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Response surface optimization and characterization of protein concentrate fraction from Cocoa Bean shell (*Theobroma cacao* L.) waste with evaluation of its antioxidant activity

Syafrika Nurlia Nafitri[1], Hariyanti[1]*, Supandi[1], Alda Ria Oktavian[2], Rizky Arcinthya Rachmania[2]

[1] Magister of Pharmaceutical Science, School of Postgraduate, Universitas Muhammadiyah Prof. Dr Hamka, Indonesia
[2] Department of Pharmacy, Faculty of Pharmacy and Science, Universitas Muhammadiyah Prof. Dr Hamka, Indonesia

*Corresponding author's e-mail: hariyanti@uhamka.ac.id

ABSTRACT

Cocoa bean shell (*Theobroma cacao* L.) waste is an underutilized agro-industrial by-product with potential as an alternative plant-based protein source. This study aimed to optimize the extraction of a protein concentrate fraction from cocoa bean shell (CBS) waste using Response Surface Methodology (RSM) and to evaluate its chemical characteristics and antioxidant activity. Protein extraction was performed by alkaline maceration with magnetic stirring varying pH, extraction time, and solvent-to-solid ratio. Optimization was conducted using a Box–Behnken Design (BBD). The quadratic regression model adequately described the experimental data, with an Adjusted R^2 of 0.9490 and a Predicted R^2 of 0.9549, while the lack of fit was not significant ($p > 0.05$). Among the extraction variables, pH showed the most significant effect on protein recovery ($p < 0.0001$). The optimum conditions were pH 12, extraction time of 90 min, and solvent-to-solid ratio of 0.104 g/mL, resulting in a predicted recovery yield of 1.33% with a desirability value of 0.996. Although the recovery yield was relatively low, the optimized conditions provide baseline information for future process improvement. The resulting protein concentrate fraction contained 1.30 ± 0.11 % yield, 13.67 ± 0.42% protein, 5.98 ± 0.31% ash, 1.88 ± 0.03% fat, and 5.30 ± 0.28% moisture content. SDS-PAGE analysis revealed five protein bands ranging from 15–78 kDa, while FTIR analysis confirmed characteristic protein functional groups and phenolic compounds. The major amino acids identified were tyrosine (6.31 mg/g), glutamate (1.72 mg/g), and aspartic acid (1.68 mg/g). Antioxidant activity was relatively weak, with IC_{50} values of 1020.38 ± 19.53 ppm and 1820.68 ± 39.12 ppm in the DPPH and FRAP assays, respectively. These findings indicate that CBS waste has potential as a sustainable source of protein-rich ingredients. However, further process intensification and post-extraction modification are required to improve protein recovery and functional properties.

Keywords:
antioxidant activity; Box–Behnken design (BBD); cocoa bean shell (CBS); protein concentrate fraction; response surface methodology (RSM).

Introduction

Improper disposal of cocoa bean shell (CBS) waste may contribute to environmental problems, including the accumulation of organic waste, unpleasant odours, increased biological oxygen demand (BOD), and soil and water contamination from the decomposition of lignocellulosic biomass. The increasing volume of cocoa-processing by-products therefore represents a significant environmental challenge for the agro-industrial sector (Soares & Oliveira, 2022). According to data from the Indonesian Ministry of Agriculture, Indonesia produced approximately 706.6 thousand tons of cocoa in 2022, indicating continued growth in cocoa processing and associated waste generation (Mas'ud & Sri, 2023). Based on geographical origin and seed morphology, cocoa is classified into three major varieties, namely Criollo, Trinitario, and

Forastero, with Forastero being the most widely cultivated due to its high productivity and resistance to environmental stress (Sanchez *et al*., 2023). The continuous increase in cocoa production inevitably leads to proportional increases in agro-industrial waste generation.

During cocoa processing, only approximately 10–15% of the total fruit weight is utilized as dried cocoa beans, while the remaining 85–90% consists of cocoa pod husk, pulp, and cocoa bean shell, which remain largely underutilized (Soares & Oliveira, 2022). It has been estimated that producing 1 ton of dried cocoa beans generates nearly 19 tons of by-products and waste (Sanchez *et al*., 2023). Currently, CBS waste is mainly used as low-value animal feed or compost and is often discarded without proper management, potentially contributing to environmental pollution and inefficient biomass utilization (Nurvabilla *et al*., 2024). These conditions highlight the urgent need for innovative valorization strategies to convert CBS waste into value-added products using scientific and technological approaches.

Recent studies have demonstrated that CBS contains substantial nutritional components comparable to those of cocoa beans. The protein content has been reported to range from 16–18 g/100 g and may reach 18.2–27.4% on a dry weight basis, exceeding that of several other cocoa by-products such as cocoa pulp (0.4–6 g/100 g) and cocoa pod husk (4–11 g/100 g) (Angioni *et al*., 2021; Soares & Oliveira, 2022; Sanchez *et al*., 2023). In addition to protein, CBS contains carbohydrates and dietary fiber ranging from 13.2–70.3 g/100 g, mainly composed of glucose, galactose, mannose, and arabinose (Angioni *et al*., 2021). From an amino acid perspective, CBS contains a considerable proportion of essential amino acids, including branched-chain amino acids, accounting for approximately 42% of total essential amino acids and 16% of total amino acids (Soares & Oliveira, 2022). Furthermore, roasting processes have been reported to decrease total protein content by approximately 10% while increasing the proportion of biologically active L-amino acids (Razola-Diaz *et al*., 2023).

Protein concentrate fractions have recently attracted considerable attention as functional food ingredients because specific amino acid residues, such as tyrosine, tryptophan, histidine, and phenylalanine, may contribute to antioxidant, metal-chelating, and radical-scavenging activities. The functionality of these fractions is strongly influenced by amino acid composition, peptide structure, molecular weight distribution, and the accessibility of hydrogen- or electron-donating groups (Pan *et al*., 2020). Nevertheless, CBS also contains antinutritional compounds such as tannins and high levels of polyphenols, which may contribute to bitter taste, protein–polyphenol interactions, and reduced protein digestibility (Sanchez *et al*., 2023). These interactions may limit the direct utilization of CBS proteins in food systems without further processing or purification. Despite these limitations, CBS's relatively high protein content is comparable to that of several plant-based protein sources, such as oat bran and rice bran, indicating its potential as a sustainable alternative protein ingredient.

Although interest in cocoa by-products has increased considerably, previous studies have predominantly focused on polyphenols, dietary fiber, and antioxidant-rich extracts. In contrast, studies concerning the extraction of protein concentrate fractions, amino acid profiling, structural characterization, and antioxidant activity of CBS-derived protein fractions remain limited (Pan *et al.,* 2020). The production of protein concentrate fractions is an important approach for concentrating protein components from CBS, thereby enabling a more detailed evaluation of their structural characteristics and functional bioactivities. The extracted protein concentrate fraction may exhibit antioxidant activity through radical-scavenging and metal-ion reduction mechanisms, which can be evaluated using DPPH and FRAP assays. The DPPH assay measures the ability of antioxidants to donate hydrogen atoms for neutralizing free radicals, whereas the FRAP assay evaluates electron transfer capacity through ferric ion reduction. The combination of these assays therefore provides a broader assessment of antioxidant mechanisms in the protein concentrate fraction.

From a process engineering perspective, optimization of the protein enrichment process is essential to maximize protein recovery and preserve functional properties. Alkaline extraction assisted by magnetic stirring was selected in this study because it is relatively simple, cost-effective, and widely applied for plant protein recovery. Compared with enzymatic hydrolysis or ultrasound-assisted extraction, alkaline extraction requires less sophisticated equipment and

simpler operational conditions (Peng *et al.,* 2020). In addition, Response Surface Methodology (RSM) using Box–Behnken Design (BBD) is considered an efficient statistical approach for optimizing extraction processes because it can evaluate interaction effects among variables while minimizing the number of experimental runs (Duwee *et al*., 2022).

Therefore, the novelty of this study lies in integrating RSM-based extraction optimisation with comprehensive characterisation of the resulting CBS protein concentrate fraction, including proximate analysis, FTIR spectroscopy, SDS-PAGE profiling, amino acid analysis, and antioxidant activity evaluation using DPPH and FRAP methods. In alignment with circular economy principles and sustainable agro-industrial waste management, the valorization of CBS into a protein concentrate fraction represents a promising strategy to enhance the economic value of cocoa by-products while reducing environmental impact (Areche *et al*., 2025). Therefore, this study aims to determine the optimum conditions for producing a protein concentrate fraction from cocoa bean shell waste using Response Surface Methodology with a Box–Behnken Design, to characterize the resulting fraction, and to evaluate its antioxidant potential for prospective applications as a sustainable plant-based functional ingredient.

Methods

Materials

The main material used in this study was cocoa bean shell (CBS) waste (Theobroma cacao L.) of the Forastero variety obtained from PT Riset Perkebunan Nusantara, Jember Regency, East Java, Indonesia. Sodium hydroxide (NaOH), hydrochloric acid (HCl), n-hexane, methanol, ethanol (95%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), o-phenanthroline, acetate buffer reagents, and phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). Coomassie Brilliant Blue (CBB) R-250 and bovine serum albumin (BSA) standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and solvents utilized were of analytical grade and employed without additional purification.

The instruments used included a magnetic stirrer (IKA®, Germany), centrifuge (Hermle®, Germany), UV–Vis spectrophotometer (Shimadzu®, Japan), FTIR spectrophotometer equipped with an ATR accessory (Shimadzu IRTracer-100, Japan), HPLC system (Shimadzu®, Japan), SDS-PAGE electrophoresis system (Bio-Rad®, USA), analytical balance (Ohaus®, USA), and laboratory oven (Memmert®, Germany).

Sample Preparation

CBS waste is manually sorted to separate damaged or contaminated material. The samples are sun-dried for approximately two days. The dried material is ground and sieved using a 60-mesh sieve. Defatting is carried out using n-hexane at a 1:5 (w/v) ratio for 6 hours, with periodic stirring. The defatted powder is then stored in an airtight vacuum container with silica gel at room temperature.

Optimization of Protein Concentrate Fraction Production Using Response Surface Methodology

Optimization was performed using a Box–Behnken Design (BBD) consisting of three variables at three levels: pH (8, 10, and 12), extraction time (30, 60, and 90 min), and material-to-solvent ratio (1:8, 1:10, and 1:12 w/v), adapted from (Bhattarai *et al*., 2022). For each experimental run, 200 g of defatted CBS powder was mixed with distilled water according to the designated solvent ratio. The pH was adjusted using 1 N NaOH solution. The mixture was stirred using a magnetic stirrer for the specified extraction time. After extraction, the mixture was centrifuged at 4000 rpm for 38 min at 4°C. The collected supernatant was filtered, and its pH was adjusted to 4.5. The solution was centrifuged again under the same conditions to obtain the recovered protein-enriched pellet. The yield of the protein-enriched fraction was calculated as the percentage ratio between the dry recovered fraction and the initial dry weight of the defatted CBS powder.

Characterization of the Protein Concentrate Fraction

Moisture content, ash content, fat content, and crude protein content were determined according to AOAC International official methods 942.05, 930.15, 989.05, and 984.13, respectively (Latimer, 2012).

Functional Group Analysis Using FTIR

Protein concentrate fraction samples were analyzed using an FTIR spectrophotometer equipped with an ATR accessory (Shimadzu IRTracer-100, Japan). Approximately 2 mg of sample was directly placed on the ATR crystal surface and scanned over the wavenumber range of 4000–450 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans. The spectra obtained were analyzed to identify characteristic functional groups associated with proteins and phenolic compounds (Subamia *et al*., 2023).

SDS-PAGE Analysis

Protein molecular weight distribution was analyzed using SDS-PAGE according to (Istiadi *et al*., 2024). Protein samples (≥10 µg/µL) were mixed with 4× LDS sample buffer and heated to 95°C for 5 min to denature. Electrophoresis was performed using 4–20% gradient polyacrylamide gel at 120–150 V for approximately 1.5–2 hours. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 and destaining with a methanol–acetic acid solution until a clear background was obtained. Molecular weight estimation was performed using a pre-stained protein marker.

Amino Acid Profile Analysis Using HPLC

Amino acid analysis was performed using HPLC according to Utami *et al*. (2016). A 25 mg protein-enriched fraction was hydrolyzed with 6 N HCl (1:10 w/v) at 110°C for 24 h, and the solution was neutralized using 6 N NaOH and filtered through a 0.45 µm membrane filter. The filtrate was derivatized using o-phthalaldehyde (OPA) before injection into the HPLC system equipped with a fluorescence detector. Separation was carried out using a reverse-phase C18 column under gradient elution conditions.

Antioxidant Activity Assay

DPPH Radical Scavenging Assay

A mixture of the protein-enriched fraction (0.1 ppm) and DPPH solution (50 ppm), both in methanol, was maintained at room temperature for 30 minutes and then analysed spectrophotometrically at 517 nm. Antioxidant activity was expressed as IC_{50} values calculated from the linear regression of inhibition percentage versus sample concentration (Hariyanti *et al*., 2024a).

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP reagent consisted of acetate buffer (0.3 M, pH 3.6), $FeCl_3 \cdot 6H_2O$ (20 mM), and o-phenanthroline (0.1%), all freshly prepared before analysis. A total of 2 mL of sample solution was mixed with 1 mL of acetate buffer, 1 mL of $FeCl_3$ solution, and 1 mL of o-phenanthroline solution. The mixture was incubated at room temperature for 30 minutes, and then analyzed spectrophotometrically at 517 nm (Hariyanti *et al*., 2024a)

Although FRAP results are commonly expressed as Fe^{2+} equivalent antioxidant capacity or Trolox equivalent antioxidant capacity, this study expressed antioxidant activity as IC_{50} values to facilitate comparison between DPPH and FRAP assays based on concentration-dependent antioxidant responses.

Results and Discussions

The raw material used in this study was botanically authenticated at Herbarium Bogoriense, National Research and Innovation Agency (BRIN), Cibinong, Indonesia. The identification confirmed that the sample belongs to the family Malvaceae, species Theobroma cacao L. Botanical authentication is an important step in natural product–based research because

variations in plant species may influence protein composition, bioactive compounds, extraction efficiency, and antioxidant activity. Official verification by Herbarium Bogoriense, therefore, supports the validity and reproducibility of this study.

Optimization of Protein Concentrate Fraction Extraction Response Surface Methodology (RSM)

Optimizing protein extraction from cocoa bean shell (CBS) waste was achieved through Response Surface Methodology (RSM) employing a Box–Behnken Design (BBD). This method was chosen to effectively assess interactions among extraction variables while reducing the number of experimental trials compared to traditional factorial techniques. Three independent variables were assessed: pH (A), extraction duration (B), and material-to-solvent ratio (C), with protein yield (%) as the outcome variable.

The experimental design and yield responses are presented in Table 1 using Design-Expert® 13 software. The protein yield varied from 0.07% to 1.33%, indicating that extraction conditions strongly affected protein recovery from the CBS matrix.

Table 1. Yield Results Obtained from Box–Behnken Design Optimization

Run	(A) pH	(B) Time (mins)	(C) Solvent ratio (g/ml)	Response Yield (%)
1	12	60	0.083	1.19
2	10	60	0.100	0.11
3	10	30	0.125	0.18
4	8	60	0.125	0.26
5	10	90	0.125	0.12
6	12	90	0.100	1.33
7	12	60	0.125	1.19
8	8	30	0.100	0.36
9	12	30	0.100	1.17
10	10	60	0.100	0.44
11	10	30	0.083	0.14
12	8	90	0.100	0.07
13	10	90	0.083	0.07
14	8	60	0.083	0.09
15	10	60	0.100	0.19

The highest yield (1.33%) was obtained at pH 12, extraction time of 90 min, and a solvent ratio of 0.100 g/mL. In contrast, the lowest yield (0.07%) occurred at lower pH conditions. Alkaline conditions improve protein solubility by increasing the negative charge density of amino acid side chains, thereby enhancing electrostatic repulsion among protein molecules and promoting protein release from the lignocellulosic matrix (Zhang *et al*., 2024). However, prolonged exposure to highly alkaline conditions may also induce partial protein denaturation and degradation. Although protein recovery from cocoa bean shell waste under the optimized conditions was relatively low, the results confirm the technical feasibility of protein extraction and provide a valuable baseline for future optimization with more advanced extraction technologies.

The relatively low yield also suggests that alkaline extraction assisted by magnetic stirring may be less effective than more advanced extraction methods, such as enzymatic hydrolysis, ultrasound-assisted extraction, or microwave-assisted extraction. Previous studies reported that ultrasound-assisted extraction improves cell wall disruption and mass-transfer efficiency, while enzymatic hydrolysis may release proteins bound within polysaccharide matrices (Bhattarai *et al.,* 2022; Areche *et al*., 2025). Nevertheless, alkaline extraction was

selected in this study because it is simple, economical, and applicable for laboratory-scale optimization.

Statistical Analysis and Model Validation

Sequential Sum of Squares analysis, as shown in Table 2, demonstrated that the quadratic regression model was the most appropriate model for describing the extraction process. Sequential Sum of Squares analysis indicated that the quadratic model was statistically superior. While the linear model was significant (p = 0.0085), inclusion of two-factor interactions (2FI) did not significantly improve model fit (p = 0.9357). The quadratic model showed a highly significant improvement (p = 0.0013), whereas the cubic model was aliased and therefore unsuitable. These findings support the statement by Piepho et al. (2018) that quadratic models are often optimal in RSM studies because they capture curvature effects not explained by linear or interaction terms.

Table 2. Model Determination Based on Sequential Sum of Squares

Source	***Sum of Squares***	**df**	***Mean Square***	**F-*value***	**p-*value***	
Mean vs Total	3.18	1	3.18			
Linear vs Mean	2.12	3	0.7061	6.52	0.0085	
2FI vs Linear	0.0579	3	0.0193	0.1361	0.9357	
Quadratic vs 2FI	**1.07**	**3**	**0.3579**	**29.70**	**0.0013**	***Suggested***
Cubic vs Quadratic	0.0010	3	0.0003	0.0112	0.9979	*Aliased*
Residual	0.0593	2	0.0296			
Total	6.49	15	0.4329			

Table 3. Model Determination Based on Lack of Fit

Source	***Sum of Squares***	**df**	***Mean Square***	**F-*value***	**p-*value***	
Linear	1.13	9	0.1259	4.25	0.2051	
2FI	1.07	6	0.1791	6.05	0.1487	
Quadratic	**0.0010**	**3**	**0.0003**	**0.0112**	**0.9979**	***Suggested***
Cubic	0.0000	0				*Aliased*
Pure Error	0.0593	2	0.0296			

Table 3 show the lack-of-fit test was not statistically significant (p = 0.9979), demonstrating excellent agreement between the experimental and model-predicted data and confirming the adequacy of the quadratic model for describing the extraction process, consistent with previous findings reported by Hariyanti et al. (2024). Furthermore, table 4 show the model exhibited strong predictive performance, as evidenced by the high values of R^2 (0.9818), Adjusted R^2 (0.9490), and Predicted R^2 (0.9549), while the close agreement between the adjusted and predicted coefficients indicates minimal overfitting and robust predictive reliability, supporting the suitability of quadratic models for response surface methodology optimization as reported by Omair et al. (2019).

Table 4. Model Determination Based on Fit Summary

Source	***Sequential* p-*value***	***Lack of Fit* p-*value***	***Adjusted* R^2**	***Predicted* R^2**	
Linear	0.0085	0.2051	0.5417	0.2879	
2FI	0.9357	0.1487	0.4004	-0.5764	
Quadratic	**0.0013**	**0.9979**	**0.9490**	**0.9549**	***Suggested***
Cubic	0.9979		0.8747		***Aliased***

An ANOVA was performed to evaluate the statistical significance of the quadratic regression model employed to forecast protein enrichment yield. Table 5 displays the ANOVA results for the yield response. The quadratic model was highly significant ($F = 29.96$; $p = 0.0008$). Among the tested factors, pH exerted the strongest influence ($F = 174.33$; $p < 0.0001$), confirming its dominant role in enhancing protein solubility. Extraction time and solvent ratio were not significant in their linear terms ($p > 0.05$), suggesting that within the tested range, their effects were secondary compared to pH. Similar findings were reported by Sangheetha et al. (2018), where pH was the dominant parameter in extraction processes. The quadratic effect of pH (A^2) was also highly significant ($p = 0.0003$), indicating curvature and an optimum region. Excessively high pH may induce denaturation or degradation. In contrast, the quadratic effects of time and solvent ratio were not significant.

Table 5. ANOVA Data for Yield Response

Source	***Sum of Squares***	**df**	***Mean Square***	**F-*value***		
Model	3.25	9	0.3611	29.96	0.0008	***significant***
A-pH	2.10	1	2.10	174.33	< 0.0001	
B-Time	0.0084	1	0.0084	0.7011	0.4406	
C-Concentration	0.0084	1	0.0084	0.7011	0.4406	
AB	0.0506	1	0.0506	4.20	0.0957	
AC	0.0072	1	0.0072	0.5994	0.4738	
BC	0.0000	1	0.0000	0.0021	0.9654	
A^2	1.00	1	1.00	82.96	0.0003	
B^2	0.0044	1	0.0044	0.3664	0.5714	
C^2	0.0264	1	0.0264	2.19	0.1988	
Residual	0.0603	5	0.0121			
Lack of Fit	0.0010	3	0.0003	0.0112	0.9979	*not significant*
Pure Error	0.0593	2	0.0296			
Cor Total	3.31	14				

Of the three main factors tested, pH (A) had the greatest influence on protein enrichment yield. This is indicated by a very high F-value of 174.33 and a p-value < 0.0001, both of which indicates high significance. The significant effect of pH indicates that small changes in pH significantly impact the solubility and efficiency of protein extraction from the cocoa bean hull matrix.

Chemically, alkaline pH conditions cause the carboxylic groups on amino acid side chains to deprotonate, increasing the protein's negative charge. This increased charge creates repulsive forces between protein molecules, ultimately increasing protein solubility in water. Furthermore, alkaline conditions can help loosen the protein's bonds to the polysaccharide and lignocellulose matrices in the cocoa bean hull, facilitating its release during the extraction process. Conversely, extraction time (B) and the w/v solvent ratio (C) did not show a significant linear effect on yield, with p-values of 0.4406, well above the 0.05 significance threshold. This indicates that within the time range (30–90 minutes) and solvent ratio (0.083–0.125 w/v) tested, changes in these two variables do not directly increase yield unless accompanied by appropriate pH conditions. This finding aligns with research by Sangheetha et al. (2018), which reported that pH was the dominant factor in pectin extraction from mango peel, while time and temperature did not have a significant effect individually.

Interactions between variables (AB, AC, and BC) generally did not significantly influence yield, as indicated by p-values above 0.05 for each. However, these interactions contributed to a more accurate response surface. The quadratic effect of pH (A^2) was highly significant, with an F-

value of 82.96 and a p-value of 0.0003, indicating a clear curvature in the relationship between pH and yield. This indicates that increasing pH to a certain point significantly increases yield, but beyond the optimum point, further increases in pH can cause protein degradation or excessive denaturation. Meanwhile, the quadratic effects of time (B^2) and solvent ratio (C^2) were not significant, indicating that neither variable has a sharp optimum within the tested range.

A lack-of-fit of 0.0112 with a p-value of 0.9979 indicates that the mismatch between the model and the experimental data is insignificant. In other words, the variation not explained by the model is still within the experimental error. The small residual value (0.0603) further confirms the high accuracy of the quadratic model used. This result aligns with the findings of (Paulo *et al*., 2022), who reported that a quadratic model with an insignificant lack of fit and a high R^2 value is a strong indicator of the validity of the RSM model in the extraction of antioxidant compounds.

Based on Table 5, the quadratic regression equation obtained for predicting protein enrichment yield is as follows:

$Y = 0.2467 + 0.5125(A) - 0.0325(B) + 0.0325(C) + 0.1125(AB) - 0.0425(AC) + 0.0025(BC) + 0.5204(A^2) - 0.0346(B^2) - 0.0846(C^2)$.

Where A is pH, B is time, and C is concentration.

The large positive coefficients for the pH (A) and A^2 variables confirm that pH is a dominant factor that linearly and nonlinearly affects yield. The negative coefficients for extraction time (B) and B^2 indicate that increasing extraction time beyond the optimum point can potentially decrease yield, possibly due to protein degradation or side reactions under prolonged alkaline conditions. This pattern aligns with findings (Nury *et al.,* 2024), which reported that excessively long extraction times in ultrasonic extraction systems lead to decreased yield due to degradation of active compounds.

The results of the model-prediction verification presented in Table 6 indicate that the optimum extraction conditions are predicted to be pH 12, approximately 90 minutes, and a w/v ratio of approximately 0.103, with a predicted yield of ±1.325% and a desirability value of 0.996. A desirability value approaching 1 indicates that this combination of conditions is highly optimal and meets the established optimization criteria.

Table 6. Design Expert Prediction Verification Data 13

Number	pH	Time (minutes)	Concentration (w/v)	Yield (%)	*Desirability*
1	**12.000**	**89.998**	**0.103**	**1.325**	**0.996**
2	12.000	89.998	0.103	1.325	0.996
3	12.000	90.000	0.104	1.325	0.996

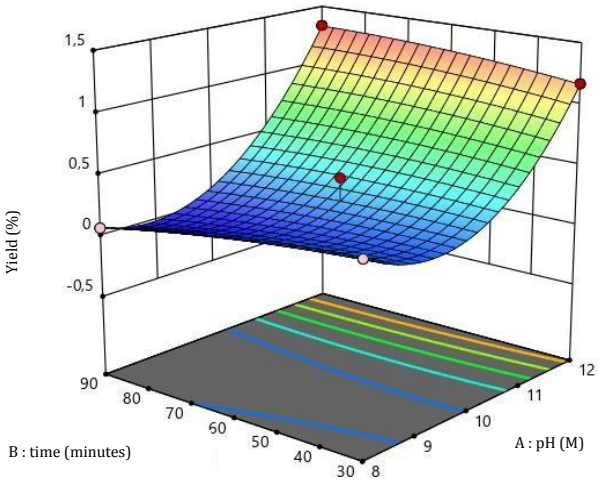

Figure 1. 3D response surface plot: the interaction effect of concentration, extraction time, and pH on protein yield.

The 3D Response Surface Plot (Figure 1) visualization shows that the interaction between pH and extraction time forms an upward-convex surface, indicating an optimum point at a combination of high pH and extraction time approaching 90 minutes. The red-to-yellow areas indicate the highest yield, while the blue areas indicate low yield at low pH and short extraction time. This pattern confirms the synergistic effect of pH and extraction time on yield. This finding aligns with a study by Duwee et al. 2022, which reported that alkaline pH and a sufficiently long extraction time significantly increased pectin extraction efficiency.

The contour plot (Figure 2) confirms these results, showing that yield increased sharply at pH ≥11, whereas varying extraction time over 40–90 minutes did not significantly change yield at low pH. This indicates that pH is the dominant factor, while time plays a supporting role. A similar pattern was also reported by Vu et al. (2019), which showed that extreme pH conditions, both very acidic and basic, increased the solubility and recovery of active compounds during extraction.

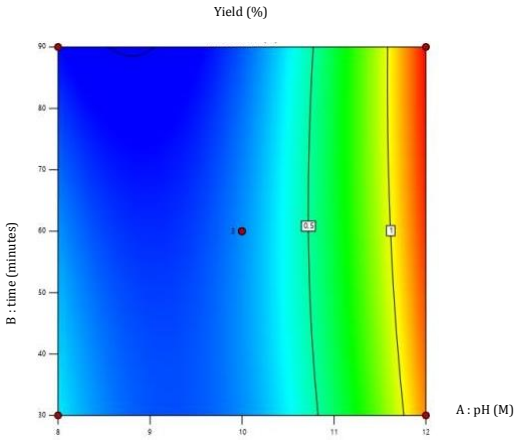

Figure 2. Contour plot showing the interaction of concentration, extraction time, and pH on protein yield.

The results of the RSM model confirmation test in Table 7 show that the predicted mean and predicted median are both 1.32303, indicating that the predicted distribution is symmetrical and unbiased. The small standard deviation (0.1098) and SE pred of 0.1454 indicate a high level of prediction precision. The actual yield (1.3015) falls within the 95% prediction interval (0.9492–1.6969), confirming that the model accurately predicts experimental results.

Table 7. Confirmation Results

Analysis	***Predicted Mean***	***Predicted Median***	**Std Dev**	**n**	**SE Pred**	**95% PI low**	**Data Mean**	**95% PI high**
Yield	1.32303	1.32303	0.109788	1	0.145441	0.949162	1.3015	1.6969

These outcomes align with research (Zakaria *et al.,* 2021) and (Mojiono & Sholehah, 2020), which reported that the success of the RSM model is demonstrated by the agreement of observed values with the predicted range, thus making the model reliable for optimizing the natural compound extraction process.

Characterization of the Protein Concentrate Fraction

Chemical and organoleptic characterization provides a preliminary overview of raw material quality, physicochemical stability, and the functional potential of the resulting protein concentrate fraction (Table 8). The concentrate fraction exhibited a brown colour, a characteristic cocoa aroma, a powder form, and a typical cocoa taste. These sensory characteristics may be associated with residual phenolic compounds and methylxanthines that have previously been

reported in cocoa bean shell materials (Soares & Oliveira, 2022; Sanchez *et al*., 2023). However, because total phenolic content was not quantitatively determined in the present study, the contribution of these compounds should be considered a plausible interpretation rather than direct experimental evidence. In addition, the observed characteristics may suggest possible protein–polyphenol interactions, which have been widely reported to influence protein solubility, digestibility, and bioactivity in cocoa by-products.

Table 8. CBS Waste Characterization Results

Chemical Characteristics	Results (Mean ± SD)
Organoleptic	Color: brown, Odor: distinctive chocolate, Form: powder, Taste: distinctive chocolate.
Yield	1.30 ± 0.11 %
Protein Content	13.67 ± 0.42 %
Ash Content	5.96 ± 0.31 %
Fat Content	1.88 ± 0.03 %
Moisture Content	5.30 ± 0.28 %

The protein concentrate fraction contained 13.67 ± 0.42% protein, indicating that cocoa bean shell waste still retains nutritionally relevant protein fractions. However, the protein content remained lower than that of commercial protein concentrate fractions, such as soy protein concentrate fraction (>80%) or pea protein concentrate fraction (>70%). This relatively low protein concentration may result from incomplete protein extraction, strong protein–polyphenol interactions, and the persistence of non-protein components within the concentrate fraction matrix.

The ash content (5.96 ± 0.31 %) remained within the Indonesian National Standard (SNI) limit of 6.0%, indicating acceptable mineral composition. The low fat content (1.88 ± 0.03 %) confirmed that the defatting process using n-hexane effectively removed most lipid fractions, while the moisture content (5.30 ± 0.28 %) suggested relatively good storage stability.

Although the physicochemical characterization demonstrated the feasibility of CBS as a plant-based protein source, this study did not evaluate protein digestibility, antinutritional compounds, or food safety aspects such as heavy metal contamination and microbiological quality. These factors are important considerations for future food application studies (Soares & Oliveira, 2022; Sabahannur *et al.,* 2023).

FTIR Analysis

FTIR analysis of the CBS protein concentrate fraction revealed typical protein absorption bands: an Amide A band at 3274 cm^{-1}, associated with N–H stretching; an Amide I band at approximately 1624 cm^{-1}, reflecting C=O stretching; an Amide II band at 1541 cm^{-1} due to N–H bending; and an Amide III band at approximately 1237 cm^{-1}, associated with C–N stretching. The presence of these bands confirms that protein constitutes one of the major recovered components within the resulting protein concentrate fraction. Furthermore, a broad O–H band in the 3300–3400 cm^{-1} range indicates the presence of phenolic groups or bound water, indicating an interaction between protein and phenolic compounds. This phenomenon is consistent with previous reports on the FTIR spectra of melanoidins and polyphenols in cocoa beans, in which similar bands were associated with the O–H groups of phenols and polysaccharides (Rahmadi *et al*., 2020). Differences in band intensity between the cocoa bean husk concentrate fraction and cocoa beans reflect variations in polyphenol content, which potentially affect protein solubility and bioactivity. Furthermore, bands in the 2922–2852 cm^{-1} range indicate the presence of aliphatic C–H stretches associated with lipid compounds or alkyl chains, consistent with the residual fat detected in the concentrate fraction. Overall, the FTIR results reinforce the findings of SDS-PAGE and HPLC that proteins in cocoa bean husks are complexed with phenolic

compounds, which has implications for the functional characteristics and bioavailability of these proteins.

SDS-PAGE Analysis

SDS-PAGE analysis of the CBS protein concentrate fraction (Figure 3) revealed five protein bands with estimated molecular weights of 78.01, 60.04, 43.93, 33, and 15 kDa. These bands indicate that the extracted fraction contains heterogeneous proteins with medium to low molecular weights. The bands observed in the 43–33 kDa and 15 kDa regions may correspond to storage protein fractions previously reported in cocoa seeds, including vicilin-like and albumin-like proteins. However, definitive protein identification cannot be established solely by molecular weight estimation and would require additional proteomic techniques such as LC-MS/MS or Western blot analysis. Previous studies have reported vicilin subunits at approximately 47, 31, and 15 kDa, whereas albumin fractions have been detected around 21 kDa (Scollo *et al*., 2018). The fermentation process, drying, and alkaline extraction conditions may also contribute to partial fragmentation of larger proteins into smaller subunits. Therefore, the assignment of these protein bands should be regarded as tentative.

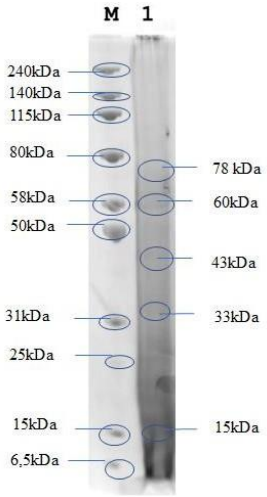

Figure 3. Markers and concentrate fraction of CBS waste

The combination of FTIR and SDS-PAGE analyses indicates that the protein concentrate fraction possesses relatively complex structural characteristics and remains associated with polyphenolic compounds. These structural properties may limit antioxidant activity because active amino acid residues remain partially embedded within the protein matrix.

Amino Acid Profile

HPLC analysis showed that free amino acids, including aspartate, glutamate, serine, and histidine, were not detected in the cocoa bean hull protein concentrate fraction. The absence of these free amino acids indicates that the protein in the cocoa bean hull is largely not in free form but is instead bound within the protein structure or complexed with other compounds. According to Sanchez *et al*. (2023), only about 1% of the protein in the cocoa bean hull is in the free amino acid form, whereas the majority is strongly bound to polyphenolic compounds through hydrogen bonds, hydrophobic interactions, or covalent bonds after phenolic oxidation. These protein-polyphenol interactions can reduce protein solubility and inhibit amino acid release, making HPLC detection without a protein hydrolysis step less than optimal.

Antioxidant Activity

The antioxidant activity of the CBS protein concentrate fraction was evaluated using DPPH and FRAP assays. The DPPH assay demonstrated weak radical scavenging activity with an IC_{50} value of 1020.38 ± 19.53 ppm. The percentage inhibition increased proportionally with increasing sample concentration, indicating a large concentration-dependent antioxidant

response. Nevertheless, the relatively high IC_{50} value suggests lower antioxidant potency than that of standard antioxidants (Utami, 2020).

Figure 4 show the FRAP assay also showed weak reducing capacity with an IC_{50} value of 1820.68 ± 39.12 ppm, indicating limited electron transfer ability. The higher IC_{50} value obtained in the FRAP assay compared with the DPPH assay suggests that the protein concentrate fraction has a relatively stronger hydrogen-donating capacity than electron-transfer capability (Zhang *et al*., 2024).

The weak antioxidant activity observed in both assays may be associated with the relatively large molecular weights and compact tertiary structures of the proteins detected by SDS-PAGE. In many protein-based systems, antioxidant activity increases substantially after enzymatic hydrolysis or fermentation because these processes generate low-molecular-weight peptides and expose amino acid residues such as tyrosine, tryptophan, histidine, and phenylalanine that can more readily donate electrons or hydrogen atoms.

Several studies have reported that peptide generation significantly enhances both DPPH radical-scavenging and ferric-reducing activities compared with intact proteins (Pan *et al*., 2020; Munteanu, 2021). Therefore, the relatively weak antioxidant activity observed in this study may reflect the limited degree of protein fragmentation achieved through alkaline extraction alone. Functional groups capable of donating hydrogen atoms or electrons, such as aromatic amino acid residues and free amine groups, may remain embedded within the protein structure, thereby reducing accessibility to DPPH radicals and ferric ions (Munteanu, 2021).

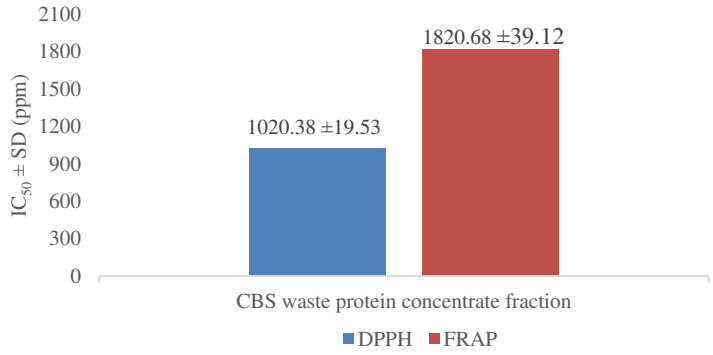

Figure 4. Comparison of IC_{50} values obtained from DPPH and FRAP antioxidant assays of CBS waste protein concentrate fraction.

Residual polyphenolic compounds may also influence antioxidant measurements. Protein–polyphenol interactions can reduce the availability of active phenolic hydroxyl groups, thereby decreasing antioxidant effectiveness. In addition, the dark brown colour of the CBS extract may have contributed to absorbance interference during spectrophotometric measurements, particularly in DPPH analysis. A blank correction was therefore applied to minimize color-related analytical interference.

Overall, the results demonstrate that cocoa bean shell waste possesses potential as an alternative, sustainable plant-based protein source. However, further optimization and structural modification are required to improve protein recovery, purity, digestibility, and antioxidant functionality for future food and nutraceutical applications.

Conclusion

This study successfully optimized protein enrichment from cocoa bean shell (CBS) waste using Response Surface Methodology, producing a maximum yield of 1.33% under optimum conditions (pH 12, 90 min, and 0.104 g/mL solvent ratio). The resulting protein concentrate fraction contained 1.30 ± 0.11 % yield, 13.67 ± 0.42% protein, 5.98 ± 0.31% ash, 1.88 ± 0.03% fat,

5.30 ± 0.28% moisture content and exhibited relatively weak antioxidant activity, with IC_{50} values of 1020.38 ± 19.53 ppm (DPPH) and 1820.68 ± 39.12 ppm (FRAP). These findings indicate that although CBS waste contains recoverable protein fractions, its antioxidant functionality remains limited, likely due to strong protein-polyphenol interactions and the predominance of intact high-molecular-weight proteins. Nevertheless, this study demonstrates the feasibility of valorizing CBS waste as a sustainable plant-based protein resource. It provides a baseline for future improvements through enzymatic hydrolysis, assisted extraction technologies, or peptide fractionation to enhance protein recovery and bioactivity.

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Conflicts of Interest

The authors declare that there are no conflicts.

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