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Optimization of xylanase production by *Streptomyces* sp. P12-137 using response surface methodology and central composite design

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Abstract Response surface methodology and central composite design were used to optimize a biosynthesis medium for the production of xylanases by *Streptomyces* sp. P12-137 in submerged fermentation culture at pH 5.0, with wheat bran as substrate. The three variables involved in this research were the wheat bran, potassium nitrate and xylose concentrations. Statistical analysis of the results showed that, in the range studied, xylose and potassium nitrate concentrations had a significant effect on xylanase production. The optimized biosynthesis medium contained (in %, w/v): wheat bran 1.0, KNO_3 1.0, xylose 0.5. This medium resulted in a 3-fold increased level of the xylanase (27.77 UA/ml) production compared to the initial level (8.30 UA/ml) after 120 h of fermentation, whereas the value predicted by the quadratic model was 26.45 UA/ml.

Keyword Xylanase · *Streptomyces* · Response surface methodology · Optimization · Central composite design

Introduction

Lignocellulose biomass represents an unexploited resource. There is increasing interest in using hemicelluloses wastes for bioconversion, with a view to obtaining unconventional energy and food resources (Rosado and Nadathur 2003; Coman et al. 2008).

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Xylan is a major component of plant cell walls, which constitute approximately one-third of all renewable organic carbon sources on Earth. Complete xylan degradation requires the combined action of different xylanolitic enzymes, such as endo-xylanase, β-xylosidase, α-arabinofuranosidase, and esterase (Techapun et al. 2002a, b). Xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is an important component in a group of enzymes that act by depolymerizing the xylan molecules into monomers that can be used by microbial systems as a primary source of sugar (Goulart and Carmona 2005). The products of xylan hydrolysis are xylose, xylobiose, xylotriose, xylotetrose and xylo-oligosaccharides. These compounds have possible applications as food additives for poultry, in wheat flour for improving dough handing and the quality of baked products, for extraction of coffee and plant oils, and in combination with pectinase and cellulase for the clarification of fruit juices and wines (Ninave et al. 2008). Xylanase has been used in bleaching during paper production, resulting in reduced use of chemicals. The alkali-stable xylanase can reduce pollution due to chloro-organo compounds in the paper pulp industry by avoiding the need for the use of chlorine which is also a significant health hazard, for bleaching (Senthilkumar et al. 2005; Buchert et al. 1994).

Culture medium optimization by the traditional "one-factor-at-a-time" technique requires a considerable amount of time and work. An alternative, statistical strategy is to use factorial experimental design and response surface methodology (RSM), which involves a minimum number of experiments and covers a large number of factors. These methods have also been employed to improve the production of microbial xylanases in submerged culture.

RSM is a popular optimization method used in recent years. There are many reports of studies based on the application of RSM in biochemical and biotechnological processes (Bas and

Boyaci 2007). RSM comprises statistically designed experimental techniques for estimating the coefficients in a mathematical model and predicting the response and checking the applicability of the model (Techapun et al. 2002a, b).

Central composite design (CCD) contains a factorial matrix with center points and "star points" around that center point that allow the curvature of the model to be established. The distance from the center of the design space to a factorial point is ±1 unit for each factor, and the distance space from the center of the design space to a star point is ±α, where |α|>1.The precise value of α depends on the number of factors used. The star point represents extreme values for each factor in the design.

The present work describes the successful optimization of a culture medium for the production of the xylanase by *Streptomyces* sp. P12-137 in submerged fermentation (SmF).

Materials and methods

Microorganism

Streptomyces sp. P12-137 was used in this study. This strain was isolated from a soil sample obtained from the Galați region and selected as a potent producer of xylanase. This strain was propagated on Gauze-agar medium at 28°C and maintained at 4°C. After growth for 72 h, spores and aerial mycelium were harvested in 10 ml sterile NaCl (0.9%) to be used as inoculum.

Production of xylanase

The unoptimized fermentation medium is composed of (g/l): birchwood xylan 10 g, K_2HPO_4 0.075 g, KH_2PO_4 1.5 g, KNO_3 4.5 g, and a trace element solution 2.7 ml/l that comprised (g/l): $ZnSO_4 \cdot 7H_2O$ 0.14 g, $MnSO_4 \cdot H_2O$ 0.16 g, $FeSO_4 \cdot 7H_2O$ 0.5 g, $CoCl_2 \cdot 2H_2O$ 0.2 g in distilled water. The pH of the medium was adjusted to 7.2 after sterilization using sterile 1 N NaOH. Erlenmeyer flasks (250 ml) containing 50 ml sterile culture medium were inoculated with 2.5 ml inoculum. The flasks were incubated at 28°C for 120 h on an orbital shaker. The extract was centrifuged at 10,000 g and at 4°C for 10 min, and the clear supernatant was assayed for xylanase activity. For optimization studies, the composition of the culture medium was varied according to the experimental data, while the pH, temperature and time of fermentation were not varied.

Xylanase assay

Xylanase activity was determined using birchwood xylan (Roth, Karlsruhe, Germany) (Bailey et al. 1992). A 0.2 ml culture supernatant was added to 2 ml xylan solution (1.5%;

pH 5.0; 200 mM acetate buffer) and incubated at 50°C. After 20 min, 2 ml 3,5-dinitrosalicylic acid reagent was added to stop the reaction, and the amount of reducing sugars released in the reaction was estimated by measuring the absorbance at 535 nm (Miller 1959). One unit (1 UA) of xylanase activity is defined as the amount of enzyme required to release 1 µmol xylose per minute under the assay conditions.

Response surface methodology

A factorial CCD for three factors with replicates at the center point and star point was used in this investigation. The variables used were wheat bran, KNO_3 and xylose each at five coded levels (−α,−1, 0, +1, +α), as shown in Table 1.

The relation between the coded forms of the input variable and the actual value of the wheat bran, KNO_3 and xylose are described in Eq. 1:

$$X_i = \frac{(Z_i - Z_0)}{\Delta Z} \tag{1}$$

where X_i is a coded value and Z_i the actual value of the factor, Z_0 the actual value of the same variable at the center point, ΔZ the step change of the variable. The CCD contained a total 15 experimental trials that included four trials for factorial design, six trials for axial points (two for each variable), and five trials for the replication of the central points (see Table 2).

These three factors, each with five coded levels consisting of 15 experimental runs, were used to analyze the experimental data to allow for a better estimate of the experimental error and to provide an extra information region (Haaland 1990). The experimental data were fitted according to Eq. 2 as a second-order polynomial regression equation, including individual and cross effect of each variable.

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} X_i X_j \tag{2}$$

where Y is the predicted response, b_0 is the intercept term, b_i is the linear effect, b_{ii} is the square effect, b_{ij} is the interaction effect, and X_i and X_j are the variables. Equation 2 was used to optimize the values of independent parameters for the response.

Table 1 Variables and their levels for the experimental central composite design (CCD)

Variable	Coded level of variable				
	−α	−1	0	+1	+α
Wheat bran (%,w/v)	0.80	1.00	1.50	2.00	2.20
KNO_3 (%,w/v)	0.04	0.20	0.60	1.00	1.16
Xylose (%,w/v)	0.02	0.10	0.30	0.50	0.78

Table 2 Experimental design and results of CCD

Run	Coded levels			Responses	
	A	B	C	Actual value (UA/ml)	Predicted value (UA/ml)
1	0	0	0	6.23	8.02
2	1	1	−1	2.52	1.2
3	1.41	0	0	9.98	11.3
4	−1,41	0	0	10.69	9.37
5	0	−1,41	0	15.99	17.31
6	0	0	0	9.17	8.02
7	1	-1	1	13.4	12.08
8	0	0	0	12.27	8.02
9	−1	1	1	27.77	26.45
10	0	0	−1,41	4.8	6.12
11	−1	−1	-1	10.69	9.37
12	0	0	0	5.88	8.02
13	0	1,41	0	9.23	10.55
14	0	0	1,41	6.51	7.83
15	0	0	0	9.22	8.02

Statistical analysis

The statistical software package Design-Expert (Stat-Ease, Minneapolis, MN) was used for a regression analysis of experimental data and to plot the response surface. Variance analysis (ANOVA) was used to estimate the statistical parameters.

Results and discussion

The results of experimental xylanase production by a complete three factor and two level factorial experiment design with five replications of the central point and six axial points are show in Table 2. The interaction term AB

Table 3 Regression analysis of CCD

| Source	Mean square	F-value	Prob>F
Model	50.03	5.43	0.0385
A-wheat bran	0.25	0.027	0.8749
B-KNO$_3$	22.85	2.48	0.1762
C-xylose	1.46	0.16	0.7068
AB	81.53	8.85	0.031
AC	31.05	3.37	0.1259
BC	57.96	6.29	0.054
A^2	25.46	2.76	0.1574
B^2	67.33	7.31	0.0426
C^2	2.11	0.23	0.6522
Lack-of-fit	18.89	2.78	0.1708

(wheat bran and KNO$_3$) ($P<0.05$) and B^2 term ($P<0.05$) were statistically significant (Table 3).

The parameters of Eq. 1 were determined by multiple regression analysis by application of the RSM method. The second-order polynomial regression equation showing the relationship between the xylanase activity (Y) and three test variables in coded units is represented in Eq. 3:

$$Y = 8.67B + 29.61C − 31.93AB − 39.40AC + 67.29BC + 7.26A^2 + 18.47B^2 − 13.09C^2 − 1.07$$

$$(3)$$

a

b
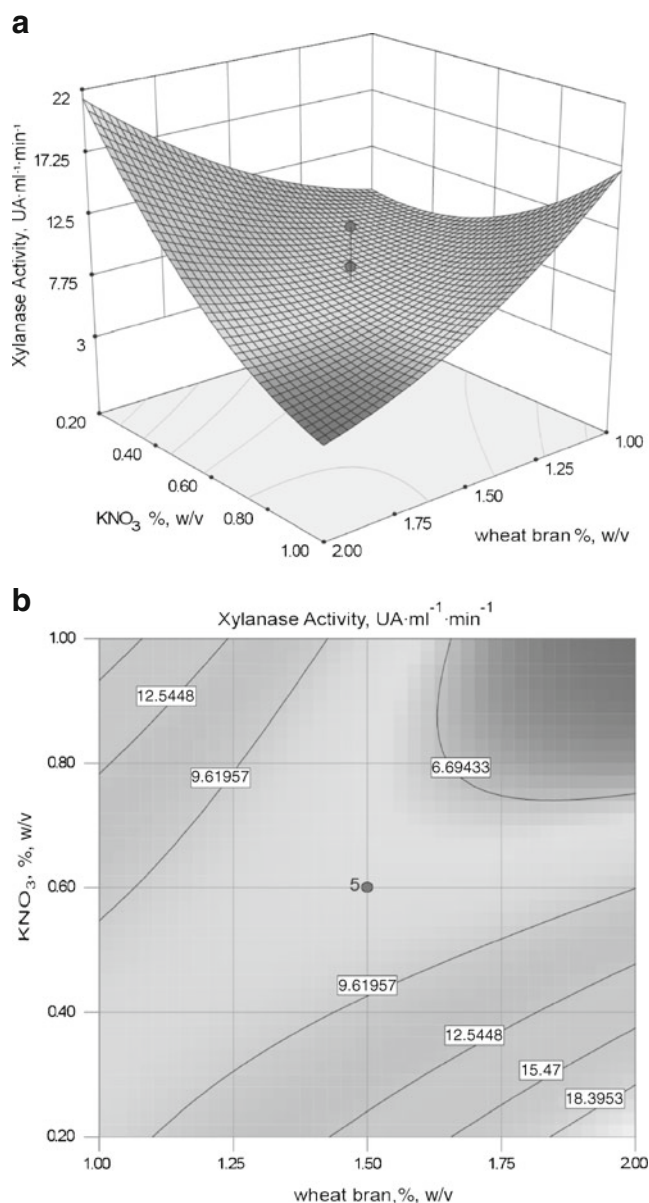
Fig. 1 Response surface curves (**a**) and contour plots (**b**) of xylanase production from *Streptomyces* sp. P12-137, showing interactions between wheat bran and KNO$_3$ concentrations after 120 h of incubation
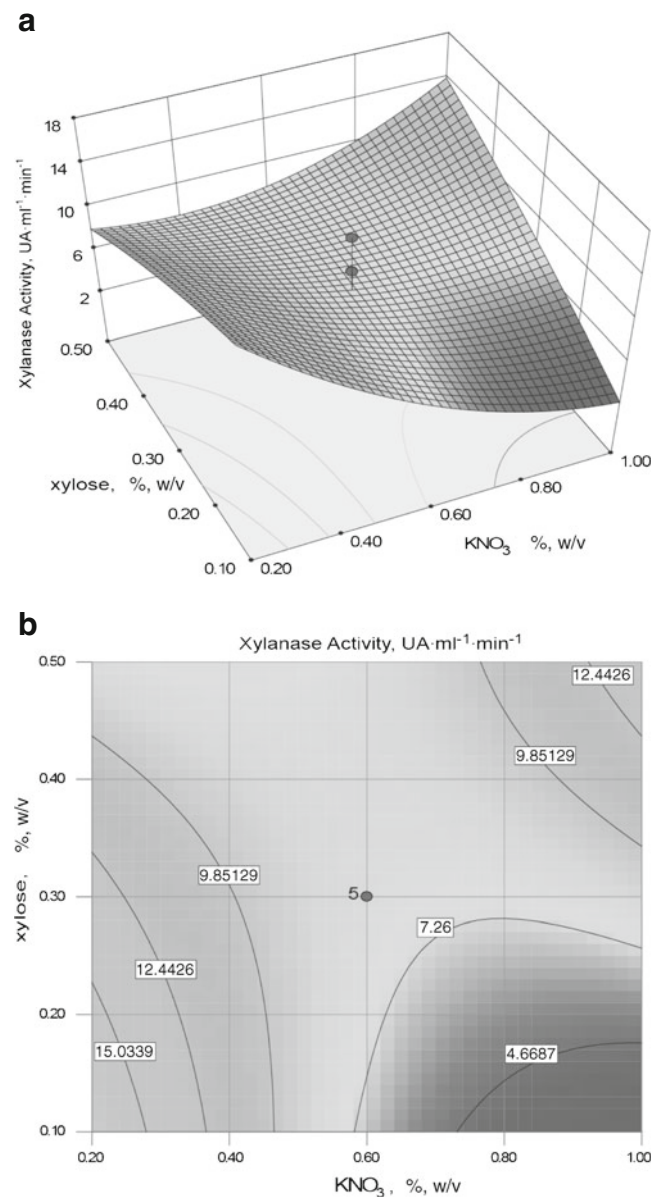
a

b
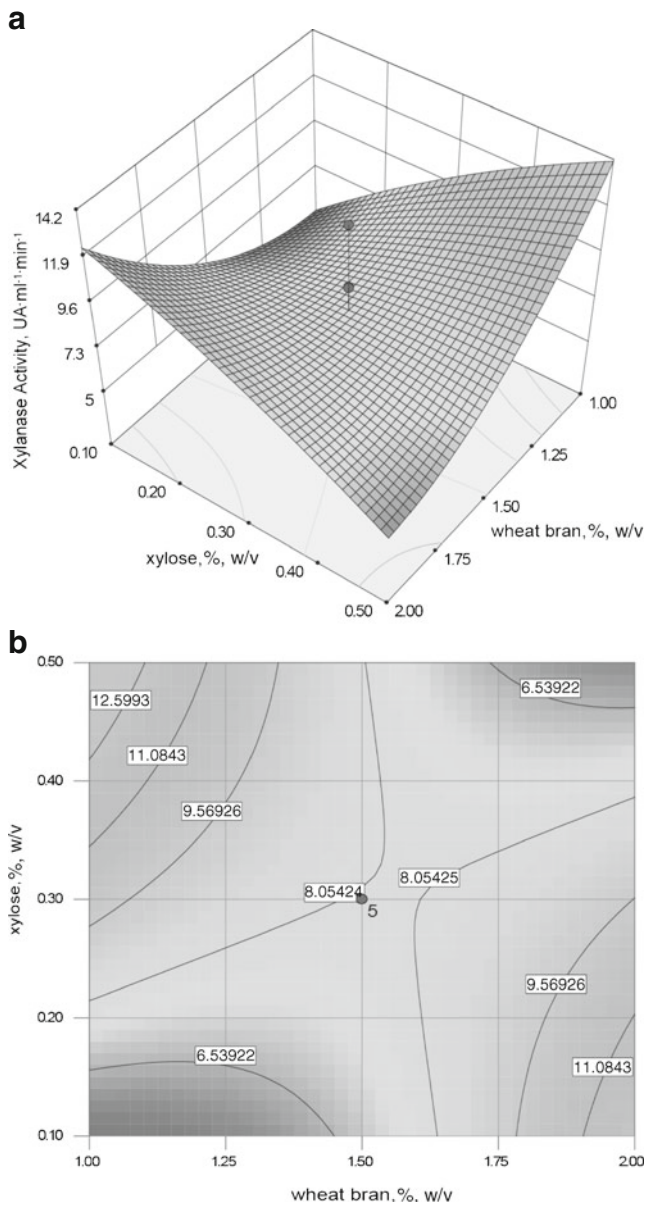
Fig. 2 Response surface curves (**a**) and contour plots (**b**) of xylanase production from *Streptomyces* sp. P12-137 showing interactions between wheat bran and xylose after 120 h of incubation

where Y is the xylanase activity (UA/ml), A is the wheat bran (g/l), B is KNO_3 (g/l) and C is the xylose (g/l). The statistical significance of the model equation was evaluated by the F-test for ANOVA, which showed that the regression is statistically significant at the 0.90 ($P<0.05$) confidence level.

A value of $P>F$ less than 0.05 indicates that the model terms are also significant. The coefficient of determination (R^2) was calculated to be 0.9072, indicating that the model could explain 90% of the variability. The "lack-of-fit F-value" of 2.78 implies that the lack of fit is significant. There is only a 0.01% chance that a "lack-of-fit F-value" this large could occur due to noise. The estimated models fit the experimental

data adequately. "Adeq Precision" is a statistical parameter that measures the signal (response) to noise (deviation) ratio. The observed ratio of 10.187 indicates an adequate signal and therefore the model is significant for the process. Three-dimensional (3D) response surfaces were plotted on the basis of the model equation in order to investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum xylanase production by *Streptomyces* sp. P12-137. Figure 1 shows the interactions with wheat bran as carbon source and KNO_3 as nitrogen source. Xylanase production increases with increasing wheat bran concentration (1 %, w/v to 2 %, w/v) and KNO_3 (0.20 %, w/v to 1 %, w/v).
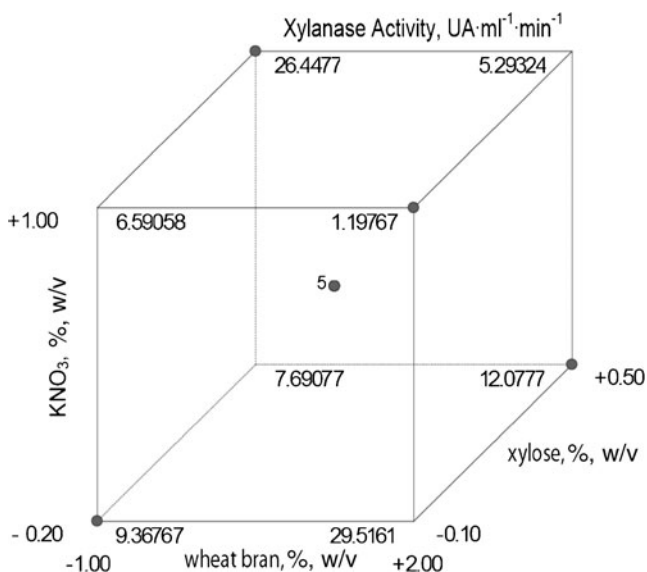
a

b

Fig. 3 Response surface curves (**a**) and contour plots (**b**) of xylanase production from *Streptomyces* sp. P12-137 showing interactions between xylose and KNO_3 after 120 h of incubation
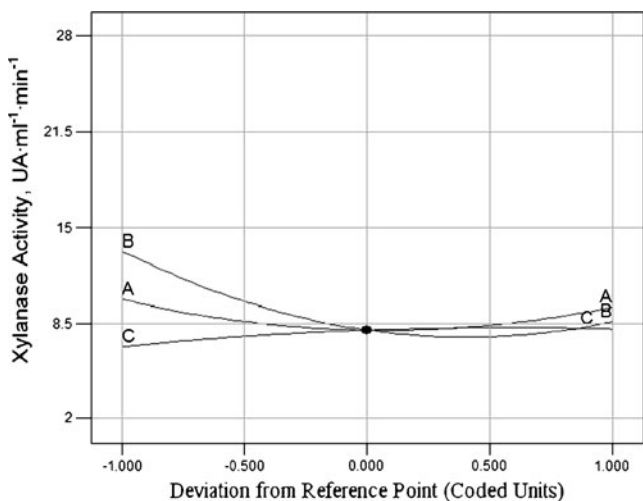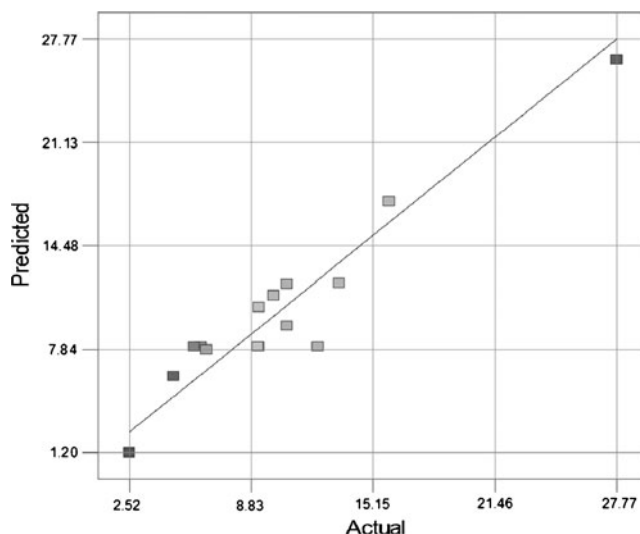
Fig. 4 Perturbation graph for three factors involved in xylanase production: *A* wheat bran, *B* KNO$_3$, *C* xylose

Fig. 6 Parity plot showing the distribution of experimental vs predicted values of xylanase production

The effects on xylanase production of changes in the concentration of wheat bran and xylose are shown in Fig. 2. Xylanase production increases with simultaneous increases in the concentrations of wheat bran and xylose from 1.5 %, w/v to 2 %, w/v and 0.20 %, w/v to 0.5 %, w/v, respectively. A decrease in xylanase activity was observed at 1.5 %, w/v wheat bran concentration and 0.30 %, w/v xylose in the gutter area of the response. Low xylanase productivity was recorded when the concentration of wheat bran and xylose was less than 1 %, w/v and 0.10 %, w/v or increased more than 2 %, w/v and 0.5 %, w/v, respectively.

The effects on the production of xylanase of variation in the concentration of KNO$_3$ and xylose, with the wheat bran concentration kept constant, are shown in Fig. 3. Increased

xylanase activity recorded at low levels of KNO$_3$ and xylose of 0.20 %, w/v and 0.10 %, w/v, respectively, but not at high concentrations of KNO$_3$ (over 1 %, w/v) and xylose concentrations of 0.50 %, w/v. Any increase of xylose concentration over 0.50 %, w/v has a repressive catabolic action on xylanase production.

Also, a steep reduction in xylanase production was recorded at low levels of xylose (< 0.10 %, w/v) and KNO$_3$ increases over 1 %, w/v. Thus, it can be concluded that the enzyme production is stimulated at relatively low concentrations of xylose and nitrogen, or with higher amounts of nitrogen source when xylose does not exceed 0.50 %, w/v.

Perturbation graphs help compare the effect for every factor on a particular point using space design. The response is

Fig. 5 Cube plot showing the influence of factors relevant to xylanase production: *A* wheat bran, *B* KNO$_3$, *C* xylose
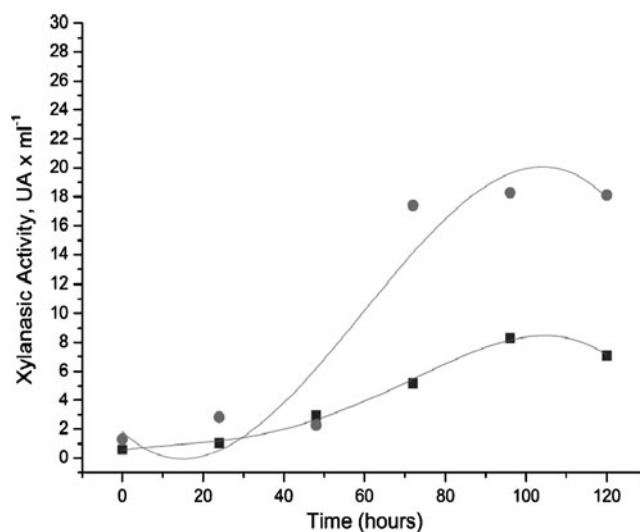

Fig. 7 Dynamics of xylanase production by *Streptomyces* sp. P12-137 in submerged fermentation (SmF) with the medium before (■) and after optimization (●). Each value is the mean value of *n*=3 experiments

shown graphically by changing one factor while other factors remain constant. One factor that was found to affect xylanase biosynthesis by streptomycetes is the source of nitrogen (Fig. 4, curve b) followed by carbon source (curve a).

The cube plot (Fig. 5) shows the maximum xylanase activity at a point (A+B−C) located in the bottom right, where a response of value 29.5161 UA/ml was predicted, corresponding to increasing levels of wheat bran (2 %, w/v) and KNO₃ (1 %, w/v) and a low concentration of xylose (0.10 %, w/v). This chart confirms the repressive action of high levels of xylose in the culture medium. The central point represents the optimal point of the combined action for the three factors, at values of 1.5 %, w/v for wheat bran, 0.6 %, w/v for KNO₃ and 0.3 %, w/v for xylose.

The parity plot showed a satisfactory correlation between experimental values and predicted values (Fig. 6), wherein the points cluster around the diagonal, which indicates the good fit of the model, since the deviation between the experimental and predicted values was small.

Design-Expert predicted the maximum xylanase yield to be 26.45 UA/ml substrate in optimized medium, which is very close to the actual level of xylanase produced in the optimized medium, which was 27.77 UA/ml wheat bran. The xylanase yield in the optimized medium, which was three-fold higher than that in the initial medium (Fig. 7). The dynamics of xylanase production in SmF using initial and optimized media showed maximum enzyme production after 90 h of fermentation.

A response surface method with a five-factor, three-level design has been used to optimize the medium components, agitation and incubation period for maximum xylanase production by *Streptomyces violaceoruber* in SmF, which resulted in a maximum production of 1,500.0 IU/ml (Khurana et al. 2007). Similarly, xylanase production by *Bacillus coagulans* BL69 has been maximized by the CCD method, with maximum xylanase yields of 1.47 IU/mg protein in SSF (Heck et al. 2005).

Little information on the optimization of xylanase production using *Streptomyces* sp. in SmF is available in the scientific literature. The aim of this work was to enhance xylanase production by *Streptomyces* sp. P12-137, which has proved to be a potentially useful strain during previous screening studies, by using CCD based on the optimization of some culture parameters (carbon source, nitrogen source, xylanase inductor production) involved in SmF processes. These factors are important in shortening fermentation periods for potential industrial applications by maintaining appropriate substrate concentration in the medium, which can make the whole process more economical and feasible.

The 3D response surfaces were plotted to work out the interactions among the selected factors, and to determine the optimum concentration for obtaining maximum xylanase production. The coordinates of the central point within the highest contour lines in each of the graphics correspond to the optimum concentration of the respective components.

The xylanase yield increased upon increasing the concentration of wheat bran (above 2.0 %, w/v). Khurana et al. (2007) found the same result using *Streptomyces violaceoruber* under SmF at 37°C for 54 h under shaking (200 rpm). The wheat bran serves as a nutrient reservoir for xylanolitic microorganisms. In a report by Beg et al. (2000), wheat bran effectively induced higher xylanase production by *Streptomyces* sp. OG -11-3. Techapun et al. (2002b) obtained the maximum yield of xylanase of about 15 IU at 50°C and pH 7, by using cane bagasse—a carbon source from the agricultural waste—and *Streptomyces* sp. Ab106. Beg et al. (2000) reported a *Streptomyces* sp. QC 113 strain that produces 7.5 IU xylanase in a wheat bran medium at 37°C at pH 8.0 after 5 days. Antanopoulos et al. (2000) reported 12 UI/ml xylanase in a xylan medium using *Streptomyces albus* at 30°C at pH 7.5 after 5 days.

Conclusion

This work has demonstrated that CCD and regression analysis methods were effective in determining the optimized carbon source, nitrogen source and inducer for xylanase activity of *Streptomyces* sp. P12-137. In this experiment, maximal activity was obtained with medium concentrations of 1 %, w/v wheat bran and 1 %, w/v KNO₃ and 0.5 %, w/v xylose. The model predicted maximal xylanase production of 29.51 UA/ml/min at 2 %, w/v wheat bran, 1 %, w/v KNO₃ and 0.10 %, w/v xylose. Under optimal conditions, the activity predicted by the model agreed very well with experimental data, confirming its validity, with a three-fold increase in xylanase activity achieved by optimization of the culture medium composition. These results suggest that the cellulase-free xylanase extract from *Streptomyces* sp. P12-137 strain may present interesting properties for industrial application in pulping and bleaching processes.

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