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Full Length Research Paper

Statistical optimization of activity and stability of β-xylanase produced by newly isolated *Thermomyces lanuginosus* THKU-49 using central composite design

Khwanchai Khucharoenphaisan[1], Shinji Tokuyama[2] and Vichien Kitpreechavanich[1]*

[1]Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[2]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529 Japan.

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A factorial design was performed to find the best conditions of pH and temperature for β-xylanase activity and to maintain its activity for prolonged periods of time of pure xylanase produced by newly isolated *Thermomyces lanuginosus* THKU-49. The central composite design (CCD) used for the analysis of treatment combinations showed that a regression models of optimization of xylanase activity and xylanase stability were good agreement to experimental results with R^2 = 0.98 and 0.99, respectively. The maximum activity of xylanase was obtained at 66°C and pH 6.3. The temperature and pH for maximum enzyme stability was 70°C and pH 7.3, respectively. Under this condition xylanase having half-life of 825 min indicated the highest thermostable xylanase.

Key words:β-xylanase, optimization, central composite design.

INTRODUCTION

Thermomyces lanuginosus has attracted considerable interest due to its production of stable enzymes, especial xylanase belonging to family 11 of glycosyl hydrolase. Several *T. lanuginosus* xylanases have been characterized. Pure xylanase from *T. lanuginosus* strains of SSBP, DSM 5826, ATCC 1645, ATCC 22083, ATCC 26909, ATCC 34626, ATCC 36350, ATCC 58160 and DSM 10635 have been reported with half-life values of 232, 201, 54, 81, 79, 126, 121, 116 and 40 min, respectively, in 50 mM of buffer pH 6.5 at 70°C (Singh et al., 2000a; Xiong et al., 2004). Intensive isolation of *T. lanuginosus* from soil samples in Thailand had been done in order to search high thermostable xylanase which is useful in biotechnological applications. Among 88 isolated strains of *T. lanuginosus*, crude xylanase that was obtained from *T. lanuginosus* THKU-49 showed the highest thermal stability having half-life at 70°C for 266 min (Khucharoenphaisan and Kitpreechavanich, 2004). The classical method of bioprocess design, one variable at a time, may be effective in some situation but fails to consider the combined effects of all involved factors (Silva and Roberto, 2001). Factorial design optimization and response surface analysis fulfill this requirement (Couto et al., 2006; Heck et al., 2006). The aims of the present work were to optimize condition of temperature and pH for maximum xylanase activity and to maintain its activity for prolonged periods of time using central composite design and response surface method.

MATERIALS AND METHODS

Cultivation and purification of xylanase

T. lanuginosus THKU-49 used in this study was selected based on high thermal stability of xylanase produced by the fungus. The strain preserved at Thailand Institute of Scientific and Technological Research (TISTR) Culture Collection Bangkok MIRCEN was identified as *T. lanuginosus* according to the manual of fungal taxonomy (Cooney and Emerson, 1964; Domsch and Gams, 1993) and Internal Transcribed Spacer (ITS) regions sequence (Sambrook et al., 1989).

*Corresponding author. E-mail: fsciwck@ku.ac.th. Tel: +81-2-5792351. Fax: +81-2-5792081.

Table 1. Code values of variables, maximum and minimum pH and temperature used in the central composite experimental design.

Independent variables	Code Levels				
	-1.414	**-1**	**0**	**1**	**1.414**
Temperature (^oC)	56	60	70	80	84
pH	5.6	6.0	7.0	8.0	8.4

Table 2. Results of the experimental plan used in CCD, showing the 13-treatment combination.

Treatment	Code setting level		Actual level		Xylanase activity (U/mg protein)		Half-life of xylanase (min)	
	Temp (^oC)	**pH**	**Temp (^oC)**	**pH**	**Actual[a]**	**Predicted**	**Actual[a]**	**Predicted**
1	-1.414	0	56	7.0	545	513	ND	ND
2	0	0	70	7.0	659	664	839	794
3	-1	1	60	8.0	451	468	ND	ND
4	0	0	70	7.0	663	664	768	794
5	0	0	70	7.0	660	664	783	794
6	1.414	0	84	7.0	276	292	171	168
7	-1	-1	60	6.0	623	657	ND	ND
8	0	0	70	7.0	668	664	783	794
9	1	1	80	8.0	401	394	322	319
10	0	0	70	7.0	663	664	809	794
11	0	-1.414	70	5.6	664	645	29	27
12	1	-1	80	6.0	413	394	292	290
13	0	1.414	70	8.4	495	499	537	535

[a]Data are average values of four replicates analysis
ND is not able to determine.

An actively growing 3-day old colony of fungus grown on yeast glucose medium was inoculated into synthetic medium (15 ml) consisting of (per liter of water) 2 g of KH_2PO_4, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4$, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and 10 g of oat spelt xylan in 50 ml Erlenmeyer flask. The flasks were shaken at 120 rpm and incubated at 45^{o}C for 5 days. The clear supernatant obtained from filtration of cultured broth through Whatman No.1 filter paper (Whatman International Ltd., USA) was used for purification. The supernatant was subjected to 80% $(NH_4)_2SO_4$ saturation. The precipitated protein was dissolved in 50 mM phosphate buffer (pH 6.0) and dialyzed. Sample from the above step was applied to a DEAE-Sepharose fast flow column, hydroxylapatite column and Sephadex G-100 column, respectively. Purified protein showed a single band with molecular weight of 24.9 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The specific activity of pure xylanase was 552 unit/mg protein. The purified xylanase was used for optimization of β-xylanase activity and its stability.

Optimization of temperature and pH on β-xylanase activity and stability

Central composite design (CCD) was used to generate 13-treatment combinations, with temperature and pH as independent variables using Minitab version 14. Table 1 showed the experimental matrix which corresponded to design with coded variables containing star points (α = 1.414) and five replications of the central point. The data of experiments were analyzed by SPSS software version 10.

For the enzymatic stability, each centrifuge tube (1.5 ml) containing 0.5 ml of xylanase (~ 120 U/ml) was incubated at various temperatures and pHs at the same above-mentioned condition, 13-treatment generated combinations. The xylanase stability was determined as value of half-life. Half-life of enzyme was the time that the activity remained 50% of its original activity. The half-life of xylanase was measured at intervals from 0 to 24 h. The data of experiments were analyzed by SPSS software version 10.

RESULTS AND DISCUSSION

Optimization of temperature and pH on β-xylanase activity

Two principal factors affecting enzymatic activity as pH and temperature were analyzed in order to determine the optimal condition of activity. The result of experimental design was shown in Table 2. The significant coefficients determined using *P*-values was shown in Table 3. High significance of temperature^2 and pH^2 indicate that temperature and pH can act as limiting factors and small variations in their values will alter xylanase activity to a considerable extent. The model clearly revealed significant interactions between temperature and pH. Treating them separately may not reflect their real influence on the xylanase activity. The equation model was obtained:

Xylanase activity (U/mg protein) = -5192.942 + 149.238*temp + 312.187*pH + 4.192*temp*pH - 1.332*temp*temp - 46.99*pH*pH

This equation model indicated that a remarkably high

Table 3. Coefficient estimates by the regression model.

Independent variables	Xylanase activity			Half-life of xylanase		
	Coefficient (β)	Standard error(β)	Significant value (*P*-value)	Coefficient (β)	Standard error(β)	Significant value (*P*-value)
Intercept	-5192.942	503.413	0.000	-50648.2	4748.911	0.000
Temperature	149.238	8.820	0.000	917.694	116.336	0.001
pH	312.187	87.213	0.002	5018.124	246.772	0.000
Temperature*pH	4.192	0.838	0.000	-16.711	2.451	0.002
Temperature^2	-1.332	0.056	0.000	-5.49	0.756	0.002
pH^2	-46.99	5.614	0.000	-261.906	12.040	0.000

correlation between observation and prediction with R^2 value (0.98). To confirm the applicability of the model, xylanase activity was determined at 66°C and pH 6.3 which were optimum values of the model suggested. In this case, the equation model predicted the enzymatic activity of 699 U/mg protein in confidential level of 95% whereas experimental activity of 712 U/mg protein was obtained.

The optimum temperature and pH of this enzyme was closed to other xylanases produced by strains of CBS 288.54, CAU44, DSM 10635, SSBP, ATCC 46882, DSM 5826 and RT9 that were temperatures in range of 60 - 75°C and pH in range of 6.0-6.5 (Alam et al., 1994; Cesar and Mrsa, 1996; Bennett et al., 1998; Singh et al., 2000b; Xiong et al., 2004; Jiang et al., 2005; Li et al., 2005). It could be observed that the enzymatic activity was notably dropped at high temperature of 80°C and alkali (pH 8.0).

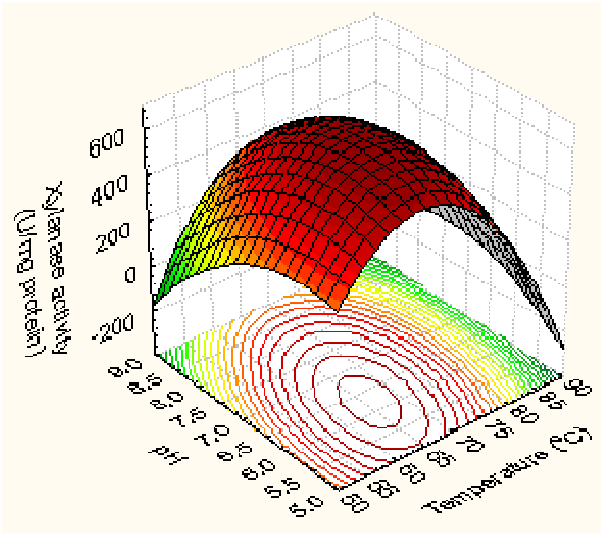

Figure 1. Response surface and contour plot for the effects of pH and temperature on xylanase activity of *T. lanuginosus* THKU-49.

Stability of xylanase

Stability is a very important aspect of industrially enzymatic bioreactor. Two principal factors, temperature and pH, affecting enzymatic stability were analyzed in order to determine the condition to maintain xylanase activity for reaction to prolonged time. The result of experimental design for enzymatic stability was shown as half-life in Table 2. The significant coefficients were determined using *P*-values as shown in Table 3. The equation model was obtained from 10 treatments without the treatments of 1, 3, and 7.

Half-life of xylanase (min) = -50648.2 + 917.694*temp + 5018.124*pH - 16.711*temp*pH - 5.49*temp*temp - 261.906*pH*pH

This equation model in range of temperatures of 70 - 84°C and in range of pH 5.6 - 8.4 indicated a remarkably high correlation between observation and prediction with R^2 value of 0.99. The condition obtained from the equation that provided the highest enzymatic stability was 70°C and pH 7.3, having half-life of 825 min. The predicted value was closed to the experiment of 812 min. The result also indicated that the stability of the enzyme decreased when the pH of enzymatic solution decreased.

The half-life of the enzyme from *T. lanuginosus* THKU-49 at this condition was higher than half-lives of xylanase from other strains of *T. lanuginosus* (Singh et al., 2000a; Xiong et al., 2004). The result showed that xylanase produced by *T. lanuginosus* THKU-49 was the highest thermalstability.

Conclusion

In this work, we demonstrated that the CCD and regression analysis method were effective to find optimized temperature and pH for xylanase activity and its stability of newly isolated *T. lanuginosus* THKU-49. The maximum activity was obtained at temperature of 66°C and pH 6.3. At the optimum condition, the predicted activity by the model agreed very well with experimental data confirming the validity of its predication. In addition, the most suitable operation conditions for great stability of this xylanase were temperature of 70°C and pH 7.3 (Figure 1). This would make possible the application of xylanase produced by this strain to long-term bioprocesses.

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