International Food Research Journal 24(6): 2632-2639 (December 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Optimization of protein enrichment of fruit peels by mixed culture of *Phanerochaete chrysoporium* and *Schizophyllum commune* as animal feed supplement

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Article history

Received: 4 August 2016
Received in revised form: 10 October 2016
Accepted: 11 October 2016

Keywords

Amino acid
Fermentation
Bacidiomycete
Process conditions
Optimization

Abstract

Optimization of the process conditions of mixed culture of bacidiomycete fungi for improved protein enrichment of fruit peels is necessary to ease replication and scale-up processes. Six-day fermentation period and temperature of 32°C were optimum for elevated protein synthesis and enzyme activities (78.99 units/ml for α-amylase and 0.36 units/ml for cellulase). A highly significant quadratic model obtained from Face Centered Central Composite Design (FCCCD) described the process optimization. Linear effect of pH and inoculum size were significant ($p < 0.05$) while pH and moisture content (MC) interact significantly. 70.2% MC, pH 5.4 and 6.1% inoculum were the optimum level for a maximum crude protein synthesis of 198.77 mg/g. The crude protein contained essential and non-essential amino acid at a comparable level with other bioprocessed materials that are currently used as animal feed supplement.

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Introduction

Fruits and vegetables are very important sources of vitamins and minerals for the well-being and survival of mankind and animals alike (Mohapatra *et al*., 2010). Several million tons of fruits are produced annually worldwide for economic advancement and improved standard of living of farmers in cultivating countries. A direct implication of the improved cultivation processes is the high through put of lignocellulosic wastes that often constitute environmental challenges (Correia *et al*., 2007; Dhanasekaran *et al*., 2011). Open air dumping and other indecriminate methods are commonly adopted in handling wastes from fruit processing industries (Ahmed *et al*., 2002). These methods could constitute environmental concerns and require several hectares of arable land to mitigate the impact.

Fruit peels constitute more than 40% of wastes emanating from fruit processing lines. Simple and complex sugars that could support microbial growth are found in high quantities within the micro and macro fibrils of fruit wastes (Lim *et al*., 2010). Banana, papaya and pineapple peels constitute larger part of wastes generated from food and hospitality industries. These peels contain sufficient carbon source that are susceptible to biological degradation (Saheed *et al*., 2013). Therefore, deployment of biotechnological techniques that involves that cultivation of white rot fungi on fruit peels offer a holistic opportunity to handle the wastes in a manner that converts them to animal feed supplement.

White rot fungi (WRF) cells are commonly used for conversion of agro-residues to single cell protein, citric acid and many other industrial products (Tran *et al*., 1999; Jamal *et al*., 2015). WRF can convert organic residues to intended products (bioethanol, animal feed etc) through their secretion of hydrolytic enzymes that could break down complex carbon molecules in the residues to simple sugars for easy assimilation. In many conversion processes involving WRF, the fungal cells are often used as monoculture but the approach is characterized with inefficient degradation and utilization of carbon constituents of organic residues (Zhong-Tao *et al*., 2009; Gad *et al*., 2010). Cultivation of compatible mixed culture of WRF on mixed fruit peels is a robust approach that could improve WRF product synthesis through efficient substrate utilization under optimized growing conditions. Extracellular enzyme production by mixed culture of WRF increased over monoculture conversion under solid state fermentation (Zhong-Tao *et al.*, 2009).

Substrate MC, inoculum size, initial pH, fermentation period and temperature are important process parameters that influence a stable microbial

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environment for improved product synthesis (Ruqayyah *et al*., 2014). Statistical optimization of growing conditions in a fermentation process is a quick and reliable approach to identify the optimum levels of process parameters necessary for maximum product synthesis. The approach remained a viable option since it reduces cost and number of experiments (Arora and Sharma, 2011; Tijani *et al.*, 2012). Face centered central composite design (FCCCD) and one-factor-at-a-time (OFAT) are commonly used approaches, but the latter often wastes time and lacks information on interaction between parameters (Ruqayyah *et al*., 2014). Therefore, this investigation focused on application of FCCCD and OFAT optimization techniques for protein enrichment of fruit peels as animal feed supplement. The quality of the synthesized protein was measured via characterization of the amino acid profile of the final product.

Materials and Methods

Fruit peels collection and preparation

Fresh banana *(Musa sapientum)* peels (Bp), Pineapple *(Ananas cosmos)* peels (PAp) and Papaya *(Carica papaya)* peels (Pp) were collected from fruit processors within Gombak area (Selangor, Malaysia). The peels were washed to remove adhering foreign materials and dried immediately in air forced oven (Memmert) at 60°C for 48 h to arrest microbial and degradation. Dried peels were milled to 2 mm particle size with a grinding machine (Model D-79219 staufen, IKA-WERKE GMBH and Co. KG Germany); the milled fruit peels were mixed at required ratio and stored in airtight container.

Microorganisms and inoculum preparation

Two white rot fungi comprising locally isolated *Schizophyllum commune (S. commune)* with accession number EU530002 (GI: 170280299) and laboratory stock of Phanerochaete chrysosporium (ATCC 20696) were selected for mixed culture bioconversion process. *S. commune* was cultivated on malt extract agar (MEA, Merck, Germany) for 7 days at 30°C in incubator (Memmert) while *P. chrysosporium* was cultivated on potato dextrose agar (PDA, Merck, Germany) for 7 days at 30°C; each strain was subcultured every fortnight. Inoculum was prepared as described (Ruqayyah *et al*., 2013) with slight modification as 15 ml of distilled water was used to wash each fungus plate before being transferred to a sterilized flask for storage at 4°C. *S.commune* and *P. chrysosporium* were mixed at pre-optimized ratio of 1.75:1.25 g/L of biomass and were thoroughly mixed before inoculation.

Solid state fermentation (SSF)

Fermentation media (20 g) was prepared and SSF was carried out in 250 ml Erlenmeyer flasks comprising varied inoculum size, initial pH and moisture content (according to the experimental design) in Table 1. Inoculums were added after sterilization of the media at 121°C for 15 min in an autoclave (Hirayama). The prepared flasks were allowed to cool to room temperature before aseptically inoculating them. Flasks were incubated at 30°C for 7 days and all experiments were carried out in triplicates.

One-factor-at-a-time

One-factor-at-a-time (OFAT) method was used to determine the optimal fermentation period and temperature for increased products (protein, cellulase and α-amylase enzyme) synthesis. Cellulase and α-amylase enzyme synthesis associated with the optimization process in achieving improved protein content was determined.

Response surface methodology (face centered central composite design)

Face centered central composite design (FCCCD) embedded in response surface methodology was used to evaluate the effect of process parameters on protein enrichment of the fruit peels. RSM was implemented in Design Expert® version 6.0.8 (State ease, Inc., Minneapolis, USA) with three factors. The boundary levels for each parameter were 4.0 g/L and 8.0 g/L for inoculum size, 4 and 7 for initial substrate pH while moisture content was between 60% and 80%. Protein content (mg/g) of each experimental run was taken as response. Twenty experimental runs with six center points were generated as shown in Table 1. The relationship between the dependent and independent variables was accounted for by fitting experimental data to a second order polynomial model by multiple linear regressions (Equation 1). Experimental results obtained were subjected to analysis of variance (ANOVA) the model was considered significant at $p< 0.05$ and the quality of fit of the model was expressed by its coefficient of determination (R^2).

$$P = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 \quad (1)$$

Where P (protein content (mg/g)) is the predicted dependent variable of the model; β_0 is intercept; β_1, β2, and β_3, linear coefficients; β_{11}, β_{22} and β_{33}, squared coefficients and β_{12}, β_{13} and β_{23} were interaction

coefficients. The response surface graphs were used to pinpoint optimal process levels as suggested by statistical software package Design Expert® version 6.0.8.

Crude protein analysis

Protein content of dried fermented product was determined in all cases using folin phenol reagent methodology (Lowry *et al.*, 1951); only protein content was used as response for optimization while other analytical procedures were limited to OFAT optimization. Ten mg of sample was extracted with 5 ml of 1 N NaOH and allowed to stand for 24 h at room temperature; centrifugation was done at 6000 rpm for 15 min and supernatant was used for the assay.

α-Amylase activity assay

Amylase enzyme activity was carried out by preparing 0.2% soluble starch (Sigma) dissolved in boiling 0.05 M KH2PO4-NaOH buffer (pH 6.0) and cooled to 40°C. Iodine reagent was prepared fresh by diluting 1 ml of stock solution (0.5% I_2 in 5.0% KI) into 500 ml of deionized water containing 5 ml of 5 N HCI. One ml of enzyme solution was placed in a test tube and warmed to 40°C in a water bath for 10 min. enzyme reaction was carried out for 10 min at room temperature. Reaction was stopped by taking a 0.2 ml sample and adding it to 5.0 ml of iodine reagent. Absorbance was measured at 620 nm against blank (0.2 ml of water in 5 ml of iodine reagent). One ml of buffer was used in place of the enzyme as enzyme blank. Amylase activity was calculated from the absorbance by using Equation 2:

$$\alpha\text{-amylase units ml}^{-1} = [(\text{control} - \text{test})/\text{control}] \times 40D \quad (2)$$

Where D is the enzyme dilution factor and 40 represents (4.0 mg of starch present in the reaction tube times 10).

Cellulase activity assay

The cellulases enzyme activity assay was conducted as outlined by the NREL LAP-006 (Adney and Baker, 1996) with modifications. Cellulases enzyme was diluted with 0.05 N Na-citrate buffer at a pH of 4.8 so that the final volume becomes 1.0 ml. All tubes were incubated in a water bath for 60 min at 50ºC. Soluble sugar released was then determined using the phenol-sulfuric acid assay (Dubois *et al.*, 1956) while cellulases activity was calculated using glucose standard curve.

Validation of the experimental model

Different solutions suggested by the point prediction feature of the statistical software package Design-Expert 6.0.8 when maximum protein was preset were used to validate the FCCCD model. Three sets of experiments involving high, mid and low values of the parameters in the predicted solutions by the software were performed and the observed results were compared.

Amino acid determination

The amino acid composition of protein was estimated using high performance liquid chromatography (HPLC) according to the hydrolysis method already described (Khan, 1994). HPLC gradient system equipped with precolumn phenylisothiocyanate (PITC) derivatization by PICO. TAG instrument (Water Inc.) was used to analyze free amino acid of the final product.

Result and Discussion

Optimization of processing conditions by OFAT

Processing temperature and bioconversion period are optimized by OFAT while FCCCD was used to optimize inoculum size, pH and moisture content and crude protein and α-amylase and cellulose enzymes secretion were measured. Results obtained showed that protein synthesis increased sharply between day two (2) and four (4) while synthesis peaked at day six (6) (Figure 1a). This product synthesis pattern showed that 6 days is enough for the fungal cells to grow and synthesize high protein. The increased protein formation could be a direct implication of catalytic activities (conversion of complex sugars to simple sugars) of extracellular enzymes on the complex substrate. This trend is consistent with several reports concerning protein production trend over a defined processing period with synthesis of hydrolytic enzymes (Saheed *et al*., 2013; Sarria-Alfonso *et al*., 2013). Fungal bio-products synthesis have been reported to peak at day 6 while others could peak at day 3 or 4 depending on the kind of product, substrate composition and organisms used (Zhong-Tao *et al.*, 2009). However, protein synthesis dropped after day 6 until the end of day 10. The decline could be due to inhibition caused by extracellular products, depletion of limiting substrates and accumulation of metabolites (Essien *et al*., 2005).

Cellulase enzyme activity increased gradually until it peaked at day 8 while α-amylase enzymatic activity increased between day 2 and 4 while maximum synthesis appeared at day 6 (Figure 1b). The peak period of protein synthesis, coincide with peak

Table 1. Experimental design and response of process optimization by FCCCD

Experimental Run	Initial substrate pH	Inoculum size (g/L)	Moisture content (%)	Protein content (mg/g)	
				Experimental	Predicted
1	4 (-1)	4 (-1)	60 (-1)	105.77	106.41
2	5 (0)	6 (0)	70 (0)	107.5	108.84
3	5 (0)	6 (0)	60 (-1)	121.78	124.52
4	7 (+1)	8 (+1)	80 (+1)	95.22	96.58
5	5 (0)	6 (0)	70 (0)	114.44	113.29
6	4 (-1)	6 (0)	70 (0)	125.89	123.36
7	5 (0)	6 (0)	70 (0)	128.12	126.99
8	5 (0)	6 (0)	80 (+1)	107.13	106.70
9	5 (0)	4 (-1)	70 (0)	174.49	173.39
10	4 (-1)	8 (+1)	80 (+1)	164.2	164.46
11	5 (0)	8 (+1)	70 (0)	172.01	173.70
12	5 (0)	6 (0)	70 (0)	176.95	174.42
13	7 (+1)	4 (-1)	60 (-1)	169.12	163.04
14	7 (+1)	6 (0)	70 (0)	166.29	171.54
15	5 (0)	6 (0)	70 (0)	200.10	198.47
16	7 (+1)	4 (-1)	80 (+1)	195.29	198.47
17	5 (0)	6 (0)	70 (0)	197.89	198.47
18	4 (-1)	8 (+1)	60 (-1)	200.49	198.47
19	4 (-1)	4 (-1)	80 (+1)	195.29	198.47
20	7 (+1)	8 (+1)	60 (-1)	200.10	198.47

α-amylase enzymatic activity and this signifies that active breakdown of cellulose and starch is necessary for improved protein synthesis by the fungal mixed culture. There are reports showing that extracellular conversion of cellulose, hemicellulose and other complex sugars (e.g starch) is a major process path of WRF towards synthesis of bio-product. Secretion of cellase and α-amylase enzymes could have converted cellulose and starch available in the fruit peels to glucose units for WRF consumption (Zhong-Tao *et al.*, 2009; Dhillon *et al.*, 2011).

The effect of fungal growing temperature on protein synthesis was investigated by varying bioconversion temperature between 28 and 36°C. Result obtained showed that protein synthesis increased as the temperature increased from 28°C to 32°C while gradual decline sets in above 32°C (Figure 1c). This trend indicates that elevated temperature impedes protein synthesis by WRF. Temperatures above 32°C has been reported to reduce bio-product synthesis by fungal cells since the mass transfer of nutrients through the fungal hypae could be hindered (Raghavarao *et al.*, 2003).

Enzymes are temperature sensitive; therefore, elevated temperature is expected to hamper activities of cellulase and α-amylase. Results obtained clearly supported the assumption as cellulase enzyme activity remained unchanged between 28°C and

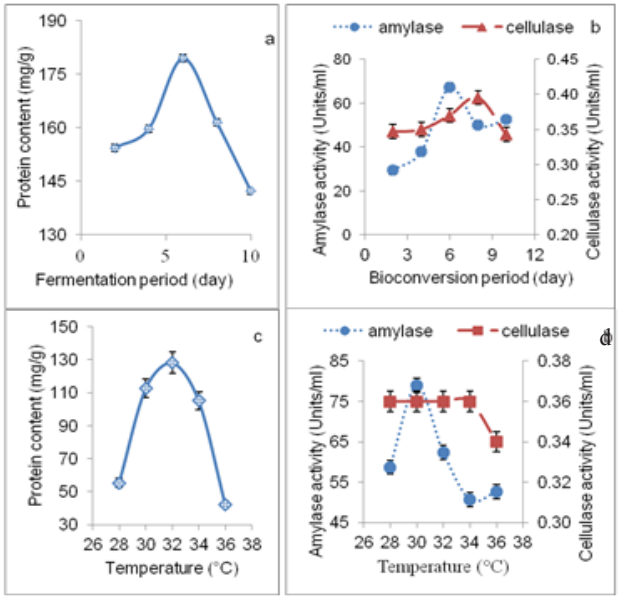

Figure 1. Effects of process conditions (a) bioconversion period on protein synthesis and (b) bioconversion period on enzymatic synthesis (c) temperature on protein synthesis and (d) temperature on enzyme activity synthesis

34°C before falling at 36°C (Figure 1d). Although α-amylase activity increased between 28°C and 30°C, its activities declined sharply afterwards. This observation showed that non thermophilic types of cellulase and α-amylase could have been synthesized by the mixed fungi. Secretion of mesophilic enzymes by WRF for nitrogen enrichment of *Opuntia ficus*

Table 2. ANOVA describing protein synthesis optimization

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	27507.56	9	3056.4	243.13	<0.0001**
A	199.45	1	199.45	15.87	0.0026**
B	1.29	1	1.29	0.10	0.7554
C	180.46	1	180.46	14.35	0.0036**
A^2	2400.27	1	2400.27	190.93	<0.0001**
B^2	1638.40	1	1638.40	130.33	<0.0001**
C^2	2674.15	1	2674.15	212.72	<0.0001**
AB	461.02	1	461.02	36.67	0.0001**
AC	29.22	1	29.22	2.32	0.1583
BC	9.7	1	9.7	0.77	0.4003
Lack of Fit	96.22	5	19.24	3.26	0.1102

R-Squared 0.995; Adj R-Squared 0.996

indica has been reported (Gad *et al*., 2010).

Optimization of processing conditions by response surface methodology

Optimum bioconversion period and 32°C temperature were fixed process parameters while pH, moisture content and inoculum size were subjected to FCCCD to identify optimal levels and nature of interaction(s). The high and low levels of the parameters were imputed into Design expert software version 6.0.8 and the center points were generated automatically. Twenty experimental runs arranged in standard order were generated (Table 1) and experiments were carried out in triplicates. Six center points are present in the design and experiments were in triplicate to facilitate adequate estimation of pure error associated with the process.

Results obtained showed that optimum protein synthesis was described by second order polynomial model. The model effectively described relationship between response (protein content) and operating parameters that was accounted for, by multiple regression analysis of the experimental data. Linear, quadratic and interaction terms of the variables that contributed to the model are retained in the reduced equation (Equation 3)

$$\text{Protein content (mg/g)} = +198.47 - 4.47^{*}A + 0.36^{*}B + 4.25^{*}C - 29.54^{*}A^2 - 24.41^{*}B^2 - 31.18^{*}C^2 - 7.59^{*}A^{*}B + 1.91^{*}A^{*}C - 1.10^{*}B^{*}C \quad (3)$$

Where A is initial pH, B is moisture content (%) and C is inoculum size (g/L)

Generally, the optimization process proceeded with comparable results of predicted and experimental results. Higher protein synthesis was recorded for all center points and this signals optimum peformance of the mixed culture at prevailing conditions in the center points. This observation showed that conversion environment offered by center points are favourable for microbial growth and product synthesis. This observation is consistent with other reports concerning performance of fungal cells under center points in a FCCCD optimization design (Ruqayyah *et al.*, 2014).

The adequacy of the quadratic model describing the optimization process could be measured through model significance, co-efficient of determination (R^2) values and Fischer's values (F-value) of parameters in analysis of variance (ANOVA). The R^2 for the model is 0.995; this indicates that 99.5% variation in the response can be accounted for by the model equation (Table 2). The lack-of-fit which is the ratio of mean square of model error to replicate error is insignificant and this suggest that observed and experimental values sufficiently fit the model (Levin *et al.*, 2008). Model F-value of 243.13 with corresponding <0.0001 p-value indicated that the model significantly account for optimum point of process parameters for protein synthesis by the fungal mixed culture.

ANOVA presented in Table 2 shows the effects of individual variables, their squared values and their interaction towards improved protein synthesis as explained by their F-values and p-values. Results showed that factor A and C are highly significant and that means these factors could be limiting factors for improving protein synthesis. The square of all the factors are highly significant while interaction of factor A and B was the only significant interaction effects. The insignificance of factor B could imply that moisture content is not limiting for improved product synthesis. The positive role of pH and moisture content in optimum production of bio-products by filamentous fungi has been reported and recorded values were consistent with present outcome (Alam *et al.*, 2008; Arora *et al.*, 2011).

Table 3. Comparison of essential amino acid profile of current study with compared *Aspergillus niger* and *Myrothecium verrucaria* bio-processed corn and sorghum waste

Amino acid	Bio-processed corn waste (%)	Bio-processed sorghum waste (%)	Current study (Bio-processed fruit waste) (%)
Isoleucine	0.37	0.40	0.29
Leucine	ND	ND	ND
Lysine	0.22	0.27	0.24
Methionine	0.09	0.05	0.04
Phenylalanine	0.44	0.46	0.30
Tryptophan	ND	ND	ND
Valine	0.37	0.47	0.37
Threonine	0.34	0.27	ND
Arginine	ND	ND	0.52
Histidine	ND	ND	0.11

ND: Not detected

Effects of interaction between pH and moisture content on protein synthesis

The effect of interaction between pH and moisture content in improving protein synthesis as depicted by 3D response surface plot showed that protein content increased as the concentration of the two parameters increased while production plummets at their high concentrations (Figure 2a). Moisture content higher than 70 % has been reported to hamper production of extracellular and cellular products accumulation of several microorganisms (Ruqayyah *et al.*, 2011; Alemu, 2013). Similarly, substrate pH of exhibit profound effects on product synthesis with fluctuations as previously reported (Ruqayyah *et al.*, 2011; Alemu, 2013).

Effects of interaction between pH and inoculum size on protein synthesis

The amount of fungal cells present in a media is a foundation for successful bioconversion process. Result obtained showed a synergistic relationship between pH and inoculum concentration used in the bioconversion process (Figure 2b). A gradual increase in both variables improved protein synthesis while their elevated concentration led to decline. Several workers have attributed low productivity and efficiency of a fermentation process to low cell population. Survival of microbial cells depends on osmotic pressure of the surrounding which may cause cells to exude cellular components into media if concentration fall below optimum (Enwefa, 1991; Essien *et al.*, 2005). Similarly, at elevated inoculum levels, swift competition among cells for space, nutrient and moisture could culminate into reduced product formation (Irfan *et al*., 2011; Alemu, 2013).

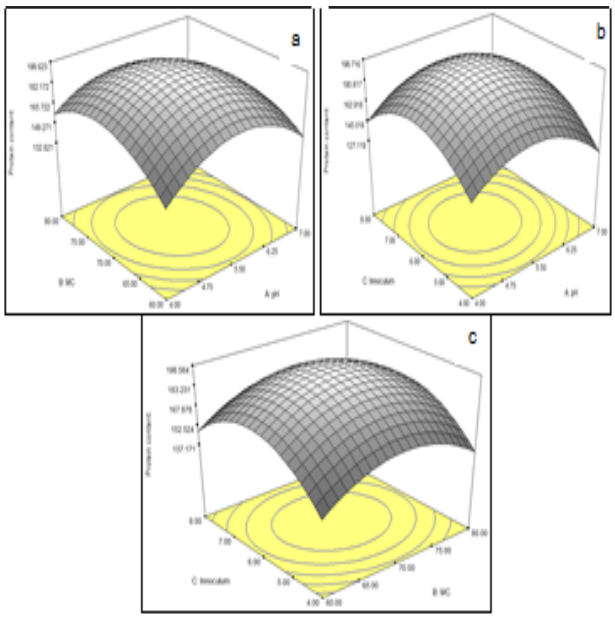

Figure 2. Response surface plots of (a) pH (b) moisture content and (c) inoculum size on protein synthesis

Effects of interaction between inoculum size and moisture content on protein synthesis

Inoculum size and moisture content are inevitable process parameters contributing to success of a fermentation process. Result obtained showed that positive interaction between the parameters trigger increased protein synthesis (Figure 2c). The optimum point for maximum protein synthesis signifies equilibrium point where amount of moisture needed by the cell is available for uptake. Increasing moisture above this optimum point meant that anearobiosis could set in due to reduced void spaces in the substrate bed which impact negatively on product formation (Raghavarao *et al.*, 2003).

Validation of the quadratic model

The quadratic model describing the values of the investigated parameters needed for optimum synthesis of product was validated by carrying out experiments at low, mid and high concentrations of the parameters as suggested by the design expert software. The predicted result for low values of processing parameters was 178.27 mg/g while the observed value was 176.37 mg/g. The medium processing values gave 198.77 mg/g and 197.08 as predicted abd observed values while 180.18 mg/g and 182.61 mg/g were predicted and observed results at high values of process parameters. The values of processing parameters leading to highest protein synthesis were pH 5.4, 70% moisture content and 6.1% inoculum size.

Characterization of the synthesized crude protein in the product

Crude protein determination is a common yardstick used for measuring protein content of bio-processed agro-residues but the method could also account for several non-protein materials in bio-processed residues (Jamal *et al*., 2009). Therefore, amino acid estimation of the product will determine protein quality of the final product (Iluyemi *et al*., 2006).

A comparison of essential amino acid content of optimized animal feed supplement showed that eight out of the ten essential amino acids were present in the product (Table 3). Although, the result showed comparable amino acid concentrations with previous study but in the present investigation, two important amino acids –Arginine and Histidine were present at required dietary level for broiler finisher feed (Mbajiorgu *et al*., 2011).

Conclusion

The optimum conversion period and growing temperature was achieved with profound synthesis of crude protein and hydrolytic enzymes. Statistical optimization produced a polynomial equation that was validated with comparable product synthesis between predicted and observed results. Amino acid profiling of the optimized product contained appreciable amount and number of essential and non-essential amino acid that complied with nutritional requirements and could serve as animal feed supplement.

Acknowledgements

The research was financially supported by a research grant EDW B13-004-0889 by the Research management Center (RMC), International Islamic University Malaysia.The authors are grateful to the RMC and Department of Biotechnology Engineering, IIUM for supporting and providing the laboratory facilities.

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