Dynamic Analysis of Bioethanol Production from Corn Stover and Immobilized Yeast

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The use of low cost and abundant corn stover in yeast fermentation can reduce product costs. In this study, bioethanol was produced from a hydrolysate of corn stover using immobilized yeast. A kinetic model was established for the total reducing sugar consumption and the production of bioethanol. The parameter estimation for kinetic modeling considered the main process variables during bioethanol production from corn stover. Total reducing sugar concentrations decreased exponentially in the bioethanol fermentation for 6 h; consumption was more than 90%. To use kinetic modelling of yeast growth for bioethanol fermentation, the value of μ_{max} reached 0.2891 h^{-1}, and the matrix inhibition constant (K_{IS}) and production inhibition constant (K_{IP}) were 8.9154 g/dm^3 and 0.00676 g/dm^3, respectively. To use kinetic modelling of fermentation time on bioethanol, the maximum ratio of bioethanol production rate (q_{max}) reached 1.427 g/g•L. However, K_{IS} was 2.813 g/dm^3, and K_{IP} was 0.0149 g/dm^3.

Keywords: Bioethanol production; Lignocellulosic biomass; Corn stover; Kinetic modeling

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INTRODUCTION

Biomass available from agricultural residue is generated from harvesting cultivated crops. Because agricultural biomass is cheaper than starch and does not compete with food sources, it is an attractive candidate for bioconversion (Alizadeh *et al.* 2005). Lignocellulosic materials in nature are the most abundant and effective feedstock for the production of second-generation bioethanol (Kristensen *et al.* 2008; Chen *et al.* 2014). Furthermore, lignocellulosic bioethanol has the potential to replace first-generation bioethanol fuels, thus avoiding the antagonistic contradiction resulting from using food crops to produce bioethanol for transport fuels (Gonzalez *et al.* 2011). In the bioethanol industry, corn stover is a lignocellulosic material that is commonly utilized for second generation energy, as it is the most abundant and effective crop residue (Turhan *et al.* 2010; Tian *et al.* 2015).

Corn stover contains a heterogeneous mixture of carbohydrate polymers (hemicellulose, cellulose, and lignin) from the stover cell walls (Tian *et al.* 2013). The other small fraction is comprised of extractives, organic acids, salts, minerals, and many other components (Steele *et al.* 2005). The most abundant biopolymers, *i.e*., cellulose and hemicelluloses, are a potential source of the total reducing sugars (mainly glucose and xylose) for bioethanol fermentation (Vishtal and Kraslawski 2011; Tian *et al.* 2016). However, there is an urgent need for bioethanol fermentation from corn stover hydrolysate, with lower costs and higher efficiency (Mabee and Saddler 2010; Han *et al.* 2011). Immobilization of yeast cells allows them to evenly distribute throughout the bioreactor, which reduces the negative impact of by-product metabolites that accumulate during

bioethanol fermentation and enhances yeast cell stability (Ban *et al.* 2002; Alvarado-Cuevas *et al.* 2013; Berlin *et al.* 2006).

The analytical method for total reducing sugars determination in residual water samples was developed based on research concerning sugarcane processing for bioethanol production (Santos *et al.* 2016). A number of kinetic models for the biomass fermentation operations for the production of bioethanol have been reported (Lee and Fan 1983; Ezhumalai and Thangavelu 2010). However, most of these do not include process variables such as operating time, concentration of residual sugar, and yeast growth rate, which would allow evaluating their influence through simulation and optimization studies (Ertas *et al.* 2014). In this way, modeling can be used in future studies to determine, for example, whether a substrate consumption step is required for enhancing yields in the immobilized yeast fermentation process (Watanabe *et al.* 2012). In a previous study, the results showed that doping corn stover pretreated by CO_2 laser increased the yield of bioethanol from 53% to 84% (Tian *et al.* 2016). In this paper, the parameter estimation for kinetic modeling considered the main process variables during bioethanol production from corn stover, which were the concentration of residual sugar, growth rate of yeast, and fermentation time.

EXPERIMENTAL

Materials

Corn stover was collected from a farm in Harbin, China. Corn stover was ground and sized through a sieve shaker of 2 mm and pretreated with a CO_2 laser. The residue was hydrolyzed by a crude cellulase preparation supplied by Gansu Hualing Biological Technology Co., Ltd., Lanzhou, China (Tian *et al.* 2011). *S. cerevisiae* AS 2607 was a gift from the China General Microbiological Culture Collection Center (Beijing, China) and was used for bioethanol fermentation experiments.

Enzymatic Hydrolysis

The corn stover, which was pretreated by CO_2 laser, was hydrolyzed for 48 h at 50 °C, pH 5.0 (0.2 M acetate buffer), and an S/L ratio of 2% (w/v) with the crude cellulase concentration of 0.12 g (8.99 FPU)/g substrate in a shaking bath (160 rpm). Hexadecylpyridinium chloride (1.5 mL, 1 mg/mL) was added to the dilute buffer solution for sterilizing the microorganism in the hydrolyzate. After enzymatic hydrolysis, the concentration of the total reducing sugars was determined by the dinitro salicylic acid (DNS) method.

Preparation and Proliferation of Immobilized Yeast Cell

Preparation of calcium alginate beads

A 48-h culture rooted in slant culture-medium was collected and blended with a sodium alginate solution. To prepare the calcium alginate beads, 3 mL of yeast seed culture and 0.2 g of pretreated corn stover residue were doped to 2% sodium alginate in 100 mL of deionized water. An alginate solution (2%) was sprayed through a thin inner nozzle (2 to 3 mm diameter at exit) into a 150 mM $CaCl_2$ solution. The calcium alginate beads, which added the pretreated corn stover, were stored after being washed three times with deionized water to remove residual $CaCl_2$. The beads were packed and stored in deionized water at 4 °C for three days for the preparation of calcium alginate beads. The undoped immobilized yeast cell was processed using the same procedure.

Proliferation of immobilized cells

The calcium alginate beads of immobilized yeast cells were placed in the immobilization proliferation medium at 30 °C and 200 rpm. The proliferation medium were replaced with fresh medium every 12 h, for a total of four times during the proliferation. The proliferated immobilized cells were then ready for bioethanol fermentation.

High Performance Liquid Chromatography (HPLC)

The mass (g) of glucose and bioethanol were determined using an Aminex HPX-87H column (Bio-Rad, Sunnyvale, CA, USA), which has a refractive index detector with a 5 mM H_2SO_4 eluent at a flow rate of 0.6 mL/min at 60 °C. The glucose and bioethanol were filtered through a 0.22-μm membrane before the HPLC analysis. The percent yield of bioethanol (X) was calculated using the following equation,

$$X\ (\%) = \frac{C_1}{C_2 \times 0.51} \times 100 \quad (1)$$

where C_1 is the concentration of bioethanol in the fermentation liquor and C_2 is the concentration of total reducing sugar in the hydrolysate.

Atomic Force Microscope Observations

Immobilized yeast beads were observed by a Multimode 8 microscope equipped with a Nanoscope V controller and a Piezo scanner AS-12 E with a 10 mm × 10 mm maximum scan size and 2.5 mm vertical range (Bruker, Billerica, MA, USA). Images were collected in air at a room temperature of about 20 °C and a relative humidity of about 30%.

Concentration of Saccharification Broth

Concentrated hydrolysates of corn stover pretreated by CO_2 laser were prepared by vacuum evaporation (Tian *et al.* 2015). The hydrolysates were evaporated at 0.5 bar and 55 °C for about 30 min under low vacuum. After evaporation, the concentrated broth was sterilized at 120 °C for 15 min and then used for yeast fermentation.

Repeated-batch Fermentation

Repeated-batch fermentation tests were carried out to evaluate the relationship between immobilized yeast and residual sugar in the concentration of saccharification liquid (Zhang *et al.* 2012). The calcium alginate beads of immobilized yeast were washed with PBS at pH 3. Fermentation experiments were conducted in the concentrated saccharification broth at 35 °C and 150 rpm with shake bottles. The fermentation period was kept at 28 h, when all fermented broth was taken out without removing the immobilized manganese alginate beads. The immobilized beads were washed with PBS at pH 3, and the same amount of the concentrated saccharification broth was immediately added to begin the next fermentation batch.

Concentration of Total Reducing Sugar and Bioethanol

The concentration of total reducing sugar and residual was measured using the DNS method. Bioethanol concentration was detected by gas chromatography (GC-8A, Shimadzu, Tokyo, Japan) with a 20% PEG column. Isopropyl alcohol was used as an

internal standard. The column temperatures at the injector and detector were 130 °C and 110 °C, respectively. The percent yield (X) of bioethanol was calculated by Eq. 1.

Description of Kinetic Modeling

The kinetic modeling was based on the assumption that factors of influence included yeast growth rate, substrate consumption rate, and bioethanol production rate in the fermentation process. Because immobilized yeast fermentation technology is efficient in bioethanol production, this technology is widely used. However, there are few studies about immobilized yeast fermentation kinetics, especially after adding corn stover residues into fixed yeast fermentation. Therefore, this paper mainly focuses on cell specific growth rate and substrate consumption rate for bioethanol fermentation kinetics of immobilized yeast.

During bioethanol production, yeast growth rate (q_s), substrate consumption rate (μ), and bioethanol production rate (q_p) were calculated using the following equations:

$$q_s = \frac{1}{X}\frac{dS}{dt} = \frac{1}{X}\lim_{\Delta t \to 0}\frac{\Delta S}{\Delta t} \tag{2}$$

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{1}{X}\lim_{\Delta t \to 0}\frac{\Delta X}{\Delta t} \tag{3}$$

$$q_p = \frac{1}{X}\frac{dP}{dt} = \frac{1}{X}\lim_{\Delta t \to 0}\frac{\Delta P}{\Delta t} \tag{4}$$

where ΔS is the change in substrate concentration (g/L) with respect to initial total reducing sugar concentration, X (g/L) is the immobilized yeast accumulated during bioethanol production, and P is the concentration (g/L) of bioethanol.

Kinetic Modeling of Immobilized Yeast Growth

Growth kinetic modeling of immobilized cells refers to a variety of dynamic models studied in this experiment (Birol *et al.* 2002). Based on the conclusions of the study, the Hinshelwood model was used to describe the fermentation process of immobilized *Saccharomyces cerevisiae*. Kinetic modeling was described by the following equation,

$$\frac{dX}{dt} = \mu_{max}\left(\frac{S}{K_{IS} + S}\right)\left(1 - K_{IP}P\right)X \tag{5}$$

where μ_{max} is the maximum growth rate of immobilized yeast. The parameters K_{IS} and K_{IP} are the inhibition constants of the substrate and production, respectively.

Kinetic Modeling of Bioethanol Production

Based on the above-mentioned Hinshelwood model, the bioethanol production process of cell growth kinetics model was described. Its production was a complex fermentation process. The initial glucose concentration of 50 g/L of corn stover hydrolyzate was generated in the process of immobilized yeast bioethanol fermentation. Kinetic modeling of bioethanol production is shown in Eq. 6,

$$\frac{dP}{dt} = q_{max}\left(\frac{S}{K_{IS} + S}\right)(1 - K_{IP}P)X \quad (6)$$

where q_{max} is the maximum rate of bioethanol production; K_{IS} and K_{IP} are the inhibition constants of substrate and production, respectively.

Statistical Analysis

The bioethanol, total reducing sugars, and substrate concentrations were measured in triplicate, and the results were expressed as mean values. The mean values were submitted to analysis of variance (ANOVA) in Origin 9.5 software (OriginLab, MA, USA), and the level of significance was set at $P < 0.05$. Multiple comparisons of means performed by Tukey test. All data are expressed as the mean ± SD.

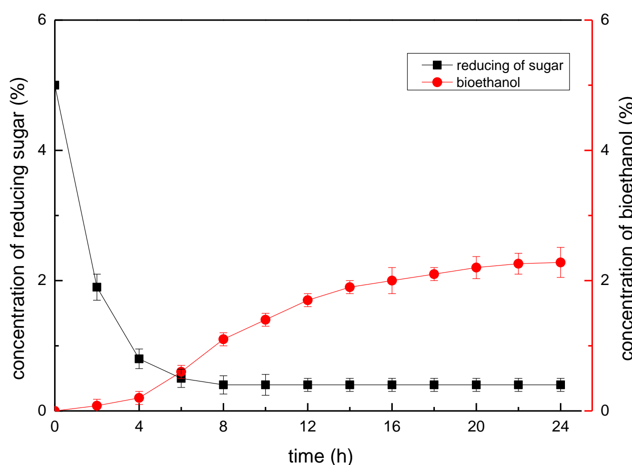

Fig. 1. The relationship between reducing sugar concentration and bioethanol concentration

RESULTS AND DISCUSSION

Effect of Reducing Sugar Consumption on Bioethanol Production

During immobilized yeast bioethanol fermentation, the total reducing sugar concentration decreased exponentially in the first 6 h; the total reducing sugar consumption was more than 90% (Fig. 1). Under these conditions, the reducing sugars produced was 98.8 mg/mL for cellulase hydrolysis. Total reducing sugars were not utilized, which was because xylose and oligosaccharides could not be used in the process. The concentration of bioethanol in the early stages of fermentation showed exponential growth, but with the passage of time, the concentration of bioethanol and fermentation level of immobilized yeast cells slowly stabilized. After about 20 h of fermentation, the bioethanol concentration reached a plateau, achieving the theoretical maximum at 24 h. After this point, increasing the fermentation time did not increase the bioethanol concentration.

Therefore, in the subsequent experiments, a 24-h fermentation time was used. Bioethanol concentration reached almost half of the total sugar concentration, which was consistent with the law of the theoretical yield of bioethanol fermentation.

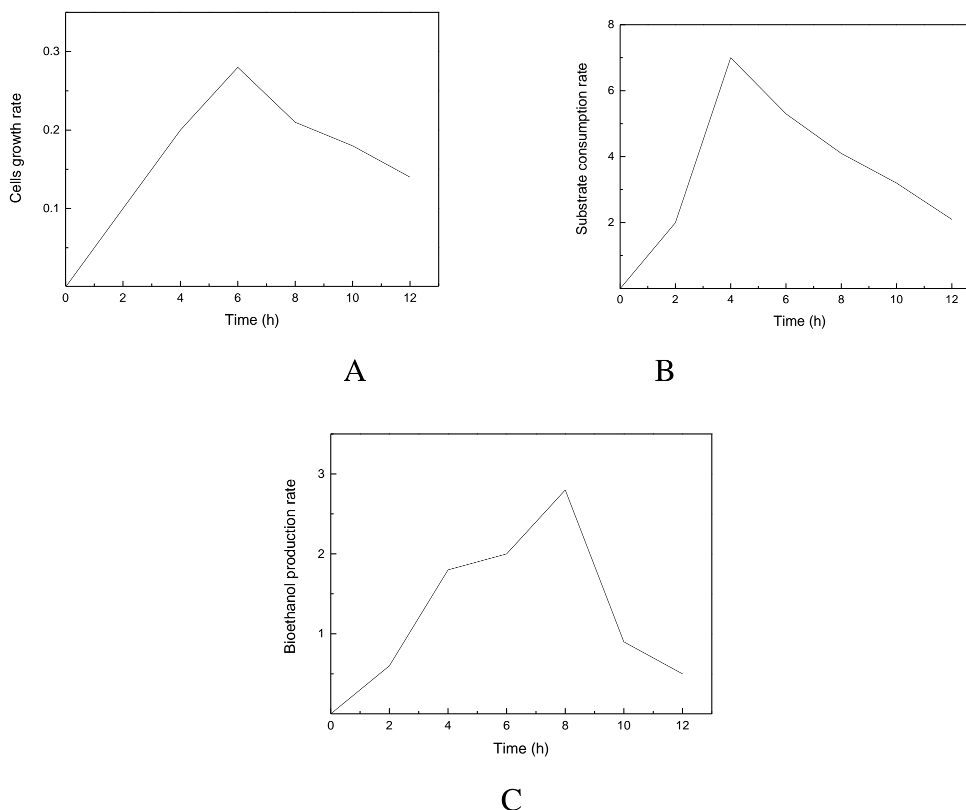

Fig. 2. Cells growth rate (A), substrate consumption rate (B), and bioethanol production rate (C) *versus* fermentation time

Nonlinear Assessment Bioethanol Fermentation Data

The effect of fermentation time on cell growth, bioethanol product synthesis, and metabolism is multifaceted. Under different conditions, fermentation time changes the nature of the medium and the activity of various metabolic enzymes (Lee *et al.* 2010). Therefore, the impact of bioethanol fermentation time is the result of the overall performance of a variety of factors. Figure 2 showed that fermentation time had a great influence on the substrate consumption rate and bioethanol production rate. Yeast growth rate, substrate consumption, and bioethanol production had a nonlinear relationship with fermentation time. Substrate consumption was significantly higher than the growth rate of cells and the bioethanol production rate. At the beginning of fermentation, the increasing rate of bioethanol was faster than that of later time. Maximum bioethanol production occurred after 4 h of fermentation. When reducing sugar consumption was completed, the yeast cells reached a maximum. The experimental data suggest dynamic kinetic modelling of yeast growth and bioethanol production.

Kinetic Modelling of Yeast Growth on Bioethanol Fermentation

Together with the nonlinear curve of Eq. 5, the data from Fig. 2 showed yeast growth kinetics parameters under different fermentation time, and the dynamic analysis of immobilized yeast growth and bioethanol production was obtained. The value of μ_{max} reached 0.2891 h^{-1}, and the fermentation reaction K_{IS} and K_{IP} values were 8.9154 g/dm^3 and 0.00676 g/dm^3, respectively. The parameter fitting coefficient of determination (R^2)

also reached 0.9979, which showed a high correlation parameter fitting. Thus, kinetic modelling for immobilized yeast cell growth had good applicability.

Kinetic Modelling of Fermentation Time on Bioethanol Production

According to the experimental data in Fig. 2, through non-linear curve fitting and evaluation, kinetic modelling of the bioethanol fermentation was obtained, and the maximum ratio of bioethanol production rate (q_{max}) reached 1.427 g/g·L. However, the matrix inhibition constant (K_{IS}) was 2.813 g/dm^3, and the production inhibition constant (K_{IP}) was 0.0149 g/dm^3. The parameter fitting correlation coefficient (R^2) also reached 0.9987, which showed that the model for the dynamics of bioethanol production had a good applicability.

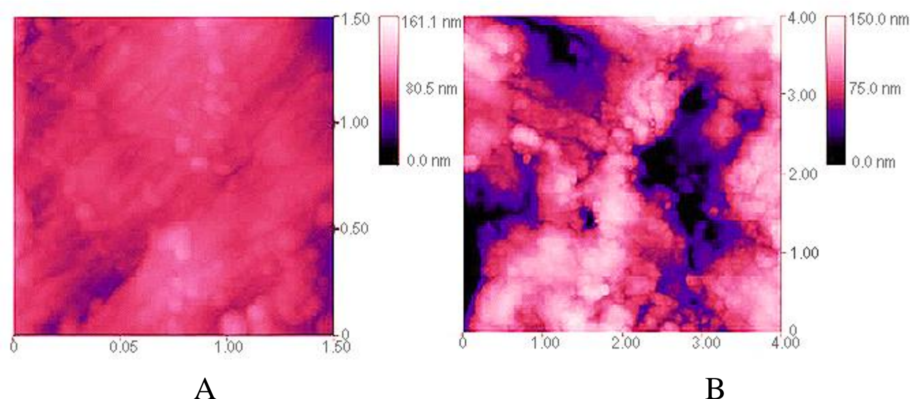

Fig. 3. AFM height image of undoped immobilized yeast (A) and doped immobilized yeast (B)

Immobilized Gel Beads Structure

The three-dimensional AFM images of undoped immobilized yeast and doped immobilized yeast are shown in Fig. 3. In Fig. 3A, the pores are not clearly distinguishable. However, in Fig. 3B the darker hues can be attributed to the presence of pores. There was evidence of tubular structures, though they were not completely defined. The structure of the doped beads may have been propitious to the binding reaction between the substrate and immobilized cells and increased the binding sites between the immobilized yeast cells and fermentable sugars in saccharification liquid.

CONCLUSIONS

1. The present study proposed kinetic modeling and parameter estimation for bioethanol production from corn stover pretreated by laser.
2. The total reducing sugar concentration decreased exponentially in bioethanol fermentation at 6 h, when the sugar consumption was more than 90%.
3. In the kinetic modelling of yeast growth and bioethanol fermentation, the value of μ_{max} reached 0.2891 h^{-1}, and the K_{IS} and K_{IP} values were 8.9154 g/dm^3 and 0.00676 g/dm^3, respectively.
4. In the kinetic modelling of fermentation time and bioethanol production, the maximum rate of bioethanol production (q_{max}) reached 1.427 g/g·L. However, the

matrix inhibition constant (K_{IS}) was 2.813 g/dm^3, and the production inhibition constant (K_{IP}) was 0.0149 g/dm^3.

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