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Effect of pH, Cultivation Time and Substrate Concentration on the Endoxylanase Production by *Aspergillus awamori* ZH-26 under Submerged Fermentation Using Central Composite Rotary Design

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Summary

The effect of substrate concentration, pH and cultivation time on the production of endoxylanase from *Aspergillus awamori* ZH-26 were optimized by statistical analysis using response surface methodology (RSM). Endoxylanase production was optimized by central composite rotary design. Statistical analysis of the results showed that the linear and quadratic terms of these three variables had significant effects. But no interactions between the three variables were found to contribute to the response at a significant level. The optimal conditions were: wheat bran 49.3 g/L, pH=4.14, and cultivation time 103.7 h. Under these conditions, the model predicted an endoxylanase activity of 28.25 U/mL. Verification of the optimization showed that endoxylanase production of 29.65 U/mL was observed under optimal conditions.

Key words: *Aspergillus awamori* ZH-26, optimization, response surface methodology, endoxylanase

Introduction

Xylanases have been widely used for clarifying fruit juices and wine (*1*), food processing in combination with cellulases (*2*), and improving the nutritional properties of agricultural silage and grain feed (*3*). Production of xylanase from a single microorganism renders its industrial application more feasible and economical. Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial application, including xylanases, whose levels in fungi are generally much higher than those in yeast and bacteria (*4*). *Aspergillus awamori* has been used for the production of enzymes such as glucoamylase (*5*), protease and xylanase (*6*). The important advantage of the application of *Aspergillus awamori* as suitable strain is that the organism has a long history of safe use for the manufacture of food products destined for human consumption and is regarded as nontoxigenic and nonpathogenic.

The cost of an enzyme is one of the main factors determining the economy of a process. Reducing the costs of enzyme production by optimization of the fermentation medium and process is the goal of basic research for industrial application. Lemos *et al.* (*7*) reported the influence of different nitrogen sources on xylanase production by *Aspergillus awamori*. Siedenberg *et al.* (*8*) investigated the effects of xylan concentration, fungal mor-

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phology, phosphate concentration and inoculum size on xylanase production by *Aspergillus awamori* on synthetic medium in shake flask cultures. However, their research was only limited to one-factor-at-a-time method and no systematic statistical design was employed to optimize the fermentation process of xylanase production by *Aspergillus awamori*.

The xylanase production by microorganisms is strongly influenced by many factors, such as carbon source (*9*), nitrogen source (*7*), growth sources, growth factors, inorganic salts (*10*), cultivation conditions (*11*), *etc*. Therefore, it is crucial to search for the key influencing factors from the many related ones. To perform such a work is extremely laborious and time-consuming, using the conventional techniques such as one-factor-at-a-time method. Moreover, it does not guarantee the determination of optimal conditions, and is unable to detect the frequent interactions occurring between two or more factors, although they often do occur. Response surface methodology (RSM) has been employed in the present study, as it is a collection of statistical techniques for designing an experiment, building models, evaluating the effects of factors, and searching for optimum conditions of factors for desirable responses. Recently, different statistical designs for medium optimization regarding xylanase production have been reported, among which factorial experiments and response surface methodology (RSM) are included (*11–14*). These statistical methods have proved to be powerful and useful tools.

To our knowledge, there is no scientific literature on the statistical optimization for the endoxylanase production by *Aspergillus awamori* ZH-26. This paper aimed at investigating the effects of substrate concentration, pH and cultivation time on the endoxylanase production by *Aspergillus awamori* ZH-26 using a response surface methodological approach.

Materials and Methods

Microorganism and inoculum preparation

Aspergillus awamori ZH-26 strain was isolated from Chinese rice wheat koji and was maintained at 4 °C on potato dextrose agar (PDA). Spore suspension was made from six-day-old cultures that had been grown on PDA slopes at 30 °C. Sterile distilled water was aseptically added to each slope and a suspension of the spores was made by lightly brushing the mycelium with a sterile wire loop. Erlenmeyer flasks (250 mL) containing 75 mL of medium were inoculated with 1 mL of $1 \cdot 10^7$ spores/mL suspension.

Cultivation media

The initial medium used for xylanase production was composed of (in g/L): glucose 10, NH_4Cl 9, KH_2PO_4 1, $NaNO_3$ 1, $MgSO_4 \cdot 7H_2O$ 1, $CaCl_2 \cdot 2H_2O$ 0.3, yeast extract 1. For the experiments of optimization, the cultivation medium was composed of different concentrations of wheat bran, with varying pH and cultivation time according to the experimental design.

Shake flask cultivation

The optimization experiments were carried out in 250-mL Erlenmeyer flasks containing 75 mL of cultivation medium. Shake flasks were seeded with inocula and then were maintained at 30 °C on a rotary shaker under 150 rev/min for 15.4, 48, 96, 144 or 176.6 h, according to the experimental design. The experiments were carried out in duplicate for each condition and the mean values were given.

Central composite rotary design

Response surface methodology (RSM) was used to optimize the submerged fermentation process for enhanced xylanase production. A Box-Behnken (*15*) factorial design with three factors and five levels, including six replicates at the centre point, was used for fitting a second order response surface. Tables 1 and 2 give the

Table 1. Coded values of variables used in central composite rotary design

Independent variables	Level				
	–1.68	–1	0	1	1.68
Wheat bran (X_1)/(g/L)	3	20	45	70	87
pH (X_2)	2.32	3	4	5	5.68
Cultivation time (X_3)/h	15.4	48	96	144	176.6

Table 2. Central composite rotary design matrix with experimental and predicted values of endoxylanase production by *Aspergillus awamori* ZH-26

Trial number	Variables			Endoxylanase production U/mL	
	X_1	X_2	X_3	Experimental	Predicted
1	–1	–1	–1	5.59	3.32
2	–1	–1	1	7.77	7.70
3	–1	1	–1	7.57	9.02
4	–1	1	1	13.97	13.40
5	1	–1	–1	11.17	10.28
6	1	–1	1	13.57	14.66
7	1	1	–1	18.37	15.98
8	1	1	1	16.11	20.36
9	–1.68179	0	0	4.38	5.32
10	1.681793	0	0	18.21	17.02
11	0	–1.68179	0	3.87	5.19
12	0	1.681793	0	16.34	14.77
13	0	0	–1.68179	10.81	13.31
14	0	0	1.681793	23.40	20.68
15	0	0	0	26.84	27.12
16	0	0	0	27.37	27.12
17	0	0	0	25.17	27.12
18	0	0	0	28.13	27.12
19	0	0	0	29.55	27.12
20	0	0	0	25.63	27.12

X_1 – wheat bran, X_2 – pH, X_3 – cultivation time

factors and their values, and the experimental design, respectively. This methodology allows the modeling of a second order equation that describes the process. Endoxylanase production was analyzed by multiple regression through the least squares method to fit the following equation:

$$Y=\beta_o+\Sigma\beta_i x_i+\Sigma\beta_{ij}x_i x_j+\Sigma\beta_{ii}x_i^2 \qquad /1/$$

where Y is the measured response variable, β_o, β_i, β_{ij} and β_{ii} are constant and regression coefficients of the model, and x_i and x_j represent the independent variables in coded values.

Data from the central composite rotary design for the optimization of endoxylanase production were subjected to a second-order multiple regression analysis using the least squares regression methodology to obtain the parameter estimates of the mathematical model. The regression analysis and analysis of variance (ANOVA) were carried out using the response surface regression (RSREG) procedure (*16*) of the SAS statistical package (version 8.1, SAS Institute, Cary, NC, USA) to fit the second order polynomial equations for all response variables. Canonical analysis, which is used to predict the shape of the curve generated by the model, was carried out as well. Response surface was made by the fitted quadratic polynomial equation obtained from the RSREG analysis, holding independent variables with one parameter at a constant value, and changing the other two variables.

Endoxylanase assay

The endoxylanase activity was determined by measuring the release of reducing sugars from oat spelt xylan (1 %, dry mass per volume) using the dinitrosalicylic acid method (*17*). Reaction mixture containing 2 mL of a solution of 1 % oat spelt xylan in citrate buffer 50 mM, pH=5.0 plus 1 mL of the diluted crude enzyme was incubated for 30 min at 50 °C. One unit of endoxylanase was defined as the amount of enzyme required to release 1 μmol of xylose from xylan in 1 min under the assay conditions.

Results and Discussion

The use of purified xylan enhances the cost of enzyme production and is a major limitation of the economically feasible bioconversion and utilization of lignocellulosic materials. Therefore, in this study, easily available agricultural residues like wheat bran were used as the main substrate in the experiment to obtain optimum levels of endoxylanase by *Aspergillus awamori* ZH-26.

Based on our earlier one-factor-at-a-time studies, substrate concentration, medium pH and cultivation time were identified as the major factors affecting endoxylanase production by *Aspergillus awamori* ZH-26. In the present work, these variables were statistically optimized using RSM.

Table 1 shows the maximum and minimum levels of variables chosen for trials in central composite rotary design. For RSM based on the central composite rotary design, which was used for the optimization of cultivation conditions for endoxylanase production, 20 experimental runs with different combinations of three factors were carried out (Table 2). The variables used for the factorial analysis were wheat bran, pH and cultivation time, named X_1, X_2 and X_3 in this design, respectively. For each run, the experimental responses along with the predicted response obtained from the regression equation for the 20 combinations are shown in Table 2, where it can be seen that there is a considerable variation in the xylanase production, depending on the substrate concentration, pH and cultivation time in the medium. The maximum endoxylanase production was 29.55 U/mL in run number 19. The centrepoint in the design was repeated for six times for the estimation of error. The replication at the centrepoint conditions resulted in higher endoxylanase production than at other levels.

By applying multiple regression analysis on the experimental data (Table 3), the following second order polynomial equation was found to explain the xylanase production regardless of the significance of coefficients:

$$Y=27.12+3.48X_1+2.85X_2+2.19X_3-5.64X_1^2-6.06X_2^2-$$
$$-3.58X_3^2+0.20X_1X_2-1.06X_1X_3-0.06X_2X_3 \qquad /2/$$

where Y is the predicted response, and X_1, X_2 and X_3 are coded values of wheat bran, pH and cultivation time, respectively.

Table 3. The least-square fit and parameters (significant of regression coefficient)

Model term	Degree of freedom	Estimate	Standard error	*t* value	$p>\|t\|$
Intercept	1	27.12	0.98	27.77	<0.0001*
X_1	1	3.48	0.65	5.38	0.0003*
X_2	1	2.85	0.65	4.39	0.0013*
X_3	1	2.19	0.65	3.38	0.0070*
X_1^2	1	–5.64	0.63	–8.94	<0.0001*
X_1X_2	1	0.20	0.85	0.23	0.8225
X_2^2	1	–6.06	0.63	–9.60	<0.0001*
X_1X_3	1	–1.06	0.85	–1.25	0.2411
X_2X_3	1	–0.06	0.85	–0.06	0.9495
X_3^2	1	–3.58	0.63	–5.68	0.0002*

*Significant at 5 % level ($p<0.05$)

Statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA) and the results are shown in Table 4. Analysis of vari-

Table 4. Analysis of variance for the response of endoxylanase production[a]

Source	Degree of freedom	Sum of squares	Mean square	F-value	p>F
Linear	3	341.95	–	19.88	0.0002
Quadratic	3	991.28	–	57.62	<0.001
Crossproduct	3	9.23	–	0.54	0.6677
Total model	9	1342.46	–	26.01	<0.001
Total error	10	57.34	5.73		–

[a]Coefficient of variation (CV)=14.34, coefficient determination (R^2)=0.9590, correlation coefficient (R)=0.9793

ance (F-test) showed that the second model is well adjusted to the experimental data. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the value of CV, the lower the reliability of the experiment. Here, a lower value of CV (14.34) indicates greater reliability of the experiments performed. The goodness of fit of the model can be checked by the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient (R^2) implies that the sample variation of 95.9 % for endoxylanase production is attributed to the independent variables, and only about 4.1 % of the total variation cannot be explained by the model. The closer value of R (correlation coefficient) to 1, the better the correlation between the experimental and predicted values. Here, the value of R (0.9793) for Eq. 2 being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. Linear and quadratic terms were significant at the 1 % level. Therefore, the quadratic model was selected in this optimization study.

The fitted contour plots for the endoxylanase production by the above model are shown in Figs. 1–3. The contour plots affirm that the objective function is unimodal in nature, which shows an optimum in the boundaries. The boundary optimum point was evaluated using gradient method in the direction of the steepest ascent. The graphical representation provides a method to visualize the relation between the response and experimental levels of each variable, and the type of interactions among test variables in order to deduce the optimum conditions. The contour plots clearly reveal that there were no saddle points within the experimental region.

Fig. 1 depicts the effect of substrate concentration and pH on the endoxylanase production, while cultivation time was fixed at its middle level. It can be seen from Fig. 1 that when the wheat bran concentration was below –0.6 (coded value), the effect of pH on endoxylanase production was not significant. When the wheat bran concentration in the medium was at a higher level, the yield of the endoxylanase production increased gradually with the increase of the pH value of cultivation medium, but decreased slowly beyond the optimal pH range (–0.2 to 0.4, coded value). As can be seen from this, increasing the substrate concentration within the tested range is beneficial to the accumulation of endoxylanase under submerged cultivation. The effect of wheat bran concentration and cultivation time on endoxylanase production while the pH was fixed at its middle level is shown in Fig. 2. This is the evidence that at low wheat bran concentration the effect of cultivation time on xylanase production was negligible. When the wheat bran concentration in the medium was at a higher level, endoxylanase production steadily increased with increasing cultivation time up to 0.2–0.3 (coded value), but decreased slowly beyond that range. This indicated that under optimal substrate concentrations and cultivation time, increase of cultivation time did not further increase the yield of endoxylanase production. These facts were important in making the whole process economically more feasible and made it possible to shorten the cultivation time in the potential industrial application. Fig. 3 shows the effect of pH and cultivation time on endoxylanase

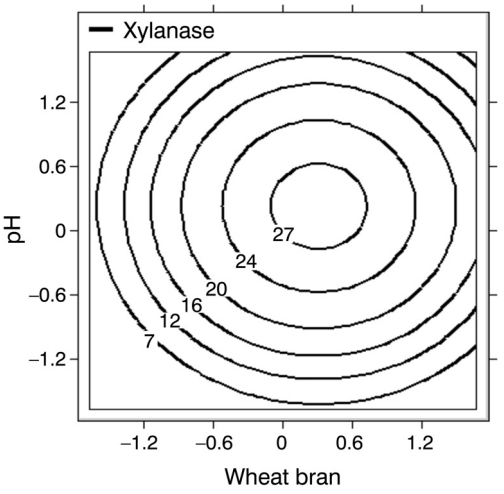

Fig. 1. Contour plot of the combined effects of wheat bran and pH on the endoxylanase production by *Aspergillus awamori* ZH-26

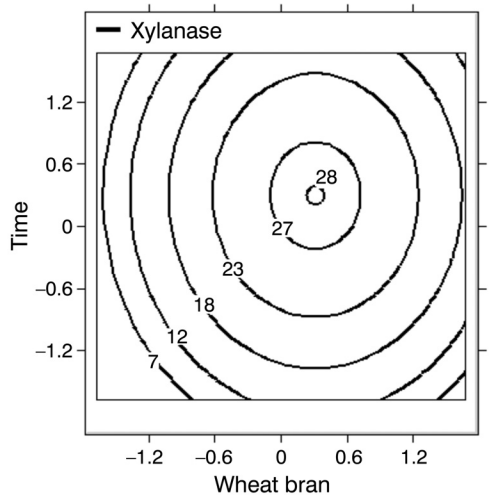

Fig. 2. Contour plot of the combined effects of wheat bran and cultivation time on the endoxylanase production by *Aspergillus awamori* ZH-26

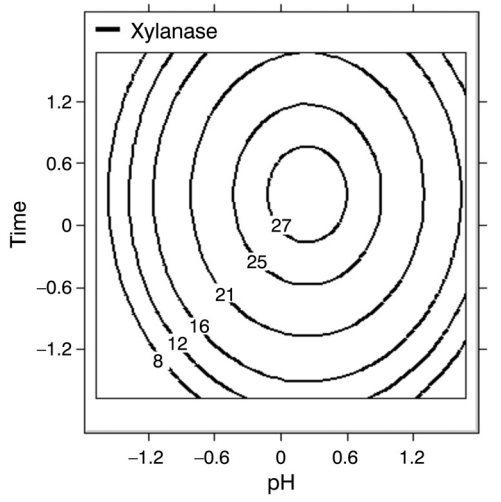

Fig. 3. Contour plot of the combined effects of pH and cultivation time on the endoxylanase production by *Aspergillus awamori* ZH-26

production while substrate concentration was fixed at its middle level. At low pH value, the effect of cultivation time on endoxylanase production was negligible. When pH in the medium was at a higher level, endoxylanase production steadily increased with increasing cultivation time up, but decreased slowly beyond that range.

The statistically optimal values of variables are obtained when moving along the major and minor axis of the contour at the centrepoint yields maximum endoxylanase production. These observations were also verified from canonical analysis of response surface. Canonical analysis revealed a minimum region for the model. The stationary point presenting a maximum endoxylanase had the following critical values: wheat bran 49.3 g/L, pH= 4.14, cultivation time 103.7 h. The predicted endoxylanase activity for these conditions was 28.25 U/mL.

The fermentation was repeated three times under optimal conditions in order to confirm the mathematical model. The maximal endoxylanase level obtained was 29.65 U/mL. This value was found to be 4.9 % higher than the predicted value. This discrepancy might be due to the slight variation in experimental conditions. The optimization resulted in 6.66-fold increase of endoxylanase production, compared to the lowest endoxylanase production of 3.87 U/mL at run 11 in central composite rotary design.

Conclusion

Statistical optimization method for fermentation process could overcome the limitations of classic empirical methods and has been proved to be a powerful tool for the optimization of endoxylanase production by *Aspergillus awamori* ZH-26. Under optimal conditions (wheat bran 49.3 g/L, pH=4.14, cultivation time 103.7 h), the predicted endoxylanase activity was 28.25 U/mL. Validation experiments were also carried out to verify the availability and the accuracy of the model, and the results showed that the predicted value agreed with the experimental value (29.65 U/mL) well.

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