Response Surface Methodology-Based Optimization of Rusip Fermentation Conditions and Characterization of Bioactive Peptide Fractions with Antioxidant and Antimicrobial Properties

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Abstract

Optimization of raw materials and processing conditions in rusip, a traditional fermented anchovy, has the potential to enhance its bioactive properties. This study consisted of two stages, including optimization of rusip processing parameters (salt, sugar, and fermentation time) using Response Surface Methodology (RSM) based on a Central Composite Design to enhance bioactivity, and physicochemical and microbiological characterization, and bioactivity evaluation of the rusip optimized formulation, including ultrafiltration-based fractionation of its water-soluble extract. The rusip optimized formulation was determined as 5% sugar, 18% salt, and 12 days of fermentation with antioxidant activity of 60.50% (DPPH assay) and inhibition zones of 4.13 mm against *Escherichia coli* and 3.27 mm against *Staphylococcus aureus* (agar well diffusion assay), with model validation ranging from 95.86% to 98.03%. Subsequently, the optimized rusip formula was analyzed for physicochemical and microbiological characteristics. The water-soluble extract of rusip optimized formulation was separated using an ultrafiltration membrane, which showed that the <3 kDa fraction demonstrated the highest antioxidant (46.09%) and antimicrobial activity (4.9 mm against *E. coli* and 4.7 mm against *S. aureus*). Proteolytic enzyme sensitivity analysis showed that exposure to enzymes significantly reduced and eliminated the bioactivity of the rusip fraction, thus indicating the compounds responsible for the bioactivity were proteins or peptides. Qualitative HPLC profiling showed the <3 kDa fraction exhibited an intense peak at 214 nm with early retention time, suggesting the presence of abundant low-molecular-weight peptide compounds. This study provides a scientifically optimized formulation and production method for rusip, revealing its potential as a functional food with natural antioxidant and antimicrobial peptides.

Keywords: rusip, optimization, antioxidant, antimicrobial, fractionation

Introduction

Anchovy (*Stolephorus indicus*) is one of the high-value fishery commodities found in coastal areas of Indonesia (Kari et al., 2022). Anchovy has a high nutritional value, containing protein (23–27%), lipid (6–7%), carbohydrate (5–6%), calcium (500 mg), iron (1.0 mg), vitamin A (47 µg), and vitamin B1 (0.1 mg) (Putri et al., 2017; Puspita et al. 2024). One form of traditional anchovy processing is rusip, a typical fermented product from Bangka Belitung, Indonesia. Rusip is produced by combining anchovy with palm sugar and salt, then undergoing fermentation in anaerobic condition for a duration of 14 to 21 days. (Kurnianto et al., 2023). Salt and palm sugar are crucial in the fermentation of rusip. Palm sugar significant influences the sensory attributes of the product and acts as a vital energy source for microbial proliferation (Susilowati et al., 2014). Meanwhile, salt plays a vital role in the fermentation, including sensory formers, attracting water and nutrients to provide a substrate for microorganism growth and creating a high-salinity environment (Y. Li et al., 2023; Xiong et al., 2016).

The environment created during rusip fermentation effectively suppresses the proliferation of pathogenic and spoilage microorganisms while promoting the growth of halophilic microorganism, particularly lactic acid bacteria (LAB), which are essential in rusip fermentation (Kurnianto et al., 2023). Some LAB genera that grow during rusip fermentation include *Lactococcus* sp., *Streptococcus* sp., and *Leuconostoc* sp. (Koesoemawardani et al., 2013; Kusmarwati et al., 2014; Kurnianto et al., 2023;). LAB is capable of producing proteolytic enzymes

that decompose proteins into peptides and amino acids, enhancing the product's sensory attributes and bioactivity (Kieliszek et al., 2021). Peptides are specific low molecular-weight fragments formed through proteolysis (Kurnianto et al., 2021, 2024). Recent research has found peptides with specific functional activities known as bioactive peptides (BP) (Kurnianto et al., 2024). Several studies have reported rusip bioactivities thought to be due to BP such as antioxidant, anti-diabetic, antihypertensive, anti-cholesterol, and antimicrobial (Kurnianto et al., 2023; Rinto et al., 2019, 2021).

Studies on the physicochemical, microbiological characteristics and potential bioactivity of rusip have been reported previously (Koesoemawardani et al., 2013b; Batubara et al., 2019; Amelia et al., 2024; 2013a; Kurnianto et al., 2026; Khairi et al., 2014; Nurhamzah, 2021; Putri et al., 2014; Putri et al., 2017). However, these studies have not evaluated the influence of salt concentration, palm sugar concentration, and duration of fermentation variation on the formation of bioactive activities, especially antioxidant and antimicrobial activities. Therefore, an optimization approach using Response Surface Methodology (RSM) can be used as an effective analytical method in assessing the interaction between factors and the influence of each variable on the observed response (Veza et al., 2023). Through this approach, it is not only possible to evaluate the influence of salt concentration, palm sugar, and fermentation duration on the bioactivity of rusip but also to determine the optimal formula combination to produce a product with the highest antioxidant and antimicrobial activity (Ain et al., 2023). The aim of this study is to optimize and analyze the influence of salt and sugar concentration, as well as duration of fermentation in the process of making rusip on bioactivity, antioxidant and antimicrobial profiles, using an optimization approach using RSM, fractionating the optimal formula fractions obtained using ultrafiltration membranes, and identifying the bioactivity and proteinaceous nature of the selected fractions produced.

Material and Methods

Preparation of rusip

Anchovies (150 g) were weighed, washed and drained. Salt (Cap Kapal, Indonesia) and palm sugar (Nira Murni, Indonesia) were added and mixed based on the treatment combination issued by Design Expert version 13 (Table 1). The mixed ingredients were then fermented anaerobically in an incubator (Memmert, Germany) at 30 ^{o}C. The finished rusip was stored in a freezer at -18 to -20 ^{o}C (Puspita et al., 2024).

Formulation preparation and RSM Design

The research study utilizing the Response Surface Methodology (RSM) was performed using a Central Composite Design (CCD), incorporating a Sequential Model Sum of Squares, Lack of Fit Tests, and Statistical Summary to evaluate model adequacy (Adamu et al., 2024). Three independent variables were considered: palm sugar concentration, salt concentration, and duration of fermentation. The responses targeted in this study were antioxidant and antimicrobial activities. The range of values for each independent variable was determined based on previous studies (Koesoemawardani et al., 2013; Amelia et al., 2024; Puspita et al., 2024). The experimental design matrix and treatment combinations were generated using Design Expert version 13 (Table 1). The optimal formulation was selected by the highest desirability value, which reflects the most favorable combination of responses. Model validation was subsequently performed to confirm that the experimental results were in close agreement with the predicted values, ensuring the reliability and robustness of the optimization process.

Extraction of water-soluble fractions

The optimized rusip formulation sample was combined with deionized water in a 1:10 ratio (w/v), subsequently subjected to homogenization for 30 minutes using a water bath shaker at 125 rpm. The homogenate was then subjected to heat treatment at 100°C for 10 minutes to inactivate endogenous enzymes, after which it was cooled to room temperature. The mixture was successively filtered through cheesecloth and filter paper under gravity to remove coarse particles. The resulting filtrate was subsequently clarified by vacuum filtration through a 0.22 µm cellulose acetate (CA) membrane to obtain a water-soluble extract (WSE) (Istiqamah et al., 2019; Amelia et al., 2024). This WSE was subsequently used for chemical composition analysis, ultrafiltration-based fractionation, bioactivity assays (antioxidant and antimicrobial), and chromatographic profiling using RP-HPLC.

Antioxidant Analysis using DPPH Methods

The samples antioxidant potential was assessed using the DPPH radical scavenging assay. In this procedure, 2 mL of 0.1 mM DPPH solution prepared in methanol was mixed with 2 mL of the WSE sample or ascorbic acid (standard) in a tube protected from light. The reaction mixture was incubated at room temperature in the dark for 30 minutes. Subsequently, absorbance was measured at 517 nm using a UV–Vis spectrophotometer (Kurnianto et al., 2021a; Munarko et al., 2021). The calculation of

the percentage of DPPH radical scavenging activity was performed using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Ac- As}}{\text{Ac}}$$

Where: Ac was the absorbance of DPPH control, As was the absorbance of DPPH in the presence of extract or standard.

Antibacterial Analysis using Agar Well Diffusion Methods

The antimicrobial activity of the WSE was analysis through the agar well diffusion method. Mueller–Hinton agar (25 mL; Oxoid, UK) was inoculated with 1% (v/v) of the test bacterial suspension (*E. coli* and *S. aureus*) adjusted to a final concentration of 1×10^6 CFU mL^{-1}. The solution was transferred into sterile Petri dishes and permitted to solidify. Wells measuring 6 mm in diameter were created in the agar utilizing a sterile cork borer, and 100 µL of each WSE sample was introduced into the wells. The Petri dishes were incubated at 37°C for 24 hours. After incubation, the antimicrobial activity was evaluated by measuring the diameter of the inhibition zones (mm) surrounding the wells. Distilled water was used as the negative control, whereas ampicillin (10 µg mL^{-1}) served as the positive control (Kurnianto et al., 2022; Kurnianto et al., 2024).

Analysis of Physicochemical and Microbiological Profile

The physicochemical profile of optimized rusip formulation sample was analyzed through proximate composition, including moisture, ash, protein, lipid, and carbohydrate contents (Puspita et al., 2024). Additional analyses included pH (Kurnianto & Munarko, 2022), soluble-protein concentration (Lowry et al., 1951), water activity (Kurnianto & Munarko, 2022), salt content, total sugar content, titratable acidity (Amelia et al., 2024), and free glutamic acid concentration (Tamam et al., 2019). Meanwhile, the microbiological profile of rusip was assessed by determining total LAB and the total plate count (TPC) counts (Kurnianto & Munarko, 2022).

Partial Purification by Ultrafiltration Membrane

The WSE of the rusip optimized formulation was fractionated based on molecular weight using ultracentrifugation with 3 and 10 kDa MWCO (molecular weight cut-off) membranes (Amicon Ultra Centrifugal Units, Regenerated Cellulose; Merck Millipore Ltd., Germany). The ultracentrifugation was carried out at 1,328.4 × g for 60 minutes at 4°C, resulting in three distinct ultrafiltration fractions: fraction of <3 kDa, fraction of 3–10 kDa, and fractions of >10 kDa. Each fraction was subsequently concentrated using a freeze dryer (Christ, Germany), reconstituted to the original sample volume with distilled water, and subjected to further bioactivity analyses, including antioxidant and antimicrobial assays (Kurnianto et al., 2021).

Reverse Phase (RP)-HPLC Profiles

RP-HPLC profile analysis of the WSE fractions was performed utilizing an Agilent 1200 Series HPLC system equipped, which was fitted with a Zorbax C18 column. (4.60 × 150 mm, 5 µm). The mobile phase consisted of HPLC-grade acetonitrile (Merck, Germany) and deionized water, applied in a gradient elution mode as outlined in the methodology presented by Utami et al., (2016). The flow rate was adjusted according to the same reference protocol. Detection was performed at two wavelengths, 214 nm and 280 nm, to monitor peptide bond absorbance and aromatic amino acid residues, respectively (Kurnianto et al., 2020).

Results and Discussion

Analysis of Antioxidant Activity Response

The concentrations of sugar and salt, as well as the duration of fermentation, are critical factors influencing the fermentation process (Puspita et al., 2024). The results identified the quadratic model as the most appropriate for describing the antioxidant response. The antioxidant activity data are summarized in Table 1. The suitability of the model is supported by a high coefficient of determination ($R^2 = 0.981$), adjusted R^2 (0.965), and predicted R^2 (0.914), indicating a robust connection between the experimental and predicted values (Bakar et al., 2023). Additionally, the model exhibited a low Prediction Error Sum of Squares (PRESS = 593.5) and a small standard deviation (3.55), suggesting the model's reliability and predictive adequacy (Bakar et al., 2023).

ANOVA confirmed that the model had a significant influence on antioxidant activity ($p < 0.05$), supporting its reliability, accuracy, and overall goodness of fit (Supplementary 1). Further, the result indicated significant interaction ($p < 0.05$) between sugar and salt concentrations, sugar concentration and duration of fermentation, and salt concentration and duration of fermentation. Although, the individual effects of each factor were not significant in statistics ($p > 0.05$). The highest antioxidant activity was observed in the R7 treatment, which employed 18% palm sugar, 20% salt, and a 14-day fermentation period. In contrast, the lowest antioxidant activity was found in R3, with 15% palm sugar, 15% salt, and 7 days of fermentation. These results suggest that the interaction among the three variables

Table 1. Research Design using Response Surface Method, and Bioactivity (antioxidant and antimicrobial activity) of rusip as responses of varying palm sugar concentration, salt concentration and time fermentation

Run	Independent Variables			Response Parameters		
	Palm sugar Conc. (%)	Salt Conc. (%)	Fermentation Time (Day)	Antioxidant Activity (%)	Antimicrobial Activity against *E. coli* (mm)	Antimicrobial Activity against *S. aureus* (mm)
1	15	25	7	29.18±0.25	2.88±0.01	1.44±0.03
2	10	20	2	45.20±0.26	3.43±0.01	2.49±0.06
3	15	15	7	3.91±0.25	2.27±0.01	3.34±0.02
4	5	15	21	28.47±0.25	2.80±0.02	2.89±0.02
5	10	20	14	49.11±0.50	3.51±0.03	3.08±0.01
6	10	12	14	6.23±0.25	2.37±0.02	3.19±0.02
7	18	20	14	60.50±0.25	4.26±0.01	3.71±0.01
8	10	20	14	56.32±0.13	4.07±0.01	3.05±0.01
9	10	20	14	54.36±0.13	4.00±0.02	3.16±0.01
10	5	15	7	54.00±0.13	4.00±0.01	3.29±0.01
11	10	28	14	6.41±0.25	2.66±0.02	1.51±0.02
12	10	20	14	50.62±0.38	3.63±0.01	2.92±0.11
13	10	20	14	58.01±0.25	4.19±0.06	3.46±0.76
14	10	20	14	51.16±0.14	3.83±0.01	3.10±0.14
15	15	15	21	39.06±0.13	3.40±0.05	1.83±0.02
16	15	25	21	56.41±0.26	4.08±0.01	1.06±0.01
17	5	25	7	34.07±0.13	3.37±0.02	1.89±0.01
18	2	20	14	50.98±0.14	3.83±0.01	2.92±0.02
19	10	20	26	43.51±0.13	3.42±0.11	0.94±0.01
20	5	25	21	9.16±0.13	2.74±0.01	2.60±0.01

plays a more critical role in enhancing antioxidant activity than the influence of each factor individually. The interactions and their influence on antioxidant response presented in Figure 1.

The influence of salt and sugar concentration, and duration of fermentation on the antioxidant activity of fermented foods has been reported. Zubaidah et al., (2020) demonstrated that increased levels of salt and sugar during cabbage fermentation enhanced antioxidant activity. This enhancement was likely attributed to an increased population of LAB and their antioxidant metabolite production (Li et al., 2023). Furthermore, the proliferation of LAB may activate specific enzymes involved in the biosynthesis of bioactive compounds (Abdul et al., 2023). Tang et al., (2024) and Wierzbicka & Janiszewska-Turak (2024) also emphasised that salt concentration affects the osmotic pressure of the fermentation medium, subsequently influencing microbial and enzymatic activity. These mechanisms are thought to facilitate the proteolytic cleavage of proteins into BP fragments through enzymatic hydrolysis (Kurnianto et al., 2023). Salt and sugar concentrations also influence the chemical properties of the fermentation medium by lowering the pH and increasing titratable acidity as a result of LAB metabolism (Liu et al., 2023a; Tang et al., 2024). The reduction in pH significantly affects the resulting antioxidant activity, as it promotes the deprotonation of hydroxyl (-OH) groups in active compounds, a mechanism that significantly enhances antioxidant function (Altunkaya et al., 2016).

Analysis of Antimicrobial Activity Response against *E. coli* and *S. aureus*

The quadratic model provided the best fit to describe the antimicrobial activity responses against *S. aureus* and *E. coli* (Table 1). The models demonstrated high R^2 (0.930 and 0.939), adjusted R^2 (0.867 and 0.885), and predicted R^2 (0.755 and 0.623), indicating good overall explanatory power. Although the predicted R^2 values were lower than the adjusted R^2, particularly for the *E. coli* model, the differences remained within an acceptable range for biological systems characterized by inherent variability. The lower predicted R^2 suggests a moderate reduction in predictive capability for new observations;

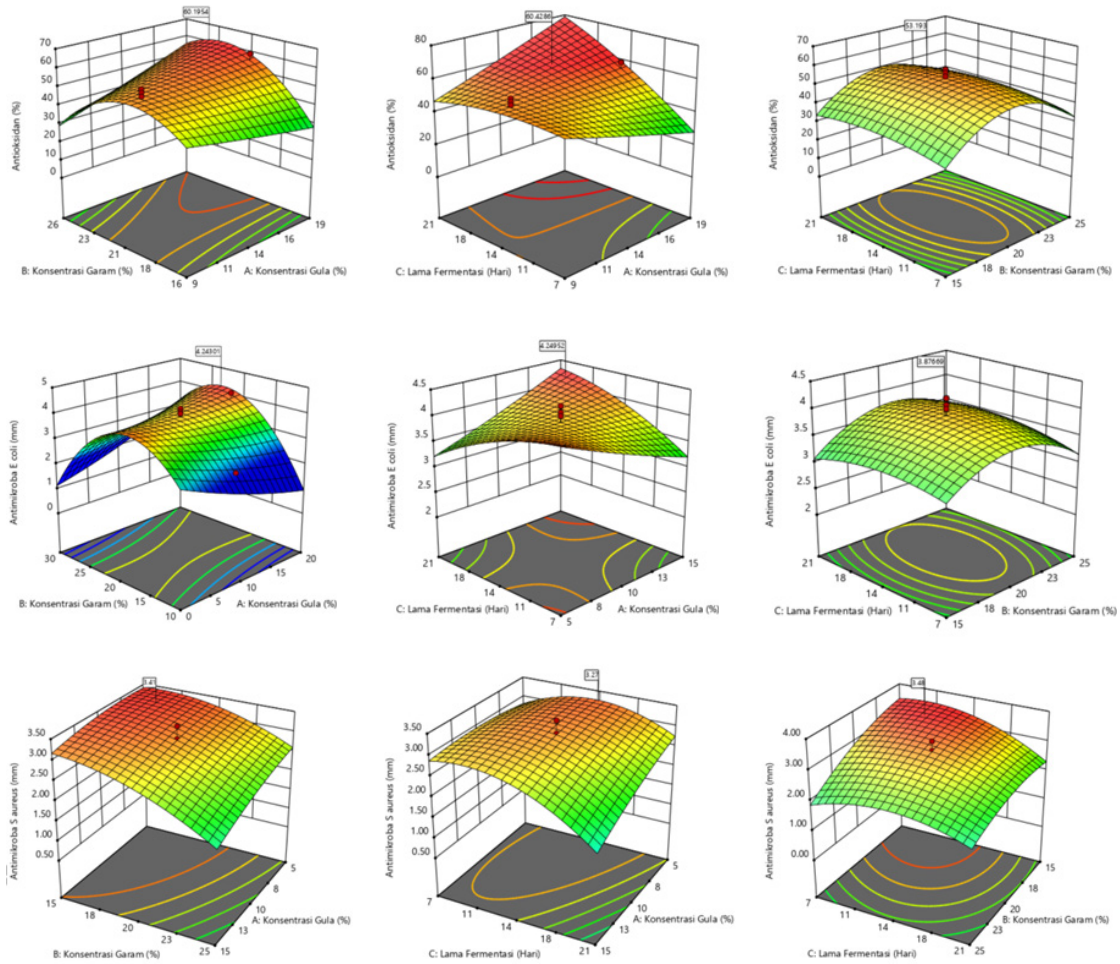


Figure 1. Diagram of the interaction between the three factors on the response of antioxidant activity (1), antimicrobial activity against *E. coli* (2) and antimicrobial activity against *S. aureus* (3); (a) salt and sugar concentrations, (b) duration of fermentation and sugar concentration, (c) fermentation time and salt concentrations

however, the values remained positive and above 0.5, supporting acceptable predictive reliability within the experimental domain. Additionally, the models showed low PRESS (1.795 and 4.56) and standard deviations (0.226 and 0.270), supporting the adequacy and reliability of the models. ANOVA confirmed the significance of both models with p-values < 0.05 (p = 0.0001), and revealed significant curvature in the responses at a 95% confidence level (Wee et al., 2020; Sari et al., 2021;).

Although no individual variables exerted significant main effects on antimicrobial responses (p > 0.05), significant interaction effects were identified. For *E. coli*, sugar–salt and sugar–fermentation time interactions were significant (p<0.05), whereas for *S. aureus*, significant interactions occurred between sugar and fermentation time, as well as salt and fermentation time (Supplementary 2 and 3). These findings indicate that antimicrobial activity was governed by synergistic and condition-dependent mechanisms rather than by independent factor effects. In fermentation systems, antimicrobial metabolites such as organic acids and bacteriocins are produced through metabolic pathways that depend simultaneously on substrate availability, osmotic conditions, and fermentation duration (Salminen et al., 2010). Sugar provides fermentable carbon sources necessary for metabolite synthesis, fermentation time allows accumulation of inhibitory compounds, and salt imposes osmotic stress that can modulate microbial metabolism and selectively favor metabolite-producing populations (Kurnianto et al., 2026). Consequently, multifactorial regulation is thought to cause significant antimicrobial responses emerging only through interaction effects rather than single-factor influences. The effects of these interactions on antimicrobial activity responses are further illustrated in the response surface plots (Figure 1).

The antimicrobial activity of WSE of rusip optimized formulation is presumed to be associated with BP

(Kurnianto et al., 2023). These peptides are produced through the proteolytic cleavage of complex protein substrates by microbial proteases produced during fermentation (Darili et al., 2017; Ramadhan et al., 2021). Extended fermentation periods have been reported to enhance proteolysis, thereby increasing the concentration of peptide fragments (Lee et al., 2022;. Li et al., 2023). LAB are considered key contributors to this proteolytic process due to their ability to produce proteases, their dominance in the fermentation microbiota, and their halophilic nature, which allows them to thrive under the high-salt conditions typical of rusip (up to 20%) (Azara et al., 2019; Ramadhan et al., 2021). Salt concentration plays a pivotal role in shaping the microbial diversity and metabolic profile during fermentation. Lower salt concentrations may promote the growth of undesirable microbes and lead to increased synthesis of biogenic amines, while excessively high salt levels can inhibit the growth and metabolic activity of LAB, resulting in suboptimal product maturation (Liu et al., 2023b; Xiong et al., 2016). The dominance of LAB also contributes to the synthesis of organic acids, which reduce pH and the establishment of an environment that is detrimental to spoilage or pathogenic bacteria (Li et al., 2023). In addition to salt, sugar concentration also modulates microbial activity and metabolite formation. Increased sugar levels provide additional carbon sources that enhance LAB growth and fermentation performance (Zubaidah et al., 2020).

Optimization and verification

After developing the mathematical models for each response variable, the optimal formulation was determined by applying multi-response optimization based on the significance of each variable (Fateha et al., 2019). The independent variables such as salt concentration, sugar concentration, and fermentation duration were assigned a high importance level, while the dependent variables such as antioxidant activity and antimicrobial activity against *E. coli* and *S. aureus* were assigned a very high importance level. The optimization analysis was conducted to identify the optimal combination of factor levels that would yield the most desirable responses. The optimization results indicated that the optimal rusip formulation consists of 5% sugar, 18% salt, and 12 days of fermentation. This combination yielded a high overall desirability value of 0.95 (95%). According to Adamu et al. (2024) and Wijayanti et al. (2024), a desirability value approaching 1.0 reflects a high probability that the predicted conditions will meet the defined optimization criteria. Under these conditions, the model predicted an antioxidant activity of 60.50%, antimicrobial activity against *E. coli* with an inhibition zone of 4.13 mm, and against *S. aureus* of 3.27 mm. The predicted antioxidant activity (60.50% DPPH scavenging) is within the range reported for fermented fish products and peptide-rich marine hydrolysates (Chan et al., 2023). Similarly, the inhibition zones observed fall within the moderate antimicrobial range reported for low-molecular-weight peptide fractions derived from fermented marine products, particularly in fractions below 3 kDa that commonly exhibit measurable activity in agar diffusion assays (Corrêa et al., 2023).

To ensure the validity of the software-generated optimal formulation, a verification experiment was conducted (Fateha et al., 2019). This step aimed to confirm the predicted responses aligned with the actual experimental results for antioxidant activity and antimicrobial activity (*E. coli* and *S. aureus*). A detailed comparison between the observed verification outcomes and model-based predictions is presented in Table 2. These findings yielded an accuracy range between 95.86% and 98.03%, demonstrating strong correlation between the model's predictions and actual measurements. The close proximity of the verification results to the predicted values confirms the adequacy and robustness of the optimization model. Furthermore, these outcomes validate the reliability of the software in predicting formulation performance and support the feasibility of applying the optimized parameters in practical settings.

Table 2. Comparison between observed verification outcomes and model-based predictions

	Sugar Conc. (%)	Salt Conc. (%)	Fermentation Time (day)	Antioxidant Activity	Antimicrobial Activity	
					E. coli	*S. aureus*
Prediction	5	18	12	60,5	4,13	3,27
Lower pred. limit	-	-	-	56,43	3,86	2,95
Upper pred. limit	-	-	-	64,56	4,4	3,6
Verification	5	18	12	58,42	3,96	3,21
Accuracy Rate (%)				96,56	95,86	98,03

Physicochemical and Microbiological Profile of rusip optimized formulation

The rusip optimized formulation (5% sugar concentration, 18% salt concentration, and a 12-day fermentation duration) was further characterized for its physicochemical and microbiological properties. The physicochemical and microbiological characteristics of the optimal rusip formulation are presented in Table 3.

The rusip optimized formulation exhibited a pH of 5.26, which aligns with previous studies (Amelia et al., 2024; Puspita et al., 2024). The relatively low pH value observed in the optimized formulation is indicative of the accumulation of organic acids, which are characteristic by-products of microbial fermentation. As the pH decreases, the titratable acidity tends to increase due to the higher concentration of dissociated acids (Puspita et al., 2024). However, in protein-rich matrices such as fermented fish products, this relationship is modulated by the intrinsic buffering capacity of fish proteins and fermentation-derived peptides (Mennah-Govela et al., 2019; Yu et al., 2024). Fish muscle proteins contain multiple ionizable groups that can resist rapid changes in pH by binding or releasing protons (Kopermsub & Yunchalard, 2012; Nemati et al., 2024). Consequently, substantial acid production is required to overcome this buffering effect before a noticeable decline in pH occurs. Therefore, the elevated TTA value (6.62%) observed in the optimized formulation reflects significant organic acid accumulation capable of surpassing the matrix buffering capacity, further confirming active fermentation and metabolite production.

In terms of proximate parameters, the optimized rusip formula contained 59.63% moisture content, 52.59% ash content (% db), 1.68% fat content (% db), 40.71% protein content (% db) and 5.07% carbohydrate (% db) (Table 3). These values are broadly in line with the findings of Amelia et al. (2024). The elevated moisture content observed in this study is likely associated with metabolic processes occurring during fermentation, particularly glycolysis and proteolysis, which generate water as a by-product (Khositanon et al., 2021). The elevated ash content in the optimized formulation is likely a consequence of the addition of 18% salt during rusip preparation, as sodium chloride constitutes the major fraction of total mineral content. This hypothesis is supported by the salt content analysis, which showed a concentration of 36.73% (Table 3). The high salt content (36.73% db) reflects the concentration effect associated with moisture loss during fermentation. When expressed on a wet basis, it corresponds to approximately 14–15%, which is consistent with the initial salt addition (18%, w/w) and typical values reported for traditional fermented fish products (Gassem, 2019; H. Li et al., 2024). Furthermore, the low-fat content observed may be explained by the inherently low-fat composition of the raw material. Boran et al., (2008) stated the fat content of anchovies ranges between 9.0–15.3% (wb), with largely influenced by seasonal factors and the fish's physiological condition.

Table 3. Physicochemical and microbiological characteristics of the rusip optimized formulation

Parameter	Value
Physicochemical	
pH	5,26 ± 0,09
Moisture (% wb)	59,63 ± 1,38
Crude Ash (% db)	52,59 ± 3,35
Crude Fat (% db)	1,68 ± 0,76
Crude Protein (% db)	40,71 ± 1,91
Carbohydrate (% db)	5,07 ± 1,14
TVB-N (mgN/100 g)	35,73 ± 0,021
Salt content (% db)	36,75 ± 1,17
Total sugar (% db)	4,54 ±1,83
Total titratable acids (% db)	6,62 ± 0,75
Free glutamic acids (% db)	7,31 ± 1,31
Microbiological	
Total BAL (Log CFU/mL)	7,42 ± 0,34
TPC (Log CFU/mL)	4,90 ± 0,68

Note: db (dry basis); wb (wet basis)

The protein analysis revealed a high protein content. Analysis of free glutamic acid showed a concentration of 7.31% (Table 3), aligning with the range reported in commercial rusip, which varies between 5.79 to 8.99% (Amelia et al., 2024). Glutamic acid is a key contributor to the development of umami flavor and its concentration is largely effected by the composition of the raw material substrate and the extent of proteolytic activity during fermentation (Koesoemawardani et al., 2018; Yang et al., 2022). Elevated levels of free glutamic acid enhance savory taste perception and overall palatability, which are critical determinants of consumer acceptance in fermented fish products (Chan et al., 2023; Yamamoto & Inui-Yamamoto, 2023). In high-salt matrices such as rusip, umami compounds play an essential role in balancing saltiness and intensifying flavor complexity, thereby improving sensory appeal (Ma et al., 2024). Regarding carbohydrate content, the analysis revealed a value of 5.07%, a result that aligns with the observations reported by Puspita et al. (2024). Another related analysis, total sugars, showed a value of 4.54%, which is in accordance with previous findings on commercial rusip which ranged from 3.28 - 5.24% (Amelia et al., 2024). The carbohydrate level in the optimized formula is largely attributable to the sugar added during processing, which serves as a carbon source for LAB and contributes to the fermentation dynamics.

In microbiological parameters, the optimized rusip formulation had a total LAB and TPC of 7.42 Log CFU/mL and 4.90 ± 0.68 Log CFU/mL, respectively. These results are consistent with those of Amelia et al., (2024), who reported that TPC values in several commercial rusip products ranged from 5.06 to 6.77 Log CFU/g. However, The total LAB count observed in the present study exceeded the levels previously reported by both Amelia et al., (2024) and Puspita et al., (2024), whose values ranged from 5.06 to 6.77 Log CFU/g and 6.31 to 6.96 Log CFU/mL, respectively. The observed higher LAB count in this study is likely attributed to the shorter fermentation time (12 days) employed in the rusip optimized formulation, compared to the longer fermentation durations in the aforementioned studies. Notably, Puspita et al., (2024) reported that the total LAB count in rusip reached its peak at 6.96 Log CFU/mL on day 14, after which it declined with prolonged fermentation time.

Antioxidant and Antimicrobial activity of Rusip Optimized Formulation

The WSE of rusip optimized formulation was separated using ultrafiltration membranes with molecular weight cut-off value of 10 kDa and 3 kDa, yielding three fractions: F>10 kDa (MW >10 kDa), F3–10 kDa (MW between 3–10 kDa), and F<3 kDa (MW <3 kDa). The selection of these cut-off values was based on previous studies indicating that most food-derived bioactive peptides possess molecular weights below 10 kDa, with enhanced biological activities frequently observed in fractions smaller than 3 kDa (Kurnianto et al. 2021; Ichim et al. 2024; Correa et al. 2023). Prior to bioactivity assays, the volume of each fraction was standardized to that of the original WSE to ensure comparability (Andayani et al., 2015). Fractionation resulted in distinct differences in protein distribution and functional activity (Figure 2). The F3–10 kDa fraction exhibited the highest soluble protein concentration (12.94 mg/mL), whereas F<3 kDa contained the lowest (4.92 mg/mL). Notably, despite its lower protein concentration, the F<3 kDa fraction demonstrated the strongest antioxidant activity (46.09%) and antimicrobial effects, producing inhibition zones of 4.7 mm against *S. aureus* and 4.9 mm against *E. coli*.

These results indicate that the low-molecular weight fraction (<3 kDa) likely contains BP with potent antioxidant and antimicrobial properties. This observation similar to the report by Takenaka et al., (2023), who demonstrated that low–molecular weight peptides (0.2–17 kDa) derived from *Aspergillus sydowii*-fermented Katsuwobushi exhibited notable radical-scavenging and ACE-inhibitory activities. Similarly, Muhialdin and Algboory (2018) reported that peptides with low-molecular weights (0.992 to 1.713 kDa) isolated from fermented camel milk exhibited notable antimicrobial activity against *S. faecalis*, *Shigella dysenteriae*, *S. aureus*, and *E. coli*. In line with these results, Taha et al., (2017) also exhibited that buffalo milk yogurt fermented with *L. acidophilus* and *L. helveticus* contained low molecular weight peptides (0.68–1.99 kDa) with promising antioxidant and antibacterial activities against *E. coli*, *S. aureus*, and *B. cereus*.

Sensitivity of rusip optimized formulation fraction to proteolytic enzyme

To confirm that the observed bioactivities were attributable to peptide compounds, a proteolytic enzyme sensitivity assay was performed using proteinase K, following the procedure outlined by Kurnianto et al., (2021b). The enzymatic treatment resulted in a significant reduction in both antioxidant and antimicrobial activities, thereby indicating that the bioactive components were peptide-based. Specifically, the antioxidant activity of the <3 kDa fraction decreased sharply from 46.09% to 5.3%, representing an 88.48% reduction. Similarly, for antimicrobial activity, the 3–10 kDa fraction lost its inhibitory effect against both *S. aureus* and *E. coli*, while the <3 kDa fraction exhibited a complete loss of activity against *S. aureus* (Table 4). These findings indicate the role of peptides as the primary contributors to the observed biological activities.

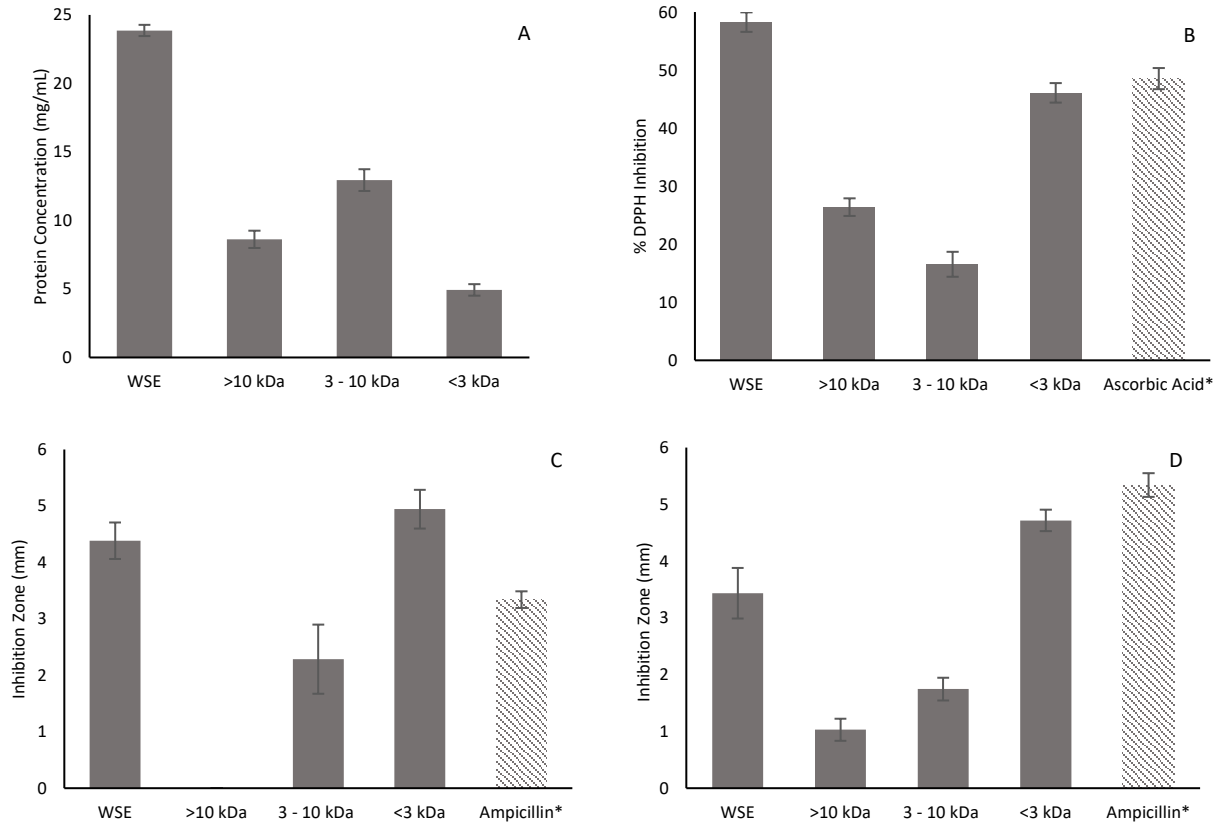


Figure 2. a) soluble-protein concentration, b) antioxidant activity (% inhibition), c) antimicrobial activity against E. coli, d) antimicrobial activity against S. aureus

Table 4. Bioactivity of rusip optimized formulation fraction after Proteinase-K enzyme treatment

Fractions	Antioxidant (%)		Antimicrobial against *E. coli*		Antimicrobial against *S. aureus*	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
WSE	58,26 ± 1,67	23,18 ± 2,23	4,38 ± 0,32	2,83 ± 0,26	3,43 ± 0,46	1,24 ± 0,18
>10 kDa	26,42 ± 1,51	12,34 ± 1,32	0,00 ± 0,00	0,00 ± 0,00	1,03 ± 0,19	0,00 ± 0,00
3 s/d 10 kDa	16,56 ± 2,15	3,39 ± 0,54	2,29 ± 0,61	0,00 ± 0,00	1,75 ± 0,20	0,00 ± 0,00
<3 kDa	46,09 ± 1,67	5,3 ± 0,45	4,94 ± 0,34	1,12 ± 0,16	4,72 ± 0,19	0,00 ± 0,00

These findings are in agreement with previous studies on antimicrobial peptides, particularly those derived from *Streptomyces variabilis* strains SCA-11 and SCA-5, which showed a significant reduction or complete loss of activity following enzymatic treatment with proteinase K, trypsin, and pepsin (Kurnianto et al., 2021b). Similarly, Moracanin et al., (2013) reported that bacteriocins lost their anti-Listeria activity after treatment with papain, pepsin, and proteinase K. Collectively, these observations reinforce the conclusion that the antioxidant and antimicrobial activities identified in the peptide fractions are primarily attributable to proteinaceous or peptidic compounds, whose functional integrity is compromised upon proteolytic degradation.

RP-HPLC profile

The most active fraction, F<3 kDa, was further analyzed for its profile using reversed-phase high-performance liquid chromatography (RP-HPLC) at wavelengths of 214 nm and 280 nm (Figure 3). According to Kurnianto et al., (2021a), HPLC profiling is a quick and precise technique for assessing the composition of complex extracts. Chromatographic analysis of the F<3 kDa fraction revealed multiple peaks eluting within the retention time of 0–15 minutes, with 13 peaks detected at 214 nm and 10 peaks detected at 280 nm. Absorbance at 214 nm is generally indicative of amido-chromophoric groups that include one or more peptide bonds (Praveen &

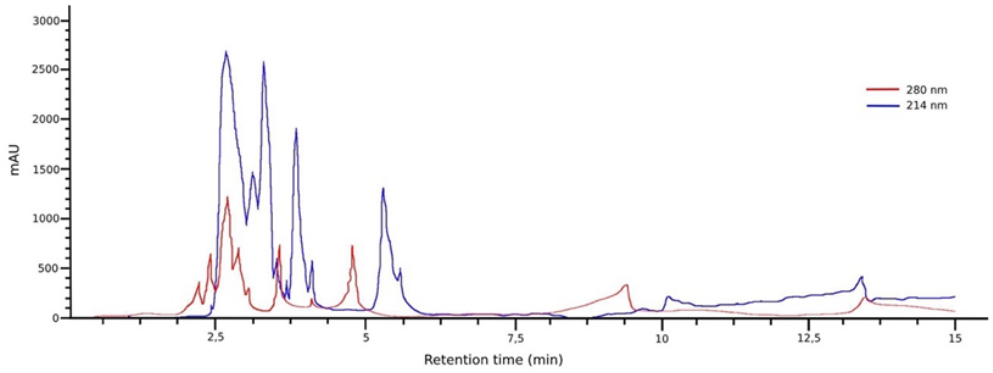


Figure 3. RP-HPLC profile from rusip optimized formulation water soluble extract detected at 214 and 280 nm.

Tripathi, 2009), while peaks detected at 280 nm indicate the presence of aromatic structures (Kurnianto et al., 2020, 2021a) Specifically, Ezra et al., (2004) identified peaks in the 208–214 nm range as corresponding to amino acid residues such as threonine, methionine, and leucine, whereas a peak at 270 nm was attributed to threonine.

The high number of intense peaks detected at 214 nm, particularly within the early retention time range of 2.18 to 5.93 minutes, indicates a substantial presence of peptide compounds in the fraction. Absorbance at 214 nm is commonly associated with peptide bonds, and higher peak intensity reflects a greater abundance of peptide-related components (Kurnianto et al., 2020, 2021a). The predominance of early-eluting peaks suggests that many of the detected peptides are relatively small and polar in nature, which is consistent with the <3 kDa fraction obtained through ultrafiltration. Peptide physicochemical properties, including molecular weight, charge distribution, and hydrophobicity, play important roles in determining bioactivity. In antibacterial mechanisms, peptides often interact with bacterial membranes through combined cationic and hydrophobic interactions, facilitating membrane disruption (Dathe & Wieprecht, 1999; Naarmann et al., 2006). Similarly, antioxidant peptides are frequently characterized by low molecular weight and structural features that enable electron or hydrogen donation (Tamam et al., 2019). Previous studies have reported that antioxidant activity in bioactive peptides is often associated with aromatic and heterocyclic residues capable of stabilizing radical species (Duan et al., 2014). Although the amino acid composition of the optimized rusip peptides was not determined in this study, the observed antioxidant activity may be related to structural characteristics commonly reported in low-molecular-weight bioactive peptides. Further peptide sequencing or amino acid profiling would be required to confirm the specific residues responsible for the observed bioactivity.

Conclusion

This study concluded that the antioxidant and antimicrobial activities of rusip were influenced by raw material composition and fermentation time, which were successfully optimized using RSM. The rusip optimized formulation (5% sugar, 18% salt, 12 days fermentation) achieved high predicted antioxidant activity (60.50%) and effective antimicrobial inhibition against *E. coli* and *S. aureus*. Ultrafiltration indicated that the <3 kDa fraction exhibited the strongest bioactivities, likely associated with low-molecular-weight peptides, as supported by Proteinase K treatment and HPLC analysis. These findings suggest that rusip may serve as a promising source of bioactive peptides with antioxidant and antibacterial potential. However, the bioactivities reported in this study were evaluated under in vitro conditions, and further studies are required to characterize peptide sequences, assess stability during gastrointestinal digestion, and validate their efficacy in vivo.

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