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Solid-state fermentation for xylanase production by *Thermoascus aurantiacus* using response surface methodology

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Abstract We investigated xylanase production by *Thermoascus aurantiacus* using semisolid fermentation. Multivariant statistical approaches were employed to evaluate the effects of several variables (initial moisture in the medium, cultivation time, inoculum level, and bagasse mass) on xylanase production. The initial moisture content and bagasse mass were the most important factors affecting xylanase activity. The xylanase activity produced by the fungus under the optimized conditions (81% moisture content and 17 g bagasse) was found to be 2700 U per gram of initial dry matter, whereas its value predicted by a polynomial model was 2400 U per gram of initial dry matter.

Introduction

Interest in thermostable xylanases has increased dramatically since resistance to thermal inactivation has become a desirable property of the enzymes used in many industrial applications. Xylanase stages have been successfully implemented in the prebleaching of kraft pulp (Viikari et al. 1986) and improvements have been achieved by attention to the use of xylanases that act at higher temperatures (Yee and Tolan 1997). Different strains of the thermophilic ascomycete *T. aurantiacus* have been well documented as a prospective source of highly thermostable xylanase, which also compared favorably with xylanase from other thermophiles (Alam et al. 1994; Grajek 1987; Yu et al. 1987; Kalogeris et al. 1998). Studies performed with a Brazilian strain of *T. aurantiacus* isolated from chip piles of *Eucalyptus* wood showed unusual characteristics compared with those described in the literature (Auer 1986). The production of phenol oxidases has been well documented (Machuca and Duran 1993; Machuca et al. 1998), but little attention has been given to studying xylanase production. Thus, the present study focused on solid-state production of xylanase activity by *T. aurantiacus* in sugar cane bagasse.

Solid-state cultivation offers advantages over liquid cultivation, especially for fungal cultures. Due to the nature of the substrates, aeration tends to require lower pressures than are needed for liquid cultivations, and vigorous agitation is not required. The metabolites so obtained are more concentrated and purification procedures are less costly (Nigam and Singh 1994; Chatterjee et al. 1996; Kumaran et al. 1997).

The aim of this work was to apply fractional factorial design followed by the response surface methodology to examine and optimize incubation time, substrate mass, moisture content, and inoculum size as variables that can affect xylanase production by *T. aurantiacus*.

Materials and methods

Microorganism

The Brazilian strain of the thermophilic ascomycete *T. aurantiacus* ATCC 204492 was cultivated in potato-dextrose agar (PDA) and incubated at 45 °C for 7 days.

Raw material

Bagasse in dry form was milled in a hammermill to pass through a 0.75 mm screen before use. The moisture content of the milled bagasse was determined and corrections were made, with all percentages on a dry weight basis.

Inoculum

Ten milliliters of distilled water was added to the tube containing the spore culture. After scraping the surface of PDA, the spore suspension was transferred to a tube and the spores were counted in a Neubauer chamber, and the medium was inoculated with an

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amount initially containing 10^4 or 10^6 spores per gram of substrate (Table 1).

Enzyme production

The fermentation medium contained in grams per 100 g: $H_2PO_4 \cdot H_2O$, 0.5; NH_4NO_3, 0.2; $MgSO_4 \cdot 7H_2O$, 0.02; $CaCl_2 \cdot 2H_2O$, 0.01; $C_6H_5Na_3O_7 \cdot 2H_2O$, sugar cane bagasse as support, at levels of 5, 10, or 15 g (see Table 1). The medium was poured into 300-ml Erlenmeyer flasks and distilled water was added to adjust the moisture to 50, 65, and 80%. The pH was measured and adjusted to 5.5. The media was then autoclaved for 60 min at 121 °C. After cooling, the flasks were inoculated with the spore suspension, and the contents, after mixing, were incubated at 45 °C under static conditions for 10, 15 or 20 days.

Enzyme extraction

After incubation, 100 ml 50 mM sodium acetate buffer at pH 5.5 was added to each flask and the mixture was homogenized and then shaken at 60 oscillations/min at room temperature for 1 h. Solids were separated under vacuum and this extraction was repeated three times and the xylanase activity of the supernatant solution assayed.

Analysis

Xylanase activity was assayed using 1% birchwood xylan (Sigma, USA), determined according to the method of Bailey et al. (1992). The release of reducing sugars was determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959) using xylose solution as a standard reference. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol reducing sugars per minute. The results of these analyses are expressed as units per gram of initial dry bagasse.

The moisture contents of solid samples were estimated from the difference in weights of samples before and after drying in an oven at 60 °C.

Experimental design

A 2^{4-1} fractional factorial design leading to eight sets of experiments, performed in duplicate, was used to verify 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. Based on the first-order model equation obtained by the fractional factorial design used, a new series of trials was performed in the direction of the steepest ascent (see Table 4).

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:

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

where y is the response variable, b the regression coefficients, and x the coded levels of the independent variable.

Statgraphics version 6.0 was used for the regression analysis of the experimental data obtained. The statistical significance of the second-order model equation was determined by Fisher's test and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

Results

The experimental design and the results of the fractional factorial design are shown in Table 2. The xylanase activity varied markedly with the conditions tested, in a range of 13–2800 U/g. The lowest values of xylanase activity were obtained when minimal levels of initial moisture and bagasse mass were used (assays 1 and 7). Xylanase activities higher than 2000 U/g were obtained when initial moisture and bagasse mass were adjusted to the highest levels. These results suggest that these variables strongly affect the xylanase activity. As can be seen from Table 3, the factors moisture content (X_1) and bagasse mass (X_4) were found to be significant at the probability level of 95% for xylanase production. The main effects of these variables were positive and there is no evidence of any interactions involving these factors. The effects of moisture content and bagasse mass increased the xylanase yield by about 1485 and 1088 U/g substrate respectively, and this was irrespective of the other variables. The values of the regression coefficients were calculated (data not shown) and an equation of the

Table 1 Range of values for the fractional factorial design

Independent variables X_i	Levels[a]		
	−1	0	+1
X_1 Initial moisture (%)	50	65	80
X_2 Incubation time (days)	10	15	20
X_3 Inoculum level (spores/g)	10^4	5.10^5	10^6
X_4 Bagasse mass (g)	5	10	15

[a] $x_1 = (X_1 - 65)/15; x_2 = (X_2 - 15)/5; x_3 = (X_3 - 10^5)/5 \cdot 10^5; x_4 = (X_4 - 10)/5$

Table 2 Experimental design and results of the fractional factorial design[a] (X_1 initial moisture (% w/v), X_2 incubation time (days), X_3 inoculum level (spores per dry weigh bagasse), X_4 bagasse mass (g); −, + mean lower and higher levels)

Run	X_1	X_2	X_3	X_4	Xylanase (U/g)	
					Assay 1	Assay 2
1	−	−	−	−	13	12
2	+	−	−	+	2250	2370
3	−	+	−	+	879	1122
4	+	+	−	−	1589	1118
5	−	−	+	+	870	997
6	+	−	+	−	1242	1505
7	−	+	+	−	13	12
8	+	+	+	+	2750	2875
9	0	0	0	0	1665	1596
10	0	0	0	0	1514	1779

[a] Experiments were performed in replicate with an average variation of ± 10%

Table 3 Estimated effects, standard errors, and Student's *t*-test results for the factorial design (2^{4-1}) with two center points

Variables	Estimated effects	Standard error	*t* Values
Average	1313.807	±58.6223	–
X_1	1485.105	±131.0836	11.33*
X_2	149.97	±131.0836	1.14
X_3	126.185	±131.0836	0.96
X_4	1088.44	±131.0836	8.30*
$X_1X_2 + X_3X_4$	166.217	±131.0836	0.88
$X_1X_3 + X_2X_4$	159.937	±131.0836	1.22
$X_1X_4 + X_2X_3$	134.102	±131.0836	1.02
Block	70.146	±117.2447	0.59

*Significant at the 95% level

first order (Eq. 3) was attained with a very high coefficient of determination, $R^2 = 0.95$.

$$Y = 1314 + 743x_1 + 544x_4 \quad (3)$$

Thus, the path of steepest ascent was to increase the moisture content and bagasse mass in order to improve the xylanase production. The incubation time (X_2) and inoculum concentration (X_3) were fixed at 10 days and 10^4 spores per gram, respectively.

The values of xylanase activity obtained in these experiments (Table 4) were very similar to those achieved in assays 2 and 8 (Table 2). These results indicate that we were working in the neighborhood of the optimum xylanase activity.

Optimization of xylanase production was carried out by two independent process variables: initial moisture (X_1) and bagasse mass (X_4) using a 2^2 full-factorial central composite design experiment with four star points ($\alpha = \pm\sqrt{2}$) and two replicates at the center point. The design of this experiment is given in Table 5, together with the results the experiment produced. Regression analysis was performed to fit the response function with the experimental data. The statistical significance of the second-order model equation was checked by an F-test (ANOVA) and the data are presented in Table 6. The fit of the polynomial model (R^2) was calculated to be 0.94, indicating that 94% of the variability in the response could be explained by the model. The response equation obtained is as follows, and represents a suitable model for xylanase production:

Table 4 Design of experiments to obtain the steepest ascent path and corresponding xylanase yields

Assay number	X_1	X_4	Xylanase activity (U/g)
1	0	0	2341
2	0.33	0.2	2062
3	0.67	0.34	1927
4	1	0.5	2262
5	1.2	0.6	2939
6	1.3	0.65	2197
7	1.4	0.7	2859

x_2 (incubation time) = 10 days, x_3 (inoculum level) = 10^4 spores per dry weight bagasse

Table 5 Experimental design and results of the 2^2 full factorial central composite design[a]

Run	Factor		Xylanase activity (U/g)	
	X_1	X_4	Assay 1	Assay 2
1	−1	−1	13	12
2	+1	−1	1416	1312
3	−1	+1	875	1060
4	+1	+1	2500	2673
5	$-\sqrt{2}$	0	305	189
6	0	$-\sqrt{2}$	1012	1012
7	$\sqrt{2}$	0	1598	1740
8	0	$\sqrt{2}$	1696	1945
9	0	0	1665	1687
10	0	0	1513	1815

[a] Experiments were performed in replicate with an average variation of ±10%

$$Y = 1669.7 + 623.8x_1 + 415.9x_4 + 66.9x_1x_4 - 345.8.08x_1^2 - 115.3x_4^2 \quad (4)$$

The three-dimensional graph obtained from the calculated response surface is represented in Fig. 1. For calculation purposes, the normalized, coded variables x_1 and x_4 were defined as: $x_1 = (X_1–65)/15$ and $x_4 = (X_4 - 10)/5$.

Table 6 Analysis of variance (ANOVA) for the full regression

Source	Sum of squares	Degrees of freedom	Mean square	*F* Ratio	*P* Value
Model	10099277	5	2019855	44.74	0.0000
Error	631985	14	45141.8		
Total	10731262	19			

R^2 = 0.94

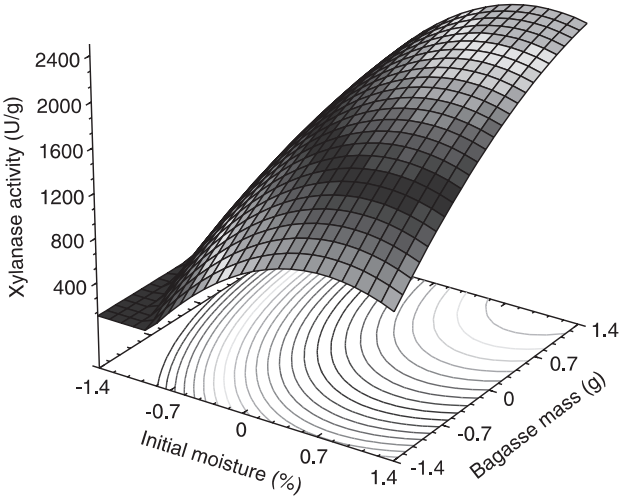

Fig. 1 Dependence of xylanase activity by *Thermoascus aurantiacus* on the initial moisture and bagasse mass. Experimental conditions: temperature 45 °C, incubation time 10 days, inoculum level 10^4 spores/g

This is a reconfirmation that the fitted surface has a maximum point which is 81% initial moisture ($x_1 = 1.1$) and 17 g bagasse ($x_4 = 1.4$). The model predicted a maximum response of 2400 U/g for this point. To confirm these results, experimental rechecking was performing using a medium representing this maximum point, and a value of 2700 U/g was obtained. The good correlation between these two results confirms the validity of the response model and the existence of an optimal point.

Discussion

The production of xylanase by *T. aurantiacus* has been under study in this laboratory with the aim of developing technology for the production of enzymes using raw materials. This particular fungus is of interest because it produces a highly thermostable xylanase in culture broth and this characteristic has a variety of biotechnological applications (Zamost et al.1991, Zentgraf 1992). Several enzymes from *T. aurantiacus*, such as xylanase (Khandke et al. 1989; Alam et al. 1994; Kalogeris et al. 1998) and arabinofuranosidase (Roche et al. 1994) have been produced by solid-state fermentation and in our laboratory sugar cane bagasse has been tested for xylanase production. The optimization of this process often presents a situation in which several variables need to be considered and yet the relative importance of each is still not known. Statistical optimization methods for solid-state fermentation have overcome the limitations of classical empirical methods (Sircar et al. 1998) and proved to be a powerful and useful tool for the optimization of xylanase production. The factor levels were defined on the basis of previous research which indicated that the moisture content, inoculum level, bagasse mass, and time of fermentation affect the fungal growth and enzyme production (Roussos et al. 1991; Kalogeris et al. 1998). In this study, the initial moisture of the medium and bagasse concentration had the most important effects on xylanase production. Increasing the bagasse mass and the initial moisture in the culture medium increases the xylanase activity. Indeed, for fermentation we propose reducing the inoculum level and time of fermentation, since these variables were not significant (Table 3), and this procedure is also interesting from an economic point of view (Mitchell and Lonsane 1992). Bagasse is a lignocellulosic substrate with 19% lignin, 46% cellulose, 24% hemicellulose, and 3% extractives (Imrie and Tilbury 1972). Moreover, owing to its low protein content and its lignocellulosic nature, bagasse is a selective source of enzyme induction, and this facilitates the recovery and purification processing of xylanase. When *T. aurantiacus* was grown in bagasse medium the xylanase activity reached a value similar to the one predicted by the model. The xylanase activity obtained in this study was compared with reference data for similar species. A detailed analysis of xylanase production by *T. aurantiacus* using beet pulp was carried out by Grajek (1987), but 26 times less xylanase activity was achieved. Biswas et al. (1988) studied the production of xylanase using wheat bran and sugar cane bagasse in solid medium with *Aspergillus ochraceus* and found that the bagasse gave lower yields than the wheat bran. From their results it can be concluded that the xylanase activity of several fungal species is directly related to the composition of the culture medium, to the cultivation conditions, and to the recovery of the enzyme from the medium. The utilization of solid lignocellulosic substrates for large-scale xylanase production has proved economically viable (Haltrich et al. 1996). Furthermore, when these substrates are used, the enzyme activities are frequently higher than those attained with pure xylan (Keskar 1992). However, the development of a solid-state fermentation process with high productivity requires a set of controlled variables. Regarding the medium composition, the choice of an appropriate substrate, suitable pretreatment, and moisture control determined the xylanase production and extraction from *T. aurantiacus*.

This paper is also an attempt to demonstrate the applicability of statistical theories to the study of solid-state fermentation processes. An optimized model was predicted and the next step for enhancing xylanase production should be production in a bioreactor under optimized conditions.

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