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Enhanced Production of Cellulase-Free Thermoactive Xylanase Using Corncob by a Black Yeast, *Aureobasidium pullulans* CBS 135684

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Abstract – Our aim was to optimize the production of cellulase-free thermoactive xylanase by *Aureobasidium pullulans* CBS 135684 with statistical methodology based on experimental designs. Among eleven variables, the nutrient sources that had significant effect on xylanase production were corncob, $(NH_4)_2SO_4$, xylose, KH_2PO_4 and tween 80, identified by the initial screening method of Plackett-Burman. The optimum concentrations of these five components were subsequently investigated using response surface methodology. The optimal concentrations ($g \cdot l^{-1}$) for maximum production of xylanase were corncob, 39.0; $(NH_4)_2SO_4$, 3.0; xylose, 1.8; KH_2PO_4 1.4; and tween 80, 1.4, respectively. An improved xylanase yield of 8.74 ± 0.84 $U \cdot ml^{-1}$ was obtained with optimized medium which is 2.1-fold higher production than previously obtained results (4.10 ± 0.10 $U \cdot ml^{-1}$) after 48 h of cultivation. In addition, the xylanase production under optimal condition reached 10.09 ± 0.27 $U \cdot ml^{-1}$ after 72 h of cultivation.

Key words: Endoxylanase, Thermo-tolerant, Black yeast, Corncob, RSM

1. Introduction

Among all xylanolytic enzymes, xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) are of significant importance that have been extensively studied due to their function and applications, especially in pulp and paper manufacturing. The pretreatment of xylanases prior to conventional bleaching of pulp was found to increase the brightness and strength of paper, while it was also efficient in the reduction of kappa number and the use of bleached chemicals [1,2]. For application of xylanase in pulp bleaching, thermal-tolerance is an ideal property for efficient operation because industrial pulping is performed at elevated temperature (70~100 °C) [3,4]. Consequently, the potent organisms capable of producing thermostable xylanase with greater yields are of special interest.

In our previous study, *Aureobasidium pullulans* CBS 135684 isolated from Thailand was found to produce cellulase-free xylanase that was relatively active and stable at high temperature (70~80 °C) with the wide ranges of pH (4~10) [5]. These properties of xylanase are hardly found in commercial enzymes, such as Cartazyme, Irgazyme 40s and Ecopulp X200 [6], and also in other yeast xylanases that would be a good potential for novel application. In addition, this strain can produce xylanases from agricultural wastes, which is practically more attractive due to the low cost of enzyme production. The highest xylanase production was found in the medium containing 1% (w/v) corncob, compared with those of wheat germ, wheat bran, rice straw and water hyacinth [5]. To obtain the highest yield with low production cost, the investigation of the optimal nutrient compositions in culture medium for xylanase production using corncob as the main substrate has gained an attention in this study.

Various nutrient compositions of carbon sources, nitrogen sources, mineral salts, inducers, and along with other nutrient parameters were found to influence the xylanase production during submerged culture. It is imperative to consider in single and combined effects on all involved parameters [8]. The classical method of one-variable-at-a-time design may be effective in some situations, but fails to consider the combined effects while the multivariate design fulfills this requirement [9]. The factorial or Plackett-Burman design is an appropriate experimental design for the first-order model to screen the significant factors among several variables [10]. Subsequently, experimental designs for second-order models, such as response surface methodology, should be used to approximate a response function or to optimize the concentration of variables [11,12]. The purpose of this study was to optimize the thermoactive xylanase production by *A. pullulans* CBS 135684 using corncob as main substrate. First, Plackett-Burman experimental design was used to determine which of the nutritional variables were significant on the production of xylanase. Sequentially, the optimum levels of the identified variables were determined through application of response surface methodology using Box-Behnken experimental design.

2. Materials and Methods

2-1. Organism and culture conditions

Aureobasidium pullulans CBS 135684 was previously isolated [5] and maintained at the fungal culture collection of the Plant Bio-

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mass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. It was deposited at the Centraalbureau voor Schimmelcultures, Netherlands (CBS number 135684). The yeast was grown in yeast malt (YM) agar medium [13] at room temperature for two days and short-term stock cultures were stored at 4 °C. For long-term storage, the strain was stored at −20°C in YM broth containing 20% (v/v) glycerol.

2-2. Xylanase production from corncob

Corncob was locally collected, cleaned, initially cut to small sizes, air dried, ground and sieved through 80-mesh screen. Seed culture was prepared by growing one colony of *A. pullulans* in YM medium at room temperature (28±2 °C) with 150 rpm shaking incubation for 72 h. The inoculum was adjusted to 2.5×10^7 cells·ml^{-1} using sterile distilled water and 100 µl was transferred into 100 ml basal medium [14] supplemented with 1% (w/v) corncob as the sole carbon source. The cultures were incubated at room temperature with 150-rpm agitation for 48 h. Cells were separated from the culture broth by centrifugation (18,000 × *g*, 10 min) at 4 °C. The supernatant was used as the crude enzyme solution for xylanase assay.

2-3. Enzyme assay

Xylanase activity was assayed at 70 °C using 1% (w/v) beech wood xylan (Fluka, USA) as the substrates in 50 mM acetate buffer (pH 6.0). The amount of released reducing sugars was determined by the 3,5-dinitrosalicylic acid (DNS) method [15]. One unit (U) of xylanase was defined as the amount of enzyme required to release 1 µmol xylose equivalent per min under the optimal conditions. Results are reported as the mean value of three replicates.

2-4. Screening of nutrient compositions using a Plackett-Burman design

To assess the effects of medium compositions on the xylanase production, the experiment was designed based on the Design Expert software (Version 8.0.2.0, Stat-Ease Inc., Minneapolis, USA) [10].

Table 1. Nutrient screening using a Plackett-Burman design

Variables Nutrient code	Nutrients	Level (g·l^{-1})	
		Low (−1)	High (+1)
X_1	Glucose	0.5	1.5
X_2	Xylose	0.5	1.5
X_3	corncob	5.0	10.0
X_4	$(NH_4)_2SO_4$	0.5	1.5
X_5	Urea	0.5	1.5
X_6	KH_2PO_4	0.5	1.5
X_7	Yeast extract	0.5	1.5
X_8	Peptone	0.5	1.5
X_9	$MgSO_4{\cdot}7H_2O$	0.2	1.0
X_{10}	$CaCl_2{\cdot}2H_2O$	0.2	1.0
X_{11}	Tween 80	0.5	1.5

A total of eleven variable components were analyzed including corncob, xylose, glucose, urea, $(NH_4)_2SO_4$, yeast extract, peptone, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, KH_2PO_4 and tween 80. All variables were investigated at two spaced intervals designated as −1 (low level) and +1 (high level) as shown in Table 1. The experimental design for the screening of the variables is described in Table 2. The Plackett-Burman experimental design was based on a first-order model:

$$Y = \beta_0 + \Sigma\beta_i X_i \quad (1)$$

where Y is the response (enzyme activity), β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable. All experiments were performed in three replicates and the mean values are given. Nutrient compositions that significantly enhanced the xylanase production were selected for further optimization.

2-5. Optimization of nutrient concentration using response surface methodology

The Box-Behnken experimental design with five factors, based on the results from Plackett-Burman experiments, in three levels including three replicates at the center point was used to fit the sec-

Table 2. The Plackett-Burman experimental design matrix for medium composition screening of xylanase production by *A. pullulans* CBS 135684

Trials	Variable factors (g.l^{-1})											Xylanase activity (U·ml^{-1})	Predicted values (U·ml^{-1})
	X_1*	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}		
1	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	2.48	2.17
2	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	3.26	3.27
3	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	4.73	4.38
4	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	3.95	4.43
5	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	6.08	6.20
6	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	3.86	3.87
7	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	3.64	3.65
8	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	2.27	2.39
9	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	5.04	5.05
10	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	3.44	3.60
11	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	6.51	6.20
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	4.62	4.67

*X_1: glucose, X_2: xylose, X_3: corncob, X_4: $(NH_4)_2SO_4$, X_5: urea, X_6: KH_2PO_4, X_7: yeast extract, X_8: peptone, X_9: $MgSO_4{\cdot}7H_2O$, X_{10}: $CaCl_2{\cdot}2H_2O$ and X_{11}: tween 80. The +1 and −1 symbols refer to the higher and lower concentrations, respectively, for each variable factor in Plackett-Burman experimental design

Table 3. Analysis of variance (ANOVA) of the Plackett-Burman experimental model developed for xylanase production by *A. pullulans* CBS 135684

Source	Sum of Squares	df	Mean square	*F*-value	*P*-value (Prob > *F*)
Model	18.0393	6	3.0065	24.7358	0.001441*
X_1-Glucose	3.9445	1	3.9445	32.4528	0.002325*
X_2-Xylose	1.1408	1	1.1408	9.3860	0.027985*
X_3-Corncob	5.9080	1	5.9080	48.6071	0.000934*
X_4-$(NH_4)_2SO_4$	2.2360	1	2.2360	18.3965	0.007796*
X_7- KH_2PO_4	2.2707	1	2.2707	18.6817	0.007555*
X_{11}-tween 80	2.5392	1	2.5392	20.8907	0.005998*
Residual	0.6077	5	0.1215		
Cor Total	18.6471	11			

R^2 = 0.967; Adj R^2= 0.928; Coefficient of variance = 8.39%; Significant at P<0.05

ond order response surface [16]. All variables were taken at a central coded value of zero. The minimum and maximum ranges of variables investigated are listed in Table 3. Upon the completion of experiments, the average of xylanase activities was taken as the response and analyzed in a statistical manner using regression. A multiple regression analysis of the data was carried out for obtaining an empirical model that related the response measured to the independent variables. Xylanase production was analyzed by multiple regressions through the least squares method to fit the following equation:

$$B = \beta_0 + \Sigma\beta_i A_i + \Sigma\beta_{ij} A_i A_j + \Sigma\Sigma\beta_{ii} A_i^2 \quad (2)$$

where B is the measured response, β_0 is the intercept term, β_i is linear coefficients, β_{ii} is quadratic coefficient, β_{ij} is interaction coefficient and A_i and A_j are coded independent variables. Statistical analysis of the data involved design package Design-Expert software (version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA) to evaluate the analysis of variance (ANOVA) and to determine the significance of each term in the equations fit. The fitted polynomial equation was then expressed in the form of three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones. In addition, the optimal concentrations of the critical variables were also obtained by these plots. The combination of different optimized variables, which yielded the maximum response, was determined to verify the validity of the model. To verify the accuracy of the predicted model, the repeated experiment was carried out using the optimized medium. Growth pattern and xylanase production of *A. pullulans* were also determined every 12 h up to 120 h after cultivation in the optimum medium compared with the previous production medium at room temperature.

3. Results and Discussion

3-1. Screening of the medium composition for xylanase production using the Plackett-Burman design

The independent variables examined in the Plackett-Burman design and their settings are shown in Table 2, while the effect of each medium component on xylanase production is shown in Fig. 1. The negative influence on the xylanase yield was found in the presence of urea

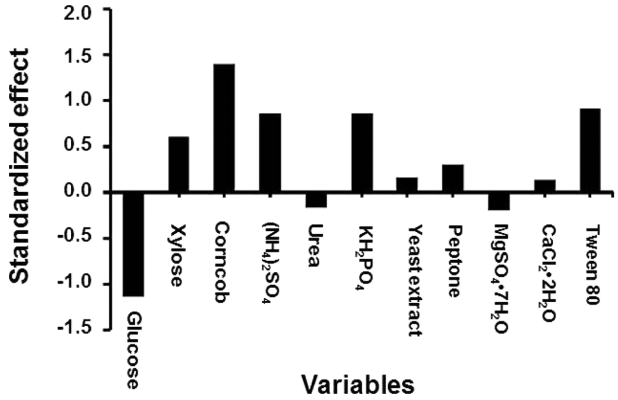

Fig. 1. Pareto chart showing the effects of medium components on xylanase production by *A. pullulans* CBS 135684.

and $MgSO_4$ due to the interfering of these residues in the cultivated broth on the measurement of enzyme activity [5], while glucose caused the repression of xylanase encoding gene according to a similar result in the previous report of *Trichoderma reesei* [17]. The strong inhibition of xylanase production in the presence of glucose (> 1.5 g·L^{-1}) was due to the cross signal between cellulase/xylanase regulatory pathways [17]. The shared-domain of carbon catabolic repressor made it able to regulate both cellulase and xylanase gene cassettes and then glucose acted as a strong repressor for xylanase production [17,18]. Therefore, low concentration of glucose (0.5 g·L^{-1}) was used to support the initial microbial growth and to study the effect on xylanase production. However, the catabolite repression caused by glucose is still dominant.

The remaining eight components, including xylose, corncob, $(NH_4)_2SO_4$, KH_2PO_4, yeast extract, peptone, $CaCl_2$ and tween 80, could enhance xylanase production. Statistical analysis (F-value and P-value) demonstrated that among the main positive effects, xylose, corncob, $(NH_4)_2SO_4$, KH_2PO_4 and tween 80 had effects above 95% level and hence were considered to significantly influence xylanase production (Table 3). As xylanases are inducible enzymes, the generally known inducers of xylanase production are xylan and its short fragments, such as xylooligosaccharides [19,20]. Such long and short chains of xylan are present in corncob [21]. For xylose, similar results were presented that it served as a strong inducer for xylanase production by *A. pullulans* CBS 58475 [22] and *A. pullulans* NCIM

1050 [23]. Tween 80 is considered to be of no antimicrobial activity as a nonionic surfactant that is widely used as amendment in the production of fungal enzymes due to the increase in permeability of cell membrane and stabilizing effect on the enzyme activity [24,25]. Therefore, the effect of tween 80 at final concentration of 0.05 and 0.15% (w/v)) on xylanase production was tested in this study within the range of typical use between 0.025 to 1% (v/v), and a positive result was also found in the same way with several studies [26-28].

With this experimental design, the role of each medium component on xylanase production by *A. pullulans* CBS 135684 was confirmed by the coefficient of determination (R^2 = 0.967) for the Plackett-Burman model and the significant probability value for regression model [(prob > F) <0.05]. The model *F*-value of 24.74 and *P* at < 0.05 also indicated that the model was significant. Regression analysis was performed on the results and a first-order polynomial equation was derived representing xylanase production as a function of the independent variables as follows:

$$Y = 4.15 - 0.57X_1 + 0.31X_2 + 0.70X_3 + 0.43X_4 + 0.44X_6 + 0.46X_{11} \quad (3)$$

where, Y is the response value or xylanase activity ($U \cdot ml^{-1}$). X_1, X_2, X_3, X_4, X_6 and X_{11} are glucose ($g \cdot l^{-1}$), xylose ($g \cdot l^{-1}$), corncob ($g \cdot l^{-1}$), $(NH_4)_2SO_4$ ($g \cdot l^{-1}$), KH_2PO_4 ($g \cdot l^{-1}$) and tween 80 ($ml \cdot l^{-1}$), respectively. Xylanase activity from the composition-adjusted medium in this experiment was found to increase about 1.6-fold (6.51±0.23 $U \cdot ml^{-1}$) compared to the previous medium containing 1% (w/v) corncob (4.10±0.10 $U \cdot ml^{-1}$) [5]. However, the xylanase activity obtained from this new medium was still slightly lower than that from the original production medium containing commercial beech wood xylan (7.23±0.15 $U \cdot ml^{-1}$). Therefore, appropriate concentrations of all five positive factors were selected to determine the optimal concentration based on Box-Behnken design.

3-2. Optimization of the medium compositions for xylanase production using response surface methodology

The observed and predicted responses from 43 experimental trials which contained different combinations of the five variables at three different concentrations are shown in Table 4. The maximum xylanase production was found to be 8.74±0.84 $U \cdot ml^{-1}$ in the trial number 8, which was very close to the predicted value (8.65±0.21 $U \cdot ml^{-1}$). The enzyme yield increased approximately two-fold when compared to the unmodified medium (4.10±0.10 $U \cdot ml^{-1}$). The second-order regression equation providing the levels of xylanase activity as the function of variables was presented in terms of coded factors as in the following equation:

$$B = 5.64A_1 + 10.39A_2 + 12.86A_3 + 18.37A_4 + 18.54A_5 - 0.14A_1A_2 - 0.03A_1A_3 - 0.22A_1A_4 - 0.20A_1A_5 - 0.05A_2A_3 + 0.39A_2A_4 + 0.09A_2A_5 + 0.13A_3A_4 - 0.10A_3A_5 + 0.02A_4A_5 - 0.06A_1^2 - 0.95A_2^2 - 3.33A_3^2 - 3.89A_4^2 - 3.61A_5^2 - 155.36$$

where, B is the response value or xylanase activity ($U \cdot ml^{-1}$). A_1, A_2, A_3, A_4 and A_5 are corncob ($g \cdot l^{-1}$), $(NH_4)_2SO_4$ ($g \cdot l^{-1}$), xylose ($g \cdot l^{-1}$), KH_2PO_4 ($g \cdot l^{-1}$) and tween 80 ($ml \cdot l^{-1}$), respectively. The model for xylanase production in this study was significant based on the *F*-value (151.97) and the model terms value of Prob > *F* less than 0.0001 (Table 6). The coefficient of determination (R^2) was calculated to be 0.9928, indicating that the model could explain up to 99% of the variability and only less than 1% of the total variance could not be explained by the model. The adjusted determination coefficient (adjusted R^2 = 0.9863) and predicted determination coefficient (R^2 = 0.9730) also confirmed the significance of the model. A lower value of coefficient of variation (CV = 3.63%) indicated a greater preciseness and reliability of experiment performed.

Comparison of predicted and experimental values revealed good correspondence between them. The plot of observed versus predicted xylanase activities under optimum conditions showed that the actual values of xylanase activity were not significantly different from the predicted response values (Fig. 2). In this case, the model showed insignificance in lack of fit (*P* = 0.7724). It indicated the second-order model equation was adequate for the prediction of xylanase production across the specified range of variables employed. It was confirmed by the coefficient of determination (R^2 = 0.9905). A lower value of coefficient of variation (CV = 3.63%) indicated a greater preciseness and reliability of the experiment performed. Thus, the estimated model fitted the experimental data adequately.

In this experiment, the linear effects (A_1, A_2, A_3, A_4 and A_5), the interactive effects (A_1A_2, A_1A_3, A_1A_4 and A_1A_5) and square effects (A_1^2, A_2^2, A_3^2, A_4^2 and A_5^2) were significant model terms for xylanase production (B), which is evident from *P*-value less than 0.05 (Table 6). To get a better understanding of the effects of the variables on xylanase production by *A. pullulans* CBS 135684, the predicted model was further assessed using RSM. Three-dimensional response plots and their corresponding contour plots were drawn based on the model equation to investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum xylanase production. The contour plots affirmed that the objective function was unimodal, which showed an optimum at the boundaries (Fig. 3~5). Xylanase production tends to increase with gradually increasing value in corncob concentration (Fig. 3 and 5). The optimal concentration of corncob (3.9% (w/v)) obtained in this study fell in the same range (1% to 4% (w/v)) reported for other strains of *A. pullulans* [29,30], *Aspergillus terricola* [31] and *A. niger* [32]. Fig. 4 shows the effects of A_3 (xylose) and A_4 (KH_2PO_4) on xylanase production. Evidently, the middle level of the KH_2PO_4 in the medium ensured more xylanase production. Similar result was found in Fig. 5

Table 4. Experimental range and levels of independent test variables used in Box-Behnken experimental design

Independent variables ($g \cdot l^{-1}$)	Variable codes	Level		
		−1	0	+1
Corncob	A1	30.0	35.0	40.0
$(NH_4)_2SO_4$	A2	2.0	3.0	4.0
Xylose	A3	1.0	1.5	2.0
KH_2PO_4	A4	1.0	1.5	2.0
Tween 80	A5	1.0	1.5	2.0

Table 5. Box-Behnken design matrix with experimental and predicted values of xylanase production by *A. pullulans* CBS 135684

Trials	Variables (g/L)					Xylanase activity (U/mL)	Predicted values (U/mL)
	Corncob (A_1)	$(NH_4)_2SO_4$ (A_2)	Xylose (A_3)	KH_2PO_4 (A_4)	Tween 80 (A_5)		
1	+1	+1	0	0	0	7.24	7.50
2	0	0	0	0	0	7.42	7.66
3	0	0	0	+1	+1	6.50	6.34
4	0	0	0	0	0	7.62	7.66
5	0	0	−1	−1	0	4.74	4.76
6	0	+1	0	0	+1	6.68	6.73
7	0	0	+1	+1	0	7.10	7.01
8	+1	0	+1	+1	0	8.74	8.65
9	0	+1	+1	0	0	7.35	7.31
10	−1	+1	0	−1	0	4.08	4.06
11	0	0	0	0	−1	4.87	5.24
12	0	−1	0	0	−1	5.04	4.97
13	0	+1	0	0	0	5.94	5.87
14	0	0	−1	+1	0	4.97	5.08
15	0	0	+1	0	−1	6.52	6.53
16	0	−1	0	−1	+1	5.52	5.60
17	−1	0	0	0	+1	3.74	3.77
18	0	0	−1	+1	−1	4.57	4.61
21	−1	0	−1	+1	0	7.44	7.56
22	0	−1	0	0	0	7.72	7.86
23	+1	0	0	−1	+1	6.40	6.32
24	+1	−1	0	0	0	7.93	7.66
25	0	−1	+1	0	0	1.80	1.64
26	0	0	0	0	0	6.02	5.93
27	−1	−1	0	+1	0	6.78	6.65
28	0	+1	0	0	−1	3.70	3.96
29	0	+1	0	−1	0	5.40	5.62
31	−1	0	+1	0	0	7.03	6.91
32	0	0	0	+1	−1	5.42	5.38
33	0	0	0	+1	+1	5.14	5.22
34	+1	0	−1	0	0	5.42	5.49
35	0	0	−1	−1	+1	2.02	2.08
36	0	−1	0	0	0	4.38	4.40
37	0	+1	−1	0	0	3.38	3.57
38	−1	0	0	0	0	2.30	2.03
39	0	−1	−1	+1	0	8.04	7.86
40	−1	0	0	0	0	8.26	8.00
41	−1	0	0	−1	−1	7.27	7.19
42	+1	0	0	0	−1	6.74	6.56
43	+1	0	0	+1	0	7.42	7.29

The −1, 0, +1 symbols are placed at one of three equally spaced values for the higher, middle and lower concentrations, respectively, for each independent variable in Box-Behnken design

that the middle level of tween-80 resulted in the higher xylanase production.

The canonical analysis revealed a maximum xylanase activity of 8.74±0.84 U.ml^{-1} under the optimal conditions of corncob, 39.0 g·l^{-1}; $(NH_4)_2SO_4$, 3.0 g·l^{-1}; xylose, 1.8 g·l^{-1}; KH_2PO_4 1.4 g·l^{-1} and tween 80, 1.4 ml·l^{-1}. The model predicted that the xylanase activity from *A. pullulans* CBS 135684 would reach up to 8.82±0.21 U·ml^{-1} when produced in the medium containing these five components at the optimized concentrations. The validity of the results predicted by the regression model was confirmed by repeated experiments under optimal conditions. The result obtained from three replications showed that the maximum xylanase production obtained (8.72±0.21 U·ml^{-1}) was close to the predicted value (8.65±0.21 U·ml^{-1}). It implied that empirical models derived from RSM can be used to adequately describe the relationship between the factors and response in xylanase production by *A. pullulans* CBS 135684. The synergistic effect of selected variables corresponded to 1.34 and 2.13-fold increase in xylanase activity (8.74±0.84 U·ml^{-1}) compared to their individual effects from Plackett-Burman model (6.51±0.23 U·ml^{-1}) and original medium (4.10±0.10 U·ml^{-1}), respectively. In addition, xylanase

Table 6. Analysis of variance (ANOVA) of the Box-Behnken experimental model developed for xylanase production by *A. pullulans* CBS 135684

Source	Coefficient factor	Sum of Squares	Df	Mean square	*F*-value	*P*-value (Prob > F)
Model	7.66	132.60	20	6.63	151.97	< 0.0001*
A_1-Corncob	2.41	92.73	1	92.73	2125.43	< 0.0001*
A_2-$(NH_4)_2SO_4$	0.52	4.33	1	4.33	99.27	< 0.0001*
A_3-Xylose	0.93	13.95	1	13.95	319.77	< 0.0001*
A_4-KH_2PO_4	0.19	0.60	1	0.60	13.84	0.0012*
A_5-tween 80	0.36	2.06	1	2.06	47.3	< 0.0001*
A_1A_2	−0.69	1.90	1	1.90	43.65	< 0.0001*
A_1A_3	−0.16	0.20	1	0.20	10.37	0.0054*
A_1A_4	−0.55	1.21	1	1.21	27.73	< 0.0001*
A_1A_5	−0.51	1.04	1	1.04	23.85	< 0.0001*
A_2A_3	−0.02	2.10E-003	1	2.10E-003	0.048	0.8285
A_2A_4	0.20	0.15	1	0.15	3.49	0.0753
A_2A_5	0.04	7.74E-003	1	7.74E-003	0.18	0.6776
A_3A_4	0.03	4.23E-003	1	4.23E-003	0.097	0.7586
A_3A_5	−0.03	2.70E-003	1	2.70E-003	0.062	0.8057
A_4A_5	5.50E-003	1.21E-004	1	1.21E-004	2.77E-03	0.9585
A_{12}	−1.45	13.51	1	13.51	309.73	< 0.0001*
A_{22}	−0.95	5.76	1	5.76	131.98	< 0.0001*
A_{32}	−0.83	4.45	1	4.45	101.92	< 0.0001*
A_{42}	−0.97	6.06	1	6.06	138.85	< 0.0001*
A_{52}	−0.90	5.21	1	5.21	119.37	< 0.0001*
Residual		0.96	22	0.04		
Lack of Fit		0.83	20	0.04	0.63	0.7724
Residual		0.13	2	0.07		
Cor Total		133.56	42			

R^2 = 0.993; Adj R^2= 0.986; Coefficient of variance = 3.63%; Significant at $P < 0.05$

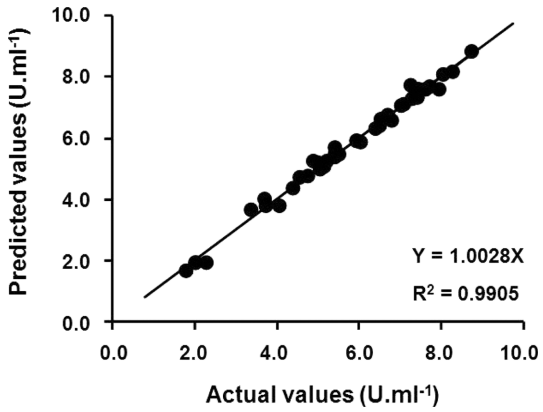

Fig. 2. Observed xylanase activity versus the predicted xylanase activity under the optimum conditions.

production from this experiment was 1.2-fold higher than that from the unmodified medium containing commercial beechwood xylan.

3-3. Xylanase production by A. pullulans before and after optimization

Two parallel experiments were conducted to study the pattern of cell growth and xylanase production in the optimized and original media (basal medium containing 1% (w/v) corncob) under the same condition. Although the cell density of *A. pullulans* CBS 135684 in optimized medium was noticeably lower than that of the basal medium,

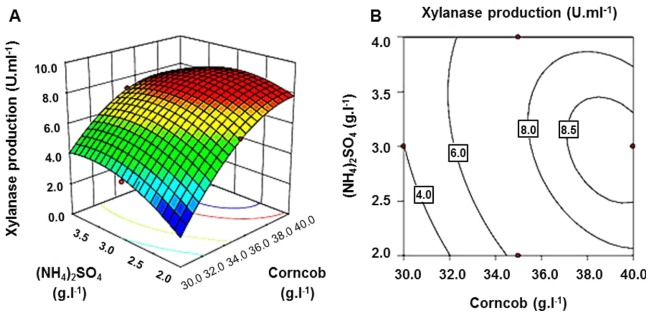

Fig. 3. Response surface plot (A) and contour plot (B) of the combined effects of corncob (A_1) and $(NH_4)_2SO_4$ (A_2) on xylanase production by *A. pullulans* CBS 135684.

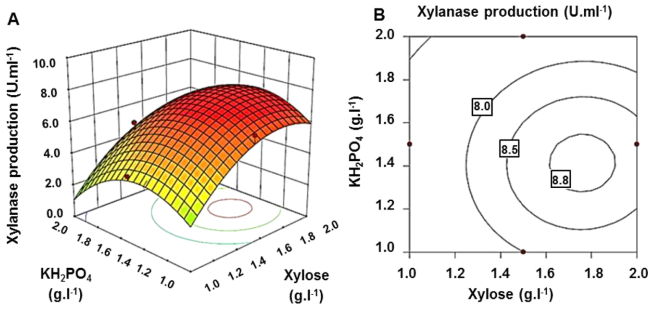

Fig. 4. Response surface plot (A) and contour plot (B) of the combined effects of xylose (A_3) and KH_2PO_4 (A_4) on xylanase production by *A. pullulans* CBS 135684.

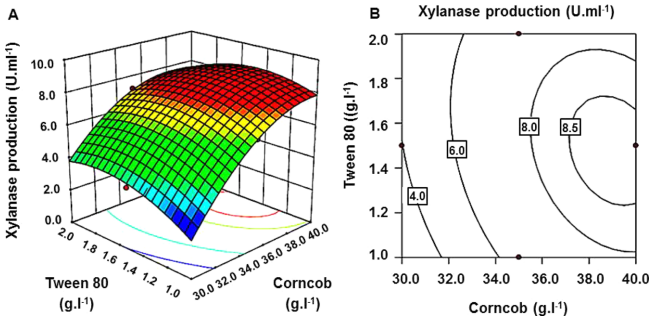

Fig. 5. Response surface plot (A) and contour plot (B) of the combined effects of corncob (A_1) and tween 80 (A_5) on xylanase production by *A. pullulans* CBS 135684.

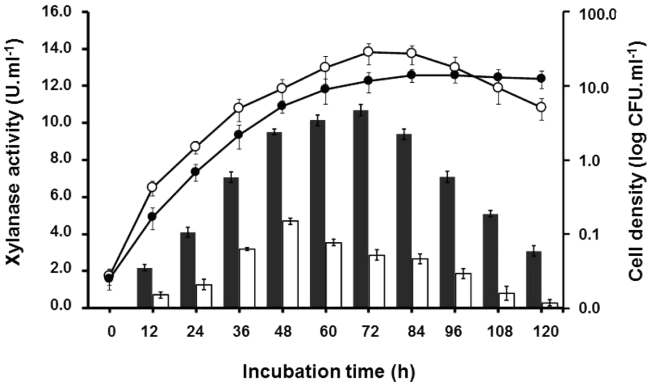

Fig. 6. Time course of growth and xylanase production of *A. pullulans* CBS 135684 at room temperature (28±2 °C) in the basal medium containing 1% (w/v) corncob and the optimized medium. Open circles (○) and solid circles (●) indicated cell density in the basal medium and the optimized medium, respectively. White bars and black bars indicated xylanase activity in the basal medium and the optimized medium, respectively. Results were shown as a mean with one standard deviation derived from three replicates.

the xylanase production was strongly increased (Fig. 6). The maximal activity (10.09±0.27 U·ml^{-1}) was found in the optimized medium after 72 h of incubation during the late log phase of the culture. In the original medium, the maximal activity was 4.10 ± 0.10 U·ml^{-1} after 48 h of incubation, which was 2.5-fold lower than that of the optimized medium.

The subsequent decrease in enzyme activity during the stationary phase was generally common for the primary metabolites and has also been noted in xylanase production by other fungi [33]. It was probably due to proteolysis of the enzyme and/or the decline of actively growing cells because of nutrient depletion [33,34]. The results also suggested that the appropriate time for harvesting the xylanase enzyme produced by *A. pullulans* CBS 135684 grown in the optimized medium was at 72 h after inoculation.

4. Conclusion

We reported the successful optimization of nutrient sources in a medium for enhancement of xylanase production by *A. pullulans* CBS 135684. Statistical methods (Plackett-Burman and Box-Behnken design) were employed, instead of the one-factor-at-a-time method, to provide the information of interaction between variables, to select significant factors and to evaluate the optimum value of variables. Corncob, $(NH_4)_2SO_4$, xylose, KH_2PO_4 and tween 80 had significant effect on xylanase production, and the optimized medium was (g·l^{-1}): 39.0, 3.0, 1.8, 1.4, and 1.4, respectively, which resulted in xylanase activity with 2.13-fold higher than that obtained from the non-optimized medium. Based on the result herein, the large scale of xylanase production from *A. pullulans* CBS 135684 should be carried out in the future study.

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