



Isolation and Optimization of Extracellular Protease Production by *Bacillus* sp. from Catfish (*Clarias* sp.) Intestine for Industrial Applications

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Abstract

A protease is one of the most used classes of enzymes in various industries. Enzyme production in Indonesia still mainly relies on imports, suggesting that local microbial resources should be investigated. In this study, isolation and optimization of protease-producing bacteria from the intestine of catfish (*Clarias* sp.) was conducted as a possible local source of industrial enzymes. Eight bacterial isolates were screened on skim milk agar. The most potential isolate was morphologically and molecularly identified as *Bacillus* sp. Strain UBL. Protease production was investigated by calculating Proteolytic Index (PI) and enzyme activity (U/mL). Optimization was performed using one factor at a time, analysing the influence of initial inoculum concentration (2–6%), pH (6–8), temperature (30–42°C), culture medium, and length of incubation periods. Maximum protease production was achieved at the following conditions: 4% Inoculum ($p < 0.05$), pH 7 ($p < 0.05$), 30°C temperature ($p < 0.05$), under TSB medium, and an incubation period of 16 h ($p < 0.05$). At pooled parameters, the obtained enzyme activity was tabulated at 21.1 ± 0.9 U/mL, having a proteolytic index value of 0.96 ± 0.04 as compared to non-optimized settings with activity being only up to 8.1 ± 4.2 U/mL and PI of just 0.37 ± 0.19 , reflecting an augmentation of about 2.6-fold in production of protease released by *Bacillus* sp. strain UBL under optimized conditions. These results indicate that fish intestinal microbiota represents a potential source of bacterial proteases.

Keywords:

Catfish, gut microbiology, protease, production.

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Introduction

Proteases are one of the most commercially relevant industrial enzymes used in food processing, detergents, medications, and leather tanning. They are responsible for catalysing the hydrolysis of the peptide bond, thereby making them applicable in various biotechnological fields. The global market for proteases is also likely to witness significant growth, due to the low cost of protease as compared to chemical catalysts and the environmentally sustainable fermentation process preferred for production. However, due to Indonesia's situation regarding its biodiversity area, enzymes still need import (Sharma et al. 2017). It is thus evident that there is a significant demand to explore indigenous microbial precursors that could serve as sustainable regional resources for the enzyme industry. Microbial proteases, particularly derived from *Bacillus* species, are exceedingly valuable as these organisms are capable of secreting large volumes of thermostable extracellular enzymes. Sun et al. (2023) investigated *Bacillus subtilis* BS-QR-052 based on optimum protease activity using the Response Surface Methodology (RSM) have produced 1780.03 U/mL of enzyme activity. Likewise,

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Hashmi et al. (2022) enhanced the activity of *Bacillus subtilis* S1 and *Bacillus amyloliquefaciens* KSM12, showing maximum activity at 37 °C and alkaline pH. The importance of this shall reflect that coordinated, precise consideration of culture factors (pH, temperature, and inoculum size) can significantly enhance enzyme productivity (Jingying and Yan, 2023; Pandey et al. 2026). However, despite extensive studies on *Bacillus*-derived proteases from terrestrial and marine environments, limited research has explored fish gut microbiota as a potential source of protease-producing bacteria. This gap is particularly evident for freshwater species such as catfish (*Clarias* sp.), where systematic studies on isolation and optimization of protease production remain scarce.

While notable advancements have been documented for protease-producing bacteria inhabiting marine and terrestrial environments, there are relatively few reports on protease-producing bacteria isolated from aquatic organisms, particularly from fish intestines (Prihanto et al. 2020). This was the main research problem, as there are still few studies that included knowledge of proteolytic bacteria able to colonize catfish intestines, and even fewer established information about parameters that maximize enzyme production. While protease-producing microbes from diverse sources have been reported earlier, no systematic study was performed for *Clarias* sp. isolates. As protease activity is significantly influenced by these environmental factors, optimization is crucial for practical production and one of the most important aspects regarding enzymes if they are to comply with industrial performance standards (Shad et al. 2024).

One way to solve this dilemma is to screen bacterial isolates that have the potential to secrete protease enzymes and optimize them under different environmental conditions. The hydrolytic effect of enzymes is indicated by proteolysis zones, but it is difficult to screen widely on normal agar. Factors like pH, temperature, and inoculum concentration are then varied systematically to evaluate the effect of each on enzyme yield. This strategy is pseudo-based, which includes qualitative and quantitative data essential for scaling microbial enzyme production.

Prior works provide techniques on how to optimize proteases. Alshehri et al. (2025) and Agustien et al. (2025) used RSM to optimize the production of alkaline protease by *Bacillus mycoides* and an endophyte isolated from it. Soil bacteria protease production is greatly influenced by culture parameters like carbon and nitrogen sources (Hamza and Woldesenbet 2017). Prihanto et al. (2021) further confirmed that *Bacillus* sp. of different types can be isolated from the intestine of catfish and that the composition of the medium affects the output of the enzyme.

A study from the catfish intestinal environment can have unique physiological and biochemical conditions for microorganisms, particularly interesting novel characteristics of enzymes such as thermostability, pH range, or substrate specificity, enabling its industrial use; thus, the deep exploration of this niche microbial ecosystem may provide us with new biocatalysts adapted to both food and non-food industries. The gastrointestinal tract of fish represents a protein-rich and competitive ecological niche that selectively enriches microorganisms capable of producing extracellular proteases with unique biochemical properties. Such host-associated microbial communities have been reported to exhibit distinct enzymatic adaptations compared to free-living counterparts, highlighting their potential as a promising source of industrial enzymes (Ray et al. 2012).

There are many studies available based on biotechnological enzyme production and also, how to optimize the yield, usually recommending single variable experiments to be carried out in which factors are adjusted separately as a part of empirical procedures, although it is common knowledge that optimization of multiple parameters simultaneously gives a better prospect regarding product yield. Studies by Zayani et al. (2025) demonstrated that protease activity depends on environmental parameters, including incubation time, temperature, and pH. Yet, the interaction of these parameters has not been well studied. Thus, bridging this knowledge gap may improve understanding of how these factors together shape enzyme production and activity across freshwater bacterial strains.

Therefore, this study aims to isolate and identify protease-producing bacteria from the intestine of catfish (*Clarias* sp.) and to optimize the culture conditions influencing extracellular protease production. In addition, this study seeks to evaluate the effect of key environmental parameters, including inoculum size, pH, temperature, and incubation time, on enzyme yield to assess its potential for industrial application.

Materials and Methods

Time, Location of Research and Material

The study was conducted in June 2025. Sampling of catfish intestines took place at Blimbing Market in Malang City, East Java, Indonesia. All materials used in this research were analytical grade.

Experimental Design

The optimization experiments were arranged in a Completely Randomized Design (CRD) and carried out in a sequential order by applying one-factor-at-a-time to each independent variable. All treatments were replicated three times for statistical significance. The independent variables were inoculum concentration (2%, 4%, and 6%), medium pH (6, 7, and 8), and incubation temperature (30°C, 37°C, and 42°C). The dependent variable was extracellular protease activity, quantified as the proteolytic index derived from the ratio between the halo diameter and colony diameter observed on skim milk agar plates.

Sample Preparation and Bacterial Isolation

Fresh catfish were dissected under aseptic conditions to extract intestinal segments. The intestinal contents were homogenized and serially diluted in sterile physiological saline. Aliquots of the dilutions were spread on Skim Milk Agar (SMA) plates to screen for protease-producing colonies. Plates were incubated overnight at 30°C. Colonies exhibiting the largest clear zones were selected for further purification.

Screening for Protease-Producing Bacteria

To confirm protease production, the bacteria were plated on the selective medium. Colonies were spotted on Skim Milk Agar (SMA). After incubation, clear zones surrounding colonies were measured in millimetres. The proteolytic index (PI) was calculated as:

$$\text{Proteolytic Index} = \frac{D_{\text{zone}} - D_{\text{colony}}}{D_{\text{colony}}}$$

where D_{zone} represents the diameter of the clear zone and D_{colony} denotes the diameter of the bacterial colony. The isolates with the highest PI values were selected as candidates for optimization experiments.

Molecular identification of the species

Genomic DNA was extracted using the method described by company's standard protocols. After extraction, sequencing was performed with the universal forward primer and the reverse primer. The resulting sequences were analyzed in FASTA format using BLAST on NCBI. Phylogenetic analysis and tree reconstruction were carried out using <https://ngphylogeny.fr> (Lemoine et al. 2019).

Optimization of Protease Production

Optimization of protease production was performed through three sequential stages. Each stage focused on one variable, namely inoculum concentration, medium pH, or temperature, while keeping the others constant at previously optimized levels.

For inoculum optimization, bacterial cultures were adjusted to three inoculum concentrations (2%, 4%, and 6%) and incubated under standard conditions (pH 7, 30°C). The medium was adjusted to pH 6, 7, or 8 using NaOH or HCl. To optimize temperature, incubation was carried out at 30 °C, 37 °C and 42 °C with the optimal inoculum and pH values. The highest PI indicated the optimal condition. Cumulative optimal conditions were then confirmed in the next phase.

Confirmation Test

Standard laboratory conditions (LB medium, pH 7, 37° C, and, 4% inoculum) and optimized conditions (TSB medium, 4% inoculum, pH 7, 30°C) were compared with regard to the enzyme production. The resulting proteolytic indices were statistically compared using a paired-sample t-test to evaluate whether the optimization produced a significant increase in enzyme activity. The significance level was set at $p < 0.05$, and results were considered statistically significant if the p-value was below this threshold

Data Analysis

All quantitative data obtained from the optimization experiments were analysed using SPSS. Prior to the main analysis, data were tested for normality and homogeneity ($p > 0.05$) to ensure the validity of parametric statistical tests.

Results

Isolation of Proteolytic Bacteria from Catfish Intestine

In the first phase of this study, bacterial colonies were successfully isolated from catfish (*Clarias* sp.) intestines. Eighteen colonies were spotted to Skim Milk Agar (SMA), in order to detect the most potential protease producer. Two colonies showed best extracellular protease producer (Figure 1). Colony No.10 has a higher proteolytic index (PI) compared to Colony No. 6. Therefore, only colony No. 10 continued to be analysed for its identity.



Figure 1. Clear Zone of Colony number 6 and number 10.

Identification of potential protease producer.

Based on 16S rRNA gene sequence analysis, the selected isolate was identified as *Bacillus* sp. strain UBL and showed close phylogenetic affiliation with members of the genus *Bacillus*. BLAST analysis indicated the highest sequence similarity with *Bacillus pseudofirmus* OF4 (CP001878.2; 99.93%) and *Bacillus lindianensis* F3R26 (PX102332.1; 98.66%), while the phylogenetic tree further confirmed its clustering with related *Bacillus* taxa. This is indicated by the relatively close proximity of the branches compared to other groups of bacteria such as *Lactobacillus* and *Salmonella* that are on separate branches.

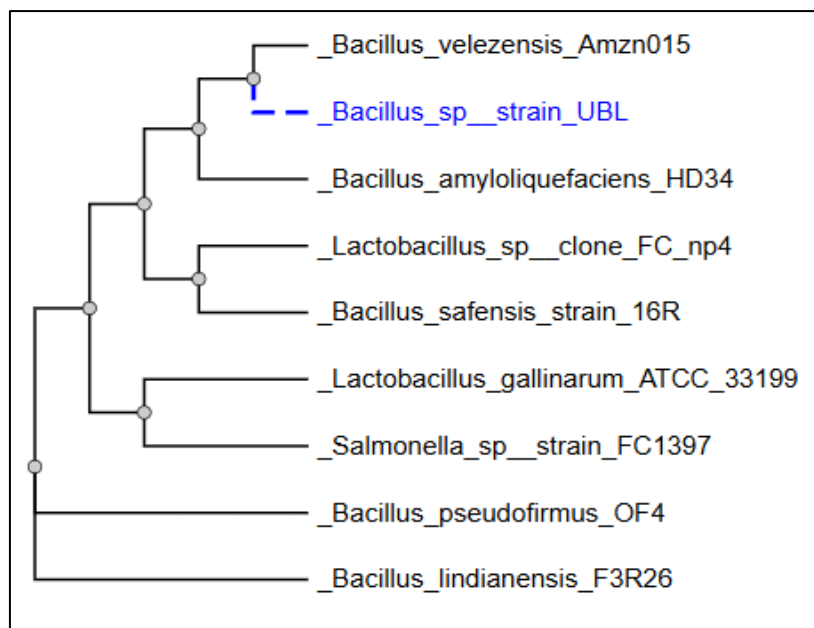


Figure 2. Phylogenetic tree analysis of the isolate that highly produces Protease (*Bacillus* sp strain UBL).

Optimization of Protease Production

Medium optimization

Among the tested media, TSB exhibited the highest protease activity (25.3 ± 0.7 U/mL), followed by LBB (22.4 ± 0.4 U/mL) and NB (21.1 ± 1.3 U/mL). The data in detail was on the Figure 3.

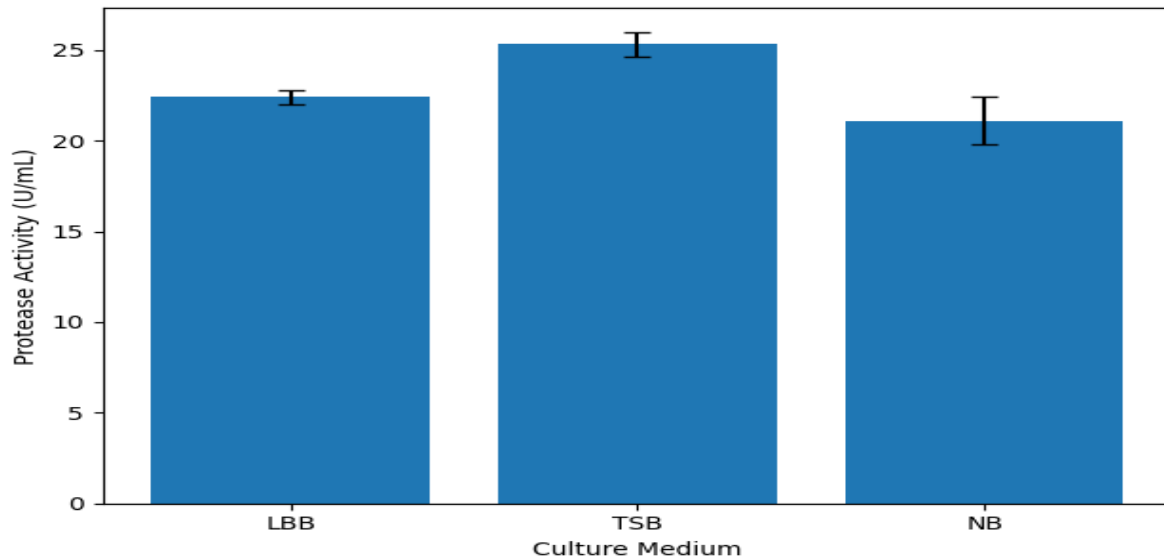


Figure 3. Effect of culture medium on Protease Production.

The optimum time for harvesting

The perfect harvest time for protease production is carried out using TSB culture media. During culture (0–36 h) proteases are produced consistently with mixed results (Figure 4). Protease production increased almost linearly, reaching a maximum at 16 h with the activity of 31.5 ± 3.2 U/mL. After this peak, enzyme activity sharply declined on 20 h. The lowest level of enzyme production was detected on 36 h (4.4 ± 0.4 U/mL).

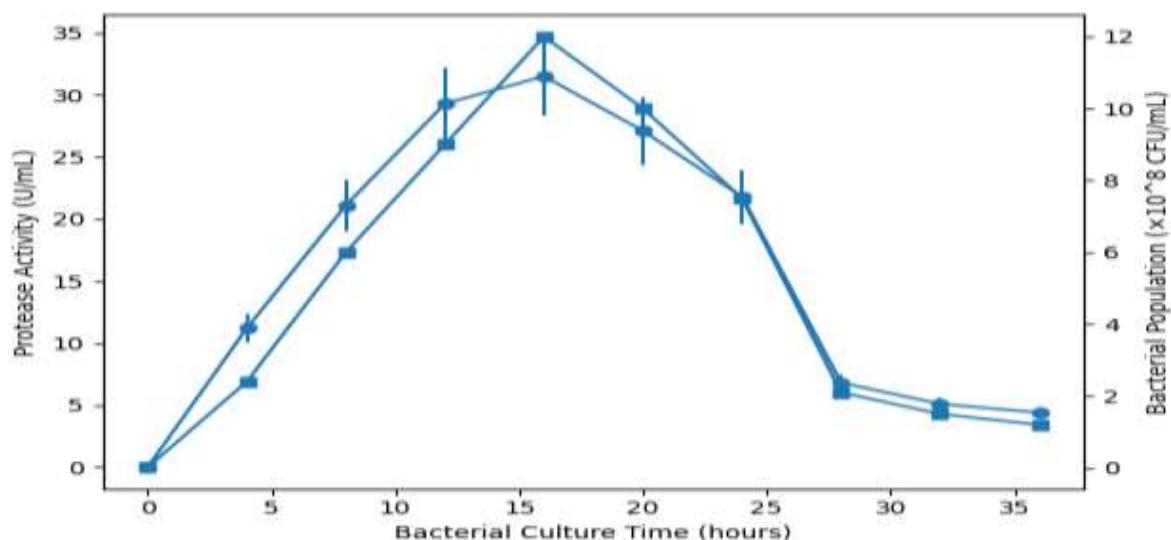


Figure 4. Time course of protease production and bacterial growth. The square dot is the bacterial population and the circle dot is enzyme activity.

The maximal peak at 16 h correlates with the maximum bacterial count (12×10^8 CFU/mL), indicating that protease production is associated with the late exponential early stationary phase.

Optimization of Inoculum Size, pH, and Temperature

The results of the optimization of inoculum size, pH and culture temperature were carried out using TSB media and were harvested at the 16th h. The optimization data is presented in Table 1.

Table 1: Effects of Inoculum Size, pH, and Temperature on Bacterial Population and Protease Activity

Parameter	Condition	Bacterial Population (CFU/mL) ($\times 10^8$)	Protease Activity (U/mL)
Inoculum	2%	6.2 ± 0.8^a	16.3 ± 2.2^a
	4%	8.3 ± 0.8^b	21.8 ± 2.2^b
	6%	6.6 ± 1.2^a	17.2 ± 3.1^a
pH	6	4.9 ± 1.0^a	12.8 ± 2.6^a
	7	6.1 ± 0.3^b	16.1 ± 0.7^b
	8	4.2 ± 1.4^a	11.0 ± 3.7^a
Temperature ($^{\circ}\text{C}$)	30	10.9 ± 0.3^b	28.6 ± 0.7^b
	37	9.8 ± 0.7^a	25.7 ± 1.8^a
	42	9.4 ± 0.3^a	24.6 ± 0.7^a

The results showed that variations in inoculum concentrations had a significant influence on bacterial populations and the activity of protease enzymes produced. Four percent of inoculum resulted in the highest bacterial population and protease activity, $8.3 \pm 0.8 \times 10^8$ CFU/mL, 21.8 ± 2.2 U/mL, respectively. At a lower (2%) and higher (6%) inoculum size, both the bacterial population and the activity of enzymes were significantly low.

The pH of Medium also shows noteworthy influence on bacterial growth and protease production. Neutral pH (pH 7) resulted in the most optimal condition for bacterial population of $6.1 \pm 0.3 \times 10^8$ CFU/mL and protease activity of 16.1 ± 0.7 U/mL. Bacterial population and enzyme activity were remarkably low under acidic pH (pH 6) and alkaline (pH 8).

Incubation temperatures exert the most significant influence on the increase in bacterial populations and protease enzyme activity. A temperature of 30°C resulted in a maximum population of $10.9 \pm 0.3 \times 10^8$ CFU/mL and a maximum protease activity of 28.6 ± 0.7 U/mL. Temperature increases to 37°C and 42°C led to a gradual decrease in both parameters.

Confirmation of Optimized Conditions

A confirmation experiment was performed to verify the interaction effect of medium, harvesting time, inoculum size, pH, and temperature on increasing protease production. The optimized conditions resulted in 21.1 ± 0.9 U/mL. The control condition (LB medium, unoptimized parameters) produced 8.1 ± 4.2 U/mL. Statistical analysis revealed a highly significant difference, $p = 0.001$ (paired t-test). The optimized fermentation process enabled a 2.6 times enhancement of protease activity in contrast to the control (Figure 5).

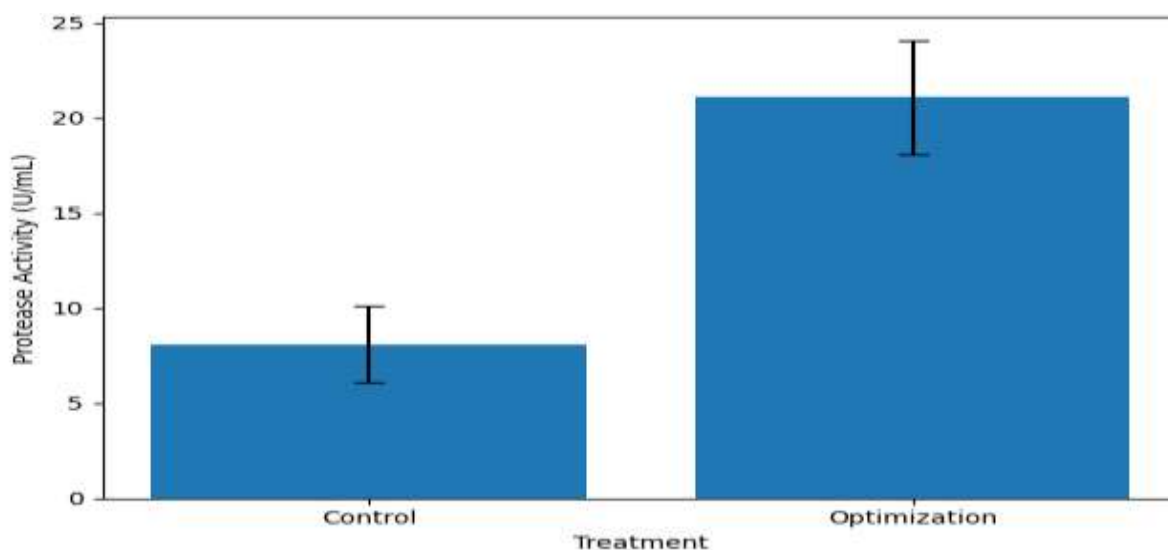


Figure 5. Comparison of control and optimized conditions for the protease activity.

Discussion

The current study isolated and optimally characterized a protease-producing bacterium classified as *Bacillus* sp. strain UBL with the identity of from the intestinal content of catfish (*Clarias* sp.). The culture environmental modification led to a 2.6-fold enhancement of protease activity. It is an indication that the environmental and nutritional regulate not only bacterial growth but also enzyme production. These findings further highlight the potential of fish gut microbiota as an underexploited source for industrially relevant enzymes.

Similar findings have been reported in other studies involving *Bacillus* spp. isolated from animal gastrointestinal tracts, where enzyme production is closely linked to host-associated environmental conditions and nutrient availability. For instance, *Bacillus* strains derived from fish gut microbiota have demonstrated significant protease activity under optimized culture conditions, highlighting their adaptive capacity and industrial potential (Ray et al. 2012; Kuebutornye et al. 2019). These comparisons reinforce that gut-derived *Bacillus* isolates represent a promising and competitive source of extracellular enzymes.

In this study, skim milk agar that has identified protease-producing isolates were used for preliminary screening of the same isolate as conducted previously (Hamza and Woldesenbet 2017; Prihanto et al. 2020). Formation of halo zones indicates the ability of extracellular proteases to hydrolyse casein into soluble peptides. The proteolytic index (PI) then semi-quantitatively validated the enzymatic performance and corresponded well to the outcomes of visual screening. Compared to the study by Prihanto et al. (2021), *Bacillus subtilis* and *Bacillus licheniformis*, the potential enzyme producer isolated from *Clarias* sp. indicated higher PI values (≥ 0.70).

The species identification revealed that the bacteria is *Bacillus* sp. Strain UBL. *Bacillus* sp. is well recognized as extracellular proteases producer (Prihanto et al, 2020; Alshehri et al. 2025). *Bacillus* sp. Strain UBL has closeness with *Bacillus subtilis*. Digestive tract of catfish represents a protein-rich ecological niche that occurs may exert a selective pressure for microbes to become accustomed by an efficient secretion of proteases during digestion. Environmental niche specialization is linked to ecologically elevated enzyme activities and systems biology (competitive survival strategies) observed in nutrient-limiting systems (Cezairliyan and Ausubel 2017). In addition, several evidence suggest that the gut-associated microbial has different physiological benefits compared to isolates isolated from soil or marine sources (Kubasova et al. 2021).

The better performance of TSB medium in the present study may be mainly due to its high levels of easily assimilable nitrogen and peptide substrates (tryptone and soy peptone). The expression of protease enzymes in bacteria, mostly controlled by nitrogen and carbon present (He et al. 2023). Several compounds of the media can act as inducers of protease expression (Cai et al. 2026). LBB and NB also promoted enzyme production; however, their lower relative activities indicate either insufficient nutrient composition or metabolic performance. These results are in agreement with those described for *Bacillus* sp. grown on nutrient-rich media, which demonstrates that protease production is significantly increased (Amin et al. 2025). Composition of the media, has been reported as a critical limit for the production of the metabolite (Rozanov et al. 2021). Therefore, medium composition plays an important role on biomass accumulation but also for controlling the expression of enzymes.

The production of the protease reached the highest on the late exponential or early stationary phase (16 h). This observation is consistent with known *Bacillus* sp. fermentation kinetics, which include stringent regulation of extracellular enzyme production associated with growth phase transitions and quorum-sensing mechanisms (Contesini et al. 2018). In this stage, low nutrient concentration activates the derepressing of genes responsible for extracellular enzyme secretion, ramping up their yield. The activity drops post 20 h may be due to the depletion of nutrients, instability of enzymes or compromised viability of cells. The same temporal trends have been observed across other *Bacillus*-based systems (Cai et al., 2019).

The effect of inoculum size, pH, and temperature has also been optimized, which further confirms their key role in the production of protease. The 4% inoculum had the highest enzyme activity, and production decreased below and above this maximum concentration. This bell-shaped response pattern has been previously reported from microbial fermentation, when too low an inoculum leads to increased length of lag phase, while too high inoculum results in nutrient competition or limited oxygen (Shafique et al. 2021; Song et al., 2023). This gives important insights into the need for proper cell density and economical metabolism.

Likewise, neutral pH (pH 7) was found to be the best condition for the production of protease. pH affects several facets of microbial physiology, including folding and secretion pathways for enzymes (Razzaq et al. 2019; Su et al. 2025). While many *Bacillus* proteases are known to be alkaline, the neutral optimal pH observed here indicates adaptation of this strain(s) to stable intestinal conditions. To bolster this theory, the origin of enzyme production is ruled by ecological factors (Contesini et al. 2018; Alamsjah et al. 2024).

Optimum temperature screening showed that both bacterial growth and enzyme production favoured at 30°C. This suggests that the isolate is mesophilic in nature, and this reflects a likely host-associated origin of the organism. Decreased enzymatic step and harmful cellular stress. Higher temperatures could increase metabolism as described by Shafique et al (2021). Evaluated in two different systems (cell lines and whole animals), the reason behind the dissemination of malignancy at high temperatures in human beings (Su et al. 2025). Thus, moderate temperatures seem to protect the enzymes from denaturation and yet allow for significant metabolic activity, thereby driving efficient syntheses of proteases.

The optimized culture condition showed a 2.6-fold enhancement in protease activity over non-optimized conditions. Similar improvement has been reported in previous studies, which have shown that culture condition optimization increases the enzyme yield significantly (Alshehri et al. 2025; Sarkar et al. 2025). Important to note, that the current study used OFAT methodology and did not consider interaction effects among variables. Advanced statistical methods such as response surface methodology (RSM) could increase production efficiency by determining optimum parameter interactions (Shad et al. 2024). While the one-factor-at-a-time (OFAT) approach offers a simple, cost-effective, and efficient strategy for preliminary optimization by isolating the effect of individual variables, it does not account for interaction effects among parameters, which may influence overall enzyme production performance. In contrast, statistical approaches such as Response Surface Methodology (RSM) enable simultaneous evaluation of multiple variables and their interactions, often resulting in more precise optimization outcomes (Bezerra et al. 2008; Bhatariwala et al. 2022).

The prospects of improving protease production with locally obtained bacterial isolates may be beneficial for lowering dependence on imported enzymes from an industrial standpoint. In addition, the use of indigenous microbial resources also promotes the establishment of bioindustries with high sustainability in biodiversity-rich countries like Indonesia.

The application of Response Surface Methodology (RSM) is recommended in future studies to better capture interaction effects and further enhance protease production efficiency. The study was mainly dealing with optimization of the process without specific characterization of the enzyme, like stability, kinetic particulars, or substrate specificity. Further, enzyme characterization such as stability, kinetic parameters, and substrate specificity is essential to evaluate its suitability for industrial applications. Recent studies on *Bacillus*-derived proteases from animal-associated systems have demonstrated that such characterization provides critical insights into enzyme functionality, performance, and potential industrial scalability (Yi et al. 2024; Liu et al. 2024)

While these results appear promising, several limitations must be addressed. This study has several limitations that should be acknowledged. First, the optimization approach employed a one-factor-at-a-time (OFAT) method, which does not account for potential interaction effects among variables and may limit the accuracy of optimal condition determination. Second, the study focused primarily on process optimization without comprehensive enzyme characterization, such as stability, kinetic parameters, and substrate specificity, which are essential for evaluating industrial applicability. Finally, the experiments were conducted at laboratory scale, and therefore, further studies are still required to assess scalability and performance under industrial fermentation conditions.

This study successfully isolated protease-producing bacterium from the intestine of catfish (*Clarias* sp.), namely, *Bacillus* sp. Strain UBL. The optimized culture condition was achieved using OFAT method. *Bacillus* sp. Strain UBL increases its protease production for up to 2.6-fold under these conditions, namely 4% inoculum, pH 7, 30 °C, TSB medium, and a harvest time of 16 h after inoculation.

These results underscore the importance of environmental and nutritional conditions to optimize microbial enzyme production. This large improvement in protease production by optimization of process conditions indicates that using these indigenous bacterial isolates has industrial potential. The results are fundamental because they support the potential to develop local and non-pathogenic enzyme producers that can reduce dependence on imported commercial strains and ultimately strengthen clean bioindustry development.

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Author Contributions:

AAP contributed to conceptualization, supervision, funding acquisition, and manuscript writing. HN and AAJ contributed to data analysis, manuscript writing, and supervision of the research activities. RLH and YA were responsible for data collection and contributed to manuscript preparation. All authors have read and approved the final version of the manuscript.

Conflict of Interest:

The authors declare that they have no conflict of interest.

Ethics Approval:

All experimental procedures involving fish samples adhered to biosafety and ethical standards established by Universitas Brawijaya.

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