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A N I N T E R N A T I O N A L J O U R N A L

Application of Response Surface Methodology for Optimizing Process Parameters in the Production of Amylase by *Aspergillus flavus*NSH9 under Solid State Fermentation

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ABSTRACT

Amylase is recognized as one of the important commercial enzymes. This group of enzymes has the ability in hydrolyzing starch into smaller oligosacharides. The present work aimed to determine the optimum fermentation conditions for maximum production of crude amylase enzyme by Aspergillus flavus *NSH9 employing response surface methodology (RSM).Central composite design (CCD) was applied to determine the optimal fermentation condition with respect to the four main process parameters such as temperature, initial moisture content, pH and the incubation period. Solid state fermentation (SSF) was performed using 5.0 g of sago hampas inoculated with $1x10^7$ sporesmL^{-1}following the experimental design obtained using CCD and further optimized by RSM. The initial moisture, pH and temperature showed significant effect on the amylase production ($p<0.05$). The maximum amylase activity produced was achieved and recorded as 1.055 ± 0.03U mL^{-1}after four days of fermentation period with 100% (v/v) moisture holding capacity, pH 6.5 and temperature at 28°C. The optimum fermentation conditions for amylase production was determined with* A. flavus*NSH9 on sago hampas.*

Key words: Solid state fermentation, *Aspergillus flavus*NSH9, *Sago hampas*, Central Composite Design, Response Surface Methodology,

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INTRODUCTION

Amylases are group of enzyme that act synergistically to break down the starch polysachharide (amylose and amylopectin) to glucose. There are four major groups of amylases according to van der Maarel et al, (2002) which were endoamylases, exoamylases, debranching enzyme and transferase. Amylase has been widely used in various industrial application, viz., detergent industry, alcohol production, food industry, textile industry and paper industry to name a few. Indutrially, amylase has been produced by submerged fermentation (SmF) system. However, in recent years, researchers have devoted some effort to use solid state fermentation (SSF) for enzymes production (Hölker et al. 2004; Souza and Magalhães, 2010). SSF can be simply defined as any fermentation process allowing the growth of microorganism on solid substrate, either on inert carriers or on an insoluble substrate that acts both physical support and source of nutrients in the absence of free flowing water (Souza and Magalhães et al. 2010). Besides, SSF possess several advantages over SmF, such as low production cost, higher product stability and lower demand of microbial contamination due to low humidity content in this system (Sun et al. 2007). Optimization processes with the one-variable-at-a-time method, in which keeping other variables at fixed levels is generally time consuming and requires number of experiments to be carried out (Hajji et al. 2008;Braga et al. 2011). Response surface methodology (RSM) is a collection of statistical and mathematical techniques that able to overcome this problem. It can be used to design the experiment and evaluate the interaction between variables and response. Inaddition, RSM is useful for optimization of the desirable response (Sharma et al. 2007;Kar et al. 2008;Shaktimay et al. 2010; Vishwanatha, et al. 2010). Therefore, the aim of this study was to evaluate the optimum fermentation process conditions for crude amylase production employing RSM by *A. flavus* NSH9 on sago *hampas*.

MATERIALS AND METHODS

Microorganism

Aspergillus flavus NSH9 was grown on Malt Extract Agar (MEA) for 7 days. The inocula of spores ($1x10^7 sporesmL^{-1}$) were prepared by flooding with 10 mL of steriled distilled water containing 0.1% (v/v) of Tween 80. Spores were scrapped off using sterile spatula and counted using microscopic under Neubauer Chamber (Rangarajan et al. 2010).

Solid state fermentation (SSF)

Solid state fermentation (SSF) was conducted using five grams of sago *hampas* inoculated with $1x10^7 sporesmL^{-1}$ of 7 days old *Aspergillus flavus* NSH9 culture. The initial moisture content was set based on the moisture holding capacity of the substrate, the sago *hampas*. Moisture holding capacity of sago *hampas* was measured using method as described by Trautmann andKrasny (1997) and Shaktimay et al. (2010). The amount of water retained was recorded and considered as 100% moisture holding capacity. The moisture holding capacity of sago hampas was calculated using the formula (1) as follows:

Water retained (mL) = Water added (mL) – Water drained (1)

The moisture holding capacity was adjusted using culture medium consisting of of the following in gram per liter: K_2HPO_4, 5; $MgSO_4$, 1; $CaCl_2.2H_2O$, 0.05; NaCl, 0.5; $(NH_4)_2SO_4$, 1.5; peptone, 5; yeast extract, 4.The fermentation was carried out under static condition.

Enzyme extraction and assay

The crude amylase was harvested using 50 mM sodium acetate buffer, pH 5 with shaking for 30 minutes at agitation speed of 130 rpm. The clear supernatant was collected and used as the crude amylase enzyme preparation. The amylase assay was performed by mixing 0.5 mL of the crude enzyme preparation with 0.5 mL of 1% (w/v) of soluble starch in 50 mM sodium acetate buffer, pH 5. The reaction mixture was incubated at 37°C for 10 minutes. The reducing sugars released were determined by the standard dinitrosalicylic acid method (Miller, 1959).

Experimental design

The central composite design (CCD) was applied to determine the optimum physical parameter of SSF for crude amylase production with respect to four variables. The four variables were incubation period, moisture holding capacity, pH and temperature. Each factor in the design was studied at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$), shown in

Table 1. A full factorial of CCD design consists of 16 factorial points, 8 axial points and 6 replicates of the central point. The coded values for independent variable were -1 (lowest level), 0 (middle level) and +1 (highest level). The factors (-α, +α) that ran along the axes drawn from the middle of the cube through the center of each face cube is coded as -1.682 and +1.682, respectively. CCD was employed in order to represent response surfaces or well known as regression equation. In general, the regression equations that involve several factors are expressed as equation (2) as follows:

$$y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j=2}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where y is the measured response (amylase activity); β_o, β_i, β_{ii} and β_{ij} are intercept term, linear, squared effect and interaction effect, respectively; x_i is the coded for independent variables (i =1,2,3…k) and ε is the error (Myers et al. 2009).Design expert software (Version 7.0, Stat-Ease, Inc) was used for regression and graphical analysis of the data obtained.

Table 1- Range of the values for central composite design (CCD)

Variables	Ranges and levels				
	-α	-1	0	+1	+ α
Incubation period (Days)	0	2	4	6	8
Moisture holding capacity (MHC) [% (v/v)]	25	40	55	70	85
pH	3.5	4.5	5.5	6.5	7.5
Temperature (°C)	28	30	33	35	38

RESULTS AND DISCUSSION

Solid state fermentation and experimental design

The optimization of solid state fermentation condition for crude amylase production by *Aspergillus flavus* NSH9 was successfully conducted in duplicate at laboratory experiments. Table 2 presents the experimental design of full factorial model (CCD) with the correspondent response (amylase activity). Based on the results, second order polynomial equation obtained as outlined in equation (3) as follows:

$$y = + 0.37 + 0.0003\ A + 0.033\ B + 0.037C - 0.045D + 0.040\ AD - 0.052A^2 \quad (3)$$

where y is the response variable which is amylase activity, A is the code for incubation period, B is for moisture holding capacity (MHC), C is for pH and D is for temperature.

The model F-value of 8.07 means that the model is significant and the Value of "Prob > F" less than 0.05 indicated that the model term also significant. There is only a 0.01% chance that the F-value large due to noise. The *lack-of-fit* F-value of 2.86 implies that the *lack-of-fit* is not significant and only 12.42% chances that this large occur due to noise. In this study, independent variables of B, C, D, AD and A^2 were significant model term at the probability level of 95%. Full model of CCD presented as 0.6779 explained that only 68% of the variability in the response. However, the predicted R^2 value (0.2430) is not close to the adjusted R^2 (0.5939) which may indicate a possible problem with the model's collection due to large block effect. Adequate precision which is used to measure the signal to noise ratio was tested. A ratio greater than 4 is desirable. Ratio of 9.840 was recorded and these indicate an adequate signal. Table 3 shows the summarized analysis of variance (ANOVA) for surface quadratic model.Coefficient of variation (CV) (standard deviation/error) of 20.19% for each dependent variable showed that the statistical quality of the experimental results was acceptable prior to evaluation of the model. Prediction Error Sum of Squares (PRESS) is another statistical test that measures the adequacy of the model to predict the response (Granato et al. 2011). Small values of PRESS are desirable. In this case, the PRESS value of 0.24 was recorded.

Table 2 - Experimental design and result of full model CCD

Standard Order	A: Incubation period (days)	B: MHC (v/v %)	C : pH	D: Temperature (°C)	Amylase activity (UmL^{-1})	
					Actual	Predicted
1	2 (-1)	40 (-1)	4.5 (-1)	30 (-1)	0.486	0.52
2	6 (+1)	40 (-1)	4.5 (-1)	30 (-1)	0.405	0.39
3	2 (-1)	70 (+1)	4.5 (-1)	30 (-1)	0.689	0.63
4	6 (+1)	70 (+1)	4.5 (-1)	30 (-1)	0.302	0.39
5	2 (-1)	40 (-1)	6.5 (+1)	30 (-1)	0.621	0.50
6	6 (+1)	40 (-1)	6.5 (+1)	30 (-1)	0.417	0.52
7	2 (-1)	70 (+1)	6.5 (+1)	30 (-1)	0.603	0.67
8	6 (+1)	70 (+1)	6.5 (+1)	30 (-1)	0.547	0.59
9	2 (-1)	40 (-1)	4.5 (-1)	35 (+1)	0.331	0.24
10	6 (+1)	40 (-1)	4.5 (-1)	35 (+1)	0.379	0.34
11	2 (-1)	70 (+1)	4.5 (-1)	35 (+1)	0.436	0.36
12	6 (+1)	70 (+1)	4.5 (-1)	35 (+1)	0.284	0.36
13	2 (-1)	40 (-1)	6.5 (+1)	35 (+1)	0.306	0.26
14	6 (+1)	40 (-1)	6.5 (+1)	35 (+1)	0.501	0.52
15	2 (-1)	70 (+1)	6.5 (+1)	35 (+1)	0.476	0.45
16	6 (+1)	70 (+1)	6.5 (+1)	35 (+1)	0.606	0.60
17	0 (-α)	55 (0)	5.5 (0)	33 (0)	0.090	0.24
18	8 (+α)	55 (0)	5.5 (0)	33 (0)	0.401	0.26
19	4 (0)	25 (-α)	5.5 (0)	33 (0)	0.358	0.43
20	4 (0)	85 (+α)	5.5 (0)	33 (0)	0.687	0.63
21	4 (0)	55 (0)	3.5 (-α)	33 (0)	0.328	0.36
22	4 (0)	55 (0)	7.5 (+α)	33 (0)	0.604	0.58
23	4 (0)	55 (0)	5.5 (0)	28 (-α)	0.789	0.71
24	4 (0)	55 (0)	5.5 (0)	38 (+α)	0.358	0.45
25	4 (0)	55 (0)	5.5 (0)	33 (0)	0.650	0.58
26	4 (0)	55 (0)	5.5 (0)	33 (0)	0.478	0.58
27	4 (0)	55 (0)	5.5 (0)	33 (0)	0.611	0.58
28	4 (0)	55 (0)	5.5 (0)	33 (0)	0.598	0.58
29	4 (0)	55 (0)	5.5 0)	33 (0)	0.594	0.58
30	4 (0)	55(0)	5.5 (0)	33 (0)	0.527	0.58

Table 3 -Analysis of variance for response sufrace quadratic model on the optimization of amylase production.

Source	Sum of squares	DF	Mean square	F value	p-value (Prob>F)	Remark
Model	0.21	6	0.035	8.07	<0.0001	Significant
A	0.0002	1	0.0002	0.058	0.8126	Not significant
B	0.025	1	0.025	5.82	0.0242	Significant
C	0.033	1	0.033	7.55	0.0115	Significant
D	0.049	1	0.049	11.31	0.0027	Significant
AD	0.026	1	0.026	5.86	0.0238	Significant
A^2	0.078	1	0.078	17.81	0.0003	Significant
Residual	0.10	23	0.0004			

Lack of fit	0.092	18	0.0005	2.86	0.1242	Not significant
Pure error	0.035	5	0.0001			
Corrected Total	1.39	29				

DF, degree of freedom; R^2 = 0.6779; predicted R^2 = 0.2430; adjusted R^2 = 0.5939

Reduced model and statistical analysis

A reduced model was proposed by eliminating the insignificant model terms. From the analysis conducted in full model, three variables which are moisture holding capacity, pH and temperature are significant model terms and therefore chosen as independent variables for the reduced experimental matrix. A new range and level of the variables were studied in order to further determine the maximum amylase production at different variable levels. The ranges of variable levels were set up based on the maximum production of amylase recorded in the full model. The ranges of values used were shown in Table 4 while the experimental design and amylase activity for reduced model was presented in Table 5.

Table 4- Range of the values for reduced model

Variables	**Ranges and levels**				
	-α	**-1**	**0**	**+1**	**+ α**
Moisture holding capacity (B) [% v/v]	45	55	70	85	95
pH (C)	3.82	4.5	5.5	6.5	7.18
Temperature (D) [°C)]	26	28	31	33	35

Based on the results in Table 5, first order equation was obtained as shown in equation (4) as follows:

$$y = +0.47 +0.037\ B +0.037\ C -0.10\ D \quad (4)$$

where y is the response variable (amylase activity), B is the code moisture holding capacity, C is for initial pH and D is for temperature.

The model F-value of 17.19 implies that the model is significant. The *lack-of-fit* F-value of 2.38 implies that the *lack-of-fit* is not significant and 17.42% chances that this large occur due to noise. In this study, independent variables of B, C and D were found to be the significant model terms at the probability level of 95%. The ANOVA for response surface linear model was summarized in Table 6. The regression coefficient equation obtained showed that the values of coefficient determination (R^2) was 0.7632, indicating that 76.32% of the variability in the response could be explained by the model. Moreover, the predicted R^2 value of 0.6048 was at reasonable agreement with the adjusted R^2 value of 0.7188. An adequate precision of 13.345 for amylase activity was recorded. A greater value of F-value in reduced model (=17.19) indicates that reduced model was highly significant model compared to full model (F-value = 8.07). According to Shi et al. (2009), the model is a good prediction of the experimental results if the calculated F value is several times greater than the tabulated F value. This was proven when the F-value (=17.19) shows five times greater values than the tabulated F-value [F (3, 16) = 3.24)] at 0.05 level. Therefore, the independent variables of B, C and D studied were significant model terms at the probability level of 95% for amylase production. The CV in the reduced model showed improved value of 12.67% thus indicating better statistical quality of the experimental results. Small PRESS values (0.093) was recorded as an indicator that the reduced model is adequate to predict the response.

Table 5 - Experimental design and result of reduced model CCD

Standard Order	**B: Mositure holding capacity(v/**	**C : pH**	**D: Temperature (°C)**	**Amylase activity (UmL^{-1})**	
				Actual	**Predicted**

	v %)				
1	55 (-1)	4.5 (-1)	28 (-1)	0.795	0.73
2	85 (+1)	4.5 (-1)	28 (-1)	0.914	0.85
3	55 (-1)	6.5 (+1)	28 (-1)	0.819	0.83
4	85 (+1)	6.5 (+1)	28 (-1)	0.935	0.93
5	55 (-1)	4.5 (-1)	33 (+1)	0.523	0.44
6	85 (+1)	4.5 (-1)	33 (+1)	0.556	0.54
7	55 (-1)	6.5 (+1)	33 (+1)	0.445	0.54
8	85 (+1)	6.5 (+1)	33 (+1)	0.55	0.64
9	45 (-α)	5.5 (0)	31 (0)	0.666	0.60
10	95 (+α)	5.5 (0)	31 (0)	0.841	0.77
11	70 (0)	3.82 (-α)	31 (0)	0.446	0.60
12	70 (0)	7.18 (+α)	31 (0)	0.863	0.77
13	70 (0)	5.5 (0)	26 (-α)	0.893	0.93
14	70 (0)	5.5 (0)	35 (+α)	0.541	0.44
15	70 (0)	5.5 (0)	31 (0)	0.767	0.68
16	70 (0)	5.5 (0)	31 (0)	0.674	0.68
17	65 (0)	5.5 (0)	31 (0)	0.605	0.68
18	65 (0)	5.5 (0)	31 (0)	0.617	0.68
19	65 (0)	5.5 (0)	31 (0)	0.626	0.68
20	65 (0)	5.5 (0)	31 (0)	0.608	0.68

Table 6 -Analysis of variance for response sufrace linear model

Source	**Sum of squares**	**DF**	**Mean square**	**F value**	**p-value (Prob>F)**
Model	0.18	3	0.060	17.19	<0.0001
Lack of fit	0.047	11	0.0003	2.38	0.1742
Pure error	0.0008	5	0.0001		
Corrected Total	0.24	19			

DF, degree of freedom; $R^2 = 0.7632$; predicted $R^2 = 0.6048$; adjusted $R^2 = 0.7188$

Response surface plotting

The response surface three dimensional diagrams were employed in order to further study the interactive effects of factors on the production of amylase enzyme. Figure 1(a) shows the response surface curves of the relationship between incubation period and temperature while the moisture holding capacity and pH were kept at 70% and pH6.5, respectively. The figure demonstrated that amylase enzyme activity was declined with the increase in the incubation period and also the temperature. Figure 1(b) represented the relationship between moisture holding capacity and pH while keeping the temperature at 28°C in the reduced model. The diagram generated from the reduced model experiment showed the linear pattern. Thus, these indicated less complex interaction between the variables and the response. The amylase enzyme activity was increased with the increase of pH and moisture holding capacity.

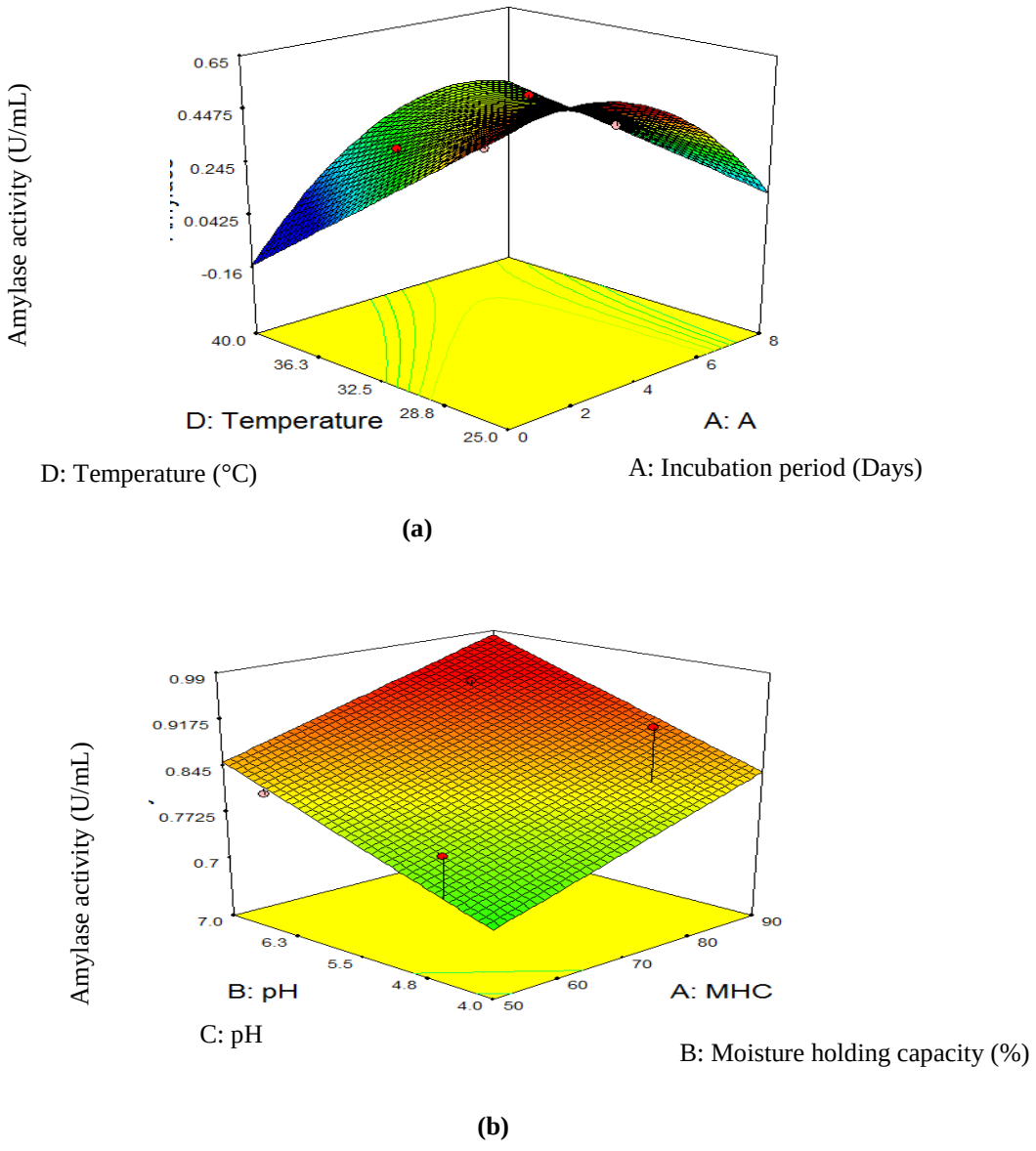

Figure 1- Response surface curve indicating the interaction between the variables on crude amylase production (a) temperature and incubation period (b) pH and moisture holding capacity. Blue, green and red colors indicate the amount amylase activity at low, intermediate and at high amount respectively.

The moisture level is main factor in SSF which often determines the success of the system. This is supported by Mitchell et al. (2000) and Neifar et al. (2011), whereby they reported that in SSF system the microorganism obtained water from the moisture held within the substrate particles which is essential for fungal growth, metabolism and the production of secondary metabolites. Moisture holding capacity has been reported as one of the important factors that influence the growth of the organism and also the enzyme production in SSF (Baysal et al. 2003; Shaktimay et al. 2010). Sun et al. (2009) further reported that moisture content was a factor that caused the swelling of the substrates thus gives effects on the gas exchange in the fermentation system. At lower level of moisture content, the degree of swelling was low and caused higher water tension thus reduces the solubility of nutrients in the system. On the other hand, higher moisture content level brings the changes on substrate particles which decreases the porosity hence promotes the development of stickiness and reduces the gas exchange. The presence of gas exchange was crucial in facilitating the utilization of substrate by the microorganism (Mahanta et al. 2008;Murdula et

al. 2011). In general, the moisture holding capacity varies between 60% - 80% that is required for an efficient SSF system (Grover et al. 2013). However, throughout this study, maximum amylase production was recorded at 100% of moisture holding capacity.

In this study, the moisture holding capacity, pH and temperature had the most effects on amylase production. The moisture level is the main factor in SSF which often determines the success of the system. According to Baysal et al. (2003), the initial moisture content of the substrate influences the growth of the organisms and enzyme production in SSF. While pH is also an important factor that affect the morphological changes and enzyme secretion by the microorganism since they are sensitive to the concentration of hydrogen ions presents in the medium (Sivaramakrishnan et al. 2006; Bhimba et al. 2011). Generally, the optimum pH required for the growth of fungi is slightly acidic (pH 4.0-6.5). In this study, pH 6.5 was recorded as the best pH for maximum crude amylase production. Balkan et al. (2005) reported that temperature plays an important role in the development of the fermentation process since it influences the protein denaturation, enzyme inhibition and cell growth. The optimum temperature for enzyme production by most microorganisms was recorded within the range of 25-37°C (Ramachandran et al. 2004; Sun et al. 2009; Abdel-Banat et al. 2010). Maximum crude amylase production by *Aspergillus flavus* NSH9 was determined at 28°C. The production of amylase enzyme was declined as the incubation temperature increases. This clearly indicates that the amylase enzyme production in solid state fermentation is significantly affected by temperature.

Optimization and verification of theoretical results

Optimization and verification experiments were done in order to find out the optimum level of process parameters by maximizing the response. The criteria were set as presented in Table 7. The confirmatory experiments were carried out to further support the optimized data as given by the statistical modelling according to the solutions determined by the reduced model with higher desirability (Table 8).

Table 7 -Optimization criteria used in this study

Parameter	Limit		Criterion
	Lower	Upper	
Moisture holding capacity [%(v/v)]	80	100	Maximize
pH	5.5	7	Maximize
Temperature (°C)	28		Target
Enzyme activity (UmL^{-1})	0.030	0.690	Maximize

The optimization and verification were carried out in triplicate for a period of four days of incubation. The error that indicates the deviation between the predicted and the actual values was calculated based on the following equation (5):

$$\text{Error} = \frac{\text{Actual value} - \text{Predicted value}}{\text{Actual value}} \times 100\% \quad (5)$$

Table 8 -Verification of theoretical results of optimized combination of fermentation parameters for amylase production

Solution	Moisture holding capacity [%(v/v)]	pH	Temperature (°C)	Predicted value, amylase activity (UmL^{-1})	Actual value, amylase activity ($U\ mL^{-1}$)	Desirability	Error (%)
1	100	7	28	1.050	1.013	1	-3.5
2	**100**	**6.5**	**28**	**1.022**	**1.055**	**0.998**	**3.2**

3	90	7	28	1.013	1.025	0.997	1.2
4	90	6.5	28	0.956	1.037	0.985	8.4
5	85	6.5	28	0.967	1.030	0.989	6.5
6	80	6.5	28	0.950	0.983	0.989	3.5

Based on the results in Table 8, the optimized fermentation parameters condition yielded the amylase production closer to the predicted values except for Solution 4 and 5. Even though, the two solutions of 4 and 5 shows error more that 5%, but they produced higher activity than predicted thus indicating that RSM is a suitable model for optimization. Therefore, from six solutions performed, Solution 2 was chosen as the optimum condition as a result of higher amylase production compared to the other solutions. Furthermore, these results corroborated with the validity of the response model and the effectiveness of the reduced model for the optimization process in the production of amylase by *Aspergillus flavus* NSH9 on sago hampas.

CONCLUSION

Optimum solid state fermentation condition for amylase production by *Aspergillus flavus*NSH9 on sago hampas was successfully achieved by using response surface methodology (RSM). The RSM is proven to be a beneficial statistical tool for designing, performing and analyzing the experiments. Besides that, this tool enables the elimination of insignificant variables thus reduces the time consumed and size of experiments run. The optimum fermentation process parameters obtained were at 100% (v/v) moisture holding capacity, pH 6.5, temperature at 28°C with incubation period of four days which gave 1.055 ± 0.03UmL^{-}1 of amylase activity.

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