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Optimization of Temperature, Moisture Content and Inoculum Size in Solid State Fermentation to Enhance Mamanase Production by *Aspergillus terreus* SUK-1 using RSM

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Abstract: Optimization of three parameters, temperature (25-35°C), moisture content (40% (w/v)-60% (w/v) and inoculum sizes (5% (w/v)-15% (w/v) were investigated and optimized by Response Surface Methodology (RSM) for optimal mannanase production by *Aspergillus terreus* SUK-1. A second order polynomial equation was fitted and the optimum condition was established. The result showed that the moisture content was a critical factor in terms of its effect on mannanase. The optimum condition for mannanase production was predicted at 42.86% (w/v) initial moisture (31°C) temperature and 5.5% (w/v) inoculum size . The predicted optimal parameter were tested in the laboratory and the mannanase activity 45.12 IU mL^{-1} were recorded to be closed to the predicted value (44.80 IU mL^{-1}). Under the optimized SSF condition (31°C, 42.86% moisture content (w/v) and 5.5% inoculum size (w/v)), the maximum mannanase production was to prevail about 45.12 IU mL^{-1} compare to before optimized (30°C, 50% moisture content (w/v) and 10% inoculum size (w/v)) was only 34.42 IU mL^{-1}.

Key words: *Aspergillus terreus* SUK-1, solid state fermentation, mannanase, response surface methodology (RSM), palm kernel cake

INTRODUCTION

Agro-industrial waste represents abundant renewable carbon source generated years from agricultural sector (Francis *et al*., 2003). Palm Kernel Cake (PKC) is one such agro-industrial waste with good potential to be used widely as a major feed ingredient for chicken feeds (Marini *et al*., 2006). Since most of PKC consists of mannan that can be degrade, the PKC has gained interest as an inexpensive substrate for the production of mannanase enzyme especially in Solid Substrate Fermentation (SSF). SSF is defined as process performed in the absence or near-absence of free water, employing a natural substrate or an inert support (Sabu *et al*., 2006). SSF has many advantages to enhance microbial enzyme production since it is economical technology due to its low capital investment and low operating expenses (Latifian *et al*., 2007). Selection of process parameters and their optimization are other key aspects of SSF including parameters such as moisture content, incubation temperature and age and size of inoculum (Manpreet *et al*., 2005).

Mannan degrading enzyme have been reported to be produced by several filamentous fungi such as *Aspergillus niger* (Abdeshahian *et al*., 2010), *Trichoderma reesei* (Atisan-Atac *et al*., 1993), *Trichoderma harzianum* (Torrie *et al*., 1990) and *Sclerotium rolfsii* (Gubitz *et al*., 1997). Filamentous fungi are commonly used in industrial enzyme since have ability to secrete large amount of protein into the growth medium. Meanwhile, *Aspergillus* species have been known as potential fungi in the production of a wide range of microbial enzyme (Gao *et al*., 2008).

Mannanase have been found practical in several industrial purpose including improving the quality of animal feed stuff, bioleaching of pulp in the paper industry, bioconversion of biomass wastes to fermentable sugar and reducing the viscosity of coffee extract (Wong and Saddler, 1993; Gubitz *et al*., 1997; Chandrakant and Bisaria, 1998; Hagglund *et al*., 2003). Furthermore, the mannooligosaccharides which derived from the hydrolysis of mannanase and mannan have been report to use as no nutritional food additives selective growth of human-beneficial intestinal micro flora, *bifidobacterium* species (Tomori, 1990).

Response Surface Methodology (RSM) is a collection of statistical techniques for experiment designing, model developing, factors evaluating and

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optimum conditions searching (Myers and Montgomery, 2002). RSM could overcome the shortcoming of the classical or empirical methods such as one-factor at-a-time-technique which is time-consuming process but also can led misinterpretation of results, especially because the interaction between different factors is overlooked (Lotfy *et al*., 2007). The aim of this study was to evaluate the effects of moisture content, temperature and inoculum size on mannanase production by our locally strain, *Aspergillus terreus* SUK-1 using RSM and to search for optimal condition to achieve a maximum mannanase production.

MATERIALS AND METHODS

Microorganism: A local isolate, *Aspergillus terreus* SUK-1 from School of Biosciences and Biotechnology, Universiti Kebangsaan Malaysia, were grown and maintained on Potato Dextrose Agar (PDA). Spore suspension of 10^7 spore mL^{-1} was prepared by harvesting from 7 days old cultures of both molds with 15 mL sterile distilled water and 10% (w/v) of inoculum (of the tested ratios) was used in all experiments.

Substrate and medium: Palm kernel cake was supplied by MARDI and used as a solid substrate and PKC was ground to a particle size of 2 mm. The medium employed (Sternberg, 1976) are shown in Table 1.

Solid state fermentation: Fermentation was carried out in Erlenmeyer flask 250 mL containing 25 g of Palm Kernel Cake (PKC) and Sternberg's medium (Sternberg, 1976). The mixtures were autoclaved at 121°C for 15 min. Each flask was then inoculated spore suspension of *A. terreus* SUK-1. All flasks were incubated for 4 days. Duplicate flasks were set up under various experiments according the experimental design.

Crude enzyme extraction: Mannanase enzyme was extracted from fermented PKC by adding 100 mL of distilled water into the flask containing 10 g of PKC and agitated at 150 rpm for 24 h at 10°C. The suspended materials and fungal biomass were then separated by filtration using Whatman filter paper No. 1. The clarified extract was used as the source of mannanase enzyme.

Enzyme assay: Mannanase activity was carried out according to the method described by McCleary (1978) using Azo Carob Galactomannan as substrate. One unit (U) of mannanase activity was defines as mannose released/min/g of substrate.

Table 1: Fermentation medium for mannanase production

Medium	Concentration (g L^{-1})
$(NH_4)_2SO_4$	1.4
KH_2PO_4,	2
$CaCl_2$	0.3
$MgSO_4$	0.3
$FeSO_4$	0.05
$MnSO_4 7H_2O$	0.0016
$ZnSO_4$	0.0014
$CoCl_2$	0.002
Protease Peptone	0.75
Tween 80	1

Table 2: Range of variables and their coded levels

	Coded value		
Independent variables	-1	0	+1
(a) Temperature (°C)	25	30	35
(b) Moisture content (% w/v)	40	50	60
(c) Inoculum size (% w/v)	5	10	15

a: Temperature at low level (-1) of 25°C and high level (+1) of 35°C, b: Moisture content at low level (-1) of 40 (w/v) and high level (+1) of 60 (w/v), c: Inoculum size at low level (-1) of 5 (% w/v) and high level (+1) of 15 (w/v)

Experimental design: The optimization of mannanase production by *A. terreus* SUK-1 was carried out using Response Surface Methodology (RSM). The variables used were temperature (a), moisture content (b) and inoculum size (c) and the coded value of variables were -1,0,1 (low, basal low, high) (Table 2). Nineteen experiments were performed for each microorganism at three levels including five replicates at the center points with three variables according second order face-centered composite design (Table 3). Analyses were carried out in duplicate. The experimental data were employed in statistical package, software Design Expert 6.0 (StatEase Inc. Minneapolis, USA) to fit a second order polynomial response surface methodology according to Eq. 1.

$$Y = X0- X_1A-X_2B- X_3C-X_{11} A^2-X_{22} B^2 + X_{33}C^2-X_{12}AB + X_{13} AC-X_{23} BC \quad (1)$$

where, Y is the mannanase activity IU mL^{-1}, X_0, X_1,....., X_{23} represent the estimated regression coefficients, X_1, X_2, X_3 represent the linear effect, X_{11}, X_{22}, X_{33} the quadratic effect and X_{12}, X_{13}, X_{23} cross product coefficient A, B and C represent variables (temperature, moisture content, inoculum size).

RESULTS AND DISCUSSION

The experimental design and result of experiment are presented in Table 3. The result of the second ordered response surface model for mannanase production in the form of Analysis of Variance (ANOVA) was given in Table 4 and 5. The Analyzed of Variance (ANOVA) was carried by Fisher's F-test and F value is the ratio of the

Table 3: Experimental Central Composite Design (CCD) with experimental value and predicted value on mannanase production by *A. terreus* SUK-1

Experiments	Temperature (°C)	Moisture content (% (w/v))	Inoculum size in concentration 10^7 Spore mL^{-1} (% w/v)	Mannanase activity (IU mL^{-1}) Experimental value	Predicted value
1	25 (-1)	40 (-1)	5 (-1)	37.33	36.09
2	35 (+1)	40 (-1)	5 (-1)	36.28	36.02
3	25 (-1)	60 (+1)	5 (-1)	24.73	24.27
4	35 (+1)	60 (+1)	5 (-1)	16.23	15.08
5	25 (-1)	40 (-1)	15 (+1)	30.58	29.75
6	35 (+1)	40 (-1)	15 (+1)	36.48	34.96
7	25 (-1)	60 (+1)	15 (+1)	14.88	13.16
8	35 (+1)	60 (+1)	15 (+1)	9.96	9.24
9	25 (-1)	50 (0)	10 (0)	25.58	29.79
10	35 (+1)	50 (0)	10 (0)	24.15	27.80
11	30 (0)	40 (-1)	10 (0)	35.23	39.80
12	30 (0)	60 (+1)	5 (-1)	16.23	20.27
13	30 (0)	50 (0)	5 (-1)	42.38	45.45
14	30 (0)	50 (0)	15 (+1)	34.58	39.36
15	30 (0)	50 (0)	10 (0)	40.73	38.02
16	30 (0)	50 (0)	10 (0)	38.80	38.02
17	30 (0)	50 (0)	10 (0)	41.58	38.02
18	30 (0)	50 (0)	10 (0)	39.98	38.02
19	30 (0)	50 (0)	10 (0)	44.73	38.02

Table 4: Analysis of Variance (ANOVA) for regression model to optimize mannanase production

Source	SS	DF	MS	F-value	Prob>F
Model	1875.17	9	208.35	10.82	0.008
Residual	173.39	9	19.27		
Lack of fit	153.36	5	30.67	6.13	0.0517
Pure error	20.02	4	5.01		
Total	2048.56	18			

SS: Sum of squares, DF: Degree of freedom, MS: Mean squares, SV = 4.39 PRESS = 689.40

Table 5: Regression coefficient for optimization of mannanase production

Model term	Coefficient estimate	Standard error	F-value	Prob>F
Intercept	38.02	1.61	10.82	0.0008
a (Temperature)	-1.00	1.39	0.52	0.4906
b (Moisture content)	-9.39	1.39	45.72	<0.0001*
c (Inoculum size)	-3.05	1.39	4.81	0.0559
a^2	-9.22	2.66	12.06	0.0070*
b^2	-8.36	2.66	9.91	0.0118*
c^2	4.39	2.66	2.73	0.1327
ab	-2.28	1.55	2.16	0.1756
ac	1.32	1.55	0.72	0.4175
bc	-1.19	1.55	0.59	0.4614

*Significant (F<0.05)

mean square due to regression to the mean square due to error (Francis *et al*., 2003).

The model F-value of 10.82 implied that the model was significant and there was only a 0.08% chance that a "Model F-value" this large could occur due to noise. The pure error was very low (20.02), indicating good reproducibility of the experimental data. The p value was used as a tool to check significant of each coefficient which in turn may indicate the pattern of the interaction between the variables (Bahceci and Acar, 2007). The smaller the p-value, the more significant is the corresponding coefficient The value probability >F less than 0.05 indicated the model term were significant.

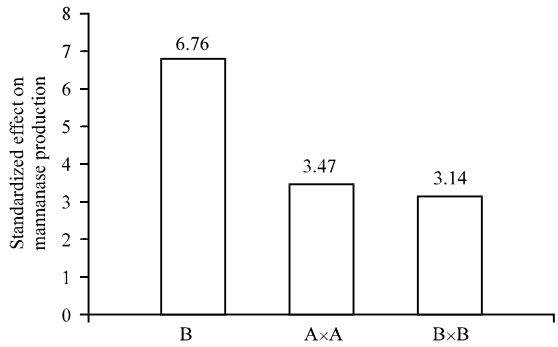

Fig. 1: Standard Pareto chart of the important of effect of variables (A: Temperature, B: Moisture content) for mannanase production by *A. terreus* SUK-1

The analyzed regression model as given in Table 5 suggested that the linear coefficient (B) and quadratic terms coefficient (A^2, B^2) were significant whereas the other terms coefficient included cross product coefficient (C, C^2, AB, AC, BC) were not significant. The significance of quadratic term of temperature (A^2) and moisture content (B^2) indicated that these factors can act as limiting factor and even small changes in value can be the cause of the big influence on mannanase production (Box *et al*., 1978).

The Importance of each effect can be determined by the size of the coefficient which have been standardized (each effect is divided by its standard error) (Fig. 1). The size of each coefficient gives a direct measurement of the important of each effect (Psomas and Kyriakides, 2007). Figure 1 clearly showed that the moisture content was the most influenced factor on mannanase production as the bar are displayed on the top correspond to the most important effect (show only model term significant).

However, there is no report available in the literature about the importance of effect moisture content on mannanase production by *A. terreus* SUK-1 but similar result was report by Latifian *et al*. (2007) that the moisture content was strongly sensitive for cellulase production by *Trichoderma reesei* mutant under SSF. The model second order polynomial equation fitted by regression analysis was:

$$Y = 38.02 - 1.00A - 9.39B - 3.05C - 9.22A^2 - 8.36B^2 + 4.39C^2 - 2.28AB + 1.32AC - 1.19BC$$

where, Y is the mannanase activity IU mL^{-1}, A is the temperature (°C), B is the moisture content (% (w/v)) and C is inoculum size% (w/v)).

The goodness of fit of the model can be checked by Lack of fit-test, Coefficient of determination R^2, Coefficient of Variance (CV), Prediction Residual Error Sum of Squares (PRESS) and adequate precession (Bahceci and Acar, 2007). The proposed model is adequate to described data when the lack of fit-test did not result in significant ($p<0.05$) (Table 3). The coefficient of determination, R^2 give the information how much of the variability in the observed data could be explained by the experiment and their interaction (Kawaguti *et al*., 2006). The Coefficient of determination, R^2 (0.9154) suggested that about 91.5% variability in the model could be explained and about 8.5% of the total variation cannot be explained by the model. The closer R^2 value to 1.0, the stronger the model to make a prediction to the response (Haaland, 1989). The good model exhibit low standard deviation, high value of R^2 and a low PRESS (Table 3). Adequate precision measures signal to noise ratio where it can compares the ranges of the predicted values at the design point to average prediction error. A ratio greater than 4 is desirable as it indicates adequate model discrimination. On this particular case, the value of 11.372 is well above 4 and the model can be used to navigate the design space.

Figure 2 shows the effect of temperature and moisture content at fixed inoculum size of 10% (w/v). An increased in production of mannanase was observed at the range moisture content of 40-50% and a temperature range of 31-28°C. Comparable results were obtained that at the 50% of moisture content, the maximum mannanase production was achieved optimization experiment by *Aspergillus niger* using PKC as a sole carbon sources (Ong *et al*., 2004). In contrast, Abdeshahian *et al*. (2010) have shown that the cultivation of *A. niger* using PKC as the sole carbon sources under SSF leads to the production of maximum levels of mannanase when moisture level increased from 40 to 60% and the level of mannanase production began to reduce when the higher level of

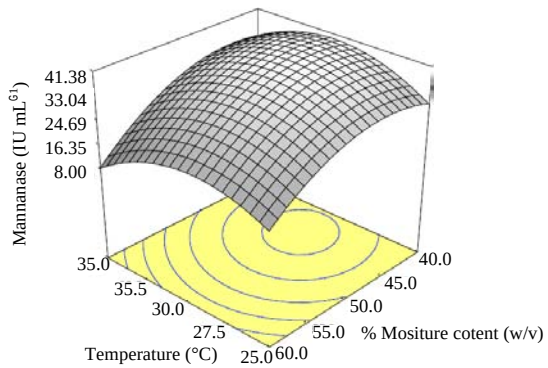

Fig. 2: The effects of temperature and moisture content on mannanase production by *A. terreus* SUK 1

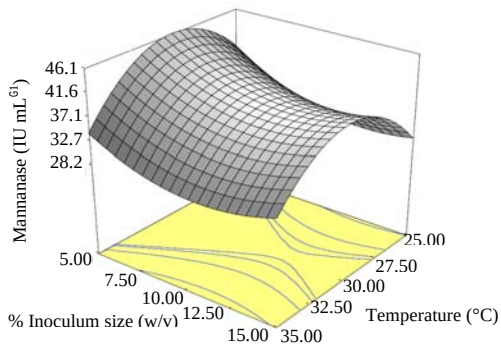

Fig. 3: The effects of inoculum size and temperature on mannanase production by *A. terreus* SUK-1

moisture content (70-80%) applied. Increasing the moisture content can leads to decrease the porosity of substrate thus limiting the oxygen transfer into the substrate all of which, in turn, result in decreases fungi growth and product formation. Meanwhile, a lower of moisture content can reduced the solubility of the nutrient of the substrate, lower degree of swelling and a higher water tension (Kheng and Omar, 2005).

Figure 3 shows the effects of inoculum size and temperature and at fixed moisture content of 50% (w/v) on mannanase production. The maximum mannanase production was obtained at the range of 29-31°C and out of from this range brings the negative effect on mannanase production. As it was reported the optimum of mannanase production under SSF was obtained at temperature of 30°C (Feng *et al*., 2003). The author reported that the mRNA of this enzyme is not stable and within a certain temperature range, an appropriate decrease in temperature would enhance the stability of the mRNA and prolong the duration of enzyme production.

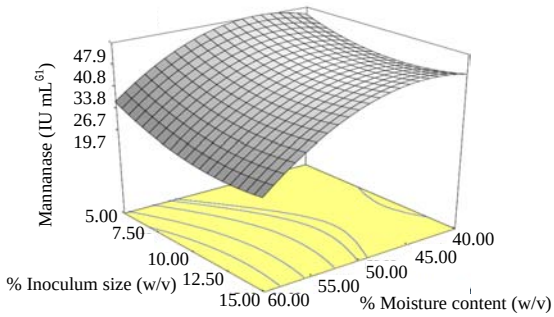

Fig. 4: The effects of inoculum size and moisture content on mannanase production by *A. terreus* SUK-1

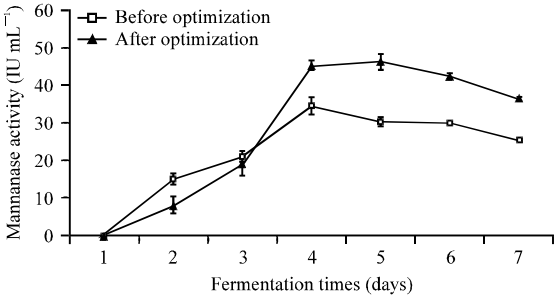

Fig. 5: Profiles of mannanase production under SSF before (30°C, 50% moisture content (w/v) and 10% inoculum size (w/v)) and after optimization ((31°C, 42.86% moisture content (w/v) and 5.5% inoculum size (w/v)) of cultural condition by RSM

This statement may be able to explain the phenomenon of inactivation mannanase enzyme out of temperature range in this study. The result of this study were consistent with the findings obtained by Abdeshahian *et al.* (2010) and Lin and Chen (2004) who reported that at 30°C leads to the production of maximum level of mannanase.

Figure 4 shows the effect of inoculum size and moisture content at fixed the temperature of 30°C on mannanase production. As shown in the Fig, the factor of moisture content had a great influenced on mannanase production than inoculum size which was not varies much can be observed on mannanase production whether increasing or decreasing the inoculum size. It can be concluded that the inoculum volume (5-15% (w/v)) of 10^7 spore mL^{-1} did not bring much significant increase in mannanase production. However, changing the inoculum concentration ranging from 1×10^4-1×10^8 spore mL^{-1} significantly influenced of mannanase production (Abd-Aziz *et al.*, 2003). Rashid *et al.* (2011) reported that the inoculum size of 1×10^7 spore mL^{-1} leads to the maximum mannanase yields by *A. niger* using PKC as the substrate under SSF. Different observation in the study of Abd-Aziz *et al.* (2003), who reported that the inoculum size of 1×10^4 was enough to enhance mannanase enzyme activity by *A. niger* under submerged fermentation. Therefore, a balance correlation between proliferating biomass and available materials are important in order to achieve maximum enzyme production (Rauf *et al.*, 2010). In the Fig. 2 and 3, even small changes value of moisture content triggers the maximum production of mannanase. The maximum of activity mannanase predicted from the model was 45.50 IU mL^{-1} under the optimal condition (31°C, 42.86% moisture content (w/v) and 5.5% inoculum size (w/v)).

To verify the predicted optimum of mannanase production, the experiment was repeated in triplicate for 96 h. The results showed that the mannanase activity was closed to the predicted value (45.50 IU mL^{-1}) to 44.80 IU mL^{-1} thus confirming the model validity. Figure 5 shows the profiles mannanase production by *A. terreus* SUK-1 before optimization and after optimization of condition by RSM. This Figure clearly shows that the optimization experiment by RSM method can improved the mannanase production by *A. terreus* SUK-1. Under the optimized condition, the highest activity mannanase enzyme was about 45.12 IU mL^{-1} than before under optimized was only about 34.42 IU mL^{-1}.

CONCLUSION

Using Response Surface Methodology (RSM), it was possible to know that the moisture content was the most influenced factor on mannanase production among the variables tested in this study. Furthermore, the optimal condition for mannanase production can be predicted by this method. By this method, the optimum of mannanase production was achieved about 44.80 IU mL^{-1} at the condition of, 31°C, 42.86% (w/v) moisture content and 5.5% (w/v) inoculum size thus confirming the model validity where the experiment data agreed fittingly at the predicted optimal condition. Under optimized condition, the maximum mannanase production was produced of 45.12 IU mL^{-1} than before optimized condition was 34 42 IU mL^{-1}.

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