Effect of sonication pretreatment parameters and their optimization on the antioxidant activity of *Hermitia illucens* larvae meal protein hydrolysates

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Abstract
The study investigated the effect of sonication conditions on antioxidant activity of *Hermitia illucens* larvae meal protein hydrolysates. Three-factor three-level: pH (7–9), time (10–30 min), and temperature (25–55°C) were optimized. Box–Behnken's design was applied to optimize sonication treatment. Ferrous ion chelating activity (ICA), DPPH-radical scavenging activity (DPPHRSA), Hydroxyl radical scavenging activity (HRSA), and cupric ion chelating activity (CCA) were considered as responses. Findings demonstrated that sonication preceding enzymolysis significantly impacted on ICA, DPPHRSA, HRSA, and CCA. ANOVA showed the determination coefficient ($R^2$) were 0.98 (ICA), 0.99 (DPPHRSA), 0.98 (HRSA), and 0.88 (CCA); demonstrating that the models were reasonably fit with experimental results. Optimum sonication conditions were pH (9), time (29.84 min), and temperature (54.93°C). For these conditions, the experimental data obtained [ICA (37.84%), DPPHRSA (43.19%), HRSA (71.01%), and CCA (68.93%)] were consistent with predicted values, higher than control, and supported by protein subunits, fluorescence spectra and microstructure.

Practical applications
With a rich nutrient profile, edible insects are potential ingredient for food applications. *Hermetia illucens* is one of the most encouraging edible insect species for incorporation in food products as it has several benefits to the environment, coupled with the already available knowhow for their rearing. With the prediction that in the next few decades insects will be reliable source of protein for humans and livestock, it is logical that the antioxidants activity of insect larvae meal proteins are investigated for new product development. Ultrasound is reported to enhance enzyme action in the preparation of bioactive hydrolysates/peptides. The present study showed that the use of ultrasonication pretreatment in the enzymatic hydrolysis of HILMP to generate hydrolysates with antioxidant constituents was efficient (likened to conventional approach). The study outcome could help the food and/or pharmaceutical industry to advance new bioactive products/ functional foods from HILMP hydrolysates.
INTRODUCTION

Over the last two decades, a growing demand among consumers across the world for bioactive elements/antioxidants from natural sources as oppose synthetic ones has resulted in the search for same by researchers for use as food ingredients or the development of functional foods. The search is driven by the continuous generation of free radicals (reactive oxygen and/or nitrogen species) in humans, as part of their usual metabolic process, which when in excess could aid the development of certain diseases (e.g., stroke, cancer, inflammation, or myocardial infarction). Free radicals may also contribute to skin pathologies or the aging of skin cells in humans (Stadtman, 2006; Umachigi et al., 2007). Antioxidants are documented as good remedies for controlling, reducing, or eliminating these diseases in humans (Lobo, Patil, Phatak, & Chandra, 2010; Ruiz-Ruiz, Dávila-Ortiz, Chel-Guerrero, & Betancur-Ancona, 2013). As a consequence the search for antioxidants from diverse natural sources by food scientists for possible use as food ingredient or the development of functional foods continuous.

Edible insects are potential ingredient in food applications, as they are rich in nutrients such as protein, lipids/ fatty acids, fiber, or micronutrients (Rumpold & Schlüter, 2013). Among the most encouraging species for the incorporation of edible insects in contemporary food is the black soldier fly (Hermetia illucens), due to its benefits to the human environment, coupled with its rich nutritional profile: 37%–63% protein, 7%–39% fat, and 9%–28% ash (Barragan-Fonseca, Dicke, & van Loon, 2017). Also, there are already technologies in place for the rearing of this insect on commercial basis for food and feed applications (Barroso et al., 2014).

The incorporation of insects such as HI (H. illucens) in the diets of humans may well enrich their food in bioactive constituents/compounds—peptides (Zielińska, Karaś, & Jakubczyk, 2017). Bioactive compounds/peptides can be generated during gastrointestinal digestion, or by in vitro proteolysis, resulting in the production of small molecular weight compounds with many health benefits to humans, such as antioxidant, antihypertensive, and anti-inflammatory benefits (Gobbetti et al., 2015; Karaś et al., 2015; Megías et al., 2007). Studies on the techno-functional properties of insect-derived protein hydrolysates are also documented (Hall, Jones, O’Haire, & Liceaga, 2017; Purschke, Meinschmidt, Horn, Rieder, & Jäger, 2018). There are also few studies on the antioxidant activity of edible insect protein hydrolysates (Zhang et al., 2016; Zielińska, Baraniak, & Karaś, 2017), but no information has been documented explaining the effect of ultrasound pretreatment on the antioxidant properties of Hermicia illucens larvae protein hydrolysate (HILPH). As a consequence, in this study for the first time, the influence of ultrasound aided enzymolysis of H. illucens larvae meal protein (HILMP) on the antioxidant activity of the hydrolysate thereof, is considered. We optimized the enzymolysis pretreatment conditions to obtain the protein hydrolysate with the optimum antioxidant activity. ICA, DPPH RSA, HRSA, and CCA were used to assess the antioxidative capacity of the hydrolysate peptides. The metals, Fe(II) and Cu(II), were considered as a result of their catalytic role in the generation of reactive oxygen species in aerobic organisms (Perron & Brumaghim, 2009).

2 | MATERIAL AND METHODS

2.1 | Sample and chemicals

H. illucens larvae meal, defatted using ethanol (Zhao, Vázquez-Gutiérrez, Johansson, Landberg, & Langton, 2016), was provided by the research team of L-507B (Jiangsu University’s School of Food and Biological Engineering, China). The larvae had been previously received from the Difei Biological Technology Company Limited (Jiangsu, China), and were microwave dried prior to milling and defatting.
Hydrogen peroxide (H₂O₂), paramagnetic solid (FeCl₂), and ferro-
zine were acquired from a chemical/reagent Company—Sinopharm Ltd (China). Enzyme (Alcalase—150,000 U/ml activity) was bought from a Biotech Company—Novozymes Ltd. (China). Remaining chemicals as well as 1, 1-DPPH (diphenyl-2-picrylhydrazyl) were of analytical rating.

2.2 | HILMP hydrolysates

*H. illucens* larvae meal was used to prepare a suspension (distilled water as solvent) with an initial substrate concentration of 14.0 g/L. The mixture was continuously stirred using a JJ-1 impeller agitator (J. Xichengxinrui Inst. Co., Jiangsu, China) set to 100 rpm, and the pH and temperature were set to the respective limits for the en-
zymolysis (Table 1). After, the respective suspensions/slurry were
pretreated by sonication using multiple frequency ultrasound (MFU) with sweep cycle of 500 ms, 5.5 L tank volume, pulse time of 15 s (on) and 5 s (off), 600 W power, and 40 ± 2 kHz frequency. The hydro-
lysation reaction was carried out according to the set conditions
(Table 1), using the mentioned enzyme (9,000 U/g). The reaction was halted after 90 min, and the enzyme was inactivated by heat (i.e., placing sample in boiling water) for a duration of 10 min. The solu-
ble fractions were collected by centrifuging at 4,000×
g for 15 min.

2.3 | Experimental design

A three-factor, three-level Box and Behnken’s design (BBD) with Box-
Wilson methodology (Response Surface) was used to investigate the
optimum sonication pretreatment conditions in preparing hydro-
lysates with best antioxidant capacity.

Thus, the sonication treatments were optimized using the fac-
tors: pH, time, and temperature under the set ultrasound parame-
ters. The results for the ICA, DPPH, HRSA, and CCA were the
dependent (response) variables.

The three factor levels were coded as: −1 (low), 0 (average), and
+1 (high), and the actual values for the factors were pH (a): 7, 8, and
9; sonication time (b): 10, 20, and 30; and sonication temperature (c):
25, 40, and 55, respectively. To expound the association between
the dependent (response) and independent variables, the responses
were fitted to a second order mathematical model:

\[ Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 \]

where, Y represent the responses (ICA, DPPH, HRSA, and CCA);
A, B, and C are independent variables; \( \beta_0 \) is the constant coefficient, \( \beta_1, \beta_2, \text{ and } \beta_3 \) are the linear coefficient; \( \beta_{12}, \beta_{13}, \text{ and } \beta_{23} \) are the cross product (interactive) coefficients; and \( \beta_{11}, \beta_{22}, \text{ and } \beta_{33} \) are the qua-
dratic coefficient.

A control experiment was also done to compare with the experi-
mental values derived from the best predictive points for all the four
responses.

2.4 | Determination of ferrous ion (Fe²⁺) chelating activity (ICA)

The ICA of the HILMP hydrolysates was estimated with the method reported by García-Moreno et al. (2014), with minor modification. The hydrolysate (3 ml) was transferred into test tube and then 0.1 ml

| TABLE 1 Box and Behnken’s design of three experimental factors with four responses |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Run | A: pH | B: Time (min) | C: Temperature (°C) | Fe²⁺ (%) DPPH (%) HRSA (%) Cu²⁺ (%) |
|-----|-------|---------------|---------------------|-----------------|-----------------|-----------------|
| 1   | 9.00  | 20.00         | 25.00               | 32.22           | 43.21           | 63.39           | 51.83           |
| 2   | 7.00  | 30.00         | 40.00               | 35.15           | 38.38           | 67.10           | 39.48           |
| 3   | 8.00  | 20.00         | 40.00               | 29.12           | 30.01           | 62.99           | 52.46           |
| 4   | 7.00  | 10.00         | 40.00               | 30.79           | 28.06           | 65.98           | 59.15           |
| 5   | 7.00  | 20.00         | 55.00               | 28.72           | 36.71           | 66.59           | 57.61           |
| 6   | 8.00  | 30.00         | 25.00               | 26.00           | 53.15           | 65.55           | 44.02           |
| 7   | 8.00  | 10.00         | 25.00               | 31.37           | 37.75           | 68.20           | 59.81           |
| 8   | 8.00  | 30.00         | 55.00               | 34.37           | 40.47           | 72.68           | 64.15           |
| 9   | 9.00  | 30.00         | 40.00               | 34.39           | 35.97           | 68.10           | 62.26           |
| 10  | 8.00  | 20.00         | 40.00               | 28.34           | 30.94           | 63.07           | 52.54           |
| 11  | 9.00  | 10.00         | 40.00               | 39.51           | 26.78           | 69.12           | 60.58           |
| 12  | 8.00  | 10.00         | 55.00               | 28.87           | 32.96           | 70.48           | 59.94           |
| 13  | 8.00  | 20.00         | 40.00               | 29.48           | 32.08           | 63.94           | 60.55           |
| 14  | 8.00  | 20.00         | 40.00               | 28.20           | 30.80           | 62.99           | 52.46           |
| 15  | 9.00  | 20.00         | 55.00               | 35.52           | 38.12           | 69.49           | 62.89           |
| 16  | 7.00  | 20.00         | 25.00               | 28.42           | 48.42           | 60.04           | 49.51           |
| 17  | 8.00  | 20.00         | 40.00               | 28.20           | 30.80           | 63.88           | 53.35           |
of FeCl₂ (ferrous chloride—2 mM) was added. After 5 min, 0.2 ml of ferrozone (5 mM) was added, vortexed, and allowed to react for 10 min at 25°C. The absorbance was then read at 562 nm, and the inhibition of Iron (II)-ferrozine complex formation was computed as follows:

$ICA(\%) = \left(1 - \frac{(A_{hs} - A_c)}{A_{wb}}\right) \times 100$

where $A_{hs}$ represent the absorbance of sample, $A_c$ absorbance of control (i.e., sample without ferrozine), and $A_{wb}$ the blank (distilled water used in place of sample).

2.5 | Determination of 1,1-DPPHRSA

The DPPHRSA of the HILMPH hydrolysates was measured with the technique reported by García-Moreno et al. (2014) with slight modification.

The hydrolysates (0.25 ml) was added to 0.5 ml of Tris buffer (pH 7.4, 50.0 mM). A 4.25 ml of fresh solution (0.1 mM) DPPH (methanol as solvent) was added, vortexed, and the mixture kept in dark (25°C, for 30 min) and the absorbance determined at 515 nm. Blank was prepared by replacing the hydrolysate with distilled water, and control was likewise prepared for each hydrolysate sample using CH₂OH (methanol) instead of the DPPH solution. The DPPHRSA was computed with the formula as follows:

$DPPHRSA(\%) = \left(1 - \frac{(A_{hs} - A_{sc})}{A_{wb}}\right) \times 100$

where $A_{hs}$ represent the sample absorbance, $A_{sc}$ control absorbance, and $A_{wb}$ the blank.

2.6 | Determination of scavenging activity—Hydroxyl radical (HRSA)

The HRSA of the hydrolysates was estimated using the method reported by Wang, Wang, Dang, Zheng, and Zhang (2013) with some modifications. Briefly, 1,000 µl of FeSO₄ solution (6 mM) was mixed with 1,000 µl of hydrolysate and 1,000 µl of H₂O₂ (6 mM) solution. The resultant mixture was vortexed and left for 15 min (25°C). Subsequently, to the mixture, 1,000 µl of a lipophilic monohydroxybenzoic acid (salicylic acid, 6 mM) was added and the absorbance (at wavelength 510 nm) was read after 30 min. The blank preparation was done by substituting the sample with distilled water (H₂O). The HRSA of the hydrolysate was calculated using equation as follows:

$HRSA(\%) = \left(1 - \frac{(A_{hs} - A_{ws})}{A_{wb}}\right) \times 100$

where $A_{hs}$ represent the absorbance of sample, $A_{ws}$ absorbance of sample devoid of salicylic acid, and $A_{wb}$ the blank.

2.7 | Cu²⁺ chelation assay

The ability of HILM hydrolysates/peptides in solution to chelate Cu²⁺ (prooxidative ion) was measured by the method reported by Zhuang, Tang, and Yuan (2013) with minor modification. For the reaction, 1 ml of CuSO₄ (2 mM) was mixed with 10% of pyridine (1 ml) and 0.1% of pyrocatechol violet (20 µl). Following the addition of the hydrolysate (1 ml), change in color (i.e., loss of blue color) was observed for 7 min, and absorbance reading was done at 632 nm. The CCA was then computed as follows:

$CCA(\%) = \left(1 - \frac{(A_{hs} - A_{wb})}{A_{wb}}\right) \times 100$

where $A_{hs}$ represent the absorbance of sample, $A_{ws}$ absorbance of sample without pyrocatechol violet, and $A_{wb}$ the blank sample (distilled water instead of sample).

2.8 | Amino acid evaluation

The protein subunits (%) of lyophilized sample (control and sonicated) at optimum condition was determined as described by Marino et al. (2010), with minor modification, using protein subunit autoanalyzer (S-433D, GmbH Sykam Co., Germany). The blank was prepared by adding 0.1% of pyrocatechol violet (20 µl). Following the addition of the hydrolysate (1 ml), change in color (i.e., loss of blue color) was observed for 7 min, and absorbance reading was done at 632 nm. The CCA was then computed as follows:

$CCA(\%) = \left(1 - \frac{(A_{hs} - A_{wb})}{A_{wb}}\right) \times 100$

where $A_{hs}$ represent the absorbance of sample, $A_{ws}$ absorbance of sample without pyrocatechol violet, and $A_{wb}$ the blank sample (distilled water instead of sample).

2.9 | Intrinsic fluorescence examination

The prepared HILMP hydrolysate samples (obtained at optimized condition—sonicated and control), freeze-dried, were subjected to intrinsic fluorescence (F₁) analysis at 24 ± 1°C using 0.05 mg/ml in 0.1 mol/L buffer (phosphate—PBS, pH 80). Cary Eclipse F, spectrophotometer (Varian-Alto Palo Incorporated, USA), with 1 cm cell as regards path length was used. The excitation and emission wavelength: 279 nm and 280–450 nm was applied; with slit width and scan-speed: 5 nm, and 10 nm/s in turn. PBS was utilized as spectrum blank, and scan spectra (10) expressed in mean scores were applied.

2.10 | Microstructure analysis

The microstructure of lyophilized HILMPH samples pretreated with and without ultrasonication, obtained at optimized condition for all the responses were analyzed using a smart light microscope (BX43-Olympus, Tokyo, Japan) installed with a V550D digital camera. The method outlined by Alenyorege and colleagues (Alenyorege et al., 2018) was used; and micrographs were captured at ambient temperature (22 ± 1°C) with 400× magnification.

2.11 | Statistical analysis

All treatments were done thrice and the results were presented as mean values. Statistical analysis was worked out using Design Expert software (v8061).
The model accuracy was evaluated using: the F-test, the lack of fit test, and $R^2$ (coefficient of determination) at 0.5, 0.001, and 0.0001% significance levels. The optimal pretreatment conditions were achieved by plotting the response surface-plots of each response (ICA, DPPHRSA, HRSA, and CCA).

3 | RESULTS AND DISCUSSION

Response surface methodology (RSM) is an arithmetic technique built on the appropriateness of quadratic (polynomial) equation to an experimental data (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). It well explains the nature of data set aiming to make arithmetic prediction/forecasting. Compared to conventionally used single-factor optimization, RSM is more favorable due to the fact that it saves time, sample usage, and space (Lee et al., 2012).

In this study, we have applied a RSM (Box–Behnken design) with three factors tested at three levels, with the aim of obtaining HILMP hydrolysates with optimum ICA, DPPHRSA, HRSA, and CCA. The values of the three factors considered in the present study are shown in Table 1 together with the experimentally measured values for the four (4) responses (DPPHRSA, ICA, HRSA, and CCA) for each experimental run as specified in the design. The measured values of the response variables were fitted to polynomial (quadratic) model, and ANOVA was utilized in assessing the influence of the studied variables, factor interactions, and significance of the model in arithmetic terms. The $p$- and $F$ values of regression coefficients for the dependent (response) variables are presented in Table 2. From the significant $p$-values of the models, it could be inferred that the quadratic polynomial model presented good estimations for the measured responses—demonstrating the fitness of the model. This was in agreement with the $R^2$ (multiple determination coefficient) values which were 0.98, 0.99, 0.98, and 0.88 for ICA, DPPHSA, HRSA, and CCA, respectively. To further confirm the model adequacy, the lack of fit values were shown to be statistically insignificant (Table 2).

3.1 | Effect of sonication parameters on ICA of HILMP hydrolysates

Metal ions (e.g., iron—Fe) are required for physiological roles in humans; but when in excess may cause serious cell/tissue damage. Excess ferrous ions (Fe$^{2+}$) in the body facilitates the formation of oxygen containing radicals and consequently causing several diseases (Lobo et al., 2010). Antioxidants will, in this regards, be needed to create a balance with the free radical production. Thus, in this study, the ICA of HILMP hydrolysates was considered.

The ICA of HILMP hydrolysates obtained through sonication pretreatments varied from 28.20–39.51% (Table 1). Figure 1 presents the influence of sonication treatment conditions on ICA of the hydrolysate. From the results (based on $p$-values), it is obvious that the principal factor influencing ICA, positively ($p < 0.0001$), was pH. Thus, ICA of the HILMP hydrolysates increased with increasing pH to a maximum PH of 9 (Figure 1a; Table 1). The influence of time was, however, not significant though positive. It was also evident in Figure 1b that the interaction between pH and temperature positively increased the ICA of HILMP hydrolysates. That is, increasing pH and temperature, together increased the ICA of the hydrolysates.

Compared to pH, temperature showed lower positive, but yet significant effect on the ICA. As illustrated in Figure 1b,c, ICA increased with increasing temperature. Temperature is one factor that is known to influence, positively, most extraction processes and/or activity of bioactive compounds; it exhibits remarkable effect on mass-transfer processes. At increased temperatures, cellular structure degradation increases which makes cells more permeable (Jovanović et al., 2017). Thus, sonication treatments at increasing temperature conditions favored the ICA of the hydrolysates. Hence,

| Table 2 | ANOVA for measured responses from Box and Behnken design |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Fe$^{2+}$ | DPPH | HRSA | Cu$^{2+}$ |
| **Model**      | 52.33    | 172.28 | 46.62 | 10.17 |
| **A (pH)**     | 100.57   | 13.27  | 32.88 | 11.95 |
| **B (Time)**   | 0.11     | 426.09 | 0.035 | 10.33 |
| **C (Temp)**   | 26.21    | 277.87 | 148.05 | 18.34 |
| **AB**         | 52.53    | 0.6    | 2.81  | 10.77 |
| **AC**         | 5.27     | 20.67  | 0.12  | 0.21  |
| **BC**         | 69.15    | 29.48  | 14.36 | 9.44  |
| **A$^2$**      | 133.27   | 7.25   | 0.058 | 0.015 |
| **B$^2$**      | 67.30    | 1.41   | 187.21 | 0.56  |
| **C$^2$**      | 12.50    | 757.13 | 25.61 | 0.64  |
| **Lack of fit**| 1.55     | 0.90   | 2.66  | 1.06  |
| **R$^2$**      | 0.9854   | 0.9955 | 0.9836 | 0.8809 |

Note: $p$-values < 0.05 are statistically significant.
from the present study findings, we can infer that ICA of HILMP hydrolysates increases with increasing sonication temperature.

Similar observation on the effect of increasing temperature has been indicated by other scholars for somewhat different studies but also on bioactive compounds (Arruda, Pereira, & Pastore, 2017; Chanioti & Tzia, 2017).

The current study results also showed that the interactions between pH and time, as well as that between temperature and time significantly influenced ICA. Furthermore, all the quadratic terms of the model showed significant effect on ICA of the hydrolysates.

To study the association between the factor and dependent variables, a second-degree quadratic equation was generated from the regression analysis. The regression equation for describing the efficiency of sonication pretreatment in achieving the optimum ICA of HILMP hydrolysates was:

$$\text{ICA} (\%) = 28.67 + 2.32A - 0.078B + 1.18C - 2.37AB + 0.75AC + 2.72BC + 3.68A^2 - 2.37B^2 - 1.13C^2$$

On the optimum conditions for obtaining maximized ICA of HILMP hydrolysates, the study results showed that sonication pretreatment conditions could be set at pH 8.99, time 10.00, and temperature 35.34°C; with a maximum predicted value of 39.78%.

3.2 Effect of sonication parameters on DPPHRSA of HILMP hydrolysates

The usual cell function is characterized by the formation of free radicals; but when in excess, may catalyze many diseases. To inhibit free radical catalyzed cell damage, antioxidants are thus needed (Young & Woodside, 2001). In this regards, 1,1-DPPH technique was applied in evaluating the antioxidant potential of HILMP hydrolysates. It (i.e., 1,1-DPPH technique) is generally used in analyzing the scavenging strength of protein-derived hydrolysates due to its precision (Laroque, Chabeaud, & Guérard, 2008).

The DPPHRSA of the HILMP hydrolysates obtained using sonication pretreatment (in this study) ranged from 26.78 to 48.42%. And the highest DPPHRSA was achieved with pH 7, sonication time of 20 min, and temperature of 25°C (Table 1). However, the lowest DPPHRSA was obtained under the sonication conditions: pH 9, 10 min, and 40°C. The investigated pretreatment conditions (pH and time) showed significant positive impact on DPPHRSA (Table 2; Figure 2a–c).

Temperature effect was also significant, but with a decreasing activity (at some point) from 25°C to the mid-ranged temperatures (37–43°C), and then increase from this point to 55°C (Figure 2b,c). Contrary to ICA, sonication treatment time showed the highest and significant positive influence on the % DPPHRSA (Figure 2c). Other studies using ultrasound aided extraction of bioactive compounds have indicated the positive effect of time in achieving best results (Rodrigues, Fernandes, de Brito, Sousa, & Narain, 2015).

Our results also showed that, the interactions between pH and temperature, as well as time and temperature significantly impacted DPPHRSA of the hydrolysates, while remaining limits were insignificant (Table 2).

The quadratic equation for predicting and/or describing the efficacy of the sonication pretreatment in achieving optimum DPPHRSA of the HILMP hydrolysates was:

$$\text{DPPHRSA} (\%) = 30.93 - 0.94A + 5.30B - 4.28C - 0.28AB + 1.65AC - 1.97BC + 0.95A^2 + 0.42B^2 + 9.74C^2$$

The present study results showed that the optimum pretreatment conditions for obtaining maximal DPPHRSA of HILMP hydrolysates were: pH 7.17, time 29.40 min, and temperature 25.09°C. The maximum predictive value, however, was 55.03%.

3.3 Effect of sonication parameters on HRSA of HILMP hydrolysate

Hydroxyl radical is a significant oxygen containing free radicals in many disease forms as they can destroy important biological molecules (e.g., DNA, fats, starches, and proteins) (Young & Woodside, 2001). To control their action in humans, antioxidants from external sources may be required.
Accordingly, the present study looked at the effect of three independent variables on the HRSA of HILMP hydrolysates. Our results showed that two of the variables, pH (A) and temperature (C), were highly significant (Table 2). Temperature level was found to have the main effect on HRSA (Figure 3c), suggesting that temperature tends to increase the HRSA of HILMP hydrolysates. Nonetheless, except for $A^2$ ($p > 0.05$), all the other quadratic terms $B^2$ and $C^2$, had significant effect on HRSA. Further, the results revealed that the interactive term $BC$ ($p < 0.05$) had positive effect on HRSA (Figure 3c); whereas the other interactions, though showed positive effect, were not statistically significant.

In the current work, the HRSA of the hydrolysates varied from 60.04 to 72.68%. The experimental conditions that showed highest HRSA were: $A = 8$, $B = 30$ min, and $C = 55^\circ$C; whereas the conditions: $A = 7$, $B = 20$ min, and $C = 25^\circ$C recorded lowest HRSA. Deducing from this is that, lower temperature and pH under pretreatment (sonication) conditions may not be desirable in generating HILMP hydrolysates with highest HRSA.

The regression equation for explaining the efficacy of the ultrasound pretreatments in generating HILMP hydrolysates with optimal HRSA is as follows:

\[
HRSA(\%) = 63.38 + 1.30A - 0.043B + 2.76C - 0.54AB - 0.11AC + 1.21BC - 0.075A^2 + 4.27B^2 + 1.58C^2
\]

The study results also showed that, the optimum conditions for sonication pretreatment could yield predictively maximal (73.57%) HRSA of the hydrolysates are: pH 8.96, time 29.91 min, and temperature 54.88$^\circ$C.

### 3.4 Effect of sonication parameters on CCA of HILMP hydrolysat

Just as in the case of Fe$^{2+}$, excess Cu$^{2+}$ in the body facilitates the production of oxygen containing radicals and accordingly may cause several tissue damages (Lobo et al., 2010). External sources of antioxidants may therefore be required to balance the action of produced free radical. This study, thus examined the CCA of HILMP hydrolysates.

Analysis of the CCA model showed that all the linear terms (A, B, and C) were significant variables. Temperature and pH had positive
effect on the CCA of the hydrolysates, indicating that an increase in the temperature and/or pH favored the chelating activity of the hydrolysates. By contrast, time showed a negated effect (Figure 4a,c) and this suggests that the CCA of the hydrolysates may be more favorable at shorter sonication (pretreatment) time.

The CCA of the HILMP hydrolysates obtained through ultrasound pretreatment varied from 39.48–64.15%; and the treatment with the highest CCA was achieved under the following condition: A = 8, B = 30 min, and C = 55°C. The least CCA was, however, obtained under the following pretreatment limits: A = 7, B = 30 min, and C = 40°C. This may be that sonication pretreatment under lower pH conditions may not be that suitable for the generation of HILMP hydrolysates with high CCA thereafter. This could be supported by the result that the second most linear term/factor influencing (positively) CCA of the hydrolysates was pH (Table 2).

Our results also indicated that the interactions among A and B (pH and time), and B and C (time and temperature) significantly influenced the CCA of the hydrolysates. Thus, as time decreases and pH or temperature increases, the CCA of the hydrolysates also increases. The quadratic levels (A, B, and C) did not statistically influence the CCA of the hydrolysates.

From our study results, the predictive equation for explaining the efficiency of the ultrasonic pretreatments in generating HILMP hydrolysates with optimum CCA was:

\[
\text{CCA} (%) = 54.27 + 3.98A - 3.70B + 4.93C + 5.34AB \\
+ 0.74AC + 5.00BC - 0.21A^2 \\
+ 1.31B^2 + 1.40C^2
\]

The optimum parameters for the ultrasound pretreatment that could yield 67.54% CCA of the hydrolysates, however, were: pH 8.89, time 27.73 min, and temperature 51.76°C.

### 3.5 | Model verification and validation

In order to verify the reliability of the model for all the investigated responses (ICA, DPPHRSA, HRSA, and CCA), an experiment was done under the optimal (predictive) sonication pretreatment conditions for the generation of hydrolysates with antioxidant activity. The optimal pretreatment/sonication conditions (for all four responses) were: pH 9, time = 29.84 min, and temperature = 54.93. The predicted responses (ICA = 38.26%, DPPHRSA = 41.39%, HRSA = 73.55%, and CCA = 72.84%) under the optimal conditions were compared to experimental values (ICA = 37.84%, DPPHRSA = 43.19%, HRSA = 71.01%, and CCA = 68.93%). The experimental data compared very well with the projected values; suggestive of the selected RSM (BBD) model was successfully applied for ultrasound pretreatment of HILMP in order to generate hydrolysates with ICA, DPPHRSA, HRSA, and CCA.

Free radicals are largely unstable and reactive. In situations where free radicals engulf an organ system’s ability to control them, it results in an oxidative stress (OS). Consequently, they (free radicals) undesirably alter the structure of proteins, fats, and DNA and initiate a couple of diseases in humans. To manage the OS, the utilization of antioxidants from external sources are recommended (Lobo et al., 2010). Also, metal ion (e.g., Fe^{2+} and Cu^{2+}) acts as a positive catalyst in the generation of reactive oxygen leading to same/similar health conditions in humans. The present study findings (DPPHRSA and HRSA, and/or ICA and CCA) suggest that the hydrolysates derived from HILMP may be beneficial in controlling OS in humans.

### 3.6 | Comparison of sonication and conventional pretreatments on ICA, DPPHRSA, HRSA, and CCA

Subsequent to validation of the model, the experimental values (ICA = 37.84%, DPPHRSA = 43.19%, HRSA = 71.01%, and CCA = 68.93%) obtained using sonication pretreatment were compared to a conventional one (control) under the set optimized conditions: pH 9, time = 29.84 min, temperature = 54.93. The conventional pretreatment (control) under the said parameters was done by replacing ultrasound with a JJ-1 impeller agitator (J. Xichengxinrui Inst. Co., Jiangsu, China). Regarding the outcome, the sonication pretreatment/method resulted in hydrolysates with higher ICA, DPPHRSA, HRSA, and CCA than the conventional method. The ICA, DDHRSA,
HRSA, and CCA using the conventional method were 28.98, 31.53, 58.71, and 56.34%, respectively. It was noted that the pretreatment by sonication resulted in higher responses than the conventional treatment.

The higher ICA, DPPHRSA, and CCA of the hydrolysate, observed with the ultrasound pretreatment, could be associated with the sonication waves facilitating the unfolding of the HILMP and consequently exposing hydrophobic ends and making it possible for an enhanced enzyme action which eventually lead to the production of hydrolysates with improved antioxidant (bioactive) activity than the traditional approach. This is in agreement with Gülseren, Güzey, Bruce, and Weiss (2007) when they reported that sonication pretreatment causes unfolding of proteins, an outcome which is good for enzyme action. Most importantly, to buttress the observed improvement in the antioxidant activity of the sonicated samples, is how ultrasound woks (i.e., it mechanism in extraction of target compounds). During ultrasonication treatment, cavitation bubbles (thin liquid sphere enfolding air/gas) swing back and forth and collapse, resulting in some physical effects such as shock waves, turbulence, microjets, and shear thrusts (Kadam, Tiwari, Álvarez, & O’Donnell, 2015; Tiwari, 2015). When the circulatory motion of the cavitation bubbles is intense as a function of the formation and collapse of bubbles (rarefaction and compression), it results in what is termed as microstreaming (Margulis & Margulis, 2004). Thus, the cavitation influence (shock waves/microjets) results in disruption (pore creation on surfaces) of cell wall of substrates/food samples, and decreased particle size; which consequently improves permeability of the food matrix, and mass transfer (Tiwari, 2015). Implicit from this is that, the cellular matrix of the sonicated samples in the current study, became more permeable, allowing solvent into its internal structure, enhancing enzyme action, causing target components to dissolve in solution, and thus facilitating the production of hydrolysates with improved antioxidant activity. This could be supported with protein subunit scores, fluorescence intensity, and microstructural data.

### 3.7 | Amino acid scores

Proteins with less than 20 or 16 down to 5 subunits demonstrate potent antioxidative activity (Zhou et al., 2015). Also, the type, amount and order of protein subunit also have effect on the bioactivity of hydrolysates. The amino acid scores of control and sonication treated samples are presented in Table 3. Glu is the main subunit in the control (conventional) hydrolysate. The HILMP hydrolysate (pretreated by sonication) showed the highest score of indispensable subunits (His, Ile, Leu, Lys, Met, Phe, Thr, and Val), likened the control. Hydrophobic units (Phe, Pro, Val, and Ile), normally linked to bioactive functions such as antioxidant activity (Megías et al., 2004), were enhanced when hydrolyzed with Alcalase, subsequent to sonication. Thus, the sonicated HILMP hydrolysate may contain protein with significant 5–16 subunits with C-terminus hydrophobic building blocks than control. This is due to the preferential cleavage of hydrophobic units at the C-terminus of proteins by Alcalase (Jia et al., 2010). The hydrophobic subunit in sonicated hydrolysate was higher likened to control (Table 3); and this support why sonication preceding enzyme hydrolysis resulted in improved antioxidative activity.

### 3.8 | 3.8 | 3.8 | F<sub>1</sub> of HIL protein hydrolysates

Spectra from F<sub>1</sub> are linked to protein subunits: Y (tyrosine), F (phenylalanine), and W (tryptophan) (Ma et al., 2011). Hence fluorescence spectra is used in illustrating the alterations of protein structure

#### Table 3 | Amino acid scores of control and sonication treated HILMP hydrolysate (%)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Control</th>
<th>Sonicated sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>7.87 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.91 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thr</td>
<td>2.31 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.05 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ser</td>
<td>1.72 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.71 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glu</td>
<td>4.80 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.51 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pro</td>
<td>0.96 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.51 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gly</td>
<td>2.38 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.98 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ala</td>
<td>7.76 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.05 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Val</td>
<td>2.98 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.17 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Met</td>
<td>2.20 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.38 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ile</td>
<td>2.36 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.31 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leu</td>
<td>5.14 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.68 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tyr</td>
<td>4.80 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.41 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>His</td>
<td>2.22 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.13 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lys</td>
<td>3.50 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.61 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arg</td>
<td>3.10 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.61 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HAA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>32.85</td>
<td>35.19</td>
</tr>
</tbody>
</table>

<sup>a</sup>Hydrophobic amino acid (Ala, Val, Leu, Ile, Phe, Pro, Thr, and Met); means with different superscript letters (in a row), are significantly different (p < 0.05).

#### Figure 5 | Fluorescence spectra of control and ultrasound treated HILMP hydrolysates
(Zhao, Dong, Li, Kong, & Liu, 2015) during product processing or storage (Keerati-u-rai, Miriani, Iametti, Bonomi, & Corredig, 2012). In this study, samples treated with ultrasound showed a shift in the maximal emission peak from 360 nm (control) to 365 nm, implying a rise in the polarity of subunits like W and F as a consequence of molecular alteration (Figure 5). Further, the F peak of the ultrasonic treated HILMP hydrolysates was intense than the conventional (control). It follows that sonication preceding enzymolysis modified the conformation of HILMP. That is, breaking hydrophobic interactions and consequently causing release (unfolding) of hydrophobic groups from the interior of molecule (Gülseren et al., 2007; Jambrak, Mason, Lelas, Herceg, & Herceg, 2008), reflecting the intense fluorescence of the HILMP hydrolysate relative to control. This is consistent with the observed variations in the antioxidant properties of the hydrolysates, with ultrasonication treated samples demonstrating enhanced activity than control.

3.9 | Microstructure

Microstructures (photomicrographs) of the HILMP samples pretreated with sonication, and without sonication (control) obtained at the optimized condition for the experimental responses are shown in Figure 6. The micrograph of the control showed distinctive compact or intact morphology/particles. However, the spaces between particles of the sonication treated samples appear separated/loose—more distracted. Deducing from this is that, the cavitation effect produced at the set ultrasonication conditions was good enough to create a sponge effect (alternating compressions and expansions) resulting in the loose structure of the samples pretreated by sonication; and this could further explain why the sonicated samples demonstrated superior antioxidant (bioactive) capacity in the current investigation.

4 | CONCLUSION

In this study, response surface methodology was successfully used to set the optimized conditions for ultrasound pretreatment of HILMP for enzymolysis. The investigated pretreatment parameters (pH, time, and temperature) impacted on the antioxidant activity (ICA, DPPHRSA, HRSA, and CCA) of the hydrolysates. With the Box–Behnken design, optimum pH, time, and sonication temperature were predicted for best ultrasound pretreatment conditions that could result in hydrolysates with ICA of 38.26, DPPHRSA of 41.39, HRSA of 73.55, and CCA of 71.01%. Compared to conventional pretreatment, the hydrolysis preceded by ultrasonication resulted in hydrolysates with higher antioxidant activity (ICA, DPPHRSA, HRSA, and CCA). The study showed that the use of sonication pretreatment in the enzymolysis of HILMP to generate hydrolysates with antioxidant components was efficient, buttressed by amino acid scores, F, spectra, and micrographs. This investigation could help the food and/or pharmaceutical industry to develop new bioactive products/functional foods from HILMP hydrolysates. Consequently, further studies on the in vivo functional properties of the HILMP hydrolysates is recommended.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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