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Optimization of FPase Activity using Sorghum Straw Planted in Malaysia by *Aspergillus terreus* SUK-1 via Solid Substrate Fermentation

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ABSTRACT

The optimum conditions for cellulase activity measured as Filter paper activity (FPase) from sorghum straw by *Aspergillus terreus* SUK-1 using Response Surface Methodology (RSM) was based on Central Composite Design (CCD). The effect of incubation temperature, initial pH and moisture content on FPase activity was carried out in Solid Substrate Fermentation (SSF). The results were analysed by analysis of variance (ANOVA) and the regression quadratic model was obtained. ANOVA analysis indicated that the model was significant ($p<0.05$) and that the effect of temperature and moisture content significantly effected on the FPase activity. The optimized conditions are; temperature 25°C, pH 4 and moisture content of 40%, while the predicted FPase activity is 0.35 U mL^{-1}. The model was validated by applying the optimized conditions and it was found that the FPase activity was 0.36 U mL^{-1} which indicate the validity of the model.

Key words: Filter paper activity, *Aspergillus terreus* SUK-1, sorghum straw, response surface methodology

INTRODUCTION

Sorghum straw as a waste in the sorghum seed production is an abundant lignocelullosic material, renewable and cheap resource, commonly used as livestock feed in the Sudan. However, few studies were conducted on sorghum straw as raw material for production of cellulase (Reddy *et al*., 2005; Zhang *et al*., 2010). Previously, sorghum straw was utilized in some biotechnological studies such as furfural production (Vazquez *et al*., 2007) and cellulase-free xylanase production in solid state fermentation (Sonia *et al*., 2005).

Cellulosic enzymes are the keys to renewable biomass processing and saccharification to generate fermentable sugars for the production of biofuel such as bioethanol, biobutanol and biohydrogen (Al-Shorgani *et al*., 2014). Thus, it is important to investigate the use of sorghum straw as the feed for biofuel production.

For a country like Sudan, sorghum straw is in abundance and thus lends itself to being potential substrate and they are composed of lignin and sugars polymerized to cellulose and hemicellulose that can be liberated by hydrolysis and then fermented to bioethanol by microorganisms (Mussatto and Roberto, 2004). Sorghum plant was planted without extra irrigation and depends only on rain- fed no chemical fertilizer was needed.

Production of enzymes is a major cost factor in the hydrolysis of lignocellulose to fermentable sugar and it is therefore, essential to improve yield and productivity of

the enzyme to make the process economically feasible (Nystrom and Allen, 1976). To enhance cellulase activity and to reduce production cost, optimization of environmental factors is chosen.

The choice of local *Aspergillus* species such as *Aspergillus terreus* SUK-1 which was isolated from palm oil mill sludge was reported has ability to improve cellulase activity (Rashid *et al*., 2011). Different studies have reported the ability of *Aspergillus terreus* SUK1 for the production of FPase (exoglucanase), CMCase (endoglucanase) and β-glucosidase which are important in the process of cell wall degradation of biomass (Yusoff *et al*., 2000). *Aspergillus* strains are also known for their ability to produce β-glucosidase with significantly higher yields than *Trichoderma* species (Damisa *et al*., 2011) make the choice of *Aspergillus* strains more attractive.

RSM can be used to determine the optimal production conditions and range of controllable variables, to generate a polynomial equation and to estimate the relationships between controllable variables and observed results (Min *et al*., 2007). For instance, RSM has been recently used for modelling an optimization of process conditions. In this study, sorghum straw planted in Malaysia is used as substrate for cellulase production using *Aspergillus terreus* SUK-1 for the economically advantages of sorghum plant. Furthermore, RSM was employed to optimize the conditions for cellulase production from sorghum straw in SSF. Central Composite Design (CCD) was applied for three variables namely; incubation temperature, pH and moisture content for optimization which resulting in twenty experimental runs for cellulase activity.

MATERIALS AND METHODS

Fungus cultivation for spore production and inoculum preparation: Local isolate, *Aspergillus terreus* SUK-1 from School of Biosciences and biotechnology, The National University of Malaysia (UKM), 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 culture of both molds with 15 mL sterile distilled water and 10% (w/v) of inoculum (of the tested ratios) was used in all experiments.

Sorghum production and medium preparation: Sorghum seeds were collected from Local market in Khartoum, Sudan. The seeds planted by drill in rows seeds in moist soil in National University (UKM) of Malaysia experimental plot Malaysia. The seeding depth was in one inch and after 70 days the sorghum were harvested (Kansas State University, 1998), the sorghum straw was firstly washed then dried and then milled into small particles using miller. The sorghum straw particles were sieved using 1 mm mesh sieve and utilized as the substrate for solid substrate fermentation. The medium used for SSF was developed by Mandels and Rees (Sternberg *et al*., 1977), the medium composition is shown in Table 1.

Enzyme production under SSF: Five gram of the sorghum straw was introduce into 250 mL Erlenmeyer flask, the Mandels medium was added to the 5 g of the sorghum straw to give different moisture ratio. Medium was sterilized for 30 min at 121°C and spore sustention was aseptically added to the flasks and mixed thoroughly, incubation temperature was set at 25-40°C for 7 days experimental run conducted based on the statistical design.

Enzyme extraction: The enzyme was extracted by a simple contact method distilled water was introduced into each fermented flask in 1:10 (v/w) ratio kept in shaker at 120 rpm for 30 min and then filtered via muslin cloth and centrifuge at 10,000 rpm for 15 min at 4°C. The clarified supernatant was used for further enzyme analysis.

Determination of enzyme activity: Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method (Hankin and Anagnostakis, 1975). Aliquots of appropriately diluted cultured filtrate as enzyme source was added to whatman No. 1 filter paper strip (1×6 cm; 50 mg) immersed in 1 mL of 0.05 M Sodium citrate buffer of pH 5.0. After incubation at 50±2°C for 1 h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar from filter paper per mililiter per min.

Experimental design: The variables used were (a) Temperature, (b) pH and (c) moisture content and the coded value variable were -1, 0, 1 (low, basal low, high) (Table 2). Twenty experiments were performed for each microorganism at three level including five replicates at the centre points with

Table 1: Medium composition

Medium component	Concentration (g L^{-1})
$(NH_4)_2SO_4$	1.4
KH_2PO_4	2.0
$MgSO_4.7H_2O$	0.3
$CaCl_2.2H_2O$	0.4
$FeSO_4.7H_2O$	0.005
$MnSO_4.7H_2O$	0.0016
$ZnSO_4.7H_2O$	0.0014
$CoCl_2.7H_2O$	0.002
Urea	0.3
Tween 80	0.2 mL
Protease peptone	0.2

Table 2: Range of variable and their coded level for the central composite design uses for FPase activity from sorghum straw using *Aspergillus terreus* SUK-1

	Coded value		
Independent variables	-1	0	+1
A-Temperature °C	25	32.5	40
B-pH	4	5.5	7
C-Moisture content (% w/v)	40	60.0	80

A: Temperature at low level (1-) of 25°C and high level (1+) of 40°C, B: pH at low level (1-) of 4 and high level (1+) of 7, C: Moisture content at low level (1-) of 40 %(w/v) and high level (1+) of 80% (w/v)

Table 3: Experimental Central Composite Design (CCD) with experimental value and predicted value on FPase activity by *Aspergillus terreus* SUK-1

	Variables			FPase activity (U mL^{-1})	
Run No.	Temperature (°C)	pH	Moisture content (%)	Experimental value	Predicated value
1	32.5(0)	7 (+1)	60 (0)	0.12	0.14
2	32.5(0)	5.5 (0)	80 (+1)	0.10	0.04
3	32.5(0)	5.5 (0)	60 (0)	0.09	0.11
4	32.5(0)	4 (-1)	60 (0)	0.20	0.17
5	32.5(0)	5.5 (0)	60 (0)	0.07	0.11
6	25 (-1)	5.5 (0)	60 (0)	0.22	0.22
7	40 (+1)	7 (+1)	80 (+1)	0.09	0.11
8	40 (+1)	4 (-1)	40 (-1)	0.15	0.15
9	25 (-1)	4 (-1)	40 (-1)	0.36	0.35
10	25 (-1)	7 (+1)	40 (-1)	0.31	0.29
11	40 (+1)	5.5 (0)	60 (0)	0.11	0.10
12	32.5(0)	5.5 (0)	60 (0)	0.08	0.11
13	32.5(0)	5.5 (0)	60 (0)	0.15	0.11
14	40 (+1)	4 (-1)	80 (+1)	0.07	0.10
15	25 (-1)	7 (+1)	80 (+1)	0.13	0.14
16	40 (+1)	7 (+1)	40 (-1)	0.17	0.16
17	32.5 (0)	5.5 (0)	60 (0)	0.13	0.11
18	25 (-1)	4 (-1)	80 (+1)	0.17	0.19
19	32.5(0)	5.5 (0)	40 (-1)	0.10	0.15
20	32.5(0)	5.5 (0)	60 (0)	0.13	0.11

three levels according second order face-centred composite design (Table 3). The experimental data was employed in statistical package, software Design Expert 6.0.0 (Stat Ease Inc. Minneapolis, USA) to fit a second order polynomial response surface methodology according to Eq. 1.

$$Y = b'0 + \sum_{i=1}^{n} biXi + \sum_{i=1}^{n} biiXi^2 + \sum_{i=1}^{n}\sum_{i>1}^{n} bijXiXj \quad (1)$$

RESULTS

The effect of the three variables on FPase activity is illustrated in Table 4. Statistical analysis by ANOVA showed that the effect of temperature and moisture content had significant effect on FPase activity. However, the effect of pH was insignificant on FPase activity (Table 4).

The experimental design and result of experiment are presented in Table 3. The results for the second ordered response surface model for FPase activity in the forms of ANOVA is shown in Table 4.

The model F-value of 7.27 implies the model is significant. There is only a 0.23% chance that a model F-value could occur due to noise. The pure error was very low (0.005207), indicating good reproducibility of the experimental data. Values of probe >F-value less than 0.0023 indicates model terms are significant.

In this case A and C are significant terms. Values that are greater than 0.05 indicate the model terms to be not significant. The fit of the model checked by the co-efficient of determination R^2 was calculated to be 0.8674 indicating that 86.74% of variables can be explained by the model and only 13.3% of variability in the response cannot be explained by the model. The multiple coefficient of determination (R^2) represent variability in the value formed response which can be explained by test factors and their interactions.

A regression model can be used to predict observation of the response (FPase) coresponding to the particular values of the regression variable (Table 5). The model equation for the individual parameters, interaction (as second order equation) can be shown by using Eq. 2:

$$\text{FPase activity U mL}^{-1} = 2.5\text{-}0.08\times\text{temperature} - 0.26\times\text{pH} - 4.11\times\text{moisture content} + 8.24\times\text{temp}^2 + 0.02\times\text{pH}^2 - 3.91\times\text{moisture content}^2 + 1.5\times\text{temp}\times\text{pH} + 1.9\times\text{temperature}\times\text{moisture content} + 3.0\times\text{pH}\times\text{moisture content} \quad (2)$$

The three dimensional response surface graphs (3D) for response surfaces are displayed in Fig. 1a-c represented the combined effects of temperature and pH (Fig. 1a), temperature and moisture content (Fig. 1b), pH and moisture content (Fig. 1c). In all 3D graphs, two variables were varied while the other variable was kept constant at its central level. The central level of temperature was 32.5°C, pH was 5.5 and moisture content was 60% as shown in Table 3.

The response surfaces obtained suggested that optimum cellulase activity by *Aspergillus terreus* SUK-1 would be obtained at a temperature 25°C, pH and 40% moisture content. The quadratic model (Eq. 2) predicted an optimum FPase activity of 0.35 U mL^{-1} under the following SSF parameters: Temperature of 25°C, pH of 4 and moisture content of 40%.

The interaction effect between temperature and pH on cellulase production was presented in Fig. 1a. The 3D graph shows that decreasing the temperature and pH around 5 leads to increase the cellulase activity. Figure 1b displays the interaction effect between temperature and moisture content on cellulase activity. The interaction effect between pH and moisture content on cellulase production was presented in Fig. 1c. It was found that pH 5 and moisture content 60% maximized the FPase activity.

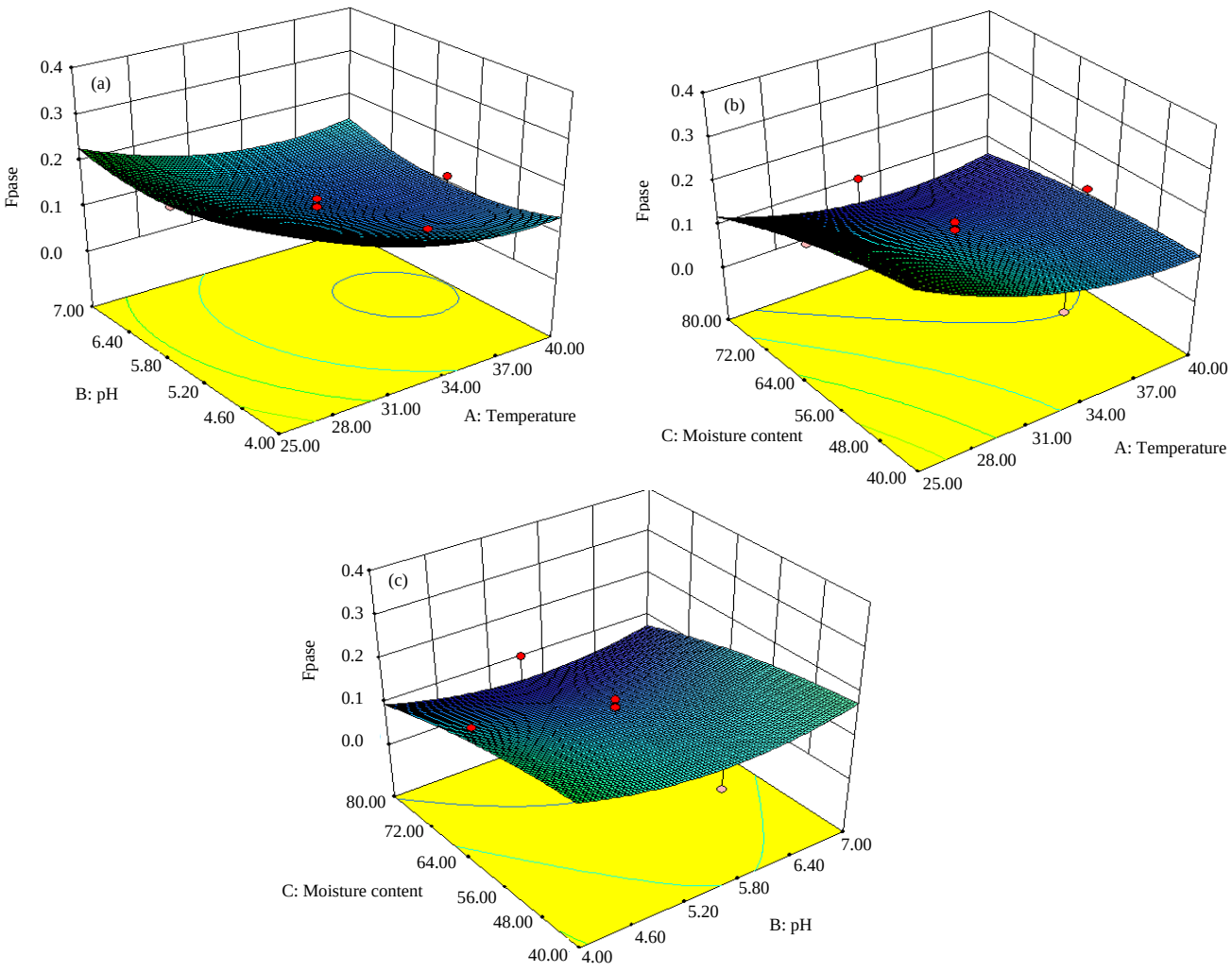

Fig. 1(a-c): Three-dimensional response surface plot for the effects of, (a) Incubation temperature and pH, (b) Moisture content and temperature and (c) pH and moisture content on FPase activity

Table 4: Analysis of Variance (ANOVA) for regression model to optimize FPase activity

Source	SS	df	MS	F-value	Prob>F
Model	0.098	9	0.011	7.27	0.0023
A-Temperature	0.034359	1	0.034358926	23.37872	0.0007
B-PH	0.001505	1	0.001504799	1.023905	0.3355
C-Moisture content	0.02821	1	0.028209805	19.1947	0.0014
A^2	0.005909	1	0.005909287	4.020835	0.0728
B^2	0.004709	1	0.004709377	3.204384	0.1037
C^2	0.000673	1	0.00067291	0.457866	0.5140
AB	0.002176	1	0.002176463	1.480923	0.2516
AC	0.006094	1	0.006094436	4.146815	0.0691
BC	0.000007	1	0.000007	0.004525	0.9477
Residual	0.014697	10	0.001469667		
Lack of fit	0.00949	5	0.001897999	1.822661	0.2630
Pure error	0.005207	5	0.001041334	23.37872	0.0007
Cor total	0.110853	19	0.034358926	1.023905	0.3355
Standerd deviation	0.038				
R-squared	0.8674				
Adj R-squared	0.2831				
Adeq precision	11.430				

SS: Sum of squares, DF: Degree of freedom, MS: Mean of squares

Table 5: Regression coefficient for optimization of FPase activity

Model term	Coefficient estimate	Standard error	F-value	Prop>F
Intercept	0.112881	0.01317907	7.27	0.0023
A-Temperature	-0.05862	0.012122981	23.37872	0.0007
B-pH	-0.01227	0.012122981	1.023905	0.3355
C-Moisture content	-0.05311	0.012122981	19.1947	0.0014
A^2	0.046355	0.023117617	4.020835	0.0728
B^2	0.041382	0.023117617	3.204384	0.1037
C^2	-0.01564	0.023117617	0.457866	0.5140
AB	0.016494	0.013553905	1.480923	0.2516
AC	0.027601	0.013553905	4.146815	0.0691

DISCUSSION

Present results are in agreement with the results of Latifian *et al.* (2007), who have reported that moisture had a significant effect on cellulase production using *T. reesei* QM 9414 and *T. reesei* MCG 77. In addition they found that the temperature was in the range of 25-30°C for optimal cellulase production.

Acharya *et al.* (2008) reported that the optimum pH and temperature for cellulase production using *A. niger* were at pH 4.0 and temperature of 28°C, respectively which is in agreement with present results. Ahmed *et al.* (2009) reported that the optimum pH for *Trichoderma viride* growth for maximal exoglucanase (EXG), endoglucanase (EG) and β-glucosidase production was found to be 5.5 at 28°C. Optimum pH for fungal cellulases varies from species to species; though in most cases the optimum pH ranges from 3.0 to 6.0 (Niranjane *et al.*, 2007; Karuppaiya *et al.*, 2009).

It is reported in the literature that solutions with higher desirability gave optimum temperature of 30°C, pH of 5 and moisture content of 70% (Oberoi *et al.*, 2011).

Moisture content in the solid state fermentation is a key factor for cellulase enzyme production. Ahmed *et al.* (2010) have optimized the moisture content by varying moisture content from 10-50% to lignocellulosic substrate wheat straw. The study indicates that maximum cellulase (301±1.16 μM mL^{-1} min^{-1}) production was observed at 40% moisture level, while further increase in moisture influenced the enzyme production negatively. They concluded that there is a positive relationship between cellulase production and moisture content (Ahmed *et al.*, 2010). Higher and lower water contents adversely affect the primary metabolic activities of microbes leading to slower enzyme formation (Wang *et al.*, 2006; Fang *et al.*, 2010).

Optimal water fractions in the solid substrate between 40-60% (by mass) under solid-state fermentation (Narasimha *et al.*, 2006). Sohail *et al.* (2009) reported that optimization of incubation temperature and initial moisture content of the medium resulted in a 6.2 fold. Increase in production from 0.605 to 3.8 U gds^{-1} of cellulase by *Aspergillus niger* MS82.

The effect of initial pH on enzyme production ranging from 3 to 7 was determined and maximum cellulase activity (348±1.29 μM mL^{-1} min^{-1}) was recovered at pH 5.5. Furthermore, it was found that maximum induction of endoglucanase was achieved at pH 5.5 as there was a correlation between the initial pH of the medium and cellulase produced by *Trichoderma reesei* Rut C-30 (Khurshid *et al.*, 2001; Xiong *et al.*, 2004).

Model validation: The model was validated by applying the optimized conditions and the obtained cellulase activity was found to be 0.36 U mL^{-1} which is slightly higher than the predicted value (0.35U mL^{-1}). This verifies the fitness of the model in predicting the combined interactions of the three independent variables (temperature, pH and moisture content) on the cellulase activity.

CONCLUSION

The optimization of FPase activity from sorghum straw under solid substrate fermentation by *Aspergillus terreus* SUK-1 was successfully conducted by using Response Surface Methodology (RSM). The temperature and moisture content was found to significant effect on cellulase activity. Optimum cellulose activity was established 0.35 U mL^{-1} at the temperature of 25°C, pH 4 and 40% moisture content.

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