Recycling Cellulase from Enzymatic Hydrolyzate of Laser-Pretreated Corn Stover by UF Membrane

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The ultrafiltration membrane reactor, utilizing a membrane module with a suitable molecular weight alleyway, retains the larger cellulase components. Smaller molecules, such as the fermentable reducing sugars and water, pass through the membrane. The purpose of this work was to investigate the capability of recycling cellulase in the UF membrane. PS30 hollow fiber membrane, an ultrafiltration method using internal pressure, was found to be an ideal membrane separation device, allowing re-use of the enzyme. A Box-Behnken experimental design (BBD) established the following optimum pretreatment parameters: operation pressure at 1.73 bar, temperature at 36.38 °C, and a pH of 5.92. Under these conditions, the model predicted a membrane flux yield of 2.3174 L/(m^2•h). The rejection rate of the UF membrane was over 95%.

Keywords: Ultrafiltration; Cellulase; Hydrolyzate; Recycling

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INTRODUCTION

The biotransformation of lignocellulosic biomass to produce bioethanol presents a remarkable opportunity for the production of recycled and environmentally friendly bioresources (Gnansounou and Dauriat 2010). However, economic constraints have so far blocked the construction of lignocellulosics to bioethanol conversion facilities on a large scale. Bioconversion process of lignocellulosic biomass mainly includes of four steps, *i.e.* pretreatment, enzymatic hydrolysis, bioethanol fermentation, and product refining (Hahn-Hägerdal *et al.* 2006). Bioconversion of renewable, inexpensive, and readily available corn stover into fermentable sugars for production of bioethanol and other bioproducts has received extensive attention by cellulase hydrolysis to release the constituent fermentable monosaccharides (Alvira *et al.* 2010).

Although extensive research projects have been devoted to maximizing cellulase production and improving cellulase performance, efficient and cost-effective enzymatic saccharification of corn stover to produce fermentable monosaccharides still remains a challenge to be addressed (Cheng and Timilsina 2011). Another means to reduce the cost of cellulase hydrolysis is to retrieve and recycle cellulase bound to the residue as well as cellulase from the reaction suspension (Qi *et al.* 2012; Lindedam *et al.* 2013). Bound cellulase on corn stover residue could be recovered by the simple contact of corn stover residue with fresh substrate (Tian *et al.* 2013). Many researchers have previously paid close attention to the use of ultrafiltration (UF) membrane to recycle cellulase in the saccharification of lignocellulosic biomass (Mores *et al.* 2001; Bae and Tak 2005). The UF membrane reactor, utilizing a membrane module with a suitable molecular weight alleyway, keeps the larger cellulase components in the UF reactor while smaller

molecules, such as monosaccharide, oligosaccharide, and water pass through the membrane (Azmi *et al.* 2013).

In the present work, the use of UF for treatment of the liquid phase of enzymatic hydrolyzate of CO_2 laser pretreated corn stover (LPCS) was proposed (Tian *et al.* 2012). The objective of this study was to investigate the possibility of recycling cellulase in the UF membrane. The UF hollow fiber membrane was selected for this work because this bioreactor type is more useful for industrial applications, owing the high flux of the UF membrane surface relative to the working solution volume (Yang *et al.* 2009). In this research, cellulase was derived from *Trichoderma reesei*. The constituents included EG Ⅰ, EG Ⅱ, EGⅢ, CBH Ⅰ, CBH Ⅱ, and GB, which have molecular weights of 50, 46, 25, 65, 58, and 75 kDa, respectively (Ma *et al.* 2013). The hollow fibers were made of polysulfone with a molecular-weight cutoff (MWCO) of 30,000 Da (PS30) (Vilakati *et al.* 2015).

EXPERIMENTAL

Materials

Corn stover was harvested in Zhengzhou, Henan, China, in 2014. It was milled and sized using a sieve shaker of 80 to 120 mesh size with a chipper mill (Tianjin Taisite Instrument Co., Ltd., Tianjin, China) and stored in the refrigerator (4 °C). All raw materials were pretreated with a CO_2 laser for 68 min at 265 W with a liquid to solid ratio of 21:1 (mL/g) (Tian *et al.* 2011). Crude cellulase powder was purchased from Hualing Biological Technology Co., China. The hollow fiber UF membrane was provided by Tianjin MOTIMO membrane technology Co., Ltd. To reuse the cellulase, the ultrafiltration membrane module was employed. A PS30 hollow fiber membrane was used as the membrane internal pressure ultrafiltration system for recycling of cellulase protein.

Methods

Measurement of total reducing sugars

The cellulase hydrolysis process was carried out in the pretreated corn stover. Total reducing sugars concentration of the residual solution was estimated indirectly by utilizing the DNS solution dosage, such as shown in Eq. 1 (Ye 2011),

$$[S_t] = [S_0] - 0.9\,[RS] \quad (1)$$

where $[S_t]$ is the theoretical residue value of the reducing sugars in the solution (g/L) during the process of recycling cellulase, $[S_0]$ is the initial concentration of the corn stover fibers in the solution (g/L), and [RS] is the total reducing sugar concentration (g / L) in the hydrolysates.

Membrane reactor

The membrane reactor consisted of a hydrolysate container, a 500 mL Berzelius beaker as the main reactor, and a PS30 hollow fiber UF module outside (Fig. 1). The reactor was kept in a water bath at 20 to 45 °C, the optimal temperature for the recycling of cellulase. The hydrolysate container was used to continuously feed fresh hydrolysate to the reactor. The pressure applied to the PS30 module was typically about 0.5 to 3 bar. The transdermal solution was collected in a 250 mL Erlenmeyer flask for flow control.

Data processing

The permeate flux of hydrolysate is defined as follows,

$$J = \frac{V}{S \times t} \quad (2)$$

where J is the membrane flux (L/(m^2•h) during recycling cellulase, V is the permeate volume (L), S is the effective membrane area (m^2), and t is the time of during the ultrafiltration process (h).

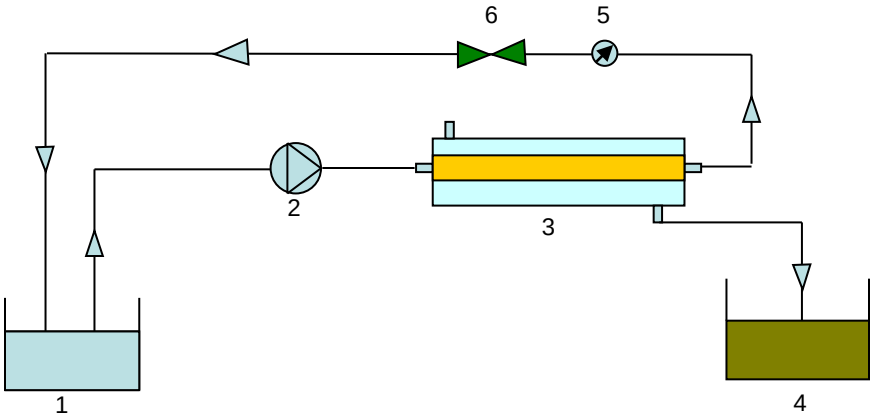

Fig. 1. Experimental set-up for membrane recovery system: 1. Hydrolysate; 2. peristaltic pump; 3. membrane module; 4. transdermal solution; 5 diaphragm; 6 relief valve

Table 1. Box–Behnken Design Matrix for the Experimental Values for the Membrane Flux

Run	Coded variable levels			Membrane flux (L/(m^2·h))
	X_1 (Bar)	X_2 (℃)	X_3 (pH)	Experimental values
1	2.0	30	6	2.08
2	1.0	35	5	1.91
3	1.5	40	5	2.05
4	1.5	30	7	1.93
5	1.5	35	6	2.29
6	2.0	35	5	2.08
7	1.5	35	6	2.27
8	1.5	35	6	2.31
9	1.5	40	7	2.01
10	2.0	35	7	2.03
11	1.5	35	6	2.28
12	1.0	40	6	1.96
13	2.0	40	6	2.29
14	1.5	35	6	2.31
15	1.0	30	6	2.03
16	1.0	35	7	1.98
17	1.5	30	5	2.09

Box–Behnken experimental design

The effect of three independent variables on the membrane flux was studied using a three-level, three-factor factorial Box–Behnken design (BBD). The three independent variable sets were operation pressure (bar, X_1), temperature (°C, X_2), and pH (X_3), and each variable was set at the three levels. The range and levels of the variables investigated are given in Table 1. A total number of 17 experiments were employed for the recycling cellulase (Table 1). Regression analysis was performed for the experiment data and fitted to the empirical second-degree polynomial model, as shown in the following equation,

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i \chi_i + \sum_{i=1}^{3} \beta_{ii} \chi_i^{\,2} + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} \chi_i \chi_j \quad (3)$$

where Y is the predicted value, b_0, b_i, b_{ii}, and b_{ij} are regression coefficients in the constant, linear, quadratic, and interaction coefficients, respectively; and X_i and X_j are the coded values of the factors.

Statistical Analysis

All experiments were performed in duplicate, and the average values were reported. Statistical significance was evaluated using the two-tailed, unpaired Student's t-test for comparisons between two means. A value of $p < 0.05$ was considered to indicate statistical significance. A software Design-Expert 8.0.5 Trial (State-Ease, Inc., Minneapolis, MN, USA) was used to obtain the coefficients of the quadratic polynomial model and estimate the response of the dependent variables.

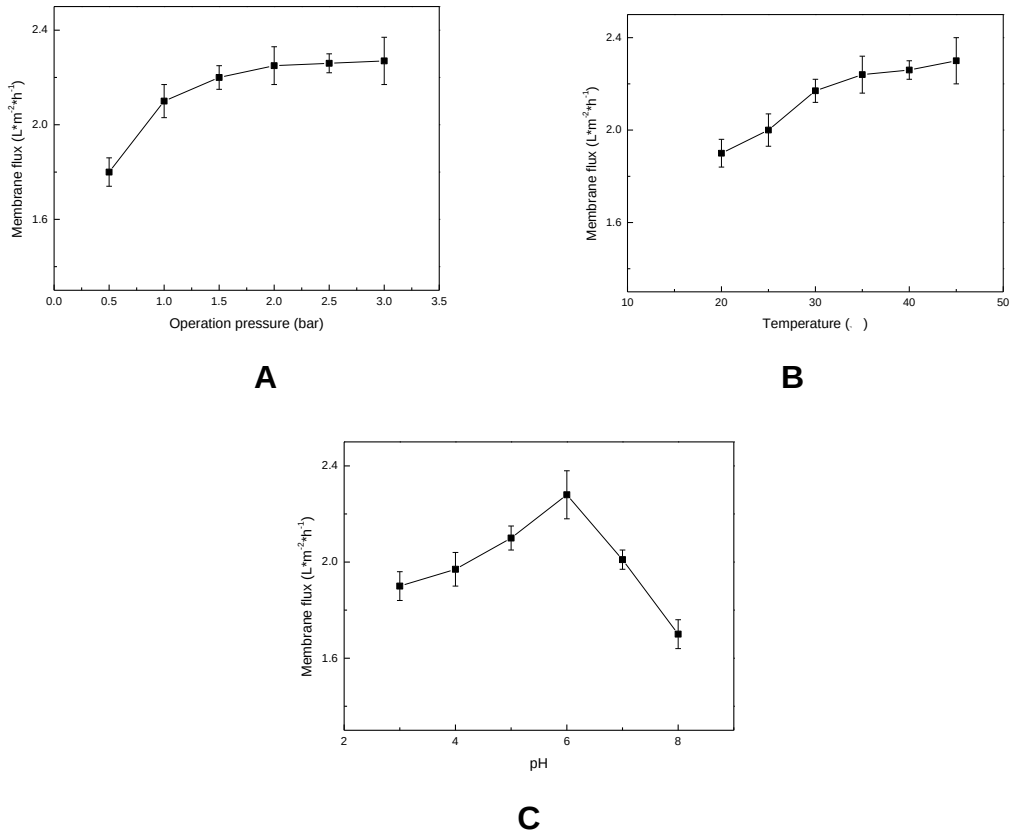

Fig. 2. Effect of different experimental parameters on membrane flux

RESULTS AND DISCUSSION

Determining levels for independent variables

The effect of different operation pressure on the recycling of cellulase was examined at 0.5, 1, 1.5, 2, 2.5, and 3 bar at 35 °C in hydrolysate (pH 6). The membrane flux during recycling cellulase with respect to operation pressure is shown in Fig. 2A. The membrane flux significantly increased from 1.74 to 2.21 L/(m^2•h). The results showed a positive correlation between the operation pressure and the membrane flux. Inactivation of cellulase was induced by high temperature and the improper pH (Ma *et al.* 2013). Therefore, temperature is one of the factors affecting membrane flux effectiveness. The influence of temperature at 20, 25, 30, 35, 40, and 45 °C was studied with an operation pressure of 1.5 bar in hydrolysate (pH 6) (Fig. 2B). The membrane flux was steady at 35 °C. Therefore, 30, 35, and 40 °C were chosen for the coded extraction time variable levels at -1, 0, and +1, respectively. The influence of pH of 3, 4, 5, 6, 7, and 8 was studied with the operation pressure of 1.5 bar and temperature of 35 °C (Fig. 2C). The highest membrane flux was observed at pH 6, where 2.21 L/m^2•h of recycling cellulase was obtained (Fig. 2C). However, enzyme activity was not inactivation in the single factor experiments.

Table 2. Analysis of Variance (ANOVA) for RSM Regression Equation

Source	Estimated coefficients	df	Mean Square	F	Prob > F
Model	2.298	9	0.0376	19.4048	0.0004
A- Operation pressure	0.075	1	0.0450	23.1959	0.0019
B- Temperature	0.023	1	0.0041	2.0876	0.1917
C-pH	-0.023	1	0.0041	2.0876	0.1917
AB	0.070	1	0.0196	10.1031	0.0155
AC	-0.030	1	0.0036	1.8557	0.2153
BC	0.030	1	0.0036	1.8557	0.2153
A_2	-0.114	1	0.0547	28.2062	0.0011
B_2	-0.094	1	0.0372	19.1774	0.0032
C_2	-0.184	1	0.1426	73.4802	< 0.0001
Residual		7	0.0019		
Lack of fit		3	0.0035	4.5455	0.0888
Pure Error		4	0.0008		
C.V. %	2.080				
R^2	0.962				

Response surface optimization of recycling cellulase conditions

Table 1 presents the BBD experimental design and the membrane flux response data. Experimental results for the analysis of variance and corresponding interactions are shown in Table 2. The coefficient of determination (R^2) of the model was 0.962, which illustrated that the model adequately represented the real relationship between the parameters chosen. The predicted second-order polynomial model was (Eq. 4):

$$Y = 2.298+0.075\chi_1+0.0225\chi_2-0.0225\chi_3+0.07\chi_1\chi_2-0.03\chi_1\chi_3+$$
$$0.03\chi_2\chi_3-0.114\chi_1^2-0.094\chi_2^2-0.184\chi_3^2 \quad (4)$$

The optima established with response surface methodology were: an operation pressure of 1.73 bar, temperature of 36.38 °C, and a pH of 5.92. Under these conditions, the model predicted a membrane flux yield of 2.317 L/(m^2•h); experimentally, a yield of 2.32 L/(m^2•h) (n = 3) was obtained, confirming the validity of the response surface methodology model. The F-test with very low probability values ($p < 0.0001$) indicated the models were highly significant.

Effect of the recycling cellulase concentration on the UF membrane

In order to obtain higher concentrations of cellulose in the progress of recycling cellulose, experimental parameters were set: operation pressure at 1.73 bar, temperature of 36.4 °C, and a pH of 5.9. As shown in Fig. 3, the membrane flux of PS30 ultrafiltration membranes exhibited a decay over 60 min of run time; therefore, the trapped fluid was collected in 24 min in subsequent work. Under these conditions, permeate samples were collected every 6 min and marked as a, b, c, and d. The trapped fluid was collected in 24 min. However, the trapped fluid was marked as J. The content of cellulase was determined by the Bradford method (Bradford 1976).

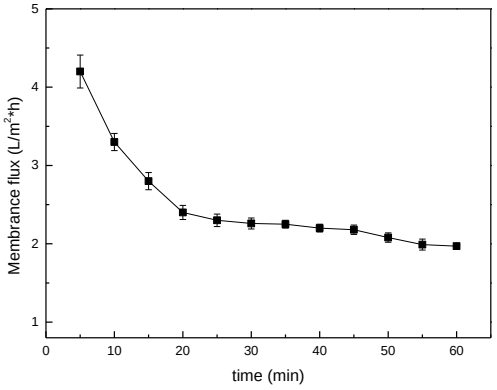

Fig. 3. The effect of the ultrafiltration time on membrane flux

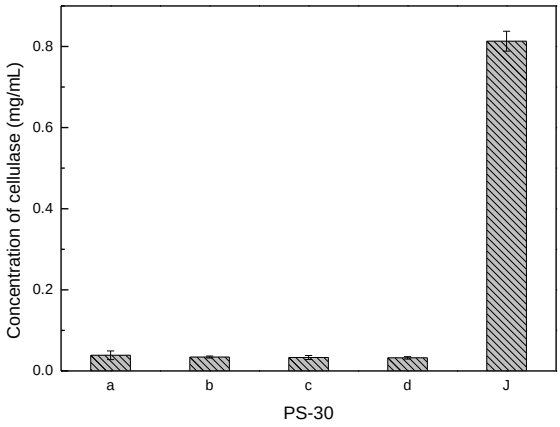

Fig. 4. The cellulase content of the permeate and retentate in the membrane separation process

Figure 4 showed preliminary results with the hollow-fiber PS30 UF membranes. The content of cellulase was insignificant, below 0.05%. The content of celluluase did not increase over time through recycling cellulose, indicating little effect on membrane separation concentrated in the ultrafiltration within 20 min, and the content of celluluase was up to 0.8 mg/mL in the trapped fluid. Therefore the retention rate of cellulase was over 95%. The results showed that the PS30 UF membrane was suitable for recycling cellulase from enzymatic hydrolyzate of laser-pretreated corn stover.

Effect of the total reducing sugars concentration on the UF membrane

In order to study the separation characteristics of PS30 hollow fiber membrane, the total reducing sugars concentration of the permeate solution and the trapped solution was determined by utilizing the DNS solution dosage. As shown in Fig. 5, the results showed that the total reducing sugars concentration of the permeate solution was a little lower than in the trapped solution. However, the total reducing sugars concentration was not significantly different in the filtrate *vs.* the retentate solution. Therefore, the PS30 hollow fiber membrane could effectively recover cellulase, but it did not affect the concentration of total reducing sugars.

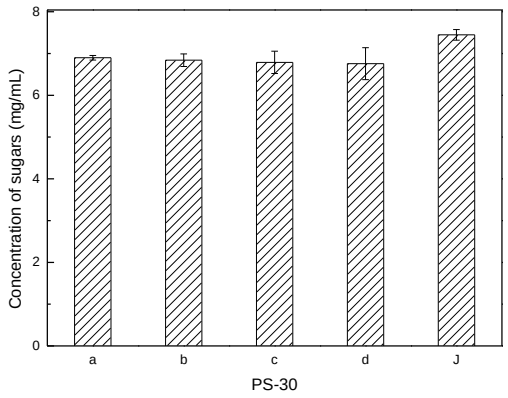

Fig. 5. The total reducing sugars content of the permeate and retentate in the membrane separation process

CONCLUSIONS

1. The PS30 hollow fiber UF membranes were suitable for recycling cellulase. The Box–Behnken design (BBD) established the following optimum pretreatment parameters: an operation pressure of 1.73 bar, temperature of 36.38 °C, and pH of 5.92. Under these conditions, the membrane flux yield reached 2.32 L/(m^2•h).
2. There was little effect on membrane separation concentrated in the ultrafiltration within 20 min, and the content of celluluase was up to 0.8 mg/mL in the trapped fluid. Therefore, the retention rate was over 95%.
3. The hollow fiber UF membrane can effectively recover cellulase; however, the concentration of total reducing sugars remains otherwise substantially unchanged.

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