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Study on the Decreased Sugar Yield in Enzymatic Hydrolysis of Cellulosic Substrate at High Solid Loading

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Abstract Current technology for conversion of biomass to ethanol is an enzyme-based biochemical process. In bioethanol production, achieving high sugar yield at high solid loading in enzymatic hydrolysis step is important from both technical and economic viewpoints. Enzymatic hydrolysis of cellulosic substrates is affected by many parameters, including an unexplained behavior that the glucan digestibility of substrates by cellulase decreased under high solid loadings. A comprehensive study was conducted to investigate this phenomenon by using Spezyme CP and Avicel as model cellulase and cellulose substrate, respectively. The hydrolytic properties of the cellulase under different substrate concentrations at a fixed enzyme-to-substrate ratio were characterized. The results indicate that decreased sugar yield is neither due to the loss of enzyme activity at a high substrate concentration nor due to the higher end-product inhibition. The cellulase adsorption kinetics and isotherm studies indicated that a decline in the binding capacity of cellulase may explain the long-observed but little-understood phenomenon of a lower substrate digestibility with increased substrate concentration. The mechanism how the enzyme adsorption properties changed at high substrate concentration was also discussed in the context of exploring the improvement of the cellulase-binding capacity at high substrate loading.

Keywords Biomass · Enzymatic hydrolysis · High substrate concentration · Cellulase adsorption · Binding capacity

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Introduction

Recently, the alternative fuels that are considered friendlier to the environment than fossil fuels are attracting remarkable attention because of both the environmental considerations and the inadequate oil availability. Ethanol, due to its advantageous properties, might become a promising alternative energy source for petroleum. At present, the ethanol produced from corn starch apparently cannot meet the fuel demand throughout the country. Only lignocellulosic biomass, such as energy crops and agricultural and forestry residues, offers the abundance and environmental attributes that can potentially support a large-scale biofuel production [1]. Current technology for biomass conversion to ethanol is mainly an enzyme-based biochemical process. The enzymatic saccharification of cellulose is catalyzed by a complex cellulase enzyme system which typically comprises three classes of enzymes: exoglucanases, endoglucanases, and β-glucosidases. The cellulase enzyme complexes from *Trichoderma viride* and *Trichoderma reesei* are two systems which have great commercial potential for application in enzymatic degradation of biomass, provided that a high sugar yield at high solid loading operation in enzymatic hydrolysis (a key step from technical and economic perspectives) can be achieved. However, at high substrate concentration, decreased sugar yield and hydrolysis rate have been observed by some researchers. Van Dyke [2] and Howell and Struck [3] first reported decrease of saccharification rate at high substrate concentration with *T. viride*-derived cellulase system. Later, Liaw and Penner [4] supported these findings in a *T. viride* cellulase system. Similarly, the enzyme complex from *T. reesei* has also been observed to exhibit decreased hydrolysis rate at lower ratios of enzyme to substrate [5–7]. All these results were observed with given enzyme concentration, where the hydrolysis rate decreased when substrate concentration was increased to a certain point.

To fully investigate the relationship between the enzymatic digestibility and loading amount of cellulosic substrates, we conducted a study in which enzyme concentration was increased proportionally with the substrate concentration. Unexpectedly, we found that the sugar yield decreased with the increased substrate concentration despite a proportional increase in enzyme concentration. Such observation raises a question: What is the underlying mechanism? Is it due to the decrease in enzyme activity or to a higher end-product inhibition under high substrate concentration? Until now, the mechanism underlying the observed behavior that sugar yield decreased at high solid loading in enzymatic hydrolysis of cellulose is still unknown. Further studies are warranted to determine the actual mechanism underlying this behavior.

In the present study, the enzymatic hydrolysis using a fixed substrate–enzyme ratio at low and high substrate concentrations was investigated. In addition, enzymatic properties of cellulase were characterized, and the adsorption kinetics and isotherm were analyzed by using Langmuir adsorption model. The possible mechanism accounting for the decrease of sugar yield under high substrate concentration and the possible approaches to improve the enzymatic hydrolysis of substrates in experimental and industrial practices were discussed in this study.

Materials and Methods

Substrates and Enzymes

Microcrystalline cellulose, Avicel pH-101 (Cat. No.11365), was purchased from Fluka Chemical Co. Cellulase enzyme Spezyme CP (lot no. 301-04075-054) was obtained from Genencor, a Danisco division (Paulo Alto, CA). Protease (P8811-1 G) and the bovine

serum albumin (BSA) protein assay reagent were procured from Sigma Chemical Co. and Bio-Rad Laboratories, Inc., respectively.

Enzymatic Hydrolysis of Microcrystalline Cellulose for Substrate Digestibility Analysis

The enzymatic digestibility tests were carried out in a 125-ml Erlenmeyer flask with total liquid volume of 50 ml. Avicel cellulose was used as substrate. The conditions of enzymatic digestibility tests were 50 mM sodium citrate buffer with pH 4.8, 50 °C, 130 rpm. Substrate concentrations ranged from 1% to 5% (*w*/*v*, based on cellulose) while the cellulase enzyme loading was kept at 15 FPU/g glucan. The reaction was initiated by the addition of enzyme solution. Samples were taken periodically during 72 h and analyzed for glucose and cellobiose by HPLC using Bio-Rad Aminex HPX-87P column. The glucan digestibility was defined as the percentage of theoretical glucose released after certain period of incubation with enzyme.

Filter Paper Assay Measurement of Cellulase Activity

The filter paper cellulase activity was determined according to the method of International Union of Pure and Applied Chemistry (IUPAC). The 3,5 dinitrosalicylic acid method [8] was used to estimate the reducing sugar released in 60 min from a mixture of 0.5 mL appropriately diluted enzyme solution, 1 mL 0.1 M acetate buffer (pH 4.8), and 50 mg Whatman no.1 filter paper, incubated at 50 °C. One filter paper unit (FPU) was defined as the amount of enzyme that releases 1 μmol glucose per minute based on 2 mg glucose released in 60 min, according to National Renewable Energy Lab procedure [9]. Activities were reported as filter paper unit per milliliter.

Kinetics and Isotherm of Cellulase Adsorption to Cellulose

All adsorption experiments were performed in 5-ml vessels with screw caps, in 50 mM sodium acetate buffer, pH 4.8. To avoid the hydrolysis of substrate by the enzyme, the experiments were performed at 4 °C at a slow agitation speed of 40 rpm. To measure the enzyme adsorption kinetics at different substrate concentrations, 15 FPU/g glucan Spezyme CP (equal to 20 mg protein/g glucan) was incubated with Avicel cellulose (1% or 5%). Aliquots (0.2 mL) were taken at 0, 15, 30, 45, 60, 75, 90, 105, 120, 180, and 240 min during the incubation. The supernatant from each sample was collected after centrifugation. The free enzyme content in the supernatant was determined using the Bio-Rad protein assay reagent with BSA as a standard. The amount of bound enzyme was calculated by subtracting the free enzyme from the initial enzyme concentration.

To determine the adsorption isotherm at different substrate concentrations, a range of concentrations of cellulase (0.04–0.5 mg/mL) with 1% (wt) Avicel cellulose and a range of concentrations of cellulase (0.2–2.5 mg/mL) with 5% (wt) Avicel cellulose, respectively, were incubated in 50 mM acetate buffer 4 °C for 90 min to reach an equilibrium. Subsequently, free enzyme content in the supernatant was assayed, and the bound enzyme was determined. Langmuir isotherm is used to describe adsorption of cellulases to cellulose [10–12].

Cellulase Effectiveness

Cellulase effectiveness was defined as the ratio of sugar yield (percent) at 72 h to the maximum cellulase adsorption capacity of Avicel cellulose. The maximum binding capacity

of cellulose for cellulase was estimated as described above. Sugar yield data for different concentration of Avicel cellulose were collected at the same enzyme loading of 15 FPU/g glucan. All hydrolysis experiments were performed as previously described.

Statistical Analysis

Unless otherwise indicated, all assays were performed in triplicate, and the statistical analyses for the described differences between treatments were conducted with a $p<0.05$ or $p<0.01$.

Results and Discussion

Effect of Substrate Concentration on the Enzymatic Hydrolysis of Microcrystalline Cellulose

T. reesei-derived, Spezyme CP, a broadly used commercial cellulase (Genencor International, Rochester, NY, USA), was used in the enzymatic hydrolysis tests of this study. The sugar yield from different concentrations of Avicel cellulose is shown in Fig. 1. Although the enzyme-to-substrate ratio was kept the same (15 FPU/g glucan), there was a marked decrease in the sugar release at higher substrate concentration. The kinetics of the sugar yield during 72 h indicated that this lower sugar yield was maintained throughout the hydrolysis process. At 5% (*w*/*v*) cellulose, 33% decrease in the sugar release was observed compared with that at 1% cellulose concentration.

The 3-h profile of the substrate–velocity curves for the different concentrations of Avicel cellulose are shown in Fig. 2. Increasing substrate concentration corresponded to increasing rates of saccharification. The effect of high substrate concentration on the initial reaction rate was not observed even at low enzyme loading, 0.1 FPU/g glucan. This is due to maintaining a constant enzyme-to-substrate ratio in these experiments where, as the substrate concentration increased, so did the enzyme concentration. The term "substrate inhibition", as used by previous researches [3–5, 7], refers to the decrease of initial reaction rate when substrate concentration increases to a certain value at fixed enzyme dosage. Our results show that the rate of saccharification did not decrease with the increase of substrate concentration when

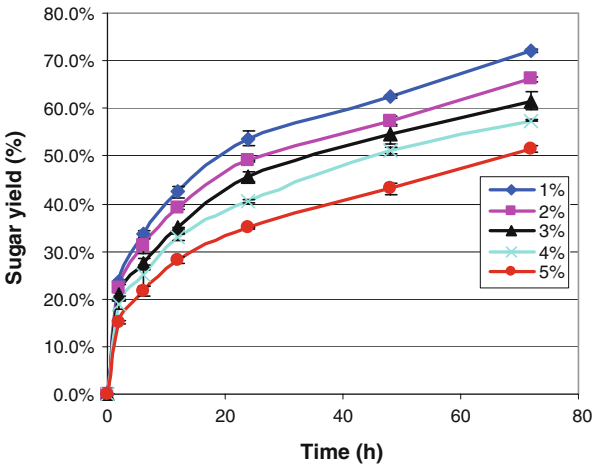

Fig. 1 Effect of substrate concentration on the sugar yield of enzymatic hydrolysis of Avicel cellulose at a fixed enzyme-to-substrate ratio. Various amounts of substrates (Avicel cellulose 1%~5%, *w*/*v*) were mixed with cellulase enzyme Spezyme CP (15 FPU, equivalent to 20 mg protein, per gram glucan) and hydrolyzed for 72 h under 50 C, pH 4.8

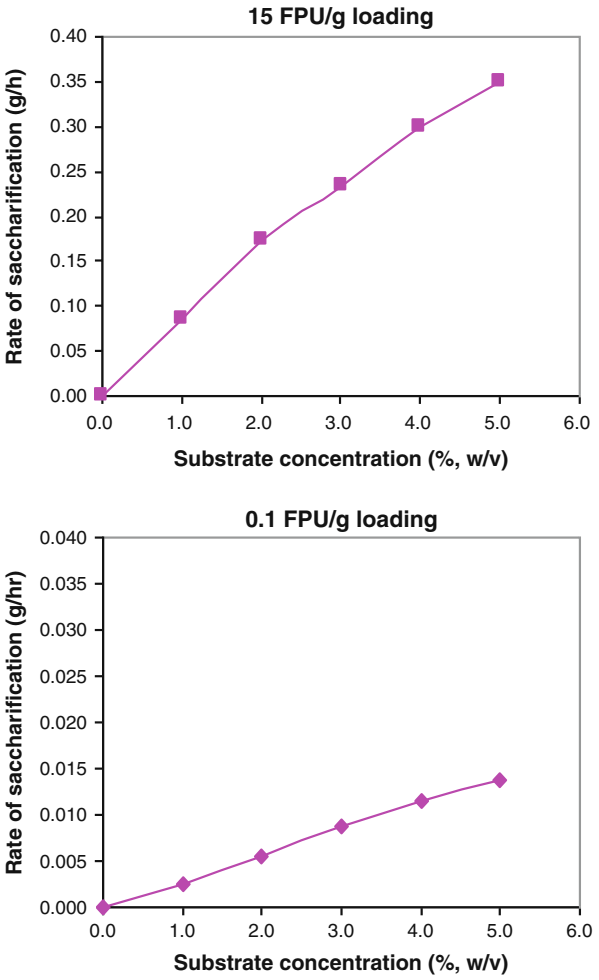

Fig. 2 Substrate–hydrolysis rate profile of Spezyme CP cellulase at different enzyme-to-substrate ratios. Conditions: incubation time 3 h; 50 C; pH 4.8

enzyme-to-substrate ratio was kept constant. However, intriguingly, as shown in Fig. 1, the sugar yield still decreased during the hydrolysis process. Could it be due to the change in enzyme activity and/or higher end-product inhibition at excess substrate?

Enzyme Activity Under Different Substrate and End-Product Concentrations

Spezyme CP cellulase (15 FPU/g glucan, equal to 20 mg protein/g glucan) was incubated with 1% and 5% Avicel cellulose, respectively, in 50 mM sodium acetate buffer (pH 4.8). At 0, 2, 6, 12, 24, and 48 h during the incubation; supersonic wave was employed to disturb the enzyme adsorption, and aliquots (0.2 mL) were taken for centrifugation. The supernatants were incubated with fresh substrate-filter paper for the determination of cellulase activity. Our data indicated no change in cellulase activity during the hydrolysis process; moreover, the activity did not decrease when substrate concentration increased to 5%, and the activity in the supernatant from 5% cellulose solution was still about five times higher than that from 1% cellulose solution. These results suggest that the decreased sugar

yield at higher substrate concentration was perhaps not due to enzyme deactivation and that there must be some other factors associated with this response.

End-product inhibition is considered to play an important role in impeding the enzymatic hydrolysis of cellulose [13–17] As a final product, glucose has a direct inhibitory effect on β-glucosidase activity. The degree of glucose inhibition on overall enzymatic hydrolysis at different substrate concentrations was investigated. The degree of inhibition in this study was determined based on the ratio of the sugar yield at 24 h with and without the presence of supplemented sugars. Three glucose levels 10, 30, and 50 g/L were chosen to supplement the three different substrate levels, 1%, 3%, and 5%, respectively. As shown in Fig. 3, the degrees of inhibition were 61.6%, 60.7%, and 59.5% corresponding to 10, 30, and 50 g/L of glucose supplementation to 1%, 3%, and 5% cellulose, respectively. These results are consistent with the previous findings by Xiao Z. Z. et al. [18]. Since the enzyme-to-substrate ratio is constant, higher substrate concentration means higher enzyme input. The comparable results we got from the experiment with three different levels of substrate and end-products indicated higher end-product inhibition cannot account for the decreased sugar yield at higher substrate concentration.

Effect of Substrate Concentrations on Cellulase Adsorption Kinetics

Cellulase (Spezyme CP) adsorption kinetics for 1% and 5% Avicel cellulose, respectively, was determined. The enzyme-to-substrate ratio was 15 FPU/g glucan (equal to 20 mg protein/g glucan), i.e., a fivefold enzyme was loaded for the 5% cellulose compared with that for the 1% cellulose. The time course of adsorption showed that the cellulase adsorption on Avicel cellulose started quickly and reached equilibrium within 1 h (Fig. 4) during which almost no cellulose was hydrolyzed at 4 °C. The adsorption profiles at two different substrate concentrations were similar because the same enzyme Spezyme CP was used although the concentration was different. However, the cellulase at lower substrate concentration was adsorbed to a higher extent than that at high substrate concentration. This indicated that the lower sugar yield in enzymatic hydrolysis under high substrate concentration may be related to the cellulase adsorption behavior.

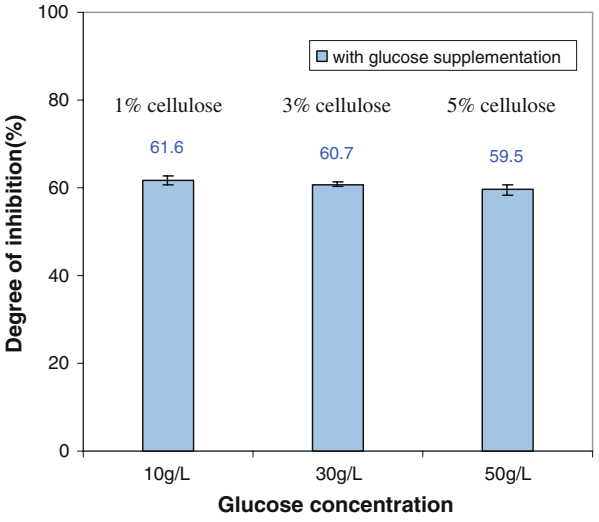

Fig. 3 Glucose inhibition on enzymatic hydrolysis of Avicel cellulose at different concentrations: 1%, 3%, and 5% (*w*/*v*). Conditions: cellulase dosage 15 FPU/g glucan; 50 C; pH 4.8

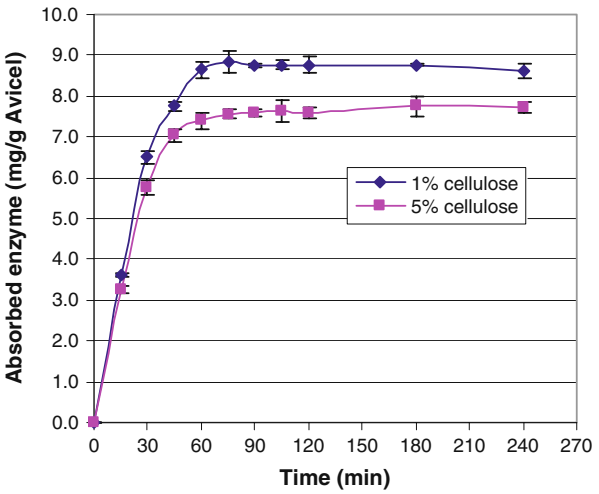

Fig. 4 Kinetics of cellulase adsorption on Avicel cellulose with a cellulase dosage of 15 FPU/g glucan

Further investigation of cellulase adsorption behavior was conducted as follows. After the above described adsorption had proceeded for 60 min, the two reaction vessels were removed, and samples were sonicated for 5 min with a supersonicator. Samples were then centrifuged, and the precipitate was suspended into 1.0 ml citrate buffer (pH 7.0) containing protease. Proteolysis was carried out overnight at 37 °C. Samples were then centrifuged, and the pellet containing 5% cellulose was diluted fivefold (to 1%) with 50 mM acetate buffer (pH 4.8). One milliliter of this cellulose suspension was taken out for the comparison of cellulase adsorption (20 mg) with fresh 1% cellulose (1 mL). After 90 min, almost the same amount of enzyme, i.e., 8.6 mg protein/g cellulose, was adsorbed to these two cellulosic substrates, indicating that the adsorption property may change with increasing substrate concentration. This result somewhat agrees with the observation of Kristensen [19], but limited adsorption studies was conducted in his research to provide further evidence.

Effect of Substrate Concentrations on Cellulase Adsorption Isotherms

Isotherm studies were performed at 4 °C in order to avoid hydrolysis of cellulose which may affect the adsorption. To determine the adsorption isotherm at different substrate concentrations, a range of concentrations of cellulase (0.04~0.5 mg/mL) with 1% (*w*/*v*) Avicel cellulose and a range of concentrations of cellulase (0.2~2.5 mg/mL) with 5% (*w*/*v*) Avicel cellulose, respectively, were incubated in 50 mM acetate buffer at 4 °C for 90 min. The amount of adsorbed enzyme was plotted against the free enzyme concentration. As shown in Fig. 5, Spezyme CP adsorbed to a higher extent on 1% cellulose than that on 5% cellulose.

Analysis of the adsorption isotherm of cellulase was performed using Langmuir model, which has previously been used to describe adsorption of cellulase to cellulose [10–12]:

$$B = \frac{n[F]}{Kd + [F]} \tag{1}$$

Where B (in milligrams per gram substrate) is the amount of bound enzyme per unit weight of substrate, and $[F]$ (milligrams per milliliter) is the free enzyme concentration in the solution. This model employs two biophysically meaningful parameters to characterize the cellulase–cellulose-binding: the binding capacity represented by the maximal number of

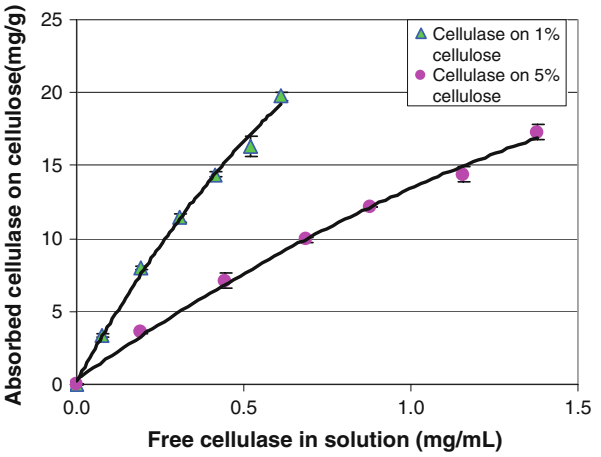

Fig. 5 Cellulase isotherms on different concentration of Avicel cellulose with a cellulase dosage of 15 FPU/g cellulose

available binding sites on substrate, n, and the dissociation constant for the enzyme–substrate complex, Kd. In this study, the parameters were obtained by non-linear regression analysis and are presented in Table 1.

The binding capacities (n) derived from Langmuir model (Table 1) reflect well the qualitative conclusion derived from Fig. 5. A comparison of the binding parameters demonstrates that the binding capacity of cellulase for 1% cellulose was 30% higher than for 5% cellulose, although the enzyme-to-substrate ratio was the same in the two experiments. In other words, the binding capacity decreased when the substrate concentration increased.

The distribution coefficient (α, milliliters per gram), expressed as below in Eq. 2, is the slope of the adsorption isotherm at the right beginning when no binding happened yet. It is another constant from Langmuir adsorption isotherm that can be used to estimate the relative affinity of enzyme to substrate [10–12]. Our results show that, at low cellulose concentrations, cellulase had a higher relative affinity for cellulose, resulting in greater cellulose conversion.

$$\alpha = \frac{n}{Kd} \tag{2}$$

The above are the adsorption studies using complete cellulase complex. To further investigate the adsorption behavior of cellulase at high solid loading, purified individual cellulase components of exoglucanase and endoglucanase are very necessary for the study of synergistic action of cellulase components in adsorption under different solid loadings. It is likely that the synergism of cellulase components in adsorption decreased at high solid loading due to jamming of molecules and lower processicity of enzymes. This work is in process.

Table 1 Langmuir parameters for the adsorption of Spezyme CP on Avicel cellulose

Cellulose concentration (%, wt)	Enzyme concentration (mg/mL)	n (mg/g cellulose)	Kd (mg/mL)	α (mL/g)
1%	0.04~0.50	49.10±1.9	1.03±0.03	48.10±1.7
5%	0.20~2.50	35.60±1.0	1.73±0.07	20.61±0.8

Effect of Substrate Concentrations on Cellulase Effectiveness

In addition to the observed differences in kinetics and isotherm of cellulase adsorption as well as cellulase affinity to cellulose at different substrate concentrations, we were also curious if the effectiveness of cellulase itself changed accordingly. Surprisingly, the measured cellulase effectiveness at low and high cellulose concentrations were nearly the same (Table 2), indicating that the catalytic efficiency of cellulase in hydrolyzing cellulose was the same after the enzyme was adsorbed to cellulose, despite the differences in the levels of cellulose loading (note that the cellulase effectiveness was defined as the ratio of sugar yield (percent) at 72 h to the maximum cellulase adsorption capacity of Avicel cellulose). Such data suggest that the binding capacity of cellulase to cellulose may be directly related to glucose release, and this is consistent with the fact that cellulosic substrate is insoluble and cellulase needs to adhere to cellulose to function. These results agree well with some previous observations, showing that binding of cellulase is related to the hydrolysis of cellulose [12, 20].

From the above results, we conclude that the decreased sugar yield at high substrate concentration is not due to the loss of activity of enzyme at high substrate concentration, nor it is due to the end-product inhibition. More likely, it is related to the lower binding capacities, i.e., lower affinity of the cellulase for cellulose.

Diffusional Limitation on the Enzymatic Hydrolysis of Cellulose and the Means to Overcome It

In addition to the enzyme–substrate binding and interactions in high solids enzymatic hydrolysis, the diffusion of enzyme is another factor that affects enzymatic hydrolysis of cellulose, especially at high loading of cellulose where there is a slower mass transfer of enzymes as well as intermediates and end-products. Lee and Fan [5] once proposed that diffusional limitation increased in the movable aqueous phase of the reaction mixture when the substrate concentration increased. Cellulase has been proposed to be capable of lateral diffusion on the cellulose surface [21]. The average time for the cellulase to migrate to a free chain end is dependent on the distance between the chain end of cellulose and the bound cellulase. In this case, low enzyme-to-substrate ratio leads to a lower enzyme surface coverage, i.e., the distance between the available chain end of cellulose and the enzyme increases. This may, conceivably, affect the synergism of enzyme components.

But, this explanation does not seem to hold in this investigation. In our study, no matter what the substrate concentration is used, enzyme-to-substrate ratio is fixed (15 FPU/g glucan), and therefore, the same surface coverage of enzyme on cellulose is expected to occur. However, in reality, the lower surface coverage may actually occur, causing less enzyme binding to cellulose, leading to a decline in cellulose conversion at higher cellulose loading as observed in our study. Likely, surface coverage limitation may be caused by the three-dimensional diffusion in solution rather than lateral diffusion of cellulase along the cellulose surface. When substrate and enzyme concentrations both increase, the viscosity of the reaction mixture increases

Table 2 Effectiveness of cellulase on cellulose of different concentrations

Cellulose concentration (%, wt)	Enzyme concentration (mg/mL)	72 h sugar yield (%)	*n* (mg/g cellulose)	Effectiveness of cellulase
1%	0.20	73.10±1.10	49.10±1.9	1.48±0.05
5%	1.00	51.59±0.82	35.60±1.0	1.45±0.06

accordingly, which may lead to a higher resistance to three-dimensional diffusion in solution rather than to a lateral diffusion. In addition, this apparent "congestion" may also hinder the movement, and thus, a proper positioning of enzyme molecules on the cellulose chain.

To test our proposal of three-dimensional diffusion limitation of cellulase at high cellulose loading, we took the advantage of the fact that three-dimensional diffusion can be enhanced by agitating the solution. Our results (Fig. 6) show that the conversion of high solid loading of cellulose at higher mixing speed is better than at low mixing speed, and the performance of enzyme at higher mixing speed under high solid loading is close to that of enzyme at low concentration of cellulose at low mixing speed.

Conclusion

Our results indicated that the decrease of cellulose conversion at high substrate concentration is neither due to the loss of activity of enzyme nor the end-product inhibition. Instead, it may be related to the change of adsorption capacity at high solid loading. The lower binding capacity of cellulase is possible to lead to a lower surface coverage of enzyme on cellulose, thus may influence the hydrolysis of cellulose. We do not deny other factors such as the slower three-dimensional diffusion of enzymes in solution, and some substances in enzyme solution maybe also related to the decreased sugar yield. Cellulase adsorption to cellulose has been thought to be much related to the hydrolysis of cellulose, and our results highlighted that more studies for a better understanding of the mechanism of the adsorption of enzyme on cellulose is necessary. In addition, the role of cellulose-binding domain and synergism of various enzyme components under high substrate concentration also need further investigation. Such research will not only help explain the changes in adsorption performance at high substrate concentration, but more importantly, it will contribute to increasing the sugar yield at high solid loading in commercial biofuel production.

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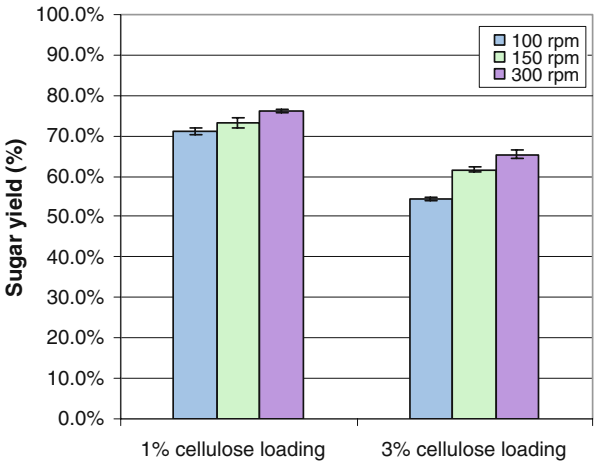

Fig. 6 Effect of different mixing speeds on cellulose conversion. Conditions: cellulase dosage 15 FPU/g cellulose; 50 C; pH 4.8

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