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Optimisation of Medium Composition and Culture Conditions of Filter-paperase by UPMC1106 using Pennisetum Purpureum

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Lignocellulosic resources are valuable and widely utilised to produce fermentable sugar. There is potential non-human consumption lignocellulosic material such as Pennisetum sp. available in the tropical and subtropical region. This study was conducted to optimise the medium composition and culture conditions of submerge fermentation of locally isolate UPMC1106, using Pennisetum sp. as the substrate in shake flasks. The Pennisetum sp. or Pennisetum purpureum sp. was collected from Pahang, Malaysia (3.793619° N, 102.523449° E) at the age of three months, processed until milled using Thomas Wiley mill and passed through a 60-mesh sieve. The powder form Pennisetum sp. was used as the sole carbon source in the cultivation medium. Four factors were chosen for the optimisation, as these factors contributing to the cellulosic enzymes production. The factors were Pennisetum sp. concentration, initial medium pH, agitation speed, and inoculum size. For the maximum filter-paperase activity, FPase, (U/mL), the Napier concentration was found to be 4.3 % (w/v), and the initial medium pH was 7.8. The optimum agitation speed and inoculum size were achieved at 170 rpm and 19.7 % (v/v). The optimum values of the respective factors gave 2.895 U/mL of FPase activity. This response was 98.8 % close to the predicted value and 30 % of improvement from the non-optimised factors.

1. Introduction

Fermentable sugar productions from cellulosic resources are currently demanding in chemicals, food, and biotechnological industries. Many studies are carried out on the utilisation of plantation and plant waste derivatives as the potential source of sugars. One of the studied cellulosic materials is sugarcane bagasse (SCB) Tovar et al. (2015) examined the correlation of Michaelis-Menten model with the experimental study of enzymatic kinetic from pre-treated SCB on the production of glucose. In Malaysia, many cellulosic resources, primarily the agricultural food waste were also being investigated (Aditiya et al., 2016). Pennisetum sp. is one of typical feedlots and currently planted by many government-linked agencies, private companies, and individual farmers. The climate and soil suitability in tropical terrestrial regions may contribute to the plants' growth. The characterisation of Napier grass found in local production and local studies show high nutritional value and suit for a various type of livestock and paper making industry (Kamarullah et al., 2015). The high carbon properties in Pennisetum sp. suggests a potential production of fermentable sugar in extended fermentation processes. In obtaining high secretion of cellulosic enzymes such as total cellulase or filter-paperase (FPase), the optimisation of cultivation medium and fermentation conditions was proposed in this study. Four factors were selected, namely, Pennisetum sp. concentration, initial medium pH, agitation speed and inoculum size. Prior to optimisation, the screening process was done using full factorial design (FFD)

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approach to evaluate the significant factors and levels. The optimisation was performed using central composite design (CCD) as it has a few advantages compared to the conventional, one-factor-at-a-time methods (Czitrom, 1999) where CCD approach can combine factors and analyse the interaction between the factors.

2. Material and methods

2.1 Microorganism

The bacterium, namely UPMC1106 was obtained from Microbial Culture Collection Unit (UNiCC), Institute of Bioscience, Universiti Putra Malaysia. The isolation and screening on the cellulase secretion were previously described by Shahirah et al. (2014).

2.2 Inoculum preparation

Inoculum preparation, a single loop of UPMC1106 from carboxymethyl cellulose (CMC) agar was inoculated into 20 mL broth medium containing peptone 10 g/L, K_2HPO_4 2 g/L, $MgSO_4$ 0.3 g/L, $(NH_4)_2SO_4$ 2.5 g/L, and CMC
10 g/L at pH 7 (Irfan et al., 2012). The culture was incubated at 37 °C for 24 h without shaking. After the fermentation, the culture was used as an inoculum source.

2.3 Filter-paperase assay

The broth cultures were withdrawn after 24 h of cultivation period and subjected to centrifugation at 4,000 rpm for 20 min. The FPase was determined by adding 0.5 mL of diluted supernatant crude enzyme to 1 cm × 6 cm Whatman filter (No.1) in test tubes. The substrate was suspended in 1 mL of 0.05 M of sodium citrate buffer (pH 4.80). Incubation was carried out for 1 h at 50 °C in a water bath. The amount of reducing sugar released was measured as glucose equivalent by the DNS method (Miller, 1959) by adding 3 mL of DNS reagent to the sample and boiled for 5 min. Subsequently, the absorbance was read at 540 nm. A standard glucose plot was used as the reducing sugar expressed in this assay. The FPase activity was calculated as 1 μmol glucose released/min/g of a substrate, wherein the Eq(1), the m and c represent the slope and intercept from the glucose standard, respectively.

$$\text{FPase activity}\left(\frac{\text{U}}{\text{mL}}\right)=\frac{\text{final absorbance}-\text{c}}{\text{m}}\times\frac{\text{dilution factor}}{\text{sample volume}}\times\frac{1}{\text{reaction time}}\times\frac{1{,}000\ \mu\text{g}}{1\ \text{mg}}\times\frac{1\ \mu\text{mole}}{180.16\ \mu\text{g}} \quad (1)$$

2.4 Submerged fermentation

The submerged fermentation was carried out in 250-mL shake flask with 20 mL working volume. A percentage of 10% (v/v) of inoculum were inoculated into medium consisting of peptone 10 g/L, K_2HPO_4 2 g/L, $MgSO_4$ 0.3 g/L, and $(NH_4)_2SO_4$ 2.5 g/L at pH 7, and incubated at 37 °C at 150 rpm for five days. The broth cultures (20 mL) were withdrawn at 24-h time interval, which was then subjected to centrifugation at 4,000 rpm for 20 min. The supernatant was collected and stored as crude enzyme at 4 °C for further analysis. A concentration of 1 g/L of Pennisetum sp. powder (leaf and stem in a ratio of 1 : 1) was used as a substrate.

2.5 Full factorial experimental design

FFD with five central points was applied as a screening method to the experimental parameters. Four parameters, which were Pennisetum sp. concentration (A), initial medium pH (B), agitation speed (C) and inoculum size (D) were selected for the optimisation study. The level was determined based on high (+1) and low (-1) values for each parameter. A total of 37 experiments, including five central points with replication were generated by Design Expert Version 6.0.6 (State Ease Inc., Minneapolis, MN, USA). In FFD, the range and the levels of the parameters investigated in this study were given in Table 1.

Table 1: Parameters and coded values of FFD

Parameters	Code	-1	0	+1
Pennisetum sp. (%w/v)	A	1	3	5
Initial medium pH	B	5	7	9
Agitation speed (rpm)	C	150	160	170
Inoculum size (%v/v)	D	10	15	20

2.6 Central composite experimental design

The same four parameters and range were used in CCD, as suggested from the current screening result of FFD. The independent variables were indicated as high (+1) and low (-1) levels, while the central point was indicated as (0). The statistical software was used for regression analysis and the graphical analysis of the data was obtained. The response of the FPase activities was subjected to the regression model of quadratic and have been expressed by the second-order polynomial as describe previously by Saini et al. (2013).

3. Results and discussion

3.1 Full factorial design

A smaller P- value is an indication of the high significance of the corresponding coefficient (Alam et al., 2008). Thus, variable with lower P- value which is close to 0.00 contributed to the model while others can be eliminated from the design. In the regression analysis, A and C with $P < 0.0001$ while B and D with $P = 0.0045$ and 0.0028 respectively, shows that the primary variables were significant factors in the production of FPase. The final order Eq(2) was generated based on the first-order model to determine the FPase production response to the cultivation screening involving the factors of Pennisetum sp. concentration (A), pH (B), agitation speed (C) and inoculum size (D).

$$\begin{aligned}\mathrm{FPase\ (U/mL)} = {} & +1.62 + 0.38 \times A + 0.039 \times B + 0.13 \times C - 0.14 \times D - 0.24 \times A \times B - 0.24 \times A \times D \\ & + 0.20 \times B \times C + 0.20 \times B \times D + 0.070 \times C \times D + 0.099 \times A \times B \times C - 0.13 \times A \times B \times D \\ & - 0.13 \times A \times C \times D + 0.19 \times B \times C \times D - 0.057 \times A \times B \times C \times D\end{aligned} \quad (2)$$

3.2 Central composite design

Optimisation of medium and cultural condition for cellulase production was carried out with the FPase activity as the response. All experiment was done in duplicates and the mean values of FPase activity were recorded as responses. From the predicted and actual value of FPase production by UPMC1106 (data not shown), an ANOVA and regression analysis was generated as shown in Table 2. The statistical analysis of the model was evaluated by the P-value based on the FPase production harvested at 72 h.

Table 2: ANOVA and regression analysis for the response surface quadratic model to produce FPase.

Sources	Sum of square	Mean square	F-value	P-value
Model	5.26	0.40	43.08	< 0.0001
A	1.70	1.70	181.85	< 0.0001
B	1.01	1.01	108.59	< 0.0001
C	0.02	0.01	1.49	0.2418
D	0.59	0.59	63.28	< 0.0001
AB	0.23	0.23	24.57	0.0002
AC	0.05	0.05	5.46	0.0338
AD	0.34	0.34	36.72	< 0.0001
BC	0.14	0.14	14.54	0.0017
BD	0.01	0.01	1.19	0.2920
CD	0.15	0.15	16.38	0.0011
A^2	1.187×10^{-4}	8.187×10^{-4}	0.09	0.7711
B^2	1.25	1.25	134.08	< 0.0001
C^2	0.17	0.17	17.93	0.0007
D^2	0.14	0.14	15.23	0.0014
Lack of fit	0.12	0.01	2.96	0.1213
R^2	0.9757			
Adjusted R^2	0.9531			
Predicted R^2	0.8641			
Adequate precision	25.34			
Coefficient of Variance (%)	4.61			

The FPase activity was fitted to the second-order quadratic model and can be explained by Eq(3).

$$\text{FPase}\left(\frac{\text{U}}{\text{mL}}\right) = 2.21 + 0.31 \times \text{A} + 0.24 \times \text{B} - 0.18 \times \text{D} - 0.69 \times \text{B}^2 + 0.25 \times \text{C}^2 + 0.23 \times \text{D}^2 + 0.12 \times \text{A} \times \text{B} + 0.056 \times \text{A} \times \text{C} + 0.15 \times \text{A} \times \text{D} + 0.092 \times \text{B} \times \text{C} + 0.098 \times \text{C} \times \text{D} \quad (3)$$

The quadratic model is highly significant as indicated by the value of $P < 0.001$. The model fitted well to the experimental design as the lack of fit is insignificant with the value of $P > 0.05$. Among the variables and their interactions, all the linear model's terms are significant except for C, while for their interactions, only A^2 and BD were reported to be insignificant. The R^2 value is close to 1 ($R^2 = 0.9757$), indicating a high degree of correlation between observed and predicted value. The 3D response surface plot obtained by the analysis of the experimental data of CCD show a relationship between two variables at a time while maintaining other variables at fixed level, as shown in Figure 1.

In details, the figure is showing, a) interaction plot of initial medium pH and Pennisetum sp. concentration (% w/v), b) interaction plot of agitation speed and Pennisetum sp. concentration (% w/v), c) interaction plot of inoculum size (% v/v) and Pennisetum sp. concentration (% w/v), d) interaction plot of agitation speed and initial medium pH, e) interaction plot of inoculum size (% v/v) and initial medium pH, and f) interaction plot of inoculum size (% v/v) and agitation speed. Pennisetum sp. concentration, initial medium pH, agitation speed, and inoculum size were found to be the critical parameters that influence the enzyme activities. The present study indicates that the optimum substrate concentration was found at 4.30 % (w/v) for maximum activities of FPase. Any concentration of less or more than the optimum levels, the enzyme activities were reduced.

In this study, Pennisetum sp. was used as a low cost cellulose source. The Pennisetum sp. was proved to be a potential substrate for cellulase production where the production of cellulase was comparable with the other reported cellulosic substrate (Pirzadah et al., 2014). The feasibility of Pennisetum sp. as the fermentation substrate would be a value added to the Pennisetum sp. plantations. The initial pH of the culture medium is one of the most critical parameters needed for a successful enzyme synthesis (Bui, 2014). The pH of the medium has a direct effect on the mineral nutrients uptakes present in a medium which can affect the enzyme activity (Khare and Upadhyay, 2011). Present data showed the optimum initial medium pH for maximum cellulase activity was found at pH 7.8. The optimum initial medium pH obtained was similar to the previous study on cellulase production by Streptomyces sp where the maximum cellulase activity occurred at a pH range of 6.0-8.0 (Prasad et al., 2013). Agitation speed is another important culture parameters. Agitation speed is applied to the fermentation process to maintain homogenous conditions and to disperse the dissolved oxygen via smaller bubbles to enhance the surface area and oxygen mass transfer rate to increase both substrate utilisation and microbial activity (Deka et al., 2013). The optimum agitation speed for this study was found to be at 170 rpm. The optimum agitation speed obtained was slightly similar to Alam et al. (2008) where the optimum agitation speed for cellulase activities by Trichoderma harzianum was found at a range of 175 - 200 rpm. The cellulase activities decreased when increasing the agitation speed.

In the present study, to determine the optimum inoculum size for maximum cellulase activities, a range of 10 - 20 %(v/v) was tested. Singh and Kaur (2012) reported that a lower inoculum size needs a longer period for the cells to multiply to sufficient number for substrate utilisation and the enzyme production. Higher number of cells in the inoculum can ensure a rapid proliferation and biomass synthesis.

3.3 Validation of optimised medium and cultural conditions

The software suggested the optimum point of Pennisetum sp. concentration (4.30 % w/v), initial medium pH (7.80), agitation speed (170 rpm), and inoculum size (19.70 % v/v) may achieve the maximum yield of FPase. The maximum response FPase predicted from the model is 2.929 U/mL. Triplicates experiments were performed to verify the predicted optimum values and a maximum of FPase activity of 2.895 U/mL was obtained. The optimised composition and conditions were also investigated against the non-optimised conditions where the activity of FPase was obtained at 2.227 U/mL. The non-optimised cultivation was conducted using Pennisetum sp. concentration at 1 %(w/v), pH 7.0, agitation speed at 150 rpm and inoculum size at 10 %(v/v).

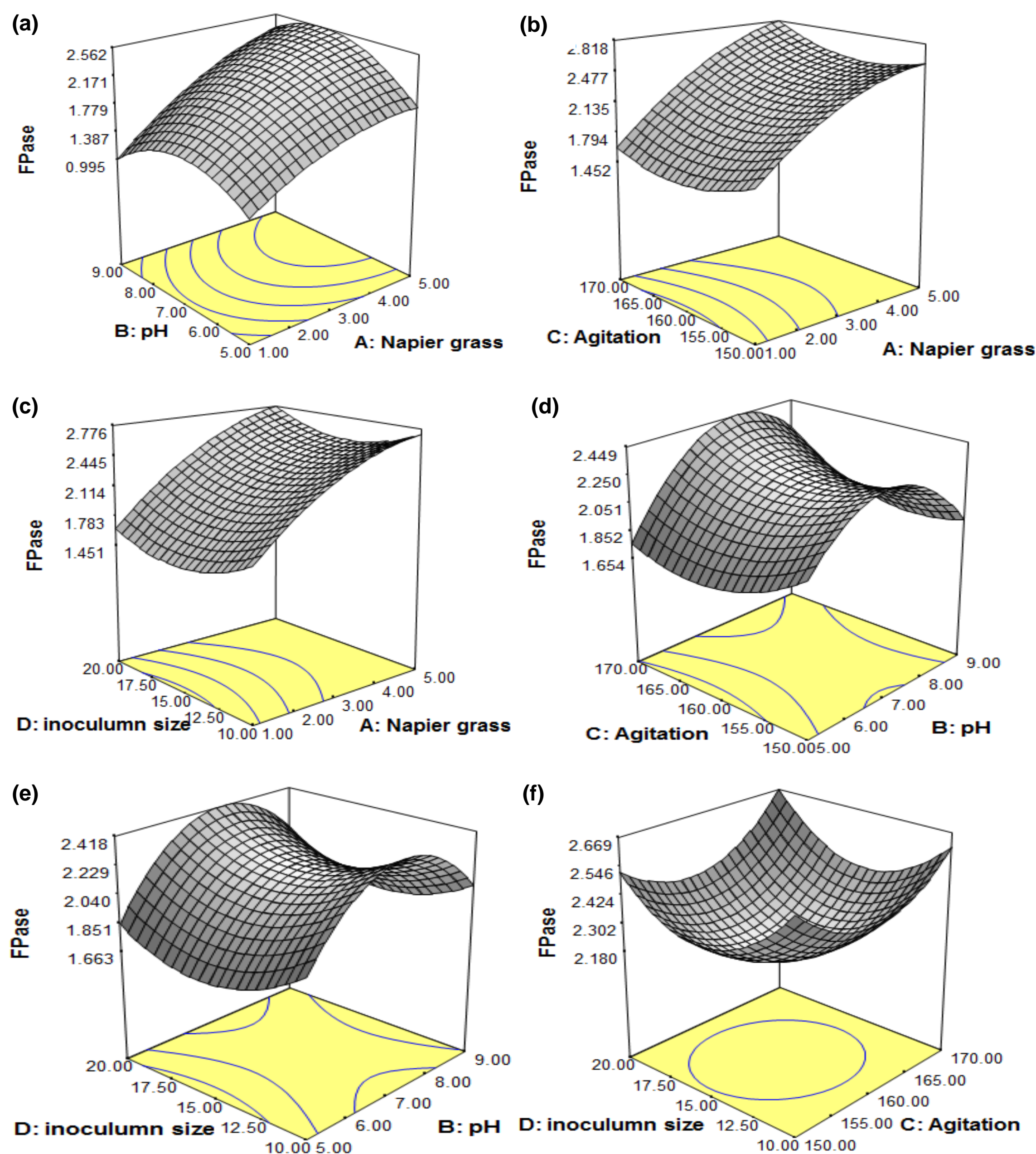

Figure 1: 3D response surface plots for FPase (U/mL) production by UPMC1106.

4. Conclusions

This study has shown the potential of Pennisetum purpureum as lignocellulosic resources with the optimisation of biological treatment via experimental design, namely FFD and CCD. As an estimated result, the optimised conditions and composition improved the production of FPase activity up to 30% than the non-optimised factors. All factors involved, except for the agitation speed, in the optimisation were determined to be significant values with P values of lower than 0.05.

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