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ORIGINAL PAPER

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Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical experimental designs

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Abstract The initial moisture content, cultivation time, inoculum size and concentration of basal medium were optimized in solid state fermentation (SSF) for the production of xylanase by an *Aspergillus niger* mutant using statistical experimental designs. The cultivation time and concentration of basal medium were the most important factors affecting xylanase activity. An inoculum size of 5×10^5 spores/g, initial moisture content of 65%, cultivation time of 5 days and 10 times concentration of basal medium containing 50 times concentration of corn steep liquor were optimum for xylanase production in SSF. Under the optimized conditions, the activity and productivity of xylanase obtained after 5 days of fermentation were 5,071 IU/g of rice straw and 14,790 IU l^{-1} h^{-1}, respectively. The xylanase activity predicted by a polynomial model was 5,484 IU/g of rice straw.

Introduction

A variety of microorganisms, including bacteria, yeast and filamentous fungi, have been reported to produce xylanase (Ken et al. 1988), of which the most potent producers are fungi (Haltrich et al. 1996). On an industrial scale, xylanases are produced mainly by *Aspergillus* and *Trichoderma* spp. Traditionally, the application of xylanases in conjunction with cellulolytic enzymes has been mainly considered for the bioconversion of lignocellulosic materials to produce fuel and other chemicals. Other potential applications include the clarification of fruit juices and wine, the extraction of plant oil, coffee and starch, the production of oligosaccharides and improvement of the nutritional value of feed (Kulkarni et al. 1999; Uma Maheswari and Chandra 2000; Wong and Saddler 1992). In recent years, interest in xylanases has markedly increased mainly due to their usage in the pulp and paper industry (Viikari et al. 1994; Wong et al. 1996).

Solid state fermentation (SSF) offers advantages over submerged fermentation. SSF processes have considerable economical potential in producing products for the food, feed, pharmaceutical and agricultural industries. More recent applications of SSF include the protein enrichment of agro-industrial residues, the production of enzymes, organic acids and other fungal metabolites, and spore production (Pandey et al. 2000; Sato and Sudo 1999). In particular, a great deal of published information is available on the production of enzymes of industrial importance such as protease, cellulase, ligninase, xylanase, pectinase, amylase, glucoamylase, etc. (Pandey et al. 1999).

The cost of an enzyme is one of the main factors determining the economics of a process. Reducing the costs of enzyme production by optimizing the fermentation medium and process is the goal of basic research for industrial applications. In general, optimization by the traditional 'one-factor-at-a-time' technique was used. This method is determined by varying one factor while keeping the other factors at a constant level. This method, although simple, often requires a considerable amount of work and time. Recently, statistical designs for optimization have been successfully employed in enzyme production (Ghanem et al. 2000; Souza et al. 1999). These statistical methods have proved to be powerful and useful tools.

The aim of this work is to evaluate the xylanase production of *Aspergillus niger* KK2 mutant grown on rice straw as a substrate in SSF. The effect of variables (initial moisture content, cultivation time, inoculum size and concentration of basal medium) on the production of en-

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zymes was studied by combining different statistical experimental designs.

Materials and methods

Microorganism

The fungal strain *Aspergillus niger* KK2 mutant (KFCC 11285, Korean Culture Collection of Microorganisms) was selected from *A. niger* KKS (ATCC 201201, Kang et al. 1994) by mutagenesis (Kang et al. 1999). The strain was cultivated in potato dextrose agar at 28°C for 7 days and stored at –20°C in 30% glycerol.

Raw material

Lignocellulosic material – rice straw – was cut into small pieces. The chopped rice straw was ball-milled and sieved with #50. The solid residue remaining above #50 was dried overnight at 70°C and used for SSF.

Inoculum

Distilled water (20 ml) was added to the slant tube containing the spore culture. The spore suspension was transferred to a beaker and the spores were counted in a haemocytometer. Each flask containing 5 g of rice straw was inoculated with an amount initially containing 10^4–10^6 spores per gram of rice straw.

Solid state fermentation

The basal medium contained, in grams per 100 g: $CoSO_4 \cdot 7H_2O$, 0.01; $CuSO_4 \cdot 5H_2O$, 0.05; KH_2PO_4, 0.5; corn steep liquor (CSL), 1.0; industrial yeast extract, 0.05. The pH of the basal medium was adjusted to 7.0. The required concentration (×1, ×5 and ×9) of basal medium was poured into individual 250 ml Erlenmeyer flasks containing 5 g of rice straw according to experimental designs and distilled water was added to adjust the initial moisture content to 50, 65 and 80%. The medium was then autoclaved for 30 min at 121°C. After cooling, the flasks were inoculated and the contents, after mixing, were incubated at 28°C under static conditions for 5, 7 or 9 days.

Enzyme extraction

After incubation, 200 ml of distilled water was added to each flask and stirred. The pH of the mixture in each flask was measured. The mixture, plus another 200 ml of fresh distilled water, was homogenized at 10,000 rpm at room temperature for 5 min. Solids were separated by centrifugation and the xylanase activity of the supernatant assayed.

Estimation of enzyme activity

Xylanase activity was assayed in 1.5 ml of a reaction mixture containing 0.5 ml of diluted enzyme solution and 1 ml of supernatant of 2.0% (w/v) birchwood xylan (Sigma) in 0.05 M citrate buffer (pH 4.8). The mixture was incubated at 50°C for 20 min. After the incubation period, the reducing sugars were determined by the dinitrosalicylic acid method (Miller 1959) with xylose as standard.

One unit of enzyme activity is defined as the amount of enzyme which releases 1 µmol of xylose in 1 min under the assay conditions.

Table 1 Range of variables at different levels for the fractional factorial design

Independent variables X_i	Levels		
	–1	0	+1
X_1 Initial moisture content (%)	50	65	80
X_2 Cultivation time (days)	5	7	9
X_3 Inoculum size (spores/gram)	10^4	5×10^5	10^6
X_4 Concentration of basal medium (magnitude)	×1	×5	×9

Experimental design

A 2^{4-1} fractional factorial design leading to eight sets of experiments, performed in duplicate, was used to determine the most significant factor affecting the xylanase activity. The variables were coded according to Eq. 1:

$$x_i = (X_i - X_0)/\Delta X_i \quad (1)$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change value. The range and the levels of the variables investigated in this study are given in Table 1. The xylanase activity was taken as the dependent variable or response, Y_i. Empirical fitting of the experimental data was by polynomial regression, based on analysis of variance (ANOVA).

In order to fit an empirical second-order polynomial model, a central composite design with five coded levels was performed. The quadratic model for predicting the optimal point was expressed according to Eq. 2:

$$y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \quad (2)$$

where y is the response variable, b the regression coefficients of the model, and x the coded levels of the independent variable. An SAS package was used for the regression analysis of the experimental data obtained.

The statistical significance of the second-order model equation was determined by F-value and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

Results

The four variables playing the most important role in SSF were chosen (see Table 1) and the effect of each variable on the production of xylanase was investigated. ANOVA was employed for the determination of significant variables. The experimental design and the results of the 2^{4-1} fractional factorial design are shown in Table 2. The xylanase activity varied markedly with the conditions tested, in the range of 168–1,177 IU/g. The lowest value of xylanase activity was obtained when maximal level of cultivation time and minimal level of concentration of basal medium were used (run 5). Xylanase activities higher than 1,000 IU/g were obtained under conditions of minimal level of cultivation time and maximal level of concentration of basal medium. These results suggest that these variables strongly affect xylanase production.

On the basis of these experimental values, statistical testing was carried out using Fisher's statistical test for

Table 2 Experimental design and results of 2^{4-1} fractional factorial design

Runs	Code levels				Xylanase activity (IU/g)
	x_1	x_2	x_3	x_4	
1	–	–	–	–	557
2	–	–	+	+	1,177
3	–	+	–	+	213
4	+	–	–	+	1,049
5	–	+	+	–	168
6	+	–	+	–	487
7	+	+	–	–	294
8	+	+	+	+	736
9	0	0	0	0	457
10	0	0	0	0	411
11	0	0	0	0	487
12	0	0	0	0	377

ANOVA. The *F*-value is the ratio of mean square due to regression to the mean square due to error and indicates the influence (significant or not) of each controlled factor on tested models. Also, the *P*-value corresponding to the *F*-value indicates the probability that differences between calculated and tabulated statistics are due only to random experimental error. When ANOVA analysis reflects the significance of a model with a confidence level greater than 99% ($P < 0.01$) in xylanase production, the *F*-value and *P*-value were 42.01 and 0.0097, respectively, as shown in Table3. Thus, the estimated models fit the experimental data adequately. The F test applied on each factor, x_2 and x_4 is statistically significant at the 1% level of significance. The *F*-values (182.2 and 146.9, respectively) of these are greater than other significant factors. Analysis of the ANOVA results showed that cultivation time (X_2) and concentration of basal medium (X_4) proved to be the two most important variables for the production of xylanase. There is no evidence of any interactions involving these factors.

Response surface methodology was introduced to determine the optimal condition of X_2 and X_4 variables. In order to obtain the optimal condition of X_2 and X_4 variables, variables X_1 and X_3 were set at 65% of initial moisture content and inoculum size of 5×10^5 spores/g, respectively. The experiment was carried out with two independent variables – cultivation time (X_2) and concentration of basal medium (X_4) – using a 2^2 full factorial design experiment with four star points ($\alpha = \pm1.414$) and four replicates at the center point. A series of experiments showed that the production of xylanase improved in proportion to increases in the concentration of basal medium. The design of the experiment and results are presented in Table 4.

Regression analysis was performed to fit the response function with the experimental data. As shown in Table 5, the *F*-value and *P*-value were 46.7 and 0.0001, respectively. The tested model is statistically significant at the 1% level of significance. The statistical significance of the second-order model equation was checked and the coefficient of determination (R^2) of the model

Table 3 Results of the regression analysis of the of 2^{4-1} fractional factorial design

Factors	Mean square	*F*-value	*P*-value
x_1	25,425	10.72	0.049
x_2	431,985	182.22	0.0028
x_3	25,878	10.92	0.0478
x_4	348,195	146.88	0.0033
$x_1 \times x_2$	89,676	37.83	0.0117
$x_1 \times x_3$	60,378	25.47	0.0181
$x_1 \times x_4$	14,365	6.06	0.0918
$x_2 \times x_3$	14,365	6.06	0.0918
$x_2 \times x_4$	60,378	25.47	0.0181
$x_3 \times x_4$	89,676	37.83	0.0117
Model	9,959	42.01	0.0097

Table 4 Experimental design and results of the 2^2 full factorial central composite design[a]

Runs	Cultivation time (day, x_2)	Concentration of basal medium (magnitude, x_4)	Xylanase activity (IU/g)
1	2 (–)	×35 (–)	1,193
2	6 (+)	×35 (–)	3,390
3	2 (–)	×65 (+)	671
4	6 (+)	×65 (+)	4,908
5	1.2 (–1.414)	×50 (0)	12
6	6.8 (+1.414)	×50 (0)	3,726
7	4 (0)	×28.79 (–1.414)	2,769
8	4 (0)	×71.21 (+1.414)	4,967
9	4 (0)	×50 (0)	4,930
10	4 (0)	×50 (0)	4,930
11	4 (0)	×50 (0)	4,833
12	4 (0)	×50 (0)	4,908

[a] X_1 (initial moisture content)=65%; X_3 (inoculum size) $=5\times10^5$ spore/g of rice straw

Table 5 Statistical analysis for models of xylanase production at different levels of cultivation time and concentration of basal medium[a]

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	37,071,482	5	7,414,296	46.69	0.0001
Error	952,881	6	952,881		
Total	38,024,362	11			

[a] R^2=0.97

was calculated to be 0.97, indicating that 97% of the variability in the response could be explained by the model. This indicates that the response equation provided a suitable model for the response surface of the experiment of xylanase production. The response equation obtained is as follows: $Y = 4{,}900.3 + 1{,}460.9\ x_2 + 513.1\ x_4 - 1{,}598.0\ x_2^2 - 598.2\ x_4^2 + 510.0\ x_2\ x_4$, where x_2= coded value of cultivation time, x_4= coded value of concentration of basal medium.

Fig. 1 shows a three-dimensional diagram and a contour plot of calculated response surface. The optimum

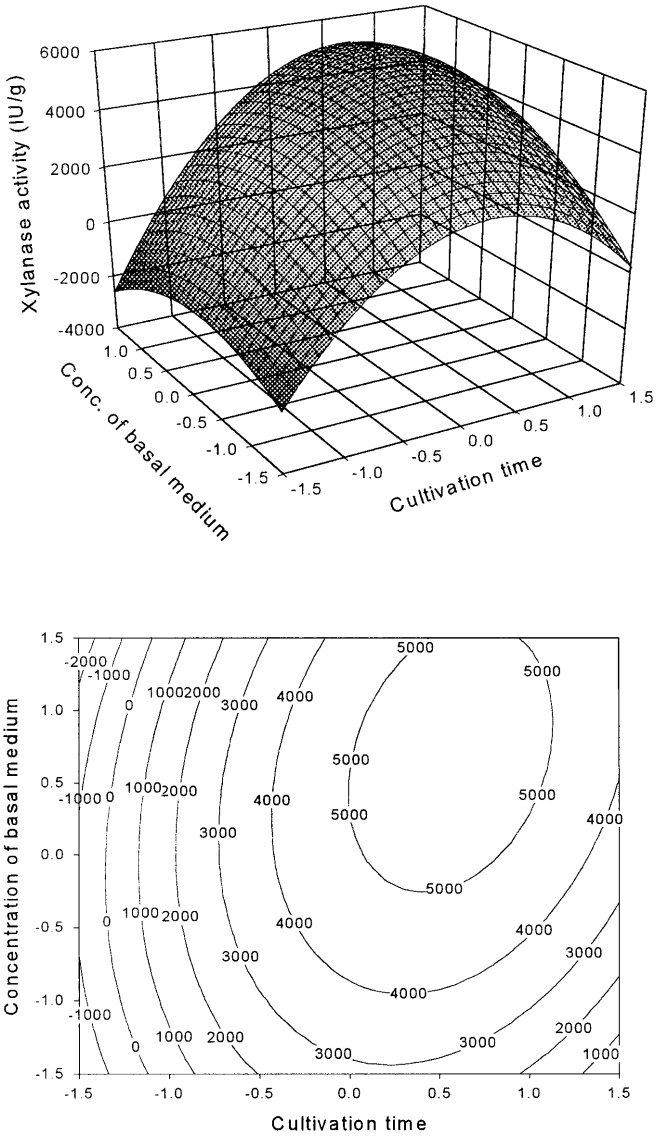

Fig. 1 Three-dimensional response surface plot of the central composite design experiment and contour plot of the calculated response surface

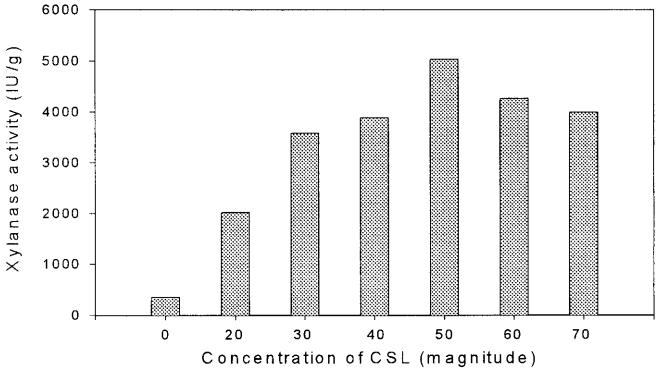

Fig. 2 Effect of concentration of corn steep liquor (CSL) on xylanase production under the optimized conditions in solid state fermentation (SSF). Concentration of basal medium without CSL was fixed at 10 times

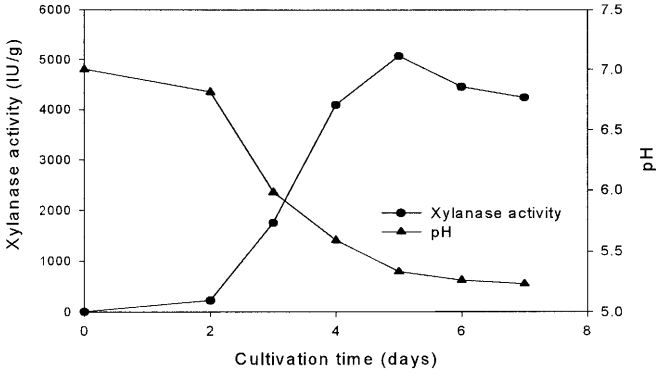

Fig. 3 Time course profile of xylanase production by *Aspergillus niger* KK2 mutant in SSF. Experimental conditions: initial moisture content 65%, inoculum size 5×10^5 spore/g of rice straw, 10 times concentration of basal medium containing 50 times concentration of CSL

values of cultivation time (x_2) and concentration of basal medium (x_4) obtained for xylanase production are the following: x_2 =0.5639, x_4 =0.6693. For calculation purposes, the normalized, coded values x_2 and x_4 were defined as: $x_2=(X_2-4)/2$ and $x_4=(X_4-50)/15$.

According to these results, optimal cultivation time and concentration of basal medium for xylanase production were calculated to be 5.1 days and 60.0 times, respectively. The maximum value of enzyme activity predicted from the model was 5,484 IU/g.

In order to determine whether CSL was an important component from among the components of basal medium, an experiment was carried out with the concentration of basal medium (without CSL) fixed at 10 times, and addition of various concentrations of CSL. With 50 times concentration of CSL, which resulted in the maximum activity of all the concentrations of CSL tested, enzyme activity was 5,034 IU/g. When CSL was not added, enzyme activity was significantly lower (354 IU/g) than if CSL was added at any of the concentrations tested (Fig. 2).

Consequently, an inoculum size of 5×10^5 spores/gram, initial moisture content of 65%, cultivation time of 5 days and 10 times concentration of basal medium containing 50 times concentration of CSL were optimum for xylanase production in SSF.

To confirm the optimal conditions, experimental rechecking was performed using conditions representing these optimal factors. The time course profile of xylanase activity is shown in Fig. 3. The strong correlation between experimental and statistical results confirms the validity of the response model and the existence of an optimal point. The highest xylanase activity of 5,071 IU/g was obtained after 5 days of fermentation.

Table 6 Comparisons of xylanase production from other strains grown on lignocellulosic materials

Organism	Substrate	Initial moisture content	Cultivation condition	Xylanase		Reference
				Activity (IU/g)	Productivity (IU $l^{-1}h^{-1}$)[a]	
Aspergillus niger CBS 11042	Wheat straw + bran (1+1), pretreated	70%	Static, 30°C, 3 days	2,500	10,400	Deschamps and Huet (1985)
Aspergillus ustus	Rice straw	75%	Static, 25–28°C, 5 days	797	1,660	Shamala and Sreekantiah (1987)
Chaetomium globosum	Wheat bran + beet pulp (8+2)	50%	Static, 35°C, 48 h	297	3,090	Wiacek-Zychlinska et al. (1992)
Melanocarpus albomyces IIS-68	Wheat straw Holocellulose from rice straw	67%	Static, 45°C, 4 days	756 1,084	2,620 3,760	Jain (1995)
Thermoascus aurantiacus	Bagasse	81%	Static, 45°C, 10 days	2,700	2,318	Souza et al. (1999)
Aspergillus niger KK2 mutant	Rice straw	65%	Static, 28°C, 5 days	5,071	14,790	This work

[a] Calculation of volumetric productivity is based on the initial moisture content

Discussion

Statistical optimization methods were used for the production of xylanase by *A. niger* KK2 mutant grown on rice straw in SSF. In this work, cultivation time and concentration of basal medium had the greatest effect on the production of xylanase. Decreasing cultivation time and increasing concentration of basal medium improved the production of xylanase. Reducing the cultivation time is promising from an economic point of view. Souza et al. (1999) have reported that initial moisture content (81%) and bagasse mass were the most important variables affecting xylanase activity produced by *Thermoascus aurantiacus*. Gonzalez et al. (1988) have shown that in SSF using *Penicillium chrysogenum*, of all culture conditions the initial moisture content is one of the most critical. Also, Castilho et al. (2000) reported that an initial moisture content of 40% provided the best conditions for production of pectinase by *A. niger*. Although many researchers have reported that the most important variable in SSF is the initial moisture content, this study showed no significant effect of this parameter (Table 3). Concentration of basal medium proved to be the important variable for xylanase production (Fig. 1). Among the components of basal medium, CSL as a nitrogen source considerably affected the production of xylanase. It was found that CSL is a good enough nutrient to support high titer xylanase production. This indicates that the amount of nitrogen present in rice straw is too low to support good growth and enzyme production. Because CSL contains vitamins, minerals and carbohydrates, it has been successfully used for other fermentations. Archana and Satyanarayana (1997) reported that CSL marginally stimulated xylanase production by thermophilic *Bacillus licheniformis* A99 in solid-state culture. Silveira et al. (2001) showed that CSL as a source of vitamins was an effective and cheap supplement for *Zymomonas mobilis* medium. For large scale use of xylanase, the cost of its preparation will certainly be the crucial factor and will be markedly reduced by using inexpensive agro-industrial wastes such as rice straw and CSL.

The maximum value of xylanase activity achieved was 5,071 IU/g of rice straw, i.e., similar to the 5,484 IU/g of rice straw predicted by the model. The productivity of xylanase by *A. niger* KK2 mutant in SSF was 14,790 IU l^{-1} h^{-1} and was compared with those reported for other xylanase-producing microorganisms (Table 6). The *A. niger* KK2 mutant used in this work had the highest yield and productivity among reported values for xylanase production from lignocellulosic materials.

In conclusion, *A. niger* KK2 mutant is a potential microorganism for the production of xylanase using SSF. No addition of expensive media is required and the use of inexpensive agro-industrial wastes will have important economic advantages.

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