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SHORT COMMUNICATION

Utilization of palm kernel cake for production of β-mannanase by *Aspergillus niger* FTCC 5003 in solid substrate fermentation using an aerated column bioreactor

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Abstract The production of β-mannanase from palm kernel cake (PKC) as a substrate in solid substrate fermentation (SSF) was studied using a laboratory column bioreactor. The simultaneous effects of three independent variables, namely incubation temperature, initial moisture content of substrate and airflow rate, on β-mannanase production were evaluated by response surface methodology (RSM) on the basis of a central composite face-centered (CCF) design. Eighteen trials were conducted in which *Aspergillus niger* FTCC 5003 was cultivated on PKC in an aerated column bioreactor for seven days under SSF process. The highest level of β-mannanase (2117.89 U/g) was obtained when SSF process was performed at incubation temperature, initial moisture level and aeration rate of 32.5°C, 60% and 0.5 l/min, respectively. Statistical analysis revealed that the quadratic terms of incubation temperature and initial moisture content had significant effects on the production of β-mannanase ($P < 0.01$). A similar analysis also demonstrated that the linear effect of initial moisture level and an interaction effect between the initial moisture content and aeration rate significantly influenced the production of β-mannanase ($P < 0.01$). The statistical model suggested that the optimal conditions for attaining the highest level of β-mannanase were incubation temperature of 32°C, initial moisture level of 59% and aeration rate of 0.5 l/min. A β-mannanase yield of 2231.26 U/g was obtained when SSF process was carried out under the optimal conditions described above.

Keywords Palm kernel cake · β-Mannanase · *Aspergillus niger* · Solid substrate fermentation · Column bioreactor

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Introduction

Palm kernel cake (PKC) is a tropical agro-industrial by-product of the palm oil industry. The fruit of the palm tree *Elaeis guineensis* contains kernels that are processed for the extraction of oil [11]. PKC is the solid residue left after the oil extraction process. Generally, PKC contains 12–18% crude fiber and 15–18% crude protein. Thus, PKC is largely used as a nutritive ingredient in feed for farm animals [3]. The dry weight of most palm seeds mostly derives from mannans, which are important compounds of hemicellulosic substances [2]. Dusterhoft et al. [7] reported that PKC contains a large number of non-starch polysaccharide components that consist of 78% mannans.

Endo-1, 4-β-D-mannanase (EC. 3.2.1.78) is a hemicellulytic enzyme that randomly hydrolyzes 1, 4-β-D-manno-pyranosyl linkages within the main chains of mannans and heteropolysaccharides such as galactomannans, glucomannans and galactoglucomannans [1]. The mano-oligosaccharides released are further hydrolyzed by β-D-mannosidase, β-D-glucosidase and α-D-galactosidase to produce mannose, glucose and galactose [4].

A variety of fungi are capable of producing mannan-degrading enzymes. *Aspergillus* species are well known for their high potency in the production of a wide range of microbial enzymes [8]. Among different *Aspergillus* species, *Aspergillus niger* has been shown to produce highly active β-mannanase in culture [1, 32].

A large number of agro-industrial residues have already been utilized in fermentative processes to produce value-added products. Among these processes, solid substrate fermentation (SSF) has been found to be an appropriate method for the utilization of agro-industrial by-products in the production of microbial enzymes [26]. The bioprocessing of agro-industrial residues by SSF provides some economic and engineering advantages over submerged fermentation (SmF). These include higher productivity per unit volume, lower capital investment, and lower downstream processing costs [21]. Environmental factors such as temperature, the moisture content of the substrate and airflow significantly affect SSF process, which in turn influences the fermentation products [25].

Although several studies have been carried out on the use of hemicellulosic compounds in the production of β-mannanase via SmF process [1, 14, 28, 29], few attempts have been made to produce β-mannanase using agro-industrial carbon sources in the SSF process [13]. On the other hand, most studies on β-mannanase production by fungi under SSF have focused on the production of enzyme in shake flasks, and there has been much less work on the use of bioreactors for the production of β-mannanase in SSF [18]. To the best of the authors' knowledge, no data on β-mannanase production by *Aspergillus niger* in column bioreactors using PKC as the sole carbon source under SSF have been published. The current study reports on the biodegradation of PKC as an inexpensive lignocellulosic substance for the production of β-mannanase by *Aspergillus niger* in SSF process using a laboratory column bioreactor. In this work, response surface methodology (RSM) as a statistical method was applied to study the combined effects of three independent variables, including incubation temperature, the initial moisture content of the substrate and the airflow rate on β-mannanase production, and to determine the optimum levels of these variables in order to achieve the maximum yield of β-mannanase.

Materials and methods

Microorganism

Aspergillus niger FTCC 5003, obtained from the culture collection of the Livestock Research Center, Malaysian Agriculture Research and Development Institute (MARDI), was used in this study. The stock culture was grown on a potato dextrose agar (PDA) slant at 30°C for seven days. A spore suspension was prepared by adding 50 ml of sterile Tween 80 (0.1%) to each slant culture. The spore suspension obtained was kept at 4°C.

Substrate and medium

Palm kernel cake was supplied by MARDI and used as a solid substrate. PKC was ground to a particle size of 2 mm and dried in an oven at 60°C for 48 h. Mandels' medium was used to moisten PKC [31]. The composition of the Mandels' medium was as follows (in g/l): $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4{\cdot}7H_2O$, 0.3; $MnSO_4{\cdot}H_2O$, 0.0016; $FeSO_4{\cdot}7H_2O$, 0.005; $ZnSO_4{\cdot}7H_2O$, 0.0014; $CoCl_2$, 0.002; protease peptone, 0.75; urea, 0.3; and Tween 80, 1.0. The initial pH of the Mandels' medium was adjusted to 5.0 with the addition of 1.0 M HCl or 1.0 M NaOH.

Experimental design and statistical model

A 2^3 full factorial, central composite face-centered (CCF) design for three independent variables, each at three levels with six star points and four replicates at the central point, was used to fit a second-order polynomial model in which a total of 18 trials were performed [10]. The experimental variables selected-incubation temperature, initial moisture content and aeration rate—were coded as X_1, X_2 and X_3, respectively. The behavior of the fitted model is defined as follows:

$$Y = a_o + a_1 X_1 + a_2 X_2 + a_3 X_3 + a_{11} X_1^2 + a_{22} X_2^2 + a_{33} X_3^2 + a_{12} X_1 X_2 + a_{13} X_1 X_3 + a_{23} X_2 X_3 \quad (1)$$

where Y is the measured response (U/g), a_o represents the intercept, a_1, a_2 and a_3 are linear coefficients, a_{11}, a_{22} and a_{33} are squared coefficients, and a_{12}, a_{13} and a_{23} are

Table 1 Independent variables and levels used in the central composite face-centered design

		Level					
		Actual range			Coded value		
Variable	Symbol	Low	Middle	High	Low	Middle	High
Incubation temperature (°C)	X_1	25	32.5	40	−1	0	+1
Initial moisture content (%)	X_2	40	60	80	−1	0	+1
Aeration rate (l/min)	X_3	0.5	1.5	2.5	−1	0	+1

interaction coefficients. The test variables were coded at three levels of −1, 0 and +1, which represent low, middle and high variable levels, respectively. The coded values and the actual levels of the variables are given in Table 1. The design matrix for the experiments is shown in Table 2. Design-Expert software (version 6.0.6 Stat-Ease, Inc.) was used for regression analysis of data and to graphically design the response surface.

Solid substrate fermentation

A glass column bioreactor that consisted of a jacketed vessel with a height of 50 cm and an inner diameter of 16 cm was loaded with 100 g of PKC (Fig. 1). The initial moisture of PKC was adjusted by the addition of Mandels' medium. The bioreactor was sterilized at 121°C for 30 min. After cooling, PKC was inoculated with 1.0 ml of spore suspension containing 10^6 spores/g dry substrate (PKC) and incubated for seven days. Incubation temperature was controlled by circulating water through the jacket of the bioreactor. The temperature of the bioreactor bed was measured using a probe inserted up to the axis of the bioreactor. Humid air was forced through the culture bed to achieve both heat removal and oxygen supply. For each trial, incubation temperature, initial moisture content and aeration rate were adjusted according to levels determined by the experimental design (Table 2).

Enzyme extraction

Enzyme was extracted from the fermented PKC by adding 100 ml of 0.05 M sodium citrate buffer (pH 5.0) to each 10 g of PKC and shaking (170 rpm) for 24 h at 4°C. The suspended materials and the fungal biomass were then separated by filter paper (Whatman No. 1) at the temperature of 4°C. The clarified extract was used as the enzyme source.

Analytical methods

β-mannanase activity was determined by mixing 0.2 ml of an appropriately diluted enzyme sample with 1.8 ml of 0.5% (w/v) locust bean gum (Sigma G-0753) in 0.05 M sodium citrate buffer (pH 5.3) at 50°C for 20 min [30]. The reducing sugar released (as mannose) was determined by the dinitrosalicylic acid (DNS) method [20]. The activities of α-galactosidase and β-mannosidase were measured using ρ-nitrophenyl-α-D-galactopyranoside and ρ-nitrophenyl-β-D-mannopyranoside, respectively, as described by Rättö and Poutanen [29]. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1.0 µmol of product per minute under the assay conditions. The activity of the enzymes produced was expressed in units per gram of dry PKC (U/g). The protein content of the culture extract was measured using bovine serum albumin (Fluka 05488) by the Lowry method [16].

Table 2 Central composite face-centered design and experimental results for the production of mannan-hydrolyzing enzymes by *Aspergillus niger* FTCC 5003 using palm kernel cake in SSF process for seven days

Run	X_1[a]	X_2	X_3	α-Galactosidase (U/g)[b]	β-Mannosidase (U/g)	β-Mannanase (U/g)	Specific activity of β-Mannanase (U/mg)[c]
1	0	0	+1	152.06	9.53	1659.18	26.45
2	0	0	0	204.24	12.82	1985.89	24.31
3	+1	0	0	80.98	5.977	1228.15	16.16
4	−1	+1	−1	7.78	0.69	43.60	2.03
5	−1	−1	+1	34.06	4.06	283.67	6.68
6	−1	−1	−1	37.96	4.70	433.75	11.01
7	−1	+1	+1	13.64	1.20	169.18	5.07
8	0	0	0	213.83	13.18	1800.95	22.04
9	+1	+1	−1	4.05	0.16	60.47	2.48
10	0	0	0	205.35	11.90	1874.77	24.07
11	0	0	0	191.75	9.55	1891.36	24.89
12	0	−1	0	27.37	2.31	481.13	12.70
13	+1	−1	−1	17.42	1.36	326.51	8.13
14	+1	+1	+1	17.12	1.58	177.67	4.78
15	0	0	−1	204.57	12.26	2117.89	27.19
16	+1	−1	+1	7.63	0.88	110.13	3.12
17	0	+1	0	41.33	3.59	470.82	10.18
18	−1	0	0	107.79	7.11	1382.91	19.15

[a] X_1, incubation temperature (°C); X_2, moisture level (%); X_3, airflow rate (l/min)

[b] Unit of enzyme activity per g of dry PKC

[c] Unit of enzyme activity per mg of protein released

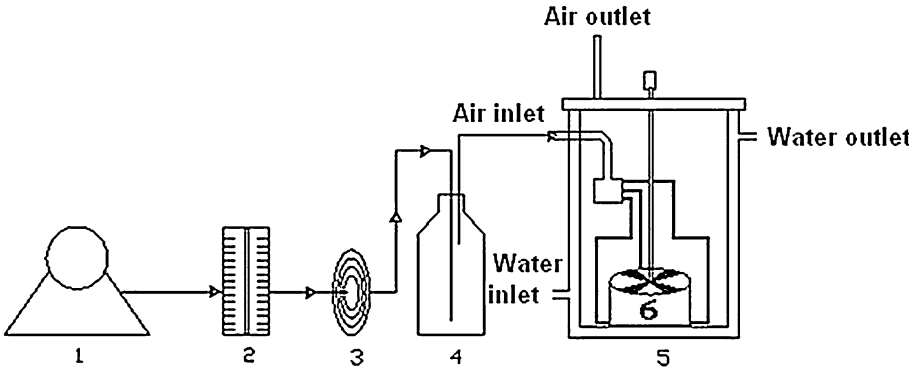

Fig. 1 Column bioreactor apparatus. *1*, Air pump; *2*, flow meter; *3*, air filter; *4*, air humidifier; *5*, bioreactor; *6*, container. The temperature of the bed was continuously controlled via a probe connected to a thermocouple. Forced air was provided by the peristaltic pump and regulated by opening the inlet air valve at a rate determined using a flow meter

Results and discussion

Production of β-mannanase in the bioreactor

Table 2 shows the yields of mannan-hydrolyzing enzymes produced by *Aspergillus niger* FTCC 5003. As can be seen, the highest β-mannanase production, with activity as high as 2117.89 U/g, was produced when *Aspergillus niger* FTCC 5003 was grown at an incubation temperature of 32.5°C, an initial moisture of 60% and an aeration rate of 0.5 l/min (Run 15). As shown in Table 2, the minimum β-mannanase activity (43.60 U/g) was produced when the fermentation process was conducted at incubation temperature, moisture content and airflow rate of 25°C, 80% and 0.5 l/min, respectively (Run 4). By applying multiple regression analysis to the test results, the following second-order polynomial equation (Eq. 2) was obtained in order to represent β-mannanase production as a function of incubation temperature, initial moisture level and airflow rate:

$$Y = 43.65 - 0.98X_1 - 2.58X_2 - 0.49X_3 - 7.73X_1^2 - 22.03X_2^2 - 0.47X_3^2 + 1.33X_1X_2 - 0.56X_1X_3 + 2.94X_2X_3 \quad (2)$$

where Y is the β-mannanase activity (U/g) and X_1, X_2 and X_3 are the coded values of incubation temperature (°C), initial moisture content (%) and aeration rate (l/min), respectively. The statistical significance of the fitted model was evaluated using Fisher's statistical test for analysis of variance (ANOVA), which is essential for determining patterns of interaction between experimental variables (Table 3). As observed, the computed model's F-value of 102.09 with a probability value (Prob > F) of less than 0.0001 indicated that the selected quadratic regression model fitted well to the experimental data ($P < 0.01$). The lack of fit F-value (7.36) implied that the lack of fit was insignificant and hence the model provided a good fit to the data. The second-order incubation temperature (X_1^2) and the second-order initial moisture level (X_2^2) were found to exert significant effects on β-mannanase production ($P < 0.01$). The statistical results also revealed that the linear effect of the initial moisture content (X_2) and the interaction effect between moisture level and airflow rate (X_2 X_3) significantly influenced the production of β-mannanase ($P < 0.01$).

In order to visualize the simultaneous effects of the variables studied on β-mannanase production and to determine the optimum conditions, three-dimensional response surface graphs of the combined effects of variables were constructed by RSM (Figs. 2–4). By analyzing the plotted graphs using Design-Expert software, the optimum conditions for attaining the highest yield of β-mannanase were evaluated. The analytical results indicated that the optimum levels of incubation temperature, initial moisture of substrate and aeration rate were 32°C, 59% and 0.5 l/min, respectively. In order to verify the accuracy of the statistical model, *Aspergillus niger* FTCC 5003 was cultivated on 100 g of PKC under optimum conditions in the bioreactor for seven days. The test results revealed that an activity of 2231.26 U/g was produced. This finding demonstrates that *Aspergillus* β-mannanase induced by PKC was significantly enhanced compared to that induced by a mixture of coffee waste and wheat bran (50 U/g) in SSF process using a bioreactor [13]. The level of β-mannanase produced by *A. niger* in the current study was substantially higher than that obtained by *A. oryzae* (800 U/g) grown on PKC under SSF conditions [5]. The β-mannanase yield obtained in the present study was satisfactorily comparable to the β-mannanase activity reported (2252 U/g) by Ong et al. [24], who utilized PKC in the production of cellulase and hemicellulase by *A. niger* in shake flasks using SSF process. Similarly, β-mannanase produced by *Sclerotium rolfsii* in a bioreactor also compared favorably with that obtained using shake flask cultivation when cellulose was used as the sole carbon source [9].

Effect of incubation temperature

As shown in Figs. 2 and 4, increasing the incubation temperature from 25°C to the optimum temperature (32°C) increased the production of β-mannanase to peak levels.

Table 3 Analysis of variance for the quadratic model of β-mannanase production by *Aspergillus niger* FTCC 5003 grown on PKC in SSF for seven days

Source	Factor	Polynomial coefficients	Standard error	Sum of squares	*df*	Mean square	*F* value	Prob > *F*
Model				3487.96	9	387.55	102.09	<0.0001*
	Intercept	43.65	0.77					
	X_1	−0.98	0.62	9.51	1	9.51	2.51	0.1521
	X_2	−2.58	0.62	66.33	1	66.33	17.47	0.0031*
	X_3	−0.49	0.62	2.39	1	2.39	0.63	0.4503
	X_1^2	−7.73	1.18	161.90	1	161.90	42.65	0.0002*
	X_2^2	−22.03	1.18	1314.97	1	1314.97	346.39	<0.0001*
	X_3^2	−0.47	1.18	0.60	1	0.60	0.16	0.7022
	$X_1 X_2$	1.33	0.69	14.05	1	14.05	3.70	0.0906
	$X_1 X_3$	−0.56	0.69	2.47	1	2.47	0.65	0.4435
	$X_2 X_3$	2.94	0.69	69.13	1	69.13	18.21	0.0027*
Residual				30.37	8	3.80		
Lack of fit				28.08	5	5.62	7.36	0.0656
Pure Error				2.29	3	0.76		

* Statistically significant at 99% probability level

$R^2 = 0.9914$; Adj $R^2 = 0.9817$; SD (square root of residual mean square) = 1.95

X_1, incubation temperature (°C); X_2, moisture level (%); X_3, airflow rate (l/min). X_1^2, X_2^2 and X_3^2 are the quadratic terms; X_1X_2, X_1 X_3 and X_2 X_3 are the interaction terms

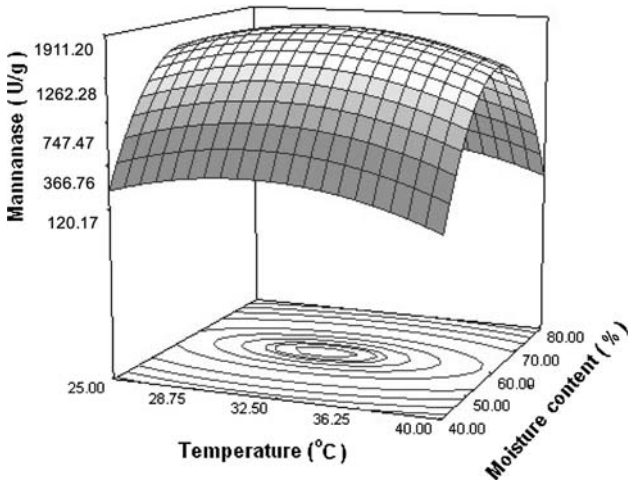

Fig. 2 Three-dimensional response surface of the combined effects of incubation temperature and moisture content on β-mannanase production by *Aspergillus niger* FTCC 5003 using palm kernel cake in SSF process for seven days

The results of this study were consistent with the findings obtained by Lin and Chen [14], who reported that peak β-mannanase activity was attained when *A. niger* was grown on β-mannanase inducer carbon sources at 30°C. In contrast, Kote et al. [12] have shown that the cultivation of *A. niger gr* at 40°C and *A. flavus gr* at 35°C in medium containing defatted copra leads to the production of maximum levels of β-mannanase in culture. As can be seen from Figs. 2 and 4, the yield of β-mannanase decreased when incubation temperature rose above the optimal level of 32°C. As reported elsewhere, reduced levels of cellulytic and hemicellulytic enzymes produced by *A.niger* were observed at temperatures higher than 32°C [27, 34].

Effect of initial moisture content

As shown in Figs. 2 and 3, increasing the initial moisture content from 40% to the optimum level of 59% led to enhanced production of β-mannanase. However, higher levels of moisture content (70–80%) reduced β-mannanase production. The adverse effect of excessive moisture levels may be attributed to the reduction of substrate porosity, lower heat and mass transmission through the culture and decreased gas exchange, all of which, in turn, result in decreased fungi growth and product formation [33]. Comparable results were obtained by Ong et al. [23], who reported that the cultivation of *A. niger* on PKC in a shake flask for ten days triggers the maximum production of β-mannanase (2281 U/g) at an initial moisture level of 60%. In contrast to this study, the growth and enzyme production of *A. niger* grown on water unextractable orange peels was enhanced when moisture level increased from 60 to 90% at an incubation temperature of 30°C under SSF [19].

Effect of aeration rate

The microbial activity of aerobic cultures is markedly affected by the air supply to the system. Durand et al. [6]

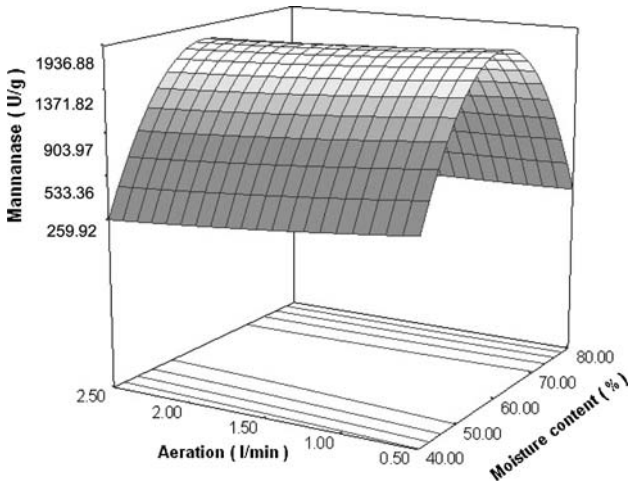

Fig. 3 Three-dimensional response surface of the combined effects of moisture content and aeration rate on β-mannanase production by *Aspergillus niger* FTCC 5003 using palm kernel cake in SSF process for seven days

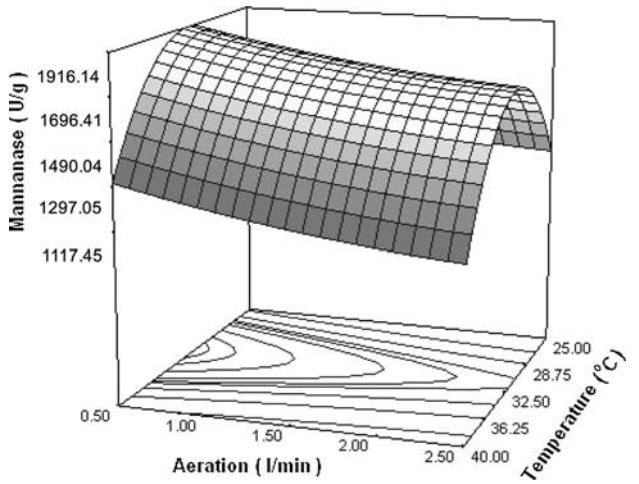

Fig. 4 Three-dimensional response surface of the combined effects of incubation temperature and aeration rate on β-mannanase production by *Aspergillus niger* FTCC 5003 using palm kernel cake in SSF process for seven days

mentioned different aspects of the aeration function for SSF, including maintenance of oxygen supply and removal of carbon dioxide from the system, as well as heat transfer and the control over moisture level. As shown in Figs. 3 and 4, increasing the airflow rate from 0.5 to 2.5 l/min led to a reduction in β-mannanase production. The deleterious effect of high aeration rate on β-mannanase biosynthesis was possibly due to the damaging effect of shear stress on the morphology of filamentous fungi and the decrease in substrate moisture content, which prevent microbial growth [15, 17, 22]. However, the three-dimensional response surface graphs plotted in Figs. 3 and 4 indicate that the decreases in β-mannanase yield were not considerable as airflow rate increased.

In conclusion, the findings of this research suggest that the bioprocessing of PKC in SSF using an aerated bioreactor results in the production of a fairly high levels of β-mannanase. This study demonstrates that incubation temperature, moisture content of substrate and airflow affect the production of β-mannanase. Hence, controlling the variables mentioned in optimal level enhances β-mannanase yield. Further research is recommended to investigate the biodegradation of PKC in a fed-batch system utilizing SSF process to further enhance the productivity of β-mannanase.

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