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Highlighting Protease-Producing Bacteria from The Shrimp Wastewater Treatment Plant as Potential Bioremediation Agents

Devi Oktavia Anjani[1,2], Wilis Ari Setyati[1*], Delianis Pringgenies[1]

[1]Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Diponegoro, Jl. Prof. H. Soedarto, S.H, Semarang, Central Java, 50275, Indonesia; [2]Department of Aquatic Resources Management, Faculty of Fisheries and Marine Science, Universitas Diponegoro, Jl. Prof. H. Soedarto, S.H, Semarang, Central Java, 50275, Indonesia

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Abstract

Shrimp cultivation waste comprises uneaten feed, residual material from digestion, exoskeletons, and metabolic byproducts. This waste is considered a potential source of bioremediation agents due to the abundance of decomposing bacteria. This study aimed to evaluate the growth kinetics and proteolytic enzyme activity of bacteria isolated from shrimp pond waste for their potential use in bioremediation. Water samples were collected from the wastewater treatment plant of a shrimp cultivation facility at the Diponegoro Science Techno Park, Jepara, Indonesia. The methods used in this study included bacterial isolation and purification, screening for protease-producing bacteria, observation of bacterial growth kinetics, measurement of protein content and total protease activity, and molecular identification using 16S rRNA gene sequencing. The results showed that 6 out of 37 bacterial isolates exhibited proteolytic activity. Two isolates, namely IT.2 and IC.1, were selected as the most promising candidates based on their high proteolytic index values (PI > 5). Growth kinetics analysis revealed that both IT.2 and IC.1 underwent logarithmic, stationary, and death phases within a 48-hour cultivation period. Isolate IT.2 exhibited the highest protease activity at 40.92 U/mL, while IC.1 reached 39.65 U/mL. Molecular identification indicated that IT.2 and IC.1 have the closest similarity to *Vibrio fluvialis* and *Pseudoalteromonas piscicida*, respectively. These findings demonstrate that bacteria isolated from shrimp pond waste have significant potential as bioremediation agents to mitigate pollution in aquaculture environments.

Keywords: Bacteria, bioremediation agent, growth kinetics, protease enzyme

1. Introduction

The high market demand for shrimp has driven a significant increase in shrimp farming operations in Indonesia (KKP, 2020). This expansion in aquaculture has led to greater volumes of waste being discharged into surrounding environments. Waste generated during the cultivation process includes undigested feed, shrimp excreta, exoskeletons, and various metabolic byproducts. The amount of waste produced is closely linked to the shrimp stocking density. According to Tampangallo et al. (2020), at a stocking density of 500 individuals/m², waste levels can reach 50.12 g of total nitrogen (TN), 15.73 g of total phosphorus (TP), and 126.85 g of carbon (C) per kilogram of feed.

Feed serves as the primary nutritional source for cultured shrimp; however, only about 22% of the total feed provided is effectively absorbed and converted into shrimp biomass. The remainder is lost in various forms: 57% dissolves in water, 14% settles at the pond bottom, and 7% is consumed by microorganisms (Jackson et al., 2003).

Protein, the most significant component of waste in shrimp ponds, undergoes degradation through aerobic and anaerobic pathways (Sanjaya et al., 2023). Under aerobic conditions, nitrogen-rich proteins are broken down into carbon dioxide (CO_2), water, and ammonium (NH_4^+), a process that increases the biological oxygen demand (BOD) in the pond. When oxygen becomes depleted, degradation proceeds anaerobically, producing ammonia (NH_3), which is highly toxic to aquatic organisms and poses a serious threat to coastal ecosystems (Xu et al., 2021).

Aerobic biodegradation agents are crucial for breaking down proteins into simpler compounds, thereby minimizing the accumulation of harmful anaerobic byproducts. Gichana et al. (2018) reported that microorganisms are effective agents for organic waste degradation and are widely used in aquaculture waste management. The addition of such microorganisms facilitates the transformation of organic matter into less harmful compounds that can be further processed by the natural environment.

Protease enzymes play a vital role in protein degradation by hydrolyzing complex peptide bonds into simpler peptides or free amino acids (Thanoon et al., 2018). This enzymatic process enhances the functional, biological, and nutritional properties of the resulting compounds. Proteases are extensively used across various industries, including pharmaceuticals, leather production, detergents, food processing, and waste treatment (Sharma et al., 2019). These enzymes can be sourced from animals,

* Corresponding author. e-mail: wilisarisetyati@yahoo.com.

plants, or microorganisms, with bacteria being the most commonly used due to their rapid growth, ease of cultivation, consistent enzyme yields, and suitability for large-scale production (Song et al., 2023). Microbial sources contribute to approximately 66% of global protease production, accounting for nearly 40% of optimized compounds used in industrial processes (Sharma et al., 2019).

Shrimp pond waste, rich in protein and other nutrients, provides a favorable environment for bacterial growth. Ariaeenejad et al. (2022) reported that bacteria isolated from various liquid and solid waste sources can degrade protein-rich materials, dissolve proteins, and survive extreme environmental conditions. Leiva-Portilla et al. (2023) further demonstrated the potential of shrimp pond-associated bacteria to produce bioactive compounds, such as antioxidants, through enzymatic hydrolysis.

Given this context, further investigation is warranted to explore the protease-producing capabilities of bacteria isolated from shrimp pond waste. Hence, the present study evaluated the enzymatic activity and growth kinetics of these bacteria in degrading proteins, thereby assessing their potential as bioremediation agents.

2. Method

2.1. Sampling

Water samples were obtained from the tanks of a wastewater treatment plant (WWTP) at a shrimp cultivation facility located at Diponegoro Science Techno Park, Jepara, Indonesia (6°37'8.18"S, 110°38'25.58"E). *Litopenaeus vannamei* was the species cultivated at the facility. Two bottle samples were collected from two different wastewater tanks, each containing 45 mL of wastewater, and stored securely in Falcon tubes. The samples were kept in a cool box and transported to the laboratory for further bacterial isolation.

2.2. Bacterial Isolation

The wastewater samples were serially diluted up to a dilution factor of 10^{-5} (Al-Zereini, 2014). Bacteria were isolated using the pour plate method, where 1 mL of the 10^{-5} dilution was added to a sterile Petri dish. Sterile Zobell Marine Agar (ZMA) medium was then poured into the dish. The inoculated Petri dishes were incubated at 37°C for 2-3 days.

2.3. Purification and Characterization of Bacterial Isolates

Bacterial isolates were purified and characterized. The isolates that appeared were streaked onto fresh media using the four-quadrant streaking method. Morphological characterization was performed by observing colony size, shape, margin, elevation, and color on the culture media (Zebua et al., 2020).

2.4. Proteolytic Activity Screening

Proteolytic activity was screened following the method described by Setyati et al. (2023). The screening medium was prepared by adding 1% skim milk to Zobell Marine Agar. Previously cultured bacterial isolates were inoculated onto sterile blank paper discs, which were then placed on top of the prepared medium. The Petri dishes were incubated for 48 hours, with observations made every 24 hours. Proteolytic activity was indicated by the formation of a clear zone surrounding the paper disc and bacterial colony.

2.5. Bacterial Growth Kinetics

The growth kinetics of protease-producing bacterial isolates were evaluated using time-course optical density measurements in liquid culture. Isolates exhibiting the highest proteolytic activity were selected and cultured in Zobell Broth. A single colony from each isolate was inoculated into 10 mL of Zobell Broth and incubated at 30°C with constant shaking at 110 rpm for 18-24 hours to obtain a preculture. A 1% (v/v) inoculum from the preculture was transferred into 250 mL of sterile Zobell Broth in a 500 mL Erlenmeyer flask. The cultures were incubated at 30°C in a shaker incubator at 110 rpm for 48 hours.

Bacterial growth was monitored by measuring optical density at 600 nm (OD_{600}) using a UV-Vis spectrophotometer at predetermined time intervals: 0, 2, 4, 6, 12, 18, 24, 30, 36, 42, and 48 hours. At each time point, 4 mL of the culture was aseptically withdrawn into a sterile Falcon tube and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded, and the remaining biomass was resuspended in 4 mL of sterile phosphate-buffered saline (PBS). The mixture was homogenized thoroughly, and OD_{600} readings were recorded to assess bacterial growth (Nguyen-Sy et al., 2020).

2.6. Measurement of Protease Enzyme Activity

Protease enzyme activity was measured using the casein assay method, following the protocols described by Anh et al. (2021) and Sher et al. (2011), with slight modifications. Bacterial cultures were prepared as described in the bacterial growth kinetics section. Enzyme activity was evaluated at intervals of 0, 2, 4, 6, 12, 18, 24, 30, 36, 42, and 48 hours. At each time point, 4 mL of culture was aseptically withdrawn into a sterile Falcon tube and centrifuged at 3000 revolutions per minute (rpm) for 10 minutes. The resulting supernatant was collected and used as the crude enzyme extract.

To perform the assay, 0.2 mL of the crude enzyme extract was transferred into a clean test tube. Afterward, 1 mL of Tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) buffer and 0.5 mL of a 1% (w/v) casein substrate were added. The mixture was vortexed and incubated at 37°C for 10 minutes to allow the enzymatic reaction to proceed. The reaction was stopped by adding 1 mL of 10% trichloroacetic acid (TCA) solution. The mixture was then centrifuged at 10,000 rpm for 10 minutes. From the resulting solution, 0.5 mL of the supernatant was transferred to a new test tube and mixed with 2.5 mL of 0.5 M sodium carbonate (Na_2CO_3) solution. This mixture was incubated at 37°C for 10 minutes.

After incubation, 1 mL of Folin-Ciocalteu reagent was added, and the solution was mixed thoroughly. The reaction mixture was incubated in the dark for 30 minutes. Absorbance was measured at 660 nanometers (nm) using a UV-Vis spectrophotometer. A blank control was prepared using the same procedure, substituting distilled water for the enzyme extract. Absorbance values were converted to micromoles of tyrosine using a tyrosine standard curve.

Protease activity was calculated using the following formula (Bhavikatti et al., 2020):

$$AP = \frac{mM}{(T \times V)}$$

(Source: Bhavikatti et al., 2020)

Notes:

AP = Protease enzyme activity (Units per milliliter, U/mL)

mM = Micromoles of tyrosine released (equivalent to µmol/mL)

T = Incubation time (minutes)

V = Volume of enzyme used (mL)

2.7. Protein Content Measurement

Protein content was determined using the Bradford assay method, originally developed by Bradford (1976) and later modified by Kruger (2009). This colorimetric method relies on the binding of Coomassie Brilliant Blue G-250 dye to proteins. A total of 400 µL of the crude enzyme extract (i.e. the supernatant from the centrifuged bacterial culture) was transferred into a clean reaction tube. Then, 4 mL of diluted Bradford reagent was added, and the mixture was vortexed to ensure uniform mixing. The solution was incubated at room temperature for 10 minutes to allow color development. After incubation, the absorbance was measured at 595 nm using a UV-Vis spectrophotometer. Protein concentrations were calculated based on a standard curve generated using Bovine Serum Albumin (BSA), and results were expressed in milligrams per milliliter (mg/mL).

2.8. DNA Extraction

DNA extraction from bacterial isolates obtained from shrimp pond wastewater was performed using the Presto™ Mini gDNA Bacteria Kit (Geneaid Ltd., Taiwan), following the manufacturer's protocol.

2.9. Polymerase Chain Reaction (PCR)

PCR amplification was conducted using universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′). Each 25 µL PCR reaction mixture contained 1 µL of 27F primer (10 µM), 1 µL of 1492R primer (10 µM), 12.5 µL of MyTaq Green Mix, 9.5 µL of DDH_2O, and 1 µL of DNA template. Thermal cycling was performed with the following conditions: initial denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension step at 72°C for 3 minutes. Amplification products were verified through electrophoresis on a 1% agarose gel, run at 100 V for 30 minutes.

2.10. Sequencing

DNA sequencing was performed using the Sanger sequencing method. The sequencing data were analyzed using MEGA 11 Software. Bacterial species were identified by comparing the obtained nucleotide sequences against the NCBI GenBank database (https://www.ncbi.nlm.nih.gov) using the BLAST (Basic Local Alignment Search Tool) algorithm.

3. Results

3.1. Bacterial Isolation

A total of 37 isolates were obtained from the isolation and purification of two wastewater samples. Of these, 21 isolates were obtained from water samples taken from Tank A of the WWTP and were assigned the isolate code "IT." Similarly, 16 isolates were obtained from Tank C of the WWTP and were assigned the code "IC."

3.2. Proteolytic Enzyme Activity

The screening results showed that six isolates exhibited proteolytic activity after 48 hours of observation. The isolate with the highest proteolytic index (PI) at the end of the observation period was IT.2 (5.83), followed by IC.1 (5.51), IC.5 (4.11), IT.9 (3.95), IT.1 (3.00), and IC.8 (2.82), which had the lowest PI. The clear zone diameter and the proteolytic index for each isolate are presented in Table 1.

Table 1. Clear Zone Diameter and Proteolytic Index of Bacterial Isolates from Shrimp Pond Wastewater

Isolate Code	Proteolytic Activity Assay			
	Clear Zone Diameter		Proteolytic Index (PI)	
	24h	48h	24h	48h
IT.1	14.7±0.084	17.975±0.7	2.45	3.00
IT.2	34.9±0.42	35±0.28	5.82	5.83
IT.9	13.95±0.63	23.7±0.42	2.33	3.95
IC.1	21.5±0.42	33.05±0.49	3.58	5.51
IC.5	11.35±0.63	24.65±0.49	1.89	4.11
IC.8	13.18±0.48	16.895±0.27	2.20	2.82

3.3. Bacterial Growth Kinetics

Growth kinetics were observed for isolates IT.2 and IC.1, demonstrating the highest proteolytic activity. The results of the bacterial growth measurements are shown in Figure 1 and Table 2. Both isolates underwent logarithmic, stationary, and death phases over the 48-hour observation period.

Table 2. Growth Rate and Generation Time of Isolate IT.2 and Isolate IC.1

Isolate Code	Growth Rate (hr^{-1})	Generation Time (hours)
IT.2	0.24	2.83
IC.1	0.26	2.68

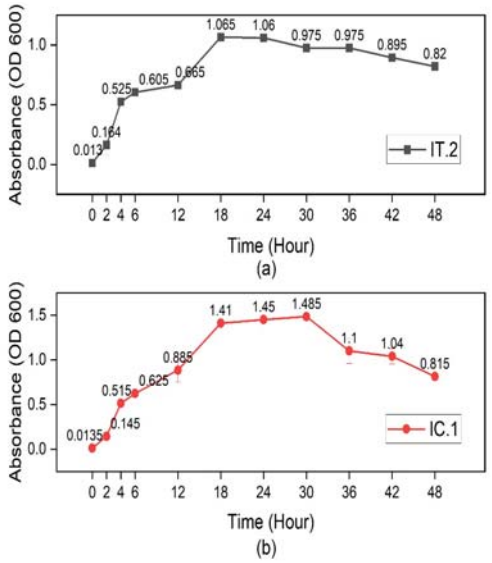

Figure 1. Growth Kinetics Curve of Isolate IT.2 (a) and Isolate IC.1 (b)

Isolate IT.2 exhibited a slower growth rate (0.24 h^{-1}) and longer generation time (2.83 hours) compared to isolate IC.1, which had a growth rate of 0.26 h^{-1} and a generation time of 2.68 hours. The cell density of IT.2 increased until the 24th hour, after which it gradually declined. As shown in Figure 1, isolate IT.2 reached its peak density at 24 hours (1.06±0.02). In contrast, isolate IC.1 followed a similar growth trend but peaked at 30 hours (1.485±0.007) before declining.

3.4. Protease Enzyme Activity

Isolate IT.2 showed increasing protease enzyme production, reaching a peak of 40.92 U/mL at the 30th hour. However, enzyme activity significantly declined afterward, reaching 17.67 U/mL by the 48th hour. Conversely, isolate IC.1 exhibited a continued increase in enzyme activity, peaking at 39.65 U/mL at the 42nd hour, with a slight decrease to 35.91 U/mL by the 48th hour. The protease enzyme production profiles of both isolates are illustrated in Figure 2.

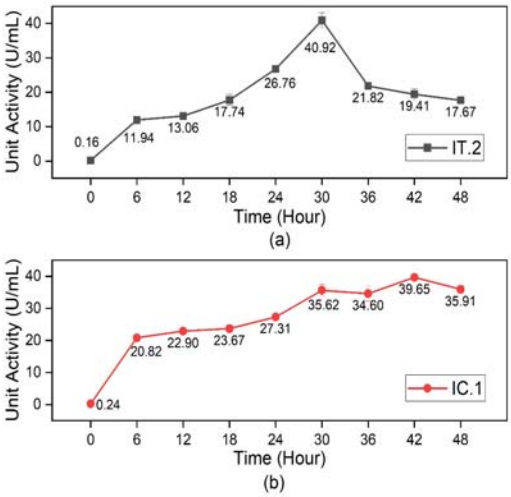

Figure 2. Protease Enzyme Production by Isolate IT.2 (a) and Isolate IC.1 (b)

3.5. Protein Content

The protein content in the growth medium was measured throughout the observation period, and the results are shown in Figure 3. Both isolates exhibited a reduction in protein concentration from the beginning to the end of the experiment. For isolate IT.2, the protein content decreased from 0.258 mg/mL at 0 hours to 0.122 mg/mL at 48 hours. Similarly, the protein content in the medium for isolate IC.1 declined from 0.317 mg/mL at 0 hours to 0.123 mg/mL after 48 hours.

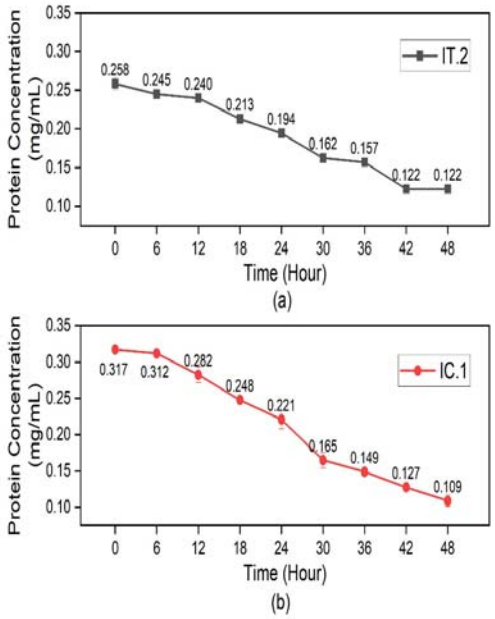

Figure 3. Protein Hydrolysis by Isolate IT.2 (a) and isolate IC.1 (b)

3.6. Bacterial Identification

Based on BLAST analysis, isolate IT.2 was identified as *Vibrio fluvialis* with a 99.65% similarity. Meanwhile, isolate IC.1 showed the closest match to *Pseudoalteromonas piscicida*, with a similarity of 99.76%. The detailed BLAST results of the bacterial sequences are presented in Table 4.

Table 4. BLAST Identification Results for Isolates IT.2 and IC.1

Isolate Code	Closest Similarity	Percent Identification	Query Coverage	Accession Number
IT.2	*Vibrio fluvialis*	99.65%	100%	PQ326252.1
IC.1	*Pseudoalteromonas piscicida*	99.76%	100%	PQ326253.1

4. Discussion

Out of the 37 bacterial isolates obtained, six demonstrated proteolytic enzyme activity, as presented in Table 1. The proteolytic index (PI) and the average diameter of the proteolytic zone for these six isolates increased between the 24-hour and 48-hour observations. Bacterial colonies produced protease enzymes that hydrolyzed proteins in skim milk into simpler peptides and amino acids. This process involved the insertion of water

molecules into the CO-NH peptide bonds, leading to their cleavage and the transformation of solid casein into a liquid or hydrolyzed form (Razzaq et al., 2019). Although all bacteria contain intracellular protease enzymes, not all are capable of producing extracellular proteases. These extracellular enzymes are secreted by bacteria to degrade complex polymers such as proteins, polysaccharides, and lipids present in their external environment. This enzymatic activity allows bacteria to access nutrients from surrounding organic materials (Rosazza et al., 2023).

Among the tested isolates, IT.2 and IC.1 displayed the highest proteolytic index values at the end of the observation period (PI > 5), signifying strong proteolytic activity. A PI value above 3.5 is generally considered high. This classification is supported by Ayuningrum et al. (2022), who defined PI values less than 1.5 as low, between 1.5 and less than 3.5 as moderate, and 3.5 or higher as high. High PI values suggest potential for further development as a protein source. Based on these classifications, except for isolates IC.8 and IT.1, four isolates demonstrated high proteolytic activity by the end of the study.

No lag phase was observed in the growth curves of either isolate, as illustrated in Figure 1. This could be due to their ability to rapidly adapt to the growth medium. Madigan et al. (2012) noted that microbes transferred to fresh media during their logarithmic phase, or those previously cultured in similar media, often adapt quickly without an adaptation period.

The logarithmic phase for isolate IC.1 was observed from hour 0 (OD_{600} = 0.0135) to hour 18 (OD_{600} = 1.41), during which absorbance increased significantly. The stationary phase occurred from hour 18 (OD_{600} = 1.41) to hour 30 (OD_{600} = 1.485), where absorbance values plateaued. A sharp decline in absorbance from hour 30 (OD_{600} = 1.485) to hour 48 (OD_{600} = 0.815) indicated the onset of the death phase. The growth pattern of isolate IT.2 closely mirrored that of IC.1, though IT.2 exhibited slightly lower absorbance values. IT.2 entered the logarithmic phase between hour 0 (OD_{600} = 0.013) and hour 18 (OD_{600} = 1.065), followed by a stationary phase until hour 36 (OD_{600} = 0.975), and finally a death phase from hour 36 to hour 48 (OD_{600} = 0.82). These growth dynamics are presented in Figure 1.

The bacterial growth cycle consists of several distinct phases: the lag phase (adaptation phase), the exponential (logarithmic) phase, the stationary phase, and the death phase (Allen & Maclaw, 2018). During the lag phase, bacteria adjust their cellular metabolism to adapt to the new environmental conditions. The duration of this phase varies depending on the bacterial species, environmental stressors, and the physiological state of cells from the previous culture medium. Interestingly, Ilmiah et al. (2018) also reported bacterial growth that bypassed the lag phase, where the logarithmic phase began immediately at hour 0 and continued until maximum growth was achieved.

The logarithmic phase is characterized by exponential cell division and heightened metabolic activity. In this phase, the bacterial culture consumes more energy and synthesizes metabolites required for cell growth. The specific growth rate remains constant, but the medium undergoes chemical changes due to substrate consumption and metabolic by-product accumulation (Allen & Maclaw, 2018). The stationary phase follows when the growth rate slows and the number of viable cells stabilizes. This stagnation is primarily due to the depletion of essential nutrients and the build-up of toxic metabolic by-products. Jaishankar and Srivastava (2017) support this condition, stating that the transition to the stationary phase typically occurs when nutrients become limiting. During the death phase, viable bacterial cells decrease as cell lysis occurs, leading to a drop in turbidity. This phase marks the end of the growth cycle and reflects unfavorable conditions for cell survival.

Based on growth analysis, isolate IT.2 exhibited a growth rate of 0.24 h^{-1} with a generation time of 2.83 hours, while isolate IC.1 demonstrated a slightly higher growth rate of 0.26 h^{-1} and a shorter generation time of 2.68 hours, as summarized in Table 2. These results indicate that the IT.2 isolate increased by approximately 24% per hour, while IC.1 grew by 26% per hour. The bacterial growth rate is defined as the rate at which a bacterial population increases over time and is typically expressed as the logarithmic change in cell count per unit of time (Allen & Maclaw, 2018). Generation time, or doubling time, refers to the duration required for a bacterial population to double in size. These two parameters are inversely related; faster growth rates correspond to shorter generation times.

The enzyme activity of isolate IT.2 increased progressively from hour 0 (0.16 U/mL), peaking at hour 30 with 40.92 U/mL. By the 36th hour, enzyme activity began to decline, reaching 17.67 U/mL at hour 48. These results indicate that the highest protease production by isolate IT.2 occurred during the stationary phase of its growth (between the 18th and 36th hours). This observation aligns with the findings by Ilmiah et al. (2018), who reported peak protease production by *Bacillus licheniformis* during the stationary phase or at the end of the logarithmic phase. Suleiman et al. (2020) further support this, suggesting that enzyme synthesis typically correlates with active cell growth and reaches maximum levels during the late exponential or early stationary phase due to the accumulation of cellular metabolites. The subsequent decline in protease activity after the 36th hour might be attributed to substrate limitation. Ramadhan et al. (2023) noted that enzyme activity declines when substrate availability decreases because it lacks sufficient substrate to interact with, preventing it from reaching its full catalytic potential.

For isolate IC.1, protease activity began at 0.24 U/mL at hour 0 and steadily increased, peaking at 39.65 U/mL at hour 42. A decrease was observed at hour 48, with activity dropping to 35.91 U/mL. Unlike isolate IT.2, which exhibited a decline in enzyme activity at the onset of the death phase, isolate IC.1 maintained increasing enzyme production even as the cell population began to decline after hour 36. This suggests that enzyme synthesis continued despite a reduction in biomass. The total protein content in the culture media of both isolates showed a declining trend from the start to the end of the observation period (see Figure 3), indicating substrate depletion over time.

A study by Ramin and Allison (2019) found that high enzyme activity can still occur when bacterial growth rates

are near zero. In such conditions, bacteria may continue to produce enzymes even when no degradable substrate is present in the medium. This phenomenon is attributed to the production of constitutive enzymes that are continuously synthesized and secreted regardless of substrate presence. The persistence of protease activity in isolate IC.1 during the death phase could thus be explained by this constitutive enzyme production mechanism. As nutrients in the environment become limited, bacterial cells may have reallocated internal resources to prioritize secondary metabolic activities, including enzyme secretion. However, since extracellular enzyme synthesis requires energy and substrates for processes such as transcription, translation, and protein secretion, the eventual decline in biomass and nutrient depletion led to reduced enzyme activity observed at hour 48 (35.91 U/mL).

Several key factors affect enzyme activity, including pH, temperature, carbon source, incubation time, and salinity. Anh et al. (2021) reported that protease activity produced by halophilic bacteria is significantly affected by variations in temperature, pH, salinity, and incubation duration. A study by Sun et al. (2023) observed that under optimized fermentation conditions, protease production by *Bacillus subtilis* increased by up to 300.72% compared to baseline levels. Similarly, Osesusi et al. (2021) found that *Bacillus ruris* produced stable alkaline proteases at pH 9 and 55°C, demonstrating that specific environmental conditions can enhance enzyme stability and activity. These findings highlight the significant effect of environmental parameters on protease expression and underscore the need for further research to optimize these conditions. Given these insights, it is essential to conduct additional studies focused on the purification, optimization, and characterization of the protease enzyme produced by isolate IC.1. Such investigations would provide a more comprehensive understanding of its catalytic properties and potential industrial applications.

Protein concentrations in the culture media for IT.2 and IC.1 isolates steadily declined throughout the study (see Figure 3). For isolate IT.2, the protein content decreased from 0.258 mg/mL at hour 0 to 0.122 mg/mL at hour 48. Similarly, for isolate IC.1, the protein concentration dropped from 0.317 mg/mL to 0.109 mg/mL over the same time period. This reduction is attributed to the proteolytic activity of the secreted enzymes, which hydrolyze proteins into smaller peptides and amino acids. Bhavikatti et al. (2020) similarly reported an inverse correlation between protease enzyme production and the remaining protein content in the growth medium. In essence, higher enzyme activity levels correlate with greater protein degradation in the surrounding medium.

Molecular identification revealed that isolate IT.2 shared the closest genetic similarity with *Vibrio fluvialis*, while isolate IC.1 was closely related to *Pseudoalteromonas piscicida*. *V. fluvialis* is a well-known marine bacterium frequently isolated from seawater and marine environments. Previous studies have shown that this species is capable of protease production. For instance, Wang et al. (2007) reported that crude extracts from *V. fluvialis* strain TKU005 exhibited protease activity totaling 34 U, with optimal activity at pH 8 and 60°C. Additionally, research by Venugopal and Saramma (2006) showed that crude proteases from *V. fluvialis* achieved a specific activity of 8.199 U/mg, which increased to 10.212 U/mg following purification via ammonium sulfate precipitation.

P. piscicida is a marine bacterium classified within the class *Gammaproteobacteria*. It is heterotrophic, rod-shaped, motile via long flagella, sporeless, and Gram-negative (Eze et al., 2023). Commonly found in marine habitats and mangrove ecosystems, *P. piscicida* is recognized for its ability to produce a wide range of biologically active compounds, including antimicrobial, antifouling, algicidal, and therapeutic agents. According to Eze et al. (2023), these bioactive properties make the bacterium of considerable biotechnological interest. Richard et al. (2017) further reported that genomic analysis of three *P. piscicida* strains revealed the presence of 13 genes encoding extracellular proteases. Detailed characterization showed that the bacterium could secrete various classes of proteases, including cysteine proteases, serine proteases, and metalloproteases.

5. Conclusion

Bacterial isolates from the wastewater treatment plant of a shrimp cultivation facility exhibited proteolytic activity. The results showed that out of 37 isolates, six of them (IT.2, IC.1, IT.1, IT.9, IC.8, and IC.5) demonstrated proteolytic activity, ranging from moderate ($1.5 < PI \leq 3.5$) to high ($PI > 3.5$) protease index levels. Growth kinetics observations of the two most potent protease-producing isolates, IT.2 and IC.1, revealed that both underwent logarithmic, stationary, and death phases within a 48-hour period. The IT.2 isolate showed the highest similarity to *Vibrio fluvialis*, producing protease enzymes with a peak activity of 40.92 U/mL. Meanwhile, the IC.1 isolate exhibited the closest similarity to *Pseudoalteromonas piscicida*, with a maximum protease activity of 39.65 U/mL.

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