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Optimization of Protease Activity of Endophytic Bacteria EUA-136 and EUA-139 from *Bruguiera gymnorrhiza* Using Response Surface Methodology

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ABSTRACT

Protease is a vital enzyme used in industries such as detergents, pharmaceuticals, and animal feed, with a growing demand in the enzyme market. Endophytic microorganisms can produce stable proteases with a rapid synthesis process. This study optimized conditions of temperature, pH, salinity, agitation, and nutrient sources for protease production by EUA-136 and EUA-139 bacterial isolates. The research used Response Surface Methodology (RSM) with a Central Composite Design (CCD) in Design Expert Software 13.1 to identify optimal conditions and the bacterial isolates. The optimum conditions for the EUA-136 bacterial isolate to produce protease were 3% inoculum at 30 ºC, pH 7, 28.5 ppt salinity, and 150 rpm agitation. For the EUA-139 bacterial isolate, the optimum conditions were a carbon source of 1% (v/v) maltose, a nitrogen source of 1% (v/v) KNO_3, casein as the inducer, and an inoculum concentration of 7.5% (v/v). Molecular identification of isolates EUA-136 and EUA-139 revealed similarities to *Bacillus cereus* strain 3TC-3 and *Bacillus paramycoides* 3665, respectively.

1. Introduction

Proteases are the enzymes that break down proteins into simpler molecules like amino acids and glycopeptides. They have valuable applications in industries such as food, livestock feed, detergents, and pharmaceuticals (Agustien *et al.* 2018; Kocabaş & Grumet 2019). Currently, proteases account for a substantial portion of the enzyme market, with demand reaching 60% (Razzaq *et al*. 2019). Market demand for the enzymes continues to increase annually up to 6.6% compound annual growth rate (CAGR) (Naveed *et al*. 2021).

Endophytic microorganisms, which utilize substrates from their host, have the potential to produce several enzymes, including protease. Enhancing protease production of these bacteria involves optimizing several factors such as carbon and nitrogen sources, inducers, inoculum concentration, pH, temperature, salinity, and agitation speed (Agustien *et al*. 2024). Determination of optimum enzyme activity was achieved using Response Surface Methodology (RSM) to model and adjust environmental variables (Beyan *et al*. 2021a).

The identification of microorganism types was based on molecular analysis using the 16S rRNA gene sequence, a widely used method for identifying microbes and studying the phylogenetic relationships among bacterial species (Anggraini *et al*. 2018). This study aimed to optimize conditions for protease production by endophytic bacterial isolates EUA-136 and EUA-139 to improve enzyme stocks for industrial use. It involved both optimization of production conditions and molecular identification of the bacteria. This research focused on bacterial isolates of endophytic protease producers from the leaves of *Bruguiera gymnorrhiza* in the Mandeh area, labeled

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EUA-136 and EUA-139. *Bruguiera gymnorrhiza* is a mangrove plant that thrives in high-salinity conditions and is capable of producing potential secondary metabolites. Therefore, it is expected to produce stable protease enzymes with high activity.

2. Materials and Methods

2.1. Reculture of Endophytic Bacteria

Endophytic bacterial isolates EUA-136 and EUA-139 were bacterial collection of the Biotechnology Laboratory. The bacteria were isolated from the leaves of the *Bruguiera gymnorrhiza* mangrove plant. Bacterial rejuvenation was done by streaking the isolates on NA medium in a test tube, followed by incubation at 30°C for 24 hours.

2.2. Growth Curve of Bacterial Isolate

As much as 1-2 loop inoculation of the isolates was inoculated onto the 100 ml of basal medium (3 g of KH_2PO_4, 3 g of K_2HPO_4, 3 g of $MgSO_4$, 5 g of NaCl, and 10 g of casein were added, followed by the addition of 1000 ml of sterile distilled water) for protease production. And the cultures were incubated at 30°C with agitation at 150 rpm for 24 hours. Afterward, a 5 ml (105 CFU/ml) aliquot was transferred to 95 ml of fresh medium. The optical density of the cultures was measured every 2 hours until a decline in bacterial growth was noted using a spectrophotometer at 600 nm.

2.3. Protease Activity

Protease activity was measured using Folin-Ciocalteu reagent. As much as 0.5 ml of casein substrate was mixed with 0.5 ml of enzyme solution and 0.25 ml of 50 mM Tris-Cl buffer, then the mixture was incubated at 30°C for 15 minutes. Afterward, 0.5 ml of TCA was added, then the mixture was incubated at room temperature for 20 minutes before being centrifuged at 6000 rpm for 20 minutes. A total of 0.375 ml of the supernatant was mixed with 1.25 ml of Na_2CO_3 and 0.25 ml of 1N Folin-Ciocalteu reagent. The optical density was measured at λ 578 nm (Takami *et al*. 1989). The standard for measurement used 0.5 ml of 5 mM tyrosine.

The formula for Calculating Protease Enzyme Activity:

Information:

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

Where:

PU : activity units of protease (Units/ml)
Asp : absorbance sample
Abl : absorbance blank
Ast : absorbance standard
T : incubation time (minutes)
P : dilution

2.4. Protein Content Measurement

Protein content was measured using several reagents as described in Lowry *et al*. (1951) The standard solution consisted of BSA (Bovine Serum Albumin) solutions with concentrations of 0, 50, 75, 100, 125, 150, 175, and 200 µg/ml.

To measure protein content, 0.1 ml of enzyme solution was mixed with 0.9 ml of distilled water, followed by the addition of 5 ml of Reagent C and incubation at 30°C for 10 minutes. Next, 0.5 ml of Reagent D was added, the mixture was homogenized, and it was incubated for an additional 30 minutes at 30°C (Lowry *et al*. 1951). Absorption was measured at 750 nm using a UV-Vis spectrophotometer, with distilled water as the control, and protein levels were determined using a BSA standard curve.

2.5. Measurement of Specific Enzyme Activity

Specific enzyme activity is measured using the following formula:

$$\text{Specific Enzyme Activity (U/mg)} = \frac{\text{UA (Enzyme Activity (u/ml))}}{\text{Protein Content (mg/ml)}}$$

2.6. Determination of Optimum Conditions for Protease Production

To determine the optimal conditions for protease production, the production medium was tested with various supplements. Carbon sources such as glucose, lactose, sucrose, and maltose were added at 1% concentration. Nitrogen sources, including KNO_3, $NaNO_3$, NH_4Cl, and $(NH_4)_2SO_4$, were also incorporated at 1%. Additionally, protease inducers like yeast extract, peptone, casein, and soy flour were used at the same concentration. The medium was incubated and sampled during the idiophase to evaluate enzyme activity.

2.7. Determination of Optimum Conditions for Protease Production

This study used Response Surface Methodology (RSM) with Design-Expert software to identify the optimal condition range based on the Response Surface Plot, with 30 experimental runs for each parameter. For the EUA-136, variations included: temperature (A) 25 to 40°C; pH was adjusted with buffer (B) from 5 to 9; inoculum concentration (C) ranged from 1% to 5%; salinity was adjusted with NaCl concentration (D) from 25 ppt to 32 ppt; and agitation (E) varied from 100 rpm to 250 rpm. Meanwhile, for the EUA-139 treatment, variations included: carbon source concentration (A) ranged from 0.5% to 1.5%; nitrogen source concentration (B) ranged from 0.5% to 1.5%; and inoculum concentration (C) ranged from 2.5% to 7.5%.

2.8. Characterization of Bacterial Isolate

Characterization of bacteria was done through macroscopic observation (colony shape, color, edges, and elevation), microscopic observation (cell morphology), gram staining, endospore staining, motility testing, and a catalase test using 3% hydrogen peroxide (H_2O_2) to observe the reaction of the isolate.

2.9. Molecular Identification

Bacterial DNA was isolated using a GeneJET Genomic DNA Purification Kit-Zymo Research, and DNA amplification was performed using the forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer (5'-GGTTACSTTGTTACGACTT-3'). The DNA amplification cycle using PCR has been optimized with an annealing temperature of 50°C. PCR fragments were analyzed with an automated DNA sequencer. The sequence results were submitted to GenBank for confirmation. DNA sequences were compared using the Clustal X and NJ Tree programs for phylogenetic tree construction. In molecular identification, a 97% similarity in 16S rRNA gene sequences typically defines bacterial species, while 99% or higher usually indicates that the strains belong to the same species.

3. Results

3.1. Growth Profile and Protease Activity

The growth profile and protease activity of bacterial isolates EUA-136 and EUA-139 were measured using optical density (OD) at 600 nm and protease activity (U/ml) after incubation at 30 ºC. Isolate EUA-136 showed a growth profile over 28 hours, including a two-hour adaptation phase, with exponential growth occurring from the 2nd to the 22nd hour (Figure 1). In contrast, isolate EUA-139 had a growth profile over 22 hours without an adaptation phase, with exponential growth from the 2nd to the 16th hour (Figure 2).

3.2. Carbon Source for Optimum Protease Production

The study found that using maltose ($C_{12}H_{22}O_{11}$) as a carbon source significantly increases protease enzyme activity to an average of 1.695 U/mg (Figure 3). Adding potassium nitrate (KNO_3) as a nitrogen source further boosts the activity to 8.171 U/mg (Figure 4). Additionally, the use of casein as an inducer results in the highest protease activity, averaging 8.972 U/mg (Figure 5), highlighting the importance of selecting optimal carbon and nitrogen sources for protease production. These optimal conditions were then used for further optimization with RSM.

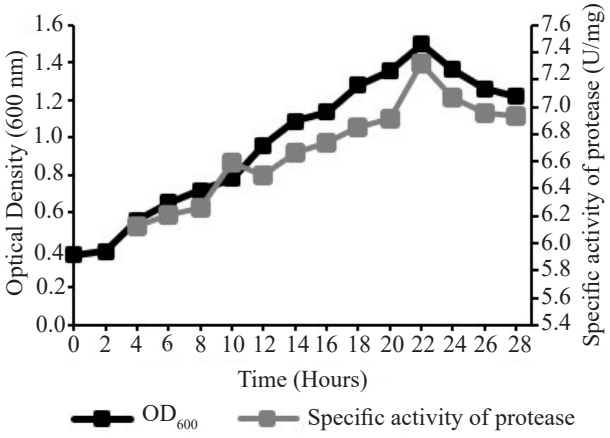

Figure 1. Growth profile and specific protease activity of the EUA-136 isolate

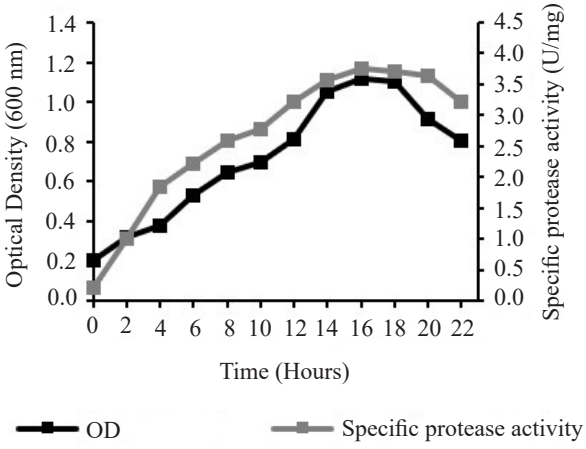

Figure 2. Growth profile and specific protease activity of the EUA-139 isolate

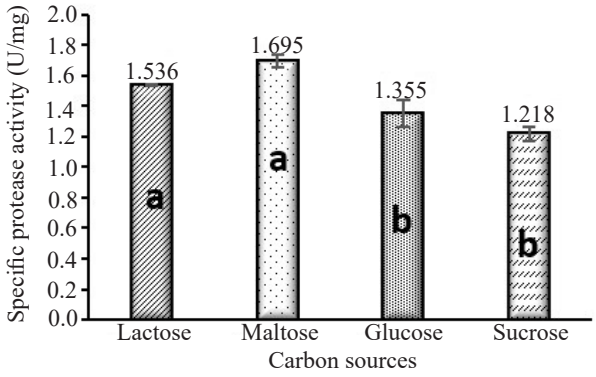

Figure 3. Histogram of specific activity of EUA-139 isolate in response to carbon sources optimization

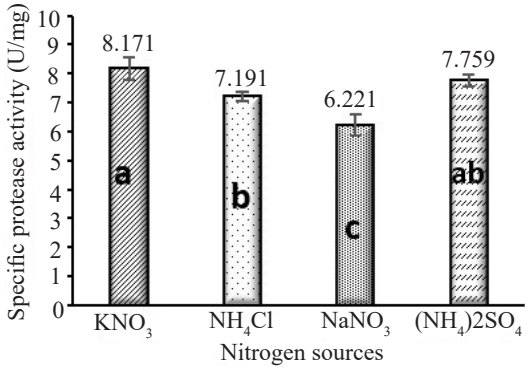

Figure 4. Histogram of specific activity of EUA-139 isolate in response to nitrogen sources optimization

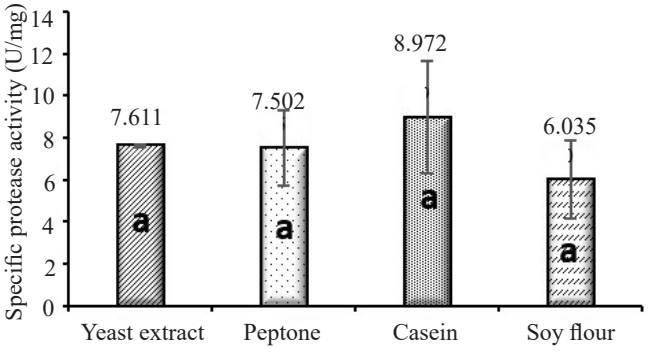

Figure 5. Histogram of specific activity of EUA-139 isolate in response to nitrogen sources optimization

3.3. Optimum Conditions For Protease Production

Optimal conditions are determined by adjusting parameters like temperature, pH, salinity, inoculum concentration, inducer, and agitation using Response Surface Methodology (RSM). The combinations of concentration for several parameters for isolate EUA-136 are presented in Table 1. Additionally, the combinations of maltose, KNO_3, and inoculum concentration (at 34 ºC, pH 7, and 150 rpm) for isolate EUA-139 are presented in Table 2.

Table 1. The results of optimization for protease production in isolate EUA-136 using central composite design (CCD)

Temperature (ºC)	pH	Inoculum (%)	Salinity (ppt)	Agitation (rpm)	Specific activity (U/mg)
40	5	5	32	100	6.187
20	9	1	32	100	9.739
20	9	5	32	250	7.633
20	9	1	32	250	8.502
20	5	5	32	100	10.971
20	5	5	32	250	6.845
30	7	3	28.5	175	13.115
20	5	5	25	250	12.34
30	7	3	32	175	12.067
20	9	5	32	100	9.690
30	7	3	25	175	9.845
30	5	3	28.5	175	13.186
40	5	1	32	250	11.541
40	9	1	25	250	11.021
40	5	5	32	250	9.947
20	5	1	32	100	10.497
40	5	1	25	250	13.547
40	5	5	25	100	7.826
20	5	1	32	250	11.377
40	9	5	25	100	8.356
30	7	3	28.5	175	13.946
30	7	3	28.5	175	14.272
20	7	3	28.5	175	10.909
40	9	1	32	250	10.580
30	7	3	28.5	175	13.359
30	7	3	28.5	250	10.203
20	9	5	25	250	10.994
30	7	5	28.5	175	13.005
40	7	3	28.5	175	11.951
30	9	3	28.5	175	10.760
40	9	1	25	100	9.2360
40	5	1	32	100	10.624
20	5	1	25	250	12.069
20	9	1	25	250	10.932
20	5	1	25	100	11.554
30	7	3	28.5	175	13.043
30	7	1	28.5	175	13.747
40	5	1	25	100	11.818
20	5	5	25	100	10.983
30	7	3	28.5	175	13.929
40	9	5	32	250	11.888
40	9	5	32	100	10.330
20	9	1	25	100	10.576
40	9	5	25	250	10.285
20	9	5	25	100	10.525
40	9	1	32	100	14.251
30	7	3	28.5	175	11.228
30	7	3	28.5	100	10.092
40	5	5	25	250	11.335
30	7	3	28.5	175	11.576

Table 2. The results of optimization for protease production in isolate EUA-139 using central composite design (CCD)

Maltose concentration (%)	KNO_3 concentration (%)	Inoculum (%)	Specific activity (U/mg)
1	1	7.5	9.171
1.5	1.5	2.5	5.311
0.5	1.5	7.5	7.877
0.5	0.5	7.5	5.619
1	1.5	5	6.007
1	1	2.5	7.811
1	1	5	9.075
1	1	5	8.678
1	1	5	8.994
1	0.5	5	5.226
0.5	1	5	8.29
0.5	1.5	2.5	6.121
1.5	1	5	6.878
0.5	0.5	2.5	6.509
1	1	5	8.775
1.5	0.5	2.5	5.576
1.5	1.5	7.5	8.446
1	1	5	7.078
1.5	0.5	7.5	6.657
1	1	5	8.428

The plot revealed the relationship between the response and variables. For the EUA-136 isolate (Figure 6), the highest protease specificity was 14.251 U/mg under optimal conditions of 30°C, pH 7, 5% inoculum concentration, 28.5 ppt salinity, and 175 rpm agitation. The model had an R^2 of 0.7172 and an adjusted R^2 of 0.0957, with an F-value and p-value of 0.0500. For the EUA-139 isolate (Figure 7), the highest protease activity was 9.171 U/mg with optimal maltose and KNO_3 concentrations of 1% and 7.5% inoculum concentration. This model had an R^2 of 0.8621, an adjusted R^2 of 0.7379, and also an F-value and p-value of 0.0500, indicating statistical significance.

3.4. Morphological Characteristics of Bacterial Isolate

Partial characterization of endophytes bacteria isolate EUA-136 and EUA-139 was performed through macroscopic and microscopic observations, as shown in Table 3 and Figures 8 and 9.

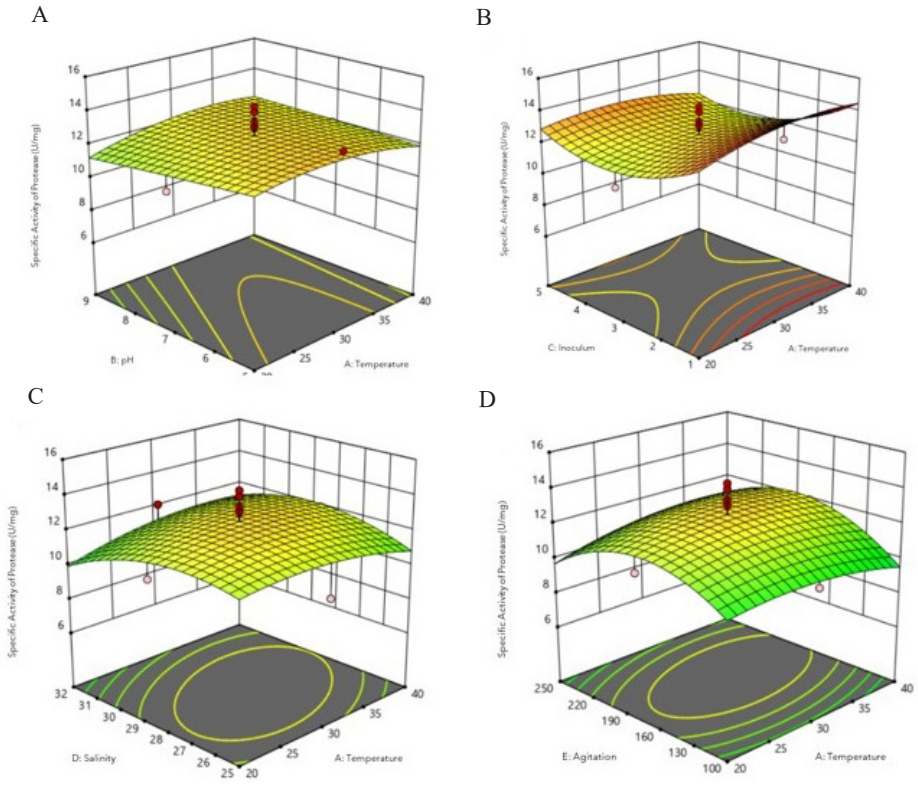

Figure 6. Response surface plots (3D) protease specific activity of (U/mg) EUA-136 (A) Temperature and pH, (B) temperature and inoculum, (C) temperature and salinity, (D) temperature and agitation)

3.5. Identification Based on 16S rRNA Gene Sequence Analysis

Amplification of isolates EUA-136 and EUA-139 using universal primers and electrophoresis revealed bands of approximately 1500 bp, consistent with a 1500 bp DNA ladder. The BLAST nucleotide sequence of EUA-136 endophytic bacteria isolates with 10 isolates contained in the gene bank has a similarity value of 99.65%-99.93 % with an E-value of 0.0, which shows more significant alignment with BLAST results. Meanwhile, the BLAST analysis of EUA-139 showed high sequence similarities compared with 10 sequences in GenBank, which is 99.51% to 100%, with E-values of 0.0 indicating highly significant alignments.

The base composition of the 16S rRNA gene was: EUA-136: T(U) = 20.9%, C = 22.8%, A = 25.7%, G = 30.6%; EUA-139: T(U) = 20.8%, C = 22.9%, A = 25.5%, G = 30.8%. Phylogenetic analysis using the NJ-method with a 1000x bootstrap value, conducted with MEGA software, showed EUA-136 was closely related to *Bacillus cereus* strain 3TC-3 (99.93% similarity) (Figure 10), while EUA-139 was closely related to *Bacillus paramycoides* 3665 (99% similarity) (Figure 11).

4. Discussion

Isolates EUA-136 and EUA-139 exponential phase starting at 22nd hours for EUA-136 and 16th hours for EUA-139. During this phase, bacteria divide rapidly and produce primary metabolites to meet their nutritional needs (Molina *et al*. 2019; Loutfi *et al*. 2020). The highest protease activity was recorded during the exponential phase, with EUA-136 showing 7.319 U/ml and EUA-139 showing 3.754 U/ml. The timing of these phases can vary based on bacterial type,

Table 3. Morphological characteristics of EUA-136 and EUA-139 bacterial isolates

Isolater's characters	EUA-136	EUA 139
Colony Morphology		
Colony Form	Circular	Circular
Margin	Undulate	Entire
Elevation	Raised	Raised
Colony Colour	Yellowish white	Yellowish white
Cell Morphology		
Cell Shape	Stem	Stem
Gram-Stain	Positive	Positive
Endospores	Present	Present
Motility Test	Positive	Negative
Catalase Test	Positive	Positive

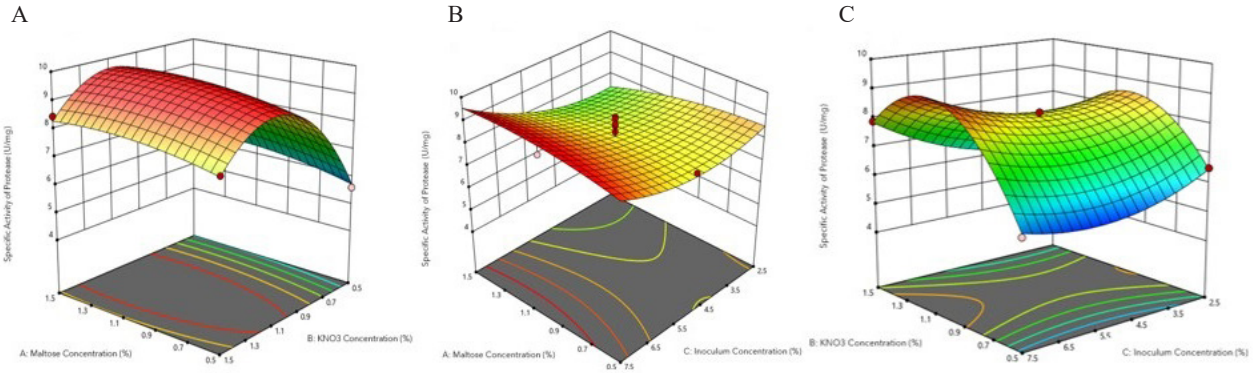

Figure 7. Response surface plots (3D) protease specific activity (U/mg) of EUA-139 (A) Maltose and KNO_3, (B) maltose and inoculum, (C) KNO_3 and inoculum)

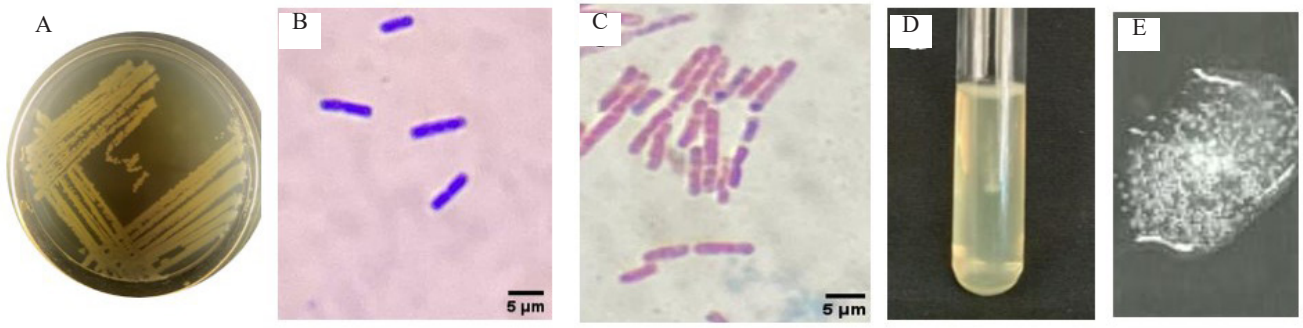

Figure 8. Partial characterization of EUA-136 (A) Morphological characteristics, (B) gram staining, (C) endospore staining, (D) motility test, (E) Catalase test

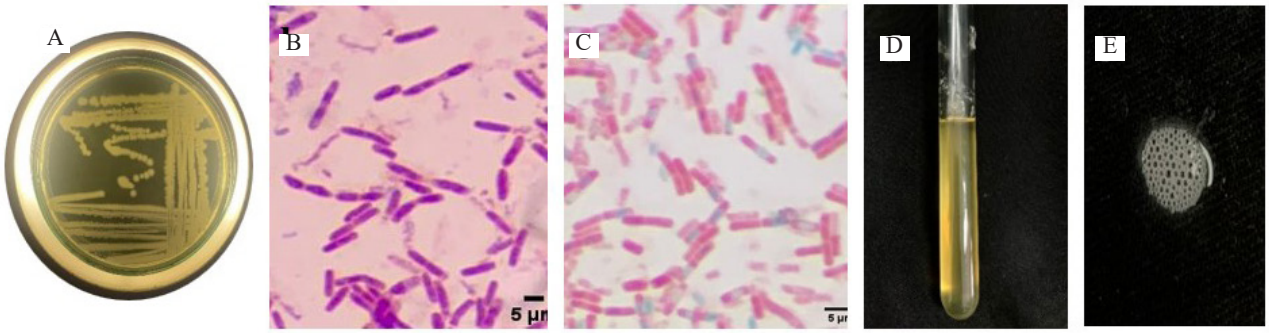

Figure 9. Partial characterization of EUA-139 (A) Morphological characteristics, (B) gram staining, (C) endospore staining, (D) motility test, (E) catalase test

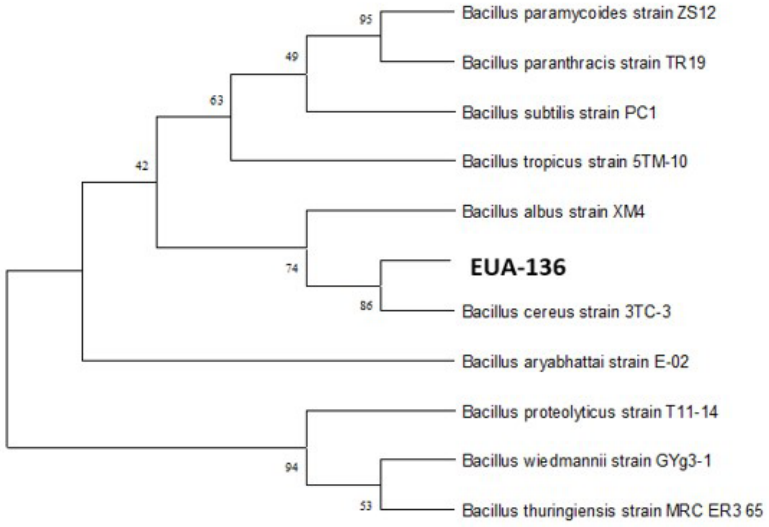

Figure 10. Phylogenetic tree of EUA-136 based on 16S rRNA analysis using the neighbor-joining (NJ) method

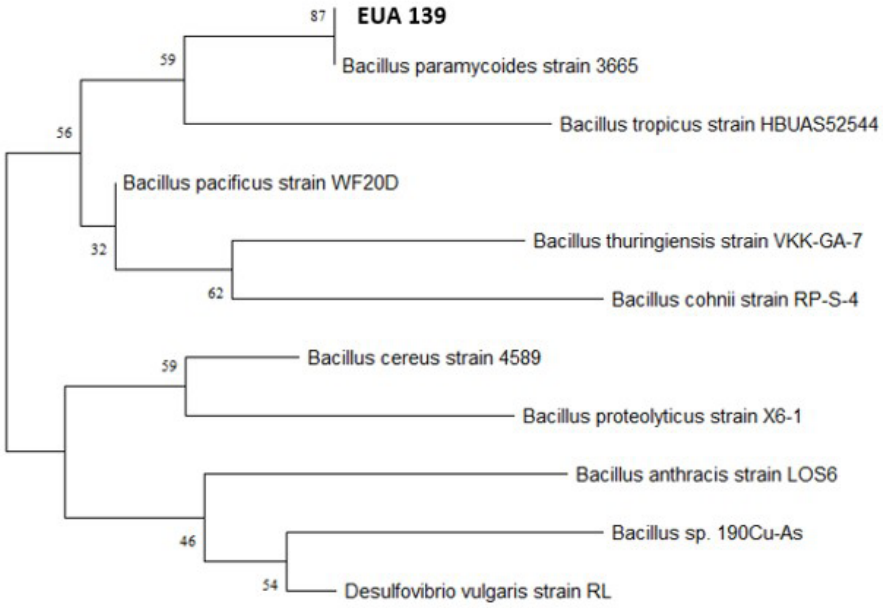

Figure 11. Phylogenetic tree of EUA-139 based on 16S rRNA analysis using the neighbor-joining (NJ) method

environmental conditions, and available substrates. (Johnson *et al*. 2022).

Protease production is influenced by temperature, with 30 ºC being more optimal than 20 ºC or 40 ºC, as enzymatic reactions slow down at these lower and higher temperatures. Higher temperatures speed up reactions by increasing molecular energy and lowering activation energy, leading to higher reaction rates until the optimum temperature is reached, where enzymatic activity peaks (Agom & Gbadebo 2024). Apart from temperature, pH also influences the speed at which enzymes catalyze reactions (Agustien *et al*. 2024). The optimal pH for enzymes is generally around pH 8, enhancing reaction efficiency and protecting the enzymes from damage. Extreme pH levels can inactivate enzymes by altering their charge and structure. Specifically, at pH 9, the increased OH^- concentration changes the enzyme's conformation, impairing substrate binding and reducing activity (Kumar *et al*. 2020).

Salinity impacts bacterial growth and metabolite production. Optimal growth is observed in a salinity range of 0% to 4%, while growth declines at 5%. Increased salinity can cause imbalances, prompting bacteria to adapt by expelling secondary metabolites to adjust (Mo *et al*. 2020). Salinity affects microorganisms by altering osmotic pressure, with organisms thriving in environments where osmotic pressure is slightly lower than inside their cells (Garcia *et al*. 2023).

Agitation is another important factor in enzyme production because it affects the homogeneity of nutrients, culture, and oxygen provision in the production medium, as well as speeds up the mixing and dissolving processes. Low enzyme production can be influenced by the speed of shaking in the production medium (Tonso *et al*. 2021).

The highest protease activity was achieved with maltose as the carbon source and KNO_3 as the nitrogen source. Bacteria use the Maltose Transport System (MTS) to uptake maltose, which then interacts with components that regulate protease activity (Suberu *et al*. 2019; Saier *et al*. 2021). KNO_3 can enhance protease activity because it provides nitrate (NO_3^-) as a nitrogen source. Bacteria can use nitrate for growth and protein synthesis. Some bacteria have metabolic pathways that allow them to assimilate nitrate from the environment and use it as a nitrogen source (Agustien *et al*. 2024).

Inducers are substances that can stimulate the production of protease enzymes in microorganisms. Casein, a protein-rich substrate, supports high protease production. Casein is an effective inducer for protease production by *Bacillus subtilis*. Therefore, it can be concluded that casein is a suitable inducer for enhancing protease activity in endophytic bacterial isolates (Swarna & Gnanadoss 2021)

Optimizing the factors influencing protease production is crucial for achieving consistency, enhancing sustainability, and minimizing variability in large-scale processes. The optimal combination accelerates production and results in high-quality protease suitable for applications in the food, textile, and detergent industries (Suberu *et al*. 2019).

Research findings indicate that isolates EUA-136 and EUA-139, with specific activity values of 14.251 U/mg and 9.171 U/mg respectively, are effective for applications in household use, food processing, and waste degradation. Their activity levels can significantly aid in the breakdown of proteinaceous materials, which is vital for waste management and various industrial processes. Future research should focus on a more in-depth characterization of the protease enzymes produced by isolates EUA-136 and EUA-139 to gain a comprehensive understanding of their potential and applications.

Response Surface Methodology (RSM) identifies optimal treatment conditions for the best response, typically visualized through response surface and contour plots. It is often referred to as "hill climbing," with the peak representing the optimal response point (Myers *et al*. 2016). Surface response analysis is visualized using contour plots on a plane defined by variables x_1 and x_2. RSM assesses the impact of individual variables to find optimal conditions in a multivariable system. Once an optimal response area is identified, RSM models this area to refine the analysis and achieve the best conditions (Beyan *et al*. 2021b).

Both bacterial isolates, EUA-136 and EUA-139, are Gram-positive, rod-shaped, and capable of forming endospores in extreme conditions (Xu & Wang 2018). In the motility test, isolate EUA-136 was found to be motile, indicating that the bacteria can spread on the growth media (Wadhwa & Berg 2022). In the catalase test, both isolates tested positive, indicating they produced the catalase enzyme. Catalase breaks down hydrogen peroxide (H_2O_2), a byproduct of aerobic respiration, into water (H_2O) and oxygen (O_2) (Shoaib *et al*. 2020).

Isolate EUA-136 is similar to Bacillus cereus strain 3TC-3, while isolate EUA-139 is similar to Bacillus paramycoides 3665. The p-distance data, which is 0.00, indicates no evolutionary distance between these

isolates and their corresponding strains. GenBank Cherry (2017) reported that the minimum p-distance for bacteria of the same species is 0.0075. Bacillus species are known for their protease production capabilities. Several neutral protease-producing Bacillus strains include *Bacillus cereus*, *Bacillus paramycoides*, *Bacillus amyloliquefaciens*, *Bacillus megaterium*, *Bacillus polymyxa*, and *Bacillus subtilis* (Pant *et al*. 2015; Adetunji & Olaniran 2020).

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