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RESEARCH PAPER

Optimized hydrolysis of tuna viscera by a spray-dried cell-free crude enzyme preparation from *Staphylococcus hominis* L1 isolated from fermented fish

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Abstract

Proteolytic enzymes are used to produce hydrolysates from fish processing wastes to recover protein for various applications. Tuna processing also generates these by-products adding to post-harvest losses and environmental pollution. Thus, this study was aimed at optimizing the proteolytic activity on yellow fin tuna (*Thunnus albacares*) viscera (YFTV) of a spray-dried cell-free crude enzyme extracted from the culture of *Staphylococcus hominis* L1 which was isolated from fermented sardines and identified using 16s rRNA gene analysis. To prepare the enzyme, the isolate was grown in a protease-producing medium supplemented with casein and peptone. The culture supernatant was harvested following fermentation and was spray-dried. The hydrolysis condition was optimized to produce hydrolysates with high total soluble protein (TSP) and high degree of hydrolysis (DH). Utilizing the central composite design of the Response Surface Methodology (RSM), hydrolysis was conducted at varying enzyme concentrations (0.5-1.5%, w/v), temperature (40-60°C) and hydrolysis times (60 to 180min). According to the RSM-generated model, the optimal conditions for achieving the highest TSP were 60 min, 40°C and 1.5% enzyme concentration. For the highest DH, the optimal conditions were 60 min, 60°C and 0.5% enzyme concentration. The predicted optimum values using the generated linear and quadratic equations were 3.68 mg/ml TSP and 30.67% DH. The lack of fit test for both responses yielded an insignificant value ($p > 0.05$), indicating that the regression coefficient was adequate for estimating both responses under various conditions. The findings of this study could be effectively applied in food production systems, particularly in downstream processing.

Introduction

Fish Tuna is one of the major species used in the production of processed seafood, and the wastes generated from tuna processing make up a significant portion of coastal waste (Sayana and Sirajudheen, 2017). In fact, most fish processing industries around the world discard more than 20 million tonnes of waste fish each year (Racioppo et al., 2021) and addressing these wastes have become a major challenge for this sector. Furthermore, fish waste contains half of the raw materials volume of the industry or processing plants. Therefore, most efforts are directed towards recovery of valuable substances from wastes by recycling them as various new products (Arvanitoyannis & Kassaveti, 2008). In tuna processing, by-products from wastes or tuna side streams can be used to produce different products such as protein hydrolysates, collagen, enzymes, tuna meal, tuna oil, and tuna bone powder. The considered wastes like fish skins, heads, viscera, and bones were discovered to have high yield, better quality and long shelf life which are promising factors for maximum utilization of fish processing wastes (Sasidharan et al., 2023).

Tuna processing generates significant quantities of waste, including heads, bones, skins, and viscera, which pose environmental and economic challenges if not properly utilized (Vasquez et al., 2022). The viscera of larger tuna species such as the yellow fin tuna is considered as a major waste in fish processing industries, but they may be used for other purposes such as fish meal and may be developed into protein hydrolysates, and applied as preservatives in bioactive packaging materials or nutraceuticals components (Wu and Chen, 2022).

In developing protein hydrolysates, various proteases may be used. Hydrolysates, produced through the enzymatic hydrolysis of these wastes (e.g. fish head, viscera, and bones), are emerging as valuable bioproducts with diverse applications across various industries (De Asis et al., 2023; Vázquez et al., 2022; Phadke et al., 2016). Rich in peptides, amino acids, and other bioactive compounds, tuna waste hydrolysates have shown great potential as functional ingredients in food products, enhancing nutritional profiles and providing health benefits. Some hydrolysates are utilized in the pharmaceutical industry for their bioactive properties (Zaky et al., 2021) and in aquaculture as high-quality, digestible feed additives (Pan et al., 2022). By converting waste into valuable resources, hydrolysates from tuna by-products contribute to sustainability and resource efficiency, offering innovative solutions for waste management and the development of high-value products.

Studies on *Staphylococcus* sp. protease enzymes highlight several relevant properties and potential biotechnological applications of these enzymes (Wang et al., 2024; Wang et al., 2022). Staphylococcal proteases are known for their robust catalytic activity and specificity (Stach et al., 2018). They exhibit a high degree of hydrolysis, which is crucial for breaking down complex protein substrates into simpler peptides and amino acids. This makes them particularly effective in processing various biological materials. Additionally, these enzymes operate efficiently under a range of conditions, demonstrating significant stability and activity at different temperatures and enzyme concentrations (Tam and Torres, 2020; Cooper et al, 1996; Demleitner and Gotz, 1994).

In the food industry, staphylococcal proteases can be utilized to enhance the nutritional value and digestibility of food products (Wang et al., 2022). Their ability to hydrolyze complex proteins into bioactive peptides can improve the functional properties of food, such as solubility, emulsification, and foaming capacity. These enzymes can be employed in the production of protein hydrolysates, which are used as flavor enhancers, nutritional supplements, and functional food ingredients. Moreover, the antimicrobial properties of bacteriocins derived from *Staphylococcus* spp. can be leveraged to develop natural food preservatives, reducing the reliance on chemical additives and extending the shelf life of food products(Wang et al., 2022). With these previous findings, the present study determined the possibility of hydrolyzing tuna viscera by using proteolytic enzymes from a bacterial strain. In this study, through Response Surface Methodology (RSM) we optimized the hydrolysis of yellow fin tuna (*Thunnus albacares)* viscera with a spray-dried preparation of the enzyme produced by *Staphylococcus* sp. isolated

from fermented sardines. The isolate was identified using 16s rRNA gene analysis. The hydrolytic capacity of its enzymes was optimized in terms of hydrolysis time, temperature and enzyme to substrate ratio with total soluble protein and degree of hydrolysis as the response variables.

Materials and Methods

Isolation, screening and identification of protease-producing bacterial isolate from fermented sardines

To allow the growth of endogenous bacteria from fermented sardines (*Sardinella spp.*), 10g of samples were placed in 100 mL of Brain Heart Infusion (BHI) broth as the enrichment medium. After incubation with shaking for 72 h, 1 mL of the culture was serially diluted up to 10^{-3} with 0.85% peptone. Then, 100 µL of each dilution was spread plated in de Man, Rogosa, and Sharpe (MRS) agar plates supplemented with 3% salt, with each dilution plated in triplicate. The plates were incubated at 37°C for 48-72 h to allow bacterial growth and colony formation. Following incubation, each colony exhibiting unique morphology was picked and re-streaked in a new MRS agar plate and allowed to grow for 18-24 h at 37°C. This isolation process was repeated twice until only one colony morphology was observed. After the final purification, isolated strains were cultured in glycerol (0.5% (v/v)) for long-term storage and on MRS slants with 3% salt for subsequent screening of protease production.

For the primary screening of protease-producing bacterial isolates, the protocol using skim milk agar (SMA) adapted from Masi et al. (2001) was used. The 1% SMA was prepared with 2.8 g of skim milk powder, 500 mg of casein enzymic hydrolysates, 250 mg of yeast extract, 100 mg of dextrose, and 1.5 g of agar in 100 mL of distilled water, adjusted to pH 8.5. The bacterial isolates were spot inoculated onto the SMA using inoculating needles and incubated at room temperature for 48-72 h. The isolates exhibiting clear zones in the SMA after incubation were selected for secondary screening. The selected isolates were cultured (1% v/v) in a nutrient broth medium composed of 0.1 g of beef extract, 0.2 g of yeast extract, 0.5 g of peptone, and 0.5 g of NaCl per 100 mL of distilled water, adjusted to pH 8.5. The cultures were incubated with agitation for 2 days at room temperature. After incubation, the cultures were centrifuged at 10,000 rpm for 15 min at 4°C to obtain the cell-free supernatant presumed to contain the extracellular proteases. The supernatant (25 µL) was inoculated in fresh SMA containing wells. The plates were then incubated at 30°C for 48-72 h and the isolate that demonstrated the highest zone of inhibition was selected for further analysis.

The isolate was identified through 16s rRNA gene analysis. For DNA extraction, the strain was cultured in Luria broth incubated for 24 h and the culture was then centrifuged at 1400 rpm for 15 min to collect the cells. Subsequently, the bacterial pellet was resuspended in 200 µL of TE buffer and the DNA was extracted by thermal lysis method by alternate incubation at 100°C and ice for 5 min each and the process repeated twice. The lysate was then collected as the resulting supernatant after centrifugation at 1500 rpm for 15 min. The supernatant served as the DNA template. The 16s rRNA gene was amplified using the universal primers 27F (5' -AGAGTTTGATCCTGGCTCAG -3') and 1492R (5'GGTTACCTTGTTACGACTT-3') which target conserved regions of the bacterial 16s rRNA gene. PCR amplification was carried out in a 25 µL reaction mixture containing 12.5 µL of 2X PCR master mix, 1 µL each of forward and reverse primers (10 µM), 1 µL of template DNA, and 9.5 µL of nuclease-free water. The thermal cycling conditions consisted of an initial denaturation of 90°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 90 sec, with a final extension of 72°C for 10 min. Amplification success was verified by agarose electrophoresis before the amplicons were sent for DNA sequencing (Kinovette Scientific Solutions Co.).

Biomass production of the bacterial protease and evaluation of its activity

For biomass production, a 24-h culture of the isolate in nutrient broth was inoculated (1% v/v) in a 3-L production medium consisted of 30 g of galactose, 15 g casein, 16.5 g of peptone, 6 g of KH_2PO_4, 30 g of $Na_2Co_3$and 6 g of $MgSO_4 \cdot 7H_2O$ at pH 8. The fermentation process lasted for 72 h at 37°C, with controlled agitation maintained at

150–200 rpm. Following fermentation, the entire culture broth was centrifuged at 6000 rpm for 15 min and then transferred to a container for spray drying. The parameters for spray drying were as follows: (1) inlet temperature at 170ºC, (2) outlet temperature at 60ºC, and (3) feed flow at 7.5 ml/min. The spray dried enzyme in powder form was then evaluated for its proteolytic activity using casein hydrolysis assay. The protocol followed was adapted from Kunitz (1947) wherein the reaction mixture was comprised 1.0 mL of 1.0% casein dissolved in 0.01N NaOH, 1.5 mL of phosphate buffer pH 7.0, and 0.5 mL of enzyme extract, making a final volume of 3.0 mL. The reaction was allowed to proceed for 60 min and stopped by adding 1.0 mL of ice-cold 5% trichloroacetic acid (TCA). The optical density of the reaction mixture was measured at 280 nm, and protease activity was expressed as grams of tyrosine released per hour per milligram of protein at 25°C under the conditions of the assay. The enzymatic activity of the sample was calculated as follows:

$$\text{Units/mL enzyme(U/mL)} = \frac{\text{(mole tyrosine equivalents released) x (total vol. of assay in mL)}}{\text{(time of assay) x (vol. of enzyme) x (vol. in colorimetric determination)}}$$

Optimization of enzymatic hydrolysis of tuna viscera using RSM

Design Expert 11 was employed to carry out the RSM with central composite design (CCD) to optimize the hydrolysis conditions of yellow fin tuna viscera (YFTV). The experimental design, illustrated in Table 3, suggested 20 experimental runs (in triplicates), each with different combinations of these factors : hydrolysis time, enzyme concentration, and temperature.

For the enzymatic hydrolysis of tuna viscera, the protocol was adapted from Ovissipour (2010). Homogenized YFTV were weighed in 50 g, placed in a 300 mL Erlenmeyer flask and were then heated at 85°C in a water bath (Memmert, Germany) for 20 min to deactivate the endogenous enzymes, and then cooled. Then the sodium phosphate buffer (1:2 w/v) was added and the mixture was maintained at pH 7 by gradual addition of 0.2 N NaOH and, if needed, 0.2 N HCl. Finally, the homogenized YFTV were added with the enzyme preparation with various concentrations as suggested by the RSM. Each experimental run was subjected to the RSM-suggested reaction time and temperature. Hydrolysis was conducted in a shaker incubator (Biobase, shaker incubator, Model: BJPX-1102L) and after the specified time, the samples were transferred to a 95°C water bath for 15 min to deactivate the enzymes, then cooled, and stored in a freezer.

Determination of the degree of hydrolysis (DH)

The protocol described by Wongsa et al. (2022) for the OPA Assay was used to determine the degree of hydrolysis. For the assay, 30 µL of the sample or standard were dispensed into each well of a 96-well microplate, followed by 250 µL of the OPA mix. The samples were incubated for 2 min and then read at 750 nm. Based on the study of Nielsen et al. (2001), the DH was computed as follows:

$$\text{DH (\%)} = \left(\frac{\text{h}}{\text{h}_{\text{tot}}}\right) \text{x } 100$$

Where h, is the number of hydrolyzed peptide bonds and h_{tot} is the total number of peptide bonds present.

Determination of the total soluble proteins (TSP)

The TSP were measured using protocol described by Lowry et al. (1951) using Bovine serum albumin (BSA) as standard. Briefly, the Lowry mix consisted of 48 ml solutions A, B, and C, wherein solution A is 2% of Na_2CO_3 in 0.1N of NaOH, 1 mL of solution B having 1% of sodium tartrate in distilled water + 1 mL of solution C having 0.5% $CuSO_4 \cdot 5H_2O$. The Folin-Ciocalteau's Phenol reagent was added with distilled water in a 1:1 ratio. For the assay, 40 µL of the samples were dispensed into a well of a microplate, followed by 200 µL of the Lowry solution. The plate was kept away from light and incubated for 10 min. Then, 20 µL of the Folin mix was added, and the samples were incubated

for an additional 30 min. The absorbance was measured at 460 nm using a spectrophotometer. Based on the study of Nielsen et al. (2001), the TSP was computed using the regression equation derived from the standard curve.

Statistical analysis

Response Surface Methodology (RSM) using Design Expert software version 11 was employed to create a completely randomized factorial design for optimizing the enzymatic hydrolysis of tuna viscera. The data were analyzed using analysis of variance (ANOVA) for mean comparison at a 95% confidence level ($p<0.05$) using Jamovi project (2022) software.

Results and Discussion

Identity of the protease-producing bacterial isolate

The strain showing the highest proteolytic activity among all the isolates (data not shown) that was used in further analysis was designated as strain L1. Its closest relatives based on BLAST homology search are *Staphylococcus* spp. as demonstrated in the phylogenetic tree showing its relationship to these species (Fig.1). In terms of sequence identity, the 16s rRNGa gene of strain L1 showed the highest homology with *Staphylococcus hominis* CM2 (Acc. No. MH174446) and *S. hominis* SA 107 (Acc. No. KY623285). It was also related to other *Staphylococcus* spp. such as *S. epidermis* PBR-9 (Acc. No. KJ806213) and *S. epidermis* (Acc. No. PX122258), *S. ptrasii (*Acc. No. NR118450), *S. brunensis* (OQ401401), *S. haemolyticus* (Acc. No. KT696497), *S. borealis* (Acc. No. MT586030). Therefore the strain was designated as putative *Staphylococcus hominis* Strain L1.

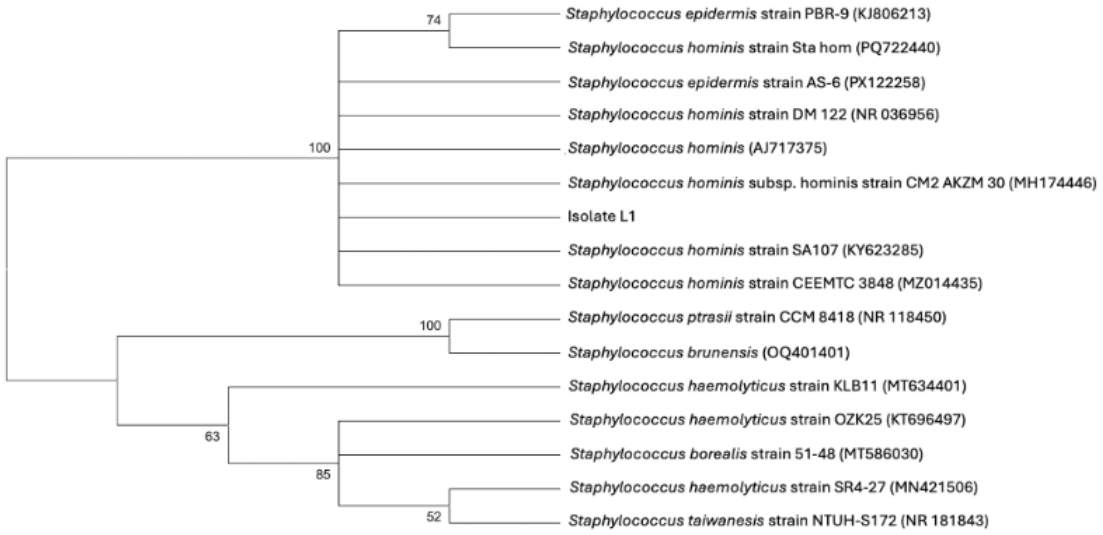

Figure 1. The phylogenetic tree showing the relationship among *Staphylococcus hominis* and closely related Staphylococcus strains. The evolutionary history was inferred using the Maximum Likelihood method. The bootstrap consensus tree was generated from 100 replicates, with branches reproduced in less than 50% of replicate trees collapsed. Bootstrap support values (%) are shown at the nodes. The initial tree for the heuristic search was selected from Neighbor-Joining and Maximum Parsimony trees, and pairwise evolutionary distances were calculated using the Kimura 2-parameter model. The *Staphylococcus taiwanensis* strain NTUH-S172, representing the most distantly related taxon, was used as the outgroup. The scale bar indicates 0.02 substitutions per nucleotide position. Phylogenetic analyses were performed using MEGA 12.

The production of extracellular proteases by *Staphylococcus hominis* has drawn growing interest because of its ecological importance and potential uses in biotechnology and the food industry. While *S. hominis* is commonly found on human skin, it is not limited to this environment and has also been isolated from various sources, including fermented foods. Unlike highly

pathogenic species such as *Staphylococcus aureus*, *S. hominis* is generally considered low-virulence, and evidence is mounting for its beneficial roles. For example, Severn et al. (2022) showed that *S. hominis* can produce antimicrobial peptides that inhibit the growth of opportunistic pathogens, suggesting it contributes more to microbial competition than to disease. Similarly, Hwang et al. (2020) found that *S. hominis* subsp. *hominis* WiKim0113 from kimchi had safe functional properties and could be used as a starter culture to produce natural pre-converted nitrite. Its proteolytic activity likely helps the bacterium adapt to different environments and utilize nutrients without being harmful. Overall, these studies support the idea that *S. hominis* is a safe species with promising applications in food processing and biotechnology.

Proteolytic activities of the crude and spray-dried enzyme from Staphyloccus hominis Strain L1

The results of the casein enzymatic assay for both crude and spray-dried protease samples of *S. hominis* L1 are presented in Table 1. The protease activities were 2.02±0.04 U/ml and 1.98±0.06 U/ml for crude and spray-dried, respectively. Results revealed no significant difference ($p>0.05$) between their activities.

This result revealed that the spray-drying process did not diminish the activity of the extracellular proteases produced by the bacterial strain. These observations underscore the importance of considering processing methods and their potential impacts on enzyme stability and performance. With the differences in protease activity between the crude and spray-dried samples being minor, spray drying remains a preferred method for sample preservation and storage convenience due to assured stability of the products (Paudel et al., 2013; Pires et al., 2021).

Table 1. Proteolytic activities of crude and spray-dried enzyme preparation from the culture of *Staphylococcus hominis* L1

Samples	Protease activity, U/ml
Crude enzyme	2.02 ± 0.04
Spray-dried enzyme	1.98 ± 0.06

Response surface plots for the total soluble protein (TSP) and degree of hydrolysis (DH)

The experimental runs for the RSM were generated using the Design Expert software, providing both actual and predicted values for two key responses: total soluble proteins (TSP) and degree of hydrolysis (DH). There were a total of 20 runs with different values for the three variables: enzyme concentration, hydrolysis time and temperature (Table 2). Results show that for TSP, the values ranged from 2.58 mg/ml to 3.68 mg/ml. The highest TSP was observed in Run 18 , which had the hydrolysis conditions as follows: 1.5% enzyme concentration, a temperature of 40°C, and a hydrolysis time of 60 minutes. Conversely, Run 7, with 0.50% enzyme concentration, a temperature of 40°C, and a hydrolysis time of 60 min, yielded the lowest TSP value.

For DH, the actual values shown in Table 2 ranged from 10.72% to 30.67%. The highest DH was achieved in Run 7, which involved a hydrolysis time of 60 min, a temperature of 40°C, and an enzyme concentration of 0.5%. On the other hand, the lowest DH was observed in Run 10, which employed a hydrolysis time of 120 min, a temperature of 50°C, and an enzyme concentration of 1.5%. Results both for TSP actual and predicted values and DH actual and predicted values were separately subjected to T-test for related samples and revealed to be not statistically significant ($p>0.05$).

The experimental runs generated by RSM shed light on the complex dynamics of enzymatic hydrolysis processes. The data provided both actual and predicted values for two key responses, TSP and DH, offering valuable insights into the efficiency of different hydrolysis conditions. The variation in TSP values, ranging from 2.58mg/ml to 26.68mg/ml, shows the effects of hydrolysis parameters on protein solubility. Notably, run 18 demonstrated the highest TSP, achieved with 1.5% enzyme concentration, a temperature of 40°C, and a hydrolysis time of 60 minutes, highlighting optimal performance under specific conditions. Conversely, run 7 yielded the lowest TSP value, reflecting the sensitivity of TSP to changes in enzyme concentration. This implies that, in this specific instance, the enzyme

concentration played a predominant role in determining TSP levels with temperature and hydrolysis time only having minimal influence on TSP under certain conditions. Various factors influence the solubility of proteins, categorized as internal, such as the type of amino acids on the protein surface, and external, including temperature, pH, ionic strength, and additives (Kramer et al., 2012). Denaturation, a significant phenomenon, affects protein solubility, with proteins generally stable under neutral pH and ambient temperature, but prone to denaturation under changing conditions like temperature, pressure, and pH, as well as during freezing-thawing and freeze-drying processes, commonly used in food and pharmaceutical processing (Izutsu, 2018).

Table 2. Experimental runs generated by RSM and the actual and predicted value of the total soluble proteins and the degree of hydrolysis

Run	Hydrolysis Time (min)	Enzyme Conc. (% w/v)	Temp. (℃)	Total Soluble Protein (mg/ml)		Degree of Hydrolysis (%)	
				Actual Value	Predicted Value	Actual Value	Predicted Value
1	180	1.5	40	3.37±0.11	3.38	12.21±0.09	11.41
2	120	1	50	3.38±0.13	3.30	27.33±0.1	26.54
3	120	1	33	3.30±0.21	3.28	15.94±0.38	17.57
4	120	1	50	3.27±0.11	3.30	28.3±0.3	26.54
5	120	1	50	3.52±0.06	3.30	28.18±1.39	26.54
6	60	0.5	60	3.10±0.09	3.08	17.85±0.34	19.10
7	60	0.5	40	2.58±0.06	2.59	30.67±0.96	29.04
8	120	1	50	3.11±0.08	3.30	27.46±0.14	26.53
9	120	1	66	3.63±0.09	3.67	18.18±0.18	15.91
10	120	1.5	50	3.60±0.11	3.58	10.72±0.14	10.29
11	60	1.5	60	3.56±0.10	3.57	13.01±0.12	13.12
12	120	1	50	3.28±0.18	3.30	26.10±0.21	26.54
13	120	1	50	3.23±0.06	3.30	21.75±0.2	26.54
14	180	0.5	40	2.87±0.16	2.85	16.88±0.12	17.23
15	220	1	50	3.13±0.05	3.16	21.85±0.14	20.66
16	180	0.5	60	3.49±0.14	3.46	16.99±0.3	17.74
17	19	1	50	3.13±0.18	3.11	24.76±0.89	25.32
18	60	1.5	40	3.68±0.04	3.70	15.89±0.21	15.59
19	120	0.1	50	2.69±0.47	2.72	20.41±0.16	20.21
20	180	1.5	60	3.38±0.10	3.36	17.31±0.58	19.39

Similar observations were made by Guo et al. (2020) with soy protein isolate, wherein short-term heating and surface modification improved solubility. Solubility typically increases with temperature or in conditions such as water saturation, but the extent depends on solvent properties like temperature and protein concentration (Carullo, 2021). Protein type can lead to varied structural changes, often significantly improving solubility or, conversely, reducing it in some cases. Haque et al. (2020) in their study on jackfruit seeds protein isolate and Liu et al. (2024) in their study on hydrolyzed/glycosylated ovalbumin, found that proteins stabilized through spray-drying exhibited enhanced solubility due to lesser denaturation at lower temperatures, increased porosity, smaller particle sizes, and more uniform distribution. The results of the present study, indicating higher enzyme concentration correlating with higher TSP suggest that increasing enzyme concentration beyond the levels tested could further enhance TSP. Thus, future experiments could explore enzyme concentrations of 2.0% and 2.5% of the total grams of tuna viscera samples to optimize enzymatic processes.

According to Daniel and Danson (2013), the discovery of an apparently universal mechanism by which enzymes lose activity as the temperature rises is of considerable interest in itself. Moreover,

changes in temperature can be independent of changes in stability and associated with changes in stability, given that conformational flexibility is often inherent in an enzyme catalytic mechanism, thus the active site might be one of the most susceptible parts of an enzyme to temperature-induced conformational changes. The present study was also in agreement with the findings of Daniel and Danson (2013), that enzyme, time and temperature does have an optimum for initial rates because enzyme activation and deactivation is rapid and initial rates decline at high temperatures and at all temperatures the activation and deactivation equilibration is faster than the time needed to start the reaction. In constant concentration and based on the Michaelis-Menten model (Peterson et al., 2004; Lee et al., 2007; Daniel and Danson, 2010), a starting point for experiments can be set at 40ºC – 60ºC for temperature and 180 min for time. Future experiments can then explore a wider range of temperatures and times.

The suggested models for TSP and DH were both quadratic, as shown in Table 3. The p-values for the responses were 0.025 in total soluble protein and 0.000061 for the degree of hydrolysis.

Table 3. Sequential model sum of squares for the total soluble proteins and degree of hydrolysis of tuna viscera protein hydrolysates

Response	Source	Sum of Squares	df	Mean	F-value	p-value	
Total Soluble Protein (mg/ml)	Mean vs Total	213.20	1	213.20			
	Linear vs Mean	1.07	3	0.36	9.08	0.00096	
	2Fl vs Linear	0.37	3	0.12	6.28	0.0072	
	Quadratic vs 2Fl	0.15	3	0.05	4.85	0.025	Suggested
	Cubic vs Quadratic	0.01	4	0.0019	0.12	0.97	Aliased
	Residual	0.10	6	0.02			
	Total	214.91	20	10.75			
Degree of Hydrolysis (%)	Mean vs Total	8478.55	1	8478.55			
	Linear vs Mean	148.26	3	49.42	1.48	0.26	
	2Fl vs Linear	111.66	3	37.22	1.15	0.37	
	Quadratic vs 2Fl	371.60	3	123.87	24.73	0.000061	Suggested
	Cubic vs Quadratic	18.68	4	4.67	0.89	0.52	Aliased
	Residual	31.41	6	5.24			
	Total	9160.16	20	458.01			

The p-values for the responses (TSP = 0.025; DH = 0.000061) were both less than 0.05, indicating that the models for TSP and DH are significant and can adequately describe the experimental data. The model p-value tests whether at least one of the regression coefficients is different from zero, indicating that the independent variables (factors) collectively have a significant effect on the response variable (Selvamuthu and Das, 2018).The lack of fit has a p-value of 0.99 for TSP and 0.67 for DH which are both not significant, lack of fit p-value tests whether the model

adequately fits the data. It compares the residual error (the difference between the observed and predicted values) to the pure error (variation observed among replicate observations). (Selvamuthu and Das, 2018). Thus, the quadratic models proposed for both TSP and DH in the current study were selected based on the principles of RSM aiming to optimize complex processes by examining the relationship between multiple variables. The choice of quadratic models signifies that the response variables, TSP and DH, exhibit non-linear behavior in response to changes in the independent factors, such as enzyme concentration, temperature, and hydrolysis time. This decision aligns with the nature of enzymatic hydrolysis processes, which often involve intricate interactions between various parameters. Furthermore, the significance of the p-values, which were less than 0.05 for both TSP (0.025) and DH (0.000061), highlights the validity of the proposed models (Mcleod, 2023). This statistical significance shows the reliability of the models in capturing the variation in TSP and DH resulting from changes in hydrolysis conditions.

Table 4 presents the analysis of variance and the coefficient of determination (R^2) for the response surface models. The p-values of 0.000062 for TSP and 0.000014. The p-values of 0.000062 for TSP and 0.000014 for DH suggest that the independent factors in enzymatic hydrolysis significantly affect both responses ($p<0.05$). The R^2 values for TSP and DH were 0.93 and 0.92, respectively, indicating a high level of correlation between the predicted and actual values, and confirming the reliability of the models in explaining the variation in the data. This suggests that the quadratic models provide a robust representation of the relationship between the independent variables and the responses.

Table 4. Analysis variance and coefficient of determination (for the response surface model for the total soluble proteins and degree of hydrolysis of tuna viscera protein hydrolysates

Response	Source	Sum of Squares	df	Mean	F-value	p-value	R^2
Total Soluble Protein (mg/ml)	Model	1.60	9	0.18	16.94	0.000062	0.93
	Residual	0.105	10	0.0194			
	Lack of Fit	0.0078	5	0.00156	0.80	0.99	
	Pure Error	0.097	5	0.0194			
	Total	1.71	19				
Degree of Hydrolysis (%)	Model	631.52	9	70.17	14.01	0.00014	0.92
	Residual	50.09	10	5.01			
	Lack of Fit	19.69	5	3.94	0.65	0.68	
	Pure Error	30.39	5	6.08			
	Total	681.61	19				

Conditions for the optimum response for the TSP

Optimization by response surface modeling provided a combination of hydrolysis conditions for tuna viscera that would yield the optimum TSP. As shown in Table 5, the model predicted that using an enzyme concentration of 1.5%, a temperature of 40°C, and a reaction time of 60 minutes would yield 3.70 mg/ml of TSP. The optimization process generated an accurate combination of hydrolysis conditions for tuna viscera to attain the maximum yield of TSP. Remarkably, the experimental yield closely matched this prediction, with an actual value of 3.68±0.04 mg/ml. Results subjected to t-test for the two values revealed to be not statistically significant ($p>0.05$) . Hence, this alignment between predicted and actual yields underscores the efficacy of the response surface model in

optimizing hydrolysis conditions for TSP

Response	Independent factors			Predicted Value %	Actual Value
	Hydrolysis time (min)	Enzyme concentration (% w/v)	Temperature (°C)		
Degree of Hydrolysis	60	0.73	44	29.10	30.67±0.96

production.

Table 5. Optimum hydrolysis conditions for the total soluble proteins in the extraction of fish protein hydrolysates

Response	Independent factors			Predicted Value %	Actual Value
	Hydrolysis time (min)	Enzyme Concentration (% w/v)	Temperature (°C)		
Total Soluble Proteins	60	1.5	40	3.70	3.68±0.04

This close agreement between the predicted and actual yields validates the effectiveness of the response surface model in optimizing the hydrolysis conditions for achieving maximum TSP yield. Fig. 2 depicts the relationships between the variables under specific optimal conditions. Fig. 2a shows the relationship between hydrolysis time and temperature when the enzyme concentration is at its optimal level of 1.5%. Fig. 2b illustrates the relationship between enzyme concentration and temperature when the hydrolysis time is fixed at its optimal duration of 60 minutes. Fig. 2c presents the relationship between enzyme concentration and hydrolysis time when the temperature is maintained at its optimal value of 40°C. These visualizations help to understand how TSP is influenced by varying pairs of variables while the third variable is kept at its optimal condition, providing insight into the dynamics of the enzymatic hydrolysis process.

Table 6. Optimum hydrolysis conditions for the degree of hydrolysis in the extraction of fish protein hydrolysates

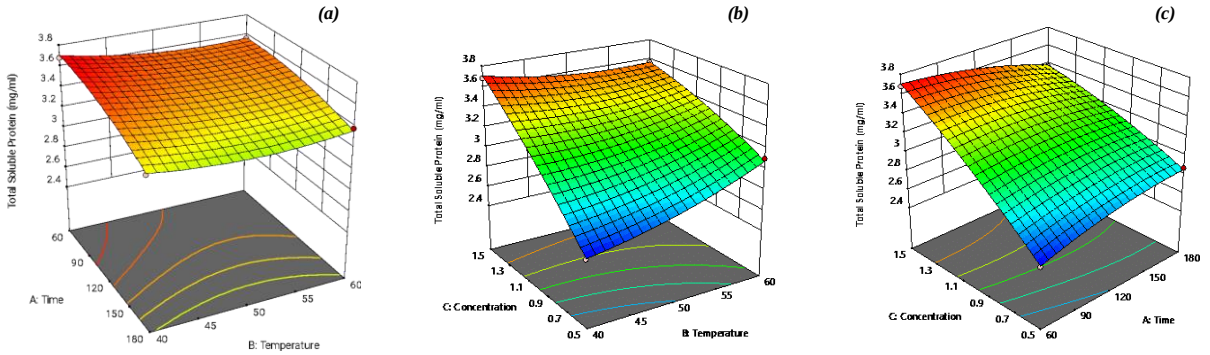

Figure 2. The relationships between variables under specific optimal conditions for total soluble proteins: (a) time and temperature in optimal enzyme concentration of 1.5%, (b) enzyme concentration and temperature in optimal reaction time of 60 min, and (c) enzyme concentration and time in optimal temperature of 40°C.

The visual representations depicted in Fig. 2 further elucidate the relationships between different variables under specific optimal conditions. In Fig. 2a, there was an observed decrease in TSP with increasing time and temperature beyond the optimal range. This suggests that prolonged hydrolysis time can diminish TSP yield due to potential denaturation or degradation of proteins. Elevated temperatures

may also affect gradually, but small changes do not affect total soluble proteins. This result corroborates with the study of Shahi et al. (2020) regarding direct correlation between the degree of hydrolysis and protein content wherein a higher degree of hydrolysis leads to greater protein degradation, resulting in production of smaller peptide fragments and amino acids. In Fig. 2b, the optimal concentration of 1.5% exhibited the highest TSP yield, regardless of temperature variations. These results highlight the critical role of enzyme concentration in driving TSP production, with higher concentrations leading to increased enzymatic activity and subsequent protein hydrolysis. This agrees with the study of Islam et al. (2021) wherein higher enzyme concentrations likely lead to increased higher protein content. In Fig. 7c, there was a decrease in TSP yield when the concentration deviates from the optimal value of 1.5%. Additionally, prolonged hydrolysis times beyond 60 minutes resulted in a decline of TSP, indicating that excessive enzymatic activity may lead to the breakdown of proteins, reducing TSP yield. These results are supported by studies of Izutsu (2018) and Guo et al (2020) in terms of prolonged time and soluble proteins decrease. Liu et al. (2024) and Zhang et al. (2017) regarding temperature increase and soluble protein decrease.

Conditions for the optimum response of the DH

According to the generated model, hydrolysis utilizing an enzyme concentration of 0.73%, a temperature of 44°C, and a reaction time of 60 min was predicted to achieve a DH value of 29.10% (Table 6). Interestingly, experimental validation under these conditions yielded a DH value of 30.67±0.96, closely mirroring the predicted outcome. Results subjected to t-test for related samples revealed no statistically significance ($p>0.05$) between the actual and predicted value. This close alignment between the predicted and actual DH values underscores the accuracy and effectiveness of the response surface model in determining the optimal hydrolysis conditions for achieving maximum degree of hydrolysis.

Fig. 3 shows the relationships among the hydrolysis parameters under specific optimal conditions. Fig. 3a illustrates the connection between hydrolysis time and temperature when the enzyme concentration is at its optimal level of 0.73%, while Fig. 3b shows the relationship between enzyme concentration and temperature when the hydrolysis time is fixed at its optimal duration of 60 min. Fig. 3c displays the relationship between enzyme concentration and hydrolysis time when the temperature is maintained at its optimal value of 44°C.

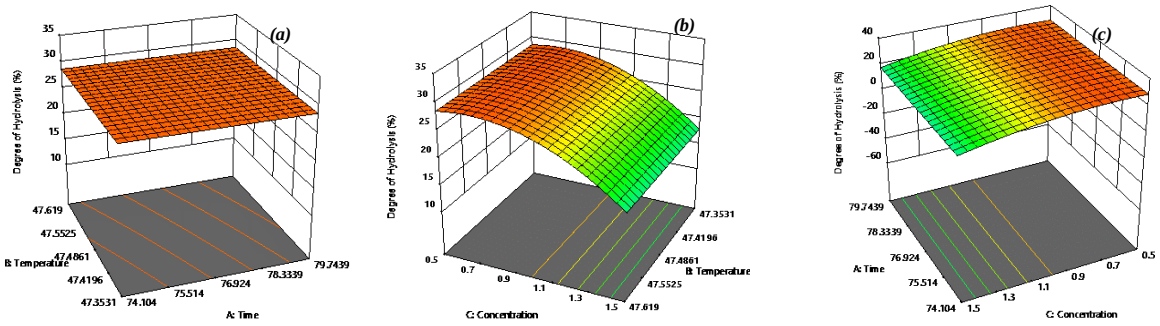

Figure 3. The relationship between variables under specific optimal conditions for degree of hydrolysis: (a) time and temperature in optimal concentration enzyme concentration of 0.73%, (b) enzyme concentration and temperature in optimal reaction time of 60 min, and (c) enzyme concentration and time in optimal temperature of 44°C.

In Fig. 3a, there were minimal variations in DH within the temperature range of 47.3°C to 47.6°C and the time range of 74.1 to 79.7 minutes. This suggests that subtle fluctuations in temperature and time within these ranges may not greatly influence the DH. Fig. 3b showed a decrease in DH with increasing enzyme concentration, while temperature remained relatively stable within the

range of 47.3°C to 47.6°C. This phenomenon suggests that higher enzyme concentrations may lead to reduced DH, highlighting the intricate balance between enzyme activity and hydrolysis efficiency. This, however, contradicts the principle of enzyme concentration in relationship with the rate of reaction (Robinson, 2015). Thus, enzyme concentration at 0.73 is the maximum if not close to the saturation with substrate, or what we call the maximal velocity.

In Fig. 3c, there was a distinct declining trend in DH as enzyme concentration increases, with an optimal concentration of 0.5%. Conversely, there are negligible changes in DH within the time range of 74.1 to 80 minutes, indicating that variations in hydrolysis time may have minimal impact on the degree of hydrolysis under optimal temperature conditions. The results for degree of hydrolysis is supported by several studies of Daniel and Danson (2010; 2013) and Peterson et al., (2004), due to the activation and deactivation and the rapid and initial rates decline at high temperatures. Moreover, based on the Michelis-Menten Model, it is possible that time and temperature does not really play a part in the hydrolysis of the tuna viscera using the enzymes from *Staphylococcus* sp., rather it is the enzyme concentration that plays a crucial role in the degree of hydrolysis.

Constraints and desirability

Shown on Table 7, are the constraints used in determining the solutions while Fig. 4 reveals the desirability of optimizing enzymes extracted from *Staphylococcus* sp., with independent variables: time, temperature, and concentration on response variables: total soluble proteins and degree of hydrolysis Results reveal that desirable time should be 82 minutes, desirable temperature is 42ºC, and desirable concentration is 0.79, as it would yield 28.06% DH and 3.02 TSP.

Table 7. Constraints used in determining the solutions

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: Time	is in range	60	90	1	1	3
B: Temperature	is in range	40	50	1	1	3
C: Concentration	is in range	0.5	1	1	1	3
Degree of Hydrolysis	is in range	25	30.67	1	1	3
Total Soluble Proteins	is in range	3	3.68	1	1	3

Shown in Fig. 10 is the Pareto plot effect and each independent variable and variable response to desired time, temperature, concentration, degree of hydrolysis, total soluble proteins, degree of hydrolysis and combined values, with range of 1.

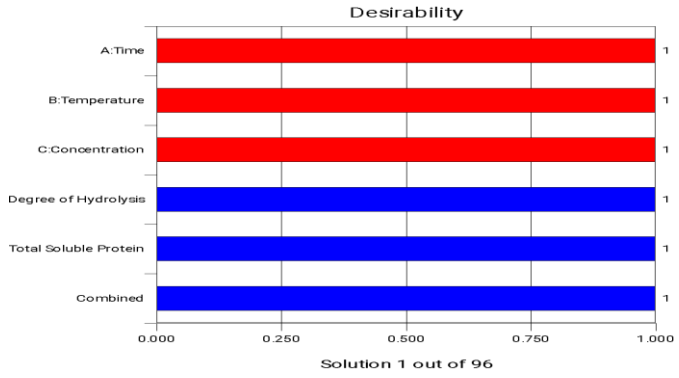

Figure 4. Pareto plots on each variable response desirability combined.

From the optimal conditions obtained on the influence of time, temperature, and concentration, the RSM analysis achieved an overall desirability of 1.0 for solution 1 out of 96 solutions, indicating the model's robustness in predicting optimal conditions. The input variables were examined within specific ranges: time ranged from 60 to 180 minutes, with the optimal desirability at 82.40 minutes; temperature varied from 40ºC to 60ºC, with 42.08ºC being the most desirable; and enzyme concentration spanned from 0.5% to 1.5%, with 0.79% yielding the highest desirability. The RSM Central Composite Design resulted in a maximum DH with values ranging from 10.72% to 30.67%, with 28.05% as the desirable outcome, and maximum TSP with values ranging from 2.58 mg/ml to 3.68 mg/ml, with 3.02 mg/ml being the desired result. The actual values are closely aligned with the model's predictions, showcasing the efficacy of RSM in optimizing enzymatic processes.

The advantages of variables include optimal time of 82.40 minutes which is relatively short, enabling efficient processing and reduced operational costs. This balance minimizes enzyme degradation and maximizes enzymatic activity. However, the disadvantages were extended times that can lead to enzyme inactivation or undesired side reactions, potentially affecting product quality. In terms of temperature, the optimal temperature of 42.08ºC is moderate, promoting high enzymatic activity while minimizing the risk of thermal denaturation. This temperature is energy-efficient and maintains enzyme stability, while the disadvantages include operating at higher temperatures which may increase reaction rates but can also lead to enzyme instability and increased operational costs for cooling. In terms of concentration, the advantage in enzyme concentration of 0.79% is economical and effective, ensuring sufficient substrate interaction without excessive enzyme use.

Conclusion

The study successfully demonstrated the hydrolysis of tuna wastes using proteases extracted from *Staphylococcus hominis* L1, specifically isolated from fermented *Sardinella sp.* The findings also show the robustness of the crude enzyme and their potential applications in the field of biotechnology. Using RSM, this study highlights the significance of enhancing the configurations of conditions for enzymes in order to optimize their performance and increase productivity. This will aid in the promotion of ecologically sustainable and streamlined fish handling practices. This work presents compelling evidence supporting the utilization of staphylococcal protease enzymes for the hydrolyzation of tuna viscera, a significant byproduct of the fish processing industry. The investigation successfully isolated and identified bacteria that generate protease, with *Staphylococcus hominis* L1 demonstrating the highest level of consistency and enzyme activity. Subsequently, the application of RSM provided a robust framework for optimizing the hydrolysis process. Key findings revealed that enzyme concentration had the most significant impact on both TSP and DH. The highest TSP was achieved at optimal conditions of 1.5% enzyme concentration, 40°C temperature, and 60 minutes of hydrolysis time. Interestingly, the DH was maximized at moderate enzyme concentrations and temperatures, indicating a non-linear relationship between these parameters and the hydrolysis outcomes. The study further demonstrates that spray-drying enzymes might serve as an effective method for preserving their activity. To further enhance the process, wider ranges of time and temperature, and higher enzyme concentrations around the identified optimal points could be tested to yield even more precise conditions. Moreover, developing more robust enzymes that retain activity at wider temperature and time could improve efficiency and confirm scalability and economic viability.

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Ethical approval

The author declares that this study complies with research and publication ethics.

Informed consent

Not available**.**

Conflicts of interest

There is no conflict of interests for publishing this study.

Data availability statement

Data are available upon a written request to the authors.

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Author contribution

Jhovian Lee Joseco **:** Writing original draft, Formal analysis, Investigation, Methodology

Sharon N. Nuñal :Conceptualization, Funding acquisition, Supervision, Writing original draft, Review, Editing

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