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Optimization of Process Conditions for the Production of High-yield and High-quality Edible Bird's Nest (EBN) Hydrolysate

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ABSTRACT

Edible bird's nest (EBN) hydrolysate is widely used in EBN downstream products. This study aimed to optimize the process conditions (combination of heat treatment and enzymatic hydrolysis) to produce high-yield and high-quality EBN hydrolysate. The effects of four factors in the process were studied by response surface methodology. The experimental factors are EBN temperature during double boiling (DB), DB duration, enzymatic hydrolysis duration, and the ratio of EBN to water. The recovery (yield) and quality (sialic acid [SA], 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid [ABTS], and 2,2-diphenyl-1-picrylhydrazyl [DPPH]) of the final product were used as response variables. The Pearson correlation coefficient showed that: EBN temperature during DB affected product recovery ($p < 0.01$) and ABTS ($p < 0.01$), DB Duration affected DPPH ($p < 0.01$), and the ratio of EBN to water affected product recovery ($p < 0.01$). The duration of enzymatic hydrolysis was not significantly correlated with any of the responses and least significant factors in the model. Two optimal conditions for the processes obtained from this study were yield (product recovery) and quality. This study also showed that EBN hydrolysate produced from EBN by-products could be used as a nutraceutical because of the antioxidant activity and high SA content.

Keywords: Edible bird's nest, enzymatic hydrolysis, heat treatment, sialic acid

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INTRODUCTION

For centuries, edible bird's nest (EBN) has been a tonic recognized by the Chinese community. The efficacy of EBN is documented in Traditional Chinese Medicine (TCM) records. Over the past 10 years, EBN has been known for its nutritional and medicinal properties, and its beneficial properties have also been confirmed by modern scientific research. EBN contains carbohydrates, protein, glycoprotein, moisture, fat, and ash. Compositional analysis of the purified EBN glycoprotein from a previous study showed that the EBN glycoprotein contained approximately 14% SA, 63% protein, and 21% total saccharide (Xu et al., 2019). SA is the signature component of EBN. The basic molecular structure of SA is an acidic amino sugar with a pyranose structure containing nine carbon atoms; it is a group of derivatives of neuraminic acid. EBN was reported to have the highest SA content in the natural world (Dai et al., 2022). Consumers always refer to the presence and percentage of SA to determine the purity of EBN. SA accounts for about 10% of EBN (Dai et al., 2021).

Before consumption, EBN must undergo a series of treatments. Different EBN products require different treatments and, therefore, different processing techniques to remove harmful substances, retain nutrients, control content, and improve taste. Processing technology is divided into three categories according to technological progress: primary processing, deep processing, and biotechnology processing. Primary processing (raw material: raw uncleaned (RUC) EBN, product: raw cleaned (RC) EBN) is a necessary process for most EBN products. RUC EBN sorting, primary washing, softening, picking, molding, drying, quality control, sterilization, packaging, and other primary processing processes. Primary processing involves laborious cleaning procedures resulting in high processing costs (Ng et al., 2020). Picking is the most tedious step, which determines the cleanliness of the EBN and the reliance on labor, so product cleanliness and recovery rates from RUC EBN to RC EBN are inconsistent. The determinants of the price of EBN products are the shape, cleanliness, and color of EBN products (Dai et al., 2021). Deep processing has opened more markets for EBN products by expanding a variety of ready-to-eat products while reducing prices. The product lineup of ready-to-eat options comprises various items such as candy, jelly, oral liquid, beverages, effervescent tablets, and nano EBN particles (Dai et al., 2021; Fan et al., 2020; Li et al., 2020; Yao, 2017). Biotechnology processing mainly obtains specific nutrients in EBN through extraction, enzymatic hydrolysis, separation, and other methods.

In general, the use of EBN in downstream products (e.g., nutraceutical and skin care products) involves only the extract and not EBN as a whole material (RC EBN) due to limitations of certain physical and chemical properties such as insolubility. The enzymatic hydrolysis technology product (EBN hydrolysate) has broad application prospects and is widely

used in EBN downstream products. After DB (heat treatment), the EBN enzymes were used to break down EBN sialylated mucin (SiaMuc) glycoproteins into simpler SiaMuc glycopeptides and free peptides. This enzymatic process improves EBN solubility, digestibility, and bioavailability (Amiza et al., 2019; Yan et al., 2021). Enzymatic hydrolysis is an alternative to the EBN cleaning process, using enzymes to improve cleaning efficiency and EBN product value (Noor et al., 2018).

The benefits of EBN extract have also been confirmed by scientific research, uncovering its nutritional value and pharmacological activity.

EBN extracts can be divided into three groups: (1) aqueous/water extract, (2) pancreatic digest extract, and (3) enzyme hydrolysis extract. The pharmacological activities have been shown with EBN water extract, which include eye care effect (Abidin et al., 2011), chondro-protective agent for human chondrocytes (Chua et al., 2013), anti-aging, anti-inflammatory, and wound-healing activities of edible bird's nest in human skin keratinocytes and fibroblasts (Hwang et al., 2020), reno-protective (Lim et al., 2021), and enhances fertility and embryo implantation rate (Albishtue et al., 2019). Previous studies have shown that using pancreatic enzymes, the pharmacological activities of EBN extract include enhancement of bone strength (Matsukawa et al., 2011), improving learning ability and memory (Careena et al., 2018), neuroprotection in Alzheimer's or Parkinson's Disease (PD) (Yew et al., 2018), and health-enhancing and antiviral activities against influenza A virus (Haghani et al., 2016).

Previous studies on EBN extract (using enzymes alcalase/papain/neutrase) and EBN hydrolysate have shown its nutritional value and pharmacological activity, which include increased angiotensin I-converting enzyme (ACE) inhibitor (Nurfatin et al., 2016) and antioxidant (Babji et al., 2018; Cao et al., 2022). EBN hydrolysate also showed higher free radical scavenging activities (antioxidant) and SA content (Cao et al., 2022; Chong et al., 2022; Yan et al., 2021, 2022).

Aqueous extracts are EBN extracts obtained by heating/boiling raw materials (RUC EBN/RC EBN/EBN by-products). Previous studies reported that protein solubility, degree of hydrolysis (DH), antioxidant activity, SA, and peptide content of EBN were positively correlated with heating time and temperature (Dai et al., 2022; Hun et al., 2015; Nasir et al., 2021; Ramachandran et al., 2017). Therefore, heating time and temperature will affect the extraction efficiency of EBN. Enzymatic hydrolysis (without heat treatment as pre-treatment) process to obtain EBN hydrolysate was reported to have: (1) influence of five factors on SA EBN extraction: pH > enzyme dosage > enzymatic hydrolysis temperature > ratio of liquid to material > enzymatic hydrolysis duration (Dai et al., 2022), and (2) influence of 4 factors on DH of EBN: pH > enzymatic hydrolysis temperature > enzymatic hydrolysis duration > enzyme concentration

(Bang et al., 2017). Factors affecting the efficacy of enzymatic hydrolysis with heat treatment as pre-treatment (double boiled at 90/100°C, 30 min) are also similar to the enzymatic hydrolysis without heat treatment, which includes enzymatic time, temperature, and enzyme dosage (Bang et al., 2017; Cao et al., 2022; Yan et al., 2022).

Optimization implies enhancing the functioning of a process, a system, or a product to get the most out of it. Optimization has become common in analytical chemistry to discover the conditions under which a procedure that produces the best response is applied (Bezerra et al., 2008). The response surface method (RSM) is an effective tool for building and parameterizing optimization models (Brightman, 1978). EBN proteolysis can be optimized to meet certain targets, such as obtaining high DH, high bioactivity, and desirable properties by using RSM (Amin et al., 2019). EBN produces a certain amount of by-products of EBN in the cleaning process, which is hard to use effectively, resulting in a certain amount of waste after primary processing (Babji et al., 2018). Adding waste or by-products of EBN to production, in other words, reduces the overall recovery of the product and ultimately increases the cost of the product. A few proteolytic enzymes that have been used in several previous studies include alcalase, pancreatin, protamex, proteases, and papain. Bromelain has been less studied, but it is also an efficient protein digesting enzyme for protein in milk and collagen (Nanda et al., 2020) and stable over a broad pH range (pH 4-8) (Ee et al., 2019).

This study aimed to optimize the process conditions (combination of heat treatment and enzymatic hydrolysis) to produce high-yield and high-quality edible bird's nest (EBN) hydrolysate. Previous studies only used heat treatment as their pretreatment for a limited period (30 min). This study includes optimizing heat treatment and enzymatic hydrolysis conditions. Bromelain's stability within a broad pH range was an advantage if the enzymatic process was applied in an industrial setting. Thus, in this study, bromelain was used in enzymatic hydrolysis process. Four factors were investigated in this study: (1) temperature of EBN during DB, (2) duration of DB, (3) duration of enzymatic hydrolysis, and (4) ratio of EBN to water. Product cost is very important in the EBN processing industry, so the raw materials used in this study are EBN by-products. One of the goals is to produce high yields of EBN hydrolysate. In addition to high yields, the quality of the product is also important. SA and antioxidant activity are important components that represent the quality of EBN products. RSM was used in this study to obtain the optimal conditions to produce high yield (product recovery) and high quality (SA, DPPH, and ABTS) EBN hydrolysate.

MATERIALS AND METHODS

Materials

The raw material here refers to the EBN by-product, which was provided by Think Birdnest (Segamat, Malaysia) Sdn. Bhd. EBN by-products are fragments of EBN

with tiny feathers. In the primary processing quality control step, EBN fragments with tiny feathers attached were picked from RC EBN to increase the cleanliness of RC EBN before sterilization and packaging. This EBN fragment is an EBN by-product here.

Chemicals used in all laboratory analysis were analytical grade, which was purchased from Sigma-Aldrich (USA) (phenol, N-acetylneuraminic acid, resorcinol, and DPPH) and Thermo Fisher Scientific (USA) (ABTS, potassium persulfate, and methanol). Other reagents and solvents were of analytical grade.

The Process to Produce EBN Hydrolysate from the By-product of EBN

The EBN hydrolysate process involves heat treatment (here, DB was used) and enzymatic hydrolysis (bromelain was used as an enzyme). The process conditions were optimized computationally by the Box-Behnken design of the RSM. The software Design Expert 13.0.0 was used. The software generated 29 runs based on 4 influencing factors, and their ranges were provided in Table 1. The range for each factor was determined through preliminary studies. Influencing factors include (A) temperature of EBN during DB, (B) duration of DB, (C) duration of enzymatic hydrolysis, and (D) ratio of EBN to water. The final product recovery rate is the response of the RSM. Most previous studies were not based on the internal DB temperature and duration of EBN itself. Thus, in this study, the internal temperature of the EBN (not the external temperature) during DB was considered, while the duration of DB was calculated after the initial temperature reached the target temperature. The samples were soaked in different proportions of water for 10 min.

Table 1
Range for each factor

Factors	Lower range	Upper range
A: Temperature of EBN during DB (°C)	85	95
B: Duration of DB (min)	20	60
C: Duration of enzymatic hydrolysis (min)	30	120
D: Ratio of EBN to water	20	100

Note. DB = Double boiling; EBN = Edible bird's nest

The samples were double-cooked at different temperatures and durations and cooled to 48°C before enzymatic hydrolysis. Bromelain (2,400 GDU/g) was added to the samples at 0.8% by EBN dry weight. Enzymatic hydrolysis was performed for different durations (give the different durations) at 48°C. The hydrolysate is then filtered through a sieve (80 mesh) to remove larger size impurities and then

sieved through smaller size WYPALL® X70 Wipers (Kimberly-Clark Professional, USA) to remove tiny impurities. After enzymatic hydrolysis, the samples were double boiled at 80°C for 20 min to denature the bromelain. Subsequently, the liquid hydrolysate and retentate (on a sieve) were fan dried in an air-conditioned room (18-20°C) for 16-24 hr. The air-dried hydrolysate was later ground to a powder and stored in a sealed plastic bag for future use.

Product and Waste Recovery

Raw material (EBN by-product) was weighed before DB. The product (hydrolysate) and the waste (retentate) were weighed after drying. The product recovery and waste recovery were calculated by using the equations below:

$$\text{Product recovery (\%)} = (\text{Product/Raw material}) * 100\% \quad (1)$$

$$\text{Waste Recovery (\%)} = (\text{Waste/Raw material}) * 100\% \quad (2)$$

A total of 9 samples out of the 29 runs were selected for quality analyses. The nine samples were the first 3 with the highest product recovery, the middle recovery, and the last 3 with the lowest recovery.

Total Polysaccharide

The phenol-sulfuric acid method (Dubois et al., 1956; Yan et al., 2022) was used to assay the total polysaccharide content in the sample. One ml of sample (EBN weight: 2.0 mg/ml) was mixed with 0.50 ml of 5% phenol solution (w/w) (Sigma-Aldrich, USA). Then, 1.5 ml of concentrated sulfuric acid (H_2SO_4) (Merck Millipore, USA) was added to the mixture. The samples were gently shaken and incubated at room temperature for 10 min. Absorbance was measured at 490 nm (Shimadzu UV-VIS Spectrophotometer mini-1240, Japan). Glucose monohydrate was used as standard.

Total SA Content

The periodate-resorcinol assay (Jourdian et al., 1971; Yan et al., 2022) was used to analyze the total SA content in the sample. A 0.5 ml (2 mg/ml) sample was mixed with 0.5 ml resorcinol reagent in a test tube. The tube was covered with chilled marble, and the sample was incubated in boiling water for 15 min. After incubation, the samples were cooled (10 min) to room temperature. Then, 2.0 ml of 1-butanol (Merck Millipore, USA) was added to the sample. The sample was vortexed vigorously for at least 10 s to form a single-phase solution. The samples were then incubated in a 37°C water bath for 3 min to stabilize the color, and the absorbance was read after cooling to room temperature. An amount of 0.22 g resorcinol (Sigma-Aldrich, USA) was mixed with 10 ml of distilled water, 80 ml of concentrated hydrochloric acid (HCl) (Merck Millipore, USA), and 0.25 ml of 0.1 M copper sulfate ($CuSO_4$, Merck Millipore, USA) solution and then top up with distilled water to make 100 ml of resorcinol reagent. Absorbance was measured at 580 nm (Shimadzu UV-VIS Spectrophotometer mini-1240,

Japan). N-acetylneuraminic acid (analytical standard) (Sigma-Aldrich, USA) was standard.

Antioxidant Activity

DPPH Assay. One ml sample (2 mg/ml) was mixed with 14 ml DPPH reagent. The DPPH reagent (0.036 mM) was prepared by dissolving 14.07 mg of DDPH powder (Sigma-Aldrich, USA) in 1 L of methanol (Thermo Fisher Scientific, USA). In the control sample, distilled water was used instead of EBN. The mixture was incubated in the dark for 30 min. The sample/control was then filtered with a polytetrafluoroethylene (PTFE) syringe filter (0.45 µm) and measured at 517 nm (Shimadzu UV-VIS Spectrophotometer mini-1240, Japan). Distilled water was used as blank. The free radical scavenging activity (%) was calculated using the equation below:

[(DPPH control absorbance - DPPH sample absorbance)/ DPPH control absorbance] * 100% (3)

ABTS Assay. A 0.2 ml sample (2 mg/ml) was mixed with 1.8 ml of ABTS reagent. The ABTS reagent was prepared by mixing 7 mM ABTS (Thermo Fisher Scientific, USA) and 2.45 mM of potassium persulfate (Thermo Fisher Scientific, USA) with a ratio of 1:1. This stock ABTS reagent was incubated for 14–16 hr at room temperature in the dark. Then ABTS reagent with absorbance 0.7 ± 0.2 was done by diluting the stock ABTS reagent with methanol (Thermo Fisher Scientific, USA). In the control sample, distilled water was used instead of EBN. The mixture was incubated in the dark for 10 min. The sample/control was then filtered with a PTFE syringe filter (0.45 µm) and measured at 734 nm (Shimadzu UV-VIS Spectrophotometer mini-1240, Japan). Distilled water was used as blank. The free radical scavenging activity (%) was calculated using the equation below:

[(ABTS control absorbance - ABTS sample absorbance)/ABTS control absorbance] * 100% (4)

Model Validation

The optimized samples were prepared according to the section 'Process to Produce EBN hydrolysate from By-product of EBN'. Samples were collected after DB/heat treatment, before enzymatic hydrolysis, and after the enzymatic hydrolysis process was completed.

Statistical Analysis

The EBN quality data were presented as mean ± standard error (SE) for at least three analyses. The IBM SPSS Statistics 28.0.0.1 calculated the Pearson correlation coefficient and the statistical relationship between two continuous variables. The software Design Expert 13.0.0 was used to calculate the analysis of variance (ANOVA) for the model.

RESULTS AND DISCUSSION

Product and Waste Recovery

Table 2 shows the conditions (factors) and product and waste recovery results for the 29 runs. The product recovery was between 54.88 and 96.22%, the waste recovery was between 0.19 and 23.31%, and the total recovery (product recovery plus waste recovery) was between 64.26 and 99.03%. Most of the runs reported a total recovery of above 90%. The overall total recovery obtained was less than 100% and may be due to (1) liquid hydrolysate being absorbed by wipers (WYPALL® X70 Wipers) during the second filtration, and (2) the remaining samples were found stuck to the glass bottle, especially sample with a ratio of EBN to water is 20. It is supported by the overall recovery results, where the samples with a ratio of 20 had the lowest recovery among the 5 samples.

Table 2

The simulated design parameters by Design Expert 13.0.0 and product and waste recovery results

Run	Condition				Recovery (%)		
	A: Temperature of EBN during DB (°C)	B: Duration of DB (min)	C: Duration of enzymatic hydrolysis (min)	D: Ratio of EBN to water	Product	Waste	Total
1	90	40	75	60	79.96	10.71	90.67
2	90	40	120	100	91.82	2.40	94.21
3	90	40	75	60	82.60	8.20	90.80
4	95	40	120	60	96.06	1.57	97.64
5	95	60	75	60	94.08	0.19	94.27
6	95	40	75	20	87.70	0.39	88.09
7	85	40	120	60	81.51	7.95	89.46
8	95	20	75	60	80.48	9.16	89.64
9	95	40	30	60	83.13	5.36	88.49
10	85	40	75	100	84.24	14.79	99.03
11	90	40	75	60	85.86	7.16	93.01
12	90	20	30	60	81.51	9.54	91.05
13	85	20	75	60	60.36	23.31	83.67
14	90	20	75	100	82.09	13.78	95.87
15	90	60	75	100	90.87	0.19	91.06
16	85	40	30	60	74.55	14.51	89.07
17	90	60	120	60	86.96	4.55	91.50

Table 2 *(Continue)*

Run	Condition				Recovery (%)		
	A: Temperature of EBN during DB (°C)	B: Duration of DB (min)	C: Duration of enzymatic hydrolysis (min)	D: Ratio of EBN to water	Product	Waste	Total
18	90	60	75	20	73.37	7.10	80.47
19	90	40	120	20	63.24	3.95	67.19
20	90	40	30	20	54.88	9.38	64.26
21	90	20	120	60	89.48	1.98	91.47
22	85	60	75	60	82.63	9.98	92.61
23	95	40	75	100	96.22	1.99	98.21
24	90	20	75	20	55.29	11.18	66.47
25	90	40	75	60	80.08	11.72	91.80
26	90	40	30	100	86.03	8.98	95.01
27	90	60	30	60	77.09	15.34	92.43
28	85	40	75	20	62.55	10.59	73.14
29	90	40	75	60	83.50	13.52	97.02

Total Polysaccharide, Total SA, and Antioxidant Activity of Selected Samples

A total of 9 samples out of the 29 runs were selected for quality analyses. The 9 samples included the first 3 samples with the highest product recoveries (runs 23, 4, and 5), middle recovery rates (runs 9, 3, and 14), and the last 3 samples with the lowest recoveries (runs 13, 24, and 20). Four response tests were performed on EBN hydrolysates, including DPPH free radical scavenging activity, ABTS free radical scavenging activity, total SA content, and total polysaccharide content.

Table 3 shows the responses of the 9 EBN hydrolysate samples. DPPH free radical scavenging activity was between 5.97 and 18.15%. Sample H3 shows a significant ($p < 0.05$) higher DPPH value, while L3 shows a significant ($p < 0.05$) lower DPPH value. ABTS free radical scavenging activity was between 67.65 and 78.35%. Among the sample, samples H1 and H2 show a significant ($p < 0.05$) higher ABTS value, while L2 shows a significant ($p < 0.05$) lower ABTS value. The total SA content in the EBN hydrolysate was between 16.03 and 20.49%. Sample M1 shows ($p < 0.05$) lower SA significantly compared to other samples. Sample H1 had the highest SA but was not significantly different ($p > 0.05$) from the H2, H2, L1, L2, and L3 samples. Thus, sample M1 was not denoted as a significantly higher sample in Table 2. The total polysaccharide content in the EBN hydrolysate was between 8.44 and 14.29%.

Among the samples, samples H2 and L1 showed significantly ($p < 0.05$) higher total polysaccharide content, while H1, H3, and M2 showed significantly ($p < 0.05$) lower total polysaccharide content. A *t*-test was performed between the two samples (rows), and there was no significant difference between the samples ($p > 0.05$).

Table 3

The analyst results of total polysaccharide content, total SA content, and antioxidant activity of nine selected samples

Run	Sample ID	Product recovery (%)	DPPH free radical scavenging activity (%)	ABTS free radical scavenging activity (%)	Total SA content (%)	Total polysaccharide content (%)
23	H1	96.22	12.60 ± 0.39	78.35 ± 0.68[a]	20.49 ± 0.73	8.88 ± 0.17[b]
4	H2	96.06	10.26 ± 0.40	77.97 ± 0.26[a]	19.21 ± 0.77	14.07 ± 0.04[a]
5	H3	94.08	18.15 ± 0.66[a]	73.99 ± 0.50	19.28 ± 0.27	8.68 ± 0.08[b]
9	M1	83.13	10.49 ± 0.62	75.13 ± 0.69	16.03 ± 0.15[b]	10.55 ± 0.00
3	M2	82.60	10.39 ± 0.36	72.25 ± 0.46	18.01 ± 0.28	8.44 ± 0.01[b]
14	M3	82.09	7.72 ± 0.82	72.94 ± 0.83	16.68 ± 0.45	13.44 ± 0.11
13	L1	60.36	7.34 ± 0.48	70.00 ± 0.65	18.61 ± 0.87	14.29 ± 0.34[a]
24	L2	55.29	10.11 ± 0.20	67.65 ± 0.60[b]	19.27 ± 0.51	10.99 ± 0.03
20	L3	54.89	5.97 ± 0.30[b]	72.78 ± 0.69	19.69 ± 0.53	12.97 ± 0.04

Note. Superscript a = Significantly higher in the same column ($p < 0.05$); Superscript b = Significantly lower in the same column ($p < 0.05$)

The IBM SPSS Statistics 28.0.0.1 calculated the Pearson correlation coefficient (Table 4) and the statistical relationship between two continuous variables. The variables included the responses and factors. Some correlations were observed from Table 4: (1) product recovery was positively correlated with ABTS ($p < 0.05$) and negatively correlated with waste recovery ($p < 0.01$), (2) DPPH was negatively correlated with total polysaccharide and waste recovery ($p < 0.01$), (3) waste recovery was negatively correlated with product recovery and antioxidant activity ($p < 0.01$), (4) EBN temperature during DB was positively correlated with product recovery rate and ABTS ($p < 0.01$), and negatively correlated with waste recovery rate ($p < 0.05$), (5) DB duration was positively correlated with DPPH ($p < 0.01$) and negatively correlated with waste recovery ($p < 0.05$), (6) ratio of EBN to water was positively correlated with product recovery ($p < 0.01$), and (7) total SA and enzyme duration were not correlated with any other variables ($p > 0.05$).

Results from Box-Behnken Design of the Response Surface Methodology (RSM)

Table 5 shows the response surface regression model analysis of variance results for the linear model of product recovery. The model *F*-value of 17.12 suggests that it is a significant model. The probability of such a large *F*-value due to noise is only 0.01%. *P*-values less than 0.0500 reveals that the model terms are significant. In this study, A, B, C, and D are significant model terms. In other words, the effects of all factors on the product recovery were significant ($p < 0.05$). In accordance with the *F*-value, the order of impact of the four factors on product recovery was the ratio of EBN to water (D) > temperature of EBN during DB (A) > duration of DB (B) > duration of enzymatic hydrolysis (C). Based on the results of simple linear regression analysis, the function of product recovery (Y, %) with temperature of EBN during DB (°C, A), duration of DB (min, B), hydrolysis time (min, C), and ratio of EBN to water (D) was established. The formula was as follows:

$$Y = 80.19 + 7.56*A + 4.50*B + 4.43*C + 11.12*D \quad (5)$$

No significant linear model can be developed for another 3 responses (SA, DPPH, and ABTS).

Enzymatic hydrolysis duration shows no significant correlation with any response (Table 4) and is the least significant factor in the model (Table 5). Cao et al. (2022) used RSM to study the optimal enzymatic hydrolysis and reported that the sequence of the impact of three factors on DH was hydrolysis temperature > enzyme concentration > hydrolysis time. EBN is affected by the degree of heat treatment, which promotes the accessibility of enzymes to the cleavage site, and the denatured protein after heat treatment is more easily hydrolyzed (Amiza et al., 2019). Duration of enzymatic hydrolysis was the least significant factor in the model ($p < 0.05$). A possible reason could be that heat-treated denatured proteins are easily cleaved by enzymes when other factors are at their optimum, so the duration of enzymatic hydrolysis is not as important. The duration tested was between 30 and 120 min.

Figures 1, 2, and 3 are the optimal conditions the software gave after inputting the data for the responses. The criteria for Figure 1 show the optimal conditions (1 out of 100 solutions) selected by RSM for response product recovery only. This solution includes 29 run conditions, as shown in Table 2. The product recovery rate was set at 100 with a 95–100% range. The optimal condition was suggested as follows: (1) EBN temperature during DB = 93.6°C, (2) DB duration = 57.5 min, (3) Enzymatic hydrolysis time = 76.4 min, and (4) ratio of EBN to water = 1:96.6. The desirability of this solution is 1.000.

Figure 2 shows the optimal conditions selected by RSM for the 4 responses. In addition to product recovery, the hallmark beneficial parameters of EBN, total SA, and antioxidant activity (DPPH and ABTS) were included as responses. This solution included 9 selected run (3, 4, 5, 9, 13, 14,

Table 4

Pearson correlation coefficient between variables

	Product recovery (%)	DPPH free radical scavenging (%)	ABTS free radical scavenging (%)	Total SA (%)	Total polysaccharide (%)	Waste recovery (%)	Temperature of EBN during DB (°C)	Duration of DB (min)	Duration of enzymatic hydrolysis (min)	Ratio of EBN to water
Product recovery (%)	1	0.647	0.823**	-0.040	-0.395	-0.718*	0.767*	0.580	0.442	0.704*
DPPH free radical scavenging (%)	0.647	1	0.317	0.224	-0.711*	-.687*	0.654	0.720*	0.242	0.211
ABTS free radical scavenging (%)	0.823**	0.317	1	0.128	-0.141	-0.709*	0.773*	0.560	0.212	0.556
Total SA (%)	-0.040	0.224	0.128	1	-0.127	-0.252	0.079	0.246	0.299	-0.219
Total polysaccharide (%)	-0.395	-.711*	-0.141	-0.127	1	0.560	-0.461	-0.574	0.167	-0.122
Waste recovery (%)	-0.718*	-.687*	-.709*	-0.252	0.560	1	-.951**	-0.809**	-0.138	-0.116
Temperature of EBN during DB (°C)	0.767*	0.654	0.773*	0.079	-0.461	-0.951**	1	0.707*	0.098	0.250
Duration of DB (min)	0.580	0.720*	0.560	0.246	-0.574	-0.809**	0.707*	1	-0.069	0.000
Duration of enzymatic hydrolysis (min)	0.442	0.242	0.212	0.299	0.167	-0.138	0.098	-0.069	1	0.294
Ratio of EBN to water	0.704*	0.211	0.556	-0.219	-0.122	-0.116	0.250	0.000	0.294	1

Note. ** = Correlation is significant at the 0.01 level (2-tailed); * = Correlation is significant at the 0.05 level (2-tailed); Number of samples for each variable = 9

20, 23, and 24) conditions. The responses were set at (1) target 100% for product recovery and a range of 95–100%, and (2) maximized total SA and antioxidant activity. The suggested optimal conditions are: (1) EBN temperature during DB = 95.0°C, (2) DB duration = 60.0 min, (3) enzymatic hydrolysis time = 117.1 min, and (4) ratio of EBN to water = 72.9. The desirability of this solution is 0.798.

Some combinations of these factors have been made to obtain higher desirability and did not significantly affect the response. (1) The setting target factor for EBN temperature during DB was 95°C, and the range was 85–95°C (same as above), and (2) the ratio of EBN to water was targeted at 80, and the range was 20–100 (same as above). The response settings are the same as in Figure 2. Figure 3 shows the optimal conditions after factor and response settings. The suggested optimal conditions were: (1) EBN temperature during DB = 95.0°C, (2) DB duration = 60.0 min, (3) enzymatic digestion time = 97.3 min, and (4) EBN to water ratio = 79.9. The desirability of this setting (0.858) is higher than the above solution (Figure 2—0.798). The response for this setup is like the solution above, with a slight decrease in DPPH from 15.18 to 14.92%. Therefore, these conditions are recommended as the optimal ones to obtain EBN hydrolysate with high yield and quality.

Table 5

Regression model variance analysis results of a response surface for product recovery linear model

Source	Sum of squares	df	Mean square	*F*-value	*p*-value
Model	2648.46	4	662.11	17.12	< 0.0001**
A: EBN temp during DB	686.67	1	686.67	17.75	0.0003**
B: DB duration	243.54	1	243.54	6.30	0.0193 *
C: Enzymatic duration	235.59	1	235.59	6.09	0.0211 *
D: Ratio EBN to water	1482.66	1	1482.66	38.33	< 0.0001**
Residual	928.43	24	38.68		
Lack of fit	903.89	20	45.19	7.37	0.0328*
Pure error	24.54	4	6.13		
Cor total	3576.88	28			

Note. ** = Correlation is significant at the 0.01 level (2-tailed); * = Correlation is significant at the 0.05 level (2-tailed)

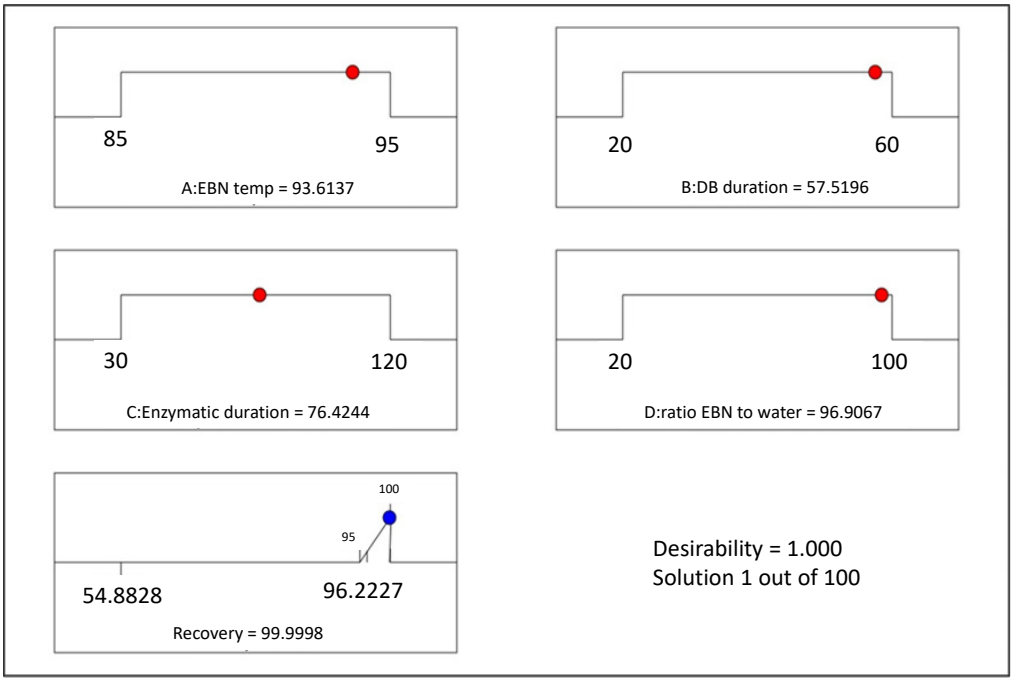

Figure 1. Optimization solution to get 100% product recovery

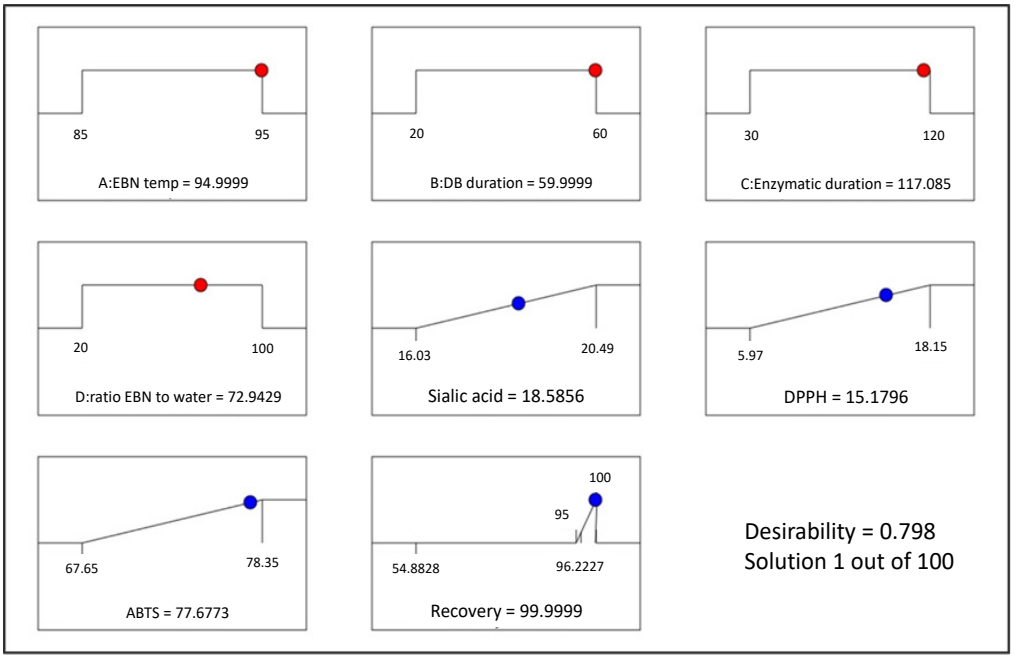

Figure 2. Optimization solution to get 100% product recovery and maximum total SA, DPPH, and ABTS

Note. SA = Sialic acid; DPPH =2,2-diphenyl-1-picrylhydrazyl; ABTS = 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid

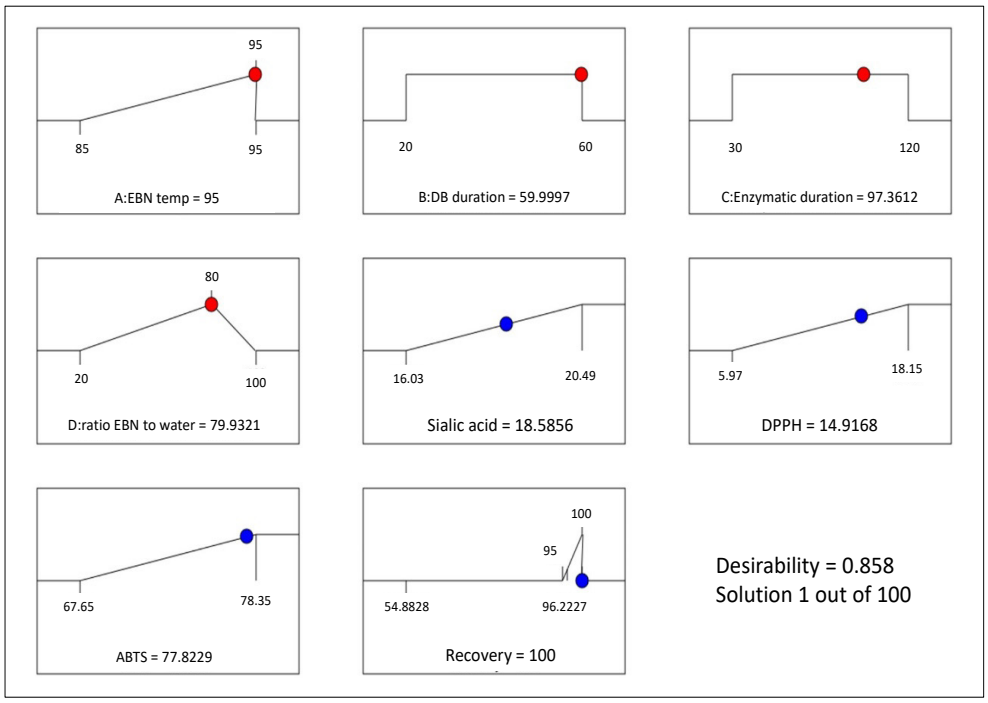

Figure 3. Optimization solution to get 100% product recovery and maximum total SA, DPPH, and ABTS. Factors EBN temperature during DB was set at 95°C, and the ratio of EBN to water was set at 80
Note. SA = Sialic acid; DPPH =2,2-diphenyl-1-picrylhydrazyl; ABTS = 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid

Model Validation

Figure 4 shows the raw material (EBN by-product) and EBN hydrolysate. Table 6 shows the results of the samples before (after heat treatment/DB) and after enzymatic hydrolysis using the optimal conditions. The optimal process parameters were: (1) temperature of EBN during DB = 95°C, (2) DB duration = 60 min, (3) duration of enzymatic hydrolysis = 97 min, and (4) ratio of EBN to water is 1:80. DPPH and ABTS assays are performed directly from liquid samples after collection without any dilution. Therefore, the antioxidant activity results shown here are for samples with 12.5 mg/ml EBN (ratio 1:80). Sample concentrations for total SA and total polysaccharide determinations were the same as above (2 mg/ml).

The antioxidant activities (DPPH and ABTS) of the samples before (after heat treatment) and after complete enzymatic hydrolysis showed similar results. Total SA and polysaccharides significantly increased after hydrolysis ($p < 0.05$). Previous studies have shown that low molecular weight EBN fractions do not affect DPPH free radical scavenging activity (Chong et al., 2022) but have positive effects on SA and total polysaccharides (Chong et al., 2022; Ng et al., 2020). It was suggested that, after heat treatment, the protein may have been

hydrolyzed to polypeptide or dipeptide, which play a role in the free radical scavenging activity. These polypeptides/ dipeptides did not increase after enzymatic hydrolysis.

The product recovery rate (96.44%) was lower than the result of the predicted model (99.99%), while the total SA concentration in the hydrolysate (19.86%) was higher than the result of the predicted model (18.59%).

Figure 4. Left = EBN by-product; Right = EBN hydrolysate after the enzymatic process
Note. EBN = Edible bird's nest

Compared to the previous study by Ling et al. (2020), which showed an 89.09% product recovery, this study demonstrated a higher product recovery (96.44%). The total SA content (a signature component of EBN) was also within the range of other studies (15–22.4%) (Chong et al., 2022; Ng et al., 2020; Yan et al., 2022). The advantage of using bromelain is that bromelain is less sensitive to pH, so no pH adjustment is required. It is especially important for the EBN industry because no additional chemicals were needed to adjust the pH, reducing processing steps. The conventional primary process for the EBN

Table 6
Product recovery, waste recovery, and quality of EBN before and after enzymatic hydrolysis

	Recovery (%)		Quality parameters			
	Product	Waste	DPPH free radical scavenging activity (%)	ABTS free radical scavenging activity (%)	Total SA content (%)	Total polysaccharide content (%)
Before hydrolysis	-	-	28.73 ± 1.08	98.59 ± 0.27	11.32 ± 0.11	6.21 ± 0.45
After hydrolysis	96.44 ± 2.94	2.77± 0.60	28.03 ± 0.78	98.68 ± 0.27	19.86 ± 0.36	9.73 ± 0.35

cleaning method was claimed to incur high processing costs caused by the laborious cleaning procedure (Ng et al., 2020). Moreover, the traditional EBN cleaning methods report losses of up to 35–40% of EBN after cleaning (Noor et al., 2018). If the raw material is an EBN by-product with many fine feathers (Figure 4), the cleaning loss may be higher than the reported value using the traditional cleaning method. This research shows that the enzymatic process has higher product recoveries (96.44%), less wastage (2.77%), and simpler cleaning procedures. This processing method saves time and does not require skilled labor, as the feathers can be removed through filtration, eliminating the need for picking. The enzymatic process demonstrated here could be a promising alternative to EBN cleaning with less reliance on skilled labor and lower product cost.

CONCLUSION

This research led to two optimal conditions, one focusing on yield (product recovery) and the other on yield and quality. Suggestions for optimal conditions for maximum yield (target 100%) are: (1) EBN temperature during DB = 93.6°C, (2) DB duration = 57.5 min, (3) duration of enzymatic hydrolysis = 76.4 min, and (4) ratio of EBN to water = 1:96.6. The optimal conditions suggested for maximum yield and quality are: (1) EBN temperature during DB = 95°C, (2) DB duration = 60 min, (3) duration of enzymatic hydrolysis = 97 min, and (4) ratio of EBN to water = 1:80. In addition, under this optimal process conditions, a 96% product recovery can be obtained, which is believed to be higher than the normal traditional cleaning process of EBN by-products (35-40%).

The factors that affect the yield and quality of EBN hydrolysate include (1) the influence of EBN temperature on product recovery and ABTS during the DB process, (2) DPPH is affected by DB Duration, and (3) the ratio of EBN to water affects product recovery rate. In this study, the duration of enzymatic hydrolysis had the least significant effect on the yield and quality of EBN hydrolysate.

This study suggests that RSM may be a good tool for determining the optimal conditions for the enzymatic process and tailoring the yield and physicochemical properties of EBN hydrolysates. This study also shows that the conversion of EBN by-products into EBN hydrolysate can be used as a nutraceutical because EBN hydrolysate was found to have high antioxidant activity and high SA content. As an extension to the current study, future studies on the degree of hydrolysis and evaluating the peptide bond cleaved after enzymatic hydrolysis will enhance knowledge of the bromelain hydrolysis process.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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