Enhanced Xylitol Production from Statistically Optimized Fermentation of Cotton Stalk Hydrolysate by Immobilized *Candida tropicalis*

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Original scientific paper
Received: January 14, 2013
Accepted: July 24, 2013

Dilute-acid hydrolysate of cotton stalk was prepared for the xylitol production, immobilized *Candida tropicalis* was used as the fermentation inoculum. The fermentative conditions were selected by single factor experiments, three factors were chosen as main conditions from five parameters to design the response surface test using a central composite design. Regression equation and analysis of variance were proposed with regard to the effects of reducing sugar concentration, initial pH value and inoculation ratio of yeast. The results showed that the correlation between actual and predicted values were significantly predicted by the model, the optimized fermentative conditions were determined as follows: reducing sugar concentration of hydrolysate was 94 g L^{-1}, inoculation ratio of the immobilized *Candida tropicalis* was 5.7 % (w/v), initial pH value was 5.1. Under the optimal conditions, the experimental concentration of xylitol was 13.02 ± 1.10 g L^{-1}, which was close to the theoretical predicted value, and shows that the model is feasible.

Key words:

Xylitol, response surface methodology, cotton stalk hydrolysate, immobilized *Candida tropicalis*, optimization

Introduction

Cotton (*Gossypium hirsutum*), which is one of the most abundant crops in the world, is cultivated widely in China, the United States, and Central Asia. The cotton stalk generated with cotton cultivation is an important source of lignocellulosic biomass. In recent years, cotton stalk has received increasing attention from researchers engaged in bioconversion areas, and some high-value products, such as bioethanol, biogas, single cell protein[1–4] have been manufactured from cotton stalk. Exploring more high-value products from bioconversion of cotton stalk may realize today's goal of utilization of lignocellulosic biomass.

Xylitol is a five-carbon sugar polyol, which is widely applied in the food, pharmaceutical and dental industries, as it has multiple properties, such as sweetness with low caloric content, anticariogenicity, tooth rehardening and remineralization, prevention of otitis.[5,6] Xylitol can be produced chemically by catalytic hydrogenation of D-xylose from hemicellulosic hydrolysates or be produced by some xylose-utilizing microorganisms as a natural metabolic intermediate.[7] Microbial xylitol production is more favorable for industrial applications due to mild fermentative conditions like atmospheric pressure and ambient temperature. Many yeasts, including *Candida tropicalis*, *Debaryomyces hansenii*, *Candida guilliermondii*, *Candida subtropicalis* species[8–11] can effectively convert xylose into xylitol. Recently, considerable attention has been drawn to the bioconversion of xylose into xylitol from lignocellulosic hydrolysate.[12,13]

However, due to the inhibitors in hydrolysate, it is difficult to obtain a high xylitol concentration in the fermentation broth, so some measures like decolorization, detoxification have been introduced to deal with the inhibitors in hydrolysate. Moreover, other measures like immobilization of inoculum cells, optimization of fermentative conditions are also needed to improve xylitol production.[14–17]

On the whole, the growing demand for health care and food industry has led to increased efforts to optimize the fermentative conditions of xylitol production.[18,19] However, few works have dealt with xylitol production from cotton stalk hydrolysate. In previous works, our lab had converted cotton stalk hydrolysate into bioethanol via microbial fermentation,[20] but that was just one method of realizing bioconversion of cotton stalk. xylitol production, as well as the study on optimization of fermentative conditions, may pave a new way for bioconversion of cotton stalk hydrolysate, which

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may improve the bioconversion rate of cotton stalk and the added value of bioproducts. Our laboratory, located in the largest cotton cultivating region of China, aims to develop multiple bioconversion methods to improve our utilization level of cotton stalk. In this paper, we statistically optimized the fermentative conditions of xylitol production by immobilized *Candida tropicalis*, in order to achieve better bioconversion of cotton stalk hydrolysate into xylitol.

Materials and methods

Preparation of cotton stalk hydrolysate

The cotton stalk (variety *Gossypium hirsutum Linn* Zhong 35), was harvested in early November 2011 from the cotton field in Xinjiang Alaer, China. The stalk feedstock was air-dried, comminuted and sifted in 20-mesh sieve, and hydrolyzed by 4 % (v/v) diluted sulfuric acid with solid-liquid ratio 1:5 at 121 °C for 30 minutes, the best hydrolysis condition reported previously by our laboratory,[2,20] which described that the main reducing sugar components in cotton stalk hydrolysate were glucose and xylose, and the changed composition of lignocellulose after pretreatment was composed of lignin 28.65 % (w/w), cellulose 43.85 % (w/w), hemicellulose 2.68 % (w/w), compared to the original contents of lignin, cellulose and hemicellulose in untreated cotton stalk (which were 38.15 % (w/w), 18.35 % (w/w), 12.91 % (w/w), separately). The cotton stalk hydrolysate was filtrated and collected, then placed at room temperature, the pH value of hydrolysate was modulated to 10.0 by calcium hydroxide solution, the supernatant was collected after centrifugation at 6000 rpm for 10 minutes, then the pH value was modulated to pH 5.0 by diluted sulfuric acid, finally, the sugar solution was decolorized by macroporous resin LS610, so the detoxified and decolorized hydrolysate was collected. We modulated the reducing sugar concentration to certain designed concentration used in xylitol fermentation by immobilized *Candida tropicalis*.

Media

Activation slant medium (per litre): malt extract 10 g, agar 20 g, initial pH natural.

Seed medium (per litre): D-xylose 10 g, glucose 10 g, yeast extract 1.5 g, peptone 2 g, malt extract 3 g, initial pH 5.5.

Fermentation medium (per litre): detoxified and decolorized hydrolysate 1000 mL (the concentration of reducing sugar was determined by experimental design), yeast extract 10 g, peptone 5 g, $MgSO_4$ 1 g, KH_2PO_4 0.2 g, initial pH value was modulated to the designed value.

Fermentation strain and preparation of immobilized yeast cell

Candida tropicalis (CICC 1779) was purchased from the China Center of Industrial Culture Collection (Beijing, China). A 250-mL Erlenmeyer flask containing 100 mL seed medium was inoculated with three loopfuls of cells taken from 2-day-old activation slant and incubated at 30 °C on a rotary shaker at 200 rpm for 18–24 hours, then put the seed stain at 4 °C overnight, deposited the cells totally to form yeast mud, the supernatant was discarded the next day, the remaining yeast cells were mixed with 2 % (w/v) sodium alginate solution in proportion of 1:3, next, the mixture was injected into 2 % (w/v) calcium chloride solution, and then calcified at 4 °C for 6 hours to form gel beads. Finally, the prepared gel beads were washed in sterilized water three to four times, and inoculated into fermentation medium.

Xylitol fermentation conditions

Five fermentative conditions of reducing sugar concentration, inoculation ratio, initial pH value, fermentation time and the rotary shaker speed were investigated in 500 mL Erlenmeyer flasks containing 100 mL of the fermentation medium, the effects of single factors were examined, based on which three main factors were chosen for the surface response methodology (RSM), which was then used to optimize the fermentative conditions.

Analysis methods

The xylose content of the hydrolysate was examined by orcinol spectrophotometer method found by Douglas (1981),[21] and the concentration of reducing sugar in the hydrolysate was determined by 3,5-dinitryl-salicylic acid reagent (DNS) method reported by van Soest *et al*. (1991),[22] then the glucose content was determined, as it was reported that the main reducing sugar components in cotton stalk hydrolysate were glucose and xylose.[20] The cell optical density (OD) of the yeast was determined at 600 nm using a spectrophotometer.

All fermentative samples were centrifuged at 4000 rpm for 10 minutes, and the supernatants were collected and filtered through 0.22 μm filters. The xylitol concentration was examined with spectrophotometer by using Potassium Periodate colorimetric method reported by Song and Arnold (1977).[23]

Statistical analysis of the single-factor experimental data was calculated by DPS 9.0 and performed with Origin 7.0 software. Design-Expert 8.0 was used for the experimental design and regression analysis of Response Surface design. Multiple comparison results were obtained from DPS 9.0 software with LSD method.

Results and discussion

Effects of fermentative conditions using single factors tests

Xylose and glucose were the main sugar components of dilute acid hydrolysate of cotton stalk, as reported in our previous research, which were proved to be with fermentative property by yeasts.[20] In this study, we modulated the reducing sugar to different concentrations to examine the effects of reducing sugar concentration on xylitol production. Concentrations of reducing sugar were set from 30 g L^{-1} to 130 g L^{-1}, the curve of xylitol concentration in Fig. 1a increased significantly with the

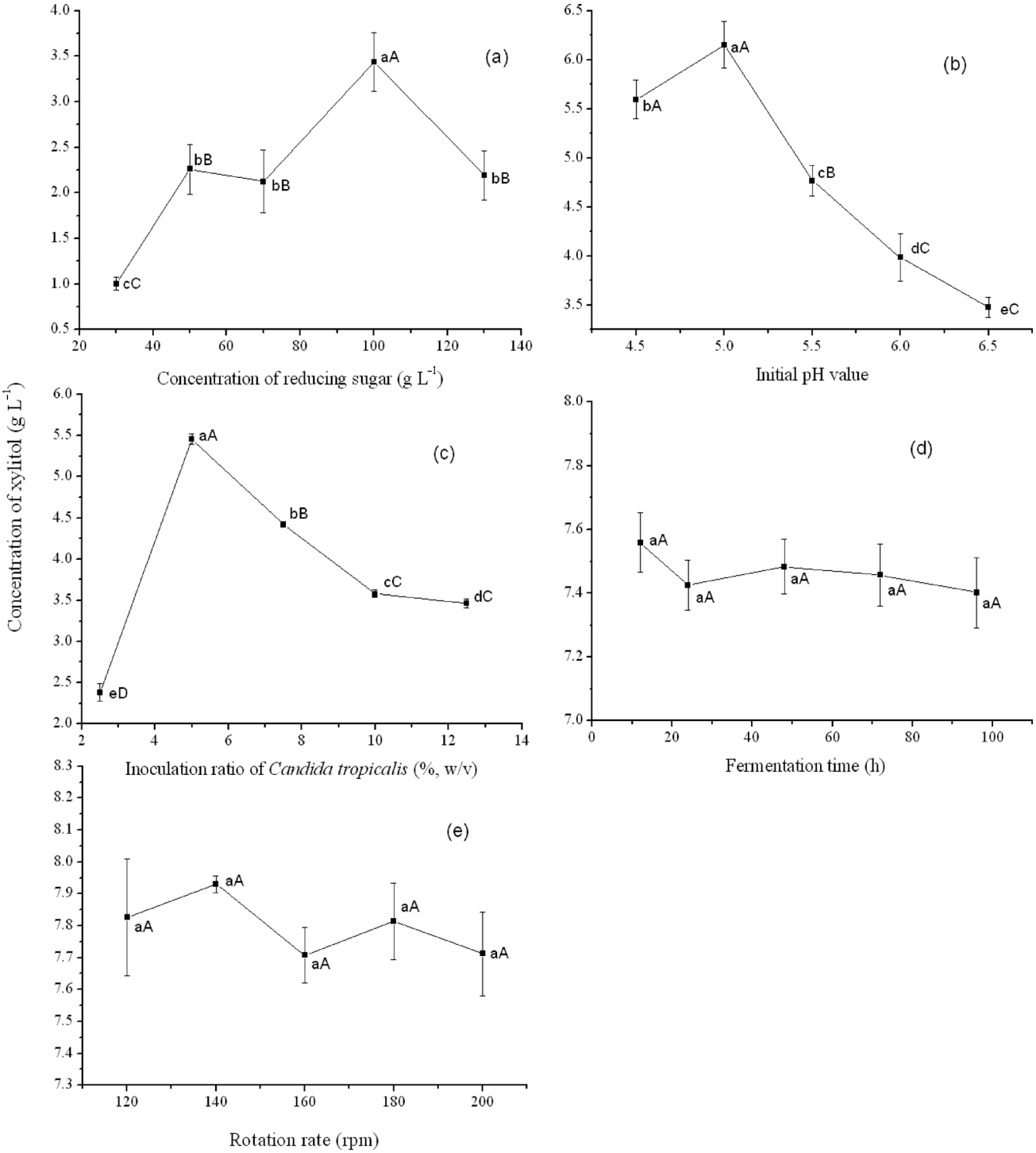

Fig. 1 – *Effects of fermentative conditions (a: Concentration of reducing sugar; b: Initial pH value; c: Inoculation ratio of Candida tropicalis; d: Fermentation time; e: Rotation rate). Data represents mean ± SEM, n = 3, letters represent results of multiple comparisons (a, b denotes differences among the average values as significant, and A, B denotes differences among the average values high significant in multiple comparison).*

reducing sugar concentration until concentration 100 g L^{-1}, the highest xylitol concentration reached, and the difference among all levels was of high significance, that is to say, the concentration modulation of reducing sugar was necessary, so the optimal reducing sugar concentration of 100 g L^{-1} was chosen as the central value in RSM design.

Some reports state that xylitol formation in cells of yeasts is a bioreduction process, which involves consumption of NADPH and regeneration of NADH.[24,25] The process may be affected by pH value in medium. The research of Attilio and José[26] revealed that pH variations strongly affected *D. hansenii* metabolism and xylitol production. Fig. 1b shows the effects of initial pH value, a plateau of maximum xylitol concentration was observed from pH 4.5 to 5.0, followed by a decrease from pH 5.5 to 6.5, and the curve also exhibited high significance.

Inoculation of immobilized *C. tropicalis* influenced the xylitol concentration high significance, as shown in Fig. 1c, the optimal inoculation ratio was 5 % (w/v), the xylitol concentration even decreased upon the further increase of the inoculation ratio. When the initial cell concentration was low, the adverse factors exerted more negative effects on cells which resulted in a long lag phase,[27] so the xylitol concentration increased with inoculation ratio. However, the yeast cells were immobilized in gel beads, the inoculation ratio should be limited to a certain value due to the limited dissolved oxygen and nutrient.

Fermentation time and rotation rate of rotary shaker showed slight but insignificant influence on xylitol concentration, all the letters of different levels of fermentation time and rotation rate were identical in multiple comparisons (Fig. 1d and e), so the effects of fermentation time and rotation rate should be minor, we could consider little about the two factors in next optimization step. As a result, we chose concentration of reducing sugar, initial pH value and inoculation ratio of *C. tropicalis* as main factors to explore the optimal fermentative conditions by surface response methodology.

Optimization of fermentative conditions using surface response methodology

The fermentative conditions for xylitol production were optimized by RSM approach. All 17 of the designed experiments were conducted to optimize the individual parameters in the current Box-Behnken design. Table 1 shows the experimental factors and levels in RSM design. The results of xylitol concentration are listed in Table 2, showing that the xylitol concentration varied in different factorial combinations. By applying multiple regression analysis on the experimental data, the response variable and the test variables were related by the following second-order polynomial equation:

$$Y = 12.52 - 0.68X_1 + 0.25X_2 + 0.27X_3 - \\ -3.08X_1^2 + 0.07X_1X_2 0.27X_1X_3 - \\ -1.66X_2^2 - 0.0044X_2X_3 - 1.12X_3^2$$

where Y is the response variable of xylitol concentration, X_1, X_2 and X_3 are the coded variables of concentration of reducing sugar, inoculum concentration of *C. tropicalis* and initial pH value.

To determine whether or not the quadratic model was significant, the statistical significance of

Table 1 – *Factors and levels of surface response methodology design*

Factors	Levels		
	–1	0	1
Concentration of reducing sugar (X_1, g L^{-1})	70	100	130
Inoculation ratio of *Candida tropicalis* (X_2, %, w/v)	3	5	7
Initial pH value (X_3)	4.5	5.0	5.5

Table 2 – *Response surface methodology design for uncoded factors and the results*

Run No.	Uncoded factor values			Results
	X_1	X_2	X_3	Xylitol concentration (g L^{-1})
1	–1	0	1	8.93
2	0	0	0	12.73
3	0	0	0	12.29
4	0	–1	1	10.03
5	0	1	1	10.55
6	–1	–1	0	8.06
7	1	–1	0	7.01
8	0	–1	–1	8.91
9	0	1	–1	9.45
10	–1	1	0	8.41
11	1	1	0	7.63
12	0	0	0	11.98
13	0	0	0	12.78
14	–1	0	–1	9.51
15	1	0	1	7.70
16	1	0	–1	7.17
17	0	0	0	12.82

regression equation was checked by *F*-test, and analysis of variance (ANOVA) for response surface quadratic polynomial model was summarized as in Table 3. The *F*-value is the ratio of the mean square derived from regression to the mean square due to residual error. The *F*-value of model was 33.36, which was greater than $F_{0.01(9,4)}$ = 14.66, meaning that there was only a 0.01 % chance that a "Model *F*-Value" this large could occur due to noise, indicating that the model was significantly greater than the unexplained variation. The "Lack of Fit *F*-value" of 2.51 implies that the Lack of Fit was not significant relative to the pure error, there was a 19.75 % chance that a "Lack of Fit *F*-value" this large could occur due to noise, showing that the model was feasible.

Table 3 – *Variance analysis and significance test of response surface methodology design*

Source	SS	DF	MS	*F*-value	*P*-value
Model	67.11	9	7.46	33.36	< 0.0001
X_1	3.65	1	3.65	16.31	0.0049
X_2	0.51	1	0.51	2.28	0.1745
X_3	0.58	1	0.58	2.61	0.1502
X_1^2	39.87	1	39.87	178.37	< 0.0001
X_2^2	11.69	1	11.69	52.28	0.0002
X_3^2	5.25	1	5.25	23.51	0.0019
X_1X_2	0.02	1	0.02	0.08	0.7902
X_1X_3	0.30	1	0.30	1.35	0.2838
X_2X_3	0.0001	1	0.0001	0.0003	0.9858
Residual	1.56	7	0.22		
Lack of Fit	1.02	3	0.3406	2.5108	0.1975
Pure Error	0.54	4	0.1357		
Total	68.67	16			
R^2	0.98				
adj. R^2	0.95				

SS: sum of square; DF, degree of freedom; MS, mean square.

The *P*-value was used as a tool to check the significance of each coefficient, which also indicated the interaction strength of each parameter. The smaller the *P*-values were, the bigger the significance of the corresponding coefficients. Here, the *P*-value of the model was smaller than 0.0001, which indicated that the model was suitable for use in this experiment. The determination coefficient (R^2 = 0.98) was close to 1, which indicated the significant correlation between actual and predicted values.[28–30] The Adj. R^2 value was 0.95, meaning that most variation (>95 %) of the xylitol concentration could be predicted by the models, while only 5 % variation could not be explained by the model.

The regression coefficients and the corresponding *P*-values are also presented in Table 3. From the *P*-values of each model term, it could be summarized that the influence of independent variable X_1, quadratic terms X_1^2, X_2^2 and X_3^2 were all of high significance (*P*-value < 0.01). However, the analysis showed that the independent variables X_2, X_3 and the interactions between two arbitrary parameters were all insignificant. The results of the study also suggested that the concentration of reducing sugar was the parameter that the most significantly influenced the xylitol concentration.

Three-dimensional response surface plots and two-dimensional contour plots are useful to examine interaction effects of the factors on the responses. The former often describes the sensitiveness of response value toward the change of variables, and the latter often illustrates significant coefficients among different variables.[31–33] Both types showed effects of two factors on the response at a time, and the third factor was kept at zero level.

The contour plots in Fig. 2(a, e) were almost cycloid, indicating that the interaction between reducing sugar concentration and inoculation ratio, as well as the interaction between inoculation ratio and initial pH value were all insignificant, which was consistent with the results of the ANOVA for quadratic model. The contour plots in Fig. 2(c) were relatively elliptical, which revealed that the interaction between reducing sugar concentration and initial pH value was relatively significant to some extent, while the interaction was insignificant by testing of ANOVA.

The purpose of this study was to find the optimized conditions for xylitol production, so the arithmetic method was used to explore the optimal conditions. Moreover, the protuberant shapes of the 3D response surfaces (Fig. 2(b, d, f)) revealed that there should be maximum value for the quadratic model. When response variable *Y* reached maximum value, the optimal parameters were extracted by solving the regression equation with its coded variables: X_1 = –0.20, X_2 = 0.14, X_3 = 0.19, the corresponding real values were: reducing sugar concentration at 94 g L^{-1}, inoculation ratio at 5.7 % (w/v), initial pH value at 5.1. The predicted maximum xylitol concentration obtained by using the above optimized conditions of the variables was 12.52 g L^{-1}. The highest xylitol concentration examined experimentally was found to be 12.73 g L^{-1} (Table 2), which was obviously in close agreement with the model prediction.

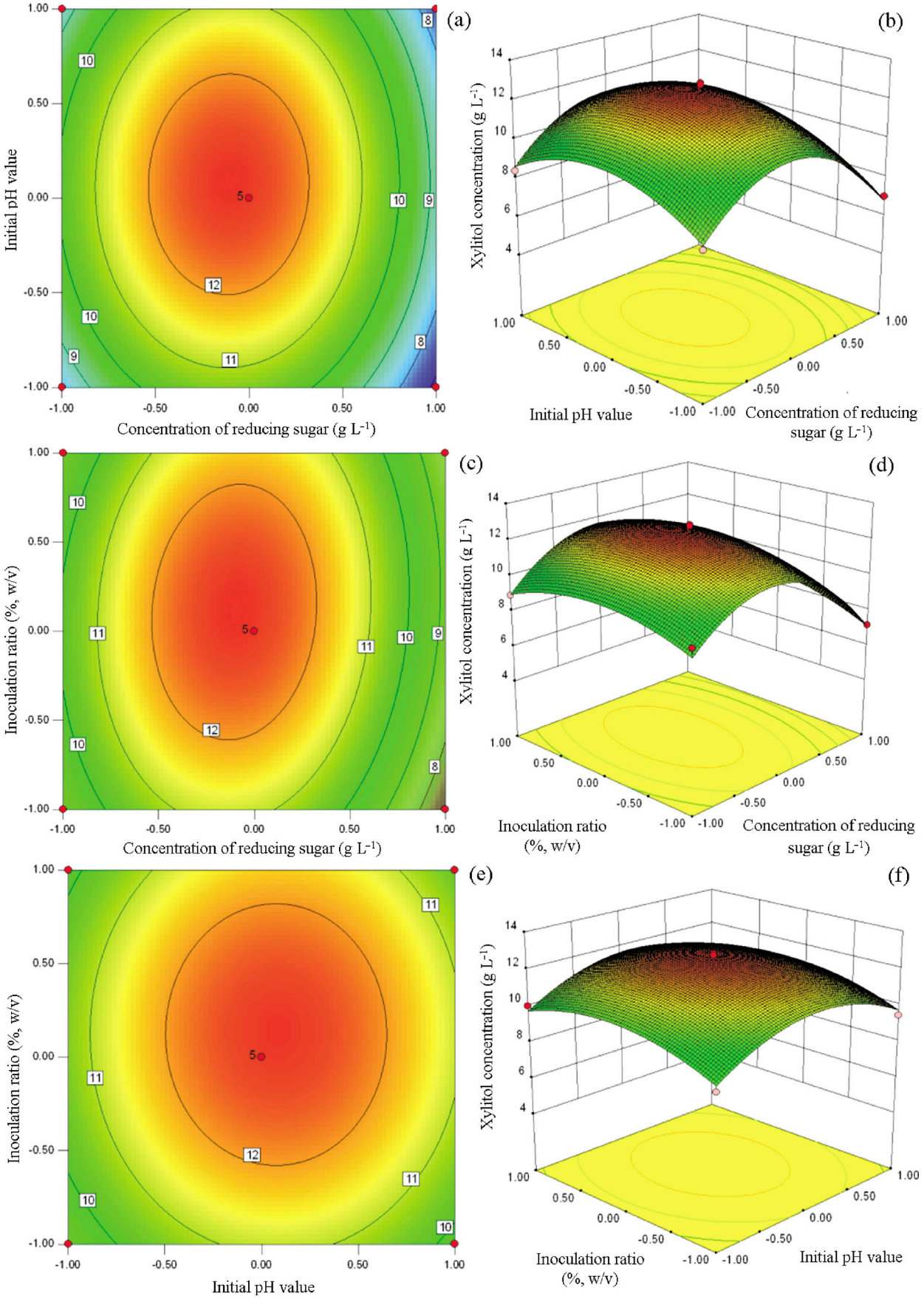

Fig. 2 – *Three-dimensional response surface plots and two-dimensional contour plots showing the effects of different fermentative conditions (X_1: Concentration of reducing sugar; X_2: inoculation ratio of Candida tropicalis; X_3: Initial pH value) on the response variable Y*

However, considering operability of optimized conditions and more persuasive conclusion, we developed the validating tests under the above-mentioned optimized conditions with three replications, the experimental concentration of xylitol was 13.02 ± 1.10 g L^{-1}, compared to the theoretical predicted value (12.52 g L^{-1}), the difference was only 0.50 g L^{-1}, indicating that the model is feasible.

Conclusion

From the single factor experiments, we have chosen three main factors (concentration of reducing sugar, inoculum concentration, initial pH value) from five factors (concentration of reducing sugar, inoculum concentration, initial pH value, fermentation time, rotation rate), as multiple comparisons are useful for factor choice over the process, so we can mostly focus on the three main factors to design the response surface test. Response surface methodology is proved to be fairly effective in predictive modeling and optimization of fermentative conditions for xylitol production, as we obtained a higher xylitol concentration than those of non-optimized conditions (seen from the results of single factor experiments). In addition, RSM test was also effective for estimating the effect of three independent variables, both the concentration of reducing sugar and the quadratic terms of three independent variables showed highly significant effects on xylitol concentration. In the validating tests, we obtained a xylitol concentration of 13.02 ± 1.10 g L^{-1}, which was close to the theoretical predicted value, which suggests that the model is feasible. On the whole, this study is the first report of xylitol production from cotton stalk hydrolysate, which proves that xylitol production could be potentially improved by statistically optimized fermentation for bioconversion from cotton stalk hydrolysate.

ACKNOWLEDGMENTS

We are grateful for the project supported by the National Natural Science Foundation (30860014) in China.

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