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Optimization and molecular identification of PUA-14 bacterial isolate from protease-producing mangrove waters

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Abstract. *Ananta Y, Alamsjah F, Agustien A. 2025. Optimization and molecular identification of PUA-14 bacterial isolate from protease-producing mangrove waters. Biodiversitas 26: 190-198.* Protease enzymes, the hydrolase enzymes that break down proteins into simpler molecules, are a vital component of the food, pharmaceutical, and other chemical industries. By selecting microorganisms as enzyme producers, we can meet the high demand for enzymes and promote sustainable production. This study aimed to determine the optimum conditions for protease production by protease-producing PUA-14 bacterial isolates from mangrove waters and to identify the isolates through biomolecular analysis. We conducted laboratory experiments to optimize protease production by varying carbon sources, nitrogen sources, trace elements, and inoculum concentrations. The optimization process employed the One Factor at A Time (OFAT) approach and the Central Composite Design (CCD) within the framework of Response Surface Methodology (RSM) using Design Expert 13 software. Furthermore, biomolecular analyses, including DNA isolation, amplification, and sequencing, were performed to identify the bacterial isolates. The study identified the optimum conditions for protease production by the PUA-14 bacterial isolate are 0.5% lactose, 2.5% $NaNO_3$, Zn as the trace element, and 2.5% inoculum concentration. Molecular analysis confirms the similarity of the PUA-14 bacterial isolate to *Bacillus pseudomycoides* strain LB-AsDX1-3. These research findings not only provide a foundation for future research but also have immediate practical implications, highlight the potential for sustainable enzyme production under optimized conditions, and support a wide range of industrial applications.

Keywords: 16S rRNA, biomolecular, optimization, protease, response surface methodology

INTRODUCTION

Enzymes are increasingly utilized in a variety of sectors, such as industry. Enzymes are replacing and catalyzing chemical reactions that were previously conducted in phases. Enzymes are essential for the biological processes that involve specific macromolecules and can accelerate biochemical reactions that occur inside and outside cells in their capacity as biocatalysts (Warshel and Bora 2016). Research on novel proteases is currently underway, as 60% of all enzymes that are commercialized worldwide are proteases. According to Naveeed et al. (2021), the protease enzyme market is expected to expand at a Compound Annual Growth Rate (CAGR) of 6.1% by 2024, with a projected value exceeding USD 3 billion. This growth is a testament to the high demand for enzymes, a demand that your work in this field is helping to meet. The high demand for enzymes is met by microorganisms, which is why they are chosen as enzyme producers. This selection supports sustainable production. Microbial enzymes are extensively employed in industrial processes due to their cost-effectiveness, high productivity, and availability (Nursyirwani et al. 2021). Microorganisms' protease production is significantly influenced by the components of the media, particularly carbon and nitrogen sources. These sources are essential for the regulation of enzyme synthesis. Protease production is influenced by physical factors, including temperature, pH, agitation, salinity, and inoculum concentration, in addition to carbon and nitrogen sources. These physical factors are critical regulators of enzyme production and substrate stability in the culture medium, as they can alter the chemical structure of the enzyme, resulting in denaturation and a loss of catalytic activity (Elgammal et al. 2020).

Response Surface Methodology (RSM) is employed for statistical optimization in modeling and analysis when the desired response is influenced by multiple variables, with the primary objective of optimizing this response (Eswari et al. 2016). This method facilitates the identification of significant parameters, the selection of optimal process conditions, and the evaluation of interactions between the response and key variables with fewer experiments (Tajabadi et al. 2015). The optimal carbon, nitrogen, and trace element sources were identified through the One Factor at a Time (OFAT) methodology. Afterward, the selected carbon and nitrogen sources and inoculum concentration were statistically optimized to increase protease production within the RSM framework using a Central Composite Design.

The study conducted on mangrove water bacteria obtained nine bacterial isolates from the Mangrove and Proboscis Bekantan Conservation Area (KKMB/*Kawasan Konservasi Mangrove dan Bekantan*), namely *Bacillus* spp., *Corynebacterium* spp., *Listeria* spp., *Enterobacteria* spp., *Pseudomonas* spp., *Aeromonas* spp., *Micrococcus* spp., *Staphylococcus* spp., and *Actinobacillus* spp. (Yulma et al. 2019). Bacteria capable of producing proteases come

from the genera *Bacillus*, *Pseudomonas*, *Steptobacillus*, *Proteus*, and *Staphylococcus* (Sukmawati et al. 2023). The Biotechnology Laboratory at Universitas Andalas, Padang District, West Sumatra, Indonesia has 38 isolates of mangrove water bacteria from the Mandeh Area, Pesisir Selatan District, West Sumatra, Indonesia four of which produce protease. PUA-14 bacterial isolate was used in this investigation, and it demonstrated the highest protease activity at 0.889 U/mL at an optimal temperature of 35°C and a pH of 8.0 (Alamsjah et al. 2024). Various factors, including the type of carbon and nitrogen source, metal ions, pH, temperature, inoculum concentration, and incubation duration, strongly influence microbial enzyme activity. Protease activity is substantially influenced by the media components, which depend on the microorganisms used. Therefore, it is imperative to optimize the concentrations of medium components (Enuneku et al. 2020). The prediction and optimization of parameters that influence the outcome can be achieved by applying RSM to model the factors and responses (Alhelli et al. 2016).

The objective of this investigation is to determine the optimal conditions for carbon sources, nitrogen sources, trace elements, inoculum concentrations, and to molecularly identify the type of PUA-14 bacterial isolate through 16S rRNA sequence analysis. Subsequently, the study's subjects were the optimization and molecular identification of PUA-14 bacterial isolates from mangrove waters that produce protease. The urgency of this research is to identify novel strains or varieties of bacteria that have been optimized and possess the potential for the production of protease that can be utilized in various applications.

MATERIALS AND METHODS

Tools and materials

The tools used are Petri dishes, test tubes, Erlenmeyer flasks, thermometer, pH meter, dropper pipette, beaker, tube needle, spirit lamp, analytical balance, measuring cup, microscope, aluminum foil, cotton, gauze, tissue, centrifuge, micropipette, microtips, Eppendorf tubes, platform shaker, shaker incubator, autoclave, magnetic stirrer, hotplate stirrer, basket, test tube rack, spatula, laminar air flow, spectrophotometer, ultraviolet lamp, thermal cycler (PCR machine), and electrophoresis set. The materials used are PUA-14 isolates from the Biotechnology Laboratory collection originating from mangrove waters in the Mandeh Area, Pesisir Selatan District, West Sumatra, Indonesia Nutrient Agar (NA), KH_2PO_4, K_2HPO_4, $MgSO_4$, NaCl, Tris-HCl, TCA, Na_2CO_3, $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, casein, maltose, glucose, lactose, folin ciocalteu reagent, agar, tyrosine, distilled water, alcohol, spirits, GeneJET Genomic DNA Purification Kit-ThermoFisher, primer 16S rRNA-27F (forward) (5' -AGAGTTTGATCCTGGCTCAG-3') and 1525R (reverse) (5'- GGTTACCTTGTTACGACTT-3').

Procedures

Growth profile of bacterial isolates

The PUA-14 bacterial isolate, a protease producer derived from mangrove waters, is part of the collection at the Biotechnology Laboratory, at Universitas Andalas, Padang, West Sumatra, Indonesia. Bacteria were isolated from mangrove waters in the Mandeh Area, Pesisir Selatan Regency, using serial dilution and streak plate methods on nutrient agar (NA). The rejuvenation of the PUA-14 bacterial isolate was done by streaking the bacterial isolate in a test tube containing NA medium. The protease production medium was prepared using a sterile distilled water solution containing 3 g KH_2PO_4, 3 g K_2HPO_4, 3 g $MgSO_4$, 5 g NaCl, and 10 g casein was used to create the protease basal media. The medium was put into an Erlenmeyer after being brought to a boil. After that, it was sterilized for around 15 minutes at 121℃ and 15 pounds of pressure in an autoclave. In 100 mL of production media, the isolate was inoculated in one or two rounds of slanted culture. Then, it was incubated for 24 hours at 30ºC and 150 rpm of agitation. Next, using ideal conditions of 35℃ and pH 8 from earlier studies, 5 mL of inoculum was pipetted into 95 mL of production medium on a 250 mL Erlenmeyer with 150 rpm agitation. The turbidity was measured with a spectrophotometer at λ 600 nm, ensuring the precision of the results. Then, 3 mL of bacterial culture was harvested. At λ 600 nm, turbidity was measured with a spectrophotometer. Samples of bacteria were cultured every two hours, and the process was stopped when the growth of the isolated bacteria had decreased.

Protease activity assay (Takami et al. 1989)

After pipetting 0.5 mL of casein substrate, adding 0.5 mL of enzyme solution, and adding 0.25 Tris-HCl buffer 50 mM pH 8.0, the mixture was incubated for 15 minutes at 30°C. Subsequently, 20 minutes were spent incubating at room temperature with 0.5 mL of TCA added. After centrifuging for 20 minutes at 6000 rpm, 0.375 mL of the supernatant was extracted, and 0.25 mL of 1 N Folin Ciocalteu reagent and 1.25 mL of Na_2CO_3 were added. At λ 578 nm, optical density measurements were. Similarly, blank values were determined by substituting 0.5 mL of pure water for 0.5 mL of the enzyme. Standard values were also determined by substituting 0.5 mL of 5 mM tyrosine for 0.5 mL of protease sample. This formula calculated protease enzyme activity:

$$UA = \frac{(Asp - Abl)}{(Ast - Abl)} \times \frac{1}{T} \times P$$

Where:
UA : Protease Activity Units (U/mL)
Asp : Sample absorbance
Abl : Blank absorbance
Ast : Standard absorbance
T : Incubation time (minutes)
P : Dilution

Determination of protein levels and specific enzyme activities

Preparation of Reagents: Reagent A: 2 g of Na_2CO_3 was dissolved in 100 mL of 0.1 N NaOH. Reagent B: 5 mL of 1% $CuSO_4.5H_2O$ solution was mixed with 5 mL of 1% Na(K)-tartrate solution. Reagent C: 2 mL of reagent B was added to 100 mL of reagent A. Reagent D: Follin Ciocelteau's

reagent was diluted 1:1 with distilled water. Standard Solution: BSA (Bovine Serum Albumin) solutions at concentrations of 0, 50, 75, 100, 125, 150, 175, and 200 µg/mL. Protein Level Determination: This process regulates the specific activity of the protease enzyme protein. 0.1 mL of enzyme solution was combined with 0.9 mL of distilled water, then 5 mL of reagent C. The mixture was incubated for 10 minutes at 30°C. Subsequently, 0.5 mL of reagent D was quickly added and mixed thoroughly, letting it sit for 30 minutes at room temperature. For the control, 0.1 mL of enzyme solution was replaced with 0.1 mL of distilled water and followed the same procedure as the sample. The absorbance was measured using a UV-Vis spectrophotometer at 750 nm. Enzyme protein levels were determined using the BSA standard curve. Specific enzyme activity is calculated as enzyme activity (Unit/mL) divided by protein content (mg/mL), expressed in units of Unit/mg (Tantray et al. 2022).

Determination of optimum conditions for protease production

Determination of carbon sources, nitrogen sources, and trace elements

The effects of different carbon sources, nitrogen sources, and trace elements on protease production were investigated using basal fermentation media in single-variable experiments. Each carbon and nitrogen source was tested at a concentration of 1%. The carbon sources tested included sucrose, lactose, maltose, glucose, and fructose. The nitrogen sources included KNO_3, $NaNO_3$, NH_4Cl, NH_4NO_3, and $(NH_4)_2SO_4$. Trace elements, specifically Ca, Zn, and Mg, were added at five ppm each in different trials. The impact of these components on protease production was assessed while maintaining constant other culture conditions. The prepared medium in an Erlenmeyer flask was incubated at 35°C, with an inoculation volume of 5%, a pH of 8.0, and an agitation speed of 150 rpm, and samples were taken at a specific time (idiophase). The production medium was centrifuged at 6000 rpm for 20 minutes to obtain the enzyme solution and measure enzyme activity.

Determination of carbon source concentration, nitrogen source concentration, and inoculum concentration

Optimization of protease production was carried out using Response Surface Methodology (RSM) with a Central Composite Design (CCD) in the Design Expert 13 statistical software. The treatment factors included varying the carbon source concentration (A) from 0.5% to 2.5%, the nitrogen source concentration (B) from 0.5% to 2.5%, and the inoculum concentration (C) from 2.5% to 12.5%. Several Erlenmeyer flasks containing the protease production medium were prepared, and the bacterial isolate inoculum was added. The inoculum medium was agitated at 150 rpm at 30°C for 24 hours. Each inoculum was then pipetted into the protease production medium based on the RSM-determined variations in carbon source concentration, nitrogen source concentration, and inoculum concentration. The production medium was incubated under the optimized conditions and sampled at a specific time (idiophase). It was then centrifuged at 6000 rpm for 20 minutes to obtain the enzyme solution and measure enzyme activity.

Characterization of bacteria

The characterization of bacteria included both macroscopic and microscopic observations. Macroscopic analysis focused on colony morphology, while microscopic observations encompassed Gram staining and endospore staining. In Gram staining, bacterial cells were fixed on glass slides, treated sequentially with crystal violet, lugol solution, and safranin, and then observed under a microscope; Gram-positive bacteria appeared purple, while Gram-negative bacteria appeared red. Endospore staining, conducted on Gram-positive rod-shaped bacteria, involved staining with malachite green under heat and counterstaining with safranin to identify endospores. Additional tests included the catalase test, where bubble formation upon the addition of 3% H_2O_2 indicated a positive result, and the motility test, in which bacterial growth spreading through the SIM medium confirmed motility. These methods provided comprehensive insights into the bacterial morphology and physiological characteristics (Welsh 2019).

Molecular identification

The molecular analysis comprised several steps to isolate and characterize bacterial DNA. First, DNA was extracted using the GeneJET Genomic DNA Purification Kit, where bacterial samples were treated with lysis buffers, proteinase K, and ethanol, followed by centrifugation and purification. Next, the DNA was amplified via PCR using specific 16S rRNA primers (27F and 1525R) with a "forward primer" (5'- AGAGTTTGATCMTGGCTCAG-3') and a "reverse primer" (5'- GGTTACSTTGTTACGACTT-3') under optimized thermal cycling conditions. The PCR products were analyzed via agarose gel electrophoresis, visualized with GelDoc, and compared against a DNA marker. Sequencing of the PCR products was performed at 1st Base Sequencing Services in Malaysia using bidirectional sequencing. The resulting forward and reverse sequences were assembled using DNA STAR software to produce complete DNA sequences. These sequences were then compared with GenBank data using BLAST to confirm their similarity to registered sequences. Finally, phylogenetic analysis was conducted using MEGA software, employing the Neighbor-Joining method with bootstrap replication to construct phylogenetic trees, providing insights into the evolutionary relationships of the bacterial isolates (Tamura et al. 2021).

Data analysis

The data obtained were analyzed descriptively and presented in subsequent tables and figures. The analysis was carried out by referring to existing references through several observation parameters, namely isolates of protease-producing bacteria, optimization using the Response Surface Methodology (RSM) type Central Composite Design (CCD) on the Design Expert 13 statistical software, and molecular identification by 16S rRNA sequence analysis.

RESULTS AND DISCUSSION

Growth profiles and specific protease activity

Observations of the growth profile and specific protease activity of PUA-14 bacterial isolates are presented in Figure 1. Figure 1 illustrates the growth profile and specific protease activity of the bacterial isolate PUA-14. By the second hour, PUA-14 undergoes adaptation and transitions to the logarithmic phase, which culminates at the 20th hour.

Optimum conditions for protease production

The optimum carbon source for protease production in PUA-14 bacterial isolates is shown in Figure 2. Figure 2 shows the influence of various carbon sources (fructose, glucose, lactose, maltose, and sucrose) on the specific protease activity of PUA-14 bacterial isolates. Lactose yields the highest activity at 2.34 U/mg.

Optimum nitrogen source

The optimum nitrogen source for protease production in PUA-14 bacterial isolates is shown in Figure 3. Figure 3 shows that the nitrogen source $NaNO_3$ yields the highest specific protease activity at 6.68 U/mg for PUA-14 bacterial isolates. It provides a suitable nitrogen source for protein synthesis and accelerates cell growth.

Optimum trace elements

The optimum trace elements for protease production in PUA-14 bacterial isolates are shown in Figure 4. The histogram shows that the highest specific protease activity for PUA-14 bacterial isolates, at 7.27 U/mg, was achieved with the trace element Zn (zinc).

Optimization of lactose concentration, $NaNO_3$ concentration, and inoculum concentration

Optimization of lactose concentration, $NaNO_3$ concentration, and inoculum concentration was based on combination randomization regulated by Response Surface Methodology (RSM) with a Central Composite Design (CCD) experimental setup in the Design Expert 13 statistical software. Table 1 presents the combination of lactose concentration (A), $NaNO_3$ concentration (B), and Inoculum Concentration (C) obtained from 20 treatments.

Figure 5 shows specific protease activity for PUA-14 bacterial isolates. The highest activity, 9.293 U/mg, is red at a lactose concentration of 0.5%, $NaNO_3$ concentration of 2.5%, and inoculum concentration of 2.5%. The blue area indicates lower activity, while the red indicates higher one.

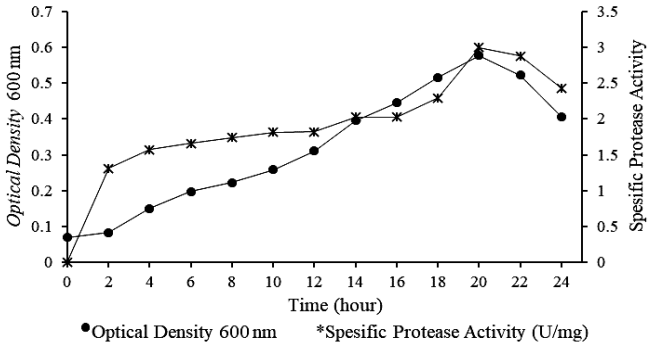


Figure 1. Growth profile and specific protease activity of PUA-14 bacterial isolates

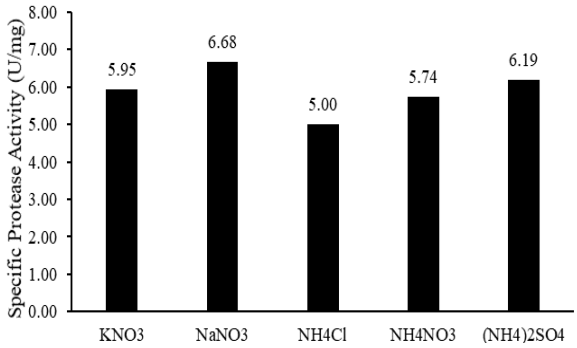


Figure 3. Effects of different nitrogen sources on protease production by PUA-14 bacterial isolate

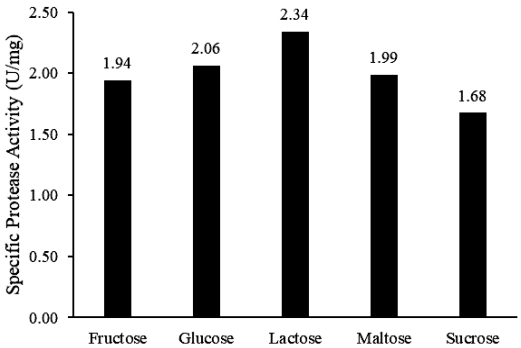


Figure 2. Effects of different carbon sources on protease production by PUA-14 bacterial isolate

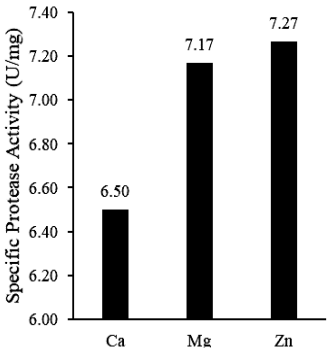


Figure 4. Effects of different trace elements on protease production by PUA-14 bacterial isolate

Table 1. Results of optimization of lactose concentration, $NaNO_3$ concentration, and inoculum concentration for protease production using Central Composite Design (CCD)

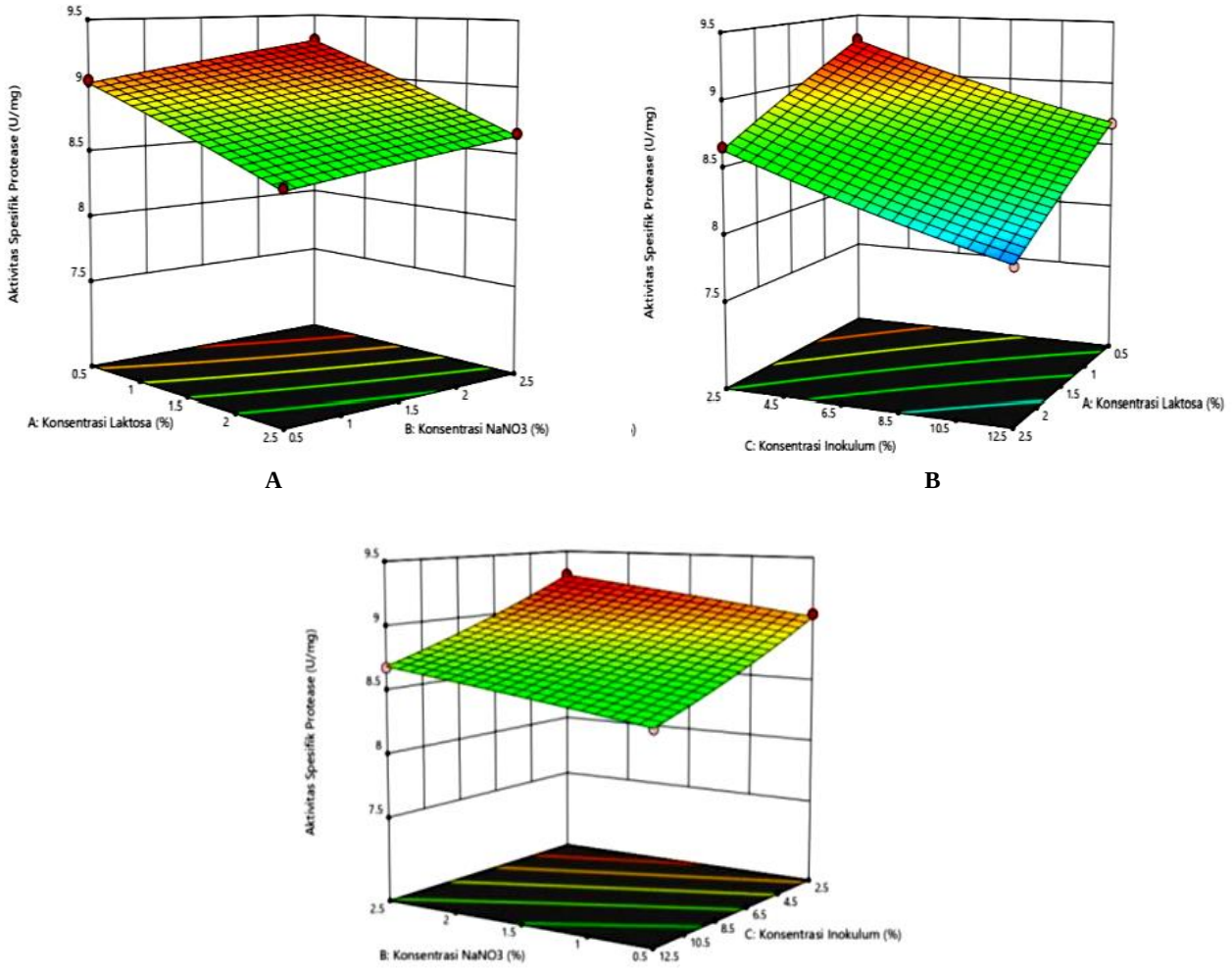
Lactose conc. (%)	$NaNO_3$ conc. (%)	Inoculum conc. (%)	Specific protease activity (U/mg)
1.5	0.5	7.5	8.345
2.5	0.5	12.5	7.746
1.5	1.5	7.5	8.486
1.5	2.5	7.5	8.628
0.5	0.5	12.5	8.394
1.5	1.5	7.5	8.528
1.5	1.5	7.5	8.513
1.5	1.5	12.5	8.255
2.5	2.5	12.5	7.963
1.5	1.5	7.5	8.502
0.5	0.5	2.5	9.042
1.5	1.5	7.5	8.486
1.5	1.5	2.5	8.793
2.5	2.5	2.5	8.655
0.5	1.5	7.5	8.817
2.5	0.5	2.5	8.438
0.5	2.5	12.5	8.677
0.5	2.5	2.5	9.293
1.5	1.5	7.5	8.608
2.5	1.5	7.5	8.141

Protease-specific activity conditions before and after optimization

Observations of protease-specific activity conditions before and after optimization are presented in Table 2. Table 2 shows that the specific protease activity of PUA-14 bacterial isolate increased by 3.1 times after optimization. Before optimization, protease production occurred at 35°C, pH 8, without added carbon, nitrogen, or trace elements. After optimization, production conditions included 0.5% lactose, 2.5% $NaNO_3$, trace element Zn, and 2.5% inoculum concentration.

Table 2. Specific protease activity conditions of bacterial isolate PUA-14 before and after optimization

Specific protease activity (U/mg)		Increased specific protease activity (times)
Before optimization	After optimization	
2.995	9.293	3.1



A **B** **C**

Figure 5. Response surface plots form three-dimensional (3D) Protease Specific Activity (U/mg) against optimization. A. Lactose concentration; B. $NaNO_3$ concentration; C. Inoculum concentration

Characterization of bacteria

Based on observations made on the PUA-14 bacterial isolate, partial characteristics of this isolate were obtained, including macroscopic observations of yellowish-white colony color, circular colony shape, raised colony elevation, and entire colony margin. Microscopic observation shows that the cells are *Bacillus*, Gram-positive, and have endospores. The motility test results showed that the bacteria were motile. A catalase test was also carried out, and a positive outcome for catalase was obtained (Figure 6).

Molecular identification

PUA-14 bacterial isolate is identified as *Bacillus pseudomycoides* through 16S rRNA gene sequence analysis using a universal primer. PCR amplification produced a 1500 bp product, matching the expected size based on the primer used. BLAST analysis of the nucleotide sequence of PUA-14 against ten isolates in the gene bank revealed a similarity of 98.52% to 98.68% with an E-value of 0.0, indicating a solid alignment. The base composition of the 16S rRNA gene of PUA-14 was found to be approximately 20.40% T(U), 21.01% C, 26.99% A, and 31.60% G. Phylogenetic analysis conducted using Neighbor-Joining (NJ) in MEGA software version 10.2.2 showed that the PUA-14 isolate is closely related to *B. pseudomycoides* strain LB-AsDX1-3, with a similarity of 98.68% (Figure 7). This is also supported by p-distance data between PUA-14 isolates and *B. pseudomycoides*. P-distance in the gene bank is 0.000, which means there is no evolutionary distance between the two. Neighbor-Joining (NJ) analysis is the basis for making a phylogenetic tree based on the difference between two sequences where a phylogenetic tree with a high bootstrap value of at least 70%.

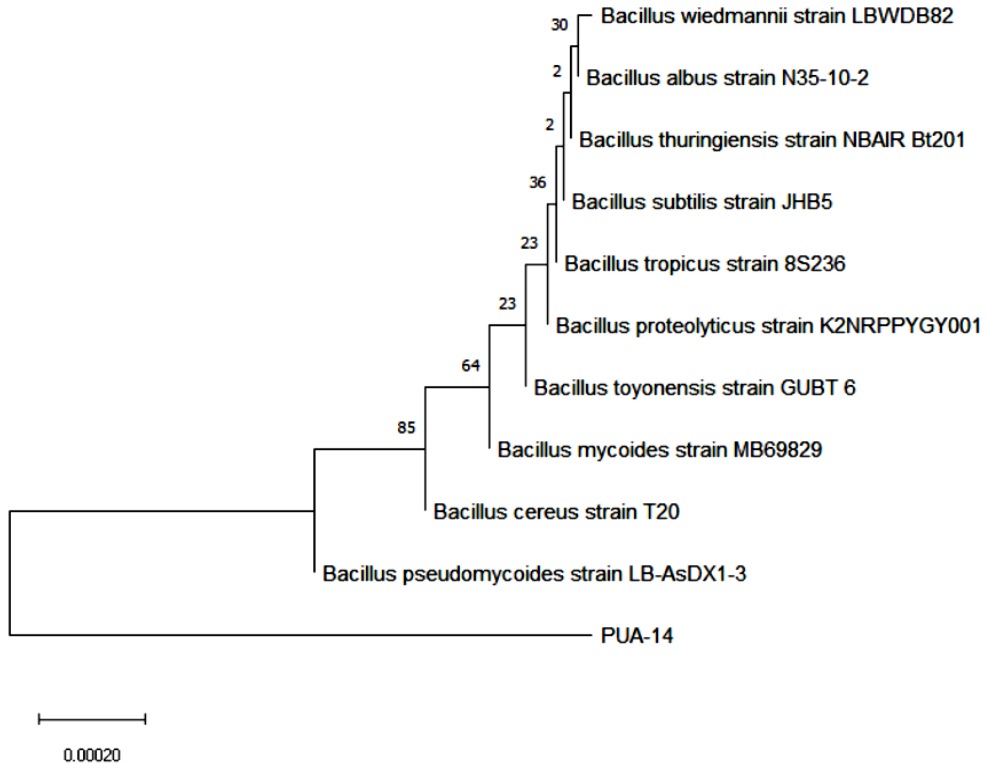


Figure 7. Phylogenetic tree of PUA-14 bacterial isolates using Neighbor-Joining (NJ) analysis based on 16S rRNA sequences

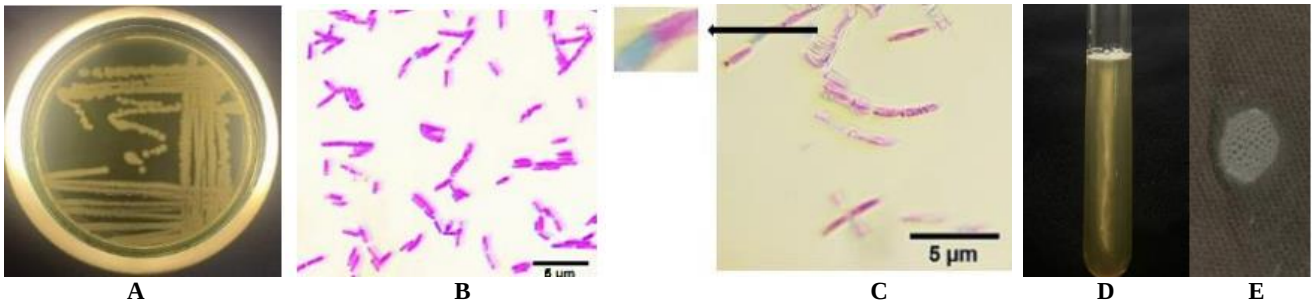


Figure 6. A. Macroscopic observation of PUA-14 bacterial isolates; B. Gram staining; C. Endospore staining; D. Motility test; E. Catalase test

Discussion

Bacterial growth is characterized by the following phases (i) the lag phase (adaptation); (ii) the exponential phase (log); (iii) the stationary phase; and (iv) the mortality phase (Agustien et al. 2024). Suberu et al. (2019) observed that bacterial adaptation is rapid as a result of similar media conditions, which is consistent with rapid growth. The logarithmic phase is characterized by the most rapid cell division and active metabolism, which are facilitated by an abundance of nutrients (Sulistijowati and Harmain 2021). Protease production commences at the 2nd hour, increases until the 20th hour, and then decreases after the 22nd hour as a result of substrate depletion and enzyme structural changes (Chen et al. 2021). The death phase occurred at the 22nd hour, and no stationary phase was detected, which is likely due to elevated metabolic activity (Jaishankar and Srivastava 2017).

Growth profiles can be determined through turbidity measurements conducted with a spectrophotometer (Pagarra et al. 2020). The transition from the logarithmic phase to the stationary phase is identified by monitoring the growth profile, which indicates the zenith of enzyme production (Alamsjah et al. 2024). Protease production reaches its maximum during the exponential or early stationary phase, which is a phenomenon strongly influenced by elevated metabolic activity and nutrient availability (Purkan et al. 2014). The exponential phase, during which metabolic activity is at its peak, is associated with optimal enzyme production (Blanco et al. 2016).

Lactose enhances protease activity through several mechanisms, including the action of β-galactosidase, which hydrolyzes lactose into glucose and galactose, providing more substrates for protease-related metabolic pathways (Nelson and Cox 2017). Additionally, lactose activates the lac operon system, lifting repression on genes encoding protease enzymes (Nelson and Cox 2017). Studies by Patel et al. (2019) also indicate that lactose is an effective carbon source for boosting protease production in *Bacillus subtilis* and *Bacillus licheniformis*. Conversely, ammonium chloride (NH_4Cl) results in the lowest specific protease activity, consistent with Sudha et al. (2018), who found NH_4Cl to have the lowest protease activity among nitrogen sources. Certain nitrogen compounds can directly interact with the enzyme, modifying its structure or active function, which in turn inhibits protease activity (Colla et al. 2023). Zn helps form and maintain protein structures and aids bacterial survival under stress. Activators like Zn increase enzymatic reaction rates, while inhibitors reduce enzyme activity (Dow et al. 2023). Khaswal et al. (2022) found that trace elements Zn and Ca boost protease activity in *Geobacillus* sp. strain GS53.

Response Surface Methodology (RSM) optimizes experimental conditions by considering multiple factors simultaneously, which are visually represented in contour and response surface plots. Here, specific protease activity (y) is influenced by lactose concentration (x1), $NaNO_3$ concentration (x2), and inoculum concentration (x3). $NaNO_3$ concentration is a critical factor in the stimulation of protease production. The ANOVA analysis indicates that the model is statistically significant, with a p-value of <0.0500.

Protease production by microorganisms is influenced by media components, mainly carbon and nitrogen sources, which regulate enzyme synthesis. Inoculum concentration also affects protease activity and substrate stability in the culture medium by impacting the chemical structure of the enzyme (Agustien et al. 2024). Carbon and nitrogen sources are essential for microbial growth and enzyme production (Sharma et al. 2017). Nitrogen supports cell growth, while carbon boosts biosynthetic energy. Optimal enzyme production requires specific inoculum concentrations, with smaller concentrations recommended for better protease production (Limkar et al. 2019). RSM optimization enhances protease production by interacting with various operational variables (Kumari et al. 2016). Subaru et al. (2019b) identified optimal protease production in *Bacillus cereus* ABBA1 using 1.5% carbon source, 2% nitrogen source, and 2.5% inoculum concentration, resulting in 159.43 U/mL protease activity. Similar studies include Aznia et al. (2014), which increased protease activity by 1.75 times in M5-24 isolates from hot springs, and (2008), which reported a 1.5 times increase in *Bacillus circulans* from soil samples. Chandramohan et al. (2019a) achieved a 3-fold increase in protease production from *B. pseudomycoides* strain MA02, with enzyme activity reaching 284 U/mL. Systematic optimization can significantly enhance enzyme activity, highlighting the importance of optimizing culture media conditions to boost enzyme activity from mangrove water bacteria (Alamsjah et al. 2024).

Mangrove waters have been relatively unexploited, and their utilization in numerous fields of biotechnology also remains unexploited. Because of their unique nature, their biological sources organisms have the ability to produce enzymes with unique properties compared to the same enzymes from terrestrial organisms (Homaei et al. 2016). The protease demonstrated activity on various modified (azocasein) and natural protein substrates, with the highest activity observed on casein. The protease can hydrolyze diverse protein sources, breaking them down into smaller peptides and amino acids. This indicates that the enzyme has a broad substrate specificity and the capability for extensive proteolysis (Dadshahi et al. 2016).

Observations of the PUA-14 bacterial isolate revealed yellowish-white, circular, raised colonies with entire margins. Microscopically, the cells are Gram-positive bacilli with endospores and motile. A positive catalase test was also obtained. Gram staining confirmed the Gram-positive nature of PUA-14, indicating thick cell walls rich in peptidoglycan, which retain the purple dye. Endospore staining showed terminal endospores, which are heat- and chemical-resistant and require special techniques. The motility test confirmed active movement, evidenced by spreading bacterial growth after media puncturing. The positive catalase test indicated the presence of the catalase enzyme, as shown by the air bubbles when H_2O_2 was added, classifying the bacteria as aerobic. This enzyme helps neutralize toxic H_2O_2, allowing aerobic bacteria to survive (Tantray et al. 2022).

Phylogenetic analysis in Figure 7, based on the Neighbor-Joining (NJ) method with 1000x bootstrap, was performed using MEGA software version 11. The PUA-14 bacterial isolate is closely related to *B. pseudomycoides* strain LB-AsDX1-3, with a similarity of 98.68% and a p-distance of 0.00, indicating no evolutionary distance. NJ analysis, which constructs phylogenetic trees based on sequence differences, considers a bootstrap value above 70% good (Kapli et al. 2020). *B. pseudomycoides* are Gram-positive rods, 3-5 μm long, single or in short chains, with white to cream colonies. They produce catalase, hydrolyze starch, casein, tyrosine, and lecithin, and grow between 15°C and 40°C, primarily isolated from soil (Chandramohan et al. 2019b). The *Bacillus* genus is a versatile microorganism with broad applications in both traditional fermentation and modern biotechnology. In traditional fermented foods, *Bacillus* species function as starter cultures, while in biotechnology, they are valued for producing enzymes, bioactive peptides, and antibiotics. *Bacillus* is also recognized as a probiotic (Todorov et al. 2022). Additionally, Bacillus can adhere to intestinal epithelial cells and exhibits significant proteolytic activity against food proteins, such as gelatin and milk, making it a promising probiotic strain for digesting food proteins (Wang et al. 2021). *Bacillus* species are also used in bacterial concentrates to reduce odors, eliminate pathogenic bacteria, and serve as potential probiotics (Lubkowska et al. 2023). The development of molecular biology techniques and metagenomic approaches has the potential to facilitate the identification of novel genes, thereby enhancing efficiency and broadening their applicability across various fields. With continuous advancements in this area, significant progress in biotechnology can be anticipated in the future (Sharifian et al. 2018).

In conclusion, the research findings indicate that the optimum conditions for the protease enzyme activity of the bacterial isolate PUA-14 effectively with 0.5% lactose as the carbon source, 2.5% $NaNO_3$ as the nitrogen source, the presence of Zn as a trace element, and an inoculum concentration of 2.5%. Based on the molecular identification of the 16SrRNA gene, it can be concluded that the PUA-14 bacterial isolate producing protease from mangrove waters exhibits similarities to the *B. pseudomycoides* strain LB-AsDX1-3. These findings provide a foundation for future studies and industrial applications of microbial proteases.

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