Optimization of enzyme complexes for efficient hydrolysis of corn stover to produce glucose

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Abstract: Hydrolysis of cellulose to glucose is the critical step for transferring the lignocellulose to the industrial chemicals. For improving the conversion rate of cellulose of corn stover to glucose, the cocktail of celllulase with other auxiliary enzymes and chemicals was studied in this work. Single factor tests and Response Surface Methodology (RSM) were applied to optimize the enzyme mixture, targeting maximum glucose release from corn stover. The increasing rate of glucan-to-glucose conversion got the higher levels while the cellulase was added 1.7μl tween-80/g cellulose, 300μg β-glucosidase/g cellulose, 400μg pectinase/g cellulose and 0.75mg/ml sodium thiosulphate separately in single factor tests. To improve the glucan conversion, the β-glucosidase, pectinase and sodium thiosulphate were selected for next step optimization with RSM. It is showed that the maximum increasing yield was 45.8% at 377μg/g cellulose Novozyme 188, 171μg/g cellulose pectinase and 1mg/ml sodium thiosulphate.

Keywords: Corn stover, glucose, cellulase, β-glucosidase, hemicellulase, pectinas.

INTRODUCTION

Lignocellulose contains cellulose and other polysaccharides that can be hydrolyzed to glucose and various other simple sugars for production of various biochemicals (Kim and Dale 2004; Hahn-Hägerdal *et al* 2006; John *et al* 2009; Abdel-Rahman *et al* 2011). Hydrolysis of the cellulose and hemicellulose to glucose and xylose with enzyme complexes is a critical step in the conversion of lignocellulose. To improve the efficiency of enzyme complexes have focused on cellulose because cellulose is the most abundant polysaccharide in lignocellulose (Zhang and Lynd 2004).

The cellulose could be hydrolyzed with cellulase, which includes cellobiohydrolases, endoglucanases and β-glucosidase. In commonly, commercial cellulase contains much endoglucanases and cellobiohydrolases, together with less β-glucosidase. As a result, the β-glucosidase is always to be added into the commercial cellulase in the hydrolyzing process of lignocellulose (Zhang *et al* 2006)

Furthermore, lignocellulose is composed of hemicelluloses, lignin and pectin besides cellulose. In the process of hydrolysis, other auxiliary enzyme, which hydrolyze other polysaccharides such as hemicellulose and pectin, should be added into the enzyme system because they can improve the hydrolysis efficiency of the cellulase even they don't hydrolyze cellulose directly (Berlin *et al* 2005; Wilkins *et al* 2007; Zhang *et al* 2013).

In general, the higher level of cellulose conversion is achieved by the synergistic interactions amongst cellulase in conjunction with hemicellulase and pectinase. As a conclusion, it is important to blend the enzyme complexes to enhance the efficiency of cellulose converting to glucose (Jøgensen *et al* 2007).

In addition of cocktail of enzyme complex, some surfactants, such as Tween 20 and PEG 4000 were added to hydrolysis system to enhance the saccharification in some research (Börjesson *et al* 2007; Kumar and Wyman 2009). The addition of surfactant can reduce wasteful cellulase adsorption on the lignin, while it improves the hydrolysis efficiency to get same degree of conversion at lower enzyme loadings (Eriksson *et al* 2002).

The cocktail of cellulase with auxiliary enzymes and chemicals which could improve the glucan-to-glucose conversion was studied in this work. A commercial cellulase supplemented with β-glucosidase, hemicellulase, pectinase, tween-80 and sodium thiosulphate was conducted to enhance the hydrolysis of corn stover. In the previous works, the cocktails of cellulase system were studied either with cellulase added auxiliary enzymes, or with cellulase added surfactants. The components include cellulase, β-glucosidase, hemicellulase, pectinase and surfactant were all considered. In addition, the sodium thiosulphate was added into the cellulase system too. With the knowledge as we known, sodium thiosulphate is the first time to be added in the lignocellulose hydrolysis system to improve the hydrolysis rate. Furthermore, the response surface methodology was employed to optimize the main components in enzyme mixture.

MATERIALS AND METHODS

Enzyme preparations

Cellulase and hemicellulase were purchased from Youtell Co. (Hunan, China). Novozyme 188 was purchased from

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Sigma, and a commercial pectinase was provided by Kejian Co. (TianJin China). All other reagents were analytical grade

Substrate preparation

The corn stover was pretreated with steam explosion. The average content of cellulose, hemicellulose and ligin in the dry substrate were 32.6%, 26.4% and 8.1% respectively. The pretreated corn stover was washed six times with water to remove soluble components. Then, the corn stover was stored at -20ºC.

Cellulose hydrolysis

Hydrolysis of corn stover was conducted with 3% (w/w) cellulose and 0.05M citrate buffer (pH 4.8) in 100ml flasks containing 40ml hydrolysis volume. The experiments were conducted according to standard methods described in the National Renewable Energy Laboratory (NREL) LAP-008 except the different combination of components in the hydrolysis system.

At first, the Tween 80 was added to the hydrolysis system before any other enzyme or chemical was added. Flasks were preincubated at 50ºC for 60 minutes in water bath at 130 rpm. The substrate was hydrolyzed with the cellulase preparation (15 FPU/g cellulose), with various addition of other components: hemicellulase, β-glucosidase, pectinase and sodium thiosulphate. The enzymatic hydrolysis experiments were run for 40 hours with sampling at the end of experiments, and centrifuged at 8000 rpm for 10 minutes. The concentration of glucose in sample was determined by the glucose oxidase-peroxidase kit (Huili, China). All experiments were run in duplicate.

Analytical methods

The cellulase activity was assayed as NREL LAP-006.

The hemicellulase activity was got to measure the increasing of reducing sugars released from hemicellulose. A 200μl aliquot of suitably diluted hemicellulase preparation was incubated in 1800μl of hemicellulose substrate (1.0g hemicellulose in 80ml 50mM Citric acid buffer, pH4.8) for 5 minutes at 50℃ .The concentration of reducing sugars were assayed with DNS method. One unit of hemicellulase activity is defined as the amount of the enzyme that released 1 μmol of xylose per minute (Bailey *et al* 1992).

The cellobiase activity was assayed to measure the increasing of reducing sugars released from cellobiase. 1ml crude enzyme was mixed with 1ml of 15mM cellobiose solution, then the mixture was incubated at 50 °C for 30min, and boiled for 5min, cooled on ice. The released glucose was assayed with glucose kit (HuiLi, changchun). One unit of cellobiase activity is defined as the amount of enzyme that liberate 2 μmol of glucose per minute (Ghose *et al* 1987).

The pectinase activity was got to measure the increasing of reducing sugars released from polygalacturonate. A 100μl aliquot of suitably diluted enzyme solution was incubated in 900μl of 0.25% (w/v) polygalacturonic acid in 50mM Tris/HCl buffer (pH 8.0) for 15 min at 45□. The reducing sugars were determinded with DNS method. One unit of pectinase activity was defined as the amount of enzyme that released 1μmol of reducing groups per minute (Klug-Santner *et al* 2006).

The protein concentration was measured by the Bardford's method with bovine serum albumin as a standard.

Response surface methodology

Response surface methodology was employed to optimize enzyme mixture, targeting maximum glucose release from corn stover. The significant factors, Novozym 188, pectinase and Sodium thiosulphate, were chosen as major factors. The variables were coded as follows:

$$xi = (Xi - Xcp) / \triangle Xi, \quad i = 1, 2, 3, \ldots k, \qquad (1)$$

where xi is the code value of an independent variable; Xi is the real value of an independent variable; Xcp is the real value of an independent variable at the center point; and △Xi is the step change of real value corresponding to a variation of a unit for the dimensionless value.

The relationship of the independent variables and the response was explained by the following quadratic equation:

$$Yi = b_0 + \sum b_i x_i + \sum\sum b_{ij} x_i x_j + \sum b_{ii} X_{ii}^2 \qquad (2)$$

In this equation, Yi is the predicted response; bi is the linear effect; bii is the squared effect; bij is the interaction effect, and xi values represent coded variables. The software package SAS (SAS Institute, Cary, NC, USA) was employed for data analysis and model building.

RESULTS

Enzyme activity

It is shown that activities of the various enzyme preparations are different (table 1). The Cellulase contained high specific cellulase activities (2.1 FPU/mg protein) and hemicellulase (0.3 U/mg protein). Novozym 188 contained mainly cellobiase activity (0.1 U/mg protein). There is high levels of pectinase activity in pectinase preparation (1 U/mg protein). Hemicellulase was determined with high hemicellulase activity (0.6 U/mg protein) with low levels of other activities (table 1).

It is known that the cost increase at the higher cellulase loading (Himmel *et al*. 1999). Therefore, 15FPU cellulase/g cellulose was selected for the successive steps to improve the hydrolysis efficiency.

Table 1: Specific activity (U/mg) of each enzyme preparation used in supplementation experiments

	FPA	Cellobiase	Hemicellulase	Pectinase
Cellulase	2.1	~0	0.3	0. 1
Novozyme 188	~0	0.1	~0	~0
Hemicellulase	~0	~0	0.6	0.1
Pectinase	~0	~0	0.6	1

Table 2: Maximum and minimum levels of the variables used in the central composite experimental design

Independent			Variable levels		
	-2	-1	0	+1	+2
X1, Novozym 188 (μg/g)	100	210	320	430	540
X2, pectinase (μg/g)	120	145	170	195	220
X3, Sodium thiosulphate (mg/ml)	0.5	0.75	1	1.25	1.5

Table 3: Experimental design and results

Run	[a]X1	[b]X2	[c]X3	[d]Increasing rate(%)
1	-1	-1	-1	29.8
2	-1	-1	+1	30.7
3	-1	+1	-1	33.5
4	-1	+1	+1	32.1
5	+1	-1	-1	43.9
6	+1	-1	+1	40.6
7	+1	+1	-1	41.8
8	+1	+1	+1	39.0
9	-2	0	0	29.3
10	+2	0	0	34.7
11	0	-2	0	20.7
12	0	+2	0	28.4
13	0	0	-2	36.9
14	0	0	+2	40.5
15	0	0	0	43.5
16	0	0	0	44.9

[a]The dimensionless value of Novozym 188 [b]The dimensionless value of pectinase [c]The dimensionless value of sodium thiosulphate [d]Increasing rate (%) is increasing rate of glucan-to-glucose conversion

Table 4: Analysis of variance (ANOVA) for the selected quadratic model

Source	Sum of Squares	DF	Mean Square	F Value	*P*
Model	620.83	9	68.98	4.80	0.03
Error	86.29	6	14.38		
Total	707.12	15			

Table 5: The least-squares fit and parameter estimates

Parameter	DF	Parameter estimate	t-value	*P*
Intercept	1	44.98	17.93	<.0001
x1	1	3.13	3.30	0.0165
x2	1	1.05	1.11	0.3105
x3	1	0.04	0.04	0.9697
x1*x1	1	-3.05	-3.22	0.0182
x2*x2	1	-4.91	-5.18	0.0021
x3*x3	1	-1.38	-1.45	0.1972
x1*x2	1	-1.10	-0.82	0.4433
x1*x3	1	-0.70	-0.52	0.6203
x2*x3	1	-0.23	-0.17	0.8722

Effect of supplementation with the tween-80

A higher level of improvement of glucan-to-glucose conversion (17.5%) was got when the cellulase preparation was supplemented with tween-80 loading of 1.7μl/g cellulose (fig. 1).

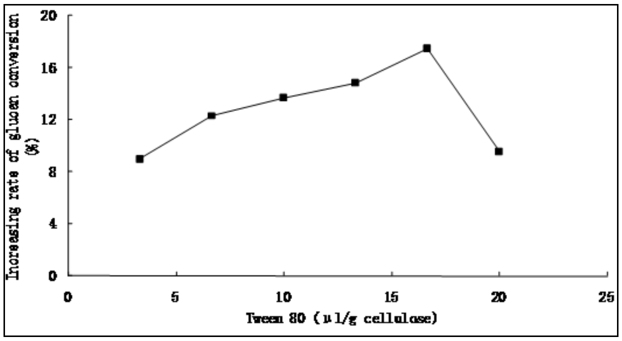

Fig. 1: The increasing rate of glucan-to-glucose conversion with the cellulase supplemented with different levels of the Tween-80.

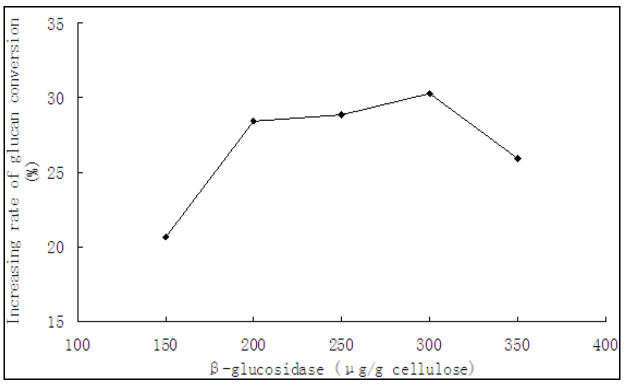

Fig. 2: The increasing rate of glucan-to-glucose conversion with the cellulase supplemented with defferent levels of the β-glucosidase.

Effect of supplementation with the hemicellulase

In this research, the different levels of hemicellulase were added in the cellulase preparation. However, it is shown that the increasing rates of glucan-to-glucose conversion were under 5% (Data not shown).

Effect of supplementation with the β-glucosidas

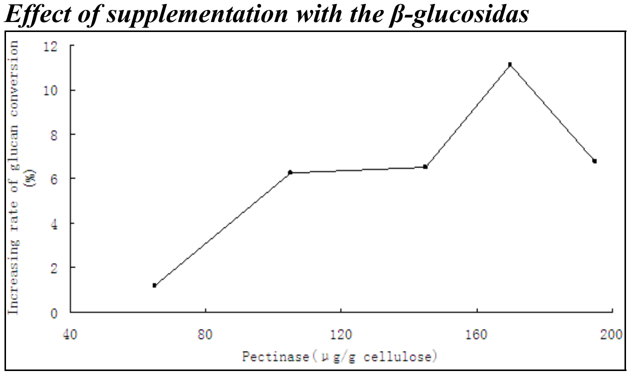

Fig. 3: The increasing rate of glucan-to-glucose conversion with the cellulase supplemented with different levels of the pectinase.

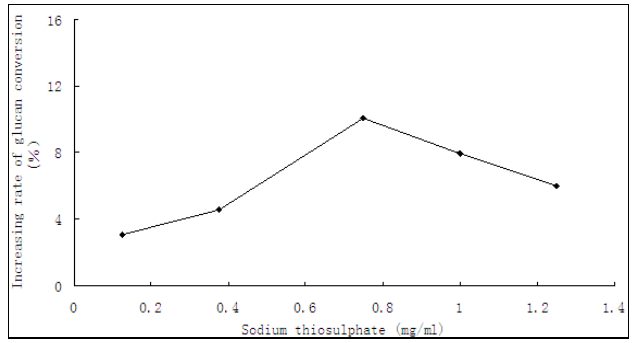

Fig. 4: The increasing rate of glucan-to-glucose conversion with the cellulase supplemented with defferent levels of the sodium thiosulphate.

The different levels of β-glucosidase were added in the hydrolysis system. The increasing rate of glucan-to-glucose conversion data are shown in fig. 2. The highest increasing rate of glucan-to-glucose conversion (30.3%) was got at 300μg β-glucosidase /g cellulose.

Effect of supplementation with the pectinase

The pectinase was added to the hydrolysis system in this work. As shown in fig. 3, supplementation of pectinase (170μg/g cellulose) brought the highest increasing rate (11.1%) of glucose yield.

Effect of supplementation with Sodium thiosulphate

The Sodium thiosulphate was added to the hydrolysis system. It is shown that the glucan-to-glucose conversion was improved with the supplementation of the sodium thiosulphate. A relatively higher level of improvement (10.0%) was got when the cellulase was supplemented with the sodium thiosulphate at the final concentration of 0.75mg/ml (fig. 4). The mechanism of the improvement of glucan-to-glucose conversion with sodium thiosulphate is unclear now.

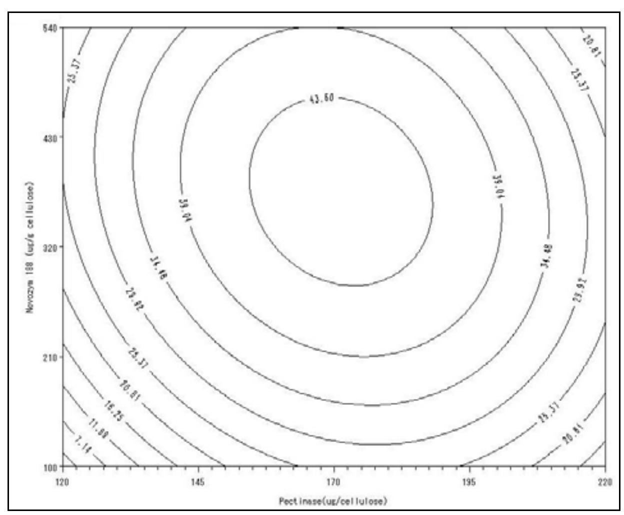

Fig. 5: Contour plot to study the effect of Novozyme 188 and pectinase on the increasing rate of glucan-to-glucose conversion at constant sodium thiosulphate coded level of zero.

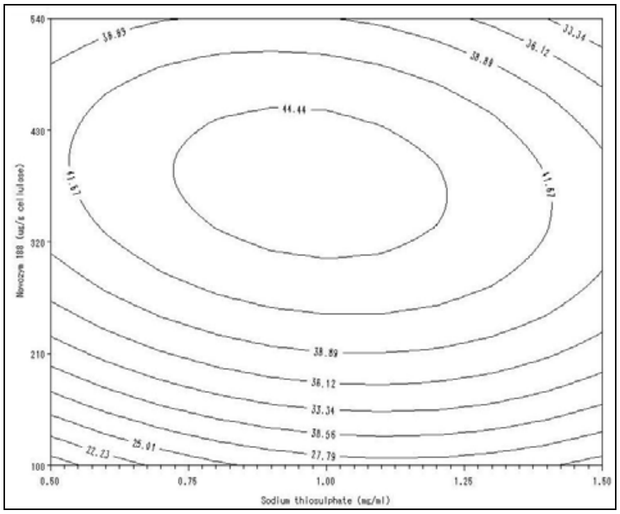

Fig. 6: Contour plot to study the effect of Novozyme 188 and sodium thiosulphate on the increasing rate of glucan-to-glucose conversion at constant pectinase coded level of zero.

Optimization of the enzyme mixture

As in the previous analysis, experiments were carried out using cellulase with other enzyme or chemical to hydrolyze the cellulose. The results indicate that the supplementation have significant influence to improve the corn stover hydrolysis. A central composite rotatable design was employed to optimize the enzyme cocktail. The Tween 80 as a surfactant, which was added to the corn stover before hydrolysis, could reduce the enzyme loadings. It has different mechanism with other factors, while it was considered separately in this study. It was added at the optimum level, 1.7μl/g cellulose, prior to other enzymes and chemical. The other three major factors, which include the Novozym 188, pectinase and the sodium thiosulphate, were sought to establish the optimal composition for corn stover hydrolysis with RSM.

Table 2 shows the range and levels of variables. The coded values, design and results of experiment were summarized in table 3. The Second-order polynomial is given with RSREG procedure from SAS as follow:

$$Y=44.98+3.13X_1+1.05X_2+0.04X_3-3.05X_1X_1-1.10X_2X_1-4.91X_2X_2-0.70X_3X_1-0.23X_3X_2-1.38X_3X_3\ (R^2=0.88)$$

The results of the second-order response surface model in the form of analysis of variance (ANOVA) were given in table 4. The Model F value (4.80) with evident from the Fisher, which implied that the model is significant. The coefficient of determination (R^2) is 88%, which indicated that 88% of the variability in the response could be explained by the model.

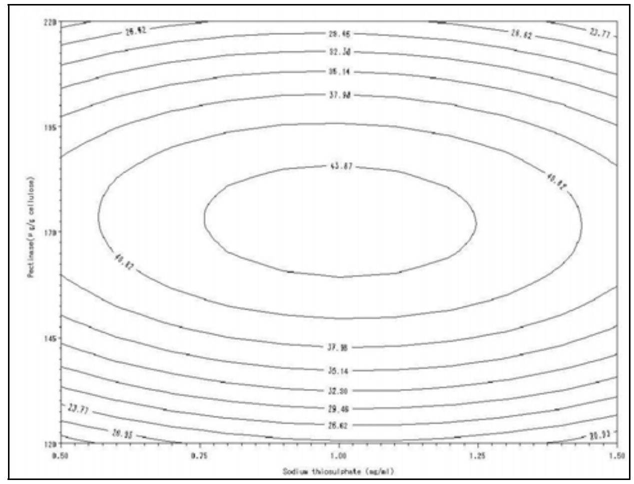

Fig. 7: Contour plot to study the effect of pectinase and sodium thiosulphate on the increasing rate of glucan-to-glucose conversion at constant Novozyme 188 coded level of zero.

The Student t distribution and the corresponding P-values, along with the parameter estimate, are given in table 5. The larger the magnitude of t test and smaller the *P*-value, the more significant was the corresponding coefficient. It is shown that both linear and quadratic relationships between Novozyme 188 and the increasing rate of glucan-to-glucose conversion are considerably important (P<0.05). Also, the effect of pectinase is considerably important because its quadratic effect is pronounced (P<0.01).

The response surface curve was plotted to understand the interaction of the factors and the optimum-increasing rate of glucan-to-glucose conversion and the optimum concentration of each factors required. The 2D contour plots were shown in figs. 5-7. From the corresponding contour plots, the optimal values of the independent variables could be observed, and the interaction between each independent variable could be easily understood.

The optimum yield was determined by RSREG analysis. The analysis indicated that maximum increasing yield was 45.8% at 377μg/g cellulose Novozyme 188, 171μg/g cellulose pectinase and 1mg/ml sodium thiosulphate. The verification of the results was checked by carrying out independent experiments. The predicted response was 45.8% and the actual response was 44.9%, which thus proves the validity.

DISCUSSION

It is reported that addition of surfactants such as Tween 80 prior to enzymatic hydrolysis, the surfactants were irreversible adsorbed on lignin to reduce enzyme loading for cellulose hydrolysis (Jørgensen *et al*. 2007). It can reduce useless binding of enzymes, therefore promote the hydrolysis with lower enzyme loading. The lignocellulose is composed primarily of cellulose, hemicelluloses and ligin. Hemicelluloses, cellulose and lignin twine around each other, which limit the accessing of cellulases to cellulose (Zhang and Lynd 2004). It has been shown that addition of hemicellulase could improved the hydrolysis efficiency of cellulases and increased glucose conversion of cellulose (Bernard and Day 2008; Pryor *et al*. 2012) One reason of lower increasing rate maybe the relative higher hemicellulase activity in the cellulase preparation, so the additional hemicellulase is not needed in this system. There is product inhibition in the cellulose hydrolysis process (Zhang *et al* 2006). The activity of β-glucosidases is always inhibited by glucose, which results in accumulation of cellobiose. The inhibition of the β-glucosidases can be reduced by addition of β-glucosidase in some extent. Pectins could be hydrolyzed by pectinase with depolymerization and de-esterification reactions (Wilkins *et al*. 2007; Zhang *et al*. 2013). A combination of cellulase and pectinase could improve the glucan-to-glucose conversion.

CONCLUSION

The significant factors that affected the hydrolysis efficiency of cellulose form corn stover with given enzyme preparations or chemicals were got by single factor tests. Then the significant factors including β-glucosidase, pectinase and sodium thiosulphate, were optimized by response surface methodology. The highest increasing rate of glucan-to-glucose conversion (44.9%) was got at the optimum concentration of β-glucosidase (377μg/g cellulose), pectinase (171μg/g cellulose) and sodium thiosulphate (1mg/ ml sodium thiosulphate). A meaningful trial was provided in this study, which could be a referential example to optimize the enzyme mixture for improving efficiency of cellulose conversion to glucose in the similar cases.

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