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A Novel Approach Coupling Optimized Enzymatic Hydrolysis Conditions with Spray Drying to Produce Functional *Acheta domesticus* Protein Powder Ingredients

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Abstract

As the global population continues to grow, so does the demand for alternative protein sources. Entomophagy, the consumption of insects, has long been practiced in many cultures worldwide and is now gaining increasing interest in Western countries. In this work, we developed novel, functional insect-based ingredients from the house cricket (*Acheta domesticus*) by utilizing optimized enzymatic hydrolysis, using two enzymes (Alcalase® or Flavourzyme®) coupled with spray drying. A Box–Behnken experimental design was used to optimize enzymatic treatments and maximize spray-drying performance and product solubility. Under optimized conditions, spray-dried hydrolyzed cricket protein (HCP) produced using Alcalase® achieved a solids recovery of 51.44% and a solubility of 58.28 ± 0.5%. In comparison, Flavourzyme®–HCP, under optimized conditions, exhibited a higher solubility of 61.25 ± 0.8%. Additional functional properties were improved for Alcalase®–HCP and Flavourzyme®–HCP, respectively, including foaming capacity at pH 4 (26.80 ± 4.0%, 36.27 ± 1.0%) and 10 (50.98 ± 2.8%, 47.06 ± 1.6%), and foaming stability in acidic conditions at pH 4 (24.18 ± 4.0%, 30.39 ± 2.9%). Moreover, the emulsion stability, especially at pH 7 (74.70 ± 3.5%, 52.04 ± 2.8%) and 10 (68.20 ± 11.3%, 69.72 ± 3.2%), was also enhanced. To the best of our knowledge, this is the first study to investigate optimized enzymatic hydrolysis coupled with spray drying to enhance the functional properties of *A. domesticus* protein powder. Overall, we established optimized processing conditions to produce spray-dried functional insect ingredients with desirable functional attributes.

Keywords: enzymatic hydrolysis; spray drying; insect protein; *Acheta domesticus*; solubility; proteolytic enzymes; functional properties

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1. Introduction

The rising global population is intensifying pressure on food security and supply, thereby increasing interest in sustainable and novel protein sources such as edible insects. Insect-based production systems offer a more sustainable alternative to conventional livestock (e.g., cattle and pork) given their lower greenhouse gas emissions, as well as their lower demand for land, water, and feed [1]. Edible insects are a good prospect for alternative proteins due to their nutritional values, specifically the protein content. Furthermore, house crickets (*Acheta domesticus*) that were freeze dried as a powder had a proximate composition (% dry matter) of protein (56.8%), fat (22.8%), and fiber (3.3%) [2].

Entomophagy, the practice of consuming edible insects, has been historically relevant in the human diet [3], and it is part of the regular diet in several countries [4], but not in most of the Western world. Among the various challenges regarding their consumption, consumer perception is a major issue due to disgust, lack of interest in insects, neophobia [5,6], and the sensory-related issues of insect-based products. Furthermore, there are also concerns about possible allergic reactions to individuals sensitive to crustaceans or dust mites, as well as potential problems of food fraud (e.g., adulteration) as the industry expands [5]. Although many edible insects are harvested from the wild, several countries regulate or prohibit this practice, including the United States of America (USA), via the Food and Drug Administration (FDA) [7]. Moreover, legislative approval of various edible insects is still in progress in the USA. Currently, there are over 2000 edible insect species, but only 4 of those insect species, including *Acheta domesticus* (house cricket), are approved for human food in the European Union [5].

Despite these barriers, there is a clear market opportunity for insect-food development. Industrial-scale insect farming is already established in Europe (for example, in France and the Netherlands) and North America (including Canada and the USA) [1]. While drying and grinding remain the predominant methods for producing insect flour [8], emerging technologies are being investigated to enhance the processing of insects and their derived ingredients. For example, enzymatic hydrolysis (EH) using proteases that selectively target and cleave the protein at different segments of the amino acid chain to produce smaller peptide chains and free amino acids could improve critical functional properties like solubility, reported as one of the major techno-functional problems of insect protein [9]. Some previous works analyzed the effect of EH utilizing Alcalase® on the functional properties of black solider fly larval protein and tropical banded cricket (*Gryllodes sigillatus*) protein, and the effect of an EH treatment utilizing both Alcalase® and Falvourzyme® on dough techno-functional properties and bread quality [10–12]. Moreover, the food industry has utilized controlled enzymatic hydrolysis to improve animal- and plant-protein functionality [8].

Additionally, spray drying is the most popular drying technique in the food industry nowadays due to its robustness, versatility, and scalability, while producing microparticles that can be incorporated into a variety of food products [13]. Additionally, its use at an industrial level is highly cost-effective [14]. However, few studies have been conducted so far to develop spray-dried insect products [15]. Some reported examples include a spray-dried emulsified mealworm (*Tenebrio molitor*) slurry [16]; spray-dried cricket powder [13]; and novel, functional spray-dried protein–polyphenol insect ingredients [17].

In this study, our objective was to produce insect-protein ingredients with improved functional properties, specifically focused on increasing the solubility of *A. domesticus* protein, a recognized technical issue for this type of protein. To accomplish this, we optimized the enzymatic hydrolysis of house-cricket protein and submitted it to spray drying to produce functional insect-derived ingredients. That said, due to the collaborative goals of this study, it was important to utilize these coupled technologies to produce the insect ingredients because of their industrial popularity in the food industry. Physicochemical and functional characteristics of the non-processed house cricket protein-powder raw control (RC) and optimized spray-dried hydrolyzed cricket protein (HCP) samples were evaluated and compared. To the best of our knowledge, this is the first study addressing the enzymatic hydrolysis coupled with spray drying applied to *A. domesticus* protein powder to improve its functional properties. Overall, we demonstrated the use of a scalable and industrially friendly technological route to produce insect-derived ingredients with enhanced attributes and multiple applications for the emerging alternative food industry.

2. Materials and Methods

2.1. Materials

The house cricket-protein powder (*Acheta domesticus*; 62.8% protein; spray-dried) used in this study was donated by Hoppy Planet Foods (Charlotte, NC, USA). Alcalase® (Protease from *Bacillus licheniformis*; 500 mL; EMD Millipore Corp., Burlington, MA, USA), and Flavourzyme® (Protease from *Aspergillus oryzae*; P6110-50mL; Sigma Aldrich, Saint Louis, MO, USA) were used in this study. All other chemicals and reagents were analytical grade (Sigma Aldrich, Saint Louis, MO, USA) unless otherwise specified. All analytical instruments were submitted to routine calibration.

2.2. Insect-Protein Preparation

Prior to processing, the house cricket (*A. domesticus*) protein powder was sieved (80 mesh), and the fraction retained in the sieve was ground for 20 s (high-speed multifunction Grinder HC-2000, cGoldenwall, Glendale, CA, USA). After, the ground material was sieved again (80 mesh). All materials were mixed to constitute a single batch and kept in cold storage (4 °C) until use. The ground, sieved, non-processed (spray-dried by provider, not hydrolyzed) cricket = protein powder was used and referred to as the raw control (RC).

2.3. Enzymatic Hydrolysis of Cricket-Protein Powder

Optimization of Enzymatic Hydrolysis Conditions

A 3^3 Box–Behnken design (BBD) for the response surface methodology (RSM) consisting of 15 experimental test trials with three central points was established to help determine optimized conditions for two enzymatic treatments consisting of Alcalase® or Flavourzyme® proteolytic enzymes (Table 1). The goal was to maximize the dependent responses, (a) solubility and (b) spray-drying solids recovery (SR %), while minimizing the independent factors solid-to-liquid ratio (S/L ratio), hydrolysis time (min), and enzyme-to-substrate ratio (E/S ratio, %). For this, the independent factors were tested in three levels: Factor A, solid-to-liquid ratio (S/L ratio; 1:4, 1:5, and 1:6); Factor B, hydrolysis time (60, 120, and 180 min); and Factor C, enzyme-to-substrate ratio (E/S ratio (%); 0.5, 1.5, and 2.5%; based on total solid-to-liquid dispersions). The three levels were chosen based on laboratory-scale spray-drying capabilities, similar work to ours that was published, and the collaborative expectations of minimizing materials and hydrolysis time. The optimal production conditions for each experimental treatment were then determined using a desirability function method to maximize solubility and SR % to obtain optimized spray-dried hydrolyzed cricket protein (HCP) for each enzyme investigated.

The enzymatic hydrolysis (EH) was conducted according to a modified protocol (Figure 1) [10]. For each trial, 35 g of non-processed cricket-protein control powder (RC) was homogenized with double deionized water in a 500 mL beaker to prepare the different S/L ratio experimental treatments (Table 1). The slurry was constantly stirred for 2 min using a 5-inch magnetic hotplate stirrer at room temperature. Optimal conditions for Alcalase® and Flavourzyme® are pH values 7–8.5 and 5–7, and a temperature of 50–60 °C and 50 °C, respectively. That said, the pH was adjusted to 8.0 with a pH meter (model Orion Star A211, Thermo Fisher Scientific, Waltham, MA, USA), coupled with a multi-stirrer, using a 5 M NaOH solution. The slurry was placed into a water bath at 50 °C, under constant stirring with a magnetic stirrer. The experimental treatments were prepared following the established amount of enzyme (Alcalase® or Flavourzyme®) and hydrolysis time (Table 1), Alcalase®-HCP was prepared following optimized conditions (Table 2, Row 16); after the initial ANOVA analysis (Table 3) and Flavourzyme®-HCP was prepared following optimized conditions(Table 4, Row 16); after the initial ANOVA analysis (Table 5). Beakers were covered with parafilm to prevent water evaporation. Following the hydrolysis,

samples were heated in a separate water bath set at 90 °C for 15 min for enzyme inactivation. The cricket-protein hydrolysates were cooled to room temperature under constant stirring with no heat and submitted to spray drying.

Table 1. BBD experimental design used to produce optimized spray-dried hydrolyzed cricket protein (HCP) using Alcalase® or Flavourzyme® enzymes.

Test	Solid-to-Liquid Ratio (S/L Ratio)	Hydrolysis Time (min)	Enzyme-to-Substrate Ratio (E/S Ratio, %)
* 1	1:5	120	1.5
2	1:6	120	0.5
3	1:5	180	0.5
* 4	1:5	120	1.5
5	1:4	120	2.5
* 6	1:5	120	1.5
7	1:6	120	2.5
8	1:4	180	1.5
9	1:5	60	2.5
10	1:5	60	0.5
11	1:6	60	1.5
12	1:6	180	1.5
13	1:4	120	0.5
14	1:5	180	2.5
15	1:4	60	1.5

* Central points.

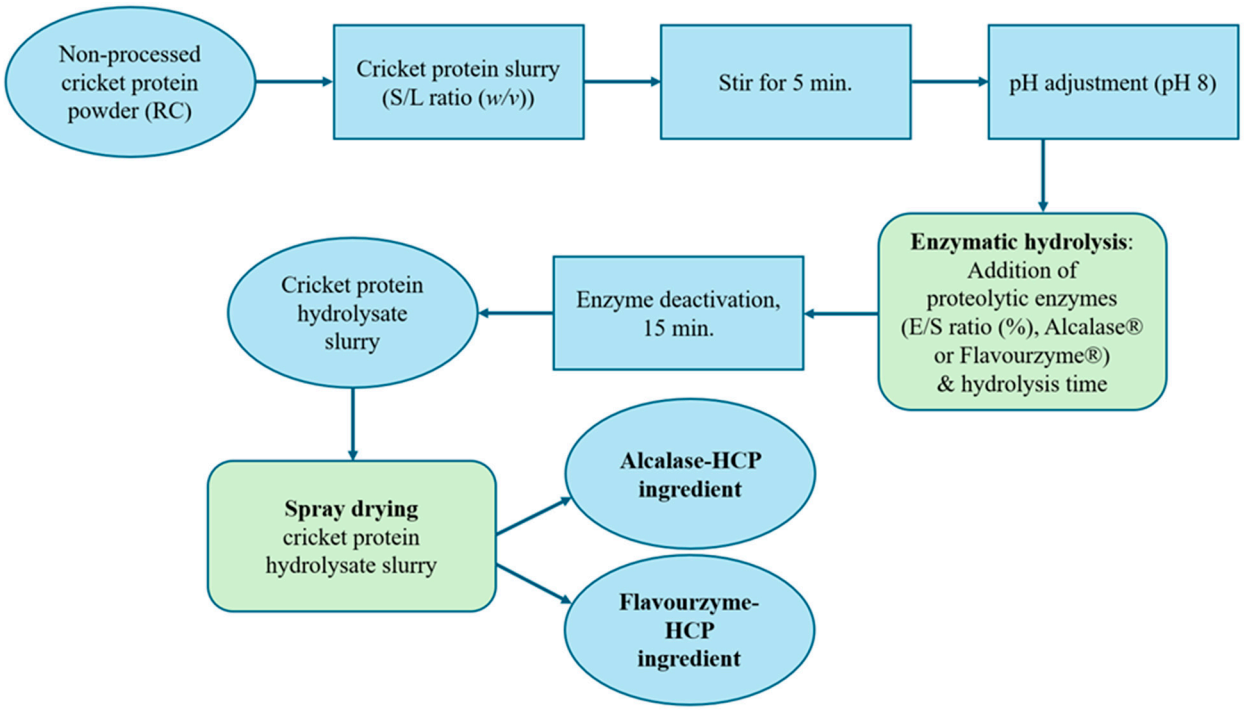


Figure 1. Process flow diagram for obtaining cricket-protein hydrolysates after enzymatic hydrolysis and subsequent optimized hydrolyzed cricket protein (HCP) ingredients post spray drying.

2.4. Spray Drying

Before spray drying, the cricket-protein hydrolysates were homogenized (high-speed homogenization, PRO Scientific PRO250, Cole Parmer, Vernon Hills, IL USA) for 4 min to eliminate any clusters of material. A lab scale spray dryer (B-290, Buchi Labortechnik AG, Flawil, Switzerland) was used under the following conditions: drying air inlet temperature 120 °C and outlet temperature 68–72 °C. The spray drying system was operated using air

co-current flow under the following conditions: 0.7 mm nozzle, 7 mL/min (controlled by a peristaltic pump), and aspirator rate of 100%. The Alcalase® or Flavourzyme® hydrolyzed cricket-protein powder (Alcalase®-spray-dried HCP or Flavourzyme®-spray-dried HCP, respectively) was collected from the collection chamber, weighed and sealed, then kept under refrigeration at 4 °C until further use (Figure 1). Solids recovery percentage (SR %) was determined according to Equation (1) [18]:

$$\text{SR } (\%) = \frac{\text{Total solids of hydrolyzed cricket protein powder(g)(after spray drying)}}{\text{Total solids content of feed solution (g)(before spray drying)}} \times 100 \quad (1)$$

2.5. Characterization of Spray-Dried Cricket-Protein Hydrolysates

2.5.1. Total Solids, Moisture, Protein Content, and Water Activity (a_w)

Total solids (%) in the spray-drying feed suspensions were assessed gravimetrically by collecting 3 mL of hydrolyzed cricket protein in 15 mL centrifuge tubes and freeze drying them (model 70020, LABCONCO, Kansas, MO, USA). A moisture analyzer (Mettler Toledo HE53, Columbus, OH, USA) was used to analyze the moisture content of spray-dried powders. The protein content (%) was measured using the Dumas test (AACC 46-30; AOAC 992.15), and the water activity was assessed using an Aqualab water activity meter (model 4TE, Meter, Palo Alto, CA, USA).

2.5.2. Hygroscopicity

Hygroscopicity was determined by transferring 0.5 g of samples to aluminum dishes. Samples were placed into a desiccator at 25 °C and 75% relative humidity (RH) for 7 days. Results were expressed as the mass of water absorbed per 100 g of sample after seven days [19].

2.5.3. Bulk and Tapped Density

Bulk density (ρB) was evaluated by adding 1 g of samples into a 10 mL graduated cylinder. The volume was recorded, and ρB was calculated (weight per volume). The tapped density (ρT) was evaluated using a modified method by [19], by gently tapping the graduated cylinder 120 times onto a rubber mat to determine ρT (weight per final volume).

2.5.4. Flowability

The Hausner ratio (HR, Equation (2)) and Carr's compressibility index (CI, Equation (3)) were used to calculate flowability according to [20].

$$\text{HR} = \rho T / \rho B \quad (2)$$

$$\text{CI} = \frac{\rho T - \rho B}{\rho T} \times 100 \quad (3)$$

2.5.5. Solubility

For all test trial samples, 0.5 g of sample was combined with 50 mL of distilled water. Samples were stirred for 30 min with a magnetic stirrer to completely hydrate the samples and transferred to 50 mL centrifuge tubes and centrifuged at 4000 rpm for 10 min at 4 °C. A 25 mL aliquot of supernatant was transferred onto an aluminum dish and vacuum oven-dried (Isotemp 285A vacuum oven, Fisher Scientific, Hampton, NH, EUA) at 105 °C to constant weight. The same procedure was performed for both optimized spray-dried HCP and RC samples, except pH was adjusted to pH 4, 7, or 10 using 1 M NaOH or 1 M hydrochloric acid (HCl) and a pH meter coupled with a multi-stirrer before samples were

stirred for 30 min. Solubility was expressed as a percentage (%) by calculating the weight of the supernatant divided by the weight of the sample [17].

2.5.6. Foaming Capacity and Stability

Suspensions were prepared by combining 0.5 g of sample with 50 mL of distilled water. Samples were stirred for 2 min, and the pH was adjusted to 4, 7, and 10 with 1 M NaOH or HCl, using a pH meter coupled with a multi-stirrer, and then stirred again for 30 min. Individual samples were poured into a graduated cylinder, and the initial volume was recorded (V_1). Samples were poured into a plastic container and homogenized with a high-shear homogenizer for 2 min at 16,000 rpm. Foamed samples were transferred back into a graduated cylinder equipped with a plastic funnel, gently pouring foam. Immediately, the volume was measured (V_2), and after 30 min, the volume was measured again (V_{30}). Results for foaming capacity and stability were calculated using Equations (4) and (5), respectively [17]:

$$\text{FC } \% = \frac{(\text{V2} - \text{V1})}{\text{V1}} \times 100 \quad (4)$$

$$\text{FS } \% = \frac{(\text{V30} - \text{V1})}{\text{V1}} \times 100 \quad (5)$$

2.5.7. Emulsifying Properties (EAI and ESI)

Emulsifying properties were evaluated using a modified procedure by [21]. For this, suspensions were prepared by combining 0.5 g of sample with 50 mL of distilled water. The pH was adjusted to 4, 7, or 10 using 1 M NaOH or 1 M HCl and measured by a pH meter coupled on a multi-stirrer plate. Then, 5 mL of sunflower oil was added to 50 mL centrifuge tubes, and 15 mL of prepared sample was transferred to centrifuge tubes containing oil. Separately, 4.9 mL of 0.1% sodium dodecyl sulphate (SDS) was added to 5 mL Eppendorf tubes. Individually, 50 mL tubes containing oil and sample were homogenized using a high-shear homogenizer at 16,000 rpm for 2 min. Aliquots (50 µL) of the emulsion were pipetted and transferred to labeled centrifuge tubes with 4.9 mL of 0.1% SDS and the procedure repeated after 10 min from the same emulsion tube. Absorbance of the individual samples was measured at 500 nm at the beginning (A_0; 0 min) and after 10 min (A_{10}; t min), using a microplate reader (model Epoch2 Microplate Reader; Biotek). The emulsifying activity index (EAI, m^2/g) and emulsion stability index (ESI, %) were calculated according to Equations (6) and (7), respectively:

$$\text{EAI} = \frac{(2 \times 2.303 \times \text{A}_0)}{0.25 \times \text{m}} \quad (6)$$

$$\text{ESI} = \frac{(\text{EAI t min})}{\text{EAI 0 min}} \times 100 \quad (7)$$

3. Statistical Analysis

The software DesignExpert (version 13; Stat-Ease, Minneapolis, MN, USA) was used to perform the BBD experimental design and data analysis. Regression models were adjusted to the experimental data, and the response surfaces were evaluated. To maximize both SR and solubility responses, a desirability function method was used. Experimental replicates were performed in either duplicate or triplicate. The statistical data analysis for the functional characterization was performed using ANOVA and Tukey's multiple comparisons test ($p < 0.05$) analysis, using GraphPad Prism software version 10 (GraphPad Software Inc., San Diego, CA, USA).

4. Results and Discussion

4.1. Optimization of Hydrolyzed Insect-Protein Powder

The main goal of this work was to obtain spray-dried hydrolyzed insect ingredients with enhanced solubility and process performance. Insect protein generally has poor solubility [22], and, therefore, improving it would enhance the functionality of insect ingredients and expand their potential application possibilities into food formulations [23]. Moreover, the solids recovery is a critical index for evaluating spray-drying performance, as it reflects the efficiency of converting feed solution into dry powder, directly impacting process economics and scalability [14,17,18].

For this, three independent regression models were built based on the experimental results for the products' solubility and spray-drying solids recovery (Tables 2 and 4). Models were built using coded factors: (A) solids-to-liquid ratio (S/L ratio), (B) hydrolysis time, and (C) enzyme-to-substrate ratio (E/S ratio) (Alcalase®: Equations (8) and (9); Flavourzyme®: Equation (10)).

$$\text{Alcalase: SR (\%)} = 54.78 - 4.70C + 2.42BC - 3.57B^2 - 2.47C^2 \quad (8)$$

$$\text{Alcalase: Solubility (\%)} = 57.37 + 3.30C \quad (9)$$

SR (%) p-values:

- C: $p = 0.0008$; BC: $p = 0.0472$; B^2: $p = 0.0138$; C^2: 0.0496.

Solubility (%) p-values:

- C: $p \leq 0.0001$.

Table 2. Experimental results of solids recovery and solubility obtained for spray-dried hydrolyzed cricket protein (HCP) using Alcalase® enzyme through BBD experimental design. Optimized enzymatic hydrolysis conditions displayed in the last row.

Test	Solids-to-Liquid Ratio (S/L Ratio)	Hydrolysis Time (min)	Enzyme-to-Substrate Ratio (E/S Ratio, %)	Solids Recovery (%)	Solubility (%)
* 1	1:5	120	1.5	56.59	56.12
2	1:6	120	0.5	57.29	53.78
3	1:5	180	0.5	53.66	54.18
* 4	1:5	120	1.5	54.20	57.60
5	1:4	120	2.5	47.31	60.16
* 6	1:5	120	1.5	53.55	58.39
7	1:6	120	2.5	48.93	60.75
8	1:4	180	1.5	50.61	57.60
9	1:5	60	2.5	38.99	60.58
10	1:5	60	0.5	54.06	53.22
11	1:6	60	1.5	52.52	55.54
12	1:6	180	1.5	51.87	58.64
13	1:4	120	0.5	56.07	54.69
14	1:5	180	2.5	48.25	60.77
15	1:4	60	1.5	50.23	58.20
Alcalase®–HCP	1:4	117	1.2	51.44	58.28 ± 0.5

* Central points.

For Alcalase® samples, SR was influenced by the E/S ratio (C) and by the linear interaction between hydrolysis time and E/S ratio (BC). Furthermore, the quadratic terms (B^2 and C^2) show that the hydrolysis time and E/S ratio dictate the curvatures of 3D response surfaces (Figure 2C). This upward curvature illustrates that the independent factors B and ntrC combined can be optimized to maximize solids recovery. As for the solubility response, it was influenced only by the E/S ratio (C) linear response (Figure 2D–F).

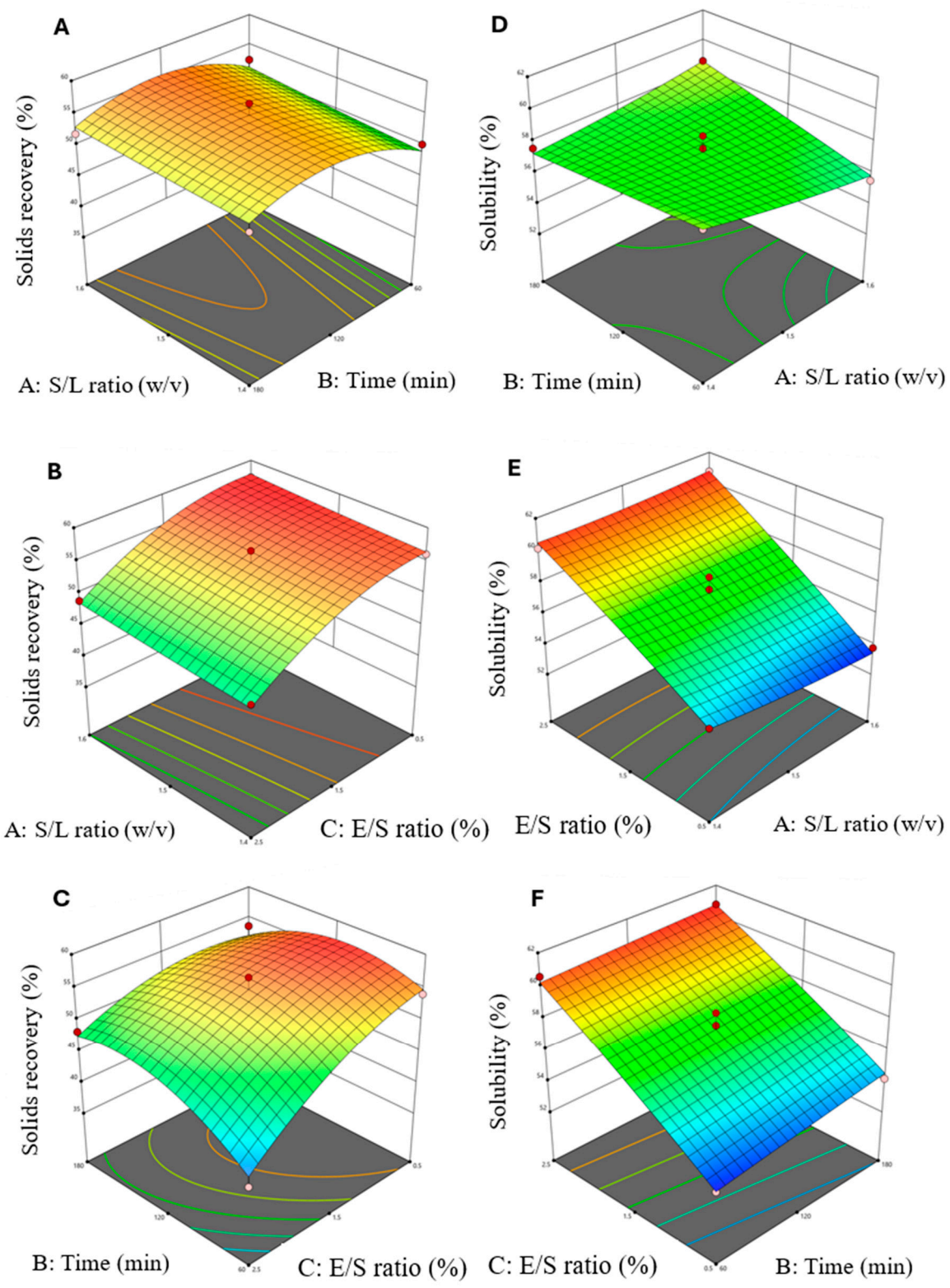


Figure 2. Three-dimensional response surfaces to obtain optimized spray-dried hydrolyzed cricket protein (HCP) using Alcalase® enzyme. (**A**–**C**) Solids recovery and (**D**–**F**) solubility as a response of (**A**,**D**): S/L ratio and hydrolysis time; (**B**,**E**): S/L ratio and E/S ratio; (**C**,**F**): hydrolysis time and E/S ratio.

For Alcalase®, the regression models are significant (Table 3), and the lack of fit (LoF) is not significant ($p > 0.05$), indicating that the data fit the model. A high coefficient of determination was found (Alcalase®: R^2, 0.9428 for SR and 0.9651 for solubility), indicating a strong agreement between the adjusted and empirical data results.

Table 3. Analysis of variance (ANOVA) of BBD models for solids recovery and solubility to produce optimized spray-dried hydrolyzed cricket protein (HCP) with Alcalase®.

Factor	SS	df	MS	F-Value	*p*-Value
Solids recovery (%)					
Model	280.64	9	31.18	9.16	0.0126
Residual	17.02	5	3.40		
LoF	11.89	3	3.96	1.55	0.4158
PE	5.13	2	2.56		
Total	297.66	14			
R^2	0.9428				
Solubility (%)					
Model	93.51	9	10.39	15.37	0.0039
Residual	3.38	5	0.6760		
LoF	0.7241	3	0.2414	0.1818	0.9008
PE	2.66	2	1.33		
Total	96.89	14			
R^2	0.9651				

SS, sum of squares; df, degree of freedom; MS, means square; LoF, lack of fit; PE, pure error; R^2, coefficient of determination.

The highest solids recovery (57.29%) for Alcalase® treatments was achieved at an S/L ratio of 1:6, hydrolysis time of 120 min, and E/S ratio of 0.5% (Table 2). However, the highest solubility (60.77%) was achieved at different conditions: S/L ratio of 1:5, hydrolysis time of 180 min, and E/S ratio of 2.5%. Therefore, optimized EH conditions were calculated using a desirability function with the goal of maximizing both responses and minimizing each factor. The obtained conditions were an S/L ratio of 1:4, hydrolysis time of 117 min, and E/S ratio of 1.2% (Table 2). The predicted value for SR was 55.10%, and solubility was 57.02%, and the actual experimental values were 51.44% for SR, and 58.28 ± 0.5% for solubility.

Regarding the solubility response for Flavourzyme® (Equation (10)), it was influenced by the hydrolysis time (B) and E/S ratio (C). The quadratic term (B^2) dictates the curvatures shown on the 3D response surface images (Figure 3A–C) and illustrates that this independent factor can be optimized to maximize solubility.

$$\text{Flavourzyme: Solubility (\%)} = 65.86 + 3.32B + 4.13C - 4.87B^2 \quad (10)$$

Solubility (%) *p*-values:

- B: $p = 0.0253$; C: $p = 0.0112$; B^2: $p = 0.0257$.

Table 4. Experimental results of solids recovery and solubility to produce spray-dried hydrolyzed cricket protein (HCP) using Flavourzyme® by BBD experimental design. Optimized enzymatic hydrolysis conditions displayed in the last row.

Test	Solids-to-liquid Ratio (S/L ratio)	Hydrolysis Time (min)	Enzyme-to-Substrate Ratio (E/S Ratio, %)	Solids Recovery (%)	Solubility (%)
* 1	1:5	120	1.5	51.52	65.15
2	1:6	120	0.5	48.68	60.50
3	1:5	180	0.5	49.53	59.45
* 4	1:5	120	1.5	50.13	68.65
5	1:4	120	2.5	49.54	64.95
* 6	1:5	120	1.5	50.41	63.77
7	1:6	120	2.5	48.01	61.63
8	1:4	180	1.5	51.46	61.84
9	1:5	60	2.5	50.31	57.23
10	1:5	60	0.5	52.19	47.90
11	1:6	60	1.5	51.90	55.43
12	1:6	180	1.5	54.45	57.17
13	1:4	120	0.5	48.62	49.75
14	1:5	180	2.5	49.54	65.48
15	1:4	60	1.5	48.77	55.46
Flavourzyme®–HCP	1:4	127	1.8		61.25 ± 0.8

* Central points.

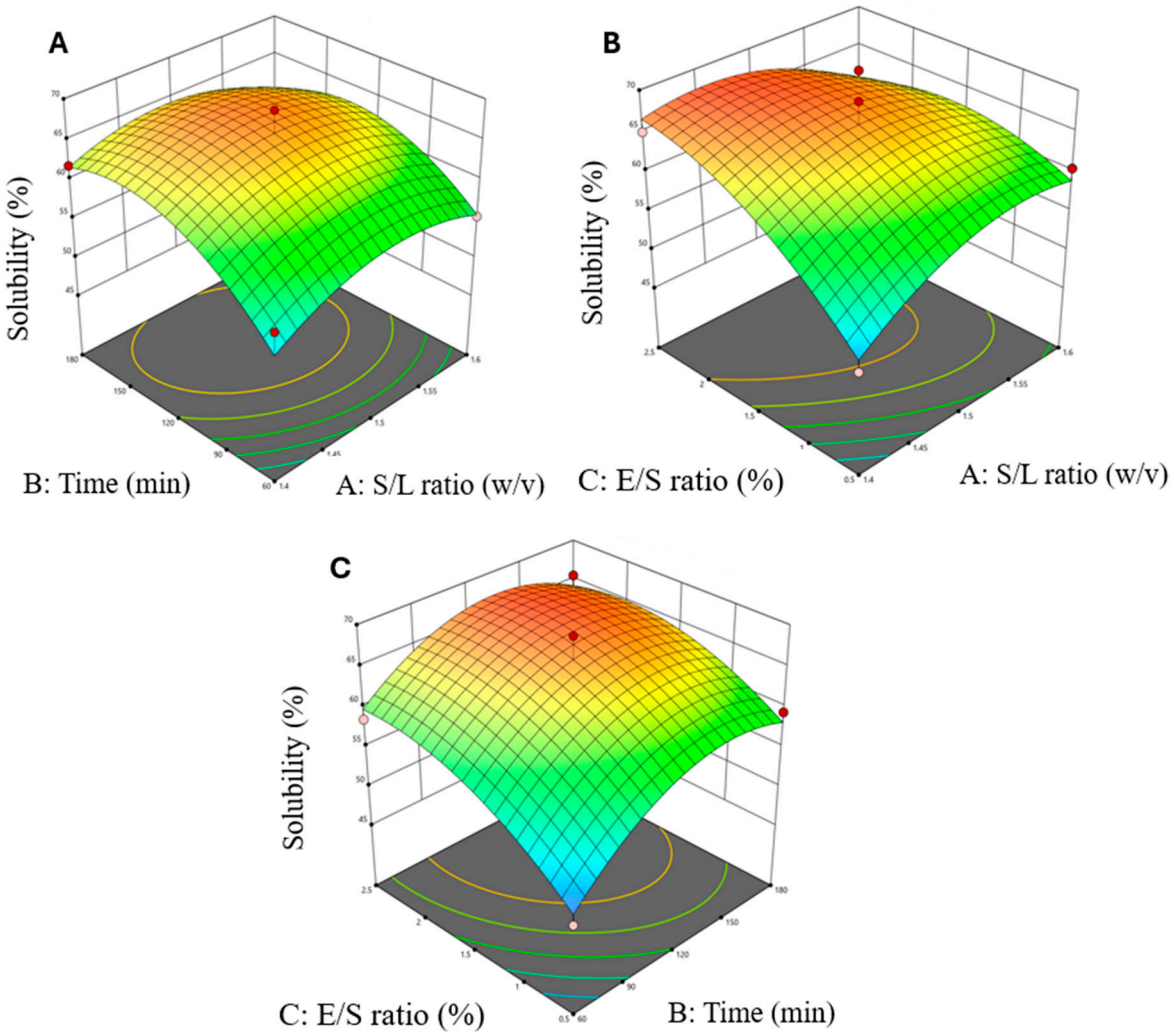


Figure 3. Three-dimensional response surfaces to obtain spray-dried hydrolyzed cricket protein (HCP) using Flavourzyme®. Solubility as a response of (**A**): S/L ratio and hydrolysis time; (**B**): S/L ratio and E/S ratio; (**C**): hydrolysis time and E/S ratio.

For Flavourzyme® treatments, the solids recovery model and LoF are not significant ($p > 0.05$), with a coefficient of determination (R^2) of 0.5563, indicating the data did not adjust well to the model (Table 5). Differently, the solubility regression model is significant, and the LoF is not significant ($p > 0.05$), showing that the data fit the model (Table 5). A high coefficient of determination was found for Flavourzyme® treatments ($R^2 = 0.9071$ for solubility), indicating a strong agreement between the adjusted and empirical data results.

As seen in Table 4, the highest solids recovery (54.45%) for Flavourzyme® treatments was achieved under the following conditions: S/L ratio of 1:5, hydrolysis time of 60 min, and E/S ratio of 0.5%. The highest solubility (68.65%) was achieved at an S/L ratio of 1:5, hydrolysis time of 120 min, and E/S ratio of 1.5%. However, because the SR model for Flavourzyme® treatments was not significant, the optimized enzymatic hydrolysis treatment conditions were calculated only for solubility. A desirability function with the goal of maximizing the solubility response and minimizing each factor determined that the optimized conditions were an S/L ratio of 1:4, hydrolysis time of 127 min, and E/S ratio of 1.8% (Table 4). The predicted value for solubility was 67.14%, and the actual experimental value was 61.25 ± 0.8%.

Table 5. Analysis of variance (ANOVA) of BBD models for solids recovery and solubility to produce optimized spray-dried hydrolyzed cricket protein (HCP) with Alcalase®.

Factor	SS	df	MS	F-Value	*p*-Value
Solids recovery (%)					
Model	22.77	9	2.53	0.6966	0.7001
Residual	18.16	5	3.63		
LoF	17.08	3	5.69	10.53	0.0879
PE	1.08	2	0.5405		
Total	40.93	14			
R^2	0.5563				
Solubility (%)					
Model	434.04	9	48.23	5.42	0.0386
Residual	44.45	5	8.89		
LoF	31.80	3	10.60	1.67	0.3950
PE	12.66	2	6.33		
Total	478.50	14			
R^2	0.9071				

SS, sum of squares; df, degree of freedom; MS, means square; LoF, lack of fit; PE, pure error; R^2, coefficient of determination.

4.2. Techno-Functional Characterization

To determine the impact of hydrolysis coupled to spray drying on the insect-protein functionality, the non-processed cricket-protein control RC and both optimized HCP treatments (using Alcalase® or Flavourzyme®) were evaluated and compared. The moisture content between the optimized Flavourzyme®–HCP sample and RC was significantly different ($p = 0.0374$). The water activity for RC and optimized samples was similar ($p > 0.05$). All samples comply with the FDA criteria for shelf-stable foods, where microbiological stability is generally ensured at values below 0.60 [17].

Powder hygroscopicity expresses the powder's ability to absorb moisture from the environment and greatly influences how food powders are handled and stored [17,24]. The hygroscopicity of optimized Alcalase®–HCP and Flavourzyme®–HCP samples was similar ($1.25 \pm 0.00\%$ and $1.28 \pm 0.01\%$, respectively) but significantly higher than that of RC ($1.14 \pm 0.01\%$, $p < 0.05$). However, the results are below 10%, and therefore all three samples are considered non-hygroscopic [25].

4.2.1. Flowability: Hausner Ratio and Carr's Compressibility Index

Table 6 shows the physical and functional properties of the non-processed cricket-protein control (RC) and the spray-dried hydrolyzed cricket protein (HCP) obtained under optimized conditions using Alcalase® and Flavourzyme®. Flowability is defined as the sample's ability to flow freely in a regular and constant manner, and it is generally measured by the Hausner ratio (HR) and Carr's compressibility index (CI) [19]. The flowability of food powders is an important property to explore because of its impact on various stages of production and overall product quality [26]. Samples were classified considering [20] the Hausner ratio and Carr's index, respectively: (excellent) 1.00–1.11, ≤10; (good) 1.12–1.18, 11.0–15.0; (fair) 1.19–1.25, 16–20; (passable) 1.26–1.34, 21–25; (poor) 1.35–1.45, 26–31; (very poor) 1.46–1.59, 32–37; and (awful) >1.60, >38. The RC and optimized Flavourzyme®–HCP had very poor flowability compared to the awful flowability of the Alcalase®–HCP sample, for both HR and CI (Table 6). Moreover, the RC was significantly different from the optimized Alcalase®–HCP for both HR and CI ($p < 0.05$). This finding is comparable to results for insect protein (*A. domesticus*) blended with pea protein complexed with polyphenols [17]. Overall, our results show that enzymatic hydrolysis with the investigated enzymes coupled with spray drying did not improve flowability, which was generally expected. This could be because of the exposure of hydrophobic groups after EH, or due

to interparticle interactions such as increased cohesive forces in the high-protein powders after spray drying [27].

Table 6. Physical and functional properties of non-processed cricket-protein control (RC) and spray-dried hydrolyzed cricket protein (HCP) obtained under optimized conditions using enzymes Alcalase® and Flavourzyme®.

Parameter	RC	Optimized Alcalase®–HCP	Optimized Flavourzyme®–HCP
Moisture (%)	2.63 ± 0.08 ^{b}	2.88 ± 0.4 ^{ab}	4.17 ± 0.4 ^{a}
Water activity	0.2351 ± 0.0002 ^{a}	0.2501 ± 0.02 ^{a}	0.2406 ± 0.03 ^{a}
Hygroscopicity (%)	1.14 ± 0.01 ^{b}	1.25 ± 0.00 ^{a}	1.28 ± 0.01 ^{a}
Hausner ratio	1.54 ± 0.06 ^{b}	1.78 ± 0.02 ^{a}	1.59 ± 0.05 ^{ab}
Carr's compressibility index (%)	35.03 ± 2.5 ^{b}	43.81 ± 0.6 ^{a}	37.01 ± 2.0 ^{ab}

Results are shown as mean standard deviation (n = 2). Superscripts with different letters (a, b) in the same row are significantly different; one-way ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

4.2.2. Solubility

Solubility is a key functional property for food ingredients, as it enables easier incorporation into product formulations and subsequent applications [17,28]. Our results showed improved solubility for both optimized hydrolyzed samples at the investigated pH range (4, 7, and 10) compared to RC (Figure 4). Alcalase®–HCP treatment had the highest solubility at pH 10 (86.64%). A significant difference was observed for pH 4 vs. 10 and pH 7 vs. 10 ($p < 0.0001$), but not for pH 4 vs. 7 (Figure 4). Similar results were observed for the optimized Flavourzyme®–HCP treatment: a significant difference for pH 4 vs. 10, pH 7 vs. 10 ($p < 0.0001$), and pH 4 vs. 7 ($p < 0.05$), with its highest solubility at pH 10 (73.04%). The same trend can be seen for RC across the investigated pH range ($p < 0.0001$), with the lowest solubility at pH 4 (26.18%) and highest at pH 10 (42.42.%). Overall, the optimized Alcalase®–HCP sample outperformed both the optimized Flavourzyme®–HCP and RC samples at all investigated pHs. Additionally, there was a significant difference between all samples at each pH ($p < 0.0001$; Figure 4).

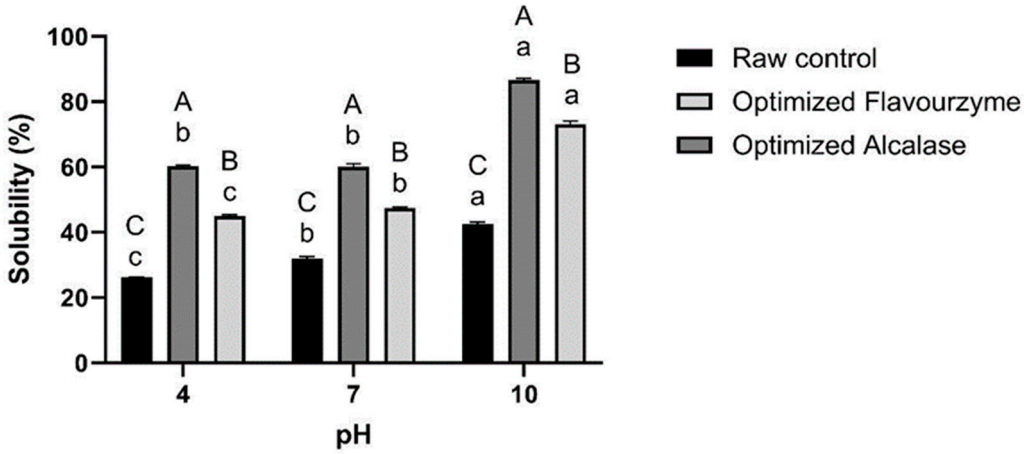


Figure 4. Solubility of non-processed cricket-protein control (RC) and optimized spray-dried hydrolyzed cricket protein (HCP) samples produced by Alcalase® and Flavourzyme®. Capitalized letters (A, B, C) represent significant ($p < 0.0001$) differences between all samples within a set pH, and lowercase letters (a, b, c) represent significant ($p < 0.0001$ or $p < 0.05$) differences among the same sample at all pHs.

Higher solubility for hydrolyzed protein using enzymes has been reported before. Authors reported that cricket-protein hydrolysates were significantly more soluble ($p < 0.05$) after EH over a range of pH values (3, 7, 8, and 10) when compared to the non-hydrolyzed control [10]. Moreover, an overall trend of increasing solubility with increasing pH was

observed. For cricket-protein hydrolyzed with Alcalase®, a significant increase ($p < 0.05$) in solubility at a range of pH (3, 7, and 8) compared to the non-hydrolyzed control was reported, with the highest solubility seen at pH 8 [29]. The increased number of smaller peptides and individual amino acids resulting from EH increases polar, hydrophilic groups, which may, in turn, justify the observed outcomes [29].

4.2.3. Foaming Properties

Protein foaming is a three-stage process. Initially, proteins diffuse to the air/water interface to reduce surface tension, followed by protein unfolding at the interface with re-orientation of hydrophilic and hydrophobic groups. Finally, polypeptides interact to form a film around bubbles to create foam [30,31]. Foaming capacity (FC) signifies the ability of a foam, in this case, a protein solution, to increase its volume by efficiently trapping air bubbles within a continuous liquid phase, and the foaming stability (FS) is the ability of the foam to sustain that volume over time [17].

For FC, there was a significant difference between all samples within a given pH ($p < 0.0001$), except between the RC and Flavourzyme®–HCP at pH 7 (Figure 5). Additionally, there was a significant ($p < 0.0001$) difference when pH was compared for each individual sample. Higher FC was observed at pH 10 for all samples, with Alcalase®–HCP having the highest result (50.98%). The FC for both optimized HCP samples was significantly higher ($p < 0.0001$) than RC at pHs 4 and 10. Interestingly, the FC of both HCP samples was the same or lower compared to RC at pH 7. Opposingly, higher results were reported for the FC of commercial cricket powder (*Gryllodes sigillatus*) at pH 7 (82%) [32].

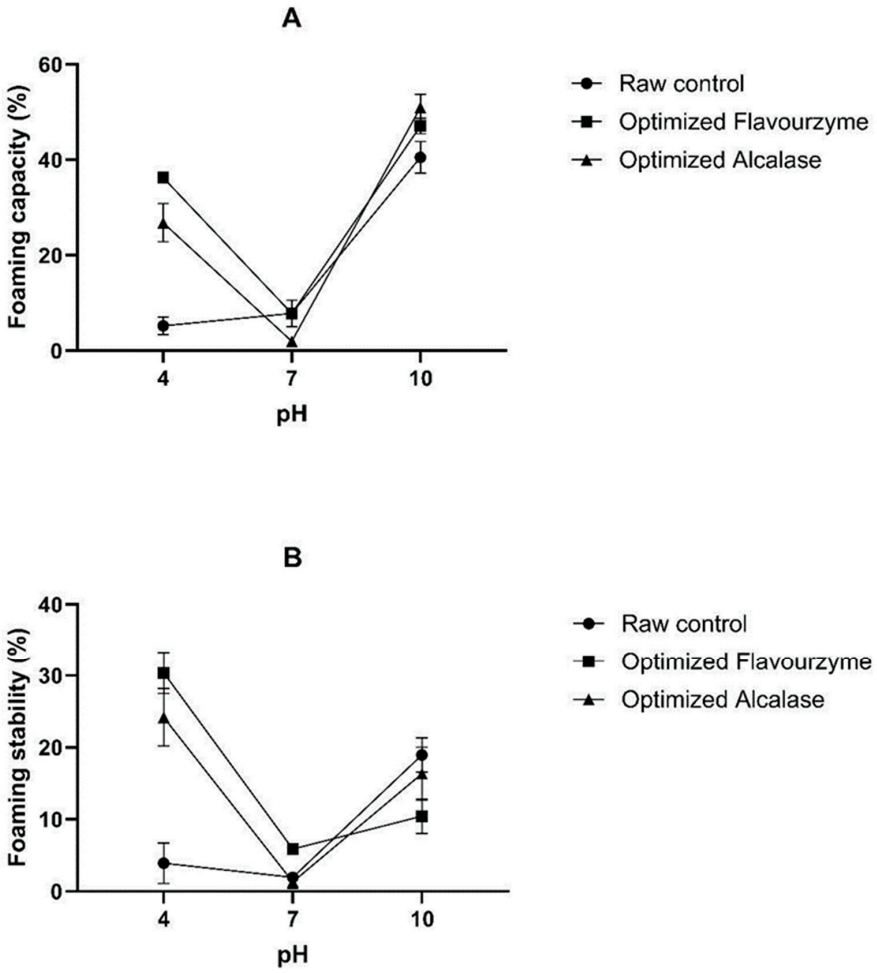


Figure 5. Foaming capacity (**A**) and foaming stability (**B**) of non-processed cricket-protein control (RC) and optimized spray-dried hydrolyzed cricket protein (HCP) samples.

Similarly to FC, there was a significant difference between the FS of all samples within a given pH ($p < 0.0001$) and when pH was compared ($p < 0.0001$) for each individual sample (Figure 5). The insect foam was more stable in acidic conditions than at pHs 7 and 10, with optimized Flavourzyme®–HCP having the highest foam stability (30.39%), while RC was more stable than optimized HCP samples in alkaline conditions. These results show that the pH influences the foaming properties of insect protein. Overall, the foaming properties of optimized samples improved after EH, mainly the FC, while some FS enhancement was shown only at pH 4. Our results indicate that EH may promote interfacial activity but affects polypeptide size and molecular weight, thus impacting foam strength [10,29]. Overall, our results provide valuable insights into determining the appropriate applications and processing conditions where these ingredients perform best.

4.2.4. Emulsification

Food protein emulsification is an important functional property because amphiphilic proteins can be used to reduce interfacial tension and stabilize oil–water interfaces in food products, since proteins can demonstrate flexibility to mitigate coalescence and flocculation during this process [33]. To measure a protein's emulsification capability, the emulsifying activity index (EAI) and emulsion stability index (ESI) were calculated. EAI is the measurement of how well a substance creates an emulsion and, more specifically to this study, the amount of oil that is emulsified by the amount of protein present in the oil/water emulsion [34], and ESI measures the stability of the emulsion over time [17].

Overall, enzymatic hydrolysis coupled with spray drying did not improve EAI compared to RC but did improve their ESI (Figure 6). This result opposes some reports for non-spray-dried hydrolyzed insect protein. Indeed, hydrolyzed, shorter peptides did not necessarily migrate to the oil/water interface quicker but were more efficient at reducing the interfacial tension, creating a stronger film around the oil droplets [35]. This could be due to an increase in protein solubility as the pH increases. Moreover, future work could take into consideration the degree of hydrolysis as a response and its effect on these properties [36]. Samples were compared within a given pH, and a single sample was compared between each pH. For samples within a given pH, the EAI was significantly different ($p < 0.05$ or $p < 0.0001$), except between RC and Flavourzyme®–HCP at pH 4 and 7 and between optimized samples at pH 10. A significant difference ($p < 0.05$ or $p < 0.0001$) was also seen when comparing pHs (4, 7, and 10) for an individual sample, except between pH 4 and 7 for both optimized samples.

For ESI, a significant difference ($p < 0.0001$) was seen between samples within a given pH, except between RC and Flavourzyme®–HCP at pH 4, and between pH values for an individual sample. At pH 10, both the EAI and ESI were significantly higher ($p < 0.0001$) than the other investigated pHs. This general trend agrees with the literature that shows that a more alkaline pH allows proteins to unfold more easily, decreasing their interfacial tension [37]. Optimized samples demonstrated better emulsion stability than RC at all investigated pHs (Figure 6B). Moreover, the stability of Alcalase®–HCP treatment peaked at pH 7 (74.70%), and there was a trend of increasing stability with increasing pH for optimized Flavourzyme®–HCP treatment.

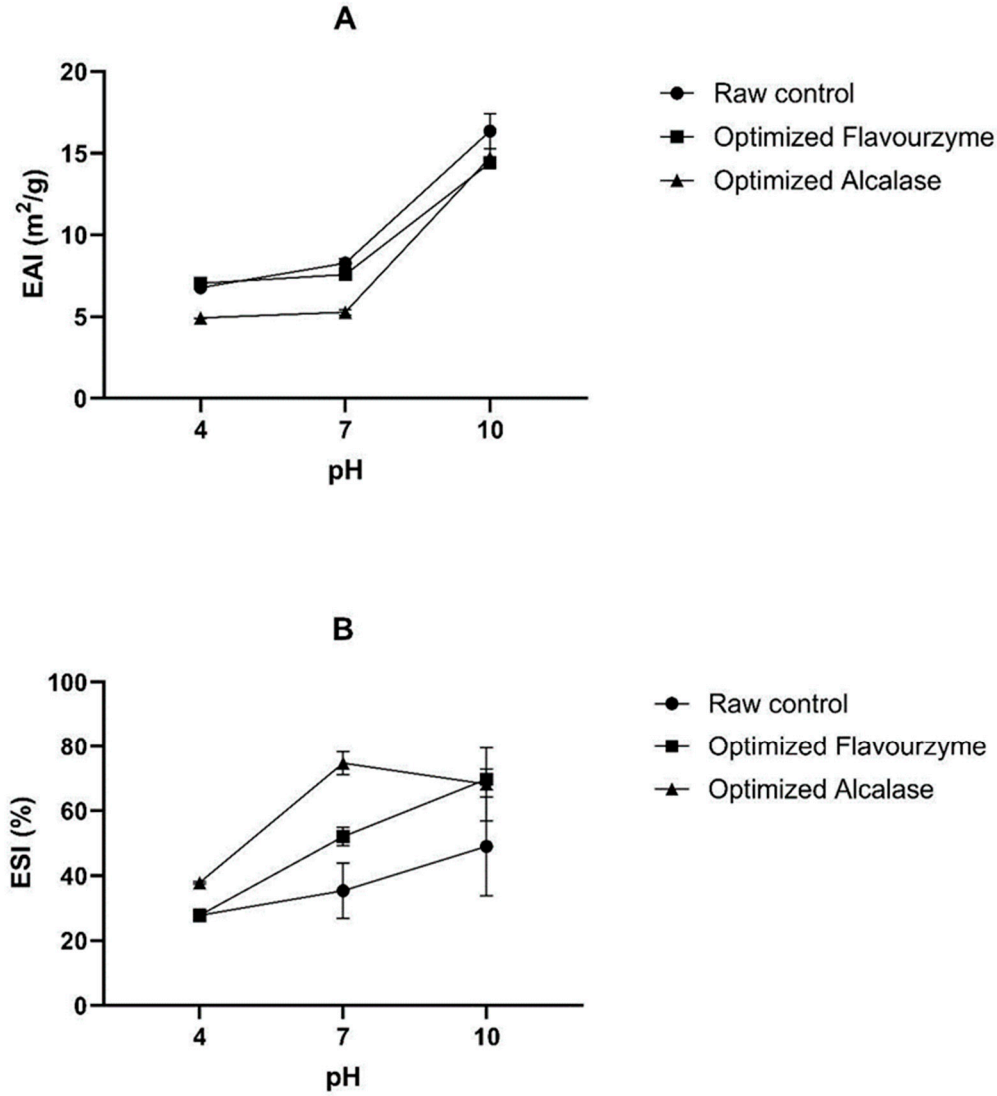


Figure 6. (**A**) Emulsion activity index (EAI, m^2/g) and (**B**) emulsion stability index (ESI, %) of non-processed cricket-protein control (RC) and optimized spray-dried hydrolyzed cricket protein (HCP) samples.

5. Limitations

Limitations to consider include consumer perception, enzyme cost, and the fact that rearing insects for use in human food is a relatively new industry in the Western world. Additionally, house crickets can contain a highly stable protein allergen called tropomyosin that could cause negative side effects after they are incorporated into food products and consumed, and enzymatic treatments may not minimize risks [38]. Future work could utilize other proteolytic enzymes to analyze the effects on functional properties of optimized ingredients. Moreover, optimizing additional responses such as the degree of hydrolysis could potentially further improve the functional and organoleptic properties of these ingredients.

6. Conclusions

This study investigated the optimization of enzymatic hydrolysis coupled with spray drying to produce insect-protein ingredients with enhanced solubility and drying performance. Optimized spray-dried hydrolyzed cricket protein (HCP) was obtained using two different proteolytic enzymes, Alcalase® and Flavourzyme®. The optimized processing conditions were established (Alcalase®: S/L for both solubility and SR: ratio (1:4), time

(117 min), E/S ratio (1.2%); Flavourzyme® for solubility: S/L ratio (1:4), time (127 min), E/S ratio (1.8%)). Furthermore, our results showed enhanced solubility at a range of pHs (4, 7, and 10), improved foaming capacity (pH 4 and 10), foaming stability (pH 4), and emulsion stability (pH 7 and 10) for both optimized insect-protein ingredients. To the best of our knowledge, this is the first report showing the use of these two technologies to produce *A. domesticus* insect-protein ingredients with improved functional properties. This work contributes to the goal of improving the quality of functional insect-protein ingredients for the emerging alternative protein market, as well as to increase their marketability and application potential in the food industry.

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Abbreviations

The following abbreviations are used in this manuscript:

HCP	Hydrolyzed cricket protein
EH	Enzymatic hydrolysis
RC	Non-hydrolyzed cricket-protein control
FDA	Food and Drug Administration
USA	United States of America
HCI	Hydrochloric acid
SDS	Sodium dodecyl sulphate
BBD	Box–Behnken design
RSM	Response surface methodology
HR	Hausner ratio
CI	Carr's compressibility index
FC	Foaming capacity
FS	Foaming stability
EAI	Emulsifying activity index
ESI	Emulsion stability index

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