MINIMIZING CELLULASE BIOSYNTHESIS FROM CELLULASE-FREE XYLANASE PRODUCTION WITH *STREPTOMYCES* SPP. P12-137 USING OPTIMIZATION BY RESPONSE SURFACE METHODOLOGY

GIGI COMAN and GABRIELA BAHRIM

"Dunărea de Jos" University, Faculty of Food Science and Engineering, Bioengineering in Food Industry Department, 800201 Galaţi, Romania

Received July 9, 2010

Response surface methodology and central composite design (CCD) were employed to optimize the biosynthesis medium for the production of cellulase-free xylanase by *Streptomyces* spp. P12-137 in submerged culture (SmF). The three independent variables were wheat bran, potassium nitrate and xylose concentrations. The statistical analyses of the results obtained over the studied range showed that wheat bran and potassium nitrate had a significant effect on minimizing cellulase production. The optimized biosynthesis medium containing 2.0 g% wheat bran, 0.2 g% KNO_3 and 0.1 g% xylose, showed a decreasing level of cellulose, with simultaneously increasing xylanase production. Cellulase-free xylanase can be used in several applications in the paper and pulp bleaching industry.

Keywords: cellulose-free xylanase, *Streptomyces*, response surface methodology, media optimization, central composite design

INTRODUCTION

Cellulose, the most abundant organic compound in the world, assures a type of renewable energy that human beings can easily utilize.[1] Each lignocellulosic substrate consists of sugar polymers in the form of cellulose and hemicellulose, cemented by lignin. Cellulose, a major polysaccharide constituent of the plant cell walls, is a β-1,4 linked linear polymer of 8000-12000 glucose units,[2,3] whose natural degradation represents an important part of the carbon cycle within the biosphere.

Xylan, a major component of hemicellulose, is a heterogeneous poly-saccharide with a backbone consisting of β-(1,4)-linked D-xylosyl residues with several side-groups attached to the main chain. Due to its complex structure, the complete hydrolysis of xylan requires the cooperation of multiple xylanases, which facilitate it.[4,5] Numerous microorganisms, including the *Streptomyces* species, produce multiple xylanases and have been found to be good thermostable cellulose-free xylanase producers.[2,4,6,15] Xylanases are used primarily for the removal of the lignin-carbohydrate complexes (LCC) generated in the kraft process, acting as physical barriers to the entry of bleaching chemicals.[7-9] Chemical bleaching uses large amounts of chlorine and chlorine-based chemicals. The by-products formed during chemical processing are toxic, mutagenic, persistent, bioaccumulating, thus causing numerous harmful disturbances in the biological systems.[7,10,11] A prerequisite in the pulp and paper industry is the use of cellulose-free xylanases, which ensure minimal damage to the pulp fibres, generate rayon grade or superior quality dissolving pulps, and minimize the use of chemical substances, thus decreasing the amount of waste released during bleaching processes.[12,13]

Response surface methodology is the most popular optimization method used in recent years. It involves three statistically designed experiments, estimating the coefficients in a mathematical model, predicting the response and checking the applicability of the model.[16]

The Central Composite Design (CCD) contains a factorial matrix with centre points

and "star points" around the centre point, establishing the curvature of the model. The distance from the centre of the design space to a factorial point is of ±1 unit for each factor, and the distance space from the centre of the design space to a star point is of ±α, where $|\alpha| > 1$. The precise value of α depends on the number of factors involved. The star point represents extreme values for each factor in the design.

In a previous work, the composition of the culture medium was optimized to achieve a high xylanase activity. Thus, the maximum level for xylanase activity[20] was of 27.77 UA/ml. The present research paper describes the successful optimization of a culture medium for the production of low-level cellulase by *Streptomyces* spp. P12-137, a new isolator of thermotoleranting microorganisms that can produce cellulase-free xylanase from agricultural waste materials, in submerged fermentation (SmF).

MATERIALS AND METHOD

Microorganisms

The *Streptomyces* spp. 12-137 strain was isolated from soil samples collected from the region of Galati (Eastern Romania), and selected as a powerful producer of xylanase and cellulase. The stock culture was maintained in a Gauze-agar medium at 4 °C.

Production of xylanase and cellulase

The initial fermentation medium was composed of (g/L): birchwood xylan – 10, K_2HPO_4 – 0.075, KH_2PO_4 – 1.5, $(NH_4)_2SO_4$ – 4.5 and a trace element solution (2.7 ml/L) made of $ZnSO_4 \cdot 7H_2O$ – 0.14, $MnSO_4 \cdot H_2O$ – 0.16, $FeSO_4 \cdot 7H_2O$ – 0.5, $CoCl_2 \cdot 2H_2O$ dissolved in distilled water. After sterilization, the pH of the medium was adjusted to 7.2, using sterile 1N NaOH Erlenmeyer flasks (250 mL) containing 50 mL sterile culture medium inoculated with a 2.5 mL inoculum. The inoculum was grown for 72 h, after which the spores were harvested in 10 mL sterile 9% NaCl. The flasks were incubated at 28 °C for 120 h on an orbital shaker at 150 rpm. 10000 g of the extract were centrifuged at 4 °C for 10 min, and clear supernatant was used for cellulase assays. 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.

Cellulase assay

The cellulase activity was performed[17] using a CMC (Carl Roth GmbH). A 0.6 mL cell-free extract was added to 6 mL of CMC solution (final concentration 0.1%) of pH 5 (0.2M acetate buffer), and incubated at 50 °C. After 20 min, 2 mL of 3,5-dinitrosalicylic acid reagent were added to stop the reaction, and the amount of reducing sugars released in the reaction was estimated by measuring[18] absorbance at 535 nm. One unit (1 UA) of cellulase activity is defined as the amount of enzyme required to release 1 μmol of glucose per minute, under assay conditions.

Response surface methodology

A factorial central composite design (CCD) for 3 factors with replicates at the centre point and star point was used. The variables were wheat bran, KNO_3 and xylose, each at 5 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 wheat bran, KNO_3 and xylose are described as Eq. (1):

$$X_i=(Z_i-Z_0)/\Delta Z \quad (1)$$

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 centre point, ΔZ – the step change of the variable. The CCD contained a total number of 15 experimental trials, including 4 trials for the factorial design, 6 trials for the axial points (2 for each variable) and 5 trials for replication of the central points.

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

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

These 3 factors, each with 5 coded levels consisting of 15 experimental runs, were used to analyze the experimental data, to allow better estimates of the experimental error and to provide extra information region.[19] The experimental data were fitted according to Eq. (2), as a second-order polynomial regression equation including the individual and cross effect of each variable:

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

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 variables. The equation was used to optimize the values of the independent parameters for the response.

Table 2
Experimental design and results of the central composite design

Run	Coded levels			Responses	
	A	B	C	Actual value ($IUml^{-1}$)	Predicted value ($IUml^{-1}$)
1	0	0	0	1.65	1.24
2	1	1	-1	0.47	0.44
3	1.41	0	0	0.69	0.72
4	-1.41	0	0	1.63	1.66
5	0	-1.41	0	1.09	1.12
6	0	0	0	1.95	1.24
7	1	-1	1	2.08	2.05
8	0	0	0	1.28	1.24
9	-1	1	1	1.02	1.05
10	0	0	-1.41	1.26	1.29
11	-1	-1	-1	1.29	1.26
12	0	0	0	0.88	1.24
13	0	1.41	0	1.38	1.41
14	0	0	1.41	1.02	1.05
15	0	0	0	0.51	1.24

Statistical analysis

The statistical software package Design-Expert 7.1.6, Stat-Ease, Inc., Minneapolis, USA, was used for regression analysis of the experimental data and for plotting the response surface. Variance analysis (ANOVA) was used to estimate the statistical parameters.

RESULTS AND DISCUSSION

The experimental results of cellulase production by a complete 3-factor, 2-level factorial experimental design, with 5 replications of the central point and 6 axial points, are shown in Table 2.

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

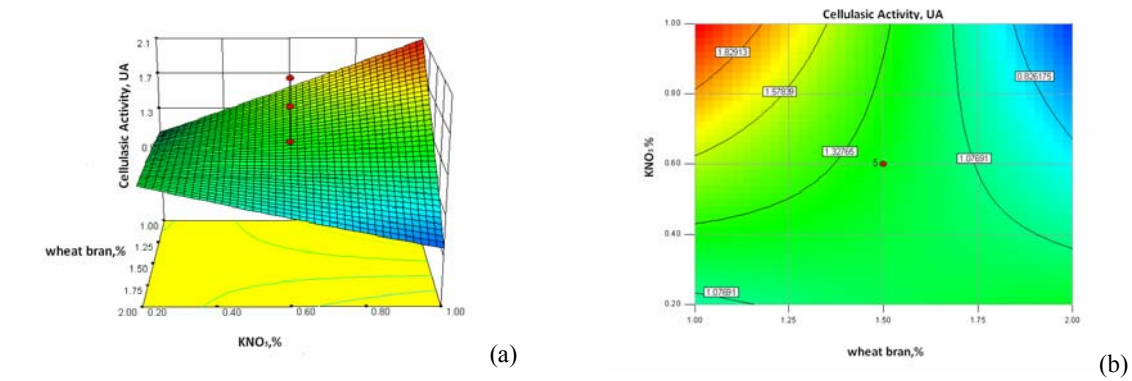
$$Y = 0.70 \cdot A + 4.80 \cdot B - 5.50 \cdot C$$
$$-2.09 \cdot AB + 5.73 \cdot AC - 4.90 \cdot BC$$
$$-0.11 \cdot A^2 + 0.06 \cdot B^2 - 0.94 \cdot C^2 - 1.71$$

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

The value of $p > F$, below 0.05, indicates that the model terms are also significant. The coefficient of determination (R^2) was calculated to be 0.58, indicating that the model could explain 58% of variability. The "lack of fit" of a p-value of 0.8829 implies that the former is not significant. There is only a 0.01% chance that a higher „lack of fit" of the p-value could occur due to noise. The estimated models fit the experimental data adequately. "Adeq precision" is a statistical parameter measuring the signal (response) to the noise (deviation) ratio. A ratio of 3.80 indicates an inadequate signal. Three-dimensional response surfaces were plotted on the basis of the model equation, to investigate the interaction among the variables and to determine the optimum concentration of each factor for minimum cellulase production by *Streptomyces* spp. P12-137. Figures 1 (a) and (b) depict the interaction between wheat bran as a carbon source and KNO_3 as a nitrogen source. A decrease in cellulase production can be seen with increasing the concentrations of wheat bran (up 2 g%) and KNO_3 (up 1 g%).

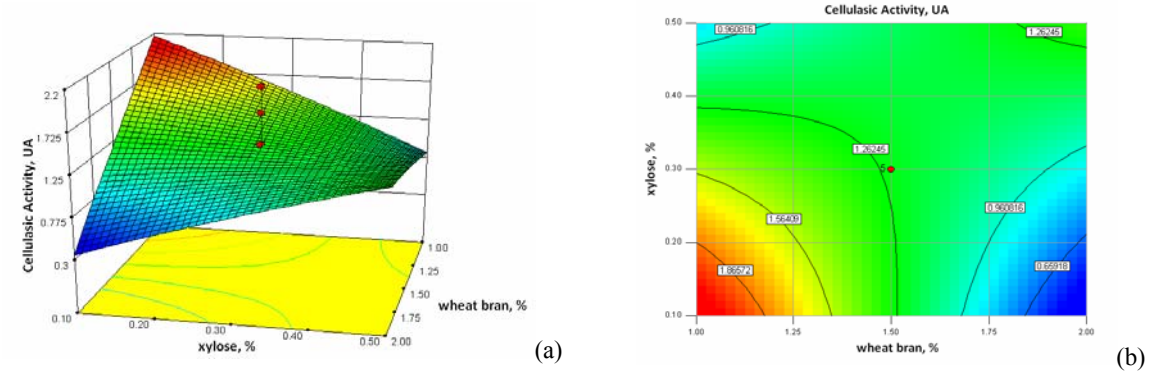
The effect of the different concentrations of wheat bran and xylose on cellulase production is plotted in Figure 2.

Cellulase activity decreased as the wheat bran concentrations increased up to 2 g%, and xylose concentrations decreased to 0.10 g%.



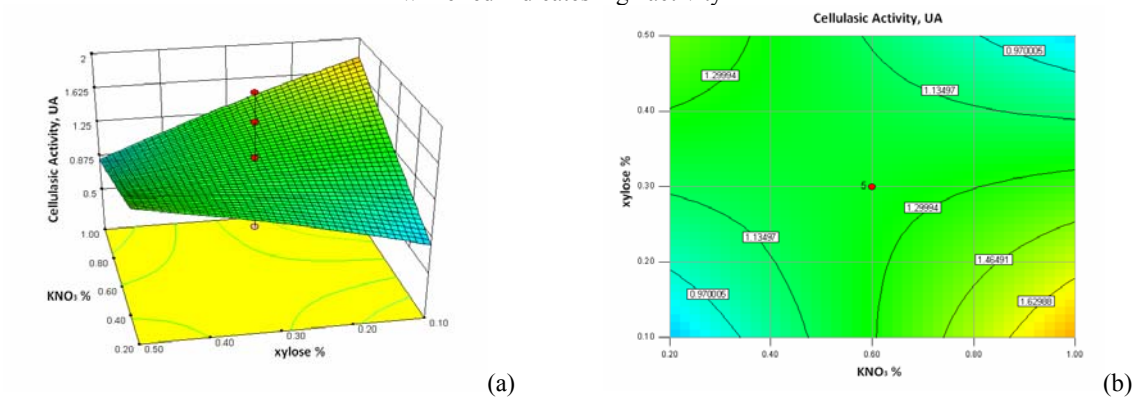
(a) (b)

Figure 1: Response surface curves (a) and contour plot (b) of cellulase production from *Streptomyces* spp. P12-137 showing interactions between wheat bran and KNO_3 after 120 h of incubation. Blue indicates low cellulase activity, while red indicates high activity



(a) (b)

Figure 2: Response surface curves (a) and contour plot (b) of cellulase production from *Streptomyces* spp. P12-137 showing interactions between wheat bran and xylose after 120 h of incubation. Blue indicates low cellulase activity, while red indicates high activity



(a) (b)

Figure 3: Response surface curves (a) and contour plot (b) of cellulase production from *Streptomyces* spp. P12-137 showing interactions between xylose and KNO_3 after 120 h of incubation

The effect of the variations in KNO_3 and xylose concentration on cellulase activity is shown in Figure 3. These two factors influenced directly and proportionally the production of cellulase. Cellulase activity was inhibited at low levels of both xylose

and KNO_3 (down to < 0.10 g% and <0.20 g%) and at concentrations up to 0.50 g% and 1 g%. Chart disturbances (Fig. 4) show that cellulase activity was significantly influenced by wheat bran (A), reflected in the pronounced slope. The concentration of nitrogen in the media influenced cellulase activity to a lower extent, while the influence of xylose concentrations on cellulase activity was minimum.

Figure 5 is a cube representation of the 3 treatments and interactions. Point 5, representing the optimum activity of cellulases, corresponds to 1.50 g% wheat bran, 0.60 g% KNO_3 and 0.30 g% xylose. An increased ratio of wheat bran and xylose leads to low cellulase activities. Wheat bran is a high-quality substrate for xylanase production by *Streptomyces* spp. P12-137, with a rich content of hemicelluloses (xylan, arabinoxylan).

The graphical representation of equation coefficients (3) showed a positive interaction (AC) between wheat bran and xylose (Fig.6). Increased concentrations of wheat bran with maximum xylose concentration increased cellulase activities.

The optimized medium to achieve the lowest cellulase activity has the following composition: wheat bran – 20 g/L, K_2HPO_4 – 0.075 g/L, KH_2PO_4 – 1.5 g/L, KNO_3 – 2 g/L, xylose – 1 g/L, trace element solution 2.7 mL/L. Note that the optimized medium allows a minimum cellulase level, which is the goal of enzyme utilization in pulp and paper industry.

The present study provides an efficient method, based on statistical tools, for minimizing the production of cellulase with the *Streptomyces* spp. P12-137 strain.

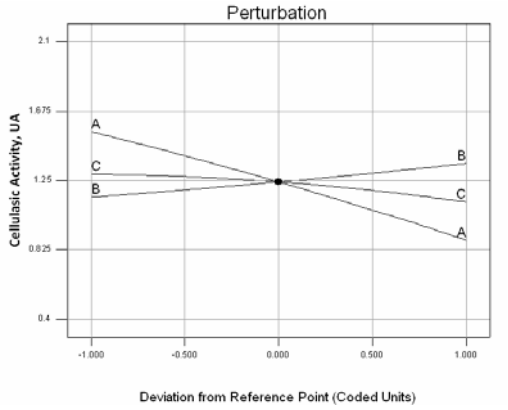


Figure 4: Perturbation graph for three factors involved in cellulase production: A – wheat bran, B – KNO_3, C – xylose

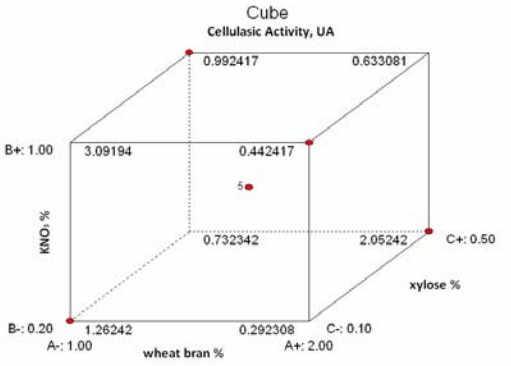


Figure 5: Cube plot showing the influence of relevant factors upon cellulase production: A – wheat bran, B – KNO_3, C – xylose

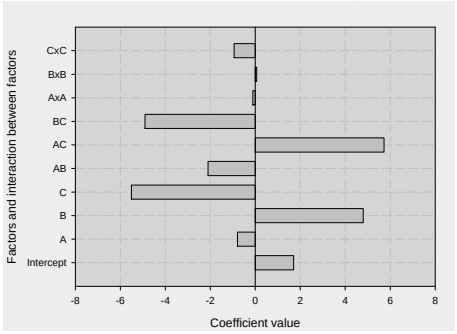


Figure 6: Equation coefficients of RSM quadratic model for cellulase activities by *Streptomyces* spp. P12-137: factors A, B and C are wheat bran, KNO3 and xylose

CONCLUSIONS

The investigation demonstrated that CCD and regression analysis methods are effective for determining the optimized carbon and nitrogen sources for a decreased cellulase activity by *Streptomyces* spp. P12-137. In the

experiment, a minimum activity was obtained for 2 g% wheat bran, 0.20 g% KNO_3 and 0.10 g% xylose. The model predicted a minimum cellulase production of 0.47 UA/mL/min at 2 g% wheat bran, 1 g% KNO_3 and 0.10 g% xylose. *Streptomyces* spp. P12-137 must be a cellulose-free xylanase producer strain. The results obtained suggest that the cellulase-free xylanase extract from the *Streptomyces* spp. P12-137 strain could have interesting properties for industrial applications in pulping and bleaching process.

ACKNOWLEDGEMENTS: The authors acknowledge the material and financial assistance of Bioaliment Platform and Specialists in Applied Biotechnology Association for this work.

REFERENCES

[1] H. D. Jang and K. S. Chen, *World J. Microb. Biot.*, **19**, 263 (2003).
[2] A. L. Grigorevski de Lima, P. N. Rodrigo, E. P. S. Bon and R. R. R. Coelho, *Enzyme Microbiol. Technol.*, **37**, 272 (2005).
[3] B. C. Saha, *Process Biochem.*, **39**, 1871 (2004).
[4] Z. Q. Jiang, W. Deng, L. T. Li, C, H. Ding, I. Kusakabe and S. S. Tan, *Biotechnol. Lett.*, **26**, 431 (2004).
[5] K. K. Y. Wong, L. U. L. Tan and J. N. Saddler, *Microbiol. Rev.*, **52**, 305 (1998).
[6] O. K. Beg, M. Kapoor, L. Mahajan and G. S. Hoondal, *Appl. Microbiol. Biotechnol.*, **56**, 326 (2001).
[7] Q. K. Bega, B. Bhushanb, M. Kapoor and G. S. Hoondal, *Enzyme Microbiol. Technol.*, **27**, 459 (2000).
[8] M. G. Paice, N. Gurnagul, D. H. Page and L. Jurasek, *Enzyme Microbiol. Technol.*, **14**, 272 (1992).
[9] A. J. Ragauskas, K. N. Poll and A. Cesternino, *Enzyme Microbiol. Technol.*, **16**, 492 (1994).
[10] P. Bajpai, *Biotechnol. Prog.*, **15**, 147 (1999).
[11] K. A. Onysko, *Biotechnol. Adv.*, **11**, 179 (1993).
[12] S. R. Senthilkumar, M. Dempsey, C. Krishnan and P. Gunasekaran, *Bioresource Technol.*, **99**, 7781 (2008).
[13] L. Jurasek and M. Paice, *Chemtech.*, **16**, 360 (1986).
[14] M. U. Maheswari and T. S. Chandra, *World J. Microb. Biot.*, **16**, 257 (2000).
[15] H. Schrempf and S. Walter, *Int. J. Biol. Macromol.*, **17**, 353 (1995).
[16] C. Techapun, T. Charoenrat, M. Watanabe, K. Sasaki and N. Poosaran, *Biochem. Eng. J.*, **12**, 99 (2002).
[17] M. J. Bailey, P. Biely and K. Poutanen, *J. Biotechnol.*, **23**, 257 (1992).
[18] G. L. Miller, *Anal. Chem.*, **31**, 426 (1959).
[19] P. D. Haaland, "Experimental Design in Biotechnology", Elsevier Science Publishing Co., New York, USA, 1990.
[20] G. Coman and G. Bahrim, *Ann. Microbiol.*, DOI 10.1007/s13213-010-0195-0 Springer (2011).