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Full Length Research Paper

Optimal conditions for maximizing production of reducing sugars from microwave-assisted $FeCl_3$ pretreated rice straw degraded by *Trichoderma viride* and *Bacillus pumilus*

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Microwave-assisted $FeCl_3$ pretreated rice straw was degraded to reducing sugars with *Trichoderma viride* and *Bacillus pumilus*. Response surface methodology (RSM) and Box-Behnken design (BBD) were used to optimize degradation conditions of pretreated rice straw with respect to initial pH, degradation temperature, *T. viride*/*B. pumilus* inoculum ratio (*T*/*B* inoculum ratio) and degradation time to achieve the highest production of reducing sugars. The maximum production of reducing sugars obtained was 7.19 g/L at the optimal conditions of initial pH, 8; degradation temperature, 32°C; *T*/*B* inoculum ratio, 3 ml/1 ml; degradation time, 60 h. This optimization strategy enhanced the production of reducing sugars from 6.62 to 7.19 g/L after optimization, which shows the beneficial effect on optimization of degradation conditions.

Key words: Microwave-assisted $FeCl_3$ pretreated rice straw, *Trichoderma viride*, *Bacillus pumilus*, degradation conditions, response surface methodology (RSM) optimization.

INTRODUCTION

China is one of the world's largest producers of rice (Fang et al., 2010). The huge quantities of rice straw cause environmental problems with the rice harvest. Utilization of rice straw converted into fermentable sugars, ethanol and hydrogen is a potential and feasible method to solve these dilemmas (Jeya et al., 2009; Zhu et al., 2005; Lo et al., 2010). Rice straw consists of cellulose, hemicellulose and lignin (Zhang and Cai, 2008).

The complex structure of lignin and hemicellulose with cellulose limits the effective conversion of rice straw to fermentable sugars (Zhu et al., 2006). Many pretreatment techniques have been developed to improve its conversion. These pretreatment methods mainly remove or alter hemicellulose and/or lignin, decrease the crystallinity of cellulose and increase the surface area (Mosier et al., 2005). Microwave pretreatment can disrupt the silicified waxy surface of rice straw, break down the lignin–hemicellulose complex and partially remove silicon and lignin (Ma et al., 2009).

Liu et al. (2009) demonstrated that pretreatment with $FeCl_3$ could easily and effectively solubilize hemicellulose into monomeric and oligomeric sugars and disrupt ether and ester linkages between lignin and carbohydrates, but did not affect delignification. Degradation of rice straw with microorganisms has been considered as an environmentally friendly and economical method that eliminates the need to utilize costly enzyme preparations and avoids enzyme recycling (Zhang and Cai, 2008).

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, which extracts the maximal information with the minimal number of runs

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Table 1. Independent variables used in the BBD and actual factor levels corresponding to coded factor levels.

Independent variable	Symbol	Actual factor level at coded factor levels		
		-1	0	+1
Initial pH	X_1	6	7	8
Degradation temperature (°C)	X_2	25	30	35
T/*B* inoculum ratio (ml/ml)	X_3	1:1	2:1	3:1
Degradation time (h)	X_4	60	72	84

(Ma et al., 2009). In the current study, response surface methodology (RSM) was employed to identify the optimal microbial degradation conditions for maximizing the production of reducing sugars from microwave-assisted $FeCl_3$ pretreated rice straw by analyzing the relationships among a number of parameters that affect the overall process. Here microwave-assisted $FeCl_3$ pretreated rice straw was degraded with *Trichoderma viride* CCTCC AF93252 and *Bacillus pumilus* CCTCC AB93225, It was hypothesized that these microorganisms would produce cellulase and xylanase, respectively, and thus would eliminate the need to utilize costly enzyme preparations. The optimal degradation conditions for maximizing the production of reducing sugars from microwave-assisted $FeCl_3$ pretreated rice straw were obtained after optimization.

MATERIALS AND METHODS

Rice straw, collected from local farmers in Ezhou, Hubei Province, China, was washed thoroughly with tap water until the wash water was clean and colorless, air-dried to a constant weight in oven at 35°C, and milled to powder with a high-speed universal disintegrator (Tianjin Taisite Instrument Co., China). The powder was sieved through a 60-mesh sieve and stored at room temperature.

Microorganisms

T. viride CCTCC AF93252 and *B. pumilus* CCTCC AB93225 were obtained from the China Center for Type Culture Collection (CCTCC), Wuhan, China and maintained as frozen cultures (-80°C) in 30% glycerol. *T. viride* was propagated on potato dextrose agar (PDA) plates at 30°C. *B. pumilus* was grown on beef extract peptone agar plates at 30°C.

Pretreatment of rice straw

Microwave-assisted $FeCl_3$ pretreatment was carried out with an ETHOS A digestion/extraction microwave labstation (Milestone Co., Italy). 5.45 g dry rice straw powder and 50 ml of 0.14 mol/L $FeCl_3$ solution were mixed together into a 100 ml reaction tube and treated by the digestion/extraction microwave labstation. A power of 800W was applied to heat the materials inside the labstation to 160°C for 19 min. After pretreatment, the slurry was filtered through a Whatman filter paper. The filtered cakes were dried at 35°C and stored for microbial degradation.

Microbial degradation

B. pumilus spores were obtained from a beef extract peptone plate and 2×10^6 spores were added to 100 ml of sterile medium containing (g/L): beef extract, 3; peptone, 5; NaCl, 5. The initial pH of the medium was adjusted to 7 before autoclaving at 121°C for 20 min. Spores of *T. viride* on a PDA slant culture were scraped off and 2×10^6 spores were added to 100 ml of medium containing (g/L): potatoes, 500; dextrose, 20. The cultures were incubated at 30°C with shaking at 150 rpm for 36 h. According to the Box-Behnken experimental design, the given amount (1, 2 and 3 ml) of the *T. viride* and 1 ml of the *B. pumilus* cultures were inoculated into a 250 ml Erlenmeyer flask containing 100 ml of medium consisting of (g/L): pretreated rice straw, 20; yeast extract, 5; peptone, 5; KH_2PO_4, 5; $(NH_4)_2SO_4$, 5; $MgSO_4\cdot7H_2O$, 5.

The initial pH of the medium was adjusted to the designated pH values (6, 7 and 8) before autoclaving at 121°C for 20 min. The cultures were incubated at different temperatures (25, 30 and 35°C) for different time (60, 72 and 84 h) with shaking at 150 rpm. The cultures were centrifuged at 8000 rpm for 10 min, and the supernatant was used for reducing sugars analysis. All the experiments were performed in duplicate and the average values were reported.

Box-Behnken experimental design and data analysis

The main factors that affect production of reducing sugars in the microbial degradation of rice straw are initial pH, degradation temperature, *T*/*B* inoculum ratio and degradation time. In order to optimize the degradation conditions, RSM and Box–Behnken statistical experiment design were used to investigate the effects of the four independent variables on the response functions. The main and interaction effects of the factors: Initial pH (X_1), degradation temperature (X_2), *T*/*B* inoculum ratio (X_3) and degradation time (X_4) on production of reducing sugars (Y) were evaluated. The production of reducing sugars was taken as the average of the duplicate. The range and levels of the independent variables are given in Table 1.

The experimental levels for each variable were selected based on literature values, available resources and results from preliminary experiments. The experimental designs with the observed responses and predicted values for production of reducing sugars are presented in Table 2. A second-order polynomial quadratic equation was fitted to evaluate the effect of each independent variable to the response:

$$Y = \beta_0 + \sum_{i=1}^{4}\beta_i x_i + \sum_{i=1}^{4}\beta_{ii} x_i^2 + \sum_{i=1}^{4}\sum_{j=i+1}^{4}\beta_{ij} x_i x_j \quad (1)$$

where x_i, x_j are the input variables, which influence the response

Table 2. BBD with the observed and predicted values for production of reducing sugars.

Run	Coded variable level				Reducing sugars, Y (g/L)	
	X_1	X_2	X_3	X_4	Observed	Predicted
1	0	0	-1	1	6.551	6.50
2	0	-1	1	0	6.227	6.33
3	1	-1	0	0	6.428	6.46
4	-1	0	0	-1	6.019	6.04
5	0	0	0	0	6.651	6.62
6	0	1	0	1	6.522	6.60
7	0	0	1	-1	6.732	6.78
8	0	0	1	1	6.818	6.81
9	1	1	0	0	6.472	6.58
10	0	-1	0	-1	6.079	6.01
11	1	0	1	0	7.166	7.09
12	-1	0	-1	0	6.03	6.11
13	0	-1	-1	0	5.931	6.01
14	-1	1	0	0	6.364	6.34
15	0	1	-1	0	6.152	6.05
16	0	1	0	-1	6.332	6.37
17	0	-1	0	1	6.408	6.37
18	1	0	-1	0	6.3	6.29
19	1	0	0	1	6.764	6.74
20	-1	0	0	1	6.536	6.57
21	-1	0	1	0	6.448	6.46
22	0	1	1	0	6.956	6.87
23	0	0	0	0	6.6	6.62
24	0	0	0	0	6.623	6.62
25	-1	-1	0	0	5.979	5.87
26	1	0	0	-1	6.718	6.68
27	0	0	-1	-1	5.938	5.94

variable Y, β_0 is the intercept, β_i is the ith linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the ijth interaction coefficient. The statistical software package Design-Expert (Version 7.1.5, Stat-Ease Inc., Silicon Valley, CA, USA) was used for the regression analysis of the experimental data and the response surface plots. Analysis of variance (ANOVA) was used to estimate the statistical parameters. Subsequently, five additional confirmation experiments were conducted to verify the validity of the statistical experimental strategies.

Analytical methods

Moisture content was measured as the weight loss of 1 g rice straw dried at 105°C for 24 h (Jeya et al., 2009). Cellulose, hemicellulose and lignin were determined as described by Liu (2004). Reducing sugars were determined using the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959).

RESULTS AND DISCUSSION

Degradation of pretreated rice straw

The initial degradation study shows that the production of reducing sugars from degradation of microwave-assisted $FeCl_3$ pretreated rice straw was 6.62 g/L under the degradation conditions of initial pH, 7; degradation temperature, 30°C; *T*/*B* inoculum ratio, 2 ml/1 ml; degradation time, 72 h. This yield was 2.9 times higher than that obtained with untreated rice straw under the same conditions (2.25 g/L).

The microwave-assisted $FeCl_3$ pretreated rice straw consisted of 59.3% cellulose, 13% hemicellulose, 9.8% lignin. The main composition of untreated rice straw was determined to be 12.8% moisture, 33.8% cellulose, 20% hemicellulose, 16.2% lignin. Compared with the chemical components of untreated rice straw, microwave-assisted $FeCl_3$ pretreatment increased the proportion of cellulose by 75.4% and decreased that of hemicellulose and lignin by 35 and 39.5%, respectively. The microwave-assisted $FeCl_3$ pretreatment was effective in fractionating the hemicellulose and lignin components. The removal of lignin and hemicellulose could have improved enzyme access to cellulose, reduced nonproductive binding of enzyme and enhanced cellulose digestion.

The higher cellulose content and decreased lignin

Table 3. ANOVA of the quadratic model for production of reducing sugars.

Source of variations	Degree of freedom	Sum of squares	Mean square	F-value	P-value	R^2
Model	14	2.61	0.19	24.51	< 0.0001	0.9662
X_1	1	0.51	0.51	66.93	< 0.0001	
X_2	1	0.25	0.25	33.39	< 0.0001	
X_3	1	0.99	0.99	130.00	< 0.0001	
X_4	1	0.26	0.26	34.74	< 0.0001	
X_1X_2	1	0.029	0.029	3.82	0.0743	
X_1X_3	1	0.050	0.050	6.60	0.0246	
X_1X_4	1	0.055	0.055	7.29	0.0193	
X_2X_3	1	0.065	0.065	8.48	0.0130	
X_2X_4	1	4.830E-003	4.830E-003	0.63	0.4410	
X_3X_4	1	0.069	0.069	9.13	0.0106	
X_1^2	1	0.027	0.027	3.49	0.0862	
X_2^2	1	0.31	0.31	41.17	< 0.0001	
X_3^2	1	0.024	0.024	3.19	0.0994	
X_4^2	1	0.012	0.012	1.51	0.2422	
Residual	12	0.091	7.608E-003			
Lack of Fit	10	0.090	8.999E-003	13.80	0.0694	

content of pretreated rice straw compared to the untreated rice straw allowed for enhanced saccharification (Lü and Zhou, 2011). Lignin removal also benefited enzymatic hydrolysis of xylan (Kumar and Wyman, 2009). The microwave-assisted $FeCl_3$ pretreatment could enhance the degradation of rice straw.

Optimization of microbial degradation conditions of pretreated rice straw

The ranges for the four main factors had been identified based on literature values, available resources and results from preliminary one-factorial experiments. These ranges were 6 to 8, 25 to 35°C, 1 to 3 ml/ml and 60 to 84 h for initial pH, degradation temperature, *T*/*B* inoculum ratio and degradation time, respectively.

Optimization of the degradation conditions was achieved by employing Box-Behnken design (BBD) and the polynomial equation describing production of reducing sugars as a simultaneous function of initial pH (X_1), degradation temperature (X_2), *T*/*B* inoculum ratio (X_3) and degradation time (X_4), is shown in Equation 2.

$$Y = 6.62 + 0.21X_1 + 0.15X_2 + 0.29X_3 + 0.15X_4 - 0.085X_1X_2 + 0.11X_1X_3 - 0.12X_1X_4 + 0.13X_2X_3 - 0.035X_2X_4 - 0.13X_3X_4 - 0.071X_1^2 - 0.24X_2^2 - 0.067X_3^2 - 0.046X_4^2 \quad (2)$$

The statistical significance of the model equation was evaluated by the F-test for analysis of variance (ANOVA) in Table 3. The P-value for the model was <0.0001, which implies that the model terms was statistically significant. The model F-value was 24.51 and the P-value for lack-of-fit was 0.0694. The high F-value and non-significant lack of fit suggest that the obtained experimental data was a good fit with the model. The coefficient of determination (R^2) of the model was 0.9662, which further indicates that the model was suitable for adequately representing the real relationships among the selected reaction variables. The high values of R^2 and adjusted R^2 of this model (Adj R^2 = 0.9268) show a close agreement between the experimental results and the theoretical values predicted by the model. The similarity between predicted and actual results presented in Figure 1 reflects the accuracy and applicability of the Box-Behnken model as a powerful method for process optimization.

The main effects plot and the interaction plots for production of reducing sugars were developed in Figures 2 and 3. Figure 2 suggests the effects of four factors on the response variable. This type of representation indicates the contribution to the response factor of changing one of the variables selected for microbial degradation process. As can be shown in Figure 2, the effects of all four factors on production of reducing sugars are positive, that is, the greater production of reducing sugars could be obtained at high level (+1) of each factor than that at low level (-1) of the factor. The slope of the plot in Figure 2 is indicative of the importance of the variable on the response factor (Zhang et al., 2010). It can show that the effects of initial pH, degradation temperature, *T*/*B* inoculum ratio and degradation time on production of reducing sugars were the same importance. The F-test suggests that the P-values for the factors of initial pH, degradation temperature, *T*/*B* inoculum ratio and degradation time were less than 0.0001. Generally,

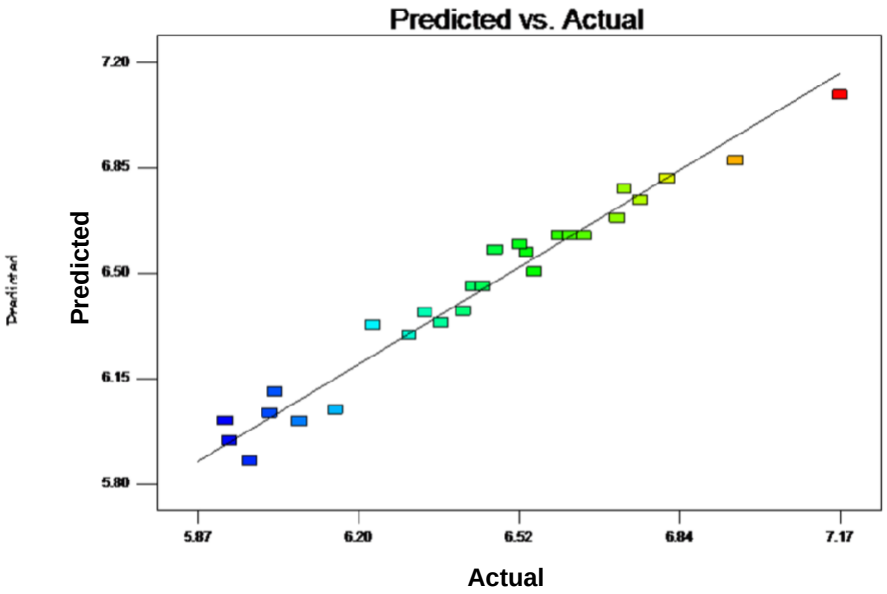

Figure 1. Actual production of reducing sugars vs. predicted production of reducing sugars.

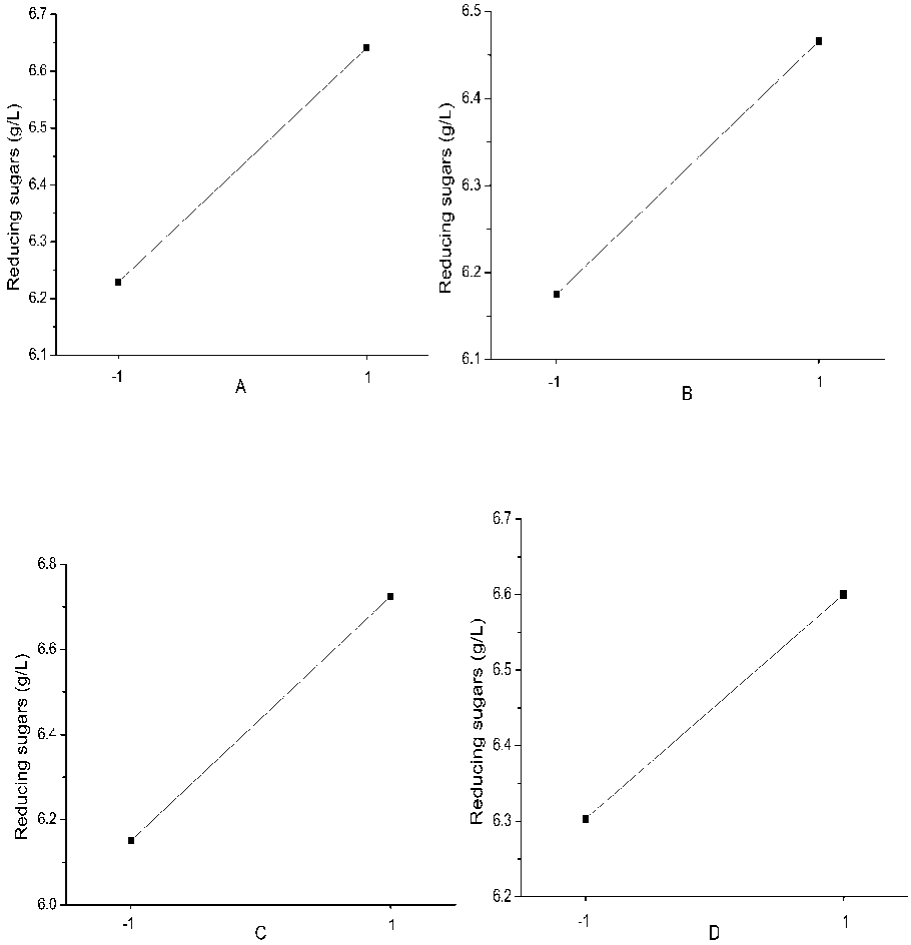

Figure 2. Main effects plot for production of reducing sugars ((A) initial pH; (B) degradation temperature; (C) *T*/*B* inoculum ratio; (D) degradation time).

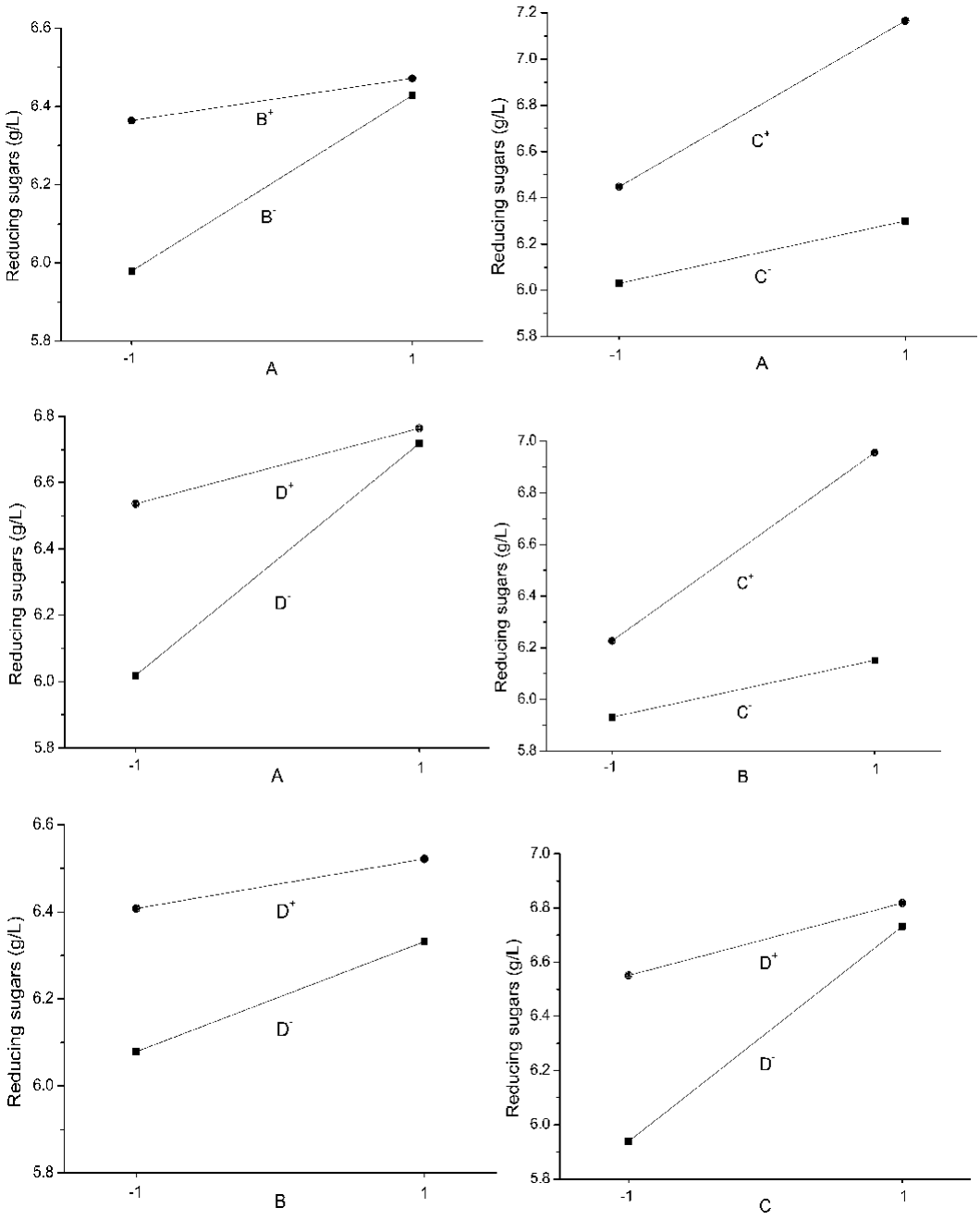

Figure 3. Interaction plots for production of reducing sugars ((A) initial pH; (B) degradation temperature; (C) *T*/*B* inoculum ratio; (D) degradation time).

P-values less than 0.05 indicate the significant model terms.

Figure 3 indicates interaction plots showing the existence or not of interaction among the factors (Zhang et al., 2010). An interaction may occur if the change in the response variable from the low level to the high level of one design variable is different from the change in the response variable at the same two levels of a second design variable (Ormad et al., 2006). In other words, the effect of one design variable depends on a second design variable (Ormad et al., 2006; Sleiman et al., 2007). The interaction effect on production of reducing sugars between degradation temperature and degradation time is insignificant due to almost parallel plot as shown in Figure 3. This was also confirmed with the high probability value (P-value > 0.1) through analysis of variance (ANOVA) (Guven et al., 2008; Korbahti and Rauf, 2008; Arslan-Alaton et al., 2009; Zhang et al.,

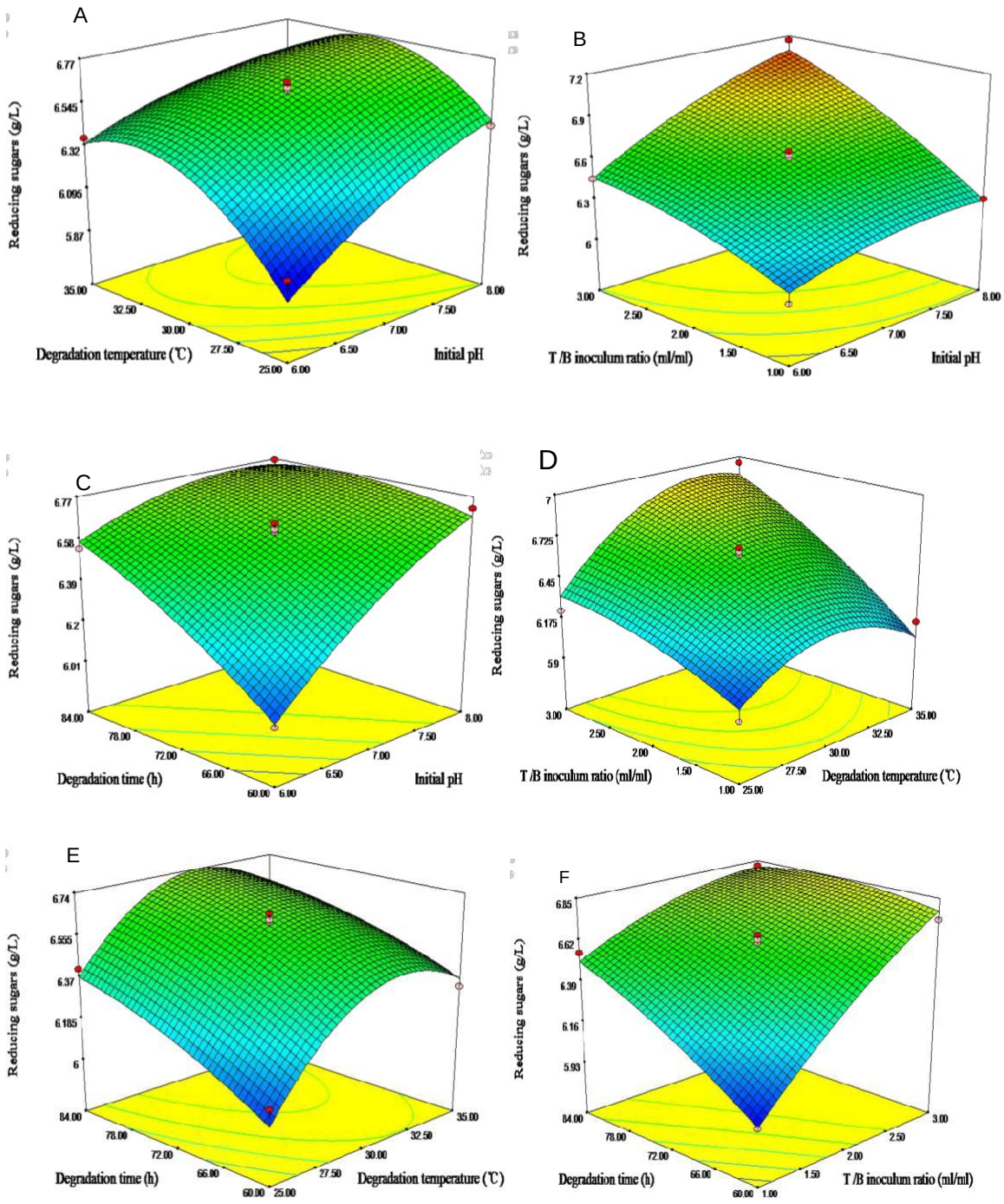

Figure 4. Response surface plots showing the interactions between the variables affecting production of reducing sugars: (A) interaction between the degradation temperature and initial pH; (B) interaction between *T*/*B* inoculum ratio and initial pH; (C) interaction between degradation time and initial pH; (D) interaction between *T*/*B* inoculum ratio and degradation temperature; (E) interaction between degradation time and degradation temperature; (F) interaction between degradation time and *T*/*B* inoculum ratio.

2010).

The 3D response surfaces and the 2D contour plots were developed based on the RSM Equation (2). The shapes of response surfaces and contour plots indicate the nature and extent of the interaction between different factors (Prakash et al., 2008). Figure 4 shows the response surface plots and their respective contour plots of the production of reducing sugars. Each figure presents the effect of two factors while the other factor was held at zero level (Liu et al., 2010). As can be seen

in Figure 4, the results show that the selected four variables (initial pH, degradation temperature, *T*/*B* inoculum ratio and degradation time) substantially affected production of reducing sugars response. Additionally, two-way interactions between variables such as *T*/*B* inoculum ratio and initial pH, degradation time and initial pH, *T*/*B* inoculum ratio and degradation temperature, degradation time and *T*/*B* inoculum ratio, were important terms in the production of reducing sugars model and significantly influenced production of reducing sugars. With P-values more than 0.05 (Table 3), the interactions between variables such as degradation temperature and initial pH, degradation time and degradation temperature, appeared not to affect production of reducing sugars significantly.

The optimal degradation conditions for maximizing the production of reducing sugars were extracted by Design Expert software though a graphical optimization. Taking both cost and efficiency into consideration, the maximum production of reducing sugars could be achieved when the initial pH, degradation temperature, *T*/*B* inoculum ratio and degradation time were set at 8, 32°C, 3 ml/1 ml and 60 h, respectively. In order to confirm the optimazation results, the suggested degradation conditions were performed in five replicates. Under these suggested conditions, the observed mean production of reducing sugars was 7.19 g/L, which was found to be largely in agreement with the predicted value. This optimization strategy led to the enhancement of the production of reducing sugars from 6.62 to 7.19 g/L, which indicates the beneficial effect on optimization of degradation conditions. There were shorter reaction time and higher production of reducing sugars after optimization than before optimization. It further confirmed the rationality and practicability of optimal conditions for microbial degradation in this work.

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