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RESEARCH ARTICLE

Optimization of fermentation medium for xylanase-producing strain Xw2

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Abstract **To improve the fermentation yield of xylanase by optimizing the fermentation conditions for strain Xw2, a Plackett-Burman design was used to evaluate the effects of eight variables on xylanase production by strain Xw2. The steepest ascent (descent) method was used to approach the optimal response surface experimental area. The optimal fermentation conditions were obtained by central composite design and response surface analysis. The results showed that the composition of the optimal fermentation medium was corn cob + 1.5% wheat bran (1:1), 0.04% $MnSO_4$, 0.04% $K_2HPO_4 \cdot 3H_2O$, and an inoculum size of 6% in 50 mL liquid volume (pH = 6.0). The optimal culture conditions were 28°C at 150 r/min for 54.23 h. The results of this study can serve as the basis for the industrial production and application of xylanase.**

Keywords enzyme activity, fermentation, Plackett-Burman test, xylan

Introduction

Hemicelluloses are polymeric carbohydrates containing polysaccharides such as xylan, arabinoxylan, xyloglucan, glucose, and mannose glycans. After cellulose, hemicelluloses are the second most abundant fraction in nature (Selinheimo et al., 2006). Xylan is a complex polysaccharide consisting of a backbone of β-1,4-D-xylopyranose residues with arabinofuranose, glucuronic acid side chains (Wong et al., 1988; Coughlan and Hazlewood, 1993), and acetyl groups. Xylans are widely distributed in nature, and occur in bacteria, fungi, snails, marine algae, and terrestrial plant tissue. They constitute the major portion of plant cell wall hemicelluloses, accounting for approximately 15%–30% and 7%–12% of the total dry biomass in angiosperms and gymnosperms, respectively.

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Xylanase (EC3.2.1.8) is an enzyme that hydrolyzes xylan, and is widely used in animal feed digestion (Bastawde, 1992), food industries (Silversides et al., 2006), and brewing science. For example, xylanases can break down the arabinoxylans present in feed ingredients, thereby reducing the viscosity of the raw material for animal feed. Similar to other hemicellulases, xylanases break down hemicelluloses present in wheat flour, helping in the redistribution of water and leaving the dough softer and easier to knead (Polizeli et al., 2005). Recently, many xylanases have been isolated and characterized from fungi and bacteria and used for application in the abovementioned fields. Feed conversion ratio and pulp bleaching had been improved by xylanase; however, the low enzyme activity of xylanase has become the main problem in the industrialized application of this enzyme.

The objective of this study was to apply Plackett-Burman and response surface methodology to optimize the fermentation parameters such as medium composition, initial pH, percentage inoculum, substrate concentration, and cultivation time to improve xylanase activity. The Box-Wilson central composite design was employed to evaluate the correctness in

a quadratic mathematical model, to provide the basis for industrial production and application of xylanase.

Materials and methods

Strain, media, and cultivation

The xylanase-producing strain Xw2 (The Xw2 strain was isolated from 50 soil samples by hydrolysis circle and transparent circle. The branching pattern was generated by neighbor-joining method, the homology of xylanase producting strain Xw2 and *Trichoderma harzianum* (HQ596967.1) up to 94%, which indicates that Xw2 belongs to *Trichoderma*) was maintained in the laboratory of Fujian Normal University. The medium used for xylanase production was composed of 1% wheat bran, 0.5% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $K_2HPO_4 \cdot 3H_2O$, 0.5% NaCl, and 0.5% $(NH_4)_2SO_4$. The microorganisms were cultured in 50 mL of medium in 250-mL Erlenmeyer flasks. After inoculation, the flasks were incubated on a rotary shaker at 28°C with shaking at 150 r/min.

Enzyme assays

Xylan solution was added to sodium dihydrogen phosphate-citrate buffer solution (pH 5.0) and incubated for 5 min (Uysal et al., 2007). The assay mixture contained 0.5 mL 1g/mL substrate and 0.5 mL 10 mg/g enzyme solution suitably diluted in buffer. This mixture was incubated at 50°C for 10 min and the reaction was stopped by addition of 1.5 mL 1g/mL dinitrosalicylic acid (DNS) reagent followed by incubating the reaction mixture in boiling water for 15 min. One unit (U) of xylanase activity was expressed as 1 μmol of reducing sugar (xylose equivalent) released in 1 min (Ding et al., 2004).

Plackett-Burman experimental design

The Plackett-Burman experimental design was used to screen the important variables that influence xylanase production (Li et al., 2007a). According to the Plackett-Burman experimental design, a total of 12 trials were required, containing 8 vialle factor and 3 factors of error analysis items (Table 1).

Central composite design

Response surface methodology (RSM) was used to optimize the fermentation parameters for enhancing xylanase production (Li et al., 2007b). RSM has four steps: procedures to move into the optimum region, behavior of the response in the optimum region, estimation of the optimal condition, and verification (Tanyildizi et al., 2005). We used the single-factor method to determine the center of the significant factors (compound nitrogen source, inoculation, and fermentation time).

Box-Behnken design

A response surface is a graph of a response variable as a function of variable factors. The typical meta-model used in a simulation application is a regression model. With the aid of RSM, a meta-model can be used to determine the optimal values for a set of factors. The results of the Plackett-Burman and central composite designs were used to determine three levels for each meaningful factor, which were then evaluated according to the Box-Behnken experimental design. The experimental results were then analyzed by SAS version 9.0, and a multivariate quadratic regression equation was obtained. The regression equation was used to map the response surface stereo analysis diagram to ascertain the best level and to determine the optimum culture conditions.

Statistics

Data are presented as means±SE. The significance of differences in mean values within and between multiple groups was evaluated using a one-way ANOVA, followed by a Tukey's multiple range test. Student's *t*-test was used to evaluate statistical significance of differences between two groups. $P < 0.05$ was considered statistically significant.

Table 1 Factors and levels of the Plackett-Burman experiment design

Variable	Parameters	Level	
		−1	+ 1
X1	Corn cob + wheat bran (% w/v,1:1)	1	1.5
X2	Yeast extract + $(NH_4)_2SO_4$ (% w/v,1:1)	0.3	0.6
X4	$MnSO_4$ (%,w/v)	0.02	0.04
X5	K_2HPO_4 (%,w/v)	0.04	0.08
X7	Initial pH 6.0	6.0	7.0
X8	Inoculums (%,v/v)	4	6
X10	Liquid volume (mL/250mL)	40	50
X11	Fermentation time (h)	48	72

Results

Optimization of the Xw2 strain fermentation conditions by the Plackett-Burman experimental design

Twelve experimental runs with different combinations of 11 factors were carried out (Table 2). The *P* values of compound nitrogen source concentration, amount of inoculum, and fermentation time (Pr > F) were < 0.1, suggesting that these three were the main factors affecting xylanase production, and their response values are very significant. Treatment run 2 showed a high level of xylanase production based on enzyme activity at 327.28 IU/m. The response values of the concentration of compound nitrogen source, percentage of inoculum, and fermentation time were found to be significant at (Pr > F) at 7.02, 4.11, and 1.43 × 10^{-2}, respectively. An R^2 value of > 0.9 was considered to denote a very strong correlation, and the R^2 value of the regression model was 0.9480. Under the condition of the fit of the equation, other factors can be neglected.

Optimization of fermentation using the Box-Behnken design

Compound nitrogen source

Various concentrations of yeast extract + $(NH_4)_2SO_4$ (1:1) (0.5%, 0.55%, 0.6%, 0.65%, 0.7%, and 0.75%) were used as the nitrogen source in the fermentation medium. The effect of different composite nitrogen concentrations on xylanase production by strain Xw2 was detected as shown in Fig. 1 (A). Maximum activity of 303.32 IU/mL was achieved when 0.6% yeast extract + $(NH_4)_2SO_4$ (1:1) was used in the medium.

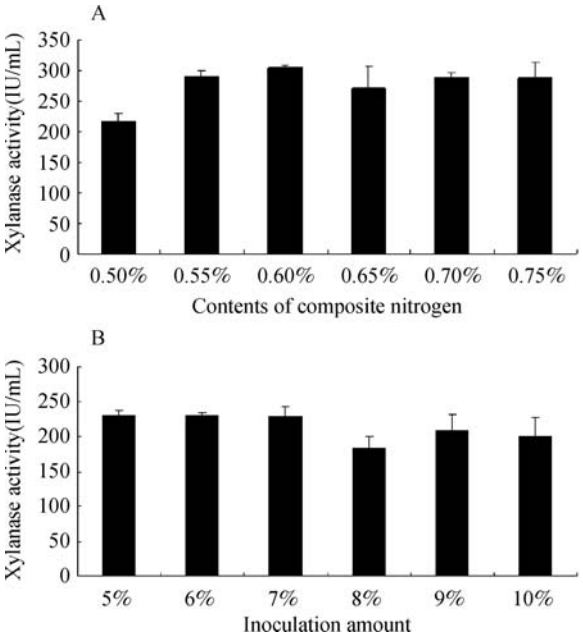

Figure 1 Effect of different values of composite nitrogen and inoculum on xylanase production from strain Xw2. Each value represents the means±SE. One-way analysis of variance (ANOVA) was used to analyze the data. Superscripts represents error bar by Tukey's mutiple-range test. *n* = 3 batches.

Amount of inoculum

The effect of various percentages of inoculum (5%, 6%, 7%, 8%, 9%, and 10%) on xylanase production from strain Xw2 was detected as shown in Fig. 1(B). While inoculum percentages of 5%, 6%, and 7% had little effect on enzyme production, 5% inoculum resulted in higher enzyme activity.

Table 2 The Plackett-Burman experimental design matrix and corresponding response values

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Yield (Y) Xylanase activity (IU/mL)
1	1	−1	1	−1	−1	−1	1	1	1	−1	1	74.54
2	1	1	−1	1	−1	−1	−1	1	1	1	−1	327.28
3	−1	1	1	−1	1	−1	−1	−1	1	1	1	43.33
4	1	−1	1	1	−1	1	−1	−1	−1	1	1	70.57
5	1	1	−1	1	1	−1	1	−1	−1	−1	1	30.02
6	1	1	1	−1	1	1	−1	1	−1	−1	−1	287.49
7	−1	1	1	1	−1	1	1	−1	1	−1	−1	234.65
8	−1	−1	1	1	1	−1	1	1	−1	1	−1	179.73
9	−1	−1	−1	1	1	1	−1	1	1	−1	1	105.74
10	1	−1	−1	−1	1	1	1	−1	1	1	−1	69.58
11	−1	1	−1	−1	−1	1	1	1	−1	1	1	76.68
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	80.53
Pr > F	42.77 (×10^{-2})	7.02 (×10^{-2})		12.88 (×10^{-2})	40.03 (×10^{-2})		19.83 (×10^{-2})	4.11 (×10^{-2})		78.25 (×10^{-2})	1.43 (×10^{-2})	
Significance	7	3		4	6		5	2		8	1	

R^2 = 0.9480

Fermentation time

The inoculum was added to the fermentation broth and incubated for 39 h. The enzyme activity of fermentation was measured every 3 h. The effect of fermentation time on xylanase production by strain Xw2 was detected as shown in Fig. 2. The results showed that enzyme activity peaked at 54 h, and started to decline thereafter.

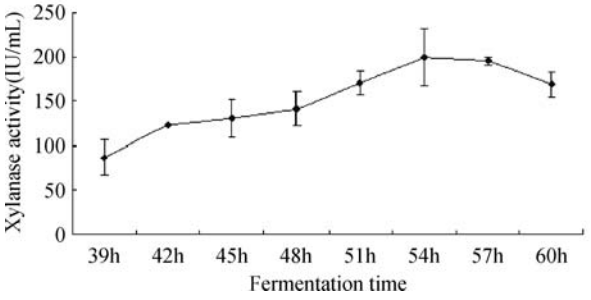

Figure 2 Effect of different fermentation times on xylanase production from strain Xw2. Each value represents the means± SE. One-way analysis of variance (ANOVA) was used to analyze the data. Superscripts represents error bar by Tukey's mutiple-range test. $n = 3$ batches.

Regression models of response

Knowledge obtained from a study was necessary for achieving a more realistic model in this method. Table 3 shows the maximum and minimum levels of variables chosen for trials in the central composite design. Fifteen experimental runs with unique combinations of the three factors were carried out in the case of RSM based on the Box-Wilson design, which was used for the optimization of cultivation conditions for the xylanase production (Table 4). Treatment runs 13–15 showed a high level of xylanase production based on the enzyme activity at 362.83, 358.83, and 351.15 U/mL, respectively. The maximum xylanase production was achieved in run 13, and the minimum xylanase production (286.20 U/mL) was observed in run 11.

By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the xylanase production regardless of the significance of coefficients:

$$\begin{aligned} Y = & 357.6033 + 5.26125 \times X1 - 8.19125 \times X2 - 5.3025 \\ & \times X3 - 24.89292 \times X1 \times X1 - 3.7125 \times X1 \times X2 \\ & - 5.08 \times X1 \times X3 - 23.01292 \times X2 \times X2 - 6.61 \\ & \times X2 \times X3 - 31.57042 \times X3 \times X3 \end{aligned}$$

Table 4 Design and results of the Box-Benhnken experiment

Trial number	X1	X2	X3	Xylanase activity (IU/mL)
1	−1	−1	0	314.70
2	−1	1	0	299.97
3	1	−1	0	326.85
4	1	1	0	297.27
5	0	−1	−1	300.46
6	0	−1	1	316.19
7	0	1	−1	303.07
8	0	1	1	292.36
9	−1	0	−1	299.76
10	1	0	−1	326.24
11	−1	0	1	286.20
12	1	0	1	292.36
13	0	0	0	362.83
14	0	0	0	358.83
15	0	0	0	351.15

where Y is the predicted response (xylanase production) and X1, X2, and X3 are the coded values denoting composite nitrogen, percentage of inoculum, and fermentation time, respectively.

The F test was used to detect statistical significance, and the analysis of variance (ANOVA) for the response surface quadratic model is shown in Table 5. It is evident that the model was highly significant, as suggested by the model F value and a low probability value (P model $> F = 0.0156$). Usually, the higher the CV (coefficient of variation) value, the lower would be the reliability of the experiment. A lower CV value (3.322%) indicated better precision and reliability of the experiments. At the same time, the R value (0.8248) indicated close agreement between the experimental results and the theoretical values predicted by the model equation. Therefore, the quadratic model was selected in this optimization study.

Student's t distribution and the corresponding P value, along with the parameter estimate, are presented in Table 6. The P values were used to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of mutual interactions between the best variables. The parameter estimates and the corresponding P values indicated that, among the independent variables, X1, X2, and X3 significantly affected xylanase production in strain Xw2.

Analysise and determination of optimum condition for enzyme production

The three-dimensional (3D) response surface plots described

Table 3 Coded values of variables used in the Box-Behnken design

Factor	Coding level		
	−1	0	1
Compound nitrogen source (X1)	0.55%	0.60%	0.65%
Inoculation amount (X2)	4%	5%	6%
Fermentation time (X3)	51 h	54 h	57 h

Table 5 ANOVA of quadratic polynomial model

Source	Sum of squares	Degree of freedom	Mean square	F value	$Pr > F$
Model	8515.65	9	912.85	8.3238	0.0156
Lack-of-fit items	477.87	3	159.29	4.5209	0.1864
Error	70.47	2	35.23		
Residuals	548.34	5	109.67		
Sum	8763.99	14			

Table 6 Significance test for regression coefficients of the quadratic model

Model term	Regression coefficients	Standard error	t	$Pr > \|t\|$
X1	5.2613	3.7025	1.4210	0.2146
X2	−8.1913	3.7025	−2.2124	0.0779
X3	−5.3025	3.7025	−1.4321	0.2115
X1*X1	−24.8929	5.4499	−4.5676	0.0060
X1*X2	−3.7125	5.2361	−0.7090	0.5100
X1*X3	−5.08	5.2361	−0.9702	0.3765
X2*X2	−23.0129	5.4499	−4.2226	0.0083
X2*X3	−6.61	5.2361	−1.2624	0.2625
X3*X3	−31.5704	5.4499	−5.7928	0.0022

by the regression model were drawn to illustrate the effects of the independent variables and the interactive effects of each independent variable on the response variables. The shape of the corresponding contour plots indicates whether or not the reciprocal interactions between the independent variables are significant. Figure 3 depicts the 3D plot and its corresponding contour plot, showing the effects of complex nitrogen source concentration, percentage inoculum, and fermentation time on xylanase production. Figure 3(A) shows that the percentage of nitrogen and percentage of inoculum affected xylanase production, while cultivation time was fixed at its middle level (540 h). When the percentage of inoculum was 5%, the xylanase production significantly increased with increasing in the percentage of nitrogen and fermentation time, as shown in Fig. 3(B). At 0.6% composite nitrogen, xylanase production significantly increased with increasing in the percentage of inoculum beyond the initial value, as shown in Fig. 3(C). From the drawings and software analysis, regression equation was found to attain a stable point, which is the maximum value of the principal factors (X1 = 0.12661, X2 = – 0.17733, and X3 = – 0.0756). According to the formula: X = intermediate coding − step × maximal corresponding to the encoded value. The formula had the following critical values: X1, 0.594%; X2, 5.177%; and X3, 54.23 h, the predicted xylanase activity for these conditions was 358.86 IU/mL.

Validation of the model

The statistical optimal values of the variables were obtained when moving along the major and minor axis of the contour, and the response at the center point yielded maximum xylanase production. These observations were also verified from canonical analysis of the response surface. The canonical analysis revealed a minimum region for the model. The stationary point with maximum xylanase activity had the following critical values: complex nitrogen source, 0.6%; inoculum, 5.0%; and fermentation time, 54.23 h. The predicted xylanase activity for these conditions was 358.86 IU/mL.

Discussion

The Plackett-Burman experimental design is a multifactorial two-level experimental design which is used to filter the main factors affecting the experiment (Francis et al., 2003). In this study, we used RSM based on the Box-Wilson design to optimize the cultivation conditions for xylanase production. The relationship between the factors and the response can be fitted by a quadratic regression equation, so as to determine the best level and cultivating conditions for optimum xylanase production.

Xylanases have a wide range of potential biotechnological applications such as in feed and food industries (Subramaniyan and Ptema, 2002). The present study intended to apply the Plackett-Burman design and RSM to optimize the fermentation parameters for the xylanase-producing strain Xw2. The effects of the following eight variables on the production of xylanase were determined using the Plackett-Burman design: corn cob + wheat bran (1:1), yeast extract + $(NH_4)_2SO_4$ (1:1), $MnSO_4$, K_2HPO_4, initial pH, inoculum, liquid volume, and fermentation time. Three factors, namely, compound nitrogen source, inoculation, and fermentation time, were found to be significant by central composite

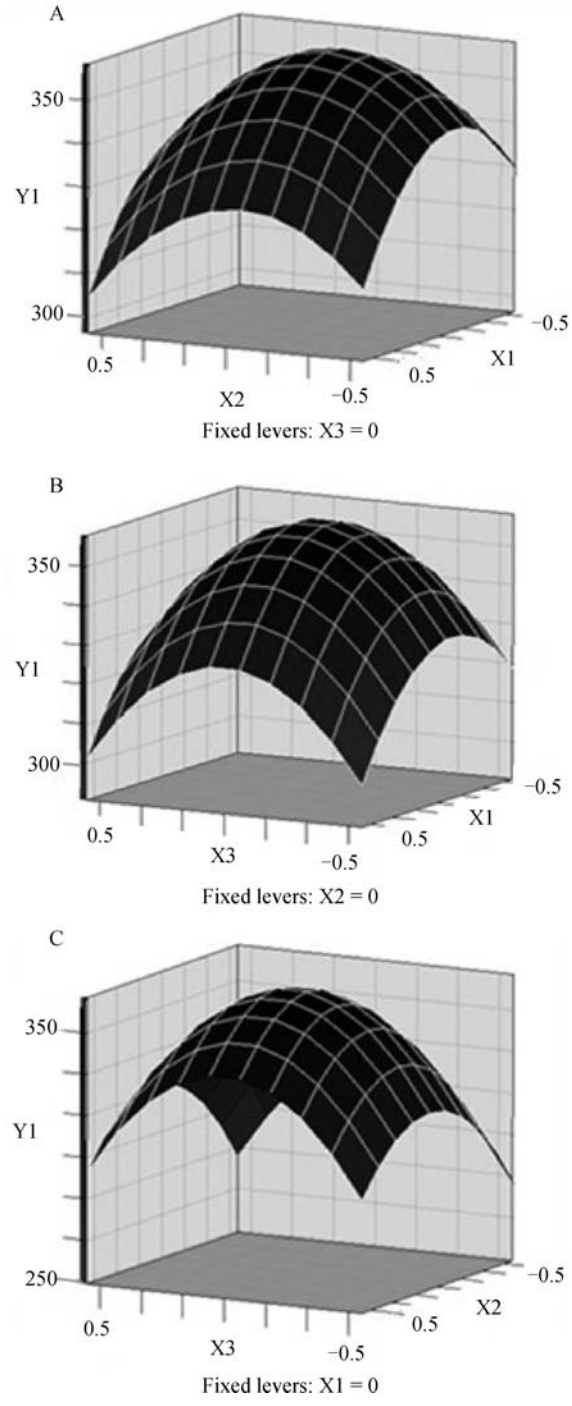

Figure 3 Response surface methodology of xylanase production using bran hydrolysate and methanol.
(A) Fixed levers: X3 = 0 (B) Fixed levers:X2 = 0 (C) Fixed levers: X1 = 0

design. Finally, a medium containing corn cob + 1.5% wheat bran (1:1), yeast extract + 0.6% $(NH_4)_2SO_4$ (1:1), 0.04% $MnSO_4$, 0.04% K_2HPO_4, at an initial pH of 6.0; 5.0% inoculum; and incubation conditions of 28°C, 150 r/min, and 54.23 h were determined as the optimal fermentation conditions for strain Xw2. Rezende et al. (2002) reported the enzyme activity of *trichoderma viride* strain up to 288 IU/mL after optimization of fermentation medium. Yue et al. (2011) reported the enzyme activity of a xylanase producing bacteria (*Pestalotiopsis*) up to 49.3 IU/mL. Under these conditions, the strain exhibited an obvious improvement in the xylanase production at 358.86 IU/mL compared with that achieved by the fermentation of the other xylanase strains.

In fact, the yield of xylanase production is very low, such as *Penicillium purpurogenum* (10.4 U/mL), *Penicillium herquei* (3.5 U/mL). So, the substrate was mainly restricted to purified high-cost xylan, limiting the production of xylanases in the industry scale. Thus, the optimization of the fermentation conditions for strain Xw2 provides the basis for the industrial production and application of xylanase. High activity of Xw2 strain is beneficial to lower cost, increase production, so as to obtain greater economic benefits. Nevertheless, xylanase has some limitations, for example, its weak thermal stability, which poses a major obstacle in production (Panbangred et al., 1983). In the future, studies should implement genetic engineering technology, such as site-directed mutagenesis and molecular cloning, to improve the thermal stability of xylanase, and consequently improve its ease of preparation and scope of application.

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Compliance with ethics guidelines

Bingying YE, Ting XUE, Shichao YE, Shengyan XU, Weiyan LI, Jihua LU, Fang WEI, Wenjin HE and Youqiang CHEN declare that they have no conflict of interest. This manuscript is a microbial research article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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