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Enhanced Enzymatic Hydrolysis of Lignocellulose by Optimizing Enzyme Complexes

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Abstract To enhance the conversion of the cellulose and hemicellulose, the corncob pretreated by aqueous ammonia soaking was hydrolyzed by enzyme complexes. The saturation limit for cellulase (Spezyme CP) was determined as 15 mg protein/g glucan (50 filter paper unit (FPU)/g glucan). The accessory enzymes (β-glucosidase, xylanase, and pectinase) were supplemented to hydrolyze cellobiose (cellulase-inhibiting product), hemicellulose, and pectin (the component covering the fiber surfaces), respectively. It was found that β-glucosidase (Novozyme 188) loading of 1.45 mg protein/g glucan [30 cellobiase units (CBU)/g glucan] was enough to eliminate the cellobiose inhibitor, and 2.9 mg protein/g glucan (60 CBU/g glucan) was the saturation limit. The supplementation of xylanase and pectinase can increase the conversion of cellulose and hemicellulose significantly. The yields of glucose and xylose enhanced with the increasing enzyme loading, but the increasing trend became low at high loading. Compared with xylanase, pectinase was more effective to promote the hydrolysis of cellulose and hemicellulose. The supplementation of pectinase with 0.12 mg protein/g glucan could increase the yields of glucose and xylose by 7.5% and 29.3%, respectively.

Keywords Lignocellulose · Cellulase · β-Glucosidase · Xylanase · Pectinase

Introduction

Lignocellulosic biomass, for its large quantities and relatively low cost, is regarded as the potential renewable energy resource for cost-effective ethanol [1]. During lignocellulose biorefineries, the most prevalent routine generally involves three main steps, including pretreatment, enzymatic hydrolysis, and fermentation. Among these steps, the efficiency of lignocellulose hydrolysis is the major factor that restricts the commercialization of related products such as fuel ethanol. During enzymatic hydrolysis of lignocelllulose, cellulose and

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hemicellulose are gradually degraded into fermentable sugars, such as glucose and xylose, under the actions from a series of enzymes with different functions.

Most commercial cellulases produced by *Trichoderma reesei*, one of the most well-known cellulase-producing fungi, is comprised of endoglucanase (EG, EC 3.2.1.4), exoglucanase, or cellobiohydrolase (CBH, EC 3.2.1.91), and β-glucosidase (BG, EC 3.2.1.21) [2–4]. However, *T. reesei* secretes low levels of BG activity, leading to the incomplete conversion of cellobiose to glucose and the inhibition to CBH. Supplement of BG activity or using novel fungi with higher BG activity can provide practically complete conversion of intermediate cellobiose to glucose [5, 6].

Hemicellulose and possibly pectin are thought to restrict the access of cellulases to their substrate in pretreated lignocellulose. Addition of xylanases and pectinases can degrade these non-cellulosic saccharides and thus increase cellulose conversion. By means of enzyme complex optimization, cellulose digestibility had reached a near-theoretic value for the acid-catalyzed steam explosion corn stover, the main composition of which was cellulose, lignin, and fewer hemicellulose [7, 8].

To date, soaking in aqueous ammonia (SAA) has been proven to be one of most feasible methods for lignocellulose pretreatment, since it can remove lignin effectively and increase cellulose digestibility at modest reaction conditions without high pressures and temperature [9, 10]. This method is somewhat less effective for pulp mill sludge from softwood, but it realized highly degrees of delignification for agricultural residues, including corncob and corn stover. SAA results in a solid fraction, consisting mainly of hemicellulose and cellulose, and an alkaline-soluble lignin. Almost all cellulose and about 85% hemicellulose can be preserved in the solid residues [11]. In order to achieve maximum substrate utilization, it is important to realize the simultaneous bioconversion of major polysaccharides including hemicellulose and cellulose. The enzymatic hydrolysis of the polysaccharides to soluble sugars (finally to fermentable sugars such as glucose and xylose) occurs under the action of different enzymes acting in concert.

In the work, the effects of multi-enzyme mixtures consisting of cellulase, β-glucosidase, xylanase, and pectinase on the enzymatic digestibility of lignocelluloses treated by aqueous ammonia were investigated. The objective of this study was to (1) optimize the multi-enzyme mixtures for the hydrolysis of SAA-treated biomass, (2) evaluate the xylose yield during the lignocellulose hydrolysis attacked by different enzyme mixtures as well as the glucose yield, and (3) determine the saturation limits for cellulase and β-glucosidase.

Materials and Methods

Materials

Corncob was obtained from a local farm (Tianjin, China). It was premilled and screened, and the fraction collected between 20 and 80 mesh was used for experiments. All other chemicals used were of analytical grade.

Cellulase (Spezyme CP) derived from *T. reesei*, xylanase (Multifect Xylanase) derived from a genetically modified strain of *T. reesei*, and pectinase (Multifect Pectinase) derived from a selected strain of *Aspergillus niger* were the kind gifts from Genencor International (Palo Alto, CA, USA). β-Glucosidase (Novozyme 188) was purchased from Sigma (St. Louis, MO, USA). The filter paper and β-glucosidase activities were determined according to the methods suggested by IUPAC [12], whereas the xylanase and pectinase activities were from [6]. Protein concentrations were measured using the Bradford assay

method using bovine serum albumin as a standard [9]. The protein concentrations and specific activities of the four enzymes were presented in Table 1.

Lignocellulose Pretreatment by SAA

Oven-dried corncob was placed in a screw-capped laboratory bottle (Pyrex bottles) and then mixed with 15 wt.% aqueous ammonia at a ratio of liquor to solid 6:1. The solid/liquid slurry was incubated in a water bath at 60 °C for 12 h with no agitation. After SAA, the solids were separated by filtering and washed with distilled water to neutral for later compositional analyses and enzymatic hydrolysis. The carbohydrate and lignin contents were determined following NREL chemical analysis and testing standard procedure.

Optimization of Multi-enzyme Mixtures for Lignocellulose Hydrolysis

Enzymatic hydrolysis of SAA-treated corncobs was performed in 25 mL 50 mM acetate buffer (pH4.8) at a glucan concentration of 2 wt%. Substrates with buffer were preincubated in the oscillation water bath at 50 °C, with shaking at 120 rpm. Subsequently, the prepared cellulase (Spezyme CP) was added into the hydrolysis system, either alone or supplemented with different combinations of three enzymes including Novozyme 188, Multifect Xylanase, and Multifect Pectinase. After 24 h digestion, the hydrolysates were immediately boiled for 10 min to denature the enzymes and then filtered. The supernatants were retained for sugar analysis by high performance liquid chromatography using an Aminex HPX-87H column (Bio-Rad, USA) operated at 65 °C with 5 mM H_2SO_4 as the mobile phase. The glucose and xylose yields were calculated by the following formulas.

$$\text{Glucose yield}(\%) = \frac{\text{Glucose released}(g) \times 0.9}{\text{Initial glucan in reactor}} \times 100 \quad (1)$$

$$\text{Xylose yield}(\%) = \frac{\text{Xylose released}(g) \times 0.88}{\text{Initial xylan in reactor}} \times 100 \quad (2)$$

Results and Discussion

Main Composition of Substrates

The main composition of intact corncob and SAA-pretreated corncob were shown in Table 2. About one third of lignin was removed after 12 h pretreatment, while most of

Table 1 Protein concentration and specific activities (U/mg of protein) of enzymes.

Enzyme	Protein concentration (mg/mL)	FPA (FPU)	β-Glucosidase (CBU)	Xylanase[a]	Pectinase[a]
Spezyme CP	35.6	3.3	0.8	0.1	0.1
Novozyme 188	44.8	0.1	20.7	0.4	0.1
Multifect Xylanase	13.4	0.1	0.1	282.7	0.6
Multifect Pectinase	23.8	0.1	62.2	8.7	25.1

[a] The specific activities were determined from reference [6]

Table 2 Main composition of corncob.

Substrate	Glucose (%)	Xylose(%)	Arabinose(%)	Lignin[a] (%)
Intact Corncob	35.9	29.2	3.5	16.5
SAA-treated Corncob	45.8	37.0	4.6	11.1

[a] Sum of Klason lignin and acid soluble liginin

glucan and xylan were preserved in the solid, indicating that SAA is effective in delignification but has no significant effect in carbohydrate composition [13]. After SAA treatment, the xylan content in the substrates was 37.0 wt.%, which is available for the biorefinery using co-fermentation of hexoses and pentoses to produce bioethanol.

Effect of Cellulase Loading

In order to evaluate the effect of cellulase on lignocellulose, β-glucosidase was overloaded to eliminate the inhibit effect of cellobiose to cellulase in this study. As shown in Fig. 1, enzymatic digestibility was directly correlated with cellulase loading. The conversions of glucan and xylan were promoted by the increase in cellulase loading and reached a high level at cellulase loading of 15 mg protein/g glucan (50 FPU/g glucan), at which the yield of glucose and xylose were 93.3% and 82.1%, respectively. Further increasing the loading of Spezyme CP beyond 15 mg protein/g glucan (50 FPU/g glucan) had no significant effect on the enzymatic digestibility.

In the case of high cellulase loading (15 mg protein/g glucan), enzymatic hydrolysis of cellulose had achieved a high degree of glucose yield, so the supplement of the accessory enzymes could not gain a significant enhancement. In order to study the influence of enzyme complex more markedly, a lower level of cellulase (9.1 mg protein/g glucan), near 30 FPU/g glucan was used for the following examination.

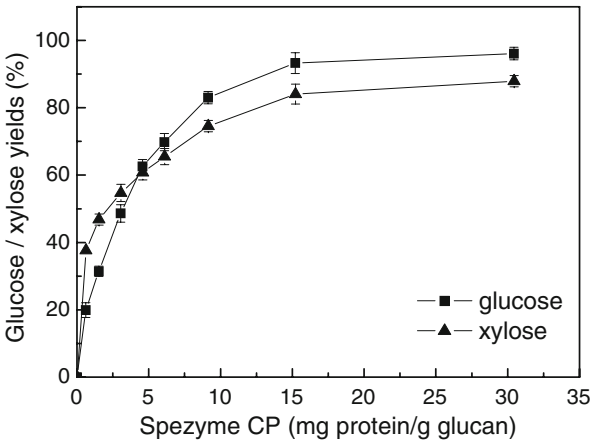

Fig. 1 The yields of glucose and xylose after 24-h hydrolysis of SAA-treated corncobs under different cellulase (Spezyme CP) loadings with the Novozyme 188 loading of 2.9 mg protein/g glucan and no other enzyme supplement

Effect of β-Glucosidase Loading

T. reesei has long been used in industry widely for its highly productive and powerful destroyers of crystalline cellulose [14]. However, the level of BG activity of *T. reesei* is low, leading to incomplete conversion of cellobiose, which inhibits the cellulose conversion [15], to glucose in the cellulose hydrolysis process. Figure 2 showed the effect of supplementation of β-glucosidase with Spezyme CP loading of 9.1 mg protein/g glucan (30 CBU/g glucan). Without β-glucosidase, the concentration of cellobiose in hydrolysate is 0.6 mg/mL, and 24-h yield of glucose was only 57.1%. It may be concluded that a little of cellobiose can significantly inhibit cellulase, especially for CBH. With the increasing β-glucosidase loading, the concentration of cellobiose decreased. The saturation limit for β-glucosidase was 2.9 mg protein/g glucan (60 CBU/g glucan), at which the yield of glucose increased to 80.2% after 24-h hydrolysis. In fact, all cellobiose could be hydrolyzed in time by the addition of β-glucosidase with 1.45 mg protein/g glucan (30 CBU/g glucan), at which the yields of glucose and xylose increased by 19.4% and 6.2%.

Supplemented with Xylanase

As displayed in Fig. 3, the supplementary of xylanase can enhance significantly the performance of cellulase and increase the bioconversions of cellulose and hemicellulose. The initial hydrolysis of cellulose was quickened, due to that the addition of xylanase increased the accessibility of cellulase to cellulose chains by removing the hemicellulose barrier and thus exposing more cellulose chains. When the cellulase complex was supplemented with xylanase (0.67 mg protein/g glucan), the 24-h yields of glucose and xylose increased by 6.5% and 26.5%, respectively (Fig. 3). With xylanase supplementation, the enzymatic digestibility of glucan and xylan has risen by 42.5% and 43.6% for SAA-treated hybrid poplar, while those increased by 4.79% and 10.74% for ammonia recycle percolation-treated corn stover, respectively [16]. Higher conversion was obtained with more xylanase loading, but the increasing trend became lower at high loading (Fig. 4). This phenomenon may be because the competition for productive binding sites between Multifect Xylanase and the *Trichoderma* sp. cellulases [17].

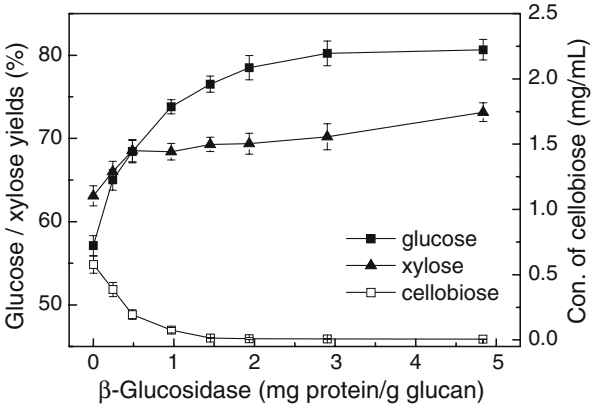

Fig. 2 The cellobiose content and the yields of glucose and xylose after 24 h hydrolysis of SAA-treated corncobs by Spezyme CP (9.1 mg protein/g glucan) supplemented with different loadings of Novozyme 188 and no other enzyme supplement

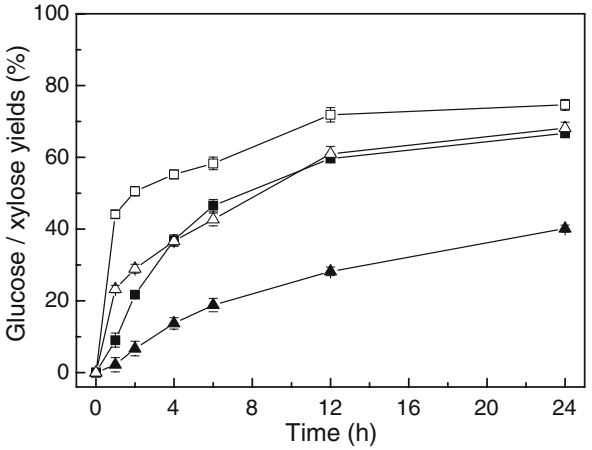

Fig. 3 The yields of glucose (*filled square*) and xylose (*filled triangle*) from enzymatic hydrolysis of SAA-treated corncobs by Spezyme CP (9.1 mg protein/g glucan) and Novozyme 188 (1.45 mg protein/g glucan), while those of glucose (*empty square*) and xylose (*empty triangle*) by such cellulases supplemented with Multifect Xylanase (0.67 mg protein/g glucan)

Supplemented with Pectinase

According to the cell wall model [17], pectin provided the steric hindrance for cellulase accessible to cellulose and then slowed down the hydrolysis of cellulose and hemicellulose. The addition of pectinase had more significant effect on the glucose and xylose yields than that of xylanase (Figs. 5 and 6). Such result was in agreement with previous results obtained from dilute acid pretreated corn stover [6]. As displayed in Fig. 5, the addition of pectinase with 0.12 mg protein/g glucan (~2.2% of total protein in solution) to the hydrolysis system increased the yields of glucose and xylose by 7.5% and 29.3%, respectively.

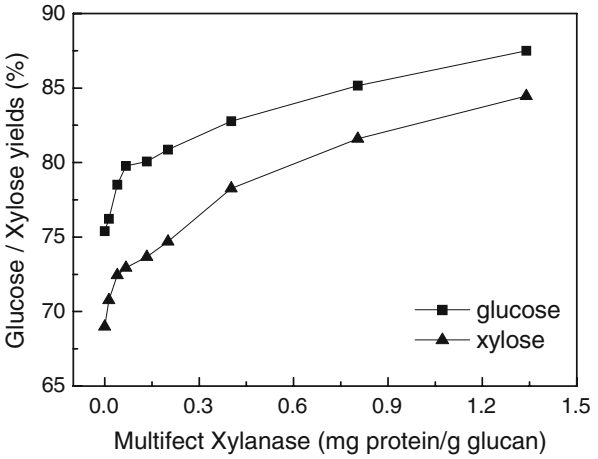

Fig. 4 The yields of glucose (*square*) and xylose (*triangle*) from enzymatic hydrolysis of SAA-treated corncobs by Spezyme CP (9.1 mg protein/g glucan) and Novozyme 188 (1.45 mg protein/g glucan) supplemented with increasing levels of Multifect Xylanase

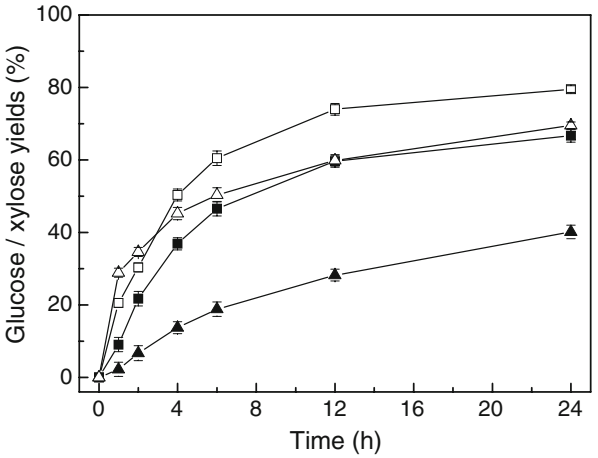

Fig. 5 The yields of glucose (*filled square*) and xylose (*filled triangle*) from enzymatic hydrolysis of SAA-treated corncobs by Spezyme CP (9.1 mg protein/g glucan) and Novozyme 188 (1.45 mg protein/g glucan), while those of glucose (*empty square*) and xylose (*empty triangle*) by such cellulases supplemented with Multifect Pectinase (0.12 mg protein/g glucan)

Conclusion

The loadings of cellulase and β-glucosidase were the key accelerant for cellulose conversion. The saturation limits of Spezyme CP and Novozyme 188 were 15 and 2.9 mg protein/g glucan, respectively. The addition of β-glucosidase with the loading of 1.45 mg protein/g glucan can convert cellobiose into glucose in lignocellulose hydrolysates in time and promoted the hydrolysis of cellulose.

Addition of xylanase and pectinase can eliminate the inhibit effect of hemicellulose and pectin, thus increasing cellulose conversion. Cellulases combined with xylanase and pectinase both increased the 24-h yields of glucose and xylose, but the latter was more effective.

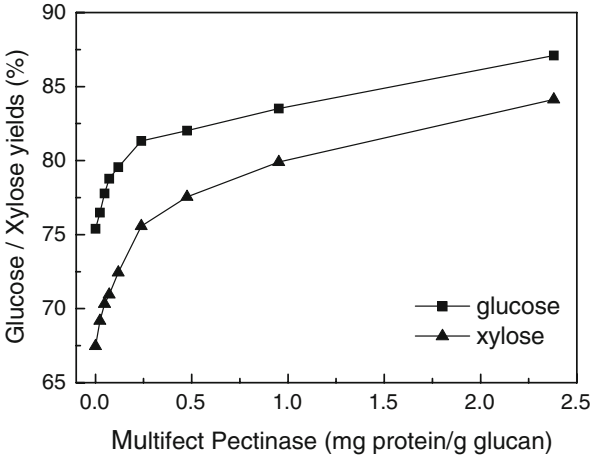

Fig. 6 The yields of glucose (*square*) and xylose (*triangle*) from enzymatic hydrolysis of SAA-treated corncobs by Spezyme CP (9.1 mg protein/g glucan) and Novozyme 188 (1.45 mg protein/g glucan) supplemented with increasing levels of Multifect Pectinase

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