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Production of Cellulosic Ethanol and Hydrogen from Solid-State Enzymatic Treated Cornstalk: A Two-Stage Process

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A two-stage process combining cellulosic ethanol and hydrogen production from solid-state enzymatic treated cornstalk was investigated in this study. A three-factor, five-level central composite design (CCD) with temperature (X_1), compound enzyme dosage (X_2) and time (X_3) as the independent variables was applied to optimizing technological parameters of solid-state enzymatic hydrolysis of cornstalk for cellulosic ethanol and hydrogen production. Experimental results showed that X_1, X_2 and X_3 all had an individual significant influence on ethanol production, but were insignificant on the subsequent hydrogen production. In the first stage, the maximum ethanol yield from cornstalk by *Pachysolen tannophilus* As2.1585 was 234.1 mg/g-total volatile solid (TVS) at 47.9 °C of temperature, 0.054 g/g-cornstalk of compound enzyme dosage and 10.46 days of reaction time. In the second stage, 66.9 mL/g-TVS of hydrogen was produced from the effluent of the first stage by mixed culture. The energy recovery of 50.9% showed that combine ethanol−hydrogen production possessed high energy efficiency. The ethanol was attributed to the bioconversion of the generated soluble sugars from the enzymatic hydrolysis of corn stalk and the hydrogen was mainly due to the biodegradation of hemicellulose and cellulose from residue of corn stalk after producing ethanol.

KEYWORDS: Cornstalk; cellulosic ethanol; cellulosic hydrogen; fermentation; solid-state enzymatic pretreatment; response surface methodology

INTRODUCTION

Due to the global environmental concerns over excessive fossil fuel usage, sustainable biomass resources have grown in importance as partial alternatives to fossil resources (*1*). Cellulosic biomass is an abundant renewable resource on earth and includes various agricultural residues. Annually, there is about 2.9 billion tons of crop stalk produced all over the world. Only in China, the annual yield of natural cellulosic biomass exceeds 0.7 billion tons, in which the amount of cornstalk is around 220 million tons (*2*). Presently, except that some of this material was used to make paper or fodder for livestock, most of it was burned after harvest or discarded, which not only has become a source of environmental pollution but also has resulted in a huge waste of a renewable resource. An alternative strategy is to convert crop straw waste to cellulosic ethanol or hydrogen as a high value-added biofuels product (*3, 4*).

Ethanol is widely recognized as an environmentally friendly and acceptable substitute for gasoline or as an additive to gasoline (*5*). Yeasts (such as *Saccharomyces* and *Pachysolen* species) and bacteria (such as *Zymomonas*) are commonly used as ethanol producers (*6*−*8*). Most of the microorganisms could only ferment soluble saccharides released from cellulosic biomass into ethanol, and very few recombinant strains could direct utilize the cellulose to form ethanol (*9*). Similarly, hydrogen, a clean energy carrier, is also accepted as a potential substitute for fossil fuels (*10, 11*). Bioconversion of cellulosic biomass into hydrogen by mixed culture has been explored recently. For instance, the bioconversion of wheat straw into hydrogen by cow dung compost was reported by Fan et al. (*12*).

As far as we know, in the bioethanol production process from cellulosic biomass, the residue of substrate after producing ethanol has to be discarded as waste, which results in both secondary environmental pollution and waste of a renewable resource. The effluent from bioethanol producing reactor is also an acceptable feedstock for biohydrogen production by mixed culture. Provided the bioethanol production from cornstalk is combined with the biohydrogen production from cellulosic residue of ethanol fermentation, it would be a one-stone−two-birds paradigm, which not only might effectively biodegrade the cellulose-rich waste via two-stage fermentation process but also produce two clean and readily usable energy products

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(ethanol and hydrogen) in a sustainable fashion at the same time. However, no information is available on the two-stage fermentation process of ethanol−hydrogen production from cellulosic biomass.

However, bioconversion of cellulosic biomass to fuels has been hindered because of its relatively refractory structure, such as the crystallinity of cellulose and the association of cellulose and hemicellulose with lignin (*13, 14*). Pretreatment was found to play a crucial role for hydrolyzing raw cellulosic biomass into fermentable saccharide which can be converted into ethanol or hydrogen by microbe. Various pretreatment methods are widely used in biohydrogen or bioethanol production from cellulosic biomass. For example, Nguyen et al. (*15*) enhanced the hydrogen yield from microcrystalline cellulose using ionic liquid pretreatment method. Viola et al. (*16*) found that ethanol production was more efficient from eel grass via combination of steam explosion and enzymatic hydrolysis. Solid-state enzymatic pretreatment might be a better option because it is environmentally friendly and does not need expensive equipment. It has been used to improve the health promising properties of several botanical materials and food production (*17*). So far, there is no report about solid-state enzymatic treatment of cornstalk for biofuels production.

The ethanol or hydrogen yield from cellulosic biomass might be improved by increasing the enzymolysis efficiency, which was significantly influenced by key technical parameters including temperature, enzyme dosage and reaction time. The general practice of determining these optima is by varying one parameter while keeping the other at an unspecified constant level. The major disadvantage of this single variable optimization is that it does not depict interactive effects among the variables (*10*). In order to overcome this problem, response surface methodology (RSM) is applied, which is time saving and minimizes the error in determining the effect of parameters (*18, 19*).

In the present study, a two-phase process combining production of cellulosic ethanol and hydrogen from cornstalk via solid-state enzymatic hydrolysis was investigated. Central composite design (CCD) was adopted in order to identify the optimal solid-state enzymolysis condition for maximum cellulosic ethanol−hydrogen production.

MATERIALS AND METHODS

Microorganism. *Pachysolen tannophilus* As2.1585 obtained from China General Microbiological Culture Collection Center (CGMCC, China) was used as ethanol-producing strain. It was maintained on a medium containing 20.0 g/L glucose, 20.0 g/L peptone and 10.0 g/L yeast extracts at 4 °C, and subcultured every month at 30 °C.

Dairy manure, as the hydrogen-producing microflora, was obtained from a cattle feedlot in the suburb of Zhengzhou City. Prior to use, the natural microflora was baked in the infrared oven for 2 h to suppress as much non-spore-forming hydrogen-consuming bacterial activity as possible while still preserve the activity of the hydrogen-producing spore-forming anaerobes, and then preincubated with sucrose in an anaerobic reactor at 36 °C for about 16 h.

Materials. Fresh-cut cornstalk was collected from a field of corn in the suburb of Zhengzhou City at harvest time. The material was milled by a vegetation disintegrator (FZ102) to pass through a 40-mesh screen, homogenized in a single lot to avoid compositional differences among aliquots, and stored in sealed plastic bags at 4 °C. Its characteristics were as follows: total solid (TS) 25.1%, total volatile solid (TVS) 22.0%, the soluble sugar (SS) 73 mg/g-TVS, cellulose 444.3 mg/g-TVS, hemicellulose 351.1 mg/g-TVS and lignin 98.3 mg/g-TVS.

The compound enzyme was provided by Beijing Zhongnongxiwang Biological Center. It consists mainly of cellulase 5000 IU/g, xylanase 350 IU/g, proteinase 300 IU/g and pectinase 400 IU/g, which were produced with *Trichoderma viride*, *Aspergillus niger*, *Bacillus amyloliquefaciens* and *Bacillus subtilis*, respectively. The enzyme activity was measured according to the manufacturer's instructions. The activity of cellulase was estimated using carboxymethyl cellulose as a substrate. One unit of enzyme activity was defined as the amount of enzyme capable of producing 1 mg of reducing sugars per min. One unit of xylanase activity is defined as the amount of enzyme that releases 1 mg of xylose per min using xylan as substrate. One unit of proteinase activity was defined as the amount of enzyme necessary to liberate 1 μg of tyrosine per hour. One unit of pectinase activity was defined as the quantity of enzyme capable of producing 1 mg of galacturonic acid in 1 min using apple pectin as substrate.

Table 1. Experimental Values and Coded Levels of the Independent Variables Utilized in the CCD

variable	label	units	level				
			−2	−1	0	1	2
X_1	temp	°C	35	40	45	50	55
X_2	compound enzyme dosage CED	g/g-cornstalk	0.03	0.04	0.05	0.06	0.07
X_3	time	days	3	6	9	12	15

Table 2. CCD with Three Independent Variables

run order	factor		
	x_1	x_2	x_3
1	0	0	−2
2	−1	−1	−1
3	2	0	0
4	0	−2	0
5	−1	−1	1
6	0	0	0
7	−1	1	1
8	0	2	0
9	0	0	2
10	1	1	−1
11	1	1	1
12	1	−1	1
13	0	0	0
14	−1	1	−1
15	0	0	0
16	−2	0	0
17	1	−1	−1

Solid-State Enzymatic Hydrolysis of Cornstalk. The solid-state enzymatic hydrolysis of cornstalk was performed by mixing ground cornstalk and compound enzyme in serum bottles and reacting under certain conditions. A three factor, five coded level CCD was used to determine the optimum conditions for enzymatic hydrolysis of cornstalk. Three independent variables, including temperature, compound enzyme dosage (CED) and time, were varied simultaneously relative to the chosen center point (temperature, 45 °C; CED, 0.05 g/g-cornstalk; time, 9 days) as shown in **Table 1**. For statistical calculations, the relation between the coded values x_i and actual values X_i are described as the following equation:

$$x_i = (X_i - X_i^*)/\Delta X_i \quad (1)$$

where x_i is a coded value of the variable, X_i is the actual value of variable, X_i^* is the actual value of the X_i at the center point and ΔX_i is the step change of variable.

Seventeen runs repeated in triplicate at each design point were done in a totally random order as shown in **Table 2**, which represents the design matrix of the variables using CCD. The behavior of the system is explained by eq (2).

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

where Y_i is the predicted response, β_0 is a constant, β_i is the liner coefficient, β_{ii} is the squared coefficient and β_{ij} is the cross-product coefficient.

The Minitab software (version 14.11, Minitab Ltd.) was used for the regression and graphical analysis of the experimental data. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour plots (*20, 21*). The solid-state enzymatic treated cornstalk was kept at −20 °C until used for analysis and further ethanol−hydrogen fermentation.

Ethanol−Hydrogen Fermentation. The two-stage bioprocess for ethanol and hydrogen fermentation was conducted bathwise using two serum bottles with a total volume of 140 mL. For ethanol production experiments, the yeast was revived in growth medium consisted of 15.0 g/L glucose, 5.0 g/L xylose, 10.0 g/L yeast extracts, 3 g/L $(NH_4)_2SO_4$, 2.0 g/L KH_2PO_4, 0.1 g/L $MgSO_4$ and 0.25 g/L $CaCl_2$ at pH 5.5. After incubation at 30 °C with shaking at 150 rpm for 24 h, the culture was used as seed culture. The ethanol fermentation medium contains (g/L) $(NH_4)_2SO_4$ 5, peptone 2.5, KH_2PO_4 2, $MgSO_4$ 0.1 and the saccharified cornstalk 160. The initial pH was adjusted to 5.5 ± 0.1 by NaOH (2 M). The bottles were covered with cotton plug and then sterilized by autoclaving at 121 °C for 20 min. The inoculums were transferred to the ethanol fermentation bottles (first bottle) aseptically containing 30 mL of the fermentation media. The cultivations were performed at 30 ± 1 °C in a gyratory incubator with a shaking speed of 100 rpm for 24 h. Samples were withdrawn periodically to determine ethanol content and stored at −20 °C prior to analysis. Afterward, the effluent of the first bottle was boiled to remove ethanol and then transferred into the second bottle for hydrogen production. The preincubated hydrogen-producing inoculums 20 mL was added to the second bottles, and the final working volume was 50 mL. The initial pH values were adjusted to 7.0 in the hydrogen production stage. The bottles were gassed with nitrogen gas to remove oxygen to keep the anaerobic environment and capped with rubber stoppers. Then, the bottles were positioned in an orbital shaker with a rotation speed of 120 rpm at 36 ± 1 °C. The biogas volume at the time interval was measured by releasing the pressure in the bottle using displacement of saturated brine, and then the hydrogen concentration was determined. All the experiments were carried out independently in triplicate.

Analytical Methods. The concentrations of ethanol and hydrogen were analyzed by gas chromatography (GC, Agilent 4890) (*22*). The soluble sugar concentration was estimated using the 3,5-dinitrosalicylic acid (DNS) method (*23*). The pH values were determined by a microcomputer pH-vision 6071. The contents of TS and TVS were determined at 105 °C in a drying oven and 600 °C in a muffle furnace, respectively. The components of cornstalk, such as cellulose, hemicellulose and lignin, were measured according to the nitric acid−ethanol method (*24*) and the Van Soest method (*25*). Hydrogen gas production was calculated from the headspace measurement of gas composition and the total volume of biogas produced, at each time interval, using the mass balance equation:

$$V = V_0\gamma_i + \sum V_i\gamma_i \tag{3}$$

where V is the cumulative hydrogen gas volume at the current (i); V_0 is the volume of headspace of vial; V_i is the biogas volume discharged from the vial at the time interval (i); γ_i is the fraction of hydrogen gas discharged from the vial at the time interval (i).

Kinetic Modeling. The cumulative volume of hydrogen produced in the batch experiments followed the modified Gompertz equation:

$$H = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \tag{4}$$

where H is the cumulative hydrogen production (mL), λ is the lag time (h), P is the hydrogen production potential (mL), R_m is the maximum hydrogen production rate (mL/h), e is 2.718281828. The values of P, R_m and λ for each batch test were estimated using the solver function in Excel (version 11.0, Microsoft) with a Newtonian algorithm (*26*). In this study, P_s (defined as mL/g-TVS) and R_m' (expressed as mL/g-TVS h^{-1}) were calculated by dividing P and R_m by the initial TVS content of the substrate, respectively.

Table 3. Characterization of the Solid-State Enzyme Treated Cornstalk Obtained from the Experimental Design[a]

run order	SS (mg/g-TVS)	hemicellulose (mg/g-TVS)	cellulose (mg/g-TVS)	lignin (mg/g-TVS)	lactic acid (mg/g-TVS)
1	114.6 ± 1.2	201.5 ± 1.9	318.2 ± 2.9	94.3 ± 0.8	3.1 ± 0.1
2	242.2 ± 2.5	177.2 ± 1.8	269.7 ± 2.5	88.7 ± 0.9	3.9 ± 0.1
3	375.3 ± 3.7	178.1 ± 1.7	224.6 ± 2.4	83.7 ± 0.8	6.3 ± 0.1
4	227.8 ± 2.4	219.6 ± 2.2	268.6 ± 2.6	92.1 ± 1.0	11.0 ± 0.1
5	254.9 ± 2.6	226.3 ± 2.1	181.3 ± 1.9	90.7 ± 0.9	23.8 ± 0.2
6	513.8 ± 5.9	179.7 ± 1.6	53.9 ± 1.0	81.7 ± 0.7	25.4 ± 0.2
7	306.2 ± 3.6	165.3 ± 1.8	203.2 ± 2.2	85.1 ± 0.8	28.9 ± 0.2
8	315.3 ± 3.1	169.5 ± 1.8	190.8 ± 2.1	93.9 ± 1.0	14.7 ± 0.2
9	330.0 ± 3.4	204.5 ± 2.1	140.8 ± 1.6	83.9 ± 0.9	30.0 ± 0.2
10	312.4 ± 3.2	209.0 ± 2.2	196.7 ± 2.2	84.4 ± 0.9	15.5 ± 0.2
11	498.0 ± 4.5	153.1 ± 1.7	96.8 ± 1.2	80.6 ± 0.8	13.2 ± 0.2
12	343.7 ± 3.5	171.9 ± 1.9	186.5 ± 2.1	84.8 ± 0.8	16.6 ± 0.1
13	509.5 ± 5.7	181.1 ± 1.8	55.8 ± 0.9	81.5 ± 0.9	27.3 ± 0.2
14	266.1 ± 2.9	157.9 ± 1.7	264.3 ± 2.8	89.2 ± 0.9	4.9 ± 0.1
15	505.1 ± 5.2	183.2 ± 2.0	58.0 ± 1.1	82.1 ± 0.7	27.6 ± 0.2
16	223.8 ± 2.4	168.9 ± 1.9	305.1 ± 2.9	92.1 ± 0.9	6.7 ± 0.1
17	296.4 ± 2.8	191.7 ± 2.1	223.0 ± 2.2	88.2 ± 0.9	18.3 ± 0.2

[a] The contents of hemicellulose, cellulose, lignin and lactic acid were calculated base on the TVS of the initial cornstalk before enzyme treatment, respectively.

RESULTS AND DISCUSSION

Characterization of the Enzyme-Hydrolyzed Cornstalk. The enzymatic saccharification of cornstalk using mixed enzymes was attempted for the efficient conversion of cornstalk into sugars. The materials obtained by solid-state enzymatic hydrolysis process on the conditions presented in the experimental design (**Table 2**) were characterized. The amounts of soluble sugar, hemicellulose, cellulose, lignin and lactic acid in the solid-state enzyme hydrolyzed cornstalk are summarized in **Table 3**.

Table 3 demonstrates the degradation behaviors of the cornstalk. The obtained SS yield of the enzyme-hydrolyzed materials ranged between 114.6 and 513.8 mg/g-TVS. The amount of the hemicellulose, cellulose and lignin changed with different degrees of reduction during the enzymatic hydrolysis process. After the enzyme treatment upon the center point reaction condition (temperature, 45 °C; CED, 0.05 g/g-cornstalk; time, 9 days), the SS yield increased from 73 mg/g-TVS to 509.4 mg/g-TVS; meanwhile cellulose, hemicellulose and lignin declined from 444.3 mg/g-TVS, 351.1 mg/g-TVS and 98.3 mg/g-TVS to 55.9 mg/g-TVS, 181.3 mg/g-TVS and 81.8 mg/g-TVS on the average, respectively. The carbohydrate solubilization and the cornstalk degradation reached maximum around the center condition. It can be seen that the hemicelulose and cellulose were solubilized or degraded greatly, but the lignin was removed little during the various treatment conditions. At the same time, lactic acid was generated during enzyme hydrolysis process. The content of lactic acid increased with the increase in reaction time, which was more helpful for the degradation of hemicellulose and cellulose by compound enzyme at around pH 4.8. Once the lactic acid reached higher levels, hemicellulose was relatively easier to be degraded compared with cellulose and lignin under this weakly acidic condition.

Ethanol Fermentation. Using central composite design and response surface analysis, the effects of three independent variables, temperature (X_1), CED (X_2) and time (X_3), were investigated to determine the optimum conditions for solid-state enzymatic hydrolysis of cornstalk to bring maximum cellulosic ethanol production. It should be noted that the effect of each variable on the response is the combination of coefficients and

Table 4. Experimental Data and Predicted Values for Ethanol−Hydrogen Fermentation under Different Pretreatment Conditions[a]

run order	ethanol yield (mg/g-TVS) exptl	ethanol yield (mg/g-TVS) predicted[b]	P_s (mL/g-TVS)	cellulose[c] (mg/g-TVS)	hemicellulose[c] (mg/g-TVS)
1	51.7 ± 0.9	60.6	65.2 ± 1.1	190.2 ± 1.9	93.8 ± 0.9
2	110.2 ± 1.0	117.7	66.8 ± 1.2	146.0 ± 1.7	71.6 ± 0.8
3	163.3 ± 1.8	167.8	53.9 ± 1.4	104.8 ± 1.2	72.4 ± 0.6
4	100.1 ± 0.9	95.1	53.0 ± 1.1	145.0 ± 1.6	110.3 ± 1.2
5	113.3 ± 1.3	117.8	55.9 ± 1.3	65.3 ± 0.7	116.4 ± 1.1
6	224.4 ± 2.1	221.0	54.1 ± 1.1	29.2 ± 0.5	73.9 ± 0.9
7	135.3 ± 1.1	149.3	53.8 ± 1.2	85.3 ± 0.8	60.8 ± 0.7
8	139.8 ± 1.2	142.3	65.3 ± 1.3	74.0 ± 0.8	64.6 ± 0.5
9	151.2 ± 1.6	140.0	58.6 ± 1.1	28.4 ± 0.4	96.5 ± 0.8
10	141.9 ± 1.3	139.9	67.9 ± 0.9	79.4 ± 0.9	100.6 ± 0.9
11	223.9 ± 2.1	219.0	52.5 ± 1.1	68.3 ± 0.8	49.6 ± 0.5
12	150.8 ± 1.7	162.8	52.4 ± 1.2	70.1 ± 0.8	66.8 ± 0.5
13	219.8 ± 2.4	221.0	54.8 ± 1.1	30.9 ± 0.4	75.2 ± 0.7
14	118.2 ± 0.9	108.8	52.3 ± 1.3	141.0 ± 1.3	54.0 ± 0.4
15	221.2 ± 2.5	221.0	55.1 ± 1.0	32.9 ± 0.5	77.1 ± 0.8
16	98.7 ± 0.8	91.7	68.9 ± 0.9	178.3 ±	64.0 ± 0.7
17	135.6 ± 1.5	124.2	60.4 ± 1.3	103.4 ± 1.0	84.8 ± 0.7

[a] The yields of ethanol, hydrogen, hemicellulose, cellulose and lignin were calculated on the basis of the TVS of the initial cornstalk before enzyme treatment, respectively. [b] Calculated from the quadratic polynomial. [c] The cellulose or hemicellulose yield of cornstalk after ethanol−hydrogen fermentation.

Table 5. ANOVA Results for the Fitted Quadratic Polynomial Model of Ethanol Yield[a]

source	sum of squares	degree of freedom	mean square	F-value	probability (P) > F
model	39298.0	9	4366.44	30.93	<0.0001
residual	988.3	7	141.19		
lack-of-fit	977.2	5	195.44	35.15	0.028
pure error	11.1	2	5.56		

[a] R^2 = 0.975, R_{adj}-squared = 0.944.

variable values as well as contribution of joint effect of variables, which cannot be observed by traditional optimization methods. From the experimental design data (**Table 2**) and its corresponding ethanol yield (**Table 4**, second column), the following second order polynomial equation was determined to explain the ethanol production:

$$Y = 220.965 + 19.025x_1 + 11.8x_2 + 19.775x_3 - 22.804x_1^2 - 25.567x_2^2 - 30.192x_3^2 + 6.175x_1x_2 + 9.625x_1x_3 + 10.1x_2x_3 \quad (5)$$

where Y is the predicted ethanol yield; x_1, x_2, and x_3 are the coded values of temperature, CED and time, respectively.

The statistical significance of eq (5) was checked by an F-test, and the analysis of variance (ANOVA) for response surface quadratic model is summarized in **Table 5**. The model F-value of 30.93 implied that the model was significant ($P < 0.0001$). There was only a 0.01% chance that it could occur due to noise. Here a determination coefficient (R^2) value of 0.975 indicated a good agreement between experimental and predicted values. Analyses of the observed versus predicted ethanol yields are shown in **Figure 1**. It can be observed that most points were near the line adjustment, which meant that the mathematical model was very reliable for ethanol production.

Furthermore, the regression coefficients for eq (5), along with the corresponding P values, are presented in **Table 6**. The P values are used as a tool to check the significance of each coefficient. The smaller the value of P, the more significant is the corresponding coefficient. It can be seen from **Table 6** that all the linear, square and interaction terms of temperature (x_1), CED (x_2) and time (x_3) had a significant effect on ethanol yield with low P-values of less than 0.1 except the interactive term of x_1 x_2.

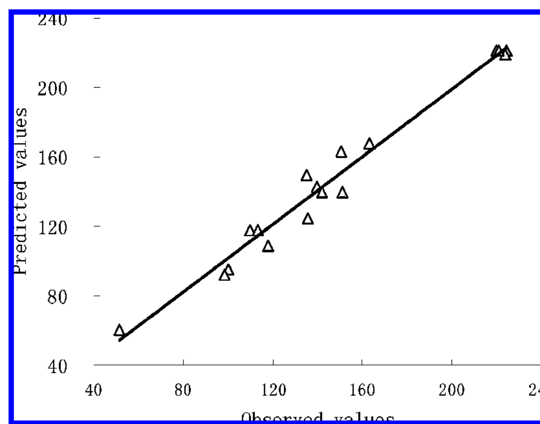

Figure 1. Plots of observed vs predicted ethanol yield values. The predicted ethanol yield values are determined by the model equations determined for CCD.

Table 6. Results of Regression Analysis of the CCD for Ethanol Production

term	coefficient	standard error	t-value	P > |t|
constant	220.965	6.555	33.709	<0.001
x_1	19.025	2.971	6.405	<0.001
x_2	11.8	2.971	3.972	0.005
x_3	19.775	2.971	6.657	<0.001
x_1^2	−22.804	2.7	−8.446	<0.001
x_2^2	−25.567	2.7	−9.47	<0.001
x_3^2	−30.192	2.7	−11.183	<0.001
x_1x_2	6.175	4.201	1.47	0.185
x_1x_3	9.625	4.201	2.291	0.056
x_2x_3	10.1	4.201	2.404	0.047

From equations derived by differentiation of eq (5), the optimal values of x_1, x_2 and x_3 in the coded units were found to be 0.5737, 0.3959 and 0.485145, respectively. Correspondingly, we can obtain the maximum point of the model, which was 47.9 °C of reaction temperature, 0.054 g/g-cornstalk of CED, and 10.46 days of reaction time, respectively. The maximum predicted value of ethanol yield was 233.6 mg/g-TVS.

Using the Minitab software, the three-dimensional response surface curves and its corresponding two-dimensional contour lines described by the regression model were constructed in **Figures 2**, **3** and **4**. Here each response surface plot represents the effect of two independent variables at an optimal level of the third variable. The shape of the corresponding contour plot indicates whether the mutual interactions between the independent variables are significant or not. As shown in **Figures 2**, **3** and **4**, the response surface of ethanol yield showed a clear peak, indicating that the optimum solid-state enzymatic hydrolysis conditions fell inside the design boundary well.

Figure 2 shows the response surface plot and corresponding contour curves based on independent variables temperature (X_1) and CED (X_2), while the third independent variable, time (X_3) was kept at an optimal level. Temperature greatly affected the enzymatic hydrolysis efficiency of cornstalk. A significantly increase on ethanol yield could be achieved when the value of temperature was increased in the range from 35 to 47.9 °C. Beyond this level, ethanol yield slightly decreased. This is mainly due to the inactivation of enzyme at the inappropriate temperature value. Usually the optimum hydrolysis temperature

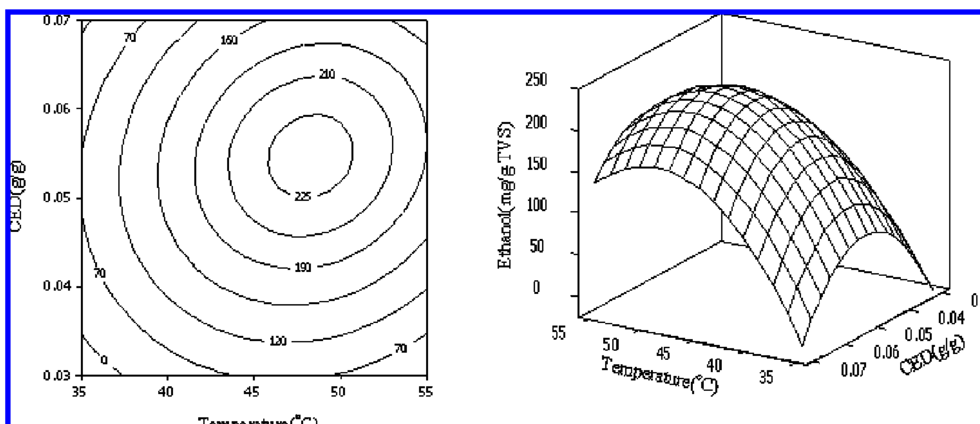

Figure 2. The response surface plot and corresponding contour plot showing the effects of temperature and CED on ethanol yield, with optimum level of time (10.46 day).

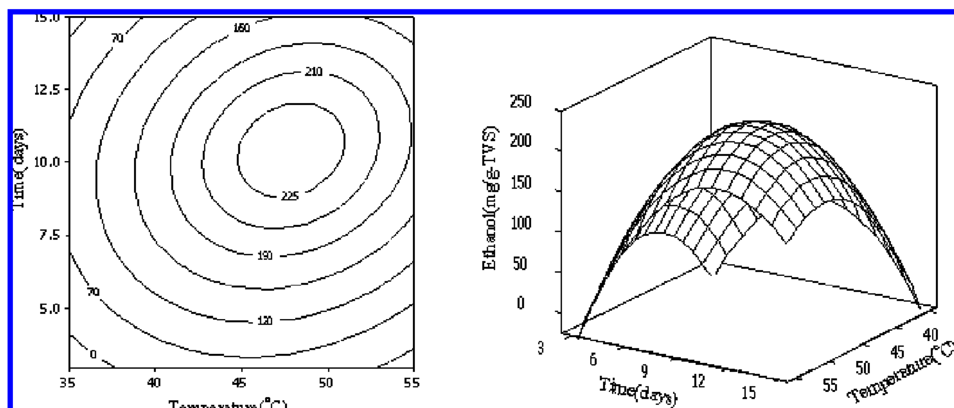

Figure 3. The response surface plot and corresponding contour plot showing the effects of temperature and time on ethanol yield, with optimum level of CED (0.054 g/g-cornstalk).

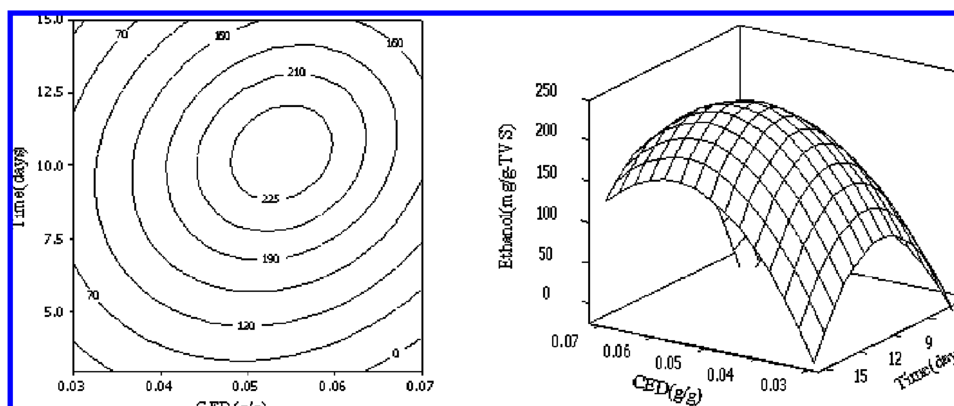

Figure 4. The response surface plot and corresponding contour plot showing the effects of CED and time on ethanol yield, with optimum level of temperature (47.9 °C).

for cellulase was found to be about 50 °C, which was dependent on the type of cellulase. The enzymatic hydrolysis efficiency was enhanced under the optimum conditions, and consequently, more soluble sugar was released and converted to ethanol. As can be seen from **Figure 2**, ethanol yield increased as the CED increased from 0.03 g/g-cornstalk to 0.054 g/g-cornstalk. But further elevating the CED showed a declining trend on ethanol yield. This observed decease of ethanol yield might be due to the product inhibition that reduced the catalytic activity of the enzymes and the conversion of generated sugars into lactic acid or other byproduct. The angle of inclination of the principal axis in **Figure 2** was slight, indicating that the positive effect of increased temperature on ethanol yield was more pronounced as CED increased. The isoresponse contour of ethanol yield showed an approximate rounded ridge running diagonally on plot, implying that temperature and CED were slightly interdependent.

Figure 3 denotes the effects of temperature (X_1) and time (X_3) for ethanol production by keeping CED (X_2) as constant. The ethanol yield was remarkably low at low values of enzymolysis temperature and time. This observation might be related mainly to the reduction of enzymolysis efficiency at this unsuitable condition. Increasing temperature or time could yield an ethanol increase in the response surface because the bioconversion of cellulose to glucose was enhanced. The response value reached its highest level at a temperature of 47.9 °C and 10.46 days of reaction time. As can be seen from **Figure 3**, ethanol yield had a sharp increase as reaction time was raised

from 3 days to about 10.46 days, beyond which ethanol yield slightly declined because the released soluble sugars were converted into lactic acid or other byproduct. The angle of inclination of the principal axis was not evidently toward either temperature or time, and this indicated that ethanol yield was nearly equally dependent on these two variables. The two-dimensional contour plot with respect to temperature and time showed a clear elongated ridge running diagonally on plot, suggesting that temperature and time were interdependent, or that there was a significant interaction on ethanol yield between temperature and time.

Figure 4 depicts the CED (X_2) and time (X_3) effects on the ethanol yield at fixed temperature (X_1) of 47.9 °C. Ethanol yield increased with increasing CED and time to optimum conditions, and then decreased with a further increase. At prolonged incubation, accumulation of the hydrolysis products would cause increased inhibition and inactivation of the enzymes. Ethanol yield was sensitive even when CED was subject to a small alteration below 0.054 g/g-cornstalk. It was obvious that the addition of compound enzyme had a particularly great effect on ethanol yield compared with the effect of time. The elliptical nature of the contour plots indicates that the mutual interactions between two independent variables (X_1, X_3) are significant.

In order to confirm the predicted result of the model, the repeated experiments under optimal conditions were carried out and a value of 234.1 ± 6.8 mg/g-TVS ($N = 5$) was obtained. The good correlation between these two results verifies the model validation and the existence of an optimal point.

Hydrogen Fermentation. As can be seen from the above statements, the utilization efficiency of cornstalk was not satisfactory in the bioconversion of the material into ethanol via the first stage fermentation. Herein, an especial interest was to attempt utilizing the effluent of ethanol fermentation to produce cellulosic hydrogen by mixed culture. The effluent of the first stage (bottle) was correspondingly transferred into the second bottle for the subsequent hydrogen fermentation. The yields of hydrogen were calculated on the basis of the TVS of the initial cornstalk before enzyme treatment. The experimental values for P_s and cellulose and hemicellulose contents under different solid-state enzymatic hydrolysis conditions (**Table 2**) are presented in **Table 4**. The regression analysis of the experimental data obtained was employed using Minitab software. The ANOVA for response surface quadratic model showed that the R^2 values for P_s was only 0.618, which meant that the model (data not shown) was not reliable for hydrogen production. The second stage of hydrogen production could not be predicted according to mathematical model.

As can be seen from **Table 4**, hydrogen yield varied between 52.3 and 68.9 mL/g-TVS in spite of the solid-state enzymatic hydrolysis conditions. After the ethanol−hydrogen fermentation stage, the hemicellulose and cellulose contents dropped respectively about 105 mg/g-TVS and 120 mg/g-TVS except for the three center point experiments, in which cellulose content decreased about 25 mg/g-TVS. These data indicated that partial cellulose and hemicellulose were disrupted by the mixed microflora during hydrogen fermentation. The hydrogen yield was mainly contributed by the direct biodegradation of the cellulose and hemicellulose during the hydrogen fermentation stage besides the utilization of the residual sugars obtained from the ethanol fermentation stage.

Figure 5 demonstrates the time course profiles of hydrogen fermentation from the effluents of the first phase under the optimized solid-state enzymatic hydrolysis conditions, which contained cumulative hydrogen yield and hydrogen content. The experiments were carried out independently in triplicate. The cumulative hydrogen production was measured with eq (3) and simulated with eq (4). With the modified Gompertz equation, P_s and R_m' were estimated as 66.9 mL/g-TVS and 2.8 mL/g-TVS h^{-1}, respectively. As shown in **Figure 5**, hydrogen production began immediately after a lag phase of 4 h and the hydrogen production rate maintained a high level at 10−25 h. The hydrogen content in the biogas increased sharply since the onset of hydrogen production and reached a maximum of 59.2 ± 1.5% at 20 h. Then after the 50 h cultivation, the cumulative hydrogen had no significant change while the hydrogen content declined slightly. Furthermore, there is no methane detected in the biogas in all runs. The high hydrogen yield and short lag time showed that the residual material was easy to be utilized by hydrogen-producing bacteria because its structure had been destroyed seriously during the solid-state enzymatic pretreatment.

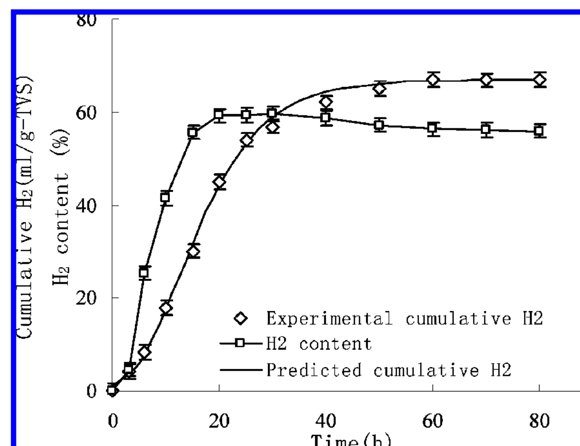

Figure 5. Time-course profile of hydrogen fermentation under optimized solid-state enzymolysis conditions.

Energy Analysis. The energy recovery rate is based on the combustion value of ethanol, hydrogen, and sugars from cornstalk. The total combustion value of cornstalk is 13.45 kJ/g-dry cornstalk. This value is based on the soluble sugar, cellulose and hemicelluloses of cornstalks, which are transformed to glucose based on the quality. The heating value of hydrogen is 285.8 kJ/mol, and the heating value of ethanol is 1367.8 kJ/mol.

In the two-stage ethanol−hydrogen fermentation, the maximum yield of ethanol and hydrogen from cornstalk were 234.1 mg/g-TVS (0.00446mol/g-dry cornstalk) and 66.9 mL/g-TVS (0.00262 mmol/g-dry cornstalk), respectively. The following energy analysis was done.

$$\text{energy recovery from cornstalk} = (0.00446 \times 1367.8 + 0.00262 \times 285.8)/13.45 \times 100\% = 50.9\%$$

The percentage of energy recovery based on the combustion values obtained in this study was found higher as compared to those calculated in the single-stage ethanol or hydrogen fermentation in terms of energy recovery from renewable biomass according to the reference (*26, 27*), which indicated that the energy efficiency can be improved by combined ethanol and hydrogen production process.

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