26–28]. Addition of 17 g/l BSA produced about the same cellulose conversion as adding Tween 20 at 2.5 g/l for saccharose phosphate synthetase, but adding Tween 20 together with BSA did not increase cellulose conversion [7]. With addition of BSA for enzymatic hydrolysis of dilute acid pretreated corn stover, cellulose conversion was increased by 10% within 72 h, whereas 30% more cellulase activity (FPU) and 65% more β-glucosidase activity (CBU) remained free in solution [17]. In the same study, it was found that BSA did not affect Avicel hydrolysis and nor did it adsorb on Avicel, but it adsorbed on pretreated corn stover with high capacity. The possible reason for the difference between Avicel and corn stover was explained that BSA could adsorb onto a lignin surface and prevent the non-productive adsorption of enzymes onto lignin of corn stover, whereas Avicel has no lignin content to adsorb the enzymes. BSA was able to increase cellulose conversion up to 70% when it was added with cellulase for hydrolyzing wheat straw, but the effect depended on the substrate features derived by pretreatment methods [3].

Both Tween 20 and 80 are non-toxic and suitable for food and/or biotechnical use, such as stimulants for enzyme production by microorganisms [29–31] and additives in the SSF process for ethanol production [2]. BSA was used to improve the enzymatic conversion of lignocellulosic biomass because of its high affinity to lignin but little affinity to cellulose [17].

Although lots of work has been done on additives for enhancement of enzymatic hydrolysis of various substrates such as wood, corn stover, and wheat straw, saline Creeping Wild Ryegrass (CWR) has not previously been studied with addition of additives. Furthermore, the mechanisms for the enhancement of enzymatic hydrolysis by additives are not clear. Thus, more in-depth studies are needed to investigate such mechanisms. Two non-ionic surfactants (Tween 20 and 80) and a non-catalytic protein (BSA) were employed to do this study.

The specific objectives of this research were to investigate (1) the effect of additives on the cellulose conversion of pretreated CWR, (2) the possible mechanism behind the effect of additives on the enzymatic hydrolysis, (3) the effect of additives on the enzyme adsorption onto several substrates, including Avicel PH101, pretreated CWR, and lignaceous residue of pretreated CWR.

Materials and Methods

Non-ionic surfactants (Tween 20 and 80) and non-catalytic protein (BSA) were used to investigate if the enzymatic hydrolysis of pretreated CWR can be improved with various surface-active additives. The enzyme protein concentration and activities in solution were measured during enzymatic hydrolysis. Based on these data, the effect of additives on enzyme protein and activity was determined. All the experiments and experimental conditions are summarized in Table 1.

Pretreated CWR and Enzyme Preparations

The CWR was pretreated by dilute sulfuric acid under selected conditions [acid concentration=1.4% (*w*/*w*), temperature=165°C, and time=8 min; 32]. The pretreated CWR

Table 1 Experimental design.

Experiments	Additives (g/g dry solid)	Substrate loading (dry %, *w*/*w*)	Enzyme loading	Measurement
Effect of additives on enzymatic hydrolysis	Tween 20, Tween 80, BSA (0.1)	Pretreated CWR (8)	(15 FPU + 15 CBU)/g cellulose	Glucose and cellobiose concentration
Effect of additives on enzyme protein concentration and activity	Tween 20, BSA (0.1)			Enzyme protein concentration and activity
Effect of additive loading on enzymatic hydrolysis	Tween 20, BSA (0, 0.05, 0.1, 0.15, 0.2)			Glucose and cellobiose concentration and enzyme protein concentration and activity
Effect of additive on enzymatic hydrolysis	Tween 20 (0.1)	Avicel (8)		
Additive added after 8 h of hydrolysis	Tween 20 (0.1)	Pretreated CWR (8)		
Effect of additives on enzyme protein concentration and activity	Tween 20 (0.1)	Lignaceous residue (3)	(15 FPU + 15 CBU)/g dry solid	Enzyme protein concentration and activity
BSA adsorption	BSA (0.1)	Avicel (8) pretreated CWR (8) lignaceous residue (3)	–	BSA protein

The temperature and pH of the mixtures were 50°C and 4.8, respectively.

was washed to remove the soluble contents and acid. The washing was stopped until the pH of the filtrate reached 4.5. The washed pretreated CWR was then treated with cellulase (Novozymes, Celluclast 1.5 l, available from Sigma, Cat. no. C2730) supplemented with β-glucosidase (Novozyme188, Sigma, Cat. no. C6105). The enzymatic hydrolysis was performed in an incubator shaker at 150 rpm, pH=4.8, and temperature=50°C with cellulase and β-glucosidase loadings of 15 FPU and 15 CBU/g cellulose, respectively. The enzymatic hydrolysis conditions described in this paper was defined as a standard condition used throughout the entire paper, unless specified otherwise.

Additives and Avicel PH101

The additives tested in this study included non-ionic surfactants (Tween 20 and 80) and non-catalytic protein (BSA). They were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Avicel PH101 was also purchased from Sigma-Aldrich.

Enhancement of Enzymatic Hydrolysis of Pretreated CWR by Additives

A batch enzymatic hydrolysis was conducted at 8% (*w*/*w*) of dry solid loading in a 50-mM citrate buffer (pH=4.8) containing 0.03% (*w*/*v*) sodium azide. The total working volume was 10 in 20 ml screw-capped vials. Before the addition of cellulase and β-glucosidase, the mixture of substrate and buffer was preheated in an incubator shaker under the temperature

of 50°C for 30 min to allow the substrate to disperse uniformly in the buffer. Tween 20, Tween 80, or BSA was then added into the vials at a loading of 0.1 g/g dry solid. The mixtures in vials were continuously heated in the incubator shaker for another 1 h for complete interaction between substrate and additives. Then, cellulase and β-glucosidase were added into vials immediately to initiate enzymatic hydrolysis. The time point of enzyme addition was recorded as time zero.

During the enzymatic hydrolysis, 1-ml aliquots were withdrawn from each vial by using micro-pippet (tips were cut) at start and at times of 8, 24, 48, and 72 h of hydrolysis to a series of 1.5-ml Eppendorf tubes. Before sampling, the vials were thoroughly shaken to make the mixture in vials as homogeneous as possible. The aliquots were immediately placed over a boiling water bath for 20 min to deactivate the enzymes as described by Helle et al. [22] and Desai and Converse [33]. After enzyme inactivation, each sample was centrifuged for 5 min at 12,000 rpm, and 500-ul supernatants were then removed to another series of 1.5-ml Eppendorf tubes. The supernatant samples were stored at 4°C for subsequent sugar analysis.

Effect of Tween 20 and BSA on Enzyme Protein Concentration and Activity

To study the effect of Tween 20 and BSA on enzyme protein concentration and activity, the enzyme protein concentration and activity were measured throughout the enzymatic hydrolysis of pretreated CWR. These experiments were conducted using the same procedure as described in "Enhancement of Enzymatic Hydrolysis of Pretreated CWR by Additives" except for the sampling procedure. In this experiment, 1-ml aliquots were withdrawn from each vial at start and times of 8, 24, 48, and 72 h using 1-ml syringes. These aliquots were then immediately filtered through syringe filters (Millex-GV4; polyvinylidene difluoride low protein-binding membrane; pore size, 0.2 μm; diameter, 4 mm, Millipore, Bedford, MA, USA) into another series of 1.5-ml Eppendorf tubes and diluted before analysis if necessary [15, 34]. The samples were stored at 4°C less than 1 h for subsequent protein concentration and enzyme activity measurements.

Effect of Tween 20 Loading on Enzymatic Hydrolysis of Pretreated CWR

The effect of Tween 20 loading on enzymatic hydrolysis of pretreated CWR was studied at five different loading levels, including 0, 0.05, 0.1, 0.15, and 0.2 g Tween 20/g-dry solid. At the end of 72 h hydrolysis, a 1-ml aliquot was withdrawn for sugars measurement. At the same time, another 1-ml aliquot was withdrawn for protein and enzyme activity measurement. For enzyme protein concentration and activity measurements, the sample pretreatment procedures before measurement were the same as described in "Effect of Tween 20 and BSA on Enzyme Protein Concentration and Activity."

Effect of Tween 20 on Enzymatic Hydrolysis of Avicel

The enzymatic hydrolysis and sampling procedures for Avicel was the same as described in "Enhancement of Enzymatic Hydrolysis of Pretreated CWR by Additives." Based on the experimental result from "Effect of Tween 20 Loading on Enzymatic Hydrolysis of Pretreated CWR," the ratio of Tween 20 to Avicel was decided to be 0.1 g Tween 20 to 1 g Avicel. A homogeneous aliquot of 1 ml was withdrawn at start and after 1, 2, 4, 8, 24, 48, and 72 h of hydrolysis. The aliquots were treated as "Effect of Tween 20 and BSA on Enzyme Protein Concentration and Activity" to obtain 500-ul supernatant samples. The

250-ul supernatant samples were then moved to other series of 1.5-ml Eppendorf tubes, which were heated in boiling water to deactivate the enzymes for sugar measurement as described in "Enhancement of Enzymatic Hydrolysis of Pretreated CWR by Additives." The rest of the 250-ul supernatant samples were used to measure enzyme protein concentration and activity.

Lignaceous Residue Preparation

Lignaceous residue of pretreated CWR was prepared by a limit enzymatic hydrolysis [13, 15, 16]. The total reaction volume was 400 ml in a 1-l Erlenmeyer flask. The pretreated CWR was hydrolyzed for 168 h with enzyme loading of 60 FPU and 75 CBU/g cellulose. These reaction conditions were enough to obtain almost complete hydrolysis of cellulose (98.5%) by monitoring the cellulose hydrolysis based on the released glucose and cellobiose concentrations in solution. The lignaceous residue after hydrolysis was sequentially washed with distilled DI water, 1.0 M NaCl, and distilled DI water. The prepared lignaceous residues were stored in the refrigerator under 4°C without drying for less than 24 h for further study.

BSA Adsorption to Avicel, Pretreated CWR, and Lignaceous Residue and Effect of Tween 20 on Enzyme Adsorption on Lignaceous Residue

To investigate the mechanism of the BSA on the improvement of enzymatic hydrolysis of pretreated CWR, the adsorptions of BSA on various substrates were studied [17]. Three sets of 20-ml vials were used to test the adsorption of BSA to Avicel, pretreated CWR, and lignaceous residue, respectively. The mixtures of citrate buffer (pH=4.8) with 8% (*w*/*w*) Avicel, 8% (*w*/*w*) pretreated CWR, or 3% (*w*/*w*) lignaceous residue were preheated to 50°C for 30 min. BSA was then added to the vials with the ratio of BSA to dry solid equal to 0.1 g BSA/g dry solid. The final working volume was 10 ml. One-milliliter aliquots were periodically withdrawn at start and after 1, 2, 4, 8, 24, 48, and 72 h. The aliquots were pretreated according to the procedures described in "Effect of Tween 20 and BSA on Enzyme Protein Concentration and Activity" for BSA protein concentration measurement.

For studying the effect of Tween 20 on the adsorption of enzymes on lignaceous residue, the mixtures of citrate buffer (pH=4.8), 3% (*w*/*w*) lignaceous residue, and Tween 20 were added to a 20-ml vial sequentially. The loading ratio between Tween 20 and lignaceous residue was 0.1 g Tween 20/g dry solid. The mixtures were then preheated to 50°C for 1 h for interaction between Tween 20 and lignaceous residue solids. Finally, the enzymes were loaded into vials with the loading of (15 FPU + 15 CBU)/g dry solid. The enzyme addition time point was set as time zero. Both enzyme protein concentration and activities were measured periodically (at start and after 1, 2, 4, 8, 24, 48, and 72 h of enzyme addition) according to the procedure described in "Effect of Tween 20 and BSA on Enzyme Protein Concentration and Activity."

Enzymatic Hydrolysis of Pretreated CWR With and Without the Presence of Tween 20 Added After 8 h of Hydrolysis

To investigate how the interaction between Tween 20 and pretreated CWR affect the enzymatic hydrolysis efficiency, the enzymatic hydrolysis of pretreated CWR with 0.1 g Tween 20/g dry solid added after 8 h of hydrolysis was studied. The results of this section were compared to those obtained in "Enhancement of Enzymatic Hydrolysis of Pretreated

CWR by Additives," in which Tween 20 was added before the addition of enzymes. During hydrolysis, 1-ml aliquots were taken at start and after 1, 2, 4, 8, 24, 48, and 72 h of hydrolysis. The sugars, enzyme protein concentration, and activity were measured.

Analytical Methods

The sugar contents such as glucose and cellobiose in the supernatant of liquid samples were analyzed on a high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan). The time constant and range were set to be 1 s and ×64 (or ×8), respectively. All samples were filtered through small syringe filters (Millex-GV4; polyvinylidene difluoride low protein-binding membrane; pore size, 0.2 μm; diameter, 4 mm, Millipore) and diluted before analysis if necessary [15, 17, 34]. The HPLC analytical column was equilibrated with HPLC grade (18 MΩ) water at a flow rate of 0.6 ml/min overnight. An Aminex HPX-87P (Bio-Rad, Hercules, CA) column was used for sugar separations at 85°C, with the HPLC grade water as the mobile phase at a flow rate of 0.6 ml/min. The HPLC system was equipped with a Carbo-P refill cartridge (Bio-Rad) as a guard column to protect the analytical column from blocking.

The protein concentration in solution was measured by the Bradford protein assay using BSA as a standard (Bio-Rad). The Bradford colorimetric method cannot distinguish the BSA protein from enzyme protein. Therefore, no differentiation was possible between BSA and enzymes in solution other than by measuring the enzyme activity. The enzyme activities in solution including FPU and CBU were measured according to the methods described by Ghose [35].

All the experimental results were the average of two replicates, unless specified otherwise.

Results

Enhancement of Enzymatic Hydrolysis of Pretreated CWR by Additives

As shown in Fig. 1, the presence of Tween 80, Tween 20, BSA or (BSA + Tween 20) improved the enzymatic hydrolysis of pretreated CWR by 8 to 14% after 72 h of hydrolysis. The initial hydrolysis rate was also increased to some extent. The cellulose conversion was increased from approximately 75% (no additives) to 89, 88, and 83% with the presence of Tween 20, Tween 80, and BSA, respectively. Of all three additives, Tween 20 was the most effective additive on the improvement of cellulose conversion, followed by Tween 80 and BSA. The treatment of Tween 20, Tween 80, or BSA gave similar results at an enzyme loading of 15 FPU/g cellulose to those at enzyme loading of 30 FPU/g cellulose but without additive addition (Fig. 1). Therefore, the addition of surfactants and/or non-catalytic protein could help save enzyme loading without decreasing the hydrolysis yield. In addition, it was found that Tween 20 and BSA gave no further increase in cellulose conversion. Thus, the effect of Tween 20 on the hydrolysis of pretreated CWR might be similar to that of BSA [7].

To determine how additions of Tween 20 and BSA before adding enzymes affected adsorption of enzymes, the enzyme protein concentration and activities (FPU and CBU) in solution were measured for pretreated CWR, as reported in Figs. 2 and 3 for an enzyme loading of (15 FPU + 15 CBU)/g cellulose. Based on a free protein of 100% for the total

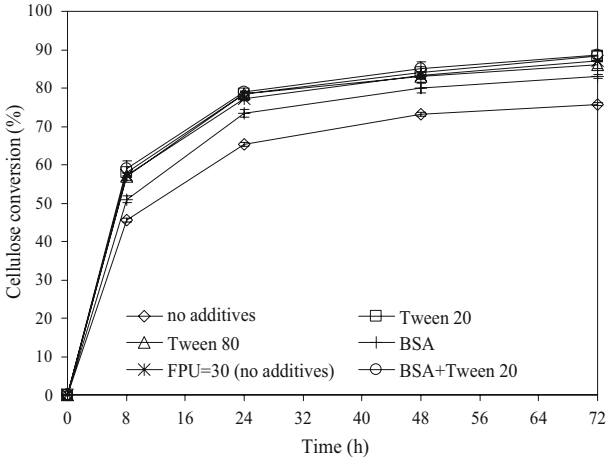

Fig. 1 Effect of additives on the cellulose conversion of pretreated CWR (the additive loading was 0.1 g additive/g dry solid and the enzyme loading was 15 FPU + 15 CBU/g cellulose)

protein concentration in solution at time zero, approximately 58% of initial enzyme protein remained in solution with the presence of Tween 20 after 72 h of interaction between enzyme and pretreated CWR (Fig. 2). However, only 15% of the initial enzyme protein remained free in solution without the addition of Tween 20. Because no differentiation was possible between BSA and enzymes in solution, enzyme protein concentration was not measured when BSA was used as an additive.

Based on a relative activity of 100% for the protein in solution at time zero, the cellulase activity (FPU) in solution dropped quickly to about 19% of its initial value within 72 h when no additives were present (Fig. 3). When Tween 20 or BSA was added to pretreated CWR before the addition of enzymes, the cellulase activity in solution only dropped to about 50% of its initial value after 72 h. With a similar trend, β-glucosidase activity (CBU) in solution dropped to approximately 5% of its initial value without additive addition but dropped far less to about 68% of the initial activity when Tween 20 or BSA was present. Based on the results from Figs. 2 and 3, a conclusion could be drawn that the Tween 20 and BSA can prevent the enzyme being adsorbed by substrate (cellulose or lignin content in the

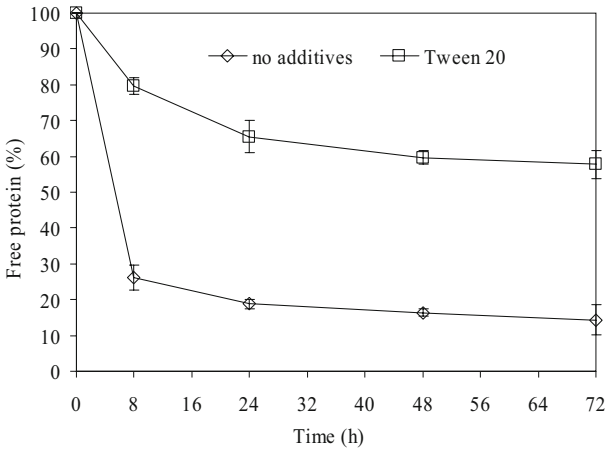

Fig. 2 Free enzyme protein in solution with and without Tween 20 during enzymatic hydrolysis

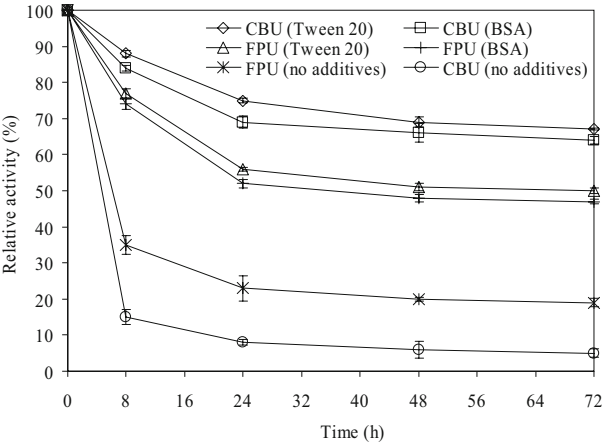

Fig. 3 Changes in relative enzyme activities with and without the addition of BSA or Tween 20 during enzymatic hydrolysis

pretreated CWR) and could increase the enzyme availability in solution for the hydrolysis of pretreated CWR.

Effect of Tween 20 Loading on Enzymatic Hydrolysis of Pretreated CWR

As shown in Fig. 4, cellulose conversion of pretreated CWR increased from 75 to 88% linearly with the increase of Tween 20 loading from 0 to 0.1 g Tween 20/g dry solid. When Tween 20 loading was higher than 0.1 g Tween 20/g dry solid, there was little increase in cellulose conversion. The changes of enzyme protein concentration and activities in solution followed similar trends to cellulose conversion (Fig. 5). With the presence of 0.1 g Tween/g dry solid, over 40% enzyme protein, 30% FPU and 60% CBU remained in solution after 72 h of hydrolysis. However, only about 6% protein, 17% FPU, and 20% CBU remained free without Tween 20 addition. The possible reason for these results of Figs. 4 and 5 was that non-productive adsorption sites in pretreated CWR were saturated by Tween 20 with the loading equal to or higher than 0.1 g Tween 20/g dry solid. Therefore, the loading of 0.1 g Tween 20/g dry solid could be a saturation value for Tween 20 to improve the cellulose conversion of pretreated CWR.

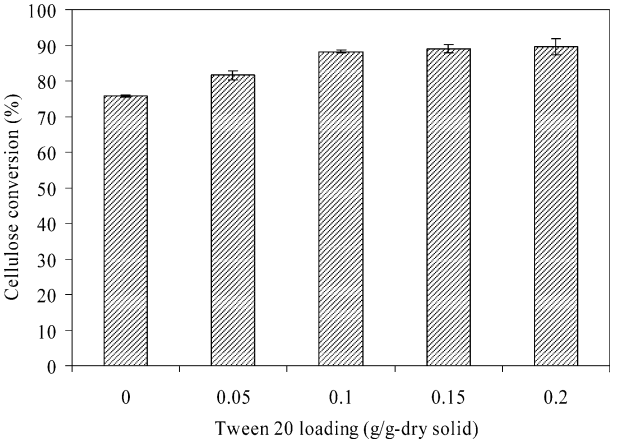

Fig. 4 Cellulose conversion of pretreated CWR after 72 h as a function of Tween 20 loading

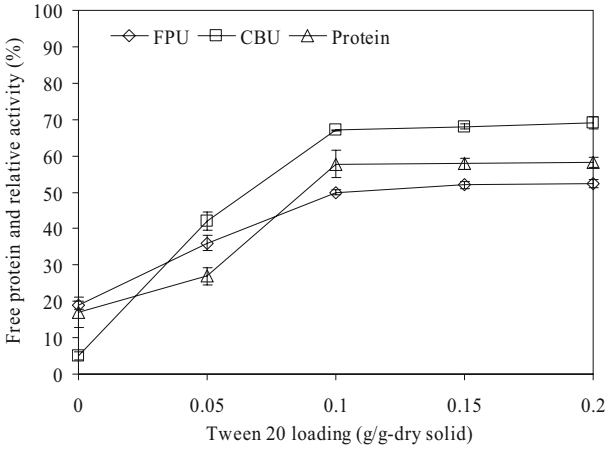

Fig. 5 Changes in free enzyme activities and protein concentration in the solution during enzymatic hydrolysis of pretreated CWR after 72 h as a function of Tween 20 loading

Considering the result from Fig. 1 that (0.1 g Tween 20 + 0.1 g BSA)/g dry solid gave no further increase of cellulose conversion compared with the addition of Tween 20 alone, a conclusion could be drawn that the mechanisms of Tween 20 and BSA on the improvement of enzymatic hydrolysis of pretreated CWR might be similar. If Tween 20 and BSA had significantly different mechanisms on the improvement of enzymatic hydrolysis, higher cellulose conversion should be expected after BSA was added to the solution with Tween 20 at saturation loading of 0.1 g/g dry solid.

Effect of Tween 20 on Enzymatic Hydrolysis of Avicel

When the substrate was Avicel, the cellulose conversion was slightly increased with Tween 20 addition, in contrast to the significant increase of cellulose conversion of pretreated CWR (Fig. 6). After 72 h, the cellulose conversion of Avicel was increased 3% with the presence of 0.1 g Tween 20/g Avicel.

The changes in enzyme protein concentration and activities in the solution during hydrolysis of Avicel were measured as functions of the addition of Tween 20 (Fig. 7).

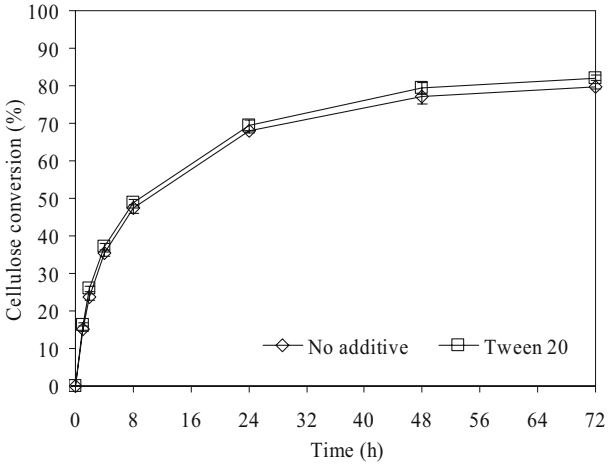

Fig. 6 Effect of Tween 20 on enzymatic hydrolysis of Avicel with 8% dry solid loading at 15 FPU + 15 CBU/g cellulose enzyme loading

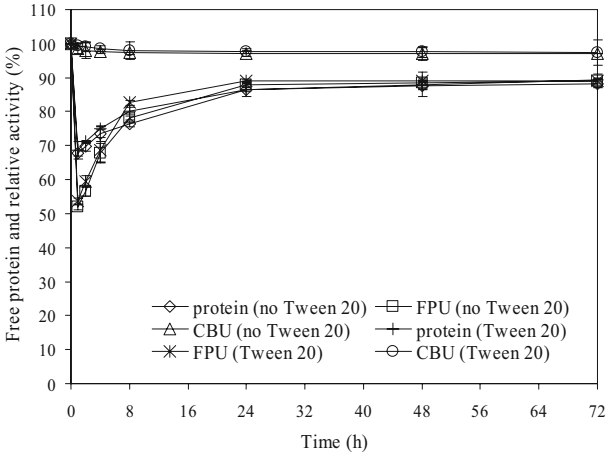

Fig. 7 Changes in free protein concentration and relative enzyme activities in solution during enzymatic hydrolysis of Avicel at 8% dry solid loading with and without Tween 20 addition

There were no significant changes about the enzyme protein, cellulase, and β-glucosidase activities with and without the addition of Tween 20, which corresponded to the performance of enzymatic hydrolysis present in Fig. 6. The free protein concentration and cellulase activity (FPU) in solution dropped rapidly to about 70 and 50% of their initial values, respectively, within the first 1 h. Both of them increased to about 80% after 8 h of hydrolysis. Then, they increased slightly until leveled off after 24 h. In addition, it was found that the β-glucosidase activity (CBU) just had few changes during the enzymatic hydrolysis of Avicel. After 72 h of hydrolysis, only about 3% β-glucosidase activity in solution was lost, which might indicate that small amount of β-glucosidase was adsorbed to Avicel, and/or that β-glucosidase activity was lost by thermal, mechanical, and/or other factors as reported by Moloney and Coughlan [36]. Ooshima et al. [37] found β-glucosidase could not adsorb on crystalline cellulose at the temperature of 50°C. If this is the case, we might conclude that the most β-glucosidase was deactivated during enzymatic hydrolysis by thermal, mechanical, and/or other factors. For cellulase, its activity loss from solution might be caused by the inactivation or adsorption on cellulose irreversibly. To our knowledge, little research has been done to distinguish between inactivation of cellulase and cellulase binding to cellulose during cellulosic hydrolysis. Mizutani et al. [38] compared the effects of Tween 20 on the hydrolysis of several cellulosic fibers with different crystallinity and found that the effectiveness of Tween 20 became higher with increased crystallinity of cellulosic substrates. Their results indicated that the surfactants could prevent some of the cellulase from adsorbing to non-productive sites on crystalline cellulose.

Comparing Figs. 2 and 3 with Fig. 7, it was found that the free enzyme protein concentration and activities in solution kept decreasing within the entire hydrolysis process of pretreated CWR, but they were slowly released back into solution after approximately 1 h hydrolysis of Avicel. Several studies have concluded that the reason for this difference was the presence of lignin content in the lignocellulosic biomass [7, 17, 22] in that the lignin adsorbed the enzymes irreversibly during the hydrolysis. The affinity of β-glucosidase to lignocellulosic biomass was much higher than that to Avicel. For cellulase, its affinity to lignocellulosic biomass was moderately higher than to Avicel. Although it is believed that lignin is a primary cause of the loss of enzyme protein and activities, the negative effect of crystallinity of cellulose might also exist. More research is needed to study the effect of cellulose crystallinity on the adsorption of hydrolytic enzymes.

BSA Adsorption to Avicel, Pretreated CWR, and Lignaceous Residue of Pretreated CWR

The adsorbed BSA on Avicel was only 2% of initial value after 72 h of interaction between BSA and Avicel (Fig. 8). When the adsorbent was changed to pretreated CWR or lignaceous residue of pretreated CWR, the free BSA concentration in the solution dropped to around 20% of the initial value within the first 1 h. For pretreated CWR and lignaceous residue, the relative adsorbed BSA were almost the same as each other. However, the adsorption capacities of lignaceous residue and pretreated CWR for BSA were calculated to be about 0.1 g BSA/g dry lignaceous residue and 0.08 g BSA/g dry pretreated CWR, respectively. In this study, the adjusted concentration of lignin content in pretreated CWR was almost the same as that of lignaceous residue in reaction vials. Based on the results of BSA adsorption on pretreated CWR and lignaceous residue, two hypotheses could be proposed. The first is that the cellulose content in pretreated CWR adsorbed little BSA, and only the lignin content in pretreated CWR could adsorb BSA. The other is that the cellulose content in pretreated CWR could adsorb some BSA, but the adsorption capacity of cellulose content was lower than that of lignin content of pretreated CWR. More research is needed to prove these hypotheses.

Effect of Tween 20 on Adsorption of Cellulase and β-glucosidase on Lignaceous Residue of Pretreated CWR

As shown in Fig. 9, the enzyme protein in the solution dropped rapidly either with or without the addition of Tween 20 within the first 1 h. From the first 1 h to the end of 72 h of adsorption, the free protein concentration in solution decreased much less rapidly, about 10% free protein concentration decrease either with or without Tween 20 addition. Over 50% more protein remained in the solution after 72 h when 0.1 g Tween 20/g dry lignaceous residue was present. The cellulase and β-glucosidase activities followed a similar change trend to free protein concentration in solution. Comparing with the adsorption without the addition of Tween 20, 60% more β-glucosidase activity and 30% more cellulase activity remained in solution after 72 h adsorption with Tween 20 addition. The results from Fig. 9 indicated that Tween 20 could prevent the non-productive adsorption of enzymes to lignin content in the pretreated CWR.

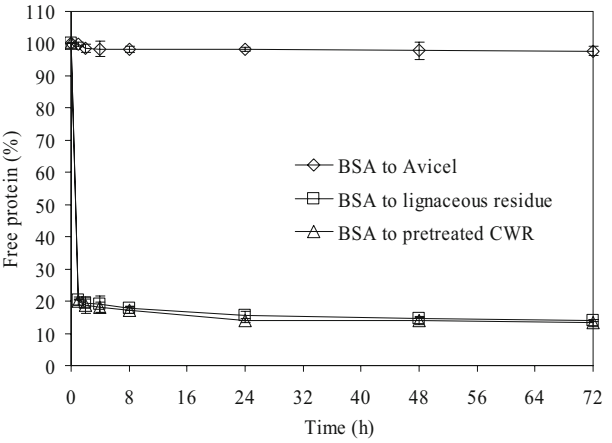

Fig. 8 BSA (0.1 g/g dry solid) adsorption to Avicel (8%, *w/w*), pretreated CWR (8%, *w/w*, containing lignin content equal to 3%, *w/w* of total reaction weight), and lignaceous residue of pretreated CWR (3%, *w/w*)

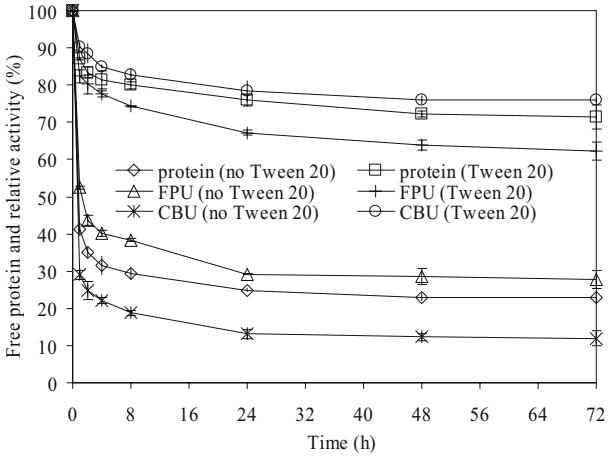

Fig. 9 Changes in free protein concentration and relative enzyme activities in solution during enzyme adsorption to lignaceous residue (3%, *w*/*w*) with and without Tween 20 addition (0.1 g Tween 20/g dry lignaceous residue)

Enzymatic Hydrolysis of Pretreated CWR With and Without Tween 20 Added after 8 h of Pretreated CWR Hydrolysis

Adding Tween 20 after 8 h of pretreated CWR hydrolysis had little effect on cellulose conversion (Fig. 10). The final cellulose conversion was improved by about 2%, which was much lower than the 14% more cellulose conversion achieved when Tween 20 was added before enzyme addition (Fig. 1).

In the same experiment presented in Fig. 10, the enzyme protein and activities were also measured, as shown in Fig. 11. The enzyme protein concentration in solution dropped quickly to about 40% of its initial value within the first 1 h of hydrolysis and to about 15% of initial value after 72 h of hydrolysis when no Tween 20 was added. At the end of 72 h, the free protein concentration in solution was increased to 17% of its initial value with Tween 20 added after 8 h of hydrolysis. When followed in a similar fashion, the cellulase and β-glucosidase activities dropped to about 20 and 5% of initial values, respectively, without the presence of Tween 20. While Tween 20 was added after 8 h of hydrolysis, only

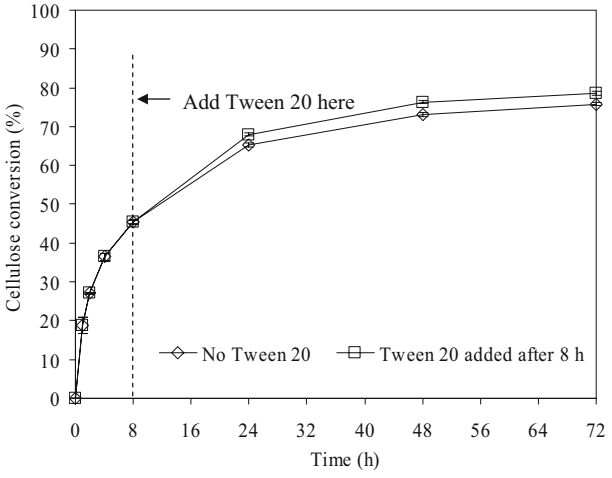

Fig. 10 Enzymatic hydrolysis of pretreated CWR (8%, *w*/*w*) with and without Tween 20 (0.1 g Tween 20/g dry pretreated CWR) added after 8 h of hydrolysis

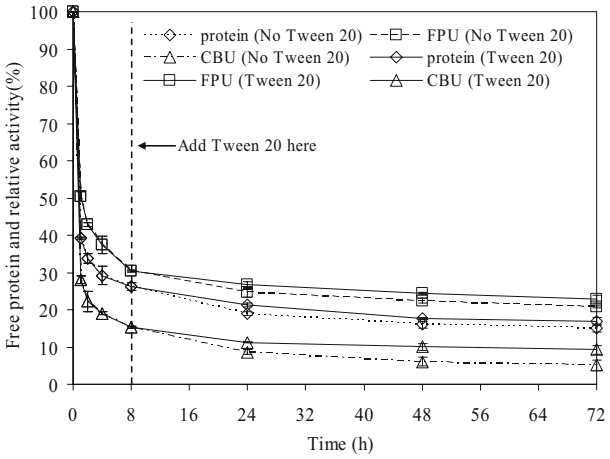

Fig. 11 Changes in free protein concentration and relative enzyme activities in solution during enzymatic hydrolysis of pretreated CWR with and without Tween 20 added after 8 h of hydrolysis presented in Fig. 10

2% more cellulase and 4% more β-glucosidase activities in solution were achieved than those without Tween 20 addition.

Considering Figs. 10 and 11, it might suggest that the primary effect of Tween 20 on cellulose conversion of pretreated CWR could be blocking non-productive adsorption of enzymes on substrate and not deactivation by some other mechanism. Comparing Figs. 2 and 3 with Fig. 11, it was found that adding Tween 20 before enzyme addition resulted in much higher enzyme protein and activities available in solution than when Tween 20 was added after enzyme addition. If Tween 20 can protect enzymes from deactivating and/or modifying the surface structure of the substrate, significantly more increased cellulose conversion should be expected. However, these results were not found in this study. Therefore, a conclusion could be drawn that the Tween 20 addition can prevent enzyme from adsorbing non-productively onto substrate, but cannot free most absorbed protein.

Discussions

Enhancement of cellulose conversion by adding various additives, including surfactants and non-catalytic proteins, to the hydrolysis mixture has been reported by many researchers. The mechanisms underlying the enhancement of enzymatic cellulose hydrolysis by the addition of surfactants and/or BSA have been the objective of intense research. Various mechanisms have been proposed that can be divided into three main categories: (1) stabilizing the enzyme by reducing thermal and/or mechanical shear forces [23, 39]; (2) changing the ultra structure of the substrate, making the cellulose more accessible to enzymes [22]; and (3) affecting enzyme-substrate interaction, e.g., enzymes are prevented from inactivation because of non-productive adsorption to lignin content in lignocellulosic biomass by steric repulsion [3, 7, 8, 17, 20, 22, 23, 40].

Kim et al. [41] reported that a significant deactivation was observed when cellulase was subjected to shear and/or exposed to air-liquid interface, which was thought to be far more severe and extensive than shear effect alone. By using sufficient additives (surfactants and non-catalytic proteins, e.g., BSA), cellulase deactivation can be, to some extent, prevented, and cellulase can be stabilized. This might be because the addition of non-ionic surfactants could reduce the contact of enzymes with the air–liquid interface because of the surface

activity of the surfactant [41]. Kaar and Holtzapple [23] proposed that the addition of Tween protected the enzymes from thermal deactivation because they found that the optimum temperature in Tween samples was 10°C higher than that of the Tween-free samples. However, Herskovits and Jaillet [42], Badley et al. [43], and Reese and Robbins [44] concluded that surfactants did not prevent heat or chemical inactivation of enzymes, and the interaction between surfactants and enzymes might lead to an increase in thermal deactivation. Employing fluorescence studies and measuring enzyme activity, Eriksson et al. [7] indicated that the difference of the denaturation temperature of enzymes was very small between with and without the addition of Tween 20, and enzymes were slightly stabilized by the addition of Tween 20 in thermal deactivation experiments. Therefore, Eriksson et al. [7] concluded that the positive effect on hydrolysis of Tween 20 could not be explained by a stabilizing effect of Tween 20 on the enzymes. In our research, results were consistent with the finding of Eriksson et al. [7, 11]. The increase of cellulose conversion became apparent immediately after additives were added (Figs. 1 and 6). Also, in contrast with models of cellulase inactivation without Tween 20, almost all cellulase (90%) and β-glucosidase (98%) were still active in solution after 72 h of hydrolysis with and/or without Tween 20 addition during the hydrolysis of Avicel (Fig. 7). Based on our results, additives had a negligible effect on the stabilization of enzymes.

Ballesteros et al. [21], Helle et al. [22], Kaar and Holtzapple [23], and Kurakake et al. [45] proposed that the surfactants could disrupt the lignocellulosic matrix and make more substrate available to the enzymes. If their theory could explain the effect of surfactants on enzymatic hydrolysis, an increase in enzyme adsorption at the beginning of enzyme addition should be expected because of more available active sites on substrate exposed to enzymes. However, this phenomenon was not detected in this research. Therefore, our findings on the changes of enzyme protein concentration and activities during hydrolysis of pretreated CWR with the presence of Tween 20 and/or BSA do not support their explanation. In contrast, increased enzyme protein concentration and activities in solution were observed with the increase of cellulose conversion in all pretreated CWR hydrolysis experiments (Figs. 1, 2, 3, 4, 5, 10, and 11). Moreover, the observed effect of BSA on the enzymatic hydrolysis of pretreated CWR was similar to surfactants, so that it is not easy to accommodate this result in a model requiring surfactant and, therefore BSA, to act by substrate disruption based on our and other's reported studies [7, 17].

Cellulose conversion of Avicel was only slightly increased by Tween 20 in contrast to that of pretreated CWR. Therefore, it may be likely that lignin content in the pretreated CWR plays a critical role in explaining the positive effect of Tween 20 on enzymatic hydrolysis because pretreated CWR has lignin content, but Avicel does not. Several findings in our study support this conclusion by studying enzyme protein and activities loss by the adsorption of enzyme on Avicel, lignaceous residue, and pretreated CWR: (1) The behavior of enzyme protein and activities loss by enzyme adsorption on pretreated CWR was similar to that on lignaceous residue with or without the addition of Tween 20; (2) BSA had the same effect as Tween 20 on the increased cellulose conversion of pretreated CWR. No additional effect was observed when BSA was added after the substrate was saturated by Tween 20. This result supports the conclusion that the effect of both Tween 20 and BSA on the enzymatic hydrolysis of pretreated CWR may be explained by the same mechanism; (3) pretreated CWR and lignaceous residue adsorbed a substantial amount of BSA, whereas Avicel adsorbed a small amount of BSA. Yang and Wyman [17] also found that BSA had little effect on the cellulose conversion of Avicel hydrolysis because Avicel adsorbed a substantial amount of cellulase but little BSA. It was proposed that the hydrophobic part of non-ionic surfactants binds to lignin on the pretreated CWR fibers through hydrophobic

interactions and/or hydrogen bonding, and the hydrophilic head group of the surfactant prevents the non-productive binding of the enzymes to lignin [8]. The modules of *Trichoderma reesei* cellulase have hydrophobic amino acids exposed on the surface [46], and these residues have been reported to be positioned for both optimal specific interaction with the cellulose [47] and non-specific interaction with lignin surfaces [16]. In addition, Berlin et al. [16] found *T. reesei* cellulase cores binding to lignin outside of the specific sites for cellulose binding. Therefore, the presence of hydrophobic residues on enzyme surfaces may result in non-specific adsorption to lignin surface. With the addition of surfactants, the hydrophobic interaction between lignin and enzymes could be disrupted by charges from surfactants [7]. Eriksson et al. [7] stated that this non-specific adsorption could have a stronger role with pretreated biomass compared to raw biomass because of the increased exposure of lignin surfaces in the pretreatment process. BSA was found to have hydrophobic sites, which could lead to the binding of fatty acids and adsorption on hydrophobic surfaces, such as lignin [28]. Therefore, the BSA effect on the hydrolysis could be explained by the similar mechanism of non-ionic surfactants, which is non-specific binding of BSA to lignin and the resulted decrease of non-productive adsorption of enzymes to lignin surfaces [17, 48].

As shown above, the improvement upon addition of surfactant of hydrolysis of Avicel was much lower than that of pretreated CWR. This result agreed well with findings by Eriksson et al. [7] who reported that surfactants and BSA were both viewed as preventing non-productive adsorption of cellulase on lignin. However, the contrary findings reported that the non-ionic surfactants could enhance the hydrolysis of cellulose such as Sigmacell 100 and Avicel and act differently from proteins [22, 25, 38]. Therefore, more research needs to be done to solve this discrepancy and better understand the mechanisms of additive effect.

Conclusions

The Tween-type non-ionic surfactants (Tween 20 and 80) and non-catalytic protein (BSA) were effective additives for enhancing the cellulose conversion of pretreated CWR. Tween 20 was the most effective among the three additives. When 0.1 g Tween 20/g dry solid was used, 14% more cellulose conversion was obtained comparing with without Tween 20 addition. With the addition of Tween 20, the cellulose conversion of pretreated CWR with the enzyme loading of 15 FPU/g cellulose was similar to that with the enzyme loading of 30 FPU/g cellulose. It is most likely possible to lower the enzyme loading by adding surfactants, e.g., Tween 20, while retaining the same degree of cellulose conversion. However, it has not been possible to perform economic evaluations on the feasibility of additive addition because of the lack of industrial scale prices of additives and enzymes. BSA and Tween 20 may have the same mechanism for improving the enzymatic hydrolysis of pretreated CWR. Enzyme protein concentration and activities were measured and could be correlated to cellulose conversion of Avicel and pretreated CWR with and without the presence of additives. When lignaceous residue of pretreated CWR was the absorbent, adsorbed protein decreased by 50% with the presence of Tween 20. At the same time, cellulase and β-glucosidase activities in solution were increased by 40 and 60%, respectively. The effect of Tween 20 on the adsorption of enzymes to lignaceous residue was similar to that on pretreated CWR. The results may be because of the prevention of non-productive adsorption of enzymes on lignin by Tween 20. However, more research is needed to fully understand the mechanisms of the effect of additive on cellulose conversion of lignocellulosic biomass.

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