Identification and Assessment of Thermotolerant Proteases from a Thermophilic *Actinomycetes* Strain Extracted from the Soil

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Introduction

One of the most important groups of soil bacteria, recognized as a source of commercially important enzymes and antimicrobials, is the *Actinomycetes*, and the diversity of *Actinomycetes* is largely underestimated. Considering the potential of discovering *Actinomycetes* that can produce enzymes and antimicrobials with industrial and medical applications, we previously screened many soil samples collected from selected sites of Ramapo campus soils. One of the several strains isolated was identified as a thermophile, and based on the colony morphology, gram staining, and diagnostic tests, it was identified to be a gram-positive spore-forming bacteria belonging to the genus *Actinomycetes*. We hypothesized that the thermophilic strain of *Actinomycetes* has great potential to produce extracellular proteases that are thermo-tolerant and alkaline in nature.

Our preliminary studies indicate that the proteases remain active in a broad range of temperatures and pH, making this a commercially important study. We are currently optimizing the conditions to maximize the protease yield in the bacterium and testing various parameters to understand the stability of the proteases. Downstream processing techniques like ammonium sulfate are used to partially purify the proteases, while the specific activity of the protease was estimated using casein as the substrate and phenol Folin–Ciolcaltea Reagent. Proteases that can remain active under extreme conditions such as high temperature, pH, and salt concentration are widely applicable in the commercial sector. Commercial applications require the proteases to resist high temperatures and alkaline conditions. The thermostable nature of these proteases makes them suitable for industrial applications requiring higher temperatures and, therefore, shorter reaction times and a low risk of contamination.

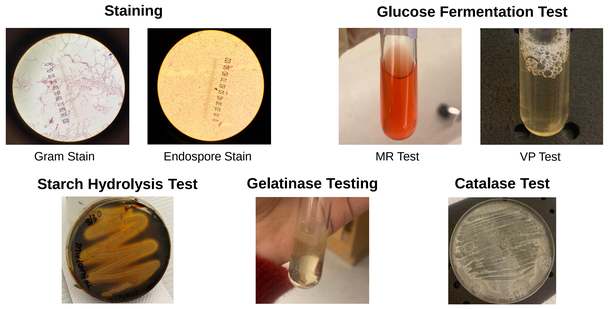
Materials and Methods

Isolation, Characterization & Screening:
Several soil samples were collected at multiple locations on the Ramapo College Campus and were subjected to a heat shock to screen for spore-forming bacteria. The samples were serially diluted, plated on Tryptic Soy Agar plates, and incubated at various temperatures. Various staining techniques and biochemical tests were done to confirm genus identity and test for the presence of enzymes like proteases.

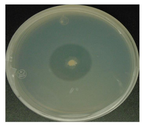
Partial Purification of the Proteases:
To obtain cell-free supernatant, the bacterial culture was centrifuged at 15,000 rpm for 10 min at 4℃. Ammonium sulfate was added slowly to the cell-free proteinase extract with gentle stirring. Ammonium sulfate fractions were then collected at 0–80% saturation, and the mixture was precipitated at 4℃ overnight. The precipitate was obtained through centrifugation at 15,000 rpm for 15 min at 4℃. The protein precipitate was re-suspended into 20 mM Tris-HCl, pH 8.

Protease Specific Assay:
5 mL of 0.65% casein dissolved in 50 mM potassium phosphate buffer (pH 7.5) was added to the test (sample) tube and to the control tube. The tubes were incubated at 37℃ for 5 min. Subsequently, 1 mL of crude protease extract was added to only the sample tube and incubated for 10 min at 37℃. The reaction was stopped with the addition of 5 mL of 110 mM trichloroacetic acid (TCA) solution in both the sample and control tubes. After the incubation was completed, protease extract was added to the control, and the mixture was filtered using a 0.45 m syringe filter. Finally, 5 mL of 500 mM sodium carbonate was added to both tubes, followed by the addition of 1 mL of 0.5 mM Folin–Ciolcaltea Reagent. The mixture was then incubated for 30 min at 37℃.

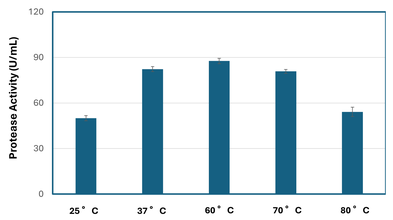
Results



Result-1: The bacterium was amylase, gelatinase, catalase, and Methyl Red positive spore-forming rod-shaped fuzzy strain identified as a **thermophile belonging to the genus of *Actinomycetes***

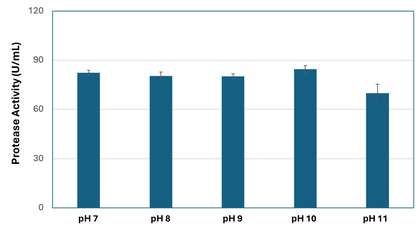


Result-2: The bacterium produced a **zone of clearance** around the skim-milk agar media indicating the presence of **bacterial proteases**



Result 3: **The bacterial protease showed specific activity at a wide range of temperatures (thermo-tolerant protease):** Various temperatures of the assay were plotted on the x-axis while the specific activity was plotted on the y-axis

Results (Continued)



Result 4: The bacterial protease showed specific activity at a wide range of alkaline pH values (alkaline protease), pH values on x-axis and the protease specific activity on y-axis

*Absorbance at 660 nM was measured using a UV-Vis, and the enzyme activity in units/mL was calculated using a tyrosine standard curve.

Discussion

- We were able to determine the specific strain of bacteria used through various diagnostic testing equipment, leading to the conclusion that it belongs to the *Actinomycetes* genus.
- The zone of clearance showcased from the casein media illustrates that this bacterial strain contains an adequate presence of proteases.
- The biochemical colorimetric assay used to determine the specific activity of bacterial proteases has been standardized and successful in this study. This assay is a universal protease assay and is helpful to ensure that bacterial proteases precisely determine activity before we purify them further.

Future Work

- To further purify the bacterial protease and examine the stability under various concentrations of salts and ions
- To send the bacterial genomic DNA for 16srRNA sequencing to identify the species

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