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Optimization of cassava peel medium to an enriched animal feed by the white rot fungi *Panus tigrinus* M609RQY

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Abstract: Media components such as wheat flour, $MgSO_4$ and particle size were screened by Placket Burman design (PBD) while the operating range was fixed by one-factor-at-a-time method (OFAT), primarily for the enrichment of cassava peels as animal feed. Optimization of the selected media components was carried out using Face–Centered Central Composite Design (FCCCD) of the Response Surface Methodology (RSM) and the responses were measured in term of protein and lignin contents. Statistical analysis of the result showed that the quadratic term of wheat flour and the interaction between wheat flour and particle size were highly significant ($P<0.01$) for protein content, while for lignin degradation, $MgSO_4$ and particle size were significant ($p< 0.05$) in their linear term. The validated result showed that the optimum media component for the production of an enriched cassava peel by the white rot fungus *Panus tigrinus* (M609RQY) was wheat flour 4.30% (w/w), 0.45 g/kg $MgSO_4$, and using 1 mm particle size. This resulted to 77.83% increase in protein and 52.62% lignin degradation, thus indicating the potency of the white rot fungus *Panus tigrinus* for the production of economical livestock feed from renewable source.

Keywords: Cassava peel, *Panus tigrinus*, optimization, animal feed, solid state fermentation

Introduction

Rapid increase in the livestock industry has demanded high consumption of livestock feeds which are predominantly from agricultural source. Moreover, the teeming human population has set up competition between man and livestock over some basic feed ingredients; this has necessitated researches into provision of economical alternative for livestock feeds, which will reduce the rivalry between animal and man in terms of feed and consequently minimize the cost of feed production. Varieties of alternative feedstock have been widely investigated and the rationalization for the various research attempts is intended to source alternative feedstocks that are readily available and have no direct importance to humans (Bisaria *et al.*, 1997; Eruvbetine *et al.*, 2003; Arora *et al.*, 2011). However, the success recorded has not been effective because the alternatives currently sourced are equally relatively expensive, thus the challenge is to source the livestock feeds from renewable and relatively inexpensive food materials such as agricultural by-product and wastes from agricultural processing such as peel, seeds and chaff.

Cassava peels, which are predominantly wastes generated during the processing of cassava have been employed as an important source of carbohydrate in livestock feeds for monogastrics in various parts of the world (Aro, 2008). However, its utilization in monogastrics feed is limited due to its fibrous nature, low protein content and high levels of hydrocyanic acid (Iyayi and Losel, 2004; Ubalua, 2007). This limitation has attracted interest, particularly for the improvement of its nutritional values with respect to protein content, reduction of its non-starch polysaccharides and lowering of its cyanide content, since the material is renewable and widely available. In most studies on microbial degradation of cassava peel (Antai and Mbongo, 1994; Iyayi and Losel, 2001, 2004; Obadina *et al.*, 2006; Oboh, 2006; Aderemi and Nworgu, 2007; Okpako *et al.*, 2008; Ezekiel *et al.*, 2010), enhancement of the protein content were given more priority and the safety of the microorganisms used were not considered. The cell wall content are either slightly degraded or not degraded at all and reduces the digestibility of the bio-converted cassava peel, thus the need for effective microorganism is highly required.

The potential application of edible white rot fungi for effective bioconversion of cassava peels is primarily required because it has been identified as perfect degrader of lignin, cellulose and hemicellulose at different rates (Shi *et al.*, 2008; Arora *et al.*, 2011). White rot fungi characteristically secretes ligninolytic enzymes which is influenced by carbon sources, nitrogen sources and inorganic salts (Jonathan and Fasidi, 2001; Mikiashvili *et al.*, 2006). The white rot fungus *Panus tigrinus* (*P. tigrinus*) belongs to a group of lignin-degrading basidiomycetes. In contrast to earlier report that the ligninolytic system of *P. tigrinus* is composed of Manganase Peroxidase

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(MnP) and laccase and no Lignin Peroxidase(LiP) (Maltseva *et al.*, 1991), *Panus tigrinus* M609RQY was observed to secrete Lignin Peroxidase, laccase and MnP (unpublished result) when grown on selected agro industrial wastes. *P. tigrinus* has been identified as a producer of peroxidase, laccase, cellobiase, endoglucanase, and xylanase which had caused an increase in biopolymer degradation during the first 10 days of its incubation (Akhmedova *et al.*, 1994) and as a selective lignin degrader of sugar cane bagasse prior to pulping (Costa *et al.*, 2002). Exploiting the above characteristics, the nutritional value of cassava could be enhanced by optimizing the media constituents through solid state fermentation, which is a better choice in the bioconversion of agro industrial wastes to animal feed considering the nature of feed (Pandey, 1992).

Optimization of media composition is a vital tool to an efficient cell growth and improved secretion of various enzymes that help in degradation. Carbon and nitrogen sources cum inorganic salts mainly influence the secretion of ligninolytic enzymes by white rot fungi. Hence, optimization of media constituents will go a long way in improving yield and reducing cost of production. Central composite design (CCD) have been described as a statistical technique that is widely used for the optimization of medium composition for growth and metabolite production (Tran *et al.*, 2010).

In this study, Face–Centered Central Composite Design (FCCD) was used to determine the influence of medium constituents and to identify the optimum level of these constituent in enriching the nutritional value of cassava peel to animal feed by the white rot fungus *Panus tigrinus* M609RQY.

Materials and Methods

Panus tigrinus *culture*

The white rot fungus *Panus tigrinus* M609RQY was obtained from the culture collection of the Department of Biotechnology Engineering, International Islamic university Malaysia. It was maintained on malt extract agar (Merck, Germany) slant at 4°C and subcultured forthnightly. Inoculum preparation was done by subculturing four malt extract agar plates for 7 days at 30°C. Mycelia suspension was prepared by washing each Petri dish with 15 ml of distilled water in 250 ml Erlenmeyer flask and the concentration of the prepared mycelia suspension was 0.865 g/l. The flasks, bent rods, funnels and distilled water were all sterilized before used.

Substrate preparation

Cassava peels were collected from Kerepek Industry in Kuala Langat, Selangor, Malaysia. They were transported to the Environmental Biotechnology Laboratory of the Department of Biotechnology Engineering, International Islamic University Malaysia, where they were immediately washed with copious amount of water to remove sand, and subsequently dried at 60°C in an air-forced oven for 48 h to avoid deterioration. The dried cassava peels (DCP) were milled and allowed to pass through 2 mm sieve before being stored in airtight container. The chemical composition of unfermented cassava peel was analyzed and recorded.

Response surface methodology (face-centered central composite design)

The Face-Centered Central Composite Design (FCCCD) under the response surface methodology was used to determine the influence of medium constituents in enriching the nutritional value of cassava peel and to identify the optimum levels. Three independent variables namely wheat flour, $MgSO_4$ and particle size were investigated at three levels (low, basal, high) coded as (-1, 0, 1). The detail experimental designs were presented in Table 1. Twenty experiments with six replications at center points were studied. The total fermentation media was fixed at 20 g, consisting of 30% solid (Dried Cassava Peel plus wheat flour) with 70% Moisture content. The concentration of wheat flour was based on the experimental design while cassava peels make up for 30% solid. The moisture content, 70% (v/w) in form of inoculum 6% (v/w), 5% mineral solution consisting of fixed minerals -$(NH_4)_2SO_4$ (1.5 g/kg DCP) $MnSO_4$ (0.05 g/kg DCP) and KH_2PO_4 (0.8 g/kg DCP) and varying concentration of $MgSO_4$ according to the experimental design and 59% distilled water (v/w). The particle size was according to the experiment design combination. The fermentation media was prepared in 250 ml Erlenmeyer flask, autoclaved at 121°C for 20 min and allowed to cool before inoculation. The prepared media was incubated at 30°C for 15 days. All experiments were carried out in triplicates Two dependent variables; protein and lignin content serve as the responses (Y). A second order polynomial equation shown below was used to determine the relationships that exist between dependent and independent variables.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y is the dependent variable (Protein and lignin content), X_1, X_2 and X_3 are the independent variables; (Wheat flour, $MgSO_4$ and particle size); β_0

Table 1. FCCCD experimental design showing coded and actual values with the experimental and predicted values for enrichment of cassava peel

Run	A: wheat flour (%w/w)	B: $MgSO_4$ (g/kg DCP)	C: Particle size (mm)	Protein (mg/g)		Lignin (%)	
				Experimental	Predicted	Experimental	Predicted
1	4 (0)	0.65 (0)	2(0)	57.60	61.58	7.29	6.79
2	4 (0)	0.65 (0)	2(0)	62.55	61.58	6.02	6.79
3	4 (0)	0.65 (0)	3(+1)	54.66	56.68	5.90	6.21
4	5 (+1)	0.45 (-1)	1(-1)	55.85	60.64	4.81	5.22
5	4 (0)	0.65 (0)	2(0)	67.32	61.58	6.74	6.79
6	5 (+1)	0.85(+1)	3(+1)	44.35	44.56	6.03	6.27
7	4 (0)	0.45 (-1)	2(0)	74.01	69.11	6.47	6.61
8	3 (-1)	0.85(+1)	1(-1)	39.11	40.32	4.90	5.14
9	3 (-1)	0.85(+1)	3(+1)	57.14	53.11	6.16	5.96
10	4 (0)	0.65(0)	2(0)	56.61	61.58	6.53	6.79
11	5 (+1)	0.85(+1)	1(-1)	55.87	54.98	5.09	5.45
12	4 (0)	0.65(0)	2(0)	62.44	61.58	6.58	6.79
13	4 (0)	0.85(+1)	2(0)	59.93	63.45	6.41	5.77
14	4(0)	0.65(0)	2(0)	60.19	61.58	6.55	6.79
15	5 (+1)	0.45(-1)	3(+1)	49.25	50.27	7.17	6.98
16	3 (-1)	0.45(-1)	1(-1)	47.70	47.58	6.32	6.42
17	3 (-1)	0.65(0)	2(0)	46.62	50.34	7.77	8.09
18	5 (+1)	0.65(0)	2(0)	58.48	53.39	8.63	7.80
19	3 (-1)	0.45(-1)	3(+1)	57.95	58.77	8.03	7.87
20	4 (0)	0.65(0)	1(-1)	58.89	55.49	5.73	4.92

is the intercept term; β_1, β_2 and β_3 are the linear coefficients; β_{12}, β_{13} and β_{23} are the interaction coefficients; and β_{11}, β_{22} and β_{33} are the square coefficients.

Statistical analysis

The statistical software package Design-Expert_6.0.8 (Stat Ease Inc., Minneapolis, USA) was used to generate the experimental design matrix, analyze the experimental data and develops the regression model. The quality of fit of the regression model expressed as the coefficients of determination (R^2), the statistical significance determined by Fisher's F test, p-value, t-test, (ANOVA) the response surface and the contour plots were all study to estimate the model as well as to determine the optimum levels/ concentration.

Analytical method

The bioconverted substrates were milled in order to have homogenized sample. This was used for analysis of protein (Lowry *et al.*, 1951), acid detergent fiber, cellulose, lignin according to the method suggested by Goering and Van Soest (1970). All results were calculated based on dry matter.

Validation of the experimental model

Different combinations predicted by the point prediction feature of the statistical software package Design-Expert_6.0.8 were used to validate the FCCCD model developed. Four combinations of the three independent variables were experimented and the observed results were compared with the predicted results. The error analysis was computed to determine the closeness between the predicted and the observed results.

Results and Discussion

Table 1 summarizes the result obtained with the experimental design which was aimed in determining the conditions that favors maximum protein increase and maximum lignin degradation in cassava peel. A second order quadratic model equations1 and 2 were fitted to the data model for predicting responses; lignin (Y_L) and protein content (Y_P).

$$Y_L = 0.068 - 1.467E\text{-}003A - 4.204E\text{-}003B + 6.433E\text{-}003C + 0.012A^2 - 5.969E\text{-}003B^2 – 0.012C^2 + 3.027E\text{-}003A\,B - 2.328E\text{-}003BC) \quad (2)$$

$$Y_P = 6158 + 1.53A - 2.83B + 0.591C - 9.71A^2 + 4.70\,B^2 - 5.49C^2 - 5.80AC \quad (3)$$

Where A is wheat flour, B is $MgSO_4$, and C is particle size (C). Model (2 and 3) gave a low probability $P_{model} > F = 0.0046$ and $P_{model} > F = 0.0010$ for lignin and protein respectively when tested by Analysis of variance (ANOVA) Table (2 and 3).

The significant lack of fit of the two models indicates that the two model equation showed a close fit with the experimental result. The goodness of fit was evaluated by the coefficients of determination (R^2), which was 0.8088 and 0.8231, revealing that 80.88% and 82.31%, variation could be accounted for by the model equation for lignin and protein respectively. The adequate precision of 8.336 and 10.76 for lignin and protein content respectively were greater than 4, which indicates the model could be used to navigate the design space.

From the ANOVA analysis, (Table 2) for lignin content as response, the linear effect of $MgSO_4$ (B) and particle size(C) were significant ($P<0.05$) while the linear effect of wheat flour was not significant ($P>0.4311$). The co-substrate wheat flour was used as supplementary nutrient for initial microbial growth (Alam, 2008). The interactive term show no significant effect ($P>0.05$), however, the linear and quadratic effects for particle size were highly significant; thus could act as limiting variable and slight variation in its concentration would alter either the growth rate or the product formation rate and, alternatively both conditions to a considerable extent. This emphasized the importance of particle size to lignin degradation as a response to enrichment of cassava peel as animal feed.

Table 2. Analysis of variance (ANOVA) for reduced response surface quadratic model for lignin

Source	Sum of Squares	Mean Square	F Value	Prob > F	
Model	0.00150	0.00019	5.82	0.0046	significant
A	0.00002	0.00002	0.67	0.4311	
B	0.00018	0.00018	5.49	0.0390*	
C	0.00041	0.00041	12.85	0.0043**	
A^2	0.00037	0.00037	11.50	0.0060**	
B^2	0.00010	0.00010	3.04	0.1090	
C^2	0.00041	0.00041	12.77	0.0044**	
AB	0.00007	0.00007	2.28	0.1596	
BC	0.00004	0.00004	1.35	0.2706	

* $P < 0.05$ indicates the model terms are significant.
** $P < 0.01$ indicates the model terms are highly significant.

No variable in the linear term showed significant effect to protein increase and this suggests that each of the variables does not individually contribute to protein increase. However, the quadratic effect for wheat flour (A^2) was highly significant ($p<0.01$) and the interactive term between wheat flour and particle size (AB) for response protein increase was found to be highly significant (Table 3). This observation indicates that interactive effects are important for true optimization rather than the one factor-at-a time (OFAT) method (Kumar and Satyanarayana, 2007).

Table 3. Analysis of variance (ANOVA) for reduced response surface quadratic model for protein content

Source	Sum of Squares	Mean Square	F Value	Prob > F	
Model	1000.56	142.94	7.98	0.0010**	significant
A	23.35	23.35	1.30	0.2759	
B	80.33	80.33	4.48	0.0558	
C	3.52	3.52	0.20	0.6654	
A^2	259.49	259.49	14.48	0.0025**	
B^2	60.81	60.81	3.39	0.0903	
C^2	82.93	82.93	4.63	0.0525	
AC	269.15	269.15	15.02	0.0022**	

** $P < 0.01$ indicates the model terms are highly significant.

The 3D response surface is the graphical representation of the regression equation in order to determine the optimum values of the variables within the ranges considered. The response surface for the second order quadratic equation for maximum protein increase with the interaction of wheat flour concentration and particle size; is shown in (Figure. 2). The result showed that at maximum concentration of wheat flour and higher particle size, protein increase was more favoured. The highest protein content (61.54 mg/g DCP) was achieved when particle size was 2 mm and wheat flour was about 4.25% (w/w). The effect of Particle size and $MgSO_4$ on lignin degradation is shown in Figure 1a. Although the interactions of these parameters was insignificant in the optimization process, it was observed that lower particle size favours lignin degradation; this is in agreement with the report of Zadrazil and Puniya (1995) on different fraction of sugar cane bagasse assessed for their potential as animal feed, where particle size <1mm gave better lignin degradation. Maximum and minimum concentrations of $MgSO_4$ favour lignin degradation (Figure 1a). An incompletely inverted dome shape was formed with an insignificant interaction between wheat flour and $MgSO_4$, the maximum lignin degradation was found around the center of wheat flour concentration. The 3D surface plot indicate that to obtain optimal condition (maximum protein and minimum lignin content), it will involve maximizing above the centre or minimizing below the centre point for $MgSO_4$ and maximizing wheat flour. For particle size, decreasing the particle size favours lignin degradation while increasing the particle size favours protein increase. Applying the model equation with the discussion of surface plots the following set of experiments were validated (Table 4). From these set of experiments, the optimum values obtained were wheat flour 4.30% (w/w), $MgSO_4$; 0.45 g/kg and 1mm particle size to

Table 4. Validation of developed quadratic model and optimum medium constituents

Wheat flour	$MgSO_4$	Particle	Lignin		Protein		
% (w/w)	(g/kg)	size (mm)	Predicted	Experimental	Predicted	Experimental	Error (%)
4.15	0.85	1	4.18	6.98	58.24	56.24	3.43
4.2	0.85	1	4.21	7.17	58.44	65.79	0.00
4.25	0.85	1	4.25	7.15	58.59	71.81	0.00
4.3	0.45	1	4.48	6.87	64.35	78.17	0.00
3.9	0.85	3	4.95	7.72	58.88	58.72	0.27

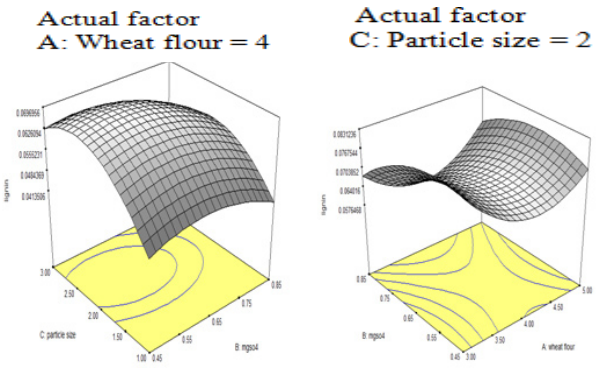

Figure 1. 3D response surface showing the effect of (a) $MgSO_4$ and Particle size on Lignin content at fixed level of wheat flour, (b)$MgSO_4$ and wheat flour on lignin content at fixed level of particle size.

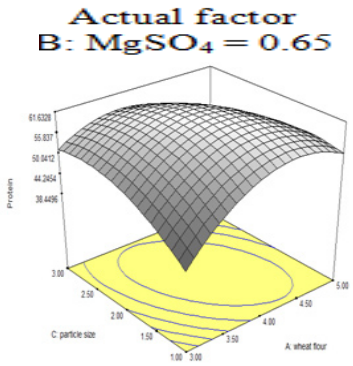

Figure 2. 3D response surface showing the effect of wheat flour (%) and particle size (mm) on protein content at fixed level of $MgSO_4$

give 6.87% lignin content and protein content of 78.17 mg/g which is 52.62% lignin degradation and 77.83% protein increase.

Conclusion

The optimum value of wheat flour, magnesium sulphate and particle size were found to be 4.30% (w/w), 0.45 g/kg and 1 mm respectively, resulting in a 77.83% enrichment in protein content and 52.62% lignin degradation in cassava peel, and suggesting a possible solution to utilization of cassava peel; thus as animal feed and also solve the environmental problem caused by their improper disposal.

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