



Aspergillus niger fermentation of cocoa pod husk for increased total crude protein utilizing response surface methodology

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Abstract

Cocoa pod husk (CPH) waste from chocolate and cocoa powder production is repurposed as raw materials for animal feed using response surface methodology (RSM) with a faced central composite design (FCCD). The investigated variables included fermentation time (ranging from 7 to 21 days), the percentage of molasses (ranging from 0 to 10%), and the liquid-to-solid (L/S) ratio ranging from 0 to 15 (mg/l). Seventeen experimental runs were executed, and the accuracy of the model was verified through ANOVA ($R^2 = 0.9878$, $R^2_{\text{adj}} = 0.9804$, $p < 0.0001$). The optimal conditions for achieving the highest predicted total crude protein content (TCPC) were determined to be as follows: 7.93% TCPC, a fermentation period of 21 days, 10% molasses, and solid-state fermentation (L/S = 0). *Aspergillus niger* were used in each run to degrade CPH, boosting TCPC compared to unfermented CPH. SEM analysis confirmed CPH degradation by the microorganism. Notably, the sample collected from these optimal conditions exhibited a reduced theobromine content of 0.58 ± 0.02 mg/100 g dry mass, along with heightened antioxidant properties. This result indicates a greater-than-expected increase in protein content, highlighting fermentation's potential to enhance the value of CPH for animal feed.

Keywords Cocoa pod husk · Theobromine content · Response surface methodology · Fermentation process · Total crude protein content · *Aspergillus niger*

1 Introduction

Cocoa pod husk (CPH) is an agricultural waste generated during the production of chocolate and cocoa powder. It is derived from the cocoa fruit [1]. The mature cocoa fruit contains CPH approximately 60–70% [2], resulting in approximately 40 thousand metric tons of waste annually in Thailand. Improper disposal of this large CPH volume can cause environmental issues and waste valuable biomass resources [2]. CPH comprises various chemical components: total crude fiber (40–70%), total crude protein (5–10%), lipids (1–5%), and ash (5–10%) on a dry basis [2]. Its substantial quantity makes it suitable for applications such as organic fertilizer production, supplementation in animal feed, and bioenergy generation [3, 4]. Despite containing antioxidant substances like polyphenols and flavonoids that offer

potential health benefits, CPH also contains theobromine ($C_7H_8N_4O_2$), a bitter alkaloid [5].

Theobromine has physiological effects, including vasodilation, diuretic properties, potential health benefits, and mood enhancement [6]. However, theobromine can be toxic to certain animals due to slower metabolism, which leads to its accumulation and adverse effects [7, 8]. Therefore, a reduction process is necessary to prevent harm to animals. Fermentation is a process commonly employed to reduce the theobromine content in agricultural products [3, 8–10]. Fermentation with an alkaline environment promotes reactions and affects enzymatic activity, leading to the degradation or conversion of theobromine [4]. Molasses, which is abundant in sugars and serves as nutrients for microorganisms, is used in fermentation to break down and metabolize theobromine [11]. This supports enzymatic processes that facilitate theobromine reduction. Furthermore, the fermentation process also contributes to an increase in the total crude protein content (TCPC) of CPH [12]. Microorganisms involved in fermentation can produce proteolytic enzymes that break down proteins into smaller peptides and amino acids [13]. These modified protein structures may exhibit

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improved nutritional properties and higher bioavailability compared to the original proteins [14].

Additionally, microorganisms such as bacteria or fungi utilized in the fermentation process have the capability to synthesize proteins through their metabolic activities and multiply during fermentation to generate new protein molecules [13, 15]. Response surface methodology (RSM) is a statistical technique used to design experiments and optimize the impact of process variables. It is based on the principles of Design of Experiments (DOE), which is a field of applied statistics focused on planning, conducting, analyzing, and interpreting controlled tests to assess factors influencing parameter values [14]. The faced central composite design (FCCD) extends the widely used central composite design (CCD) for research in engineering and science. It adds “face” points on the design space boundaries, improving coverage for exploring response surface curvature [16]. The FCCD models the relationship between input variables and the response variable. By strategically collecting data, regression models describe this relationship. It combines factorial, axial, and face points to evaluate main effects, interaction effects, and curvature effects. The FCCD optimizes process exploration, aids in prediction under different conditions, and efficiently studies the impact of input variables on the response of interest [17].

The aims of this study were to enhance the TCPC in cocoa pod husk (CPH). Fermentation was employed to increase TCPC content in CPH, considering independent parameters such as fermentation time, % molasses, and liquid-to-solid ratio (L/S). DOE and RSM were utilized to optimize the single response variable, employing a FCCD. Analysis of variance (ANOVA) was carried out to validate the experimental results and ensure the acceptability of the developed model. Following this, the optimal conditions were applied to investigate the theobromine content and antioxidant substances that supported these findings, as compared to unfermented CPH.

2 Material and methods

2.1 Materials and chemical reagents

Cocoa mature fruits [Pa₇+Na₃₂] were obtained from Tah-Sala in Nakhon Si Thammarat Province, Southern Thailand. The fruits were washed with tap water, and the seeds were removed to obtain cocoa pod husk (CPH). The CPH was ground to a small size using a commercial grinder machine and then kept in a vacuum bag. It was stored in a freezer at 0°C until use. The chemical reagents used in this research were of analytical grade. Standard theobromine (purity 98.5%), absolute ethanol, acetonitrile, phosphoric acid, quercetin, the 2,2-diphenylpicrylhydrazyl (DPPH), gallic

acid, sodium carbonate, Kjeldahl catalyst, sodium hydroxide (98%), and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich Pte Ltd. (Nucleos, Singapore).

2.2 Growth of *A. Niger* in a controlled environment

The *A. niger* TISTR3013 used in this study was purchased from Thailand Institute of Scientific and Technological Research, Thailand. To culture *A. niger*, the laboratory utilized potato dextrose agar for cultivation, followed by an incubation at 30°C for a period of 5 days. The spores of *A. niger* were collected through gentle tapping of the inverted plate. Utilizing the Fuch-Rosenthal technique [18, 19], spore quantification was conducted using a hemacytometer, resulting in an estimated count of 10⁸ spores/ml.

2.3 Utilizing scanning electron microscopic for microanalysis

Fermented and unfermented CPH' samples were analyzed for their structure by scanning electron microscopic (SEM) that was performed in a SEM SU3900 device (Hitachi Instruments), operating at 15 keV. Before analysis, samples were vacuum dried and coated/metallized with a layer of gold in a sputter coater and then dried.

2.4 The increase in total crude protein in CPH

RSM DOE was employed to examine the effects of fermentation conditions on the increase in TCPC. Fermentation time (X_1), percentage of additional molasses (X_2), and the ratio of additional water to CPH (X_3) were the parameters of interest in this study. The alpha coefficient value (α) was set to face the center, resulting in 17 experimental runs obtained from the DOE. The effects of these three independent variables were represented as “-1” (low level), “0” (normal level), and “1” (high level), as shown in Table 1. The axial or star points in the FCCD variables were statistically examined. Multiple regressions were used to fit a quadratic polynomial model to the experimental design data, as shown in Eq. (1).

$$Y = b_0 + \sum_{i=1}^{i=3} b_i X_i + \sum_{i=1}^{i=3} b_{ii} X_i^2 + \sum_{i=1}^{i=2} \sum_{j=i+1}^{j=3} b_{ij} X_i X_j \quad (1)$$

Table 1 Three independent variables and their FCCD levels

Variables	Level		
	-1	0	1
X_1 : Fermentation time (days)	7	14	21
X_2 : molasses (%)	0	5	10
X_3 : L/S ratio (mL/g)	0	7.5	15

where Y is the predicted crude protein content and b_0 , b_1 , b_{ii} , and b_{ij} are the regression coefficients for the model intercept, linear quadratic, and interaction terms, respectively. The independent variables in the model were fermentation time (X_1), percentage of molasses (X_2), and liquid-to-solid ratio (X_3).

The FCCD design was carried out using Design-Expert® software for Windows, version 13. The acceptability of the model was assessed based on the correlation coefficient (R^2), adjusted correlation coefficient (R^2_{adj}), and the lack-of-fit test. Regression analysis and three-dimensional (3D) response surface plots were utilized to determine the optimal condition for protein content.

2.5 Preparation of fermented CPH sample

CPH was blended into a paste, then weighed (50g) and placed into 1000-ml Duran bottles to serve as the fermentation reactor. The fermentation process involved mixing the CPH paste with 1 ml of the *A. niger* solution (10^8 spores/ml) in each reactor. This fermentation procedure was conducted under aerobic conditions in a room maintained at temperatures between 30 and 40°C, with fermentation times determined based on the experimental design.

2.6 Determination of TCPC

The TCPC of the fermented samples was determined using the modified Kjeldahl method [20]. Two grams of dried CPH samples was placed in a Kjeldahl flask, and 20 ml of sulfuric acid and 2 g of Kjeldahl catalyst were added. The flasks were then placed in a block heater at 420°C for 3 h. The resulting solutions were diluted by adding 30 ml of distilled water, followed by the addition of 30 ml of 40% sodium hydroxide (w/v). The flasks were placed on the heater blocks and connected to a condenser for distillation. The distillates were collected using 30 ml of 4% boric acid (w/v) solution and were analyzed by titration with 0.1 N HCl as the titrant. A few drops of methyl red were used as an indicator. The total nitrogen content of CPH from the Kjeldahl method was calculated according to Eq. (2) below:

$$N\% = (V_1 \times n_1 \times F_1 \times M_w) / (w_s \times 10) \quad (2)$$

where V_1 is the volume of HCL used in the titration (ml), n_1 is the normality of HCL, F_1 is the acid factor, and M_w is the molecular weight of nitrogen. To calculate the TCPC, Eq. (3) below was used.

$$TCPC (\%) = N\% \times \text{Factor} \times F_2 \quad (3)$$

where factor value of 6.25 was used for food and feed, and F_2 is dilute factor in the dilution.

CPH extract preparation

The fermented CPH sample with the highest TCPC was extracted. The chosen sample was dried in a convection oven, and precise amounts of 1.5 g were then placed into 250-ml Erlenmeyer flasks. The flask was subsequently filled with 45 ml of 55% ethanol and placed in an ultrasonic bath at 50°C for 60 min. Afterwards, the sample was centrifuged for 10 min at the centrifugation force of 4430g at room temperature. The resulting supernatants, containing the extract solutions, were collected in individual vials and stored at 0°C until they were used to determine the theobromine content and antioxidant properties.

2.7 Determination of theobromine content

The theobromine content in the CPH extracts was analyzed by an Agilent 1200 HPLC system based on the modified method of Nguyen et al. [21]. In summary, theobromine samples (100 µg/mL in methanol) and extracts were filtered through 0.45-µm nylon membranes. Then, 50 µL of each sample was injected using an auto-injection system. A calibration curve for theobromine ranged from 0 to 400 mg/L, utilizing a C18 column (Hypersil ODS, 5 µm, 4.0 × 250 mm cartridge). The column was maintained at 35°C. Isocratic elution was achieved with a mobile phase of 80% distilled water (A) and 20% pure acetonitrile (B) at a flow rate of 1 mL/min. Detection of theobromine content in the extracts was performed at 274 nm using an Agilent Multi λ fluorescence detector (EX:330, EM:440 nm). The theobromine content in the extracts was quantified as mg/100 g dry mass using Eq. (4).

$$\text{Theobromine content} = (A \times B) / C \quad (4)$$

where A is the peak area of sample/slope of calibration curve, B is the weight of extract obtained per 100g of dry mass, and C is the weight of extract diluted in 55% ethanol before HPLC analysis.

2.8 Determination of total phenolic content

The total phenolic content (TPC) of the sample was determined using the Folin-Ciocalteu method [22]. The Folin-Ciocalteu reagent was diluted at a ratio of 1:10 with pure distilled water. Sodium carbonate with a purity of 99.5%, weighing 57.5 g, was diluted with distilled water to a final volume of 500 ml. In a test tube, 0.1 ml of CPH extract sample was added to 5 ml of Folin-Ciocalteu reagent, followed by the addition of 3.5 ml of the sodium carbonate solution. The mixture was incubated in a water bath at 40°C for 1 h. Subsequently, the samples were measured using a UV-vis spectrophotometer at a wavelength of 738 nm. Gallic acid, at concentrations ranging from 0.05 to 0.5 mg/ml, was used as the standard to generate a standard curve. The results were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of sample dry mass (d.m.).

2.9 Determination total flavonoid content

The quantification of the total flavonoid content in the selected sample was accomplished by employing a modified methodology, which was derived from the approach initially proposed by Hu et al. [23]. In a screw-capped tube, 1 mL of CPH extract solutions was diluted to 10 mL with 55% ethanol. Then, 0.5 mL of each sample extract was added, followed by the addition of 0.1 mL of 5% NaNO₂ solution. Next, 1 mL of 10% AlCl₃ solution was added to the tube. Finally, 1.0 mL of 1M NaOH solution and 3 mL of distilled water were added to the tube. The flavonoid content in each extract sample was determined using a UV-Vis spectrophotometer at a wavelength of 510 nm. The experiment was conducted in triplicate, and no sample was utilized as a blank. The results were reported as milligrams of quercetin equivalent (QE) per 100 g of dry mass (d.m.), with quercetin used as the standard to generate a calibration curve.

2.10 Determination of %DPPH scavenging activity

The DPPH radical scavenging activity of the extract samples was examined and adapted following the method of Thabti et al. [24]. Firstly, 3.0 mL of DPPH solution (200 mM DPPH in 95% ethanol) was thoroughly mixed with 1.0 mL of extract solution at concentrations of 333, 666, and 1332 µg/mL in a screw-capped tube. The absorbance was measured at 517 nm using a UV-Vis spectrophotometer. The DPPH scavenging activity (%) was calculated according to Eq. (5) below:

$$\text{Scavenging DPPH\%} = 100\% \times (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100\% \quad (5)$$

where A_{control} is the light absorption value of the DPPH solution, and A_{sample} is the light absorbance value of the extract mixed with DPPH solution.

2.11 Statistical analysis

All experiments were performed in triplicate. The data were presented as mean \pm standard deviation, with statistical analysis conducted using Design-Expert® software (version-13). One-way ANOVA was used to analyze results, with a significance level set at less than 5% ($p \leq 0.05$).

3 Results and discussion

3.1 Statistical analysis of the model by ANOVA

The experimental results obtained in this study demonstrated that the desired dependent variable was influenced by the main parameters of fermentation time (days), percentage of molasses, and liquid-to-solid ratio (L/S). The mutual and simultaneous effects of these parameters were found to be

significant in relation to the TCPC (%). The p-value of the model for the desired parameter was <0.0001 , indicating the significance of the model and the obtained results. After excluding the quadratic polynomial models, Eq. (6) represents the most significant empirical relationship between the predictors (independent process variables) and their response. This relationship was determined based on various criteria, such as lack of fit, p-value, highest R², predicted R², and adjusted R² values. The statistical parameters are provided in Table 4.

$$Y = 4.60 + 0.0814 X_1 + 0.2563 X_2 + 0.0378 X_3 - 0.0045 X_1 X_2 - 0.0088 X_1 X_3 - 0.0033 X_2 X_3 \quad (6)$$

Table 2 presents the values of various terms related to Eq. (6), encompassing fermentation time, the percentage of added molasses, and the liquid-to-solid ratio for TCPC determination. The model showcased in this table holds significance, supported by a low p-value (<0.0001) and a high F-value (134.47). A commendable model accuracy is indicated by the R² value of 0.9878, aligning with the theoretical approach that favors lower p-values, higher F-values, and elevated R² values to establish a substantial and well-fitting model. Moreover, the lack of fit test for TCPC ($p = 0.4852$) revealed that the quadratic models retained statistical significance and could be applied for further analysis. Additionally, a robust correlation between the experimental and predicted values was observed, substantiated by R², adjusted R², and predicted R² values all surpassing 0.95. This underscores the adequacy and goodness of fit of the models [25].

3.2 Accuracy check of the model using diagnostic charts

The assessment of data normality involves examining the alignment of data points around a straight line. In the normal distribution plot, shown in Fig. 1a, the correlation between variables is depicted. The results closely adhere to the straight-line pattern, confirming the normal distribution of the data. Additionally, upon comparing the predicted versus residuals data in Fig. 1b and predicted versus run in Fig. 1c, it becomes apparent that the residuals versus actual plot closely aligns with a straight line. This suggests that the results obtained from the experiments closely resemble the data generated from the runs, as illustrated in Fig. 1d. These findings underscore the consistency between predicted values and the values acquired from experiments, thereby affirming the accuracy of the designed model.

3.3 Contour plots of variants parameters

The outcomes derived from the experiments and model predictions are displayed in Table 3. The influence of chosen input

Table 2 RSM quadratic model analysis of variance (ANOVA) of CPH fermentation experiments

TCP content (%).						
Source	Sum of squares	df	Mean square	F-value	p-value	
Model	15.02	6	2.50	134.47	< 0.0001	Significant
X_1 -Time	0.9283	1	0.9283	49.88	< 0.0001	
X_2 -%molasses	2.27	1	2.27	121.71	< 0.0001	
X_3 -water-to-solid ratio	0.1109	1	0.1109	5.96	0.0348	
X_1X_2	0.1984	1	0.1984	10.66	0.0085	
X_1X_3	1.71	1	1.71	91.95	< 0.0001	
X_2X_3	0.1200	1	0.1200	6.45	0.0294	
Residual	0.1861	10	0.0186			
Lack of fit	0.1576	8	0.0197	1.38	0.4852	Not significant
Pure error	0.0285	2	0.0142			
Cor total	15.20	16				
Std. dev.	0.1364	Mean	5.94	C.V. (%)	2.30	
R^2	0.9878	Adjusted R^2	0.9804	Predicted R^2	0.9683	
Adeq precision	43.7059					

df degrees of freedom

variables and their interactions on TCPC percentage was simulated. This simulation is illustrated in Fig. 2, showcasing how TCPC varies between two variables while keeping the third one constant at its mean value. In Fig. 2a–c, distinct conditions are depicted for TCPC. Notably, the fermentation time, %molasses, and L/S ratio were found to exert a significant impact on TCPC. Increasing %molasses from 0 to 10% led to a rise in TCPC from approximately 5 to 8%. Conversely, an initial increase in the L/S ratio resulted in a decrease in TCPC from about 8 to 5%. Furthermore, following 21 days of fermentation, the TCPC escalated from roughly 5 to 8%. The initial TCPC in fresh CPH was approximately 4.75%. These findings conclusively demonstrate that the chosen input variables particularly fermentation time, %molasses, and L/S ratio play a pivotal role in determining TCPC.

Response surface plots and effects of variants variables

The model equation indicates a positive correlation between TCPC and fermentation time as well as % molasses, while the L/S ratio has a negative effect. Graphs depicting the actual results against the predicted values for TCPC were generated using RSM in Design-Expert® software. The 3D surface plot illustrates the impact of fermentation time and % molasses on TCPC in Fig 3. The TCPC increases significantly with longer fermentation time (from 7 to 21 days) and the addition of molasses (from 0 to 10%). Natural microorganisms play a crucial role in biodegradation, breaking down organic materials into simpler compounds, and the addition of molasses provides a sufficient food source to activate their growth [12]. Microorganisms synthesize proteins using the nutrients derived from the degradation of organic matter, and they also produce enzymes for biodegradation, many of which are proteins themselves [12]. The variety of microorganisms

participating in the fermentation process can also impact the protein content. Various microorganisms might exhibit differing rates of protein synthesis and specific requirements, collectively contributing to an overall rise in protein levels. Conversely, as the fermentation progresses, microorganisms could selectively target constituents of CPH, and molasses based on their metabolic capabilities. Consequently, this selective utilization of carbohydrates and other nutrients can lead to an enhanced synthesis of proteins [12]. A high L/S ratio, referred to as submerged fermentation, significantly affects protein synthesis. Insufficient or imbalanced nutrients in the medium can limit the microorganisms' ability to efficiently produce protein, resulting in reduced TCPC. In this study, TCPC ranged from 4.12 to 7.85%. Through modeling calculations, the maximum predicted TCPC was 7.93% at 21 days of fermentation time with the addition of 10% molasses, and without additional liquid media.

3.4 Optimal state validation

The optimal conditions were determined to be a fermentation time of 21 days, with the addition of 10% molasses, and employing solid-state fermentation (L/S=0). To validate the model, the optimal predicted values generated by Design-Expert® software were compared with the actual values. The optimal predicted values are accompanied by a 95% confidence interval (CI). The actual value of TCPC was $7.85 \pm 0.02\%$, whereas the predicted value stood at 7.93%. For a 95% confidence interval, the lower bound value was calculated at 7.67%, while the upper bound value was determined to be 8.19%. The optimal conditions, namely 10% molasses addition and a fermentation period of 21 days,

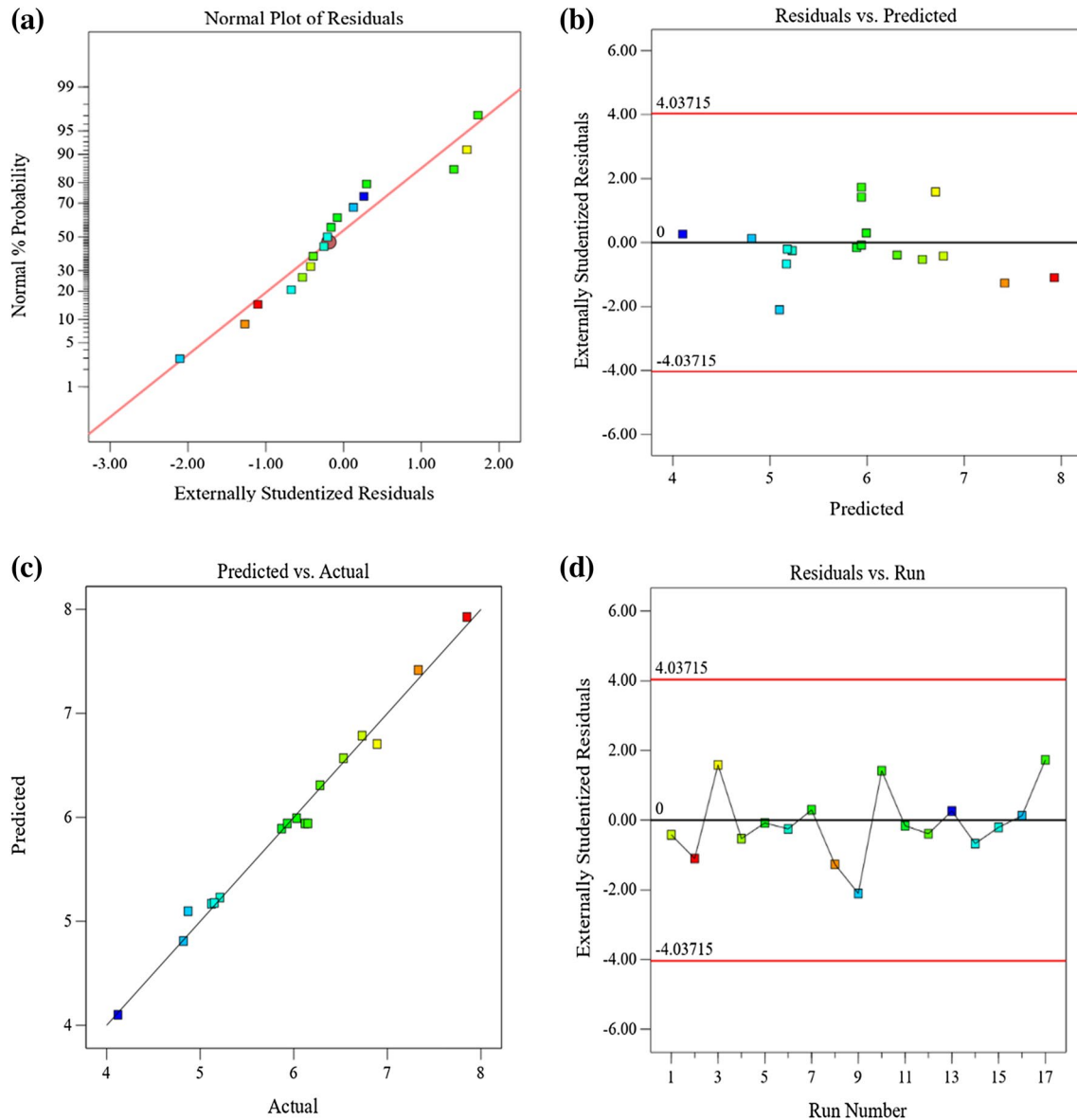


Fig 1 Distribution of data for total crude protein content (TCPC); **a** normal plot of residuals; **b** predicted vs. residuals; **c** predicted vs. run; **d** actual vs. residuals

conducted under solid-state ($L/S = 0$) fermentation, were successfully scaled up to a 20-l fermentation system. TCPC of the optimal condition was measured at $7.99\% \pm 0.08\%$. The observed value exceeded the predicted value obtained through RSM, indicating that the experimental conditions yielded a more favorable outcome than initially anticipated.

3.5 Examination of the structural configurations of CPH using SEM

Figure 4a–c present SEM images of unfermented cocoa pod husk (CPH) samples, while Fig 4d–f display SEM images of fermented CPH samples at a 1000 \times magnification. These

images reveal significant alterations in surface texture, porosity, and the exposed structural characteristics.

In Fig 4a, we observe the epicarp, which is the outer layer of the CPH where the deposition of epicuticular waxes on the fruit takes place. Turning our attention to Fig 4d, it becomes evident that the epicarp has undergone substantial changes following the fermentation process, showcasing the effective decomposition of epicuticular waxes by microorganisms.

Fig 4b and e provide cross-sectional views of fresh CPH and fermented CPH, respectively. A marked contrast is observable when comparing fresh CPH, characterized by gaps between porous cell walls due to the rapid evaporation

Table 3 Experiments designed for the total crude protein content (TCPC) using the FCCD design-response surface method

Run order	Time (days) (X_1)	% Molasses (X_2)	L/S ratio (X_3)	Actual value	Predicted value
1	14	10	7.5	6.73	6.79
2	21	10	0	7.85	7.93
3	14	5	0	6.89	6.71
4	7	10	15	6.53	6.57
5	14	5	7.5	5.93	5.94
6	21	10	15	5.21	5.23
7	7	5	7.5	6.03	5.99
8	7	10	0	7.33	7.42
9	14	0	7.5	4.87	5.10
10	14	5	7.5	6.12	5.94
11	21	5	7.5	5.87	5.89
12	21	0	0	6.28	6.31
13	21	0	15	4.12	4.10
14	7	0	0	5.12	5.17
15	14	5	15	5.15	5.18
16	7	0	15	4.82	4.81
17	14	5	7.5	6.15	5.94

of moisture during the drying process while preserving the overall cell structure, with fermented CPH. The latter exhibits clear signs of digestion by microorganisms, resulting in the fragmentation of the mesocarp layer. Notably, fungal growth was also detected during the fermentation process.

Lastly, when analyzing the endocarp layer of the CPH, a striking disparity emerges between picture (c), representing the state before fermentation, and picture (f), illustrating the post-fermentation condition. In picture (f), decomposition is evident, manifesting as a rough and cracked surface [26]. Consequently, it is reasonable to conclude that the fermentation process has indeed led to the decomposition of CPH, a phenomenon in line with the observed increase in the TCPC value.

A. niger employs diverse mechanisms to effectively break down and utilize CPH, encompassing both plant biomass and industrial byproducts. The fungus secretes specific extracellular enzymes, including cellulases, hemicellulases, pectinases, amylases, and proteases, to target components within the CPH. This process results in the decomposition of cellulose and hemicellulose into simpler sugars like glucose, xylose, and mannose [27]. Pectinases work on complex polysaccharides such as pectin, transforming them into sugars and oligomers. Amylases play a crucial role in the hydrolysis of starch into maltose and glucose, while proteases break down proteins into amino acids present in the CPH [28]. *A. niger* strategically utilizes the breakdown products from CPH as sources of carbon and energy for its metabolic processes. The fungus demonstrates versatility by engaging in fermentation processes under suitable conditions, leading to the production of organic acids, notably citric acid, contributing to its energy supply [13]. This capacity to generate

organic acids enhances the acidic environment, supporting the efficiency of enzymatic activities specific to the CPH. The fungal hyphae of *A. niger* exhibit their ability to penetrate CPH, facilitating efficient exploration and utilization of available nutrients. Crucially, *A. niger* exemplifies a sophisticated regulatory mechanism in enzyme production, adjusting its enzymatic toolkit based on the unique composition of CPH. This dynamic interplay of enzymatic degradation, metabolic utilization, and regulatory adjustments underscores the fungus' effectiveness in breaking down and assimilating CPH.

3.6 Theobromine reduction

The HPLC analysis of the theobromine standard and fermented CPH extracts revealed a peak at 2.509 min. As depicted in Fig 5, the standard theobromine peaks and theobromine contents were observed under the optimal conditions of fermented CPH samples. In Fig 5a, the initial theobromine content in unfermented CPH was quantified at 59.50 ± 1.22 mg/100 g dry mass. Following fermentation with 10% molasses, without additional water, and a fermentation period of 21 days, as shown in Fig 5b, the theobromine content in CPH decreased to 0.58 ± 0.02 mg/100 g dry mass. This reduction can be attributed to the proliferation of indigenous microorganisms during fermentation, which facilitates the breakdown of theobromine into smaller molecules [29]. Xanthine, theophylline, and methylxanthines are some of the compounds formed through the destruction of theobromine [6]. Although these compounds share structural similarities, they possess distinct chemical properties. Under

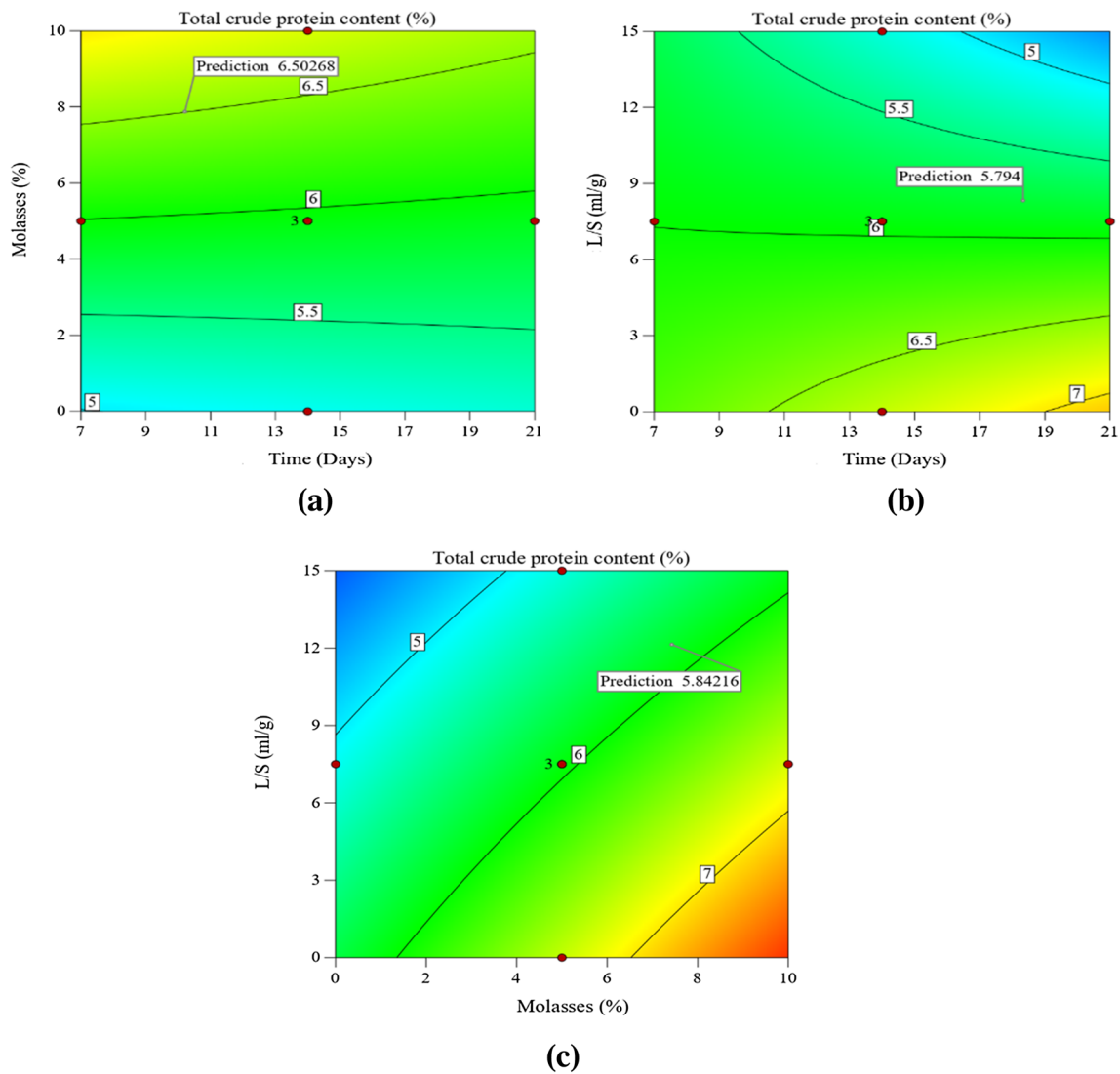


Fig 2 Contour plots depicting the combined effect of two different variables on total crude protein content (TCPC), **a** time vs. % molasses, **b** time vs. L/S ratio, and **c** % molasses vs. L/S ratio

the fermentation conditions of excess molasses and a solid-state setup ($L/S=0$) and conditions, the microorganisms, predominantly *A. niger*, exhibited growth under these optimal conditions. *A. niger* is recognized for its capacity to generate diverse enzymes, including cellulases and hemicellulases, which play a crucial role in breaking down complex carbohydrates such as fibers. Furthermore, *A. niger* exhibits the capability to produce organic acids, with citric acid being a prominent example. According to findings reported by other study, it has been observed that when the pH of the fermentation process is decreased, there is a significant reduction in the theobromine content during the fermentation of CPH [30–32]. The reduction in theobromine content of cocoa pod husk (CPH) after the fermentation process enhances

the efficiency of CPH as an alternative animal feed material, thereby mitigating potential harmful effects on animals [33].

3.7 Total phenolic content

The value of total phenolic content from optimum conditions was determined equal to 2127.76 ± 19.29 mgGAE/100g dry mass. The presence of molasses in the fermentation process can facilitate the breakdown of complex organic compounds into simpler compounds, leading to an increase in the total phenolic content [34, 35]. This breakdown process allows larger phenolic compounds to be transformed into smaller, and more bioavailable forms. The increase in total phenolic content observed after *A. niger* fermentation is a result of

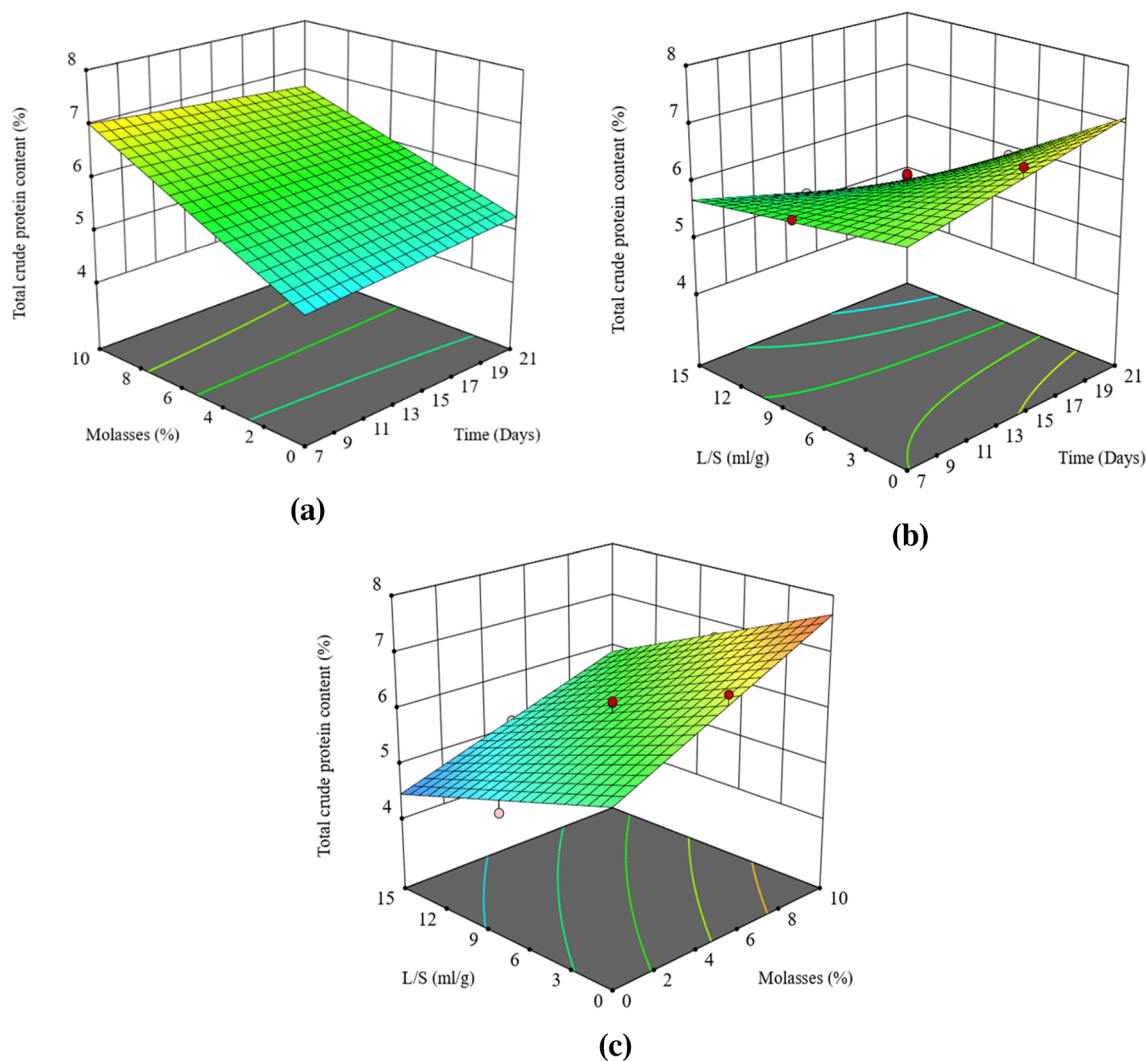


Fig 3 Response surface plots of the effects variants variables to total crude protein content (TCPC) (%), **a** times vs. % molasses, **b** time vs. L/S ratio, and **c** % molasses vs. L/S ratio

various factors working together. These include enzymatic activity, bioconversion, substrate metabolism, release from the matrix, and microbial synthesis. *A. niger* utilizes enzymatic processes to break down complex compounds in the substrate, releasing phenolic compounds that were previously bound in the cellular structure of the raw material. Additionally, *A. niger* can convert precursor compounds into phenolic compounds with enhanced antioxidant activity, leading to an accumulation of total phenolic content in the final product. As *A. niger* metabolizes substrates for growth, the breakdown of these substrates generates intermediate products, some of which may be phenolic compounds, contributing to an overall increase. Furthermore, phenolic compounds that were bound or conjugated in the raw material matrix can be released by microbial activity, making them more available and adding to the observed rise in total phenolic content. The microbial synthesis of phenolic

compounds during metabolic processes further contributes to the overall accumulation of phenolic content in the fermented product. These complex mechanisms highlight the dynamic nature of changes in phenolic composition facilitated by *A. niger* during fermentation. A study conducted by Ouattara et al. investigated the effect of fermentation with *Lactobacillus plantarum* on the phenolic content, antioxidant capacity, and bioactive compounds of cocoa pod husk from Ghana. Their results showed that the total phenolic content increased from 626.66 ± 22.01 mg GAE/100g dry mass to 2127.76 ± 19.29 mg GAE/100g dry mass after fermentation [36].

3.8 Total flavonoid content

The total flavonoid content value obtained under the optimal conditions was determined to be 4198.30 ± 24.71

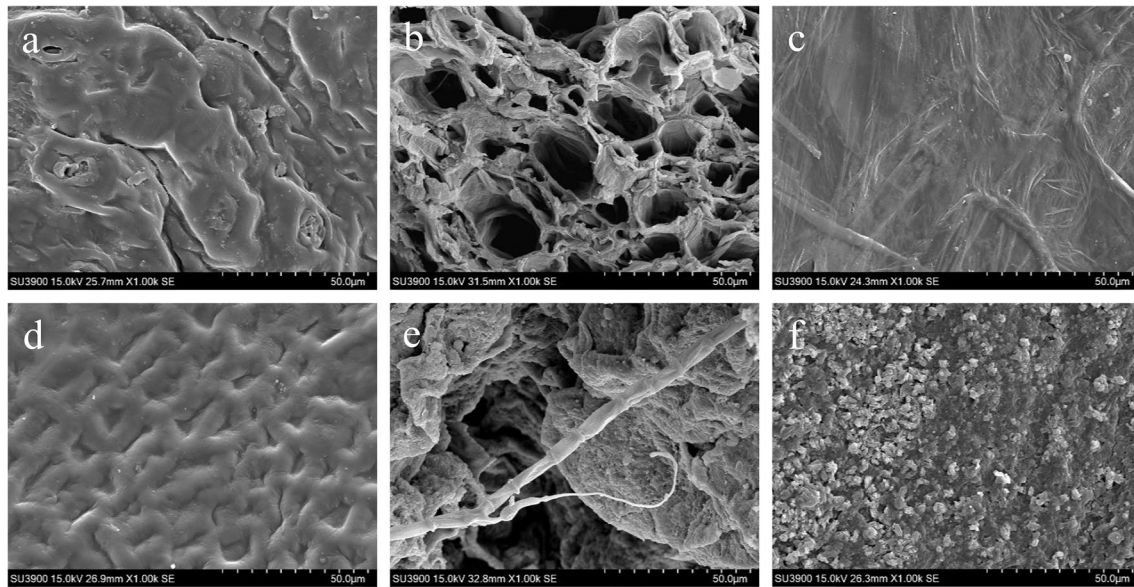


Fig 4 Scanning electronic microscopic photo of cocoa pod husk (CPH) sample: **a** outer layer (epicarp) of fresh CPH; **b** middle layer (mesocarp) of fresh CPH; **c** inner layer (endocarp) of fresh CPH; **d**

outer layer (epicarp) of fermented CPH; **e** middle layer (mesocarp) of fermented CPH; **f** inner layer (endocarp) of fermented CPH, all magnified at 1000 \times

mg/100g dry mass. The highest total flavonoid content was observed in the optimal conditions. This discovery suggests that the fermentation process has the potential to

enhance the overall flavonoid content, with the addition of 10% molasses (w/w) contributing to the highest value. Flavonoids, encompassing flavanols, flavones, and condensed

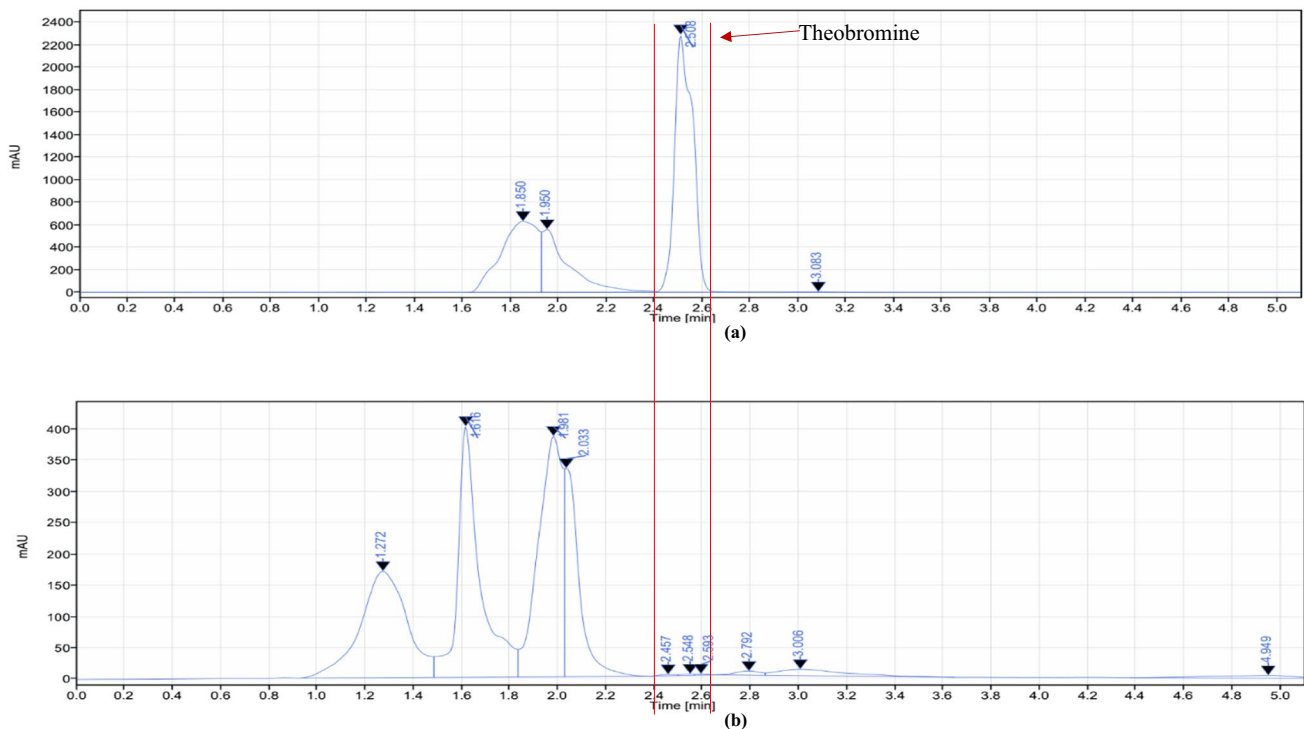


Fig 5 High performance liquid chromatography (HPLC) output of cocoa pod husk (CPH) for **a** calibration peak of theobromine substance at approximately 200 ppm, **b** CPH fermented with 10% of molasses, 21 days of fermentation period, and at solid-state fermentation (L/S = 0)

tannins, are recognized to experience an increase during the fermentation process due to the proliferation of microorganisms. *A. niger* play a role in catalyzing the conversion of flavonoids into flavonoid derivatives [22, 37, 38] Additionally, fermentation can influence the extraction and solubility of flavonoids within the fermented matrix. It has been observed that some flavonoids become more soluble in solvents compared to their non-fermented counterparts, which contributes to an increase in the measured total flavonoid content [39].

3.9 %DPPH scavenging

In this investigation, the extracts for free radical scavenging potential was studied using the DPPH assay. The %DPPH values from the optimal conditions was $88.33 \pm 4.32\%$. The addition of 10% molasses strongly influenced the change in %DPPH, as the molasses acted as a nutrient source for natural microorganisms, thereby activating their growth. The increased presence of microorganisms involved in fermentation can produce metabolites with antioxidant properties [40]. The increase in %DPPH can also be attributed to the formation of new bioactive compounds during fermentation or the breakdown of larger antioxidant molecules into smaller, more bioavailable forms, which enhances the %DPPH scavenging activity [41].

Essentially, antioxidants play a crucial role in maintaining optimal animal health by neutralizing free radicals and preventing oxidative stress. Their anti-inflammatory effects help preserve tissues, support the immune system, and provide essential cellular protection. With links to cardiovascular health and disease prevention, antioxidants are vital for promoting overall well-being. Incorporating these compounds into animal nutrition is essential to reduce the risks associated with oxidative stress and ensure a healthier life [42–44]. The antioxidant properties of the optimal conditions determined through RSM were compared with those of unfermented CPH, as depicted in Table 4.

4 Conclusion

The optimal conditions for fermentation to enhance TCPC were achieved through 21 days of fermentation period, with 10% molasses, an L/S ratio of 0, and 1 ml of *A. niger* solution (10^8 spores/ml). The successful application of response surface methodology (RSM) enabled the identification of key variables influencing TCPC increase. This was further supported by statistical parameters confirmed through ANOVA. The quadratic model, boasting an R^2 value of 0.9878, R^2_{adj} value of 0.9804, and a p-value of <0.0001 , effectively demonstrated its predictive capacity. SEM analysis indicated that the CPH was decomposed by *A. niger*, leading to an enhancement in the production of simpler substances, especially TCPC. Fermentation time and the percentage of molasses exhibited positive impacts on TCPC, while the L/S ratio exerted a notably strong negative influence. As a result of these conditions, the theobromine content saw a significant reduction from 59.50 ± 1.22 to 0.58 ± 0.02 mg/100 g dry matter compared to the unfermented CPH sample. Remarkably, these optimized conditions not only minimized the theobromine content but also maximized antioxidant activities, including TPC (total phenolic content), TFC (total flavonoid content), and %DPPH (percentage of DPPH radical scavenging), which measured 2127.76 ± 19.29 mg GAE/100g dry mass, 4198.30 ± 24.71 mg QE/100g dry mass, and $88.33 \pm 4.32\%$, respectively. The fermentation of CPH using *A. niger* can increase the TCPC while simultaneously reducing the amount of theobromine, which may cause harm to animals. Furthermore, it provides a comprehensive understanding of the variables that positively influence the experiment. Moreover, it was discovered that the fermented CPH exhibited a higher level of antioxidant substances compared to the samples that did not undergo the fermentation process. These findings carry significant implications for potential CPH utilization across various industries, particularly in the realms of food and agriculture. The developed model serves as a valuable tool for future research and holds the potential for application on a larger scale within the industrial domain.

Table 4 Quantification of antioxidants substance

Sample	Mean \pm SD		
	TPC (mg GAE/100g dry mass)	TFC (mg QE/ 100g dry mass)	%DPPH
Unfermented	626.66 ± 22.01	702.07 ± 124.53	79.58 ± 0.78
Fermented	2127.76 ± 19.29	4198.30 ± 24.71	88.33 ± 4.32

TPC total phenolic contents, SD standard deviation ($n = 3$), GAE gallic acid equivalent, TFC total flavonoid content, QE quercetin equivalent

Author contribution NS was responsible for writing the original draft and conducting formal analysis, while PP and JC were responsible for conceptualization, reviewing, and editing.

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Data availability All obtained data during this work are included in this manuscript.

Declarations

Ethical approval The manuscript is prepared in compliance with the Publishing Ethics Policy.

Competing interests The authors declare no competing interests.

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