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Production Optimization and Characterization of Antioxidant Protein Hydrolysate From *Piaractus brachypomus* Fish Meat By Probiotic *Bacillus* Strain Isolated From Chicken Gizzard

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ABSTRACT

The present study aimed to optimize the conditions for antioxidant potential hydrolysate (RPMPH) production from *Piaractus brachypomus* fish (RBPF) meat using the probiotic *Bacillus* (SNGC) isolated from the chicken gizzard. The RBPF meat exhibited 79.25% moisture, 14.06% protein, 5.08% fat, and 0.60% ash content. RSM generated optimum fermentative hydrolysis conditions was obtained at 48h fermentative time, 2% sucrose concentration, and 30% RBPF meat concentration for the DH (49.41%), DPPH activity (83.14%), ABTS activity (92.02%), and ferric reducing antioxidant power (273.87 μM TE/ g), and Fe^{2+} chelating) activity (68.87%). Furthermore, RPMPH displayed strong antibacterial properties against *L. monocytogenes* and *S. aureus*, while they were weak against *E. coli* and *S. enteritidis.* SDS-PAGE patterns of RPMPH revealed light and diffused low molecular weight bands ranging from 8- 25kDa. RPMPH exhibited slightly higher alanine, glycine, and serine content than the native RBPF meat protein. RPMPH showed solubility at a wide range of pH. RPMPH presented the highest emulsification at 2% concentration and foaming at 3% concentration. The water and oil absorption capacities of RPMPH were 2.55 and 1.74mL/ g, respectively. Therefore, RPMPH produced by the probiotic *Bacillus* (SNGC) strain could be used as a bio-functional ingredient or preservative in food, pharmaceuticals, and cosmetic preparations.

INTRODUCTION

Meat product development and consumption have significantly increased in recent decades due to being rich in nutrients and high net protein utilization. Meat proteins are called complete proteins containing all essential amino acids and are recognized as key ingredients for health and nutrition. In recent years, meat proteins have become the more popular supplement for sports and athletes. Fish are considered a highly nutritious food since they are rich in essential nutrients for health maintenance and protection from different diseases. Moreover, fish are believed to be a significant source of animal protein for human consumption due to their good protein digestibility, palatability, and

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availability. Consequently, fish production increased dramatically throughout the world. However, fish are a perishable food commodity that needs immediate consumption or proper preservation. Preservation techniques increase the shelf life and retain fish's quality and nutritional profile. Besides, developing value-added fish meat products is also possible to improve the traditional fish trade and profitability. Value addition to fish and fish products involves altering their nature to increase selling profits and enhance nutritional value. Therefore, the development of a value-added product from fish is growing in a more significant space. As a result, various value-added fish products were introduced into the global market. Nowadays, increasing trends have emerged in the development of fish products that can be easily stored, distributed, and retailed at ambient temperatures. Additionally, such product must retain or enhances the nutritional content in the diets of the targeted consumers. Therefore, fish protein hydrolysate is a product in high demand in the market.

Fish meat protein hydrolysate obtained via hydrolysis could be a good source of biologically active peptides with various biological functions **(Jemil *et al.,* 2014; Sripokar *et al.,* 2019)**. Amino acid composition and sequence of peptides determine its biological potential. Several studies have reported fish meat protein hydrolysate's antioxidant, ACE-inhibitory, and antimicrobial potentials **(Jemil *et al.,* 2014;Jemil *et al.,* 2016;Sripokar *et al.,* 2019)**. The degree of hydrolysis influences the bioactivities and functional properties of protein hydrolysate **(Sripolar *et al.,* 2019)**. Generally, protein solubility, water holding, oil binding, emulsification, and foaming properties of protein hydrolysate are mainly influenced by the composition, size, and surface charges of peptides **(Kudre *et al.,* 2018; Sripokar *et al.,* 2019)**. Protein hydrolysis is generally performed by chemical, enzymatic or microbial methods. Enzymatic hydrolysis is the most common method for preparing fish protein hydrolysates. In recent years, greater attention has been paid to producing protein bioactive protein hydrolysate by fermentative hydrolysis using probiotic bacteria. The probiotic bacterial fermentation of meat protein improves the biomass's digestibility, quality, physicochemical characteristics and provides probiotic substances' health benefits **(Bethi *et al.,* 2021)**. Moreover, fermentation is one of the best biotechnological preferable methods, chiefly in tropical countries, due to the availability of favorable temperature, carbohydrate sources in addition to being economically inexpensive. Protein hydrolysate prepared by the fermentative process offered better antibacterial, antioxidative, antihypertensive, and immunomodulatory properties **(Jemil *et al.,* 2014; Bethi *et al.,* 2021)**. **Jemil *et al.* (2014)** reported that the composition, functional properties, and in vitro antioxidant and antibacterial activities of protein hydrolysates are prepared with a proteolytic bacterium, *Bacillus subtilis* A26, through fermentation of fish proteins. The red-bellied pacu (*Piaractus brachypomus*) is a freshwater fish with a pomfret-like shape. It is also known as the red pomfret or freshwater pomfret. Furthermore, amino acid content, peptide sequence, molecular weight, and structure are all linked to antioxidant activity **(Wang *et***

***al.,* 2018**). Ironically, no studies were noted on the preparation of antioxidant and antibacterial protein hydrolysate from the RBP fish meat using probiotic fermentative hydrolysis. Therefore, the present investigation aimed to study the effect of fermentative hydrolysis (RBP fish meat concentration,sucrose concentration, and fermentation time)on antioxidant activity of red-bellied pacu fish meat protein hydrolysate by probiotic *Bacillus* strain isolated from the country chicken gizzard. Furthermore, optimized RBP fish meat protein hydrolysate (RPMPH) was evaluated for the antibacterial properties against *L. monocytogenes*, *S. aureus*, *E. coli*, and *S. enteritidis* and functional properties. Box–Behnken design of the response surface methodology (RSM) was used to perform the optimization of RPMPH. The RSM has been widely used to optimize the conditions for hydrolysate preparation with bioactivities.

MATERIALS AND METHODS

1. Chemicals

The deMan Rogosa Sharpe (MRS) media were purchased from HiMedia Laboratories Pvt. Ltd, Mumbai- India. L-leucine, TNBS (Trinitrobenzene Sulfonic Acid), DPPH (2, 2-diphenyl-1- picrylhydrazyl), ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6- sulphonic acid)] and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were procured from Sigma-Aldrich, USA. TPTZ (2,4,6- Tri- (2-pyridyl) -5 triazine), ferrozine monosodium was procured from Sisco Research Laboratories Pvt. Ltd. (SRL) - Mumbai- India. Pathogen microbial strains were purchased from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India.

1.1. Fish collection and preparation of minced meat

Piaractus brachypomus Cuvier 1818 (red-bellied pacu) fish were collected from the local fish market, Mysuru, Karnataka, India. Samples were packed in a polyethylene bag and transported to the laboratory within 30min in a chilled condition. Upon arrival, the fish samples were washed twice with tap water and distilled water. The cleaned fish fillets were minced using a vertical cutter (robotcoupe, France). Minced meat was defatted using n-hexane. Briefly, n-hexane was added to minced meat at a 1:3 ratio (meat: hexane, w/v). The resultant mixer was stirred for 30 minutes and subjected to centrifugation at 9000rpm for 25 minutes. Minced meat pellets were collected and placed in polyethylene bags and stored at –20°C until used, but not longer than one month. Prior to producing fish protein hydrolysate, the frozen minced fish meat was thawed using running tap water until the core temperature reached 28- 30°C. The proximate composition (moisture, protein, ash, and fat content) of minced meat was performed according to the methods of **AOAC (2000)**.

2.2 Preparation of red-bellied pacu protein hydrolysate

2.2.1 Micro-organism and inoculum preparation

The probiotic strain used in this study was previously isolated from chicken gizzard on an MRS agar plate. The isolated strain was identified as belonging to the *Bacillus* strain (SNGC), which is close to *Bacillus subtilis*. For inoculum preparation, isolated probiotic *Bacillus* culture (SNGC) was transferred from stock slant to MRS agar plate and incubated at 37°C for 24h. Subsequently, the grown culture on the MRS plate was inoculated into MRS broth and incubated at 37°C for 24h under constant shaking (50rpm). The resultant grown culture was used as inoculum for the fermentative hydrolysis of red-bellied pacu fish proteins.

2.2.2 Fermentation and protein hydrolysate preparation

Different ratios of minced meat from red-belied pacu fish (RBPF) were mixed with distilled water in 250mL-Erlenmeyer flasks, and the final volume of the mixture was 100ml. To these mixtures, different sucrose levels were added as a carbon source. These resultant mixtures were heated at 90ºC for 15min to inactivate the endogenous enzyme and native micro-organisms. Afterwards, the mixture was cooled to room temperature (27–30°C) and inoculated with 10ml of 24h old *Bacillus* inoculums in aseptic condition. The fermentation was carried out by incubating the flask at 37ºC with constant agitation (50rpm) at different times. The fermented RBPF meat (hydrolysate) was centrifuged at 9000rpm for 25min at 4°C. The supernatant was collected and freeze-dried using CoolSafe^{TM} Freeze Dryer (Labogene, Denmark) operated at -54°C, while the vacuum was set at 0.250mbar. The freeze-dried RBPF meat protein hydrolysate was subjected to analysis.

2.3 Optimization of fermentative hydrolysis of RBPF meat using the response surface model and experiment design

The Box-Behnken design (BBD) **(Box & Behnken, 1960)** of the response surface methodology (RSM) was adopted in the present study to evaluate the effect of three independent variables on the five dependent variables (responses) and determine the optimum conditions for the preparation of antioxidant RBPF meathydrolysate by *Bacillus* fermentation. Based on the preliminary experiments, three main independent variables were chosen for the study: sucrose concentration (A), meat concentration (B), and fermentation time (C). While, DH, DPPH, ABTS, FRAP, and Fe^{2+}chelating activity were selected as response factors. Table (1) displays the experimental ranges corresponding to the process variables. The three levels for the process factors were coded as –1, 0, and +1. Three levels of process variables were: sucrose concentration (1, 2, and 3%, w/v), meat concentration (20, 30, and 40% w/v), and fermentation time (24, 48, and 72h.). As per BBD design, 17 experimental runs were generated, including 12 design points and 5 replications at the center points (Table 1). All the experimental runs were performed in triplicates. Multiple regression analysis was applied to the responses obtained from the

experimental design to fit the quadratic polynomial model. The quadratic polynomial model used is expressed as follows:

$$Y = \beta_O + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j$$

Where, Y is the response variable; β_0 is a constant,β_i; β_{ii}, and β_{ij} represent linear, quadratic, and interaction coefficients, respectively; k is the number of variables, while X_i and X_j are the independent variables.

Table 1. Reaction condition variables and levels for BBD

Code	Numerical factor	Level		
		–1	0	+1
A	Sucrose concentration (%)	1	2	3
B	Meat concentration (%)	20	30	40
C	Fermentation time (h)	24	48	72

2.4 Degree of hydrolysis (DH)

DH of RBPF meat protein hydrolysate (RPMPH) was performed according to the method of **Benjakul and Morrissey (1997)**. The DH was calculated using the following formula:

$$\mathrm{DH} = \frac{(L_t - L_0)}{(L_{max} - L_0)} X\ 100$$

Where, L_t is the amount of α-amino group released at time t; L_0 is the amount of α-amino group in the supernatant at 0 h., and L_{max} stands for the total amount of α-amino obtained after acid hydrolysis.

2.5 *Determination of antioxidant* activities

2.5.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical-scavenging activity of RPMPH was measured following the method of **Wu *et al.* (2003)**, with slight modification. The following formula is the description of the activity used:

$$\text{DPPH radical scavenging (\%)} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

2.5.2 2'-azino-bis-3 ethylbenzthiazoline-6-sulfonate (ABTS) radical scavenging activity

The ABTS radical scavenging activity, as described by **Binsan *et al.* (2008)**, was determined. ABTS radical scavenging activity formula was expressed as follows:

$$\text{ABTS radical scavenging (\%)} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

2.5.3 Ferric reducing antioxidant power (FRAP)

FRAP was measured according to **Benzie and Strain (1996),** with minor modifications. Trolox in concentrations ranging from 10 to 400M was used to create the standard curve. The activity was expressed as µmol Trolox equivalents/g RPMPH sample.

2.5.4 Fe^{2+} chelating activity

Chelating activity towards Fe^{2+} of RPMPH was performed according to the method of **Thiansllakul *et al.* (2007)**, with slight modification. The Fe^{2+} chelating activity was calculated using the following equation:

$$\text{Fe}^{2+}\text{chelating activity(\%)} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

2.6 Antibacterial activity of RPMPH using well diffusion method

Antibacterial activities of RPMPH were assessed using the agar well diffusion method. The inhibition zones were reported in millimeters (mm). Freshly grown pathogenic bacteria *Listeria monocytogenes* (ATCC 19111), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 8739), and *Salmonella enteritidis* (ATCC 1211) were used as references for the antibacterial assay. Penicillin (1000µg/ml) was used as a positive control against *L. monocytogenes, S. aureus, E. coli,* and *S. enteritidis*. The zone of inhibitions (ZI) produced by the RPMPH around the well was measured in millimeters, and the established formula calculating the 'activity index' (AI) is as follows:

$$\text{Active Index (AI)} = \frac{\text{Inhibition zone of the sample}}{\text{Inhibition zone of the standard}}$$

2.7 Electrophoretic analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) of RBPF and RPMPH was carried out following the method of **Laemmli (1970)**. The protein content of RBPF and RPMPH was determined using the method of **Lowry *et al.* (1951)**, with bovine serum albumin as a standard. Wide range-molecular-weight protein markers were used to determine the molecular weight of the peptides.

2.8 Amino acid analysis

The amino acid composition of RBPF and RPMPH was determined using Sykam GmbH amino acid analyzer system (Serial#:0.10820 Model: S-433D; Germany), as described by **White *et al.* (1986)**. Briefly, the digestion of samples was carried out by mixing the dried RBPF and RPMPH with 1.0mL of 6 N HCl in 0.1% phenol at 110°C for 24h. The

injection volume was 50µL for both standard and samples. The amino acid detection was performed at 570nm. The amino acid composition was expressed as a gramof amino acid/ 100g of crude protein.

2.9 Functional properties of RPMPH

2.9.1 Solubility

The effect of different pH (3, 5, 7, and 9) on the solubility of RPMPH was evaluated as described by **Kudre *et al.* (2018)**. The protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{(Protein content in supernatant)}}{\text{(Total protein content in sample)}} \times 100$$

2.9.2 Emulsifying properties

To assess the emulsifying properties of RPMPH, the emulsifying activity index (EAI) and the emulsion stability index (ESI) were used. EAI and ESI were determined using the method of **Pearcr and Kinsella (1978),** with a slight modification. The following formulas were used to calculate EAI and ESI:

$$\text{EAI } (\text{m}^2/\text{g}) = \frac{(2 \times 2.303 \times A \times DF)}{l\phi C}$$

Where, A is the absorbance of the sample at 500; DF is the dilution factor (100); l is the path length of the cuvette (m); ϕ is the oil volumetric fraction (0.25), and C is a concentration of RPMPH in the aqueous phase (g/m^3)

$$\text{ESI (min)} = \frac{A_0 \;x{\times}\Delta_t}{\Delta A}$$

Where, ΔA is $A_0 - A_{10}$; A_0 = absorbance at time of 0 min; A_{10} = absorbance at time of 10 min, and Δ_t = 10 min.

2.9.3 Foaming properties

The foam expansion (FE) and foam stability (FS) of RPMPH were determined at 0.5%, 1.0%, 2.0%, and 3.0% concentrations according to **Kudre *et al.* (2018)**, with minor changes. The following equations were used to calculate FE and FS:

$$\text{FE (\%)} = \frac{VT}{V_0} \times 100$$

$$\text{FS (\%)} = \frac{V_t}{V_0} \times 100$$

Where, VT is the total volume after whipping; V_0 is the original volume before whipping, and V_t is the total volume after leaving at room temperature for different times (60min).

2.9.4 Water and oil absorption capacity

According to the method of **Junianto *et al.* (2020)**, water and oil absorption capacity was determined. The water/ oil absorption was calculated as follows:

$$\text{Water or oil absorbation (mL/g)} = \frac{\text{Initial water or oil volume} - \text{Unabsorbed water or oil volume}}{\text{Sample weight}}$$

3. Statistical analysis

All the experiments were carried out in triplicate, a completely randomized design (CRD) was used, and the data were analyzed using ANOVA. A comparison of means was carried out using Duncan's multiple-range tests. The analysis was performed using an SPSS package (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA). The ANOVA and regression coefficients and significance of the response surface quadratic model were carried out using Design Expert 12.0.12.0 software.

RESULTS AND DISCUSSION

1. Proximate composition of red-bellied pacu fish meat

The proximate composition values (moisture, crude protein, crude fat, and total ash) of red-bellied pacu fish were 79.25, 14.06 , 5.08, and 0.60%, respectively. These findings match those of **Ahmed *et al.* (2022)** who stated that, the main components of fish are 66%−81% water, 16%−21% protein, 1.2%−1.5% mineral, 0.2%−25% fat, and 0%−0.5% carbohydrate.

1.1. Optimization of RBPF meat hydrolysate preparation by BBD

Optimal conditions for the production of bio-functional RPMPH were performed by BBD of RSM. The influence of three process factors (sucrose concentration, meat concentration, fermentation time) on responses (DH, DPPH, ABTS, FRAP, and Fe^{2+}chelating activity) was investigated. The experimental design matrix and the responses for the preparation of bio-functional RPMPH are displayed in Table (2). The regression coefficients for each model were determined by ANOVA. The ANOVA results for all the responses are shown in Table (3). Based on the F-value and *P*-value, the significance of the model was validated. The high F-value and low *P*-value for the responses reveal that the model is statistically significant. The application of regression analysis on responses yielded three linear coefficients (A, B, C), three quadratic coefficients (A^2, B^2, C^2), and three cross-product coefficients (AB, AC, BC). In the ANOVA results (Table 3), the coefficient of all quadratic terms (A^2, B^2, C^2) was significant ($P< 0.05$) for four responses (DH, DPPH, FRAP, and Fe^{2+}chelating activity). Moreover, the coefficient of linear terms (A, B, C) was significant ($P< 0.05$) for FRAP and Fe^{2+}chelating activity. Besides, the co-efficient of interactive terms AC and BC ($P< 0.05$) were significant for FRAP activity. Furthermore, the coefficient of determination (R^2) for all responses was noticed to be R2>0.95, and the adjusted R^2 for all responses was R^2>0.88, as shown in Table (3), which revealed the high degree of precision and reliability ofF-experimental values. Moreover, the coefficient of variation values for all the responses indicates that the fitted model is more reproducible.

1.1.1 Interactive effects of the process parameters on the responses

The 3D response surfaces in Fig. (1) depict the impact of the independent variables (sucrose concentration, meat concentration, and fermentation time) and their interactions on the DH and antioxidant activities (DPPH, ABTS, FRAP, and Fe^{2+}chelating activity) of RPMPH. Each figure illustrates the effect of two process factors on the DH and antioxidant activities, while the other process factor was maintained at the center (0 levels).

1.1.2 Effect of process parameters on DH

The DH values for RPMPH were recorded in the range of 5.40 to 49.41% under studied experimental conditions. The 3D response figures for the interactive effects of process variables on the degree of hydrolysis (DH) are displayed in Fig.(1a- c). It was noted that, the DH increased with the increase in the sucrose concentration from 1% to 2%. Further increase in the sucrose concentration (above 2.5%) resulted in a gradual decrease in DH (Fig. 1a, b). In contrast, a significant increase in the DH values was observed with the rise in the meat concentration from 20 to 30%. However, when the meat concentration was increased from 30 to 40%, the DH values dropped significantly (Fig. 1a, c). The effect of fermentation time on DH values followed similar trends as the effect of meat concentration. A significant increase in the DH values was noticed with the increase in the fermentation time from 24 to 48h. At 48h, the hydrolysis time and the maximum DH were observed. Conversely, the increment in fermentation time from 48h to 72h decreased the DH values significantly. The marked decrease in the DH might be due to the extensive hydrolysis of fish proteins by the bacterial enzyme **(Jemil *et al.,* 2016)**. These results are in the same line with those of **Jemil *et al.* (2016)** who explained that, the DH is an indicator of the efficiency of the hydrolysis, and generally, after a quick initial reaction phase, the rate of hydrolysis tends to diminish, subsequently entering the stationary phase. The higher DH of RPMPH corresponds to higher peptide bond breakage by proteolytic enzymes. Therefore, the probiotic *Bacillus* strain (SNGC) might release a higher content of proteolytic enzymes at 2% sucrose concentration, 30% meat concentration, and 48h of fermentation time, resulting in higher DH of RBPF meat protein. The polynomial equation obtained after applying multiple regression analysis on DH results was found as:

$$\mathrm{DH} = +47.30 + 8.21A - 0.038B - 0.3501C - 1.46AB + 0.1495AC - 0.1829BC - 7.34A^2 - 10.59B^2 - 28.90C^2$$

Where, A is sucrose concentration (%); B is meat concentration (%), and C is fermentation time (h). The model's coefficient of determination (R^2) was 0.9898, and the coefficient of variance (CV) was 10.8%. Both values signify an agreeable model fit.

1.1.3 Effect of process parameters on DPPH radical scavenging activity

The BBD matrix results for DPPH antioxidant activity of RPMPH were in the 65.33% to 83.14% range in studied experimental conditions. The 3D-response surface figures for the interactive effects of the process variables on the DPPH radical scavenging activity of RPMPH are shown in Fig. (1d- f). The Fig. (1d, e) represents the interactive effect of sucrose concentration and meat concentration on the DPPH activity of bioactive peptides. The DPPH activity was noticed to increase significantly with the increase in sucrose concentration from 1% to 2%. Above 2% sucrose concentration, the DPPH activity was observed to decrease gradually with the increase in the meat concentration. The result indicated that a 2% sucrose concentration might be the maximum concentration to produce the DPPH scavenging peptides from RBPF meat protein by probiotic Bacillus strain (SNGC). Besides, the increase in the meat concentration from 20% to 30% resulted in a gradual increase in the DPPH activity, and the maximum DPPH activity was noted at 30% meat concentration. Further, the DPPH activity significantly decreased with the increase in the meat concentration from 30% to 40%. The result suggested that 30% could be the maximum RBPF meat concentration for producing DPPH scavenging peptides by probiotic Bacillus strain (SNGC). Therefore, generated RBPF meat peptides can potentially donate electrons to free radicals, converting them to more stable products and ending the radical chain reaction. Furthermore, it was observed that DPPH radical scavenging activities increased as DH increased ($P< 0.05$). The effect of fermentation time on the DPPH activity of bioactive peptides could be very well interpreted from Fig. (1e, f). The DPPH activity increased by increasing the fermentation time up to 40h. However, the DPPH activity was observed to decrease significantly above 40h. In the case of 40% RBPF meat, the hydrolysates prepared after 72h showed relatively low DPPH scavenging activity. This result may be due to the dilution of the new peptides with high radical scavenging activity in a diluted matrix of intact peptides with lower antioxidant activity. The polynomial equation corresponding to DPPH activity was found as:

$$\mathrm{DPPH} = +81.32 + 1.49A - 1.44B - 4.56C - 0.1104AB + 0.9056AC - 0.0399BC - 2.18A^2 - 2.61B^2 - 5.64C^2$$

Where, A is sucrose concentration (%); B is meat concentration (%), and C is fermentation time (h). The coefficient of determination of model (R^2) was 0.9497, and the coefficient of variance (CV) was 2.29, revealing a significant model fit.

Table 2. Experimental design matrix for red-belied pacu fish meat (RBPFM) hydrolysate preparation

Run	A: Sucrose concentration (%)	B: Meat concentration (%)	C: Fermentation time (h)	R1: DH	R2: DPPH	R3: ABTS	R4: FRAP	R5: Iron chelating activity
1	0	1	1	6.93	66.88	83.57	92.70	32.36
2	-1	-1	0	17.30	77.69	85.41	162.31	42.40
3	0	0	0	45.51	80.10	92.02	271.15	69.78
4	1	0	1	17.02	72.80	86.06	97.29	39.43
5	1	1	0	38.52	75.13	78.77	174.82	41.66
6	-1	1	0	20.69	75.05	77.20	160.45	37.82
7	1	0	-1	17.21	79.84	88.35	140.36	43.98
8	0	0	0	49.41	80.15	90.06	273.87	66.84
9	-1	0	1	4.61	65.33	85.32	98.15	32.95
10	0	0	0	48.87	82.11	89.11	271.56	68.87
11	0	-1	1	7.78	69.85	88.91	105.82	37.54
12	0	1	-1	8.20	76.36	83.29	119.18	36.92
13	-1	0	-1	5.40	75.99	85.93	99.17	37.05
14	0	0	0	47.43	81.08	91.93	270.11	70.82
15	1	-1	0	40.96	78.21	84.68	178.53	47.51
16	0	0	0	45.26	83.14	90.27	268.37	66.65
17	0	-1	-1	8.32	79.16	89.26	121.68	39.46

Values are mean ± SD (n=3)

Table 3. ANOVA results and fit statistics of various models for red-belied pacu fish protein hydrolysate (RPMPH) production using *Bacillus* strain (SNGC)

Source	DH, R1			DPPH, R2			ABTS, R3			FRAP, R4			Chelating agent , R5		
	df	co-efficient	F-value	df	co-efficient	F-value	df	co-efficient	F-value	df	co-efficient	F-value	df	co-efficient	F-value.
Model	9	47.30	75.75	9	81.32	14.69	9	90.68	23.56	9	271.01	1561.48	9	68.59	149.04
A	1	8.21	75.26	1	1.49	5.81	1	0.4995	1.55	1	8.87	107.01	1	2.81	25.81
B	1	-0.0038	0.0000	1	-1.44	5.40	1	-3.18	62.75	1	-2.65	9.55	1	-2.26	16.69
C	1	-0.3501	0.1319	1	-4.56	54.57	1	0.3706	0.8535	1	-10.81	158.95	1	-1.89	11.70
AB	1	-1.46	1.14	1	-0.1104	0.0160	1	0.5762	1.03	1	-0.4637	0.1464	1	-0.3411	0.1905
AC	1	0.1495	0.0120	1	0.9056	1.08	1	0.4220	0.5531	1	-10.51	75.24	1	-0.1143	0.0214
BC	1	-0.1829	0.0180	1	-0.0399	0.0021	1	0.1555	0.0751	1	-2.65	4.79	1	-0.6595	0.7122
A^2	1	-7.34	30.48	1	-2.18	6.59	1	-4.50	66.33	1	-51.54	1903.67	1	-12.24	258.23
B^2	1	-10.59	63.51	1	-2.61	9.41	1	-4.66	71.03	1	-50.44	1823.19	1	-14.03	339.05
C^2	1	-28.90	472.89	1	-5.64	43.91	1	0.2417	0.1910	1	-110.72	8784.67	1	-18.00	558.08
Residual	7			7			7			7			7		
Lack of fit	3		3.53	3		2.81	3		0.5569	3		2.03	3		0.0.3843
Pure error	4			4			4			4			4		
Cor total	16			16			16			16			16		
						Fit statistics of various models									
R2		0.9898			0.9497			0.9680			0.9995			0.9948	
C.V. %		10.80			2.29			1.31			1.42			3.27	

1.1.4 Effect of process parameters on ABTS activity

The interactive effects of the independent variables on the ABTS activity of RPMPH are shown in Fig.(1g- i). The effect of sucrose concentration on the ABTS activity exhibited a similar trend for DPPH and DH. Higher ABTS radical scavenging activity ($P< 0.05$) is associated with higher DH for hydrolysates, indicating the formation of new peptides capable of removing free radicals from the $ABTS^{+\bullet}$. Peptides with a lower molecular weight or a higher DH have a greater ability to scavenge since mononuclear cells produce reactive oxygen species. The maximum ABTS activity was observed at a 2% sucrose concentration. The result implied that *Bacillus* strain (SNGC) required a maximum of 2% of sucrose to release the ABTS activity peptides from the RBPF meat protein. The effect of RBPF meat concentration displayed a different trend than sucrose concentration. The ABTS activity of fish bioactive peptides increased with the increased meat concentration, but maximum activity was noted between 20%-30% meat concentration. The result endorses that close to 30% meat could be the maximum concentration to produce the ABTS radicals scavenging peptides. However, above that point, the ABTS activity decreased significantly, indicating that above 30% meat concentration, the ABTS scavenging peptides could be diluted and hence lose the radical scavenging property. From Fig. (1g, i), it can be noted that fermentation time didn't significantly affect the ABTS activity of RPMPH. The polynomial equation derived after the application of regression analysis on ABTS results was

$$\mathrm{ABTS} = +90.68 + 0.4995A - 3.18B - 0.3706C + 0.5762AB - 0.4220AC + 0.1555BC - 4.50A^2 - 4.66B^2 + 0.2417C^2$$

1.1.5 Effect of process parameters on FRAP activity

In the case of FRAP, the studied ranges of sucrose concentration, meat concentration, and fermentation time yielded bell-shaped response surface curvatures (Fig.1j- l). From the 3D response surface plots, it is evident that the FRAP activity was maximum at a sucrose concentration of 2%, RBPF meat concentration of 30%, and fermentation time of 48h. This result showed that higher content of iron-reducing peptides was produced by the *Bacillus* strain (SNGC) at 2% sucrose, 30% meat, and 48h fermentative time. Beyond these points (> 2% sucrose, 30% meat, and 48h fermentative time), the FRAP activity decreased significantly. This could be due to the diluted new peptides formed in intact hydrolysates. The polynomial equation corresponding to the FRAP activity of fish meat bio-functional peptides is expressed as:

$$\mathrm{FRAP} = +271.01 + 8.87A - 2.65B - 10.81C - 0.4637AB - 10.51AC - 2.65BC - 51.54A^2 - 50.44B^2 - 110C^2$$

Where, A is sucrose concentration (%); B is meat concentration (%), and C is fermentation time (h). The coefficient of determination of the model (R^2) was 0.9995, and the coefficient of variance (CV) was 1.42, revealing a significant model fit.

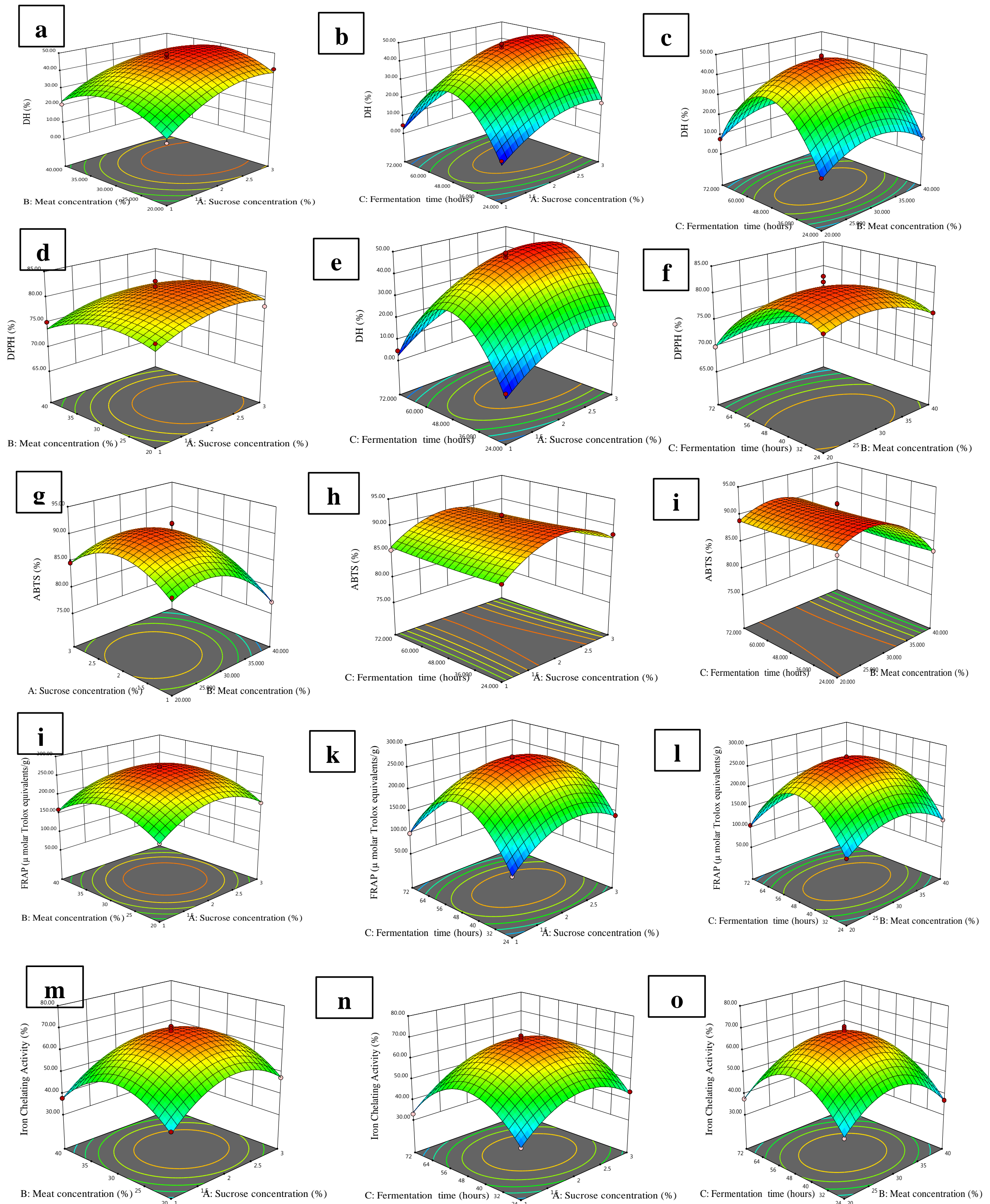

Fig.1. Response surface 3D plots for the effects of variables for red-belied pacu fish protein hydrolysate (RPMPH) production using *Bacillus* strain (SNGC) on the degree of hydrolysis (DH), DPPH, ABTS, FRAP, and Fe^{2+}chelating activity showing: (A) Sucrose concentration (%), (B) Meat concentration (%), (C) Fermentation time (h).

1.1.6 Effect of process parameters on Fe^{2+}chelating activity

Fig.(1m – o) illustrates the effect of the process variables on the Fe^{2+}chelating activity of the RPMPH. It is evident from the response surface curvatures that the Fe^{2+}chelating activity of the bioactive peptides increased with the increase in the values of sucrose concentration from 1 to 2%, meat concentration from 20 to 30%, and fermentation time from 24h to 48h. At 2% sucrose concentration, 30% meat concentration, and 48h fermentation time, RPMPH exhibited maximum chelating activity toward Fe^{2+}. The presence of histidine residues and a higher concentration of amino groups (NH_2-) and carboxylic groups (COOH-) in the side chain of the basic and acidic amino acids in RPMPH may be the cause of the Fe^{2+} ion chelating activity **(Chalamaiah *et al.,* 2015)**. Above 2% sucrose concentration, 30% meat concentration and 48h fermentation time, the Fe^{2+} chelating activity of RPMPH markedly reduced. The reduced Fe^{2+}chelating activity might be due to RPMPH's inability to form the complex with metals.

$$Fe^{2+}\text{chelating activity} = +68.59 + 2.81A - 2.26B - 1.89C - 0.3411AB - 0.1143AC - 0.6595BC - 12.24A^2 - 14.03B^2 - 18.00C^2$$

Where, A is sucrose concentration (%); B is meat concentration (%), and C is fermentation time (h). The coefficient of determination of model (R^2) of the model was 0.9948, and the coefficient of variance (CV) was 3.27, revealing a significant model fit.

2. Antimicrobial activity

Antagonistic activity zone of inhibition (ZI) and activity index (AI) of RPMPH had strongly antagonistic activity against *L. monocytogenes* (ZI: 15.33± 0.58mm; AI: 0.74± 0.20mm) and *S. aureus* (ZI: 14.67± 0.57mm; AI: 0.75± 0.01mm). However, RPMPH had weak antagonistic activity against *E. coli* (ZI: 3.66±1.73mm; AI: 0.16± 0.04mm) and *S. enteritidis* (ZI: 4.33± 0.58mm; AI: 0.17± 0.02mm). The result revealed that RPMPH exerted strong antagonistic activity against Gram-positive bacteria. It has been reported that Gram-negative bacteria are generally not sensitive to bacteriocins due to the outer membrane, which is less permeable **(Jemil *et al.,* 2014; Karaman *et al.,* 2020)**. Besides, Gram-positive bacteria are more prone to bacteriocins **(Karaman *et al.,* 2020)**. Captivatingly, RPMPH had the strongest zone of inhibition against *S. aureus*. The *S. aureus* is well known for being resistant to most phytochemical compounds. Moreover, *S. aureus*is a major human pathogen producing enterotoxins that cause gastroenteritis. Antagonistic properties posed by fermented products are mostly bacteriocin produced by probiotic bacteria or peptides produced from substrate protein **(Jemil *et al.,* 2014)**. However, some studies revealed that in the case of a protein hydrolysate, small molecular weight (< 20kDa) peptides mainly exerted antagonistic properties against the bacteria **(Jemil *et al.,* 2014; Jain & Anal 2017; Karaman *et al.,* 2020)**. Moreover, it has been demonstrated that hydrophobic amino acids and positively charged amino acids of

peptides illicit antibacterial activity **(Jain & Anal, 2017)**. In the present study, RPMPH exhibited several low MW peptides that could impart highly antibacterial properties against *L. monocytogenes* and *S. aureus.* Protein hydrolysates from fermented fishes such as goby (*Zosterizessor ophiocephalus*) **(Narsi *et al.,* 2013)**, ray (*Dasyatis pastinaca*), and sardinelle (*Sardinella aurita*) **(Jemil *et al.,* 2014)** presented the antimicrobial activity. Therefore, RPMPH can act as a natural preservative against food-borne pathogens.

3. Amino acid analysis of RBPF meat and RPMPH

The amino acid profile of RBPF meat and RPMPH are shown in Table (4). A slight variation was detected in the amino acid profile of RBPF meat and RPMPH. The RPMPH presented a lower content of total amino acids compared to RBPF meat protein. This result indicated that the fermentation process influenced the amino acid profile of RBPF meat protein. This result is in line with **Vidotti *et al.* (2003)**, who reported a decrease in the total amino content of fish silage (salt water and freshwater fish) and the Nile tilapia filleting residues fermented by *Lactobacillus plantarum*. Methionine and tyrosine showed the highest destroyed amino acids during the RBPF meat fermentation, as evidenced by the lower content of methionine and tyrosine in RPMPH compared to native RBPF meat samples. Besides, RPMPH exhibited slightly higher alanine, glycine, and serine content than the native RBPF meat protein. Both RBPF meat and RPMPH samples showed glutamic acid, lysine, aspartic acid, and alanine as the dominant amino acids. In general, glutamic acid, lysine, aspartic acid, and alanine were the prominent amino acids of fish muscle proteins **(Liu *et al.,* 2015)**. Furthermore, RPMPH constituted lower content of total essential amino acid (EAA) (34.19%) and higher content of total non-essential amino acid (NEAA) (65.81%) compared to the amino acid composition of RBPF meat protein. This result demonstrated that fermentative hydrolysis of RBPF meat protein led to a slightly decreased essential amino acid content. Nevertheless, RPMPH scored better EAA except for methionine compared to the recommended FAO/WHO amino acid requirements **(Riyadi *et al.,* 2019)**. It has been stated that the presence of tyrosine, methionine, histidine, lysine, and tryptophan in protein hydrolysate generally contributes to the antioxidant activity of the protein hydrolysate **(Liu *et al.,* 2015)**. RPMPH exhibited higher total hydrophobic amino acids (57.37%) than RBPF meat protein (41.99%). Fermentative hydrolysis might liberate the higher content of hydrophobic amino acids predominantly present in the interior of the RBPF meat protein. The higher antioxidant of RPMPH is probably related to a higher content of hydrophobicamino acids**. Liu *et al.* (2015)** demonstrated that with an antioxidant activity their isgenerally a hydrophobicity of peptides. Overall, the results suggested that the amino acid profile of RBPF meat was slightly affected by the fermentative hydrolysis of the probiotic *Bacillus*(SNGC) strain.

4. Electrophoresis pattern of RBPF meat and RPMPH

SDS-PAGE was used to determine the molecular weight of RBPF meat and RPMPH. Fig. (2) shows SDS-PAGE patterns of RBPF meat protein and RPMPH. Electropherograms of RBPF meat samples exhibited several high-intensity bands of

myofibrillar proteins ranging from 22- 200kDa and above. The protein bands > 11 kDa MW might correspond to the subunits of myosin protein. The band with MW of ~ 45kDa represents the actin. The bands lower than 40kDa MW belongs to the tropomyosin, troponin, and myosin light chain. RPMPH showed the disappearance of myosin, actin, tropomyosin, and troponin bands, and a concomitant appearance of small MW peptide bands (< 25kDa) was noticed. Moreover, RPMPH exhibited faint and diffused bands compared to the RBPF meat protein bands. This result depicted noticeable degradation of RBPF meat proteins during fermentation. *Bacillus*strain (SNGC) might release the extracellular proteolytic enzymes that cleavage myofibrillar proteins into smaller MW peptides. Other studies reported similar results for protein hydrolysate prepared from eggshell membrane protein **(Jain & Anal, 2017)** and fish protein **(Khiari & Mason, 2018)** by bacterial fermentation. The peptide bands ranged from ~18- 25kDa in RPMPH and were more likely derived from troponin hydrolysis. Furthermore, RPMPH had an 8-12kDa denser band that might be arisen from the hydrolysis of myosin light chains by *Bacillus*strain (SNGC) during the fermentation. Overall, the SDS-PAGE pattern of the RPMPH revealed the hydrolysis of RBPF meat protein occurred during the fermentation by *Bacillus*strain (SNGC).

5. Functional properties

5.1. Solubility

Fig. (3A) shows the solubility of RPMPH at different pHs (3.0, 5.0, 7.0, and 9.0). The protein solubility of RPMPH is influenced by different pHs and ranges from 80.88-94.94%. The lowest solubility of RPMPH ($P< 0.05$) was noted at pH-5 (80.88%). Lowest solubility RPMPH at pH-5 since fish meat protein exhibited an isoelectric pH of around 5.0. **Li *et al.* (2012)** also obtained more than 80% protein solubility of grass carp (*Ctenopharyngodon idellus*) protein hydrolysates at pH (3–8). Generally, protein molecules obtain the net zero charge at isoelectric point pH and increase the protein-protein interactions, reducing protein solubility. Several studies reported that fish protein or protein hydrolysate demonstrated the minimum solubility between pH 4 and 6 **(Sripokar *et al.,* 2019)**. Furthermore, RPMPH exhibited increased solubility ($P< 0.05$) on either side (pH 3 and pH 7 & 9) of isoelectric pH. RPMPH exhibited the highest solubility at pH 9. However, no significant difference ($P> 0.05$) of RPMPH solubility was detected at pH 7 & 9. The change in solubility can be attributed to the amino acid residues' net charge after hydrolysis, which increases as the pH moves away from the isoelectric point, promoting hydrophobic interaction aggregation **(Taheri *et al.,*2014)**. In addition, hydrophobic and ionic interactions are two major factors influencing protein solubility. Protein-protein interactions are promoted by hydrophobic interactions, which result in decreased solubility, whereas protein-water interactions are promoted by ionic interactions, which result in increased solubility. Ionic residues on peptide and protein surfaces cause electrostatic repulsion between protein molecules and hydration shell repulsion around ionic groups, both of which contribute to increased protein solubility

(Hordur & Barbara 2000; Junianto *et al.*, 2020). Overall, RPMPH has a good solubility over a wide pH range.

Table 4. Amino acid profile of red-belied pacu fish meat (RBPFM) and RBPFM protein hydrolysate (RPMPH)

Amino acid	RBPFM (g/100g)	RPMPH (g/100g)	Reference protein[a]	Chemical score for RBPFM [b]	Chemical score for RPMPH [b]
Histidine (His)	2.02	1.62	1.6	1.26	1.01
Isoleucine (ILe)	4.26	2.66	1.3	3.28	2.05
Leucine (Leu)	8.44	6.20	1.9	4.44	3.26
Lysine (Lys)	9.70	8.99	1.6	6.06	5.62
Methionin(Met)	3.10	0.51	1.7	1.82	0.3
Phenylalanine (Phe)	4.09	3.35	-	-	-
Tyrosine (Tyr)	3.44	0.50	-	-	-
Threonine (Thr)	4.83	3.11	0.9	5.37	3.46
Arginine (Arg)	6.83	4.15	-	-	-
Valine (Val)	5.09	3.10	1.3	3.92	2.38
Total of essential amino acids	**51.80**	**34.19**			
Asparagine (Asp) + aspartate	8.88	8.87	-	-	-
Glutamine (Glu) + glutamate	18.36	11.20	-	-	-
Serine (Ser)	3.94	4.18	-	-	-
Glycine (Gly)	5.52	22.46	-	-	-
Alanine (Ala)	7.67	16.65	-	-	-
Proline (Pro)/ hydroxy proline	3.83	2.45	-	-	-
Cysteine (Cys)	ND	ND	-	-	-
Total of non-essential amino acids	**48.20**	**65.81**	-	-	-
Total hydrophobic amino acids (%)	**41.99**	**57.37**	-	-	-
Total hydrophilic amino acids (%)	**21.09**	**16.65**	-	-	-

[a]Suggested profile of essential amino acid requirements for adults (WHO/ FAO/ UNU, 2007); [b]Chemical score is calculated with the FAO / WHO reference protein as the base (WHO/ FAO/ UNU, 2007). ND: Not detected

5.2. Emulsifying properties

Emulsifying properties including EAI and ESI of RPMPH at various concentrations (0.5, 1.0, 2.0, and 3.0% w/v) are depicted in Fig. (3B). Emulsifying properties are linked to the surface properties of proteins or peptides because of their hydrophilic and hydrophobic groups. Emulsification of proteins or peptides generally occurs through the deformation and disruption of droplets, increasing the emulsion's specific surface area and stabilizing the newly formed interface by an emulsifier or surfactant (**Klomkao *et al.,* 2013; Kudre *et al.,* 2018; Sripokar *et al.,*2019**). In the present study, both EAI and ESI increased as RPMPH increased, and the highest values were noted at 2% RPMPH ($P < 0.05$). The result endorsed that, up to 2% RPMPH, the enhanced interaction between the oil and aqueous phases resulted in a stable emulsion. Further, with an increase in the RPMPH concentration (3%), a decrease in EAI and ESI was noted ($P < 0.05$). At 3% RPMPH concentration, peptides might accumulate at a higher level in the aqueous phase, decreasing emulsification. A similar result was reported by **Sripokar *et al.* (2019)** for starry triggerfish (*Abalistes stellaris*) muscle protein hydrolysate. Therefore, 2% RPMPH could be the appropriate concentration for forming viscoelastic films in the oil-water interphase and stable emulsion.

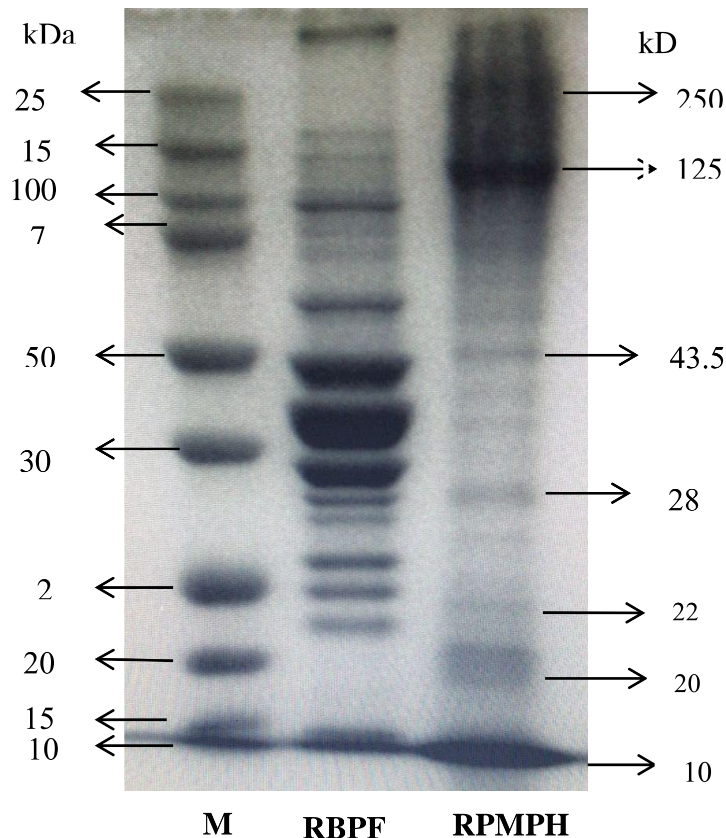

Fig. 2.SDS-PAGE pattern of red-belied pacu fish meat (RBPFM) and RBPFM protein hydrolysate (RPMPH). M: marker

5.3. Foaming properties

Foam expansion (FE) and foam stability (FS) of RPMPH at different concentrations (0.5, 1.0, 2.0, and 3.0%) are displayed in Fig.(3C). In the present study, FE and FS of RPMPH increased as RPMPH concentration increased ($P< 0.05$). The result suggested that a higher concentration of peptides might form a thick film at the air-water interface, resulting in denser and more stable foam. **Sripokar *et al.* (2019)** reported a similar result patternfor starry triggerfish (*Abalistes stellaris*) muscle protein hydrolysate. Easy to denature, soluble, and surface hydrophobic low molecular weight peptides are ideal for foam-forming and stabilizing processes. FE and FS of RPMPH at 3% concentration were 114.58% and 102.65%, respectively. Amphiphilic peptides could help in the formation hydrophobic portion of the protein to extend into the air and the hydrophilic portion to extend into the aqueous phase **(Hordur & Barbara, 2000)**. Protein hydrolysates from toothed ponyfish muscle produced with hybrid catfish viscera extract produced a similar result **(Klomklao *et al.,* 2013)**.

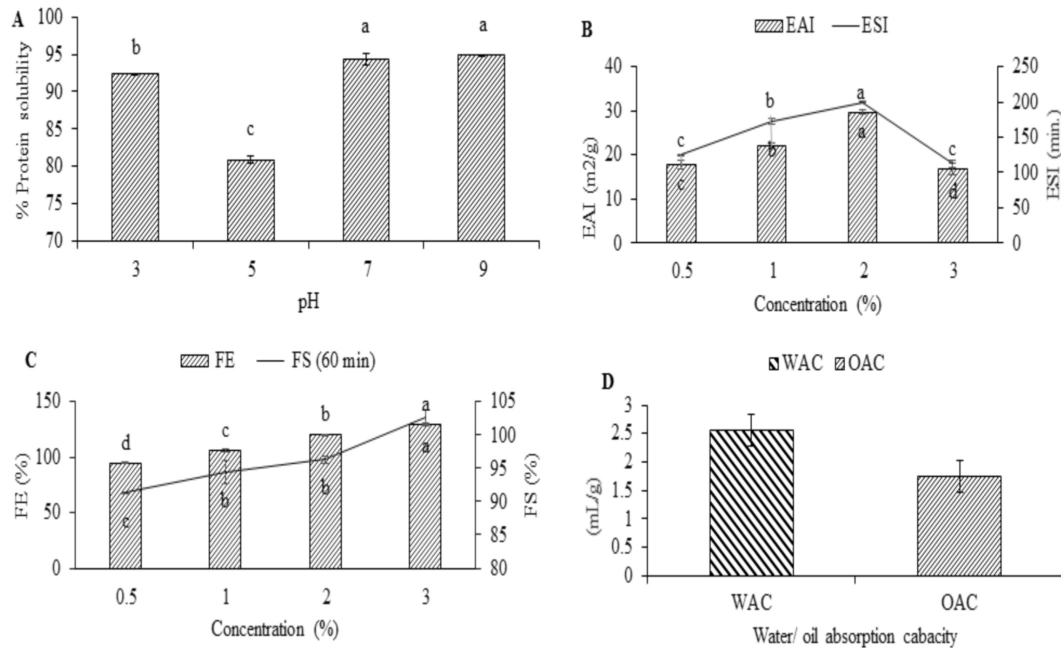

Fig. 3. Protein solubility at different pH values (A), emulsifying activity index (EAI), emulsion stability index (ESI) (B), foam expansion (FE) and foam stability (FS) (C), water absorption capacity (WAC) and oil absorption capacity (OAC) (D) of red-bellied pacu protein hydrolysate (RPMPH). Values are mean ± SD (n=3). The different lowercase letters indicate significant differences ($P< 0.05$)

5.4.Water and oil absorption capacity

The water absorption of RPMPH was 2.55mL/g (Fig.3D), which was slightly higher than the Nile tilapia (2.1mL/g) **(Foh *et al.,* 2011)**, however it was less that recorded in the rainbow trout (5.1mL/g) **(Taheri *et al.,* 2014)** and bony barb fish protein hydrolysate (3.1mL/g) **(Junianto *et al.,* 2020)**. The results obtained suggest that RPMPH can absorb more water due to its more hydrophilic polar side chains. Proteins interact with water in the presence of polar amino acid groups like the carbonyl, hydroxyl, amino, carboxyl, and sulfhydryl groups, which causes water absorption.

Oil absorption is the capacity of RPMPH to absorb oil. RPMPH exhibited a 1.74mL/ g oil absorption capacity (Fig. 3D), which was slightly lower than that of the Nile tilapia (2.27mL/ g) **(Foh *et al.,* 2011)** and bony barb fish protein hydrolysate (1.94 mL/g) **(Junianto *et al.,* 2020)**.The taste and palatability of a product in the food industry are greatly influenced by the oil absorption of protein hydrolysate **(Junianto *et al.,* 2020)**.

CONCLUSION

The probiotic *Bacillus* (SNGC) isolated from country chicken gizzard has the potential to produce antioxidant and antibacterial protein hydrolysates from red-bellied pacu fish (RBPF) meat. The Box-Behnken response surface methodology (RSM) design revealed an efficient statistical tool to optimize the fermentative hydrolysis of conditions for RBPF meat. The RSM provided optimized conditions to obtain maximum antioxidant (DPPH, ABTS, FRAP, and Fe^{2+}chelating activity) protein hydrolysate from 30% RBPF meat concentration,2% sucrose concentration, and 48h fermentation time at 37°C and 50rpm. Additionally, RPMPH exhibited antibacterial activities against *L. monocytogenes, S. aureus, E. coli,* and *S. enteritidis.* The protein pattern ofRPMPH endorsed that probiotic *Bacillus* stain released the proteolytic enzymes that may degrade RBPF meat protein. The probiotic *Bacillus* strain fermentation slightly affects the amino acid profile of RBPF meat protein. High solubility at a wide pH indicates that RPMPH can be used in different food applications. Additionally, RPMPH exhibited desirable emulsification (2% concentration) and foaming (3% concentration) water and oil absorption properties. Therefore, the present finding could enhance the possible application of RPMPH produced by probiotic *Bacillus* (SNGC) strain as a bio-functional ingredient or preservative in food, pharmaceuticals, and cosmetic preparations.

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Authors' contributions

AMAH: Investigation, methodology, formal analysis, and writing original manuscript; SSK assisted in conducting experiments; TGK: Conception, design, and implementation of the research, data analysis, manuscript writing, and overall supervision of the project.

Ethics Declarations

Conflicts of interest/Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the research presented in this manuscript.

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