Fed-Batch Mode Optimization of SSF for Cellulosic Ethanol Production from Steam-Exploded Corn Stover

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To achieve a desired ethanol concentration and maximize substrate concentration, the fed-batch simultaneous saccharification and fermentation (SSF) process was performed on steam-exploded corn stover using the yeast strain *Saccharomyces cerevisiae* Y5. The fed-batch SSF experiments were conducted with feed loading and scheduled feed time conditions that were optimized with response surface methodology (RSM). The overall ethanol yield (based on the raw material cellulose content) in 48 h was as high as 64.0%, which was achieved with a final substrate loading of 26%(w/w), enzyme loading of 7 FPU/g cellulose, and dry yeast loading weight of 2.0 g/L. No additional yeast cells or enzymes were added during solid substrate fermentation.

Keywords: Overall ethanol yield; Fed-batch SSF; Steam-exploded corn stover; High solid loading; Response surface methodology

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INTRODUCTION

Agricultural residue is a popular material to bioconvert to ethanol due to its low lignin content and low-bulk density. Using this abundant and renewable carbohydrate source in place of fossil fuels is one of the most effective ways to fight both the energy crisis and environmental problems caused by biowaste accumulation and carbon dioxide emissions (Lin and Tanaka 2006; Gnansounou 2010; Limayem and Ricke 2012; Sanchez and Cardona 2008; Balat and Balat 2009; Öhgren *et al.* 2006a).

Many technological barriers prevent the economical production of ethanol from lignocellulosic biomass. For cellulosic ethanol production to be economically viable on an industrial scale, it is of great practical importance to maximize ethanol yield from the cellulose fraction, and the ethanol concentration must be above 4% (v/v) in the fermentation broth (Manzanares *et al.* 2011; Wingren *et al. 2003;* Öhgren *et al.* 2006b). For most types of lignocellulosic materials, this requires operating at dry mass concentrations of about 15% to achieve sufficiently high cellulose levels (Jørgensen *et al.* 2007). However, the amount of dry matter (DM) required presents a challenge to the simultaneous saccharification and fermentation (SSF) procedure. A high substrate concentration also increases the concentration of inhibitors such as furfural, hydroxymethylfurfural (HMF), and acetic acid, which significantly affect enzyme and yeast performance (Liu *et al.* 2010). In addition, high viscosity results in lower mixing and heat transfer efficiencies (Varga *et al.* 2004; Georgieva *et al.* 2008; Wang *et al.* 2011). Most of the reported studies on batch SSF at high substrate loadings resulted in low conversion efficiencies unless high cellulase and yeast loading levels were used (Öhgren *et al.* 2006a; Chu *et al.* 2012; Sassner *et al.* 2006). For example, an enzyme loading of 21.5 FPU/g glucan and yeast loading of 5 g/L dry weight produces an ethanol concentration of 25 g/L with an SSF

efficiency of approximately 68% when steam-exploded corn stover is used at a solids loading of 20% (Zhang *et al*. 2010).

Instead of adding all of the substrate at the start of the reaction, adopting a fed-batch strategy can alleviate the high viscosity. Fresh substrate is added only when the viscosity has been decreased after several hours of reaction. A suitable feed rate also may allow the continuous conversion of inhibitors, which results in lower enzyme and yeast inhibition.

In this study, the fed-batch SSF process was applied to a high-solid load of steam-exploded corn stover. The fed-batch method factors, including the amount of feeding substrate and feeding time, were optimized by response surface methodology (RSM) to increase ethanol concentration and shorten the ethanol production period. The results suggest that it is possible to use fed-batch SSF to meet a desired ethanol concentration using a high solid concentration of pretreated lignocellulosic material with low enzyme and yeast loading.

EXPERIMENTAL

Raw Materials and Chemicals

Non-detoxified, steam-exploded, pretreated corn stover was provided by Henan Tian Guan Group Co., Ltd (Henan, China). The natural corn stover was chipped to 1 to 2 cm and steam-exploded at 2.0 MPa and 205 ºC for 5 min. Steam-exploded corn stover was air dried at room temperature and then used as the substrate for enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF). The moisture content of the substrate was 10%.

The composition of corn stover was determined using a previously reported method (Sluiter *et al.* 2008) (Table 1). Celluclast 1.5 L and Novozym 188 (β-glucosidase) were supplied by Novozymers A/S (Bagsværd, Denmark). Other chemicals, including culture medium ingredients and sodium acetate, were from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). All of the chemicals were of analytical quality.

Table 1. Chemical Compositions of Untreated and Treated Corn Stover (% of DM)

Composition	Raw material	Steam-explosion pretreated
cellulose	38.9	45.7
hemicelluloses	23.2	12.0
lignin	19.1	23.0

Microorganism, Medium, and Culture Conditions

Saccharomyces cerevisiae Y5 is a strain newly developed in our laboratory (Patent No.:ZL200810222897.7, CGMCC2660). Its detailed ethanol production profiles have been reported elsewhere (Tian *et al.* 2010). The yeast was cultured on YPD plates containing 10 g/L yeast extract, 20 g/L peptone, 50 g/L glucose, and 20 g/L agar. The Y5 colony was grown in filter-sterilized media containing 3.0 g/L yeast extract, 5.0 g/L peptone, and 50 g/L glucose. The inoculate culture was prepared using freshly grown cells harvested at the logarithmic growth phase and incubated with agitation at 200 rpm for 18 h at 30 ºC. Cells were harvested by centrifugation (10,000 rpm for 5 min), washed with sterile, deionized water, and adjusted to an initial cell concentration of 2 g/L dry

weight by standard curves that related 600 nm absorbance to cell concentration (Agilent 8453, UV-visible Spectroscopy system, Agilent Technologies, Santa Clara, CA, USA).

SSF Experiments

Batch mode

SSF experiments were performed in 250-mL Erlenmeyer flasks at 10 to 18% solids content (w/w) in a sodium acetate buffer (pH 4.80). The total reaction mass was 100 g. A mixture of Celluclast 1.5 L with an activity loading of approximately 20 FPU/g cellulose and Novozyme188 with an activity loading of approximately 20 IU/g cellulose was used. The experiments were initiated by enzyme addition and pre-hydrolysis for 24 h at 50 ºC in a shaker set at 100 rpm. The temperature was reduced to 35 ºC, and the yeast was added to the slurry to the same initial cell concentration of 2 g/L, which converted the process into SSF. The time of yeast addition was referred to as time 0. Samples of the fermentation broth were taken periodically and stored at -4 ºC until they were analyzed for sugar and ethanol content. Additional nutrients were added in all fermentation experiments (1 g/L yeast extract and 1 g/L peptone).

Fed-batch mode

The fed-batch SSF experiments were initiated with 14% DM and an enzyme loading of 15 FPU/g cellulose. Twenty-four-hour prehydrolysis at 50 ºC and 100 rpm was performed. The first 6% feed loading of non-detoxified steam-exploded corn stover was added at 5.5 h, and then the second 6% of the substrate was added at 12 h. After both feedings, fed-batch SSF at a final DM (w/w) of 26%. The fermentation conditions, cell mass concentration, and nutrient concentrations were the same as in the batch experiments. No additional yeast cells or enzymes were added with the addition of solid substrate during fed-batch SSF.

Response surface analysis

The response surface method (RSM; Design Expert software version 7.1.3, Stat-East Inc., Minneapolis, USA) was used to optimize the fed-batch mode parameters. The central composite design (CCD) is one of the most commonly used response surface designs for fitting second-order models (Kim *et al.* 2008; Yan *et al.* 2011). In the present study, feed time (X_1) and feed loading (X_2) were chosen as independent variables. The variable feed time was defined as the substrate addition time in fed-batch mode, and the variable feed loading was defined as the amount of feeding substrate. The levels of the two variables were designated as -1.41, -1, 0, 1, and 1.41, respectively, which were given in the variable levels X_i, coded as x_i according to the following equation,

$$x_i = (X_i - X_i^*)/\Delta X_i \quad , \tag{1}$$

where x_i is the coded value, X_i is the actual value, X_i^* is the value of X_i at the center point of the investigated area, and ΔX_i is the step change of the variable. The ranges and levels of the two variables in the experimental design are shown in Table 2.

To obtain optimal fed-batch model parameters, a second-order model was used to fit the response of ethanol concentration (Y_{ec}). The quadratic regression equation for optimization is expressed as follows,

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + b_4X_{12} + b_5X_{22} \quad , \tag{2}$$

where Y is the predicted response, b_0 is the intercept, b_1 and b_2 are the linear coefficients, b_3 is the cross-product coefficient, and b_4 and b_5 are the quadratic coefficients.

Table 2. Experimental Design and CCD Results

Run	Coded value		Actual value		Results	
	x_1	x_2	X_1(h)	X_2 (%)	Y_1 (g/L)	Y_2 (g/L)
1	-1	-1	4.58	4.82	36.0	41.2
2	1	-1	7.41	4.82	35.3	41.5
3	-1	1	4.58	7.78	35.7	39.8
4	1	1	7.41	7.78	35.2	40.4
5	-1.41	0	4	6.30	35.6	39.7
6	1.41	0	8	6.30	35.1	40.6
7	0	-1.41	6	4.20	35.5	41.6
8	0	1.41	6	8.40	35.7	39.8
9	0	0	6	6.30	38.1	42.5
10	0	0	6	6.30	38.4	42.6
11	0	0	6	6.30	38.1	42.2
12	0	0	6	6.30	37.0	41.5
13	0	0	6	6.30	37.3	41.9

The theoretical overall yield of ethanol was calculated as follows,

$$Overall\ ethanol\ yield = \frac{Ethanol\ (g)}{Cellulose_s\ (g) \times 1.1 \times 0.51} \times 100\% \quad (3)$$

where $Cellulose_s$ is the amount of cellulose in the substrate.

An analysis of variance (ANOVA) was employed to evaluate the statistical significance of the model using the same software, and Fisher's F-test was applied.

Analytical Methods

Ethanol analysis of the cellulosic substrate fermentation broth was carried out using a gas chromatograph (GC, model 7890A, Agilent Technologies) through a headspace sampler (HS, model 7694E, Agilent Technologies) using an external standard for calibration. The chromatograph was equipped with a flame ionization detector and an Agilent HJ-PEG column of 30 m with an internal diameter of 0.32 mm. Samples were run under the following conditions: column oven at 120 ºC and front injection port at 200 ºC, with N_2 as the carrier gas at a flow rate of 4 mL/min. The sugar concentration was measured using HPLC equipped with a KNAUER NH_2 column (5-mm particle size, 250 mm × 4.6 mm) and a KNAUER RI detector (model K-2301). Samples were run at a temperature of 30 ºC and a mobile phase of acetonitrile/ultrapure water at a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Batch SSF

SSF can eliminate sugar inhibition under high solid loading conditions by maintaining a low glucose concentration in SSF broth, which enhances cellulose saccharification (Cantarella *et al.* 2001; Ask *et al.* 2012; Olsson *et al.* 2006). As shown in Fig.

1a, the ethanol concentrations in the reaction system increased correspondingly when the substrate loading increased from 10% to 12% to 14%. The maximum ethanol concentration reached 28.6 g/L after 72 h with a substrate loading of 14% (Fig. 1a). However, when the solids content in SSF was increased, the ethanol yield tended to decrease (Fig. 1b). In practice, it is difficult to achieve good ethanol yields above a substrate loading of around 14% in batch experiments. When the substrate concentration was further increased to 16%, the substrate in the reaction system could not be liquefied within 72 h. High viscosity was a significant problem with high substrate loading and resulted in mass and heat transfer problems. In fact, during the batch process, more than 90% of the ethanol was produced in the first 48 h. The negative impact of the increase in substrate loading was obvious, and a substrate concentration of 14% was the limit for this reaction system.

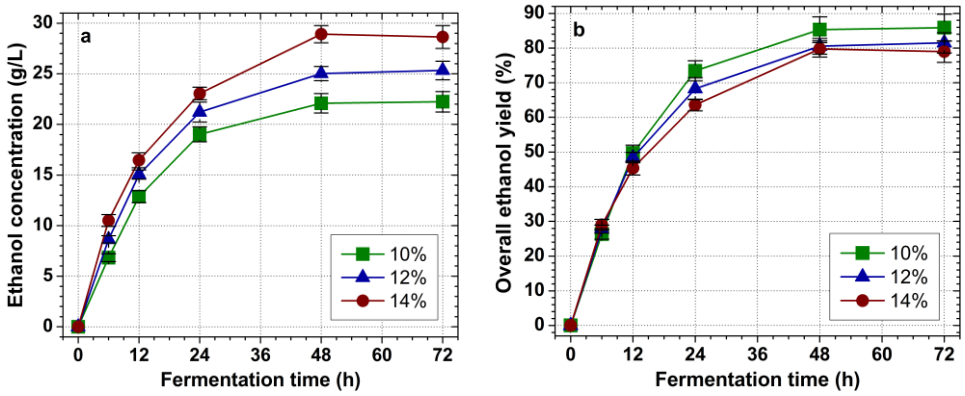

Fig. 1. Ethanol concentration (a) and overall yield (b) in batch SSF at different substrate loadings (%, DM)

The effect of enzyme reduction on overall ethanol yield in batch SSF was studied at 14% solid loading. When enzyme loading was reduced from 20 FPU/g to 15 FPU/g cellulose, the overall ethanol yield reduced slightly, from 79.0% to 76.8%, with an ethanol concentration decrease of 0.8 g/L after 72 h (Fig. 2). Further reduction resulted in lower ethanol yields, with a decrease of more than 10%. The mean values from all SSF experiments were used for parameter presentation in the fed-batch mode.

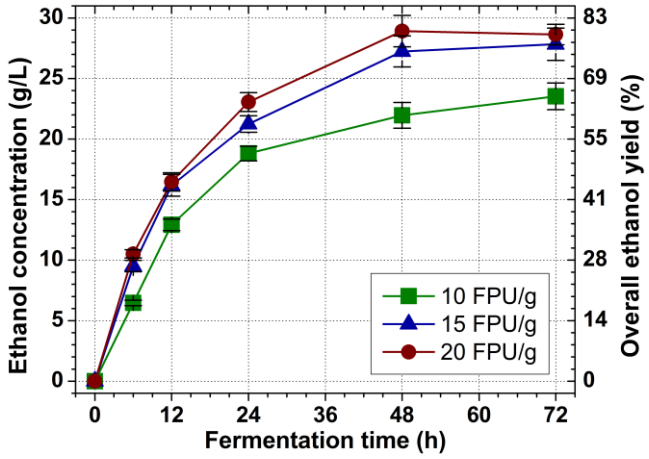

Fig. 2. Ethanol concentration and overall yield in batch SSF with different enzyme loadings

Fed-Batch SSF

Based on the initial DM concentration of 14% and enzyme loading of 15 FPU/g cellulose, the fed-batch method was used in the SSF process. The feed time and loading were optimized by RSM (Table 2). Two quadratic regression equations were obtained from the results of the CCD experiments as follows,

$$Y_1 = -0.62314 + 6.72704\ X_1 + 5.94840\ X_2 + 0.03261\ X_1X_2 - 0.59210\ X_1^2 - 0.48752\ X_2^2$$

$$Y_2 = 17.59530 + 5.38064\ X_1 + 2.91818\ X_2 + 0.03634\ X_1\ X_2 - 0.45230\ X_1^2 - 0.28308\ X_2^2\ ,$$

where Y_1 is the response at the first feed time and Y_2 is the response at the second feed time.

Table 3 shows the ANOVA results for the models. In this study, the ANOVA of the quadratic regression model indicated that the models fit the data well, with additional support from the F-test analysis.

Table 3. ANOVA for the Response Surface Quadratic Model

Source	Sum of squares	Degree of freedom	Mean squares	F-value	Probe >F
Ethanol concentration (by the first feeding) R^2=0.9136, CV=1.29%,					
Model	16.25	5	3.25	14.80	0.001
Residual	1.54	7	0.22		
Lack of fit	0.10	3	0.03	0.09	0.961
Pure error	1.44	4	0.36		
Total	17.8	12			
Ethanol concentration (by the second feeding) R^2=0.9164, CV=0.93%					
Model	11.33	5	2.27	15.35	0.001
Residual	1.03	7	0.15		
Lack of fit	0.20	3	0.07	0.32	0.811
Pure error	0.83	4	0.21		
Total	12.36	12			

A low probe >F value indicates that it is unlikely that the significant results occurred due to experiment noise because the independent variables significantly contribute to the responses. All probe >F values in the four calculated models are far less than the 0.05 significance level, which demonstrates the appropriateness of the model. Additionally, all the coefficients of determination for R^2 are greater than 90%, and all the coefficients of variation for CV are less than 2%, which further imply the precision and reliability of the experiments.

Two-dimensional (2D) contour curves and related 3D-response surface plots of the central composite design for the optimization of the fed-batch model are shown in Fig. 3. As shown in Fig. 3A with the first substrate addition, the maximum ethanol concentration production was observed with relatively high feed loading and a relatively short feed time. After a long feed time interval and with low feed loading, the ethanol concentration approached the maximum for the second fed-batch process (Fig. 3B). The optimum values of the studied variables were determined by solving the regression equations, which gave the following results in terms of coded values: X_1 = -0.103, X_2 = -

0.002 for the first feeding, and X_1= 0.121, X_2 = -0.504 for the second feeding. The actual values obtained by substituting the respective optimum values in equations were as follows: first feed loading 6.3%, feed time 5.85 h; second feed loading 5.6%, feed time 12.02 h. The maximum response value for ethanol concentration was estimated to be 42.3 g/L.

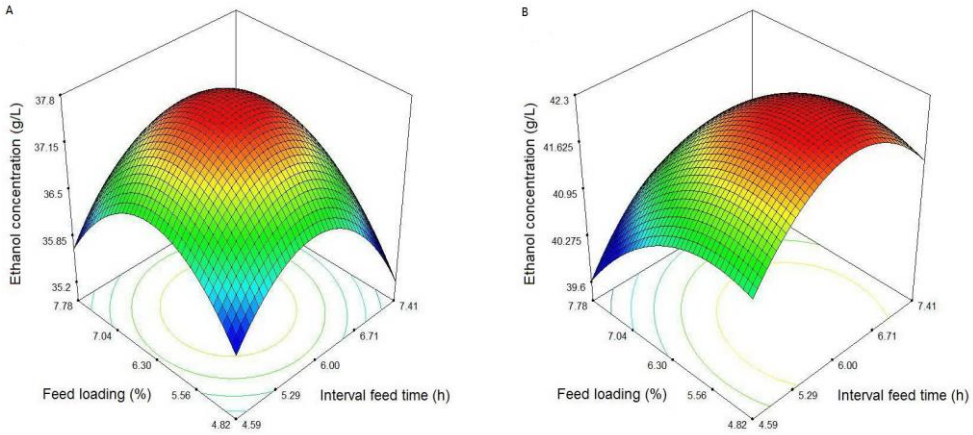

Fig. 3. Contour and response surface plots of the CCD for fed-batch SSF optimization: effects of feed time and loading during the first feeding (A); effects of feed time and loading during the second feeding (B)

To confirm the predicted optimum conditions, fed-batch SSF experiments were conducted in triplicate under theoretically optimum conditions. Based on the regression equations, we first added 6% of the substrate at 5.5 h, and then added the second 6% of the substrate at 12 h (Fig. 4). After both feedings, fed-batch SSF at a final DM (w/w) of 26% produced the highest ethanol concentration, of 41.7 g/L, from steam-exploded corn stover, corresponding to an overall ethanol yield of 64%. The initial glucose concentration in the fermentation broth was 13 g/L after presaccharification. The glucose consumption rate for the fed-batch SSF in the first 2 h was rapid, at about 7.7 g/L/h. After 2 h, no residual glucose was detected. We did not supplement yeast cells or enzymes after the addition of solid substrate during fed-batch SSF in the present study. Thus, the enzyme dosage during the whole process decreased more than two-fold, from 15 FPU/g cellulose to 7 FPU/g cellulose.

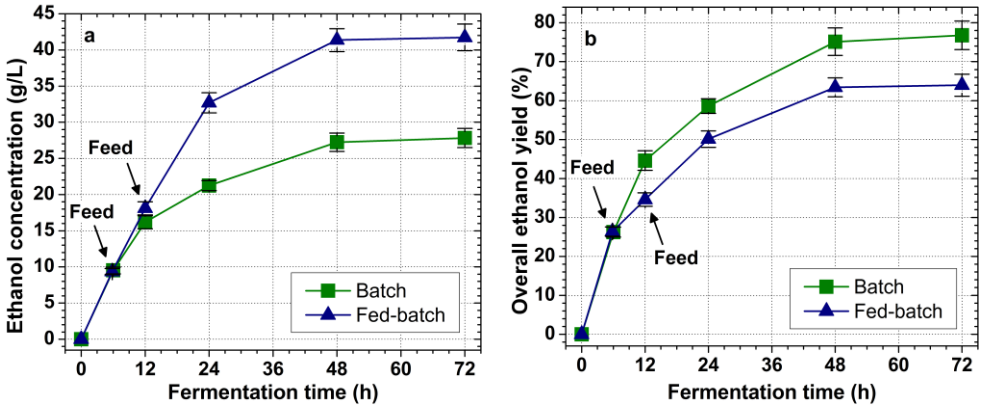

Fig. 4. Ethanol concentration (a) and overall ethanol yield (b) in batch SSF and fed-batch SSF

Enzyme loading levels are clearly important for the economy of the process. Techno-economical calculations have indicated that a 50% reduction in enzyme loading is beneficial if the yield decreases less than 6 to 7% (Sassner *et al.* 2008). These values meet the requirements for economically viable, industrial-scale production of ethanol from lignocellulose. However, enhanced absorption and obstruction of cellulose caused by increased lignin concentration can reduce material fluidity and result in deteriorated conditions for enzymatic hydrolysis and fermentation. As shown in Table 4, there were differences in overall ethanol yield between the batch and fed-batch models.

Table 4. SSF Conditions and Ethanol Production Parameters in Batch and Fed-Batch Modes

Fermentation mode	Feed time (h)	Total Substrate conc. (% of DM)	Enzyme loading (FPU/g cellulose)	Ethanol conc. (g/L)	Overall ethanol yield (%)
Batch	n	14	15.0	27.8	76.8
Fed-batch (by first feed)	5.5	20	9.4	37.4	71.4
Fed-batch (by second feed)	12	26	7.0	41.7	64.0

CONCLUSIONS

1. To obtain high ethanol production at high substrate concentrations with low enzyme and yeast loadings, the feed loading and scheduled feed time during the fed-batch SSF experiments were conducted under theoretical optimum conditions determined by RSM.
2. An ethanol production above 40 g/L was achieved from steam-exploded corn stover by employing fed-batch simultaneous enzymatic saccharification and fermentation with a total solids loading of 26% (w/w) with 7 FPU/g cellulose and 2 g/L yeast.
3. No additional yeast cells or enzymes were added with the solid substrates, and approximately 95% of the ethanol was produced during the first 48 h of fermentation.
4. Our results suggest a potential for decreasing enzyme dosage and yeast cell concentration while maintaining a feasible ethanol yield in a short production period.

ACKNOWLEDGMENTS

The authors acknowledge the financial support received from the National Key Technology R&D Program (No. 2013BAD22B03), the National Natural Science Foundation of China (No. 31100578), and the Education Committee of Beijing (No. KZ201310028034). We thank the Henan Tian Guan Group Co., Ltd., for providing the steam-exploded corn stover.

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Article submitted: July 17, 2013; Peer review completed: August 21, 2013; Revised version received and accepted: September 10, 2013; Published: September 25, 2013.