Optimization of Multi-enzyme Production by Fungi Isolated from Palm Kernel Expeller using Response Surface Methodology

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Response surface methodology (RSM) was used to optimize the co-production of a mixture of crude cellulosic and hemicellulosic enzymes (endoglucanase, xylanase, and mannanase) by *Aspergillus terreus* K1 in solid-state fermentation (SSF) using palm kernel expeller (PKE) as the sole carbon source. These enzymes have gained renewed interest due to their efficacy to improve the digestibility of PKE for use in diets of mono-gastric animals (poultry, pigs, and fish). The results showed that temperature, moisture, inoculum concentration, and initial pH had significant ($P< 0.05$) effects on the enzymes production. Using PKE as a solid substrate, maximum endoglucanase, mannanase, and xylanase (17.37, 41.24, and 265.57 U/g DM, respectively) were obtained at 30.5 °C, 62.7% moisture, 6% inoculum, and pH 5.8. The enzyme activities recorded were close to the predicted values (19.97, 44.12, and 262.01 U/g DM, respectively).

Keywords: Solid-state fermentation; Aspergillus terreus; Palm kernel expeller; Response surface methodology

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INTRODUCTION

Escalating demand for traditional feed ingredients such as corn, soybean, and other grains for animal feed over the last two decades has resulted in their scarcity and created competition for food with the human population (Vasta *et al.* 2008). Thus, the search for alternative feed ingredients, including agro-industrial byproducts such as palm kernel expeller (PKE), has been given considerable attention in recent years. PKE, a by-product of the palm oil industry obtained through the screw-pressing process, is a good source of energy and protein for ruminant animals, but it is sparingly used in poultry feed (Soltan 2009) because it contains a high level of non-starch polysaccharides (NSPs), mainly mannans and some cellulose, xylose, and other polysaccharides (Sundu *et al.* 2006) that monogastric animals (poultry, pigs, and fish) cannot digest. This suggests that at least three main cellulolytic and hemicellulolytic enzymes are needed to digest the glycosidic bonds of mannan, cellulose, and xylan to improve the nutritive value of PKE if it is to be used more efficiently in diets for poultry and other monogastric animals. Previous studies in Malaysia (Ng *et al.* 2002; Saenphoom *et al.* 2011) showed that commercial enzymes could be used to pre-treat PKE to degrade these fibrous compounds

to usable monosaccharides to enhance their metabolizable energy before feeding them to fish and poultry. However, most of the enzymes tested were imported and not specifically designed to break down the lignocellulosic components in PKE (Ibrahim 2008).

Discovery of more robust local fungi for the production of enzymes to specifically digest PKE may be more effective and economical. Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications. Those belonging to the genus *Aspergillus* are commonly used for the production of cellulase (Bakir *et al.* 2001), mannanase (Kurakake and Komaki 2001; Lin and Chen 2004; Puchart *et al.* 2004), and xylanase (Lu *et al.* 2003).

Solid-state fermentation (SSF) has gained renewed interest in recent years for the production of enzymes due to its lower operating costs and energy requirements, as well as requiring simpler plant equipment compared to submerged fermentation (Mitchell and Losane 1992; Pandey 2003). Nevertheless, the efficacy of SSF depends on several factors, such as initial pH, temperature, moisture content, and inoculum size (Mitchelle and Losane 1992; Baysal *et al.* 2003). Response surface methodology (RSM) is a collective statistical technique that has recently been used to model and optimize several bioprocess, including fermentation, enzymatic reactions, product recovery, and enzyme immobilization techniques (Ismail 2005; Levin *et al.* 2008; Bonugli-Santos *et al.* 2010; Su *et al.* 2011; Zhang *et al.* 2011). A second-order model like the central composite design (CCD) is widely used in RSM, because it can take on a wide variety of functional forms, and this flexibility allows it to predict the true response surface more closely. This approach has been successfully employed to maximize production of enzyme in SSF (Abdeshahian *et al.* 2010; Abdeshahian *et al.* 2011; Coman and Bahrim 2011). However, most of the above studies focused on the optimization of single enzyme production. In this study, CCD-based RSM was used to optimize the fermentation parameters for co-production of endoglucanase, mannanase, and xylanase using *A. terreus* K1 (which was isolated in this study) in SSF using PKE as the sole carbon source.

EXPERIMENTAL

Sample Collection

PKE was collected from two commercial kernel oil extraction mills in Malaysia; Klang in Selangor and Kuantan in Pahang from the west- and east-coast of Peninsula Malaysia, respectively. The fresh samples were divided into two equal portions; one portion was immediately packed and stored at 4 °C for the isolation of fungi, and the other was ground and passed through a sieve of 2.5 mm and stored at 4 °C to be used for SSF studies (Saenphoom *et al.* 2011).

Isolation, Screening, and Identification of Potential Isolates

Serial dilution technique was used for the isolation of effective fungal strains. For this technique, 0.1 mL diluent was pipetted onto potato dextrose agar plates, spread with a glass spreader, and incubated at 30 °C for 5 to 7 days for observation. Each formed colony was transferred onto a fresh PDA plate, sub-cultured, and maintained on PDA slant at 4 °C with periodic (30 days) sub-culturing.

Spore suspensions were prepared by adding Tween-80 (0.1%) to 5-day-old cultures grown on PDA slant at 30 °C and gently brushing the mycelium with a sterile

wire loop. Spores were counted using a hemocytometer, and the concentration of the spore suspension was adjusted to a final spore count of 1.0×10^7 spores/mL.

To screen for the best enzyme producer, each isolate was grown in SSF at 30 °C for 7 days, using PKE as the sole carbon source. The activities of endoglucanase, mannanase, and xylanase for each isolate were assessed. The best enzyme producer was identified by the analysis of its genomic internal transcribed spacer, ITS-region, using the standard methodology of White *et al.* (1990) and subsequently compared with sequences in the public databases of GenBank.

Optimization of Enzyme Production

Response surface methodology was used to optimize the SSF process and enhance the endoglucanase, mannanase, and xylanase production. Design-Expert® software (version 8.0) was used for the statistical design of experiments and data analysis. A CCD with four factors and five levels using six replicated center points was employed. The range and center point values of the four independent variables are presented in Table 1. The full experimental design with respect to the real value of the independent variables and attained values for the response (endoglucanase, mannanase, and xylanase activity) is presented in Table 2. The experiment was carried out in duplicate, and the mean enzyme activity was taken as response Y. Data from the CCD (Table 2) were analyzed by the least squares method to fit the following second-order polynomial equation,

$$Y = B_o + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_{11}X_{12} + B_{22}X_{22} + B_{33}X_{32} + B_{44}X_{42} + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{14}X_1X_4 + B_{23}X_2X_3 + B_{24}X_2X_4 + B_{34}X_3X_4 \quad (1)$$

where Y is the measured response; B_0 is the intercept term; B_1, B_2, B_3, and B_4 are linear coefficients; B_{11}, B_{22}, B_{33}, and B_{14} are quadratic coefficients; B_{12}, B_{13}, B_{23}, and B_{24} are interaction coefficients; and X_1, X_2, X_3, and X_4 are coded independent variables.

The statistical analysis of the model was performed using an analysis of variance (ANOVA) generated by the Design-Expert software.

Table 1. Coded Values of Variables Used in Central Composite Design

Independent variable	Level				
	-2	-1	0	1	2
Temperature (°C)	25	30	34	37	42
Moisture (%)	40	50	60	70	80
pH	3.0	4.5	6.0	7.5	9.0
Inoculum (%)	3	6	9	12	15

Solid-state Fermentation

Ground PKE (2.5 mm) was moistened with different volumes of distilled water to achieve the moisture content (weight of liquid/total weight of liquid plus solid) and adjusted to the initial pH as shown in Table 2. This medium was sterilized by autoclaving prior to treatment. SSF was carried out in 500-mL Erlenmeyer flasks containing 30 g of PKE, which was inoculated with different concentrations of inocula and incubated for 7 days at different temperatures, according to the experimental design.

Table 2. Central Composite Design with Experimental and Predicted Values of Enzyme Produced by *Aspergillus terreus K1*

Trial No.	Variable				Endoglucanase (U/g)		Xylanase (U/g)		Mannanase (U/g)	
	X_1	X_2	X_3	X_4	Expt.	Pred.	Expt.	Pred.	Expt.	Pred.
1	-2	0	0	0	10.83	11.38	252.87	262.02	17.79	18.57
2	-1	-1	-1	-1	17.21	16.62	242.67	247.35	33.92	36.16
3	-1	1	-1	-1	11.18	11.55	276.22	276.76	23.74	24.09
4	-1	-1	1	-1	15.89	15.93	197.74	195.60	22.12	21.52
5	-1	1	1	-1	13.32	13.42	240.70	282.64	29.41	28.22
6	-1	-1	-1	1	10.45	11.07	243.27	248.18	30.30	30.31
7	-1	1	-1	1	9.89	10.51	206.78	207.25	47.68	48.43
8	-1	-1	1	1	12.83	13.70	239.22	137.63	22.98	24.49
9	-1	1	1	1	14.12	14.16	247.32	239.44	31.62	30.12
10	0	0	0	-2	9.08	8.33	134.99	127.83	45.76	46.29
11	0	0	-2	0	18.03	17.34	338.35	347.04	28.04	33.61
12	0	-2	0	0	6.55	5.60	230.15	225.68	11.61	10.95
13	0	0	0	0	16.82	15.88	168.54	169.79	37.77	37.34
14	0	0	0	0	17.08	16.62	252.94	247.35	38.02	36.16
15	0	0	0	0	10.72	10.89	231.98	227.89	10.24	9.21
16	0	0	0	0	7.74	7.61	161.96	169.59	25.93	25.38
17	0	0	0	0	11.81	11.51	121.86	119.65	24.99	24.99
18	0	0	0	0	16.72	16.62	241.62	247.35	38.23	36.16
19	0	2	0	0	15.92	16.62	255.04	247.35	33.79	36.16
20	0	0	2	0	11.62	12.00	248.46	28.97	34.67	33.86
21	0	0	0	2	10.64	10.75	259.25	248.57	16.83	18.16
22	1	-1	-1	-1	16.92	16.62	229.10	247.35	37.47	36.16
23	1	1	-1	-1	12.21	12.59	249.79	246.02	26.94	26.50
24	1	-1	1	-1	15.88	16.62	264.99	247.35	35.11	36.16
25	1	1	1	-1	15.84	15.65	284.57	279.88	12.68	12.56
26	1	-1	-1	1	9.99	10.07	200.73	202.51	29.84	29.81
27	1	1	-1	1	11.34	10.78	101.50	110.16	18.12	17.78
28	1	-1	1	1	8.66	9.15	146.11	150.39	19.26	18.93
29	1	1	1	1	13.34	13.10	214.03	218.01	12.49	12.43
30	2	0	0	0	13.71	13.78	168.94	166.94	15.47	16.86

Expt. = experimental
Pred. = predicted

Enzyme Extraction and Enzyme Assays

Enzymes were extracted by shaking the PKE in 50 mM citrate buffer (pH 5) at 4 °C for 24 h, centrifuging at 10,000 rpm for 10 min, and filtering through Whatman No. 1 filter paper. The filtrate was used for the analysis of endoglucanase, xylanase, and mannanase.

Endoglucanase (carboxymethylcellulase, endo-1,4-b-D-glucanase; EC 3.2.1.4) activity was determined according to Grajek's method (1987), whereas xylanase activity was estimated using the method of Bailey *et al.* (1992). The concentration of free carboxymethyl glucose and xylose units that reacted with dinitrosalicylic acid reagent was estimated using the DNS method (Miller 1959). Endoglucanase and xylanase activities were expressed in international units (IU), where one IU is the amount of enzyme required to release 1 µmol of reducing sugar (glucose or xylose) equivalent in 1 mL of enzyme solution in one minute.

The β-mannanase assay was performed using the procedure specified by Megazyme (Ireland) with a slight modification. About 0.2 mL of the previously prepared

PKE filtrate was added to 0.2 mL of the substrate (Azo-Carob galactomannan) solution, stirred for 5 s on a vortex stirrer, and incubated at 40 °C for 10 min. After that, 1 mL of ethanol (~95%) was added to the mixture, which was stirred continuously for another 10 s on the vortex stirrer. The mixture was allowed to equilibrate to room temperature for 10 min and was then centrifuged at 3,000 rpm for 10 min. The supernatant solution was poured directly from the centrifuge tube into a cuvette, and the absorbance was measured using a spectrophotometer (Barnstead Turner SP-380 plus, USA) at 590 nm. Different concentrations of pure endo-1,4-β-mannanase (Megazyme, Ireland) were used for the standard curve, following the same procedure as previously described.

Enzyme activity assays were carried out in triplicate, where the average enzyme activity obtained was used as the response.

RESULTS AND DISCUSSION

Isolation and Identification of Lignocellulosic-Degrading Enzyme Producers

Lignocellulosic-degrading enzymes are necessary for the degradation of the biomass cell wall. The ability of different fungi to produce these enzymes varies, depending on the carbon source and the microorganisms used. In this study, palm kernel expeller (PKE) was used as the sole carbon source. Ten fungi were initially isolated from PKE, which was obtained from commercial kernel oil extraction mills, using potato dextrose agar. Table 3 shows the enzyme activities (endoglucanase, mannanase, and xylanase) of the ten fungal isolates, with isolates F4, F3, and K1 produced the highest endoglucanase (10.32 U/g), mannanase (43.37 U/g), and xylanase (81.54 U/g) activity, respectively. However, because the endoglucanase and mannanase activities of K1 were the same as ($P < 0.05$) that of isolate F4 and isolate F3, respectively, isolate K1 was selected for the subsequent optimization process. The analysis of the ITS-region of Isolate F3 showed similarity to the ITS region of known *Paecilomyces variotii* while both ITS sequences of Isolate F4 and Isolate K1 showed similarity to *Aspergillus terreus* sequence deposited in the Genbank.

Table 3. Enzyme Production in Fungi Isolated from Palm Kernel Expeller

Isolates	Endoglucanase (U/g)	Mannanase (U/g)	Xylanase (U/g)
F1	6.42 ± 0.11^{a}	40.79 ± 0.38^{a}	63.42 ± 3.30^{a}
F2	4.58 ± 0.08^{b}	23.28 ± 0.26^{d}	36.51 ± 7.17^{b}
F3	7.27 ± 0.09^{c}	43.37 ± 0.35^{c}	65.60 ± 2.05^{a}
F4	10.32 ± 0.08^{d}	42.07 ± 0.54^{d}	59.63 ± 3.55^{a}
F5	4.76 ± 0.13^{be}	21.15 ± 0.26^{e}	46.32 ± 3.67^{bc}
K1	10.01 ± 0.05^{d}	42.64 ± 0.08^{cd}	81.54 ± 2.10^{d}
K2	5.87 ± 0.09^{f}	30.49 ± 0.18^{f}	55.54 ± 1.71^{ac}
K3	4.54 ± 0.04^{b}	19.33 ± 0.12^{g}	39.40 ± 0.15^{b}
K4	5.16 ± 0.03^{g}	21.50 ± 0.23^{be}	63.00 ± 2.64^{a}
K5	5.04 ± 0.03^{eg}	22.40 ± 0.04^{bd}	40.43 ± 0.44^{b}

*Results are mean values ± SD (n=3)
** $^{a-g}$ Values on the same column with different superscript differ significantly ($P < 0.05$)

Optimization of Enzyme production

To obtain the optimum production of lignocellulosic enzymes by *Aspergillus terreus* K1, the four parameters that most significantly affected enzyme production were statistically optimized using RSM. The parameters were as follows: temperature (X1), moisture (X2), medium pH (X3), and inoculum concentration (X4). The maximum and minimum levels of these parameters for the tests in the CCD are shown in Table 1. To improve the accuracy of the regression model, the center point was replicated six times. A total of 30 experiments were performed following the experimental design. The experimental results and predicted activities for each enzyme as estimated from the model equations are shown in Table 2. This approach was chosen to preserve the significance of the interaction effects, which would have been lost if variables were examined one at a time while keeping the other variables constant.

Optimization of endoglucanase production

The ANOVA summary for endoglucanase production is presented in Table 4. The model validity was estimated as a function of its coefficients of determination (R^2), which provided a measure of variability in the observed response values that could be explained by the experimental factors and their interactions. In this experiment, a R^2 value of 0.974 indicated that the model was appropriate and could be used for quantitative prediction of endoglucanase production. In addition, the large model's F-value (39.97) implied that the model was significant ($P < 0.01$), and the lack of fit test result of 1.76 implied that it was insignificant relative to pure error ($P > 0.05$).

Table 4. Analysis of Variance (ANOVA) Table for Endoglucanase Production

Source	Sum of squares	Df	Mean square	F-value	Prob > F
Model	284.92	14	20.35	39.97	< 0.0001
X_1- temperature	77.58	1	77.58	152.36	< 0.0001
X_2- moisture	6.17	1	6.17	12.12	0.0033
X_3- pH	5.84	1	5.84	11.48	0.0041
X_4- inoculum	12.42	1	12.42	24.39	0.0002
X_1X_2	4.96	1	4.96	9.75	0.0070
X_1X_3	0.51	1	0.51	1.01	0.3309
X_1X_4	24.38	1	24.38	47.89	< 0.0001
X_2X_3	9.78	1	9.78	19.21	0.0005
X_2X_4	0.26	1	0.26	0.50	0.4894
X_3X_4	0.30	1	0.30	0.59	0.4546
Residual	7.64	15	0.51		
Lack of fit	5.95	10	0.59	1.76	0.2768
Pure error	1.69	5	0.34		
Cor. Total	292.55	29			
Std. dev.	0.71		R^2		0.9739
C.V. %	5.54		Adjusted R^2		0.9495
			Predicted R^2		0.8556

Analysis of the P-values was used to check the significance of each coefficient. This analysis was required to understand the pattern of the mutual interactions between the independent variables. The smaller the magnitude of the P, the more significant is the corresponding coefficient. This implies that all of the first order main effects, and the interaction terms X_1X_2, X_1X_4, and X_2X_3, are highly significant ($P < 0.01$). On the other

hand, the interaction terms between temperature and pH (X_1X_3), moisture and inoculum (X_2X_4), and pH and inoculum (X_3X_4) are not significant; there is thus no correlation between each of these variables, and their interactions did not contribute to endoglucanase production. The contour plot of these interactions shows a relatively broad plateau region (Fig. 1), indicating only small changes in endoglucanase activity when these factors were varied.

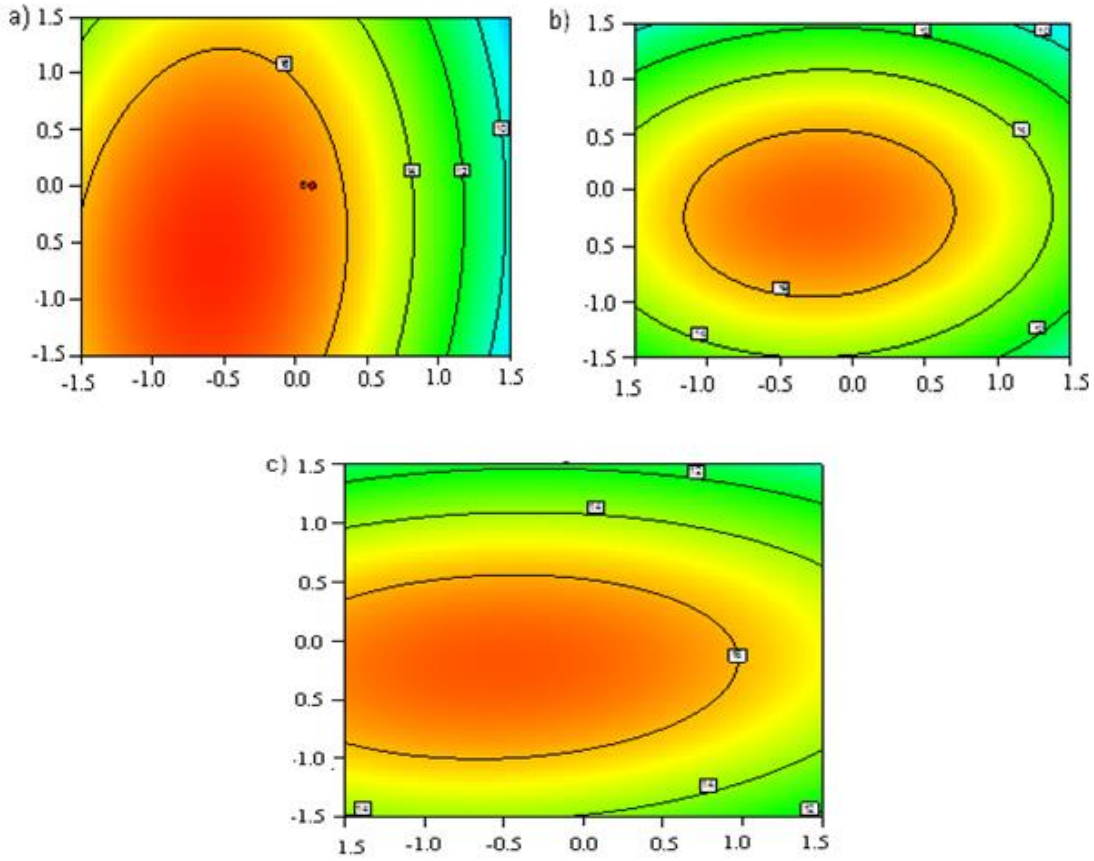

Fig. 1. Contour plot showing the effect of (a) temperature and pH, (b) moisture and inoculum, and (c) pH and inoculum on the production of endoglucanase

By applying multiple regression analysis to the experimental data, a second-order polynomial equation was found to explain endoglucanase production, regardless of the significance of coefficients (Table 5).

Table 5. Predictive Second-Order Polynomial Equation Describing the Relationship between Various Enzyme Activities

Endoglucanase	$16.88 - 2.03\ X_1 - 0.51\ X_2 - 0.49\ X_3 - 0.72\ X_4 - 1.81\ X_1^2 - 1.17\ X_2^2 - 0.48\ X_3^2 - 1.8\ X_4^2 - 0.67\ X_1X_2 + 0.22\ X_1X_3 + 1.49\ X_1X_4 - 0.78\ X_2X_3 + 0.13\ X_2X_4 + 0.14\ X_3X_4$
Mannanase	$36.62 - 3.39\ X_1 + 1.13\ X_2 + 1.11\ X_3 - 0.75\ X_4 - 4.73\ X_1^2 - 5.22\ X_2^2 - 5.36\ X_3^2 + 2.80\ X_4^2 + 2.58\ X_1X_2 - 0.28\ X_1X_3 + 1.79\ X_1X_4 - 4.38\ X_2X_3 - 2.22\ X_2X_4 - 1.74\ X_3X_4$
Xylanase	$244.98 + 22.01\ X_1 + 13.95\ X_2 - 30.90\ X_3 - 23.79\ X_4 - 15.83X_1^2 - 3.52\ X_2^2 - 5.99\ X_3^2 - 19.95\ X_4^2 - 53.62\ X_1X_2 + 22.67\ X_1X_3 + 33.42\ X_1X_4 - 20.45\ X_2X_3 + 2.88\ X_2X_4 - 16.22\ X_3X_4$

The results predicted by the model equation showed that adjusting the fermentation conditions to 30.4 °C, 60.5% moisture, pH 5.3, and 7.5% inoculum would favor maximum endoglucanase yield (18.05 U/g), which was close to the experimental endoglucanase activity of 20.12 U/g. The coefficients for temperature were larger than the coefficients for other factors, indicating that temperature had the most significant effect on endoglucanase production. In Table 5, X_1, X_2, X_3, and X_4 are the coded values for temperature, moisture, pH, and inoculum, respectively.

Optimization of mannanase production

The R^2 value of 0.9884 indicated that 98.84% of the total variability in the response could be explained by the second-order polynomial equation (Table 5). The large model F-value (85.18) indicated that the model was highly significant ($P < 0.01$). In addition, the small value obtained from the lack of fit test (1.52) implied that it was insignificant relative to pure error, and the small CV (5.84%) indicated the reliability of the experiment performed. All of these statistical results (Table 6) showed good agreement between the experimental and predicted values and implied that the mathematical models were suitable for the simulation of mannanase production in the present study. Based on the statistical analysis, only the interaction between temperature and pH had no significant effect ($P < 0.05$) on mannanase production. The plot (Fig. 2) was used to represent the interaction effect of all independent variable. A non-perfectly ellipse contour plot means that there were fewer interaction effect between the independent variable (Fig. 2b, 2c, 2e, and 2f) on mannanase production (Muralidhar *et al.* 2001). The results predicted by the model equation showed that the optimal values for mannanase production of the four variables in un-coded units were 31.2 °C, 60.8% moisture, pH 6.4, and 6.00% inoculum. Under the optimum conditions, the predicted maximum mannanase production was 42.03 U/g, which was lower than the actual experimental mannanase activity of 46.07 U/g.

Table 6. Analysis of Variance (ANOVA) Table for Mannanase Production

Source	Sum of squares	Df	Mean square	F-value	Prob > F
Model	2967.30	14	211.95	85.18	< 0.0001
X_1- temperature	197.75	1	197.75	79.48	< 0.0001
X_2- moisture	27.29	1	27.29	10.97	0.0051
X_3- pH	26.32	1	26.32	10.58	0.0058
X_4- inoculum	11.88	1	11.88	4.77	0.0464
X_1X_2	62.40	1	62.40	25.08	0.0002
X_1X_3	0.76	1	0.76	0.30	0.5899
X_1X_4	29.91	1	29.91	12.02	0.0038
X_2X_3	322.98	1	322.98	129.81	< 0.0001
X_2X_4	68.08	1	68.08	27.36	0.0001
X_3X_4	41.76	1	41.76	16.79	0.0011
Residual	34.83	14	2.49		
Lack of fit	13.67	9	1.52	0.36	0.9139
Pure error	21.17	5	4.23		
Cor. total	3002.13	28			
Std. dev.	1.58		R^2		0.9884
C.V. %	5.84		Adjusted R^2		0.9768
			Predicted R^2		0.9558

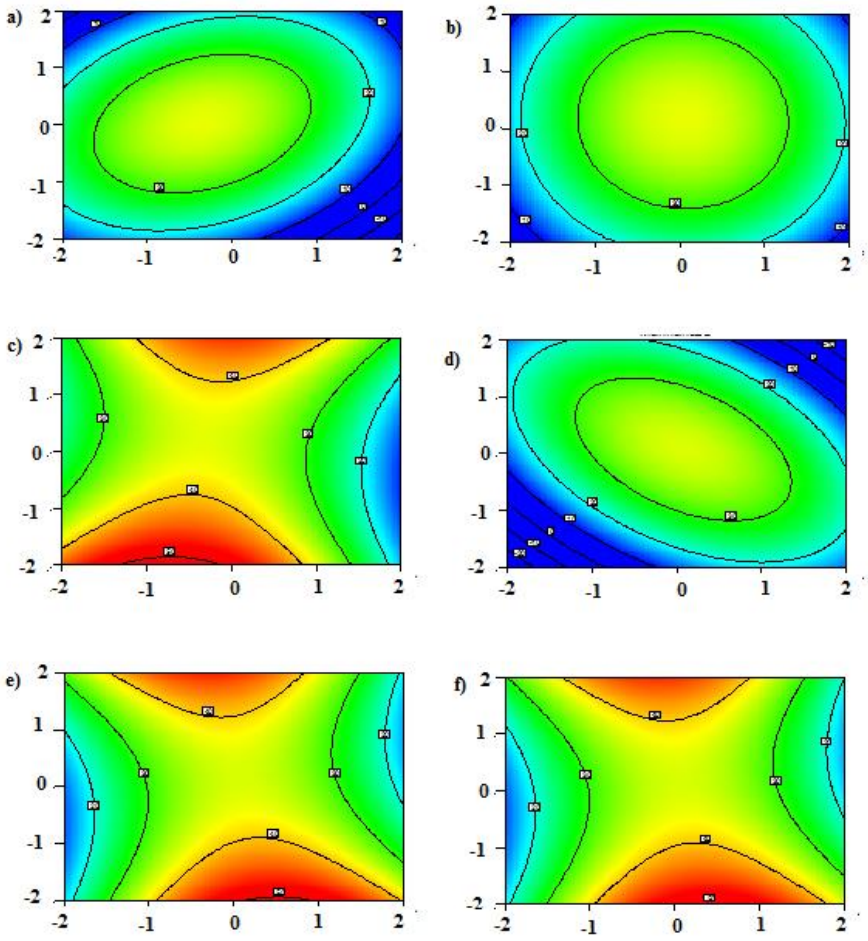

Fig. 2. Contour plot showing the effect of (a) temperature and moisture, (b) temperature and pH, (c) temperature and inoculum, (d) moisure and pH, (e) moisture and inoculum, and (f) pH and inoculum on the production of mannanse

Optimization of xylanase production

The statistical significance of the fitted model, essential for determining patterns of interaction between experimental variables, was evaluated (Table 7). The computed model's F-value (44.85) with a probability value of $P < 0.01$ indicated that the selected quadratic regression model fit well to the experimental data. The lack of fit F-value (0.61) also implied that the model provided a good fit to the data.

Yields of xylanase produced by *Aspergillus terreus* K1 are shown in Table 2. The most xylanase (339.80 U/g) was produced when the fungus was cultured at 30 °C, initial moisture of 70%, pH 4.5, and inoculum size of 6% (run 11), whereas the minimum xylanase activity (101.50 U/g) was produced when the fermentation process was conducted at incubation temperature, moisture, pH, and inoculum of 30 °C, 70%, 7.5, and 12%, respectively (run 27). By applying multiple regression analysis to the test results, the second-order polynomial equation representing xylanase production was obtained (Table 5). Using the Design-Expert software, the optimal conditions for xylanase production were predicted to be 29.3 °C, 69.6% moisture, pH 4.6, and 7.7% inoculum, with a yield of 339.68 U/g xylanase, which was close to the actual xylanase activity of 343.07 U/g.

Table 7. Analysis of Variance (ANOVA) Table for Xylanase Production

Source	Sum of squares	Df	Mean square	F-value	Prob > F
Model	76371.82	14	5455.13	44.85	< 0.0001
X_1- temperature	4155.09	1	4155.09	34.16	< 0.0001
X_2- moisture	3559.38	1	3559.38	29.26	0.0002
X_3- pH	17455.90	1	17455.90	143.51	< 0.0001
X_4- inoculum	10989.40	1	10989.40	90.35	< 0.0001
X_1X_2	21884.58	1	21884.58	179.92	< 0.0001
X_1X_3	3903.29	1	3903.29	32.09	0.0001
X_1X_4	9179.71	1	9179.71	75.47	< 0.0001
X_2X_3	5138.91	1	5138.91	42.25	< 0.0001
X_2X_4	94.97	1	94.97	0.78	0.3943
X_3X_4	3001.72	1	3001.72	24.68	0.0003
Residual	1459.61	12	121.63		
Lack of fit	670.80	7	95.83	0.61	0.7354
Pure error	788.81	5	157.76		
Cor. Total	77831.42	26			
Std. dev.	11.03		R^2	0.9812	
C.V. %	5.03		Adjusted R^2	0.9594	
			Predicted R^2	0.8944	

Optimization of multi-enzyme production

Co-production of endoglucanase, mannanase, and xylanase can be found in many enzyme production systems when microorganisms are grown in agro-wastes. Nevertheless, the efficient production of this enzyme mixture is dependent on various factors such as temperature, moisture, pH, and inocula (Abdel-Sater and El-Said 2001; Facchini *et al.* 2011). Thus, it is important to optimize the production system to enhance the enzyme production capability of the isolated fungal strain. The results of this study showed that each individual enzyme is typically produced under a different set of conditions. To find the best environment for the co-production of these enzymes (with an emphasis on mannanase production), optimization was carried out using Design-Expert software, and the activities of all three enzymes were used as responses. It was predicted that by incubating PKE at 30.5 °C, 62.7% moisture, pH 5.8, and 6% *A. terreus* K1 spores (1.0×10^{-7} spores/mL), maximum endoglucanase, mannanase, and xylanase could be obtained (17.37, 41.24, and 265.57 U/g DM, respectively). Verification of this predicted condition was conducted in triplicate, and the enzyme activities obtained (19.97, 44.12, and 262.01 U/g DM, respectively) were close to the predicted values.

It has been reported that enzyme production is subject to induction or catabolic repression (Abdel-Sater and El-Said 2001). Because PKE constitutes mainly mannan polymers, mannanase was expected to be the major enzyme produced (Lee 2007). However, our results showed otherwise. The higher xylanase activity obtained in this study could have been induced by both xylan and cellulose present in the PKE (Biely 1985; Royer and Nakas 1989) or through induction by the end-products of mannanase during fermentation of PKE, which will release glucose, mannose, or xylose (Sachslehner *et al.* 1998). Alternatively, the production of mannanase is growth-dependent, and it is an induced enzyme (Feng *et al.* 2003). That is, in the presence of an appropriate inducer, mannanase will be produced, but once depleted or when the cell is in the stationary phase, production will cease immediately.

Temperature is one of the main factors affecting the growth of fungi, although these microorganisms have been shown to be able to tolerate a wide range of temperatures, typically from 30 to 40 °C, and some are able to survive in extreme temperatures (≥ 50 °C) (Michael 1972; Smith and Wood 1991; Lin and Chen 2004; Wang *et al.* 2006; Chellapandi and Jani 2009; Sohail *et al.* 2009; Facchini *et al.* 2011). Despite the wide temperature tolerance, the optimum temperature predicted was 30 °C, a temperature that is close to the natural habitat where this fungus was isolated. Similar to the production of endoglucanase, the production of mannanase was more affected by changes in temperature (a temperature above 32 °C marked a decrease in mannanase productivity). It has been proposed that the mRNA involved in mannanase synthesis is only stable within a certain temperature range. Thus, a decrease in temperature will gradually stabilize and prolong the production of this enzyme, but production will cease with further drops in temperature due to decreased biochemical processes (Feng *et al.* 2003).

Unlike mannanase and endoglucanase production, xylanase production was more significantly affected by changes in pH (as shown by the larger value of coefficient estimation, X4). Based on the time course of enzyme production (Lee 2007), it was observed that the production of mannanase occurred during the fungal growth stage, whereas the optimum production of endoglucanase and xylanase occurred later. During the growth of fungi, the pH will initially decrease and then increases slightly with incubation time due to the accumulation of organic acid and soluble sugars (Kurakake and Komaki 2001). Thus, too low of an initial pH will affect the production of mannanase, but the reverse will lead to an environment that is too alkaline and might deter subsequent endoglucanase or xylanase production due to the increase in pH along incubation period.

In this study, a local fungus isolated from PKE was used for the production of multiple enzymes using SSF. Though several fungal strains have been isolated and characterized for the production of enzyme (Abdeshahian *et al.,* 2010; Abdeshahian *et al.*, 2011; Coman and Bahrim, 2011), study on the co-production of enzymes specifically for hydrolysis of PKE using PKE as the sole substrate is limited. Through optimization, 19.97, 44.12, and 262.01 U/g of endoglucanase, mannanase, and xylanase activity were obtained. These values are higher than those (1.20, 24.0, and 1.90 U/g of endoglucanase, mannanase and xylanase activity, respectively) reported by Lee (2007) using *A. wentii TISTR 3075* to ferment Palm Kernel Meal (a by-product of palm kernel oil extraction using solvent extraction method). Nevertheless, direct comparison of enzyme activities among studies is not always possible due to the lack of standardized enzyme assay conditions and variation in source of substrate used as shown in the Table 8.

Table 8. Enzyme Production by Different Fungi in SSF

Microorganism	Carbon source	Enzyme activity (U/g DM)			References
		CMCase	Mannanase	Xylanase	
A. Terreus K1	PKE	19.97	44.12	262.01	This study
A. wentii TISTR 3075	PKM	1.20	24.00	1.90	Lee (2007)
A. niger	PKC		1781.00		Ong *et al.* (2004)
A. niger USM F4	PKC		918.00		Rashid *et al.* (2012)

CONCLUSIONS

1. Production of multi-enzymes (endoglucanase, mannanase, and xylanase) by locally isolated *A. terreus* K1 using PKE as the sole substrate was successfully optimized. It was demonstrated that temperature, moisture, pH, and inoculum affect the production of each enzyme. Thus, by controlling these variables at an optimal level, the enzyme yield can be increased
2. Results of the study provide a viable option for production of an economical enzyme mixture using indigenous fungi isolated from the target substrate (PKE). The enzyme mixture, specifically designed to digest PKE, can be used to enhance the digestibility of PKE to be used more efficiently in feed for poultry and other monogastric animals.

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