

# Isolation, identification, and enzyme optimization of proteolytic lactic acid bacteria from tuna viscera by-products

YOGA DWI JATMIKO<sup>✉</sup>, ARLISA M. R. MUARIF

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Jl. Veteran, Malang 65145, East Java, Indonesia.  
Tel.: +62-341-575841, ✉email: jatmiko\_yd@ub.ac.id

Manuscript received: 2 March 2025. Revision accepted: 27 May 2025.

**Abstract.** *Jatmiko YD, Muarif AMR. 2025. Isolation, identification, and enzyme optimization of proteolytic lactic acid bacteria from tuna viscera by-products. Biodiversitas 26: 2762-2772.* Fish processing by-products, particularly protein-rich viscera, are underutilized despite their potential to be converted into bioactive peptides using protease enzymes produced by Lactic Acid Bacteria (LAB). This study aimed to isolate proteolytic LAB from tuna viscera, identify the strain with the highest proteolytic activity, and optimize the growth conditions for enhanced protease enzyme production. Proteolytic LAB were isolated from tuna viscera and screened for enzyme activity using skim milk agar and tyrosine-based assays. The isolate with the highest proteolytic activity was identified through 16S rDNA sequencing. Protease production was then optimized using Response Surface Methodology (RSM) based on a Central Composite Design (CCD) with Design-Expert software, evaluating the effects of pH, tuna viscera concentration, and incubation time. The proteolytic potential of 12 LAB isolates from tuna viscera was obtained. The isolate of VT11 showed the highest enzyme activity of 0.55 U mL<sup>-1</sup> among the proteolytic LAB strains. After conducting 16S rDNA sequencing, VT11 was identified as *Pediococcus pentosaceus*, exhibiting 99.72% similarity. Furthermore, RSM analysis identified 24 hours of incubation at pH 5 with 15% tuna viscera as the optimal conditions for protease production by *P. pentosaceus* VT11.

**Keywords:** Lactic acid bacteria, proteolytic activity, response surface methodology, tuna viscera

## INTRODUCTION

Processing of fishery products invariably generates substantial by-products, such as heads, viscera, skin, and fins, constituting 50-70% of the fish's total weight (Thirukumaran et al. 2022). These nutrient-rich wastes can stimulate microbial metabolism and are suitable for fermentation. Fermentation using microorganisms has proven effective in valorizing fish waste, particularly for protease enzyme production, with whole fish parts and wastewater identified as promising substrates (Ramkumar et al. 2016). Tuna (*Thunnus* sp.) is a prominent fishery commodity in Indonesia, boasting high nutritional value, including complete essential amino acids, vitamins, minerals, and low-fat content (Hadinoto and Idrus 2018). However, tuna processing generates visceral waste, which is often disposed of directly into the environment, leading to pollution. For instance, a single tuna fish of 75-90 kg can generate approximately 5 kg of visceral waste. Notably, tuna viscera are characterized by their high protein content, which renders them a promising alternative material for producing bioactive compounds with a high economic value (Hadinoto and Idrus 2018).

Given its high protein content, fish waste has the potential for conversion into enzymes, bioactive peptides, and biopolymers (A'yunin et al. 2021; Saravanan et al. 2023). Specifically, bioactive peptides derived from fish waste hold significant potential in a variety of applications, including functional foods, pharmaceuticals, and cosmetics. These peptides have been shown to possess antioxidant,

anti-inflammatory, antimicrobial, and antidiabetic properties (Zamora-Sillero et al. 2018). The production of bioactive peptides involves the use of protease enzymes during the hydrolysis process. Protease production accounts for 70% of total enzyme production and has exhibited a Compound Annual Growth Rate (CAGR) of 6% from 2016 to 2021. Protease holds significant economic value due to its wide application across various industries, and microorganisms are preferred producers owing to their favorable biochemical traits, genetic accessibility, and high productivity (Dubey et al. 2024).

Among the spectrum of microorganisms capable of producing protease, Lactic Acid Bacteria (LAB) have garnered particular interest (Ter et al. 2024). These organisms are Generally Recognized as Safe (GRAS) and have been demonstrated to be beneficial to human health. Lactic Acid Bacteria (LAB) are naturally present in the microflora of fish guts, where they contribute to antimicrobial protection and digestion by breaking down complex nutrients (Migaw et al. 2014). These bacteria enhance the immune system, preventing diseases and promoting fish health. The presence of proteolytic LAB strains, including *Lactiplantibacillus plantarum*, *Pediococcus acidilactici*, and *Enterococcus faecalis*, has been identified in the viscera of fish (Jini et al. 2011).

Tuna processing generates a significant amount of visceral waste, which has been identified as a promising source for bacterial protease production due to its high protein content (Rebah and Miled 2013; Moranda et al. 2018). This makes tuna viscera a suitable alternative substrate for LAB-

mediated protease production. As the industrial demand for protease continues to rise, optimizing its production under the specific growth conditions required by LAB is crucial to improve efficiency and reduce production costs (Kieliszek et al. 2021; Ter et al. 2024). Response Surface Methodology (RSM) is a widely used approach for optimizing enzymatic production. This methodology enables the identification of critical parameters, the determination of ideal conditions, and the assessment of interactions between variables (Adetunji and Olaniran 2020). Studies have confirmed that RSM optimizes enzyme production, maximizing yields while minimizing costs (Lisdiyanti et al. 2021; Emon et al. 2024). This study aimed to isolate proteolytic LAB strains from tuna viscera, identify the strain with the highest proteolytic activity, and determine optimal growth conditions to enhance enzyme production using tuna viscera-supplemented media.

## MATERIALS AND METHODS

### Sample collection

Samples of fish waste were obtained from the waste of the smoked fish industry process at Plaza Ikan Tuban, East Java Province, Indonesia. The samples were collected in the form of solid waste, specifically gills and viscera, which encompass intestines, spleen, liver, and pancreas. The fish used in this study was yellowfin tuna. The samples were collected and stored in zip lock plastic bags within a cooling box (4-6°C) before being transferred to the Microbiology Laboratory at the Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia.

### Procedure

#### *Sample preparation*

The preparation of tuna viscera as a substrate for protease enzyme production followed the methods established by Ellouz et al. (2001) and Fahmy and El-Deeb (2021). The tuna fish waste, in the form of solid visceral waste, was subjected to boiling. The tuna viscera were then filtered to remove water, followed by drying in an oven at 80°C for 24-48 h. The dried tuna viscera were ground into a powder, filtered once more, and finally sterilized using an autoclave at 121°C for 15 minutes. The tuna viscera powder was analyzed for protein content based on the Indonesian National Standard (SNI) 01-2354.4:2006 and subsequently used as a substrate for protease production.

#### *Isolation of lactic acid bacteria*

Lactic Acid Bacteria (LAB) isolation was conducted to obtain isolates that potentially exhibited proteolytic activity. Samples of yellowfin tuna viscera were collected in amounts of up to 25 g, then dissolved in 225 mL of a sterile saline solution (NaCl 0.85%), and homogenized using vortex mixer. The resultant suspension was a  $10^{-1}$  dilution. Subsequently, serial dilution was executed by extracting 1 mL of the suspension from the  $10^{-1}$  dilution and introducing it into 9 mL of sterile saline solution. The mixture was then vortexed until homogeneity was achieved. This process

was repeated for all dilutions until the  $10^{-6}$  dilution. Next, 0.1 mL of the suspension from each dilution was inoculated into a Petri dish containing de Man, Rogosa and Sharpe (MRS) agar plates supplemented with 1%  $\text{CaCO}_3$ . Each dilution was plated with in triplicate on separate Petri dishes. The samples were then inoculated using the spread plate technique, employing a sterile glass spreader until the samples were fully absorbed into the media. Finally, the samples were incubated at 37°C for 24 hours. Bacterial colonies that exhibited a clear zone on MRS agar were selected for further experiments (Linh et al. 2018; Govindaraj et al. 2021).

#### *Phenotypic characterization of LAB*

Lactic acid bacteria colonies exhibiting clear zones were subjected to morphological (phenotype) characterization, encompassing both macroscopic and microscopic (Gram staining) characterizations, in addition to biochemical tests (catalase test). Macroscopic characterization was conducted by observing colony morphological characters, including the following: (i) the overall shape of the colony (round, irregular, filamentous); (ii) periphery/configuration (thorough, grooved, lobate, erose); (iii) surface (flat, ascending, convex, umbonate, pulvinate, papillate convex, and wrinkled convex); (iv) texture (rough, contoured, wrinkled); (v) consistency (thin membrane-like, limpid, dry); (vi) optical characteristics (opalescent, faint, shiny); and (vii) pigmentation (red, yellow, cream, white, colorless) (Prastujati et al. 2022).

Lactic acid bacteria were purified by streaking a loopful of colonies onto MRS agar supplemented with 1%  $\text{CaCO}_3$  using the four-quadrant streak method and incubated at 37°C for 24 hours to obtain single colonies. Cell morphology was examined microscopically via Gram staining, and Gram-positive isolates were selected for further analysis, including the catalase test. The catalase test was performed by dripping 3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) onto each isolate. Gram-positive isolates that tested negative in the catalase test (i.e., no gas bubble formation) were selected for proteolytic activity screening. Phenotypic data derived from morphological and biochemical characteristics of each isolate were grouped into a dendrogram using PAST software version 4.12b, employing the UPGMA clustering algorithm (Prastujati et al. 2022).

#### *Screening of LAB-producing protease enzymes*

The proteolytic activity screening was conducted following the methods established by Amin (2018) and Matti et al. (2019), with minor modifications. In this method, two inoculation loops of LAB isolates obtained from tuna viscera were taken from the stock culture and then inoculated into 5 mL of MRS broth. The inoculated broth was then incubated at 37°C for 36 hours. The absorbances of the isolates were measured at a wavelength of 600 nm, and the OD values were then standardized to 0.6. Subsequently, Skim Milk Agar (SMA) media was divided into four quadrants, and wells were made using a 6 mm diameter cork-borer. A total of 25  $\mu\text{L}$  of LAB inoculum was added to each well, after which the plates were incubated at 37°C for 24 hours. The presence of a clear zone around the colonies was taken as an indication of proteolytic activity.

Subsequently, the proteolytic index was determined by using Equation 1, which was previously established by Ramadhan et al. (2021).

$$\text{Proteolytic index} = \frac{\text{Clear zone diameter} - \text{Colony diameter}}{\text{Colony diameter}} \dots [1]$$

The tyrosine standard curve was prepared according to the protocol outlined by Sigma-Aldrich (Cupp-Enyard 2008). The standard curve was prepared by diluting the tyrosine stock solution to a concentration of 1.1 mM. Then, a series of tyrosine solutions with varying concentrations were prepared by the addition of 0.05 mL of tyrosine to 1.95 mL of distilled water, 0.1 mL of tyrosine to 1.9 mL of distilled water, 0.2 mL of tyrosine to 1.8 mL of distilled water, 0.4 mL of tyrosine to 1.6 mL of distilled water, 0.5 mL of tyrosine to 1.5 mL of distilled water, and a blank solution of 2 mL of distilled water. Consequently, the concentration of tyrosine solution was determined to be 0.0275 mM, 0.055 mM, 0.11 mM, 0.22 mM, 0.44 mM, and 0.88 mM, respectively, as determined from these dilutions. The solution was then subjected to incubation at a temperature of 37°C for 30 minutes. Subsequently, each solution was augmented with 5 mL of Na<sub>2</sub>CO<sub>3</sub> and 1 mL of Folin-Ciocalteu's phenol reagent, followed by incubation at 37°C for an additional 30 minutes. Next, the spectrophotometer was utilized to measure the sample's absorbance at a wavelength of 660 nm. The resulting values were then graphed, with the absorbances assigned to the y-axis and the solution concentrations assigned to the x-axis. The trendline was subsequently displayed to obtain the regression equation and R<sup>2</sup> value.

Furthermore, the screening of proteolytic activity was quantitatively conducted, referring to the method of Luong et al. (2023), with modifications. Isolates of similar density were inoculated onto MRS broth augmented with 2% skim milk, with three replicates performed. The inoculum was then subjected to incubation at 37°C for a duration of 24 h. Subsequently, samples were collected in volumes up to 1 mL, followed by centrifugation at 10,000 rpm for 10 minutes at a temperature of 4°C. The supernatant was extracted as a volume of 0.5 mL. Then, 2.5 mL of casein (0.65% in 50 mM potassium phosphate buffer, pH 7.5) was added, and the mixture was incubated at 37°C for 10 minutes. Next, 2.5 mL of 110 mM trichloroacetic acid (TCA) reagent was added and the mixture was incubated at 37°C for 30 minutes. The sample was then subjected to centrifugation at 10,000 rpm for 10 minutes at 4°C. Subsequently, 1 mL of supernatant was mixed with 2.5 mL of a 440 mM Na<sub>2</sub>CO<sub>3</sub> solution and 0.5 mL of a Folin-Ciocalteu's phenol reagent, which had been threefold diluted, and then incubated at 37°C for 30 minutes. Next, the absorbances of the samples were measured using a spectrophotometer at a wavelength of 660 nm. The resulting value was then substituted into the regression equation of the tyrosine standard curve, thus yielding the tyrosine concentration. Subsequently, the enzyme activity was measured using Equation 2 by calculating the total volume of the test, the incubation time during the test, the volume of enzyme obtained, and the volume used in colorimetric

detection to obtain the value of proteolytic enzyme activity in units per milliliter (U mL<sup>-1</sup>) (Luong et al. 2023).

$$\text{AE} = \frac{[\text{Tyrosine}]}{C} \times \frac{V}{p \cdot q} \dots [2]$$

Where: AE: Enzyme activity (U mL<sup>-1</sup>); [Tyrosine]: Concentration of tyrosine; C: Volume of solution for spectrophotometry (mL); V: Total volume (mL); p: Volume of protease crude extract (mL); q: Reaction time (minutes).

#### Identification of lactic acid bacteria

Lactic acid bacteria isolates with the highest proteolytic activity were identified based on 16S rDNA sequences. The DNA extraction process was conducted using the ZR Fungal/Bacterial Miniprep Kit (Zymo Research), which was adjusted according to the protocol (Ojo-Okunola et al. 2020). The extracted DNA was assessed for purity using a NanoDrop™ spectrophotometer, with absorbance measured at 260 nm and 280 nm. An A<sub>260/280</sub> ratio between 1.8 and 2.0 indicated acceptable DNA purity for downstream molecular analysis. Subsequently, 16S rDNA amplification was performed using universal primers (27F: 5'-AGAGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-CCGTCAATTCCTTTGAGTTT-3'). Amplification was performed in a volume of 50 µL. The composition of the PCR mix was as follows: My Taq® HS Red Mix 2x, forward and reverse primers (10 pmol), DNA template (88.15 ng µL<sup>-1</sup>), and ddH<sub>2</sub>O. The PCR process was carried out on a thermal cycler, with the following amplification conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 35 seconds, annealing at 49°C for 30 seconds, extension at 72°C for 90 seconds, and final extension at 72°C for 5 minutes (Sulistiyantini et al. 2023).

The amplicons (6 µL) were electrophoresed on a 1.5% agarose gel at 100 V for 30 minutes and were visualized using a UV transilluminator. Subsequently, the amplicons were subjected to sequencing at First Base Laboratories in Malaysia. The obtained sequencing results were then analyzed using Sequence Scanner and BioEdit software. Furthermore, the nucleotide bases of the isolates were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). The construction of the phylogenetic tree was facilitated by the utilization of MEGA 11.0 software, using the Neighbor-Joining algorithm with the Tamura-Nei model, and evaluated by bootstrap analysis with 1000 bootstrap replications, as previously outlined by Suberu et al. (2019). *L. plantarum* (GenBank accession no. NR\_042394) was used as the outgroup.

#### Protease production and proteolytic activity test

The media for protease production was formulated using MRS broth, which was augmented with three different concentrations of tuna viscera (5%, 10%, and 15%). The production of protease was conducted on a single isolate that exhibited the highest degree of proteolytic activity. The method of making starter culture was modified from the approach described by Fitriana and Asri (2022) and

began with the transfer of two loops of the LAB stock culture were transferred into 50 mL of MRS broth and incubated at 37°C in a shaking incubator at 120 rpm for 24 hours. Following the incubation, the cell density was determined using a hemocytometer, yielding a concentration of  $1.53 \times 10^8$  cells/mL. This culture was then used as a starter culture, with 2% (v/v) inoculated into each medium. The cultures were subsequently incubated in a shaker incubator at 37°C and 120 rpm to achieve the optimal protease enzyme production. The proteolytic activity was measured at 24, 36, 48, and 72 hours of incubation to determine the time point at which the highest activity was achieved (Luong et al. 2023). The proteolytic activity of LAB was tested following the Sigma-Aldrich protocol (Cupp-Enyard 2008). The cultures were centrifuged at 10,000 rpm for 10 min at a temperature of 4°C. The cell-free supernatant was then used to measure the protease activity. The proteolytic activity assay was performed using the same method as the proteolytic activity measurement in the quantitative screening.

#### *Medium optimization design with response surface methodology*

Response Surface Methodology (RSM) is a statistical and mathematical analysis that optimizes the response of several factors or independent variables was determined. This methodology can provide information about the relationship between variables and their optimal conditions, thereby enabling the expeditious attainment of sensitive results (Ruby-Figueroa 2016). In the context of optimizing the proteolytic activity of LAB, an experimental design incorporating RSM with two factors was employed: X1 (pH 5, 6, and 7) and X2 (concentration of tuna viscera 5%, 10%, and 15% (w v<sup>-1</sup>) added to the medium) to yield the response in the form of the highest proteolytic activity (U mL<sup>-1</sup>). The experimental design for optimization followed a Central Composite Design (CCD) and included 30 experimental setups. This experimental design consists of minimum and maximum values, or factorial values, which were coded as -1 and +1, respectively, along with center points coded as  $n_0$ , and axial points coded as  $-\alpha$  and  $+\alpha$  (Breig and Luti 2021; Djimtoingar et al. 2022). The independent variables used in this study were pH and the addition of tuna viscera substrate concentration, resulting in axial points at 4, with a center point of 6. The experimental design was obtained for two factors using Design Expert software version 13.0.5.0 (Stat-Ease Corp., Minneapolis, MN, USA.). The RSM analysis in this experimental model was conducted using Equation 3, which was developed by Breig and Luti (2021).

$$Y = \beta_0 + \sum_{i=1}^k \beta_{0i} x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k \beta_{ij} x_i x_j [3]$$

Where: Y: Prediction of the obtained results;  $\beta_0$ : Constant;  $\sum_{i=1}^k \beta_{0i} x_i$ : Linear coefficient;  $\sum_{i=1}^k \beta_{ii} x_i^2$ : Coefficient R<sup>2</sup>;  $\sum_{i=1}^{k-1} \sum_{j=2}^k \beta_{ij} x_i x_j$ : Coefficient of relationship among variables

#### **Data analysis**

Proteolytic index and proteolytic activity data were analyzed using one-way ANOVA (IBM SPSS v.27) to identify significant differences ( $p < 0.05$ ), followed by Tukey's post hoc test. Enzyme activity values from selected LAB isolates were then input into Design Expert v13.0.5.0 for Response Surface Methodology (RSM) analysis. This included model significance testing, generation of contour and 3D surface plots, and numerical optimization to determine the optimal conditions for maximum enzyme activity.

## **RESULTS AND DISCUSSION**

#### **Characteristics of LAB isolates derived from tuna viscera**

Lactic Acid Bacteria (LAB) were successfully isolated from tuna viscera, resulting in 12 distinct isolates, each exhibiting unique morphological characteristics. The LAB isolates were characterized by the presence of a clear zone surrounding their colonies. As noted by Rahayu (2021), this clear zone indicates the ability of LAB to metabolize glucose into primary metabolites, particularly lactic acid. The lactic acid produced reacts with CaCO<sub>3</sub> in the MRS agar, converting it into Ca-lactate, which results in the surrounding medium becoming transparent. The results of Gram staining revealed that all isolates were identified as Gram-positive bacteria. Concurrently, the catalase test results indicated that all isolates were catalase negative. This result aligns with the observations reported by Ter et al. (2024), which documented that LAB are phenotypically classified as Gram-positive bacteria, manifesting as cocci or bacilli, and are catalase negative. According to Ismail et al. (2018), LAB do not produce the enzyme catalase, which is responsible for breaking down hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into water and oxygen. Each isolate obtained from the tuna viscera was assigned a code, namely VT1, VT2, VT3, VT4, VT5, VT6, VT7, VT8, VT9, VT10, VT11, and VT12. Most of the LAB isolates exhibited a round shape, well-rounded edges, convex elevation, contoured texture, and shiny optical characteristics. The analysis of bacterial phenotypic characters presented in a dendrogram, demonstrating that each isolate exhibited morphological similarities, with higher similarity values corresponding to more pronounced morphological character similarity among the bacterial colonies (Figure 1).

#### **Screening of proteolytic LAB**

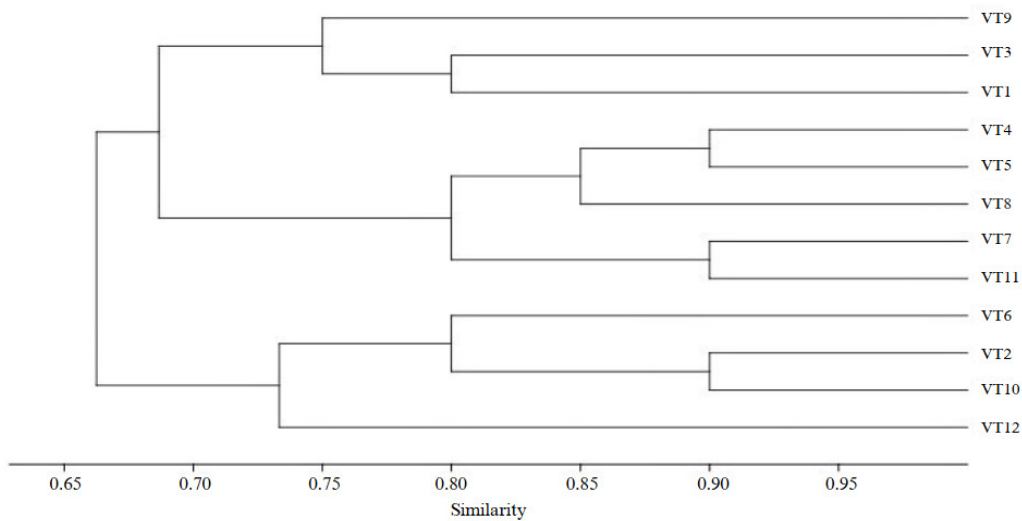
The screening of LAB proteolytic activity was conducted using two methods: qualitative and quantitative. Isolates showing the highest proteolytic index and enzyme activity in these screenings were subsequently selected for optimization. The qualitative screening results, obtained through the agar well diffusion method, with subsequent observation after a 24-hour incubation, showed that all isolates exhibited proteolytic activity. This activity was indicated by the clear zone around the colonies in the SMA media. The qualitative screening of LAB originating from fish viscera yielded proteolytic index values ranging from 0.09 to 0.27. The results of the qualitative screening revealed that the LAB isolate designated VT11 exhibited

the highest proteolytic index. This distinction was marked by the clear zone (Figure 2) surrounding the bacterial colony and the highest proteolytic index value among the other isolates, which was 0.27. This finding suggests that isolate VT11 exhibits elevated proteolytic activity (Figure 3). According to Kabense et al. (2019), the proteolytic index is a measure of a bacterium's capacity to produce protease enzymes.

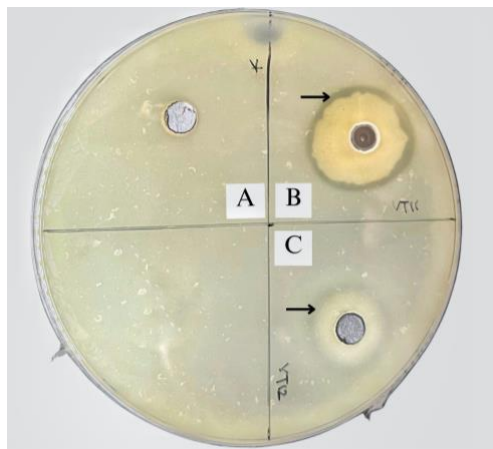
The presence of a clear zone surrounding the colony signifies the breakdown of casein present in SMA medium into simple amino acids and peptides by the protease enzymes produced by LAB. The variation in the size of the clear zones observed among the various LAB isolates shows the differential capacity of these isolates to produce protease enzymes (Birahy et al. 2025). The presence of a clear zone on the media serves as an indication of the extracellular protease enzymes produced by bacteria. These

enzymes play a pivotal role in the production of free amino acids and peptides, which are essential for bacterial growth and the process of acidification during fermentation (Phupaboon et al. 2023).

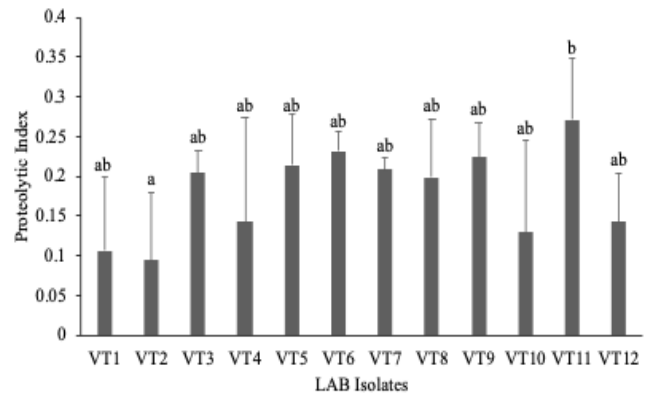
The results of the quantitative screening of LAB isolates revealed that the isolates exhibited enzyme activity values ranging from 0.38 to 0.55 U mL<sup>-1</sup> (Figure 4). The isolate with the highest enzyme activity among the other isolates was VT11 (p<0.05), with an average value of 0.55 U mL<sup>-1</sup>. It is noteworthy that this quantitative screening result exhibited concurrence with the qualitative screening results. Conversely, the isolate with the lowest enzyme activity was VT3, with an enzyme activity of 0.38 U mL<sup>-1</sup> (Figure 4). Therefore, the isolate VT11 was selected for further analysis to identify its species and to optimize its protease enzyme production.



**Figure 1.** Phenotypic dendrogram of LAB isolates from tuna viscera based on morphological and biochemical characteristics, generated using PAST software with the UPGMA clustering method



**Figure 2.** The clear zone of LAB proteolytic activity on Skim Milk Agar. A. Control (MRS broth), B Isolate VT11, C. Isolate VT12. The black arrow indicates the clear zone



**Figure 3.** Proteolytic index of LAB isolates. Different letter notations serve to indicate significant differences between isolates with a significance level of p<0.05

The protease enzyme activity of each LAB isolate exhibited different values. This variation can be attributed to the multifaceted nature of protease enzyme production, which is influenced by various factors, including bacterial species and environmental conditions such as pH, temperature, and aeration (Rosnawita et al. 2015; Ter et al. 2024). For instance, pH can exert an effect on protease enzyme activity, as each protease enzyme possesses an optimal pH range. The bacterial species also contribute to this variation; as different species have varying capacities to produce protease enzymes. Temperature is another factor that can affect protease activity, as it can affect the synthesis and secretion of extracellular protease enzymes from bacteria (Vinoth et al. 2014).

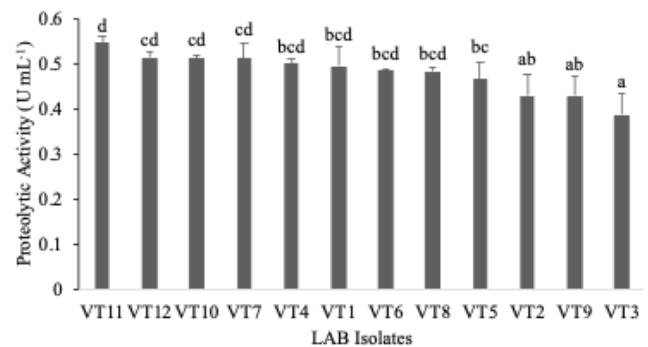
**Identification of LAB with the highest proteolytic activity**

Based on the highest proteolytic activity, isolate VT11 was selected for identification. The subsequent DNA extraction process yielded a DNA concentration of 88.15 ng  $\mu\text{L}^{-1}$  and a purity value of 1.87. BLAST analysis of the 16S rDNA sequence showed that isolate VT11 shared 99.72% similarity with *Pediococcus pentosaceus* Mees, 1934 CMGB-L16, confirming its identification as *P. pentosaceus* (Figure 5). *P. pentosaceus* is a Gram-positive, immotile, facultative anaerobic lactic acid bacterium that typically appears as pairs or tetrads. It grows optimally at 25-40°C and pH 6.0-6.5 (Raccach et al. 2014), and has been isolated from various natural fermentation products, including seafood such as fish, lobster, and octopus (Xing et al. 2013; Gómez-Sala et al. 2015; Lim and Lee 2016). Moreover, *P. pentosaceus* is well-documented for its probiotic, anti-inflammatory, anticancer, and antioxidant properties (Jiang et al. 2021; Rosales Cavaglieri et al. 2025). Consistent with this, Birahy et al. (2025) demonstrated that a *P. pentosaceus* IL13 showed strong proteolytic and probiotic properties. Tuna waste hydrolysate produced by

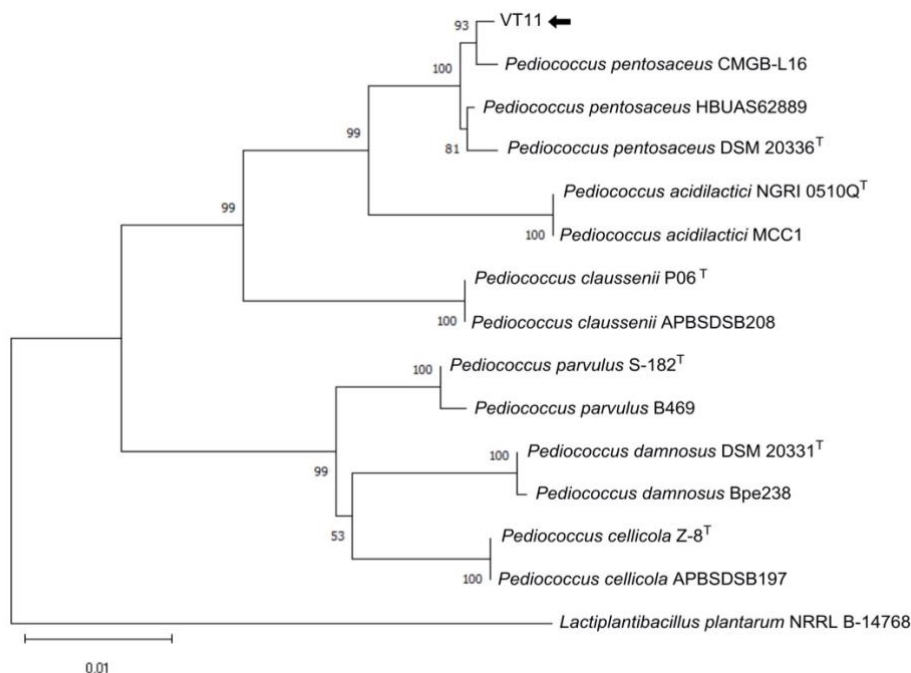
*P. pentosaceus* IL13 showed moderate antioxidant activity and was rich in glutamic acid.

**Optimum composition of protease enzyme production media with Response Surface Methodology (RSM)**

Isolate VT11, identified as *P. pentosaceus*, exhibited the highest proteolytic activity value and was utilized in the optimization process. The optimization process entailed the addition of a substrate, namely sterile tuna visceral flour with a protein content of 45.09%. In a separate study, Nugroho et al. (2020) found that the protein content of the tuna viscera used was 53.52%. The protein content of tuna viscera was found to exceed that of other tuna components, including the head (18.75%), dorsal skin (22.75%), tail fin (23.80%) (Dilini Jayaweera 2023), and skin (37.45%) (Nurjanah et al. 2021). According to Garofalo et al. (2023), the differences in protein content found in tuna viscera could be influenced by several factors, including the tuna species, diet, and age of tuna.



**Figure 4.** Proteolytic activity of LAB isolates. Different letter notations serve to indicate significant differences between isolates with a significance level of  $p < 0.05$



**Figure 5.** Phylogenetic tree of isolate VT11 and reference isolates based on 16S rDNA similarity, as determined by Neighbor-Joining algorithm and Tamura-Nei model with 1000 bootstrap replicates

Optimization procedures were also conducted to adjust the initial pH of the growth medium prior to the addition of the inoculum. This was done to ascertain the optimal point of protease enzyme production with the addition of substrates, thereby ensuring the maximization of protease enzyme yield. The experimental design for the isolate *P. pentosaceus* VT11, as determined by the Design Expert software for response surface methodology with the central composite design model (Table 1), encompassed 30 experimental replicates.

The following results correspond to the 24<sup>th</sup> hour incubation time point, using 30 experimental runs. ANOVA results from Design Expert indicated an R<sup>2</sup> value of 0.86, suggesting a strong model fit and a high degree of correlation between the predicted and observed values. This implies that both pH and tuna viscera concentration had a significant influence on the proteolytic activity of *P. pentosaceus* VT11 at this time point. Further analysis using contour and 3D surface plots confirmed that the optimal conditions were achieved at pH 5 with 15% (w/v) tuna viscera (Figure 6.A). These optimal conditions were visually represented by a red zone on the plots, indicating maximum enzyme activity (Mugendiran et al. 2014). The model at this time point was statistically significant (p < 0.0001) and followed a quadratic pattern, supporting its use in predicting protease production behavior. Based on this analysis, Equation 4 was established to describe the optimization model for the 24<sup>th</sup> hour incubation.

$$Y = 0.7883 - 0.0077A + 0.1160B.....[4]$$

Where: Y: Enzyme activity (U mL<sup>-1</sup>); A: pH; B: Concentration of tuna viscera (%)

The optimization process for *P. pentosaceus* VT11 at the 36<sup>th</sup> hour sampling point was conducted using 30 experimental runs. The analysis identified the quadratic model as the best fit, with a highly significant p-value (p < 0.0001; Figure 6.B). ANOVA revealed an R<sup>2</sup> value of 0.81, suggesting a strong model fit. These results indicate that pH and tuna viscera concentration significantly affected enzyme production at this time point. The resulting Equation 5 was used to predict protease activity, showing a proportional increase in enzyme response with increasing pH and substrate concentration, further supported by a positive regression coefficient (Nurmiah et al. 2013).

$$Y = 0.9880 + 0.0224A + 0.1271B.....[5]$$

Where: Y: Enzyme activity (U mL<sup>-1</sup>); A: pH; B: Concentration of tuna viscera (%)

At the 48<sup>th</sup> hour sampling interval, optimization was also applied using 30 experimental trials. The linear model provided the best statistical fit, with a significant p-value (p<0.0001; Figure 6.C). However, the R<sup>2</sup> value was 0.56, indicating a relatively weak correlation and suggesting that pH and tuna viscera concentration had minimal impact on

enzyme yield at this time point. Equation 6 was derived from this model and used to estimate response behavior under the tested conditions.

$$Y = 0.8273 + 0.0136A + 0.1127B.....[6]$$

Where: Y: Enzyme activity (U mL<sup>-1</sup>); A: pH; B: Concentration of tuna viscera (%)

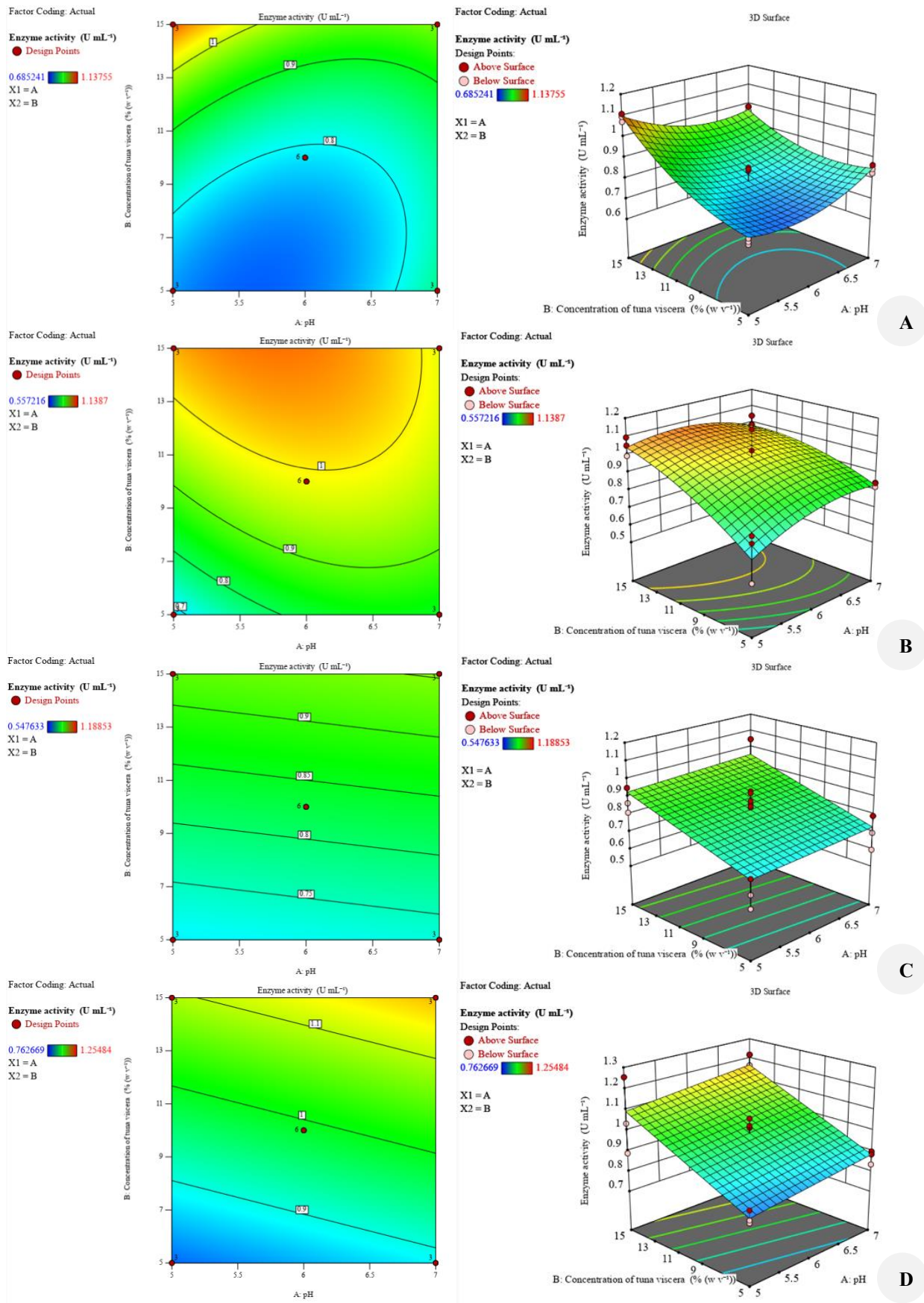
For the 72<sup>th</sup> hour sampling point, a similar optimization scheme involving 30 experimental sets was implemented. The most suitable model selected was linear, showing strong statistical relevance (p<0.0001; Figure 6.D). ANOVA results yielded an R<sup>2</sup> value of 0.80, reflecting a good fit between predicted and actual values. This suggests that both pH and tuna viscera concentration significantly influenced protease production at 72<sup>nd</sup> hour. Based on these findings, Equation 7 was formulated to represent the predictive model for this time point.

$$Y = 0.9886 + 0.0355A + 0.1401B.....[7]$$

Where: Y: Enzyme activity (U mL<sup>-1</sup>); A: pH; B: Concentration of tuna viscera (%)

**Table 1.** Proteolytic activity of isolate VT11, with adjustments to pH and the addition of tuna viscera at varying concentrations based on the design of RSM

Run	pH	Tuna viscera (%)	Proteolytic activity (U mL <sup>-1</sup> )			
			24 h	36 h	48 h	72 h
1	6	3	0.672	0.586	0.650	0.633
2	5	15	0.919	0.871	0.673	1.040
3	6	17	0.824	0.879	0.985	0.984
4	4.6	10	0.810	0.605	0.653	0.841
5	7	15	0.817	0.818	0.699	1.010
6	5	15	0.888	0.908	0.789	0.858
7	6	17	0.943	0.879	0.783	1.010
8	7	15	0.800	0.722	0.731	1.010
9	6	17	0.899	0.944	0.800	0.929
10	5	15	0.908	0.823	0.717	0.737
11	5	5	0.598	0.635	0.586	0.656
12	6	10	0.708	0.763	0.714	0.878
13	7	5	0.718	0.699	0.657	0.692
14	5	5	0.621	0.462	0.455	0.667
15	7.4	10	0.766	0.767	0.568	0.826
16	6	10	0.584	0.793	0.773	0.730
17	5	5	0.608	0.666	0.515	0.704
18	4.6	10	0.848	0.717	0.759	0.782
19	6	3	0.665	0.579	0.614	0.669
20	7	5	0.685	0.697	0.576	0.745
21	6	3	0.569	0.583	0.573	0.665
22	6	10	0.702	0.944	0.702	0.878
23	7.4	10	0.708	0.749	0.653	0.826
24	7.4	10	0.766	0.749	0.794	0.858
25	6	10	0.696	0.849	0.763	0.841
26	7	5	0.702	0.680	0.495	0.734
27	6	10	0.589	0.797	0.696	0.849
28	6	10	0.642	0.771	0.727	0.841
29	7	15	0.817	0.863	0.869	0.963
30	4.6	10	0.688	0.711	0.620	0.737



**Figure 6.** Contour plots and 3D response surface graphs illustrating the effects of pH and tuna viscera concentration on protease activity of *P. pentosaceus* VT11 at different incubation time points: A) 24<sup>th</sup> hour; B) 36<sup>th</sup> hour; C) 48<sup>th</sup> hour; D) 72<sup>nd</sup> hour. Optimal conditions are indicated on each surface

The application of RSM yielded optimal conditions for maximizing protease enzyme activity. Specifically, the highest protease enzyme activity was observed at an initial media pH of 5 and a tuna viscera concentration of 15% for an incubation duration of 24 hours (Figure 6). The pH of the culture medium is a critical factor influencing enzymatic activity and the transport of nutrients and metabolites across the bacterial cell membrane, which in turn affects cell growth and product formation (Sodagar et al. 2024). In this study, the initial pH was found to play an essential role in optimizing protease production by supporting efficient nutrient uptake and metabolic activity. Based on the pH range within which they are most active, protease enzymes can be classified into three types: acidic, neutral, and alkaline proteases (Lim et al. 2019). A study by Sun et al. (2019) demonstrated that the maximum protease activity of *P. pentosaceus* was attained at an initial media pH of 4, with an incubation time duration of 36 hours. Xu et al. (2015) reported that the protease produced by *P. pentosaceus* 220 exhibited high activity within the acidic pH range of 3.0-4.5, with maximum activity at pH 4.0. Protease activity decreased sharply between pH 4.0 and 6.5, and was nearly undetectable at pH 6.5. In line with this, the current study found that the highest proteolytic activity of the selected *P. pentosaceus* VT11 was achieved at an initial medium pH of 5.0. Although slightly higher than the previously reported optimum, this pH still falls within the acidic range and may reflect strain-specific characteristics or substrate influences such as tuna viscera. These findings indicate that the enzyme produced by *P. pentosaceus* functions optimally under acidic conditions, classifying it as an acidic protease.

Each LAB species exhibits a distinct proteolytic activity under varying pH conditions. This finding suggested that the proteolytic activity of each LAB species is profoundly influenced by the prevailing environmental conditions and the composition of the media used. The conditions optimized through the RSM approach, specifically pH and tuna viscera concentration were found to effectively support the growth of *P. pentosaceus* VT11 and to maximize its proteolytic enzyme production. This result aligns with the findings of a study by Lim et al. (2019), which reported that *P. pentosaceus* exhibited the highest proteolytic activity at an initial pH of 5 in the media. The optimal pH conditions are known to induce conformation in the active site of the protease enzyme that matches the substrate. This configuration maximizes the proton donor and acceptor clusters, which are important in the catalytic ionization of the enzyme. Consequently, the protease enzyme activity obtained was found to be high (Sulthoniyah et al. 2015). Escobar-Sánchez et al. (2022) observed that *P. pentosaceus* exhibited the highest proteolytic activity at pH 5.5, with a decline in activity at higher pH values. This finding suggests that *P. pentosaceus* can produce the most potent proteolytic enzymes under acidic conditions.

The optimization results with RSM at 48<sup>th</sup> hour and 72<sup>nd</sup> hour of incubation time points did not attain the optimum point for protease enzyme production. However, RSM yielded solutions for each condition of each incubation time point. The highest protease enzyme activity at the 48<sup>th</sup>

hour incubation time point was observed in the initial condition of media with a pH of 7 and the addition of a 15% tuna viscera concentration, yielding an enzyme activity of 0.95 U mL<sup>-1</sup>. Furthermore, at the incubation time point of 72<sup>nd</sup> hour, RSM identified conditions that included an initial media pH of 7 and the addition of 15% tuna viscera concentration. This result was likely due to a decline in the initial media pH (set at 7.0) to a more acidic range by the 48<sup>th</sup> and 72<sup>nd</sup> hour of incubation, caused by lactic acid production from *P. pentosaceus* VT11. As reported by Yin et al. (2003), the pH of media initially adjusted between 5.0 and 7.0 can drop to approximately 3.9 after 48 hours of fermentation, which supports this explanation.

Among the four incubation time points tested (24<sup>th</sup>, 36<sup>th</sup>, 48<sup>th</sup>, and 72<sup>nd</sup> hour), the highest protease activity was achieved at 24 hours at a pH of 5 and 15% tuna viscera concentration. This finding aligns with the study by Toe et al. (2019), which reported that the proteolytic activity of *P. pentosaceus* reached its peak at the incubation for 24 hours. Our study demonstrates that tuna viscera are a potential source of proteolytic LAB. All isolated LAB demonstrated protease enzyme production potential, with *P. pentosaceus* VT11 exhibiting the highest activity at 0.55 U mL<sup>-1</sup>. The application of RSM indicated that the highest proteolytic activity was achieved at a pH of 5 with 15% (w v<sup>-1</sup>) tuna viscera and 24 hours of incubation with the model prediction agreed with the experimental data (R<sup>2</sup> = 0.86). These findings highlight the potential of using *P. pentosaceus* VT11 for sustainable protease enzyme production from tuna viscera by-products, and suggest its applicability in the fermentation of fish-based food products.

## ACKNOWLEDGEMENTS

We thank Tri Ardyati, Ph.D., and Dr. Sri Widyarti for their constructive feedback on the research, and Prof. Salam Ibrahim for his helpful suggestion on the manuscript.

## REFERENCES

- A'yunin Q, Sulistyono DA, Syawli A, Rahmawati A, Intyas CA, Alviyanti D, Wiratno EN, Setyawan FO, Supriatin FE, Djamaludin H, Tambunan JE, Rihmi MK, Wardani NH, Rijal SS, Anitasari S, Ma'rifat TN, Sari WK. 2021. Sustainable Fisheries. UB Press, Malang. [Indonesian]
- Amin M. 2018. Marine protease-producing bacterium and its potential use as an abalone probiont. *Aquac Rep* 12: 30-35. DOI: 10.1016/j.aqrep.2018.09.004.
- Adetunji AI, Olaniran AO. 2020. Statistical modeling and optimization of protease production by an autochthonous *Bacillus aryabhatai* Ab15-ES: A response surface methodology approach. *Biocatal Agric Biotechnol* 24: 101528. DOI: 10.1016/j.cbab.2020.101528.
- Birahy DC, Sunarti TC, Meryandini A. 2025. Selection of proteolytic lactic acid bacteria with probiotic properties for fish protein hydrolyzate production. *Jurnal Ilmu Pertanian Indonesia* 30 (1): 31-39. DOI: 10.18343/jipi.30.1.31.
- Breig SJM, Luti KJK. 2021. Response surface methodology: A review on its applications and challenges in microbial cultures. *Materials Today: Proceedings* 42: 2277-2284. DOI: 10.1016/j.matpr.2020.12.316.
- Rosales Cavaglieri LA, Isgro MC, Aminahuel C, Parada J, Poloni VL, Montenegro MA, Alonso V, Falcone RD, Cavaglieri LR. 2025. Exploring the potential of lactic acid bacteria to produce postbiotics with antimicrobial and antioxidant properties: Focus on the probiotic

- strain *Pediococcus pentosaceus* RC007 for industrial-scale production. *Intl J Food Sci Technol* 60 (1): vvae003. DOI: 10.1093/ijfood/vvae003.
- Cupp-Enyard C. 2008. Sigma's non-specific protease activity assay-casein as a substrate. *J Vis Exp* 19: 899. DOI: 10.3791/899.
- Djimtoingar SS, Derkyi NSA, Kuranchie FA, Yankyera JK. 2022. A review of response surface methodology for biogas process optimization. *Cogent Eng* 9 (1): 2115283. DOI: 10.1080/23311916.2022.2115283.
- Dubey CK, Mishra J, Nagar A, Gupta MK, Sharma A, Kumar S, Mishra V, Pandey HP. 2024. Microbial Protease: An Update on Sources, Production Methods, and Applications. In: Mishra V, Mishra J, Arora NK (eds.). *Bioactive Microbial Metabolites*. Academic Press, Massachusetts. DOI: 10.1016/C2022-0-00301-9.
- Ellouz Y, Bayouhdh A, Kammoun S, Gharsallah N, Nasri M. 2001. Production of protease by *Bacillus subtilis* grown on sardinelle heads and visceral flour. *Bioresour Technol* 80 (1): 49-51. DOI: 10.1016/S0960-8524(01)00057-8.
- Emon TH, Hakim A, Chakraborty D, Azad AK. 2024. Enhanced production of dehairing alkaline protease from *Bacillus subtilis* mutant E29 by consolidated bioprocessing using response surface modeling. *Biomass Convers Bioref* 14: 19501-19517. DOI: 10.1007/s13399-023-04244-3.
- Escobar-Sánchez M, Carrasco-Navarro U, Juárez-Castelán C, Lozano-Aguirre Beltrán L, Pérez-Chabela ML, Ponce-Alquicira E. 2022. Probiotic properties and proteomic analysis of *Pediococcus pentosaceus* 1101. *Foods* 12 (1): 46. DOI: 10.3390/foods12010046.
- Fahmy NM, El-Deeb B. 2023. Optimization, partial purification, and characterization of a novel high molecular weight alkaline protease produced by *Halobacillus* sp. HAL1 using fish wastes as a substrate. *J Genet Eng Biotechnol* 21 (1): 48. DOI: 10.1186/s43141-023-00509-6.
- Fitriana N, Asri MT. 2022. Proteolytic activity of protease enzymes from rhizosphere bacteria of soybean plants (*Glycine max* L.) in Trenggalek. *LenteraBio: Berkala Ilmiah Biologi* 11 (1): 144-152. DOI: 10.26740/lenteraBio.v11n1.p144-152. [Indonesian]
- Garofalo SF, Cavallini N, Demichelis F, Savorani F, Mancini G, Fino D, Tommasi T. 2023. From tuna viscera to added-value products: A circular approach for fish-waste recovery by green enzymatic hydrolysis. *Food Bioprod Process* 137: 155-167. DOI: 10.1016/j.fbp.2022.11.006.
- Govindaraj K, Samayanpaulraj V, Narayanadoss V, Uthandakalaipandian R. 2021. Isolation of lactic acid bacteria from intestine of freshwater fishes and elucidation of probiotic potential for aquaculture application. *Probiotics Antimicrob Proteins* 13 (6): 1598-1610. DOI: 10.1007/s12602-021-09811-6.
- Hadinoto S, Idrus S. 2018. Proportion and proximate analysis parts of body yellowfin tuna (*Thunnus albacares*) from Maluku. *Majalah BIAH* 14 (2): 51-57. DOI: 10.29360/mb.v14i2.4212. [Indonesian]
- Ismail YS, Yulvizar C, Mazhitov B. 2018. Characterization of lactic acid bacteria from local cow's milk kefir. *IOP Conf Ser: Earth Environ Sci* 130: 012019. DOI: 10.1088/1755-1315/130/1/012019.
- Dilini Jayaweera D. 2023. Biorefinery for yellowfin tuna (*Thunnus albacares*) byproducts for characterization and identification of value-added materials. GRÓ Fisheries Training Programme under the auspices of UNESCO, Iceland. Final project. [https://www.grocentre.is/static/gro/publication/1730/document/DiliniJayaweera22\\_prf.pdf](https://www.grocentre.is/static/gro/publication/1730/document/DiliniJayaweera22_prf.pdf).
- Jiang S, Cai L, Lv L, Li L. 2021. *Pediococcus pentosaceus*, a future additive or probiotic candidate. *Microb Cell Fact* 20 (1): 45. DOI: 10.1186/s12934-021-01537-y.
- Jini R, Swapna HC, Rai AK, Vrinda R, Halami PM, Sachindra NM, Bhaskar N. 2011. Isolation and characterization of potential Lactic Acid Bacteria (LAB) from freshwater fish processing wastes for application in fermentative utilization of fish processing waste. *Braz J Microbiol* 42: 1516-1525. DOI: 10.1590/S1517-838220110004000039.
- Kabense R, Ginting EL, Wullur S, Kawung NJ, Losung F, Tombokan JL. 2019. Screening of the proteolytic bacteria symbiont with algae *Gracillaria* sp. *Jurnal Ilmiah PLATAX* 7 (2): 421-426. DOI: 10.35800/jip.7.2.2019.24487.
- Kieliszek M, Pobięga K, Piwożarek K, Kot AM. 2021. Characteristics of the proteolytic enzymes produced by lactic acid bacteria. *Molecules* 26 (7): 1858. DOI: 10.3390/molecules26071858.
- Lim ES, Lee NG. 2016. Control of histamine-forming bacteria by probiotic lactic acid bacteria isolated from fish intestine. *Korean J Microbiol* 52 (3): 352-364. DOI: 10.7845/kjm.2016.6041.
- Lim YH, Foo HL, Loh TC, Mohamad R, Abdullah N. 2019. Comparative studies of versatile extracellular proteolytic activities of lactic acid bacteria and their potential for extracellular amino acid productions as feed supplements. *J Anim Sci Biotechnol* 10: 15. DOI: 10.1186/s40104-019-0323-z.
- Linh NTH, Sakai K, Taoka Y. 2018. Screening of lactic acid bacteria isolated from fermented food as potential probiotics for aquacultured carp and amberjack. *Fish Sci* 84: 101-111. DOI: 10.1007/s12562-017-1150-9.
- Rohmatussolihat, Lisdiyanti P, Sari MN, Sukara E. 2021. Response surface methodology for optimization of medium components for extracellular protease production by *Enterococcus faecalis* InaCC B745. *IOP Conf Ser: Earth Environ Sci* 762: 012078. DOI: 10.1088/1755-1315/762/1/012078.
- Luong HQ, Le TN, Lee PH, Hsieh PC. 2023. Optimization of nonspecific protease activity fabrication by *Bacillus subtilis* N30 isolated from Taiwan using different models of response surface methodology. *Biocatal Agric Biotechnol* 50: 102686. DOI: 10.1016/j.cbac.2023.102686.
- Matti A, Utami T, Hidayat C, Rahayu ES. 2019. Isolation, screening, and identification of proteolytic lactic acid bacteria from indigenous chao product. *J Aquat Food Prod Technol* 28 (7): 781-793. DOI: 10.1080/10498850.2019.1639872.
- Migaw S, Ghrairi T, Belguesmia Y, Choiset Y, Berjeaud JM, Chobert JM, Hani K, Haertlé T. 2014. Diversity of bacteriocinogenic lactic acid bacteria isolated from Mediterranean fish viscera. *World J Microbiol Biotechnol* 30 (4): 1207-1217. DOI: 10.1007/s11274-013-1535-6.
- Moranda DP, Handayani L, Nazlia S. 2018. Utilization of yellowfin tuna (*Thunnus albacares*) skin waste as gelatin: Hydrolysis using HCl solvent with different concentrations. *Acta Aquatica: Aquatic Sci J* 5 (2): 81-87. DOI: 10.29103/aa.v5i2.850. [Indonesian]
- Mugendiran V, Gnanavelbabu A, Ramadoss R. 2014. Parameter optimization for surface roughness and wall thickness on AA5052 aluminum alloy by incremental forming using response surface methodology. *Procedia Eng* 97: 1991-2000. DOI: 10.1016/j.proeng.2014.12.442.
- Nugroho G, Ekawati AW, Kartikaningsih H. 2020. Characteristics of tuna viscera (*Thunnus* sp.) protein hydrolysate fermented by *Bacillus licheniformis*. *Res J Life Sci* 7 (2): 101-107. DOI: 10.21776/ub.rjls.2020.007.02.4.
- Nurjanah N, Baharuddin TI, Nurhayati T. 2021. Collagen extraction of yellowfin tuna (*Thunnus albacares*) skin using pepsin and papain enzymes. *Jurnal Pengolahan Hasil Perikanan Indonesia* 24 (2): 174-187. DOI: 10.17844/jphpi.v24i2.35410. [Indonesian]
- Nurmiah S, Syarif R, Sukarno S, Peranginangin R, Nurmata B. 2013. Application of response surface methodology on optimization of Alkali Treated Cottonii (ATC) processing conditions. *Jurnal Pascapanen dan Bioteknologi Kelautan dan Perikanan* 8 (1): 9-22. DOI: 10.15578/jpbkp.v8i1.49. [Indonesian]
- Ojo-Okunola A, Claassen-Weitz S, Mwaikono KS, Gardner-Lubbe S, Zar HJ, Nicol MP, du Toit E. 2020. The influence of DNA extraction and lipid removal on human milk bacterial profiles. *Methods Protoc* 3 (2): 39. DOI: 10.3390/mps3020039.
- Phupaboon S, Hashim FJ, Phumkhaichorn P, Rattanachaikunsopon P. 2023. Molecular and biotechnological characteristics of proteolytic activity from *Streptococcus thermophilus* as a proteolytic lactic acid bacteria to enhance protein-derived bioactive peptides. *AIMS Microbiol* 9 (4): 591-611. DOI: 10.3934/microbiol.2023031.
- Prastujati AU, Hilmi M, Khusna A, Arief II, Makmur S, Maulida Q. 2022. Isolation and identification of lactic acid bacteria of bekamal (Banyuwangi traditional fermented meat). *IOP Conf Ser: Earth Environ Sci* 1020: 012026. DOI: 10.1088/1755-1315/1020/1/012026.
- Raccach M. 2014. *Encyclopedia of Food Microbiology (Second Edition)*. Elsevier, Amsterdam.
- Rahayu HM. 2021. Isolation and identification of lactic acid bacteria from *tempoyak* for making yogurt by morphological and biochemical characterization. *EPiC Ser Biol Sci* 1: 118-124. DOI: 10.29007/vvbj.
- Ramadhan AR, Bachruddin Z, Erwanto Y, Hanim C. 2021. Isolation and selection of proteolytic lactic acid bacteria from colostrum of dairy cattle. *IOP Conf Ser: Earth Environ Sci* 788: 012077. DOI: 10.1088/1755-1315/788/1/012077.
- Ramkumar A, Sivakumar N, Victor R. 2016. Fish waste-potential low cost substrate for bacterial protease production: A brief review. *Open Biotechnol J* 10: 335-341. DOI: 10.2174/1874070701610010335.
- Rebah BF, Miled N. 2013. Fish processing wastes for microbial enzyme production: A review. *3 Biotech* 3 (4): 255-265. DOI: 10.1007/s13205-012-0099-8.
- Rosnawita M, Agustien A, Nasir A. 2015. Effect of abiotic factors on protease production from bacterial isolate ML-23. *Jurnal Biologi Universitas Andalas* 4: 45-49. DOI: 10.25077/jbioua.4.1.%25p.2015. [Indonesian]
- Gómez-Sala B, Muñoz-Atienza E, Sánchez J, Basanta A, Herranz C, Hernández PE, Cintas LM. 2015. Bacteriocin production by lactic

- acid bacteria isolated from fish, seafood and fish products. *Eur Food Res Technol* 241: 341-356. DOI: 10.1007/s00217-015-2465-3.
- Saravanan A, Yuvaraj D, Kumar PS, Karishma S, Rangasamy G. 2023. Fish processing discards: A plausible resource for valorization to renewable fuels production, optimization, byproducts and challenges. *Fuel* 335: 127081. DOI: 10.1016/j.fuel.2022.127081.
- Sodagar N, Jalal R, Najafi MF, Bahrami AR. 2024. A novel alkali and thermotolerant protease from *Aeromonas* spp. retrieved from wastewater. *Sci Rep* 14 (1): 26000. DOI: 10.1038/s41598-024-76004-w.
- Suberu Y, Akande I, Samuel T, Lawal A, Olaniran A. 2019. Optimization of protease production in indigenous *Bacillus* species isolated from soil samples in Lagos, Nigeria using response surface methodology. *Biocat Agric Biotechnol* 18: 101011. DOI: 10.1016/j.bcab.2019.01.049.
- Sulistiyantini C, Mustafa I, Jatmiko YD. 2023. Isolation and Identification of Chitinolytic Bacteria as Biocontrol Agents of Fungal Pathogens in Cocoons of *Cricula trifenestrata* (Lepidoptera: Saturniidae). [Thesis]. Institut Pertanian Bogor, Bogor 13 (1): 29-34. DOI: 10.21776/ub.jels.2023.013.01.05. [Indonesian]
- Sulthoniyah STM, Handoko, Nursyam HH. 2015. Characterization of extracellular protease lactic acid bacteria from shrimp paste. *J Life Sci Biomed* 5 (1): 1-5.
- Sun F, Hu Y, Chen Q, Kong B, Liu Q. 2019. Purification and biochemical characteristics of the extracellular protease from *Pediococcus pentosaceus* isolated from Harbin dry sausages. *Meat Sci* 156: 156-165. DOI: 10.1016/j.meatsci.2019.05.030.
- Ter ZY, Chang LS, Babji AS, Zaini NAM, Fazry S, Sarbini SR, Peterbauer CK, Lim SJ. 2024. A review on proteolytic fermentation of dietary protein using lactic acid bacteria for the development of novel proteolytically fermented foods. *Intl J Food Sci Technol* 59 (3): 1213-1236. DOI: 10.1111/ijfs.16888.
- Thirukumaran R, Anu Priya VK, Krishnamoorthy S, Ramakrishnan P, Moses JA, Anandharamakrishnan C. 2022. Resource recovery from fish waste: Prospects and the usage of intensified extraction technologies. *Chemosphere* 299: 134361. DOI: 10.1016/j.chemosphere.2022.134361.
- Toe CJ, Foo HL, Loh TC, Mohamad R, Abdul Rahim R, Idrus Z. 2019. Extracellular proteolytic activity and amino acid production by lactic acid bacteria isolated from Malaysian foods. *Intl J Mol Sci* 20 (7): 1777. DOI: 10.3390/ijms20071777.
- Vinoth J, Murugan S, Stalin C. 2014. Optimization of alkaline protease production and its fibrinolytic activity from the bacterium *Pseudomonas fluorescens* isolated from fish waste discharged soil. *Afr J Biotechnol* 13 (30): 3052. DOI: 10.5897/AJB2014.13863.
- Ruby-Figueroa R. 2016. Design of Experiments. In: Drioli E, Giorno L. (eds) *Encyclopedia of Membranes*. Springer, Berlin, Heidelberg. DOI: 10.1007/978-3-662-44324-8\_1997.
- Xu Y, Dai M, Zang J, Jiang Q, Xia W. 2015. Purification and characterization of an extracellular acidic protease of *Pediococcus pentosaceus* isolated from fermented fish. *Food Sci Technol Res* 21 (5): 739-744. DOI: 10.3136/fstr.21.739.
- Xing CF, Hu HH, Huang JB, Fang HC, Kai YH, Wu YC, Chi SC. 2013. Diet supplementation of *Pediococcus pentosaceus* in cobia (*Rachycentron canadum*) enhances growth rate, respiratory burst and resistance against photobacteriosis. *Fish Shellfish Immunol* 35 (4): 1122-1128. DOI: 10.1016/j.fsi.2013.07.021.
- Yin LJ, Wu CW, Jiang ST. 2003. Bacteriocins from *Pediococcus pentosaceus* L and S from pork meat. *J Agric Food Chem* 51 (4): 1071-1076. DOI: 10.1021/jf025838f.
- Zamora-Sillero J, Gharsallaoui A, Prentice C. 2018. Peptides from fish byproduct protein hydrolysates and its functional properties: An overview. *Mar Biotechnol (NY)* 20 (2): 118-130. DOI: 10.1007/s10126-018-9799-3.