Appl Biochem Biotechnol (2011) 164:23–33
DOI 10.1007/s12010-010-9111-0

Hydrolytic Enzyme of Cellulose for Complex Formulation Applied Research

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Received: 27 April 2010 / Accepted: 11 October 2010 /
Published online: 24 October 2010

Abstract To improve the enzymatic hydrolytic efficiency and reduce the supplementation of enzymes, the mixture designed experimental approach was used to optimize the composition of enzyme mixture and promote the hydrolysis of ball-milled corn stover. From the experimental results, a synergistic effect was found when combinations of the three enzymes, two kinds of cellulases and a kind of xylanase, were used. The optimal hydrolysis of pretreated corn stover accorded with enzymes activity ration of FPU/CMCase/β-glucosidase/xylanase=4.4:1:75:829, and the hydrolysis efficiency of corn stover increased significantly compared with using individual enzyme. The results indicated that the mixture design experiment could be an effective tool for optimized enzyme mixture for lignocellulose hydrolysis.

Keywords Experimental mixture design · Cellulose · Complex formulation · Cellulase · Xylanase

Introduction

Lignocellulosic biomass has long been recognized as a potential sustainable source of mixed sugars for fermentation to fuels and other chemicals. In response, many countries have initiated extensive research and development programs in biofuels, a sustainable and renewable energy resource that can provide liquid transportation fuels. In future biorefineries, biofuels will be produced from biomass resources such as agricultural residues, forestry wastes, waste paper, and energy crops [1–4].

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The most efficient means to produce fermentable sugars from lignocellulosic biomass is by enzymatic hydrolysis. There are two general categories of enzymes necessary to convert cellulose and hemicellulose into soluble sugars: cellulases and hemicellulases. Crystalline cellulose is hydrolyzed by the synergistic action of endo-acting (with respect to the cellulose chain) enzymes known as endoglucanases, and exo-acting enzymes, known as exoglucanases. The endoglucanases locate surface sites at locations, probably found at random, along the cellodextrin and insert a water molecule in the β-(1,4) bond, creating a new reducing and non-reducing chain end pair. β-D-glucosidases (cellobiases) act to hydrolyze cellobiose, the product of cellulase action, and thus relieve the system from end-product inhibition [5–7].

In order to obtain fast enzymatic hydrolysis of biomass with a high sugar yield (for both hexose and pentose), the two main protective coats around cellulose–hemicellulose and lignin need to be removed or altered without degrading the hemicellulose sugars. In an attempt to overcome the obstacles to complete enzymatic hydrolysis, additional xylanase was added to the cellulase mixture in order to increase the hemicellulose hydrolysis and thus increase cellulase accessibility. Increased hydrolysis rate should also lead to decreased hydrolysis time and hence reduced process cost [8–13].

All these enzymes work synergistically to hydrolyze cellulose by creating new unit accessible sites for each other to remove obstacles and to relieve product inhibition [14]. Individual cellulases have very limited hydrolytic activity while a mixture of cellulases can exhibit a synergistic effect where the hydrolytic activity of the cellulase mixture is greater than the sum of the hydrolytic activities of the individual enzyme [15, 16]. Extensive research is being done on improving the hydrolytic efficiency of such enzymes [17–19].

Several studies have reported that the synergism between cellulases is tightly connected with the type and ratio of the individual enzyme [20, 21], it has also been reported that synergistic effects were evident among several components and the significant change of the rate of hydrolysis of cellulose as well as the synergism depend on the components and the ratio of them. The hydrolytic efficiency of a multi-enzyme mixture in the process of lignocellulose saccharification depends both on properties of individual enzymes and their ratio in the multi-enzyme cocktail [14, 22]. Corn stover, an abundant agricultural by-product was used as the raw material in this study. It was designed to find an optimal ratio of cellulase and hemicellulase mixture to improve the efficiency of cellulose hydrolysis into sugars.

In the present study, we used three enzymes (two cellulases and one xylanase) to construct an optimized cellulase mixture-by-mixture-designed experiments. This method is a powerful tool for optimization and analysis of the effects of each component as well as interactions between components. Several researchers have used this technique for optimization of bioprocesses [23, 24]. In a mixture design, the factors are components or ingredients of a mixture and the sum of proportions of mixture components should be equal. In mixture experiments, the measured response is assumed to only depend on the relative proportions of the components in the mixture, but not on the amount of the mixture. The aim of this study is to construct a cellulase cocktail for a more efficient enzymatic hydrolysis of corn stover and a more rational utilization of enzymes.

Materials and Methods

Enzyme and Substrate Preparations

The corn stover used in this study was purchased from Jiangsu province in China. After collecting, the stover was chopped, air-dried, milled to pass through a 1.0-mm screen,

stored in plastic Ziploc bags and kept at room temperature. In the pretreatment process, corn stovers were milled with a planetary ball mill (XQM, Chishun China) as previous studies described [25, 26]. The optimum parameters for milling process are speed 350 r/min, liquid ratio 1:10, raw material particle size 1.0 mm, grinding media 20% (steel ball, Φ=10 mm), 30 min. The sugar and lignin contents of the raw material were determined according to standard methods of the National Renewable Energy Laboratory [27]. The compositions of the corn stovers are presented in Table 1.

Enzyme Activity Assays

Cellulase (ZSL-1300) and Xylanase were provided by Shandong Zesheng Biotechnology Co., Ltd (Shandong, China), Cellulase (Accellerase 1000) was provided by Genencor International Inc (Wuxi, China). The study was conducted by using three enzyme products. The developmental enzymes were in liquid form. In measuring enzyme activity, the filter paper activity of the cellulase preparations was determined by applying the procedure recommended by the International Union of Pure and Applied Chemistry [27]. The endoglucanases activity assay was performed in sodium citrate buffer (50 mM, pH 4.8) consisting of carboxymethycellulose-Na (CMC-Na, Sigma, 1.0%, *w*/*v*). The released reducing sugars were measured by the dinitrosalicylic acid (DNS) method using glucose as a standard. *p*-Nitrophenyl-glucose was used as the substrate for the activity assays of β-1,4-glucan cellobiohydrolase. The released *p*-nitrophenol was measured at 400 nm with a spectrophotometer after adding Na_2CO_3 to a final concentration of 60 mM [6].

Xylanase (1,4-β-D-xylanase) activity was determined under similar conditions as described above, except that 1% xylan solution (birch wood xylan Sigma Co. Ltd) was used as the substrate in place of CMC. The released reducing sugars were determined by the DNS. All activities were expressed in international units (IU) defined as 1 μmol of glucose or xylose produced per minute [6].

Protein determination, the protein concentrations were determined by the Coomassie brilliant blue method using bovine serum albumin as the standard, and monitored at A_{280}nm [28].

The protein concentrations and activities of the three enzymes were presented in Table 2.

Enzymatic Hydrolysis

The enzymatic hydrolysis of corn stover was conducted with individual cellulase (Genencor, G; Zesheng, Z) loading of 20 FPU/g glucan, and xylanase (X) loading of 9,500 IU/g glucan. Xylanase (X) as a supplemental enzyme was assessed in combination with each of the cellulase to increase the yield of enzymatic hydrolysis, these xylanase loadings were designated as Z+X, X+G and G+Z, respectively, each enzyme was a half loading amount of individually. The total amounts of enzyme activity for each cellulase and xylanase loading are summarized in Table 3.

Samples were hydrolyzed at 50 °C with gentle agitation (120 rpm) for a period of 95 h. The hydrolysis was conducted in 100-mL shake flasks and the solid concentration of 10%.

Table 1 Compositions of pretreated corn stover expressed as percent of dry matter

Glucan (%)	Xylan (%)	Ligin (%)	Ash (%)
36.8	21.5	17.5	9.3

Table 2 Protein concentration and activities of enzymes (per mL)

Activity	Genencor	Zesheng	Xylanase
Filter paper activity (FPU)	93	77	35
CMC activity (IU)	7.3	17.3	13
β-glucosidase activity (IU)	1,632	1,356	872
Xylanase activity (IU)	849	997	38,026
Protein (mg/ml)	38.6	37.8	19.8

Sugar (glucose and xylose) in the liquid were analyzed by HPLC (Dionex, P680 HPLC Pump System) equipped with a Bio-Rad Aminex HPX-87P column and a refractive index detector. The mobile phase was water at a flow rate of 0.6 mL/min and column temperature used for HPLC analysis was 85 °C.

The sugar yield of enzymatic hydrolysis of corn stover after ball milling pretreatment was calculated according to the following equation:

$$\text{Sugar yield}(\%) = \frac{[\text{glucose}] + [\text{xylose}]}{1.111[\text{glucan}] + 1.136[\text{xylan}]} \times 100\% \quad (1)$$

Glucose Glucose concentration (g/L)
Xylose Xylose concentration (g/L)
Glucan Glucan concentration at the beginning of the hydrolysis (g/L)
Xylan Xylan concentration at the beginning of the hydrolysis (g/L)

Experimental Design

The primary objective of the experiment was to identify three enzymes mixture as either having a negative or a positive effect. Our secondary objective was to identify the interaction of three enzymes. In an experimental mixture design the independent factors were proportions of different components in a mixture. The total proportions of the different factors had to be 100%, which is 2.0 mL using three components (Table 4). The measured response of hydrolysis rate was assumed to be dependent on the relative proportions of the components in the mixture.

The influence of the components on enzymatic hydrolysis was studied using a Simplex Centroid Design with constraints. The Design Expert software (Version 7.0, Stat-Ease Inc.) was used for regression and graphical analysis of the data obtained. The experimental

Table 3 Total combined amounts of cellulase and xylanase activity IU/g glucan

Enzyme (designation)	FPU	CMCase	β- glucosidase	Xylanase
Zesheng (Z)	20	3.72	292	214
Genencor (G)	20	1.57	351	183
Xylanase (X)	14	5.75	218	9,500
Zesheng/2+Genencor/2(Z+G)	20	2.65	321	198
Zesheng/2+Xylanase/2(Z+X)	17	4.74	255	4,860
Genencor/2+Xylanase/2(G+X)	17	3.66	284	4,845

Table 4 Experimental mixture design of components and enzymes loading

Code	Factors	Low ratio (mL)	High ratio (mL)
X	Xylanase	25% (0.50)	50% (1.00)
Z	Zesheng (cellulase)	25% (0.50)	50% (1.00)
G	Genencor(cellulase)	25% (0.50)	50% (1.00)

design generated is shown in Table 5. The factor interactions were studied using 3D-response surface, contour area graphs. These graphs allow one to study the interactions of any three of the factors in terms of their effect on enzyme production. The graphs given in Fig. 2 provide predicted values based on a quadratic fit. The best-fitting mathematical model was selected based on the comparisons of several statistical parameters including the determination coefficient (R^2), and the F value provided by analysis of variance. All responses providing a significant F value generated the response surfaces.

Results and Discussions

Enzymatic Hydrolysis

Synergism is often defined as the ratio of the rate or yield of a single product released by the simultaneous action of enzymes to the sum of rate or yield of these products when produced by the action of individual enzymes when used separately in the same amounts as in the mixture. However, since this study is followed by the release of two products, glucose and xylose on the loadings of xylanase which can enhance xylan and glucan hydrolysis, these results were in agreement with the previous report [8, 29]. As displayed in Fig. 1a, b, the xylose and glucose yield increased highly because supplementary 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 the addition of xylanase which increased the accessibility of cellulase to cellulose chains by removing the hemicelluloses barrier and thus exposing more cellulose chains and supporting the hypothesis that residual hemicellulose interferes with cellulose hydrolysis. Increased hemicellulose hydrolysis as a result of xylanase supplementation therefore improves cellulose hydrolysis [11, 30, 31].

Table 5 Mixture design for the centroid simplex design and sugar yield

No.	Z (%)	G (%)	X (%)	Sugar yield (%)
1	50	25	25	85.3
2	25	50	25	91.4
3	25	25	50	73.4
4	38	38	25	75.0
5	38	25	38	94.9
6	25	38	38	69.3
7	33	33	33	69.8
8	42	29	29	76.2
9	29	42	29	73.8
10	29	29	42	78.6

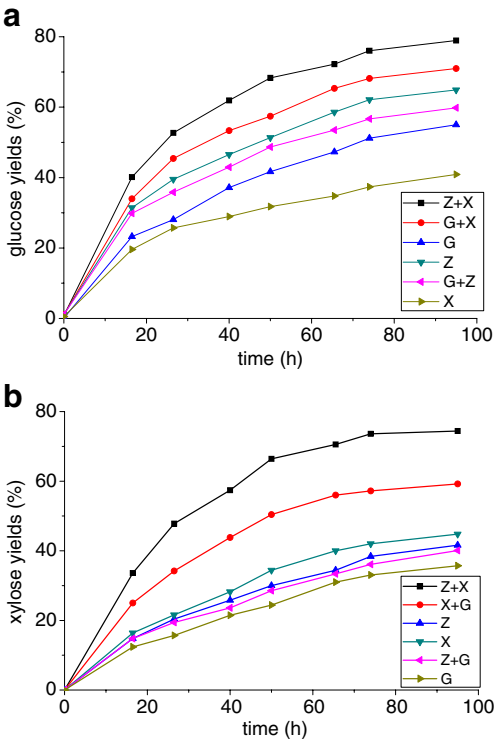

Fig. 1 **a** Glucose yields in hydrolysis of pretreated corn stover. The enzymatic hydrolysis was performed with control enzyme loading and xylanase supplementation. **b** Xylose yields in hydrolysis of pretreated corn stovers. The enzymatic hydrolysis was performed with control enzyme loading and xylanase supplementation

Three types of cellulose activities are needed to deconstruct cellulose into glucose. These include endoglucanase (filter paper activity), exoglucanase (CMCase) and cellobiohydrolase (β-glucosidase activity). In the hydrolysis process, endoglucanase first randomly cleaves different regions of crystalline cellulose, producing chain ends. Exoglucanase then attaches to the chain end and threads it through its active site, cleaving off cellobiose units. The exoglucanase also acts on regions of amorphous cellulose with exposed chain ends without the need for prior endoglucanase activity. Finally, β glucosidase breaks the bonds between the two glucose sugars of cellobiose to produce monomers of glucose [4, 32]. Proper optimization of the ratio between enzyme components can enhance the cellulose hydrolyzation if not the cellulase hydrolysis process will be slowed down.

Screening Experiments

Enzymes from different strains of *Trichoderma* sp. have long been considered the strongest destroyers of cellulose. However, the multi-enzyme cocktail secreted from the strains may be sub-optimal for application in biotechnological process, because some of the most critical cellulases may be expressed at a level that is insufficient for highly effective hydrolysis or the secreted cellulase mixture may be not well balanced by individual enzymes. The initial ratio of each component activities in crude enzyme preparation was not the same [14, 33].

As in the previous analysis, experiments were carried out using one or two enzymes to hydrolyze the cellulose and to explore the existence of synergistic effect between two enzymes. The results indicate that enzymes mixture have significant influence to improve the

lignocellulose hydroxylation. We performed a mixture design for the three enzymes and sought to establish the optimal composition of the enzyme mixture to be used in the lignocellulose hydrolysis process. Table 5 presents the design and the sugar yield for each experiment.

The prescription mixture experiment design in Table 5 was analyzed by multivariate regression analysis, to observe improvement in the lignocellulose hydroxylation using cellulase and xylanase. The results were analyzed by the Design Expert software to determine the most suitable correlation between the variable parameters and responses. The regression models can be applied to screening crucial enzyme components. To facilitate the optimum location, the data are fit to a special cubic model:

$$\text{Sugar yield} = +84.23{*}Z + 91.46{*}G + 74.61{*}X - 55.18{*}Z{*}G + 62.49{*}Z{*}X - 49.69{*}G{*}X - 223.77{*}Z{*}G{*}X \quad (2)$$

Table 6 lists the main statistical properties of the regressions obtained with the analysis of variance. The model F value of 11.52 implies the model is significant. Values of "Prob> F" less than 0.0500 indicate model terms are significant. In this case ZG, ZX, GX are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Based on the data obtained (Table 5), regression equations were designed for the properties analyzed at each cellulose ratio, these equations are referred to the enzyme used, Z, G, and X represent the fractions of Zesheng (cellulose), Genencor (cellulose), Xylanase, respectively.

Figure 2a, b, respectively, represent cellulase and xylanase mixture design data. The compound after the three-producing isogram interactions and 3D response surface plot can be seen from the graph. The synergies among these cellulases and xylanase are key factors to promote the efficient and complete degradation of cellulosic substrates and to result in an enhanced efficiency of hydrolysis. In fact, synergism between cellulases has been described either for enzymes of the same microorganism or from different microorganisms [14]. In this study, we obtained the efficient cellulase mixture for cellulose hydrolysis by statistically designed experimental method, which provided a great deal of flexibility in experimental design and operation. The multi-enzyme mixtures based on cellulases and xylanase to gain the more refined results in evaluating the synergism process. In the three important components cellulase (Zesheng), cellulase (Genencor) and xylanase were screened out. Maximum synergism was achieved at a volume ratio of approximately

Table 6 Analysis of variance for the centroid simplex design regression model

Source	Sum of Squares	*df*	Mean Square	*F* value	*p* value prob>*F*
Model	676.3	6	112.7	11.52	0.035
Linear Mixture	61.4	2	30.7	3.14	0.184
ZG	128.6	1	128.6	13.14	0.036
ZX	164.9	1	164.9	16.86	0.026
GX	104.3	1	104.3	10.66	0.047
ZGX	48.7	1	48.7	4.97	0.112
Residual	29.3	3	9.78		
Cor total	705.6	9			
R square	0.958				
Adj *R* square	0.875				

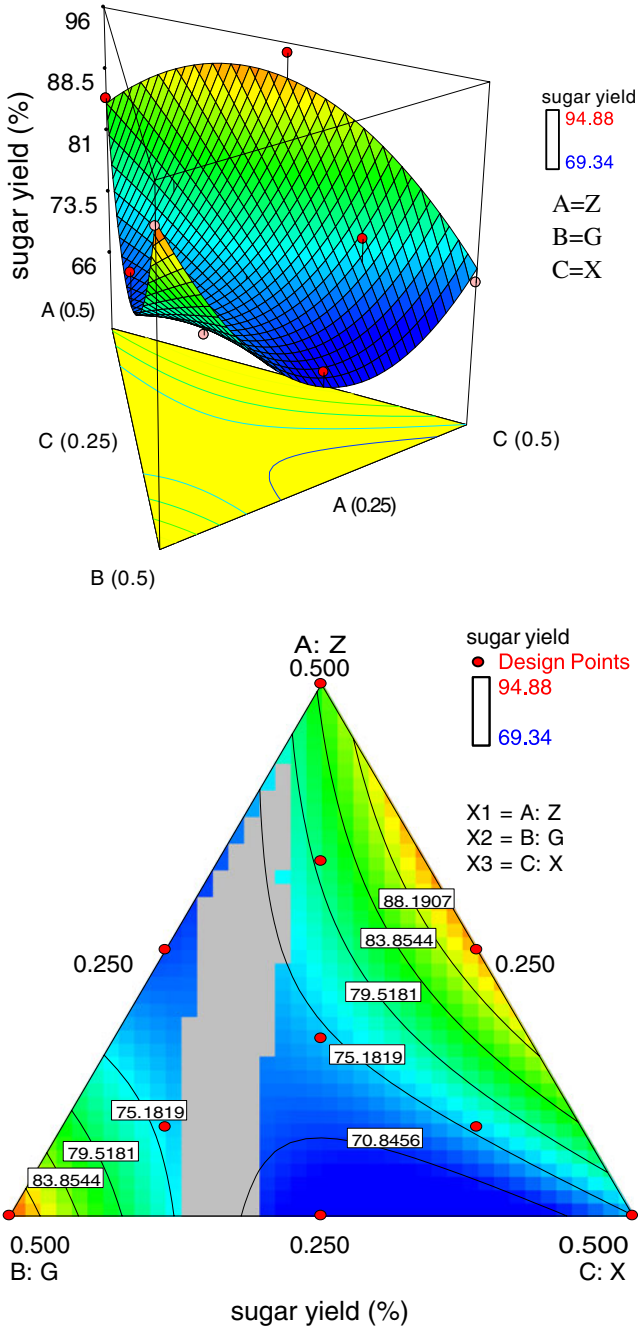

Fig. 2 **a** Response surface plot showing relative effect of enzymes parameters on sugar yield. **b** Contours diagram illustrating effect of enzyme parameters on sugar yield

8:5:7, that is, filter paper activity (FPU)/CMC activity (IU)/β-glucosidase (IU)/xylanase activity (IU)=4.4:1:75:829, when the hydrolysis of corn stover achieved the highest efficiency with the total sugar yield 95%. By experimental verification in the same conditions using a single enzymatic hydrolysis the results are as follows: yield of total sugar cellulase (Zesheng) 65%, cellulase (Genencor) 59%, and xylanase 45%. As can be seen by comparing the above data cellulase hydrolysis efficiency increased significantly.

The cellulases could be divided into two classes, one attacks the non-reducing ends of cellulose molecules and the other attacks the reducing ends. Moreover, the cellulases synergism between them only occurs when the two cellulases come from different classes. The detailed explanation has been given by Michael et al. [5].

Xylanase plays an important role during the hydrolysis processes. The cellulose microfibrils are coated with other polysaccharides such as hemicellulose or xyloglucans. Depending on the plant species, 20–40% of the plant cell-wall polysaccharides are hemicellulose. Like cellulose, hemicellulose could be converted into fermentable sugars by enzymatic hydrolysis for the production of cellulosic ethanol.

In addition, the parabolic effects on hydrolysis should be further noted when both enzymes are over certain concentration values (Fig. 2a, b). Single enzyme has limited hydrolytic activity while mixed-enzyme can exhibit a synergistic effect in lignocellulose hydrolysis, where the hydrolytic activity ratio of the mixed-enzyme is better than that of the single enzyme. By optimizing enzyme mixture ratio, it could be possible to attain a higher rate and more effective hydrolysis of pretreated corn stover. Based on the results of single enzymes hydrolysis, the parabolic effects of mixed-enzyme on hydrolysis should be further explored. Supposedly, this negative effect was observed because of competition for productive binding sites among enzymes and saturation of the individual enzyme. The parabolic effect has also been observed by several researchers when they investigated the optimization of other cellulases ratios [14, 22, 34].

Conclusions

The statistical design of mixture experiments and the response surface methodologies had been proved to be powerful tools for designing and analyzing experiments to ascertain the influence of complex formulation for cellulose hydrolyzation. In this study, we obtained an efficient cellulase mixture for cellulose hydrolysis by statistically designed experimental method which provided a great deal of flexibility in experimental design and operation. Addition of xylanase can eliminate the recalcitrant effect of lignocelluloses on enzymatic hydrolyzation, thus increase cellulose conversion. Cellulases combined with xylanase can increased the yields of glucose and xylose. The three enzyme components were all for optimization usage, which helped us to interpret the effects of each components as well as interaction among the different components very well. Comparing the ratio of individual and mixture enzymes activity, we found that it was necessary to enhance the amount of xylanase activity in the mixture for the maximum of saccharification. It was essential for us to select a typical pretreated lignocellulose, such as pretreated corn stover, as a target substrate to evaluate the enzyme performance.

Acknowledgements This work was financially supported by China Petroleum & Chemical Corporation (No. 207035), National Natural Science Foundation of China (No. 20876078), the Key Program of National Natural Science Foundation of China (No. 20936002), United Foundation of NSFC and Guangdong Province (No. U0733001), National Hi-tech Research and Development Program of China (No. 2009AA02Z08) and National Basic Research Program of China (No. 2007CB707805).

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