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Isolation of Protease Producing Microorganism from Soil and Characterization of Partially Purified Enzyme

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Abstract: Proteases represent a cornerstone class of enzymes in modern biotechnology, constituting approximately 60% of the total enzyme market due to their remarkable versatility and applicability. These specialized biocatalysts function by hydrolyzing peptide bonds in proteins, facilitating their degradation into smaller peptides and amino acids. With an emphasis on process parameter optimization to increase enzyme yield, this thesis investigates the microbial fermentation method of producing proteases. By analyzing how temperature affects protease synthesis, the study determines the ideal circumstances for maximizing enzymatic activity. A number of bioreactor systems are examined for their function in effectively increasing production, including fed-batch fermentation. In commercial enzyme synthesis, the results highlight the significance of regulated environmental conditions and reactor topologies. Thus, this study offers important new information about economical and sustainable methods for producing proteases on a big scale. [21]

Keywords: *Casein, Yeast Extract Peptone, Folin – Ciocalteus Reagent, Carbon Source, Fermentation.*

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I. INTRODUCTION

While proteases naturally occur throughout all biological kingdoms—plants, animals, and microorganisms—industrial-scale enzyme production increasingly favors microbial sources due to their economic viability and technical advantages.. Both commercial uses and natural biological processes depend on these enzymes. Proteases play a key role in vital physiological processes in nature, including digestion, cell signaling, and protein recycling. They have become more well- known from an industrial perspective because of their wide substrate specificity, operational stability, and economical production techniques, especially microbial fermentation.[2] Proteases are used in many different industries. They improve stain removal in the detergent business by dissolving protein-based stains. They are employed in the food industry to hydrolyze proteins, tenderize meat, and prepare dairy products[13]. They are used by the pharmaceutical sector to formulate drugs, debride wounds, and provide digestive aids.

Furthermore, among their many applications, proteases aid in the production of animal feed, leather processing, and photographic film recovery. [3]

Since microbial sources can be genetically engineered to improve enzyme properties and provide high yields, they are frequently used for large-scale production, particularly specific strains of Bacillus, Aspergillus, and Streptomyces. In commercial protease production, fed- batch fermentation is one of the most widely utilized techniques because it maximizes enzyme synthesis and minimizes inhibitory effects by providing perfect control over growing conditions and substrate concentration.[10]

Significant research and development is still being done on the generation of proteases through microbial fermentation, as the need for sustainable and environmentally friendly bioprocesses grows. Proteases' efficiency, stability, and specificity are regularly increasing due to developments in strain engineering, process optimization, and bioreactor design, which increases their value in contemporary biotechnology and industrial processes.[4][5]

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II. MATERIALS AND METHODS

➢ *Techniques of Experimentation*

- *Protease Production Screening*

To evaluate protease secretion capability, bacterial isolates were cultured on nutrient agar plates containing 1% casein as the protein substrate. Following 24- hour incubation at 37°C, we examined the plates for characteristic clear zones surrounding bacterial growth, indicating casein hydrolysis and confirming protease production.

- *Culture Medium Preparation*

After adjusting the pH to 6.8, a liquid medium made of peptone and yeast extract was created. To stop the spores from sticking together, marble pieces were added. Following autoclaving, a bacterial culture was added to the medium, and it is shaken at 120 rpm for 24 hours at 30°C.[8]

- *Substrate Processing*

Wheat bran, corn cobs, leftover bread, and wheat straw are examples of agricultural by- products that have been studied as inexpensive substrates for solid-state fermentation. After being crushed, oven-dried, and sieved to 40 and 80 mesh sizes, these materials were put away in airtight containers.[7]

- *Fermentation Optimization*

In order to increase product yield and efficiency, fermentation optimization involves adjusting parameters such as pH, temperature, nutrient levels, One-factor-at-a-time or statistical approaches are frequently used to enhance microbial growth or enzyme production. Efficient bioprocessing and increased productivity are guaranteed by proper optimization.[12][6]

✓ Time spent incubating: 24–48 hours Warmth: 25 to 45°C
✓ pH range: 6–12
✓ Ratio of substrate to moisture: 1:0.5 to 1:1

➢ *Enzyme Recovery*

Post-fermentation, we extracted enzymes by adding distilled water to the solid substrate and incubating the mixture with agitation at 30°C for one hour. The liquid fraction was separated by filtration and centrifugation (10,000 rpm, 15 minutes, 4°C), with the resulting supernatant stored as crude enzyme preparation at 4°C.

III. EXAMINATION OF ENZYMES

➢ *Protease Activity Measurement*

Enzyme activity was determined using casein (0.65% in Tris-HCl buffer, pH 8.5) as sub- strate. The reaction mixture, containing prewarmed substrate and enzyme solution, was incubated at 37°C for 30 minutes before termination with trichloroacetic acid. After filtra- tion, we quantified released tyrosine by reaction with Folin-Ciocalteu reagent, measuring absorbance at 660 nm. [20][1]

➢ *Tyrosine Standard Preparation*

We generated a standard curve using serial dilutions of L-tyrosine (0.2 mg/mL). Each standard solution was treated with sodium carbonate and Folin's reagent, with absorbance readings taken after 20 minutes at 660 nm..[17]

IV. ENZYME CHARACTERIZATION

➢ *pH Activity Profile*

We assessed enzyme performance across pH 6-12 using appropriate buffer systems, main- taining other optimal conditions constant during 20-minute incubations.

➢ *Temperature Activity Profile*

The thermal stability was evaluated between 25-75°C at the predetermined optimal pH, with reactions proceeding for 30 minutes.

➢ *Protein Quantification*

Total protein content in crude extracts was measured by the Lowry method using bovine serum albumin as the protein standard.

➢ *Specific Activity Determination*

We calculated specific activity by normalizing total enzyme activity (U/mL) to protein concentration (mg/mL), expressing results as U/mg protein..[11][16]

➢ *Purification by Enzymes*

Crude enzyme extracts were subjected to stepwise ammonium sulfate precipitation (10- 80% saturation). After 4-hour incubation at 4°C and centrifugation, pellets were resus- pended in buffer and both fractions were analyzed for protease activity.[14][19]

V. RESULT AND DISCUSSION

➢ *Protease Production Screening in Bacillus Subtilis*

Bacillus subtilis was tested for the generation of proteases is explained in the paragraph.

Casein agar plates were used for the screening, and the existence of transparent hydrolysis halos surrounding the bacterial colonies suggested that the bacteria were secreting extracellular protease enzymes that could degrade casein protein as shown in.

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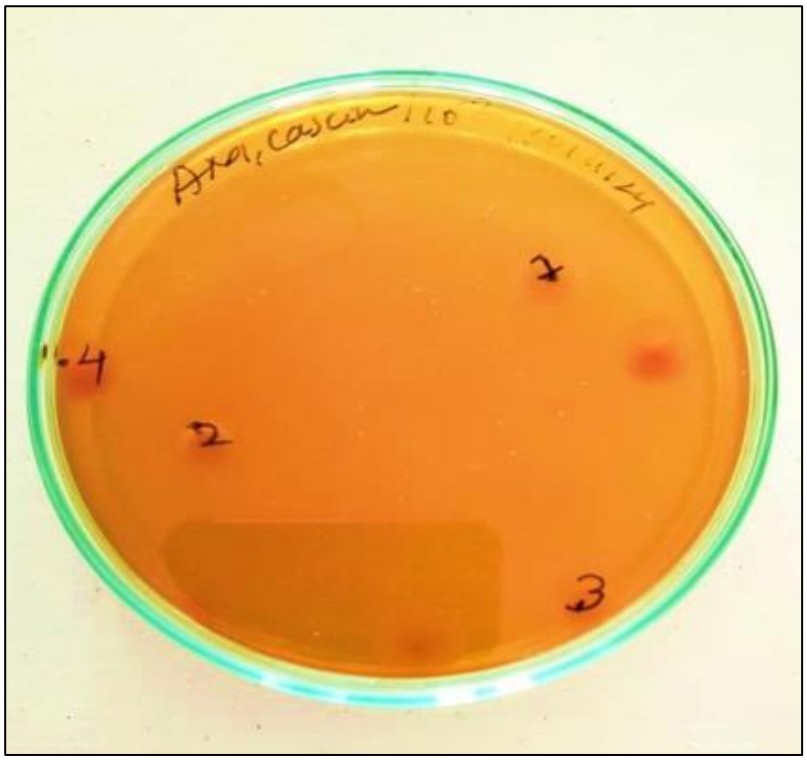

Fig 1 Proteolytic Activity Assessment of *Bacillus Subtilis* on Casein Agar Medium

➢ *Temperature Influence on Enzyme Yield*

Enzyme production exhibited temperature-dependent variation, peaking at 35°C (71.38 U/mL) as shown in Figure 3.2. The observed decline beyond this optimum temperature correlates with protein denaturation processes, consistent with previous findings on micro- bial protease thermostability. Our results align with established literature reporting optimal production between 35-37°C for *Bacillus* proteases as shown in figure 2.

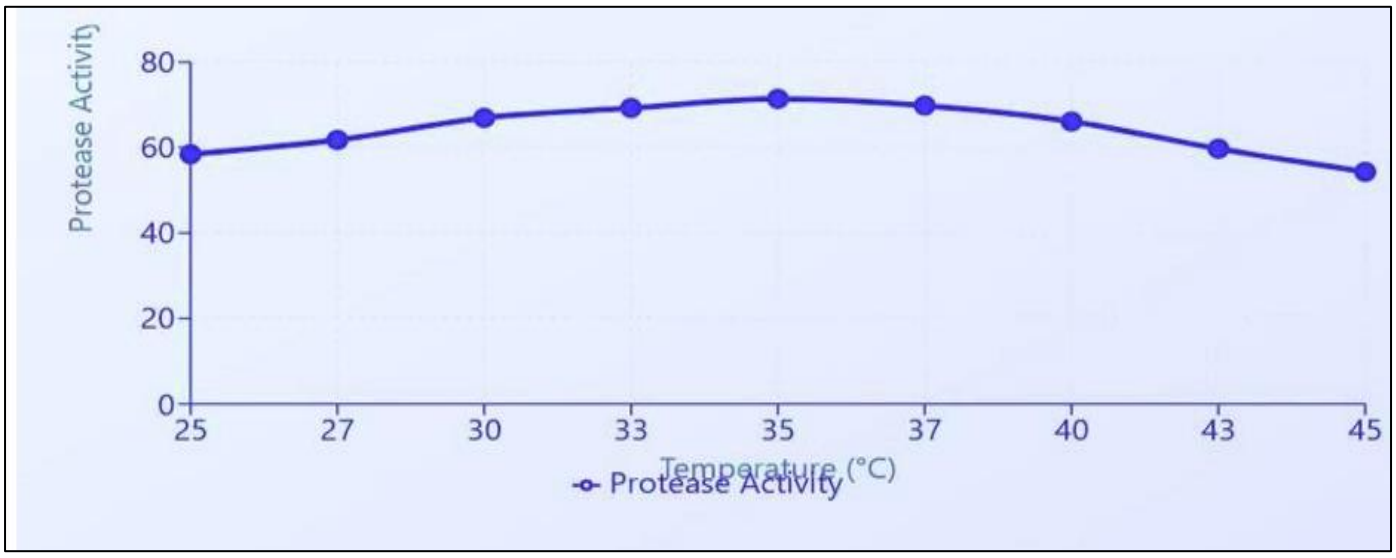

Fig 2 Temperature-Dependent Variation in Protease Biosynthesis

➢ *pH-Dependent Production Characteristics*

The alkaline nature of the protease became evident through pH optimization studies, with maximum yield (72.09 U/mL) occurring at pH 9 (Figure 3). This alkaliphilic preference matches previous characterizations of *Bacillus*-derived proteases , though some variation exists among different strains.

➢ *Incubation Period Optimization*

Time-course analysis revealed maximal enzyme production (72.42 U/mL) after 36 hours incubation as shown in figure 4, corresponding with late logarithmic growth phase. The sub- sequent decline likely reflects nutrient depletion, matching established patterns of microbial enzyme production dynamics

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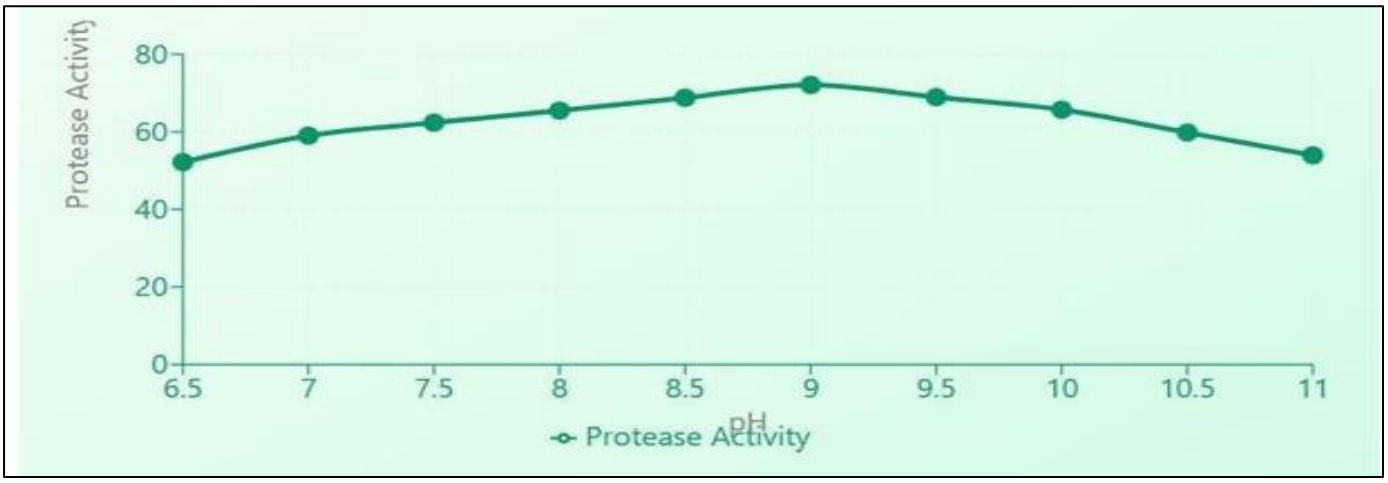

Fig 3 pH Optimization Profile for Protease Biosynthesis

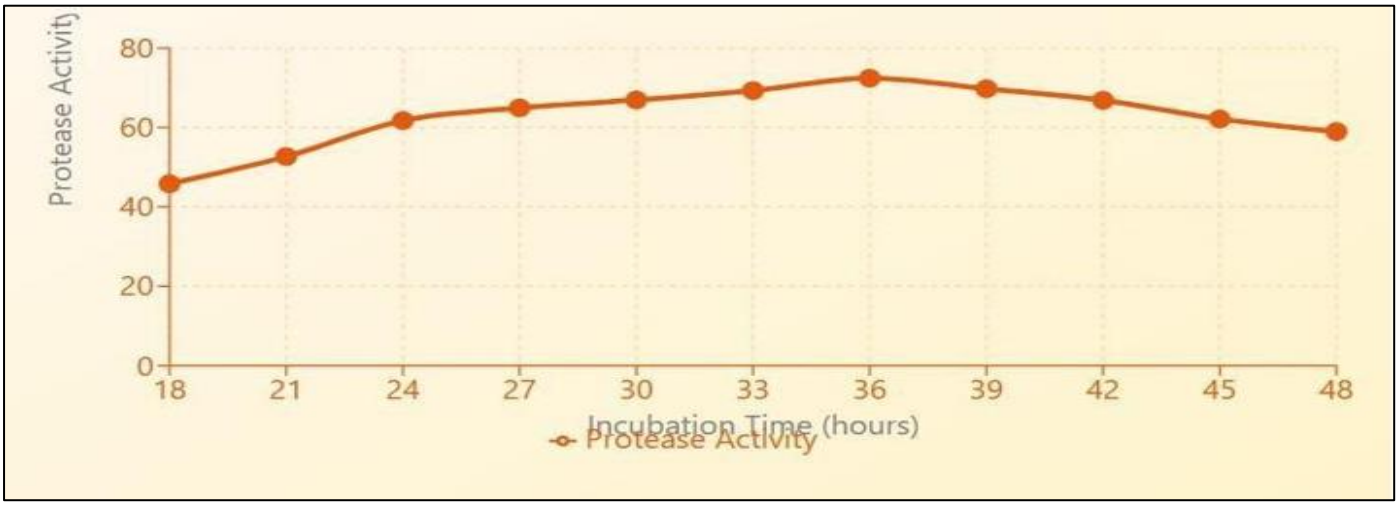

Fig 4 Temporal Pattern of Protease Accumulation during Culture Incubation

➢ *Moisture Content Effects in SSF*

Solid-state fermentation optimization demonstrated 75% moisture content as optimal (71.43 U/mL), with reduced yields at higher moisture levels (Figure 5). This moisture-dependent pattern reflects oxygen diffusion limitations in water-saturated substrates.

➢ *Substrate Selection Analysis*

Among various agricultural residues evaluated, wheat bran supported superior protease production (70.21 U/mL) compared to wheat straw (17.73 U/mL) (Table 3.1, Figure 6). These findings support the economic viability of using cereal byproducts for industrial enzyme production.

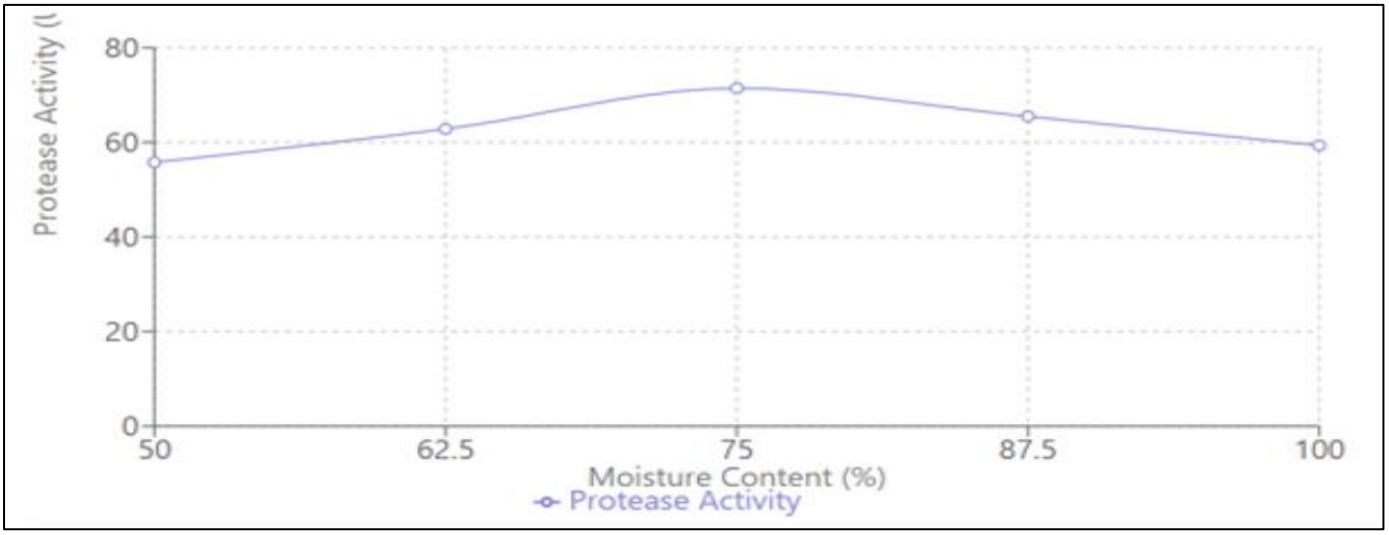

Fig 5 Impact of Moisture Content on Protease Production in Solid-State Fermentation

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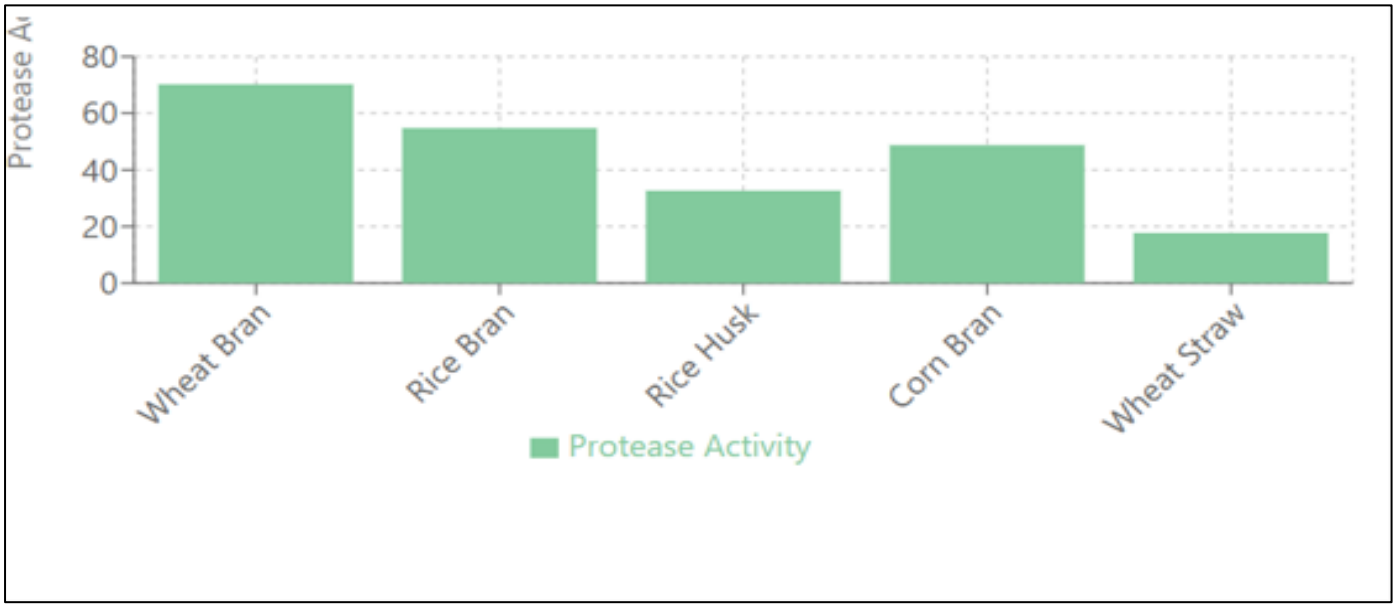

Fig 6 Substrate-Dependent Variation in Protease Production Efficiency

➢ *Process Validation Studies*

Four consecutive fermentation batches under optimized conditions demonstrated consis- tent protease yields (71.18, 68.67, 70.76, and 69.93 U/mL, Table 1), confirming process reliability and reproducibility.

Table 1 Consistency Evaluation of Protease Production Batches

Batch No	Protease Activity (U/mL)
1	71.18
2	68.67
3	70.76
4	69.93

➢ *Enzyme Characterization*

- *Thermal Properties*

The crude protease demonstrated optimal activity at 60°C (96.32% relative activity) with notable thermal stability between 50-60°C (Figure 8), character- istic of thermotolerant bacterial enzymes.

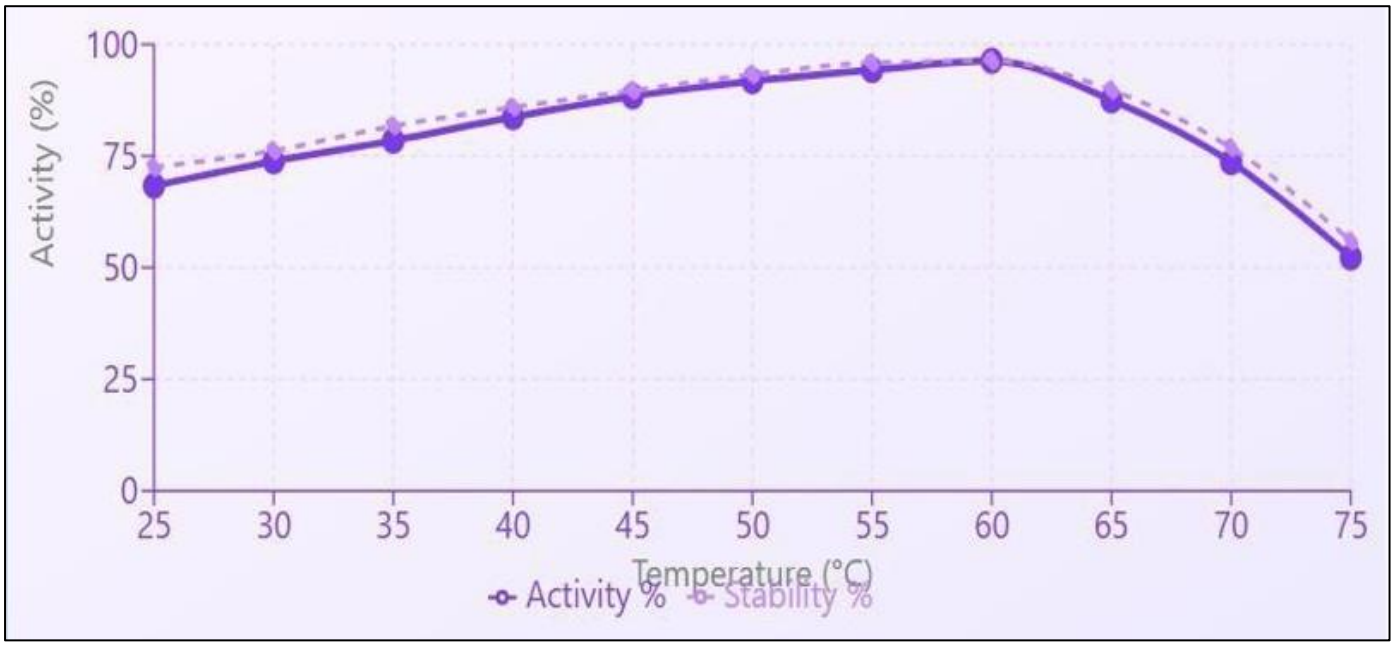

Fig 8 Thermal Activity and Stability Profile of the Protease Enzyme

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➢ *pH Activity Profile*

Maximum enzymatic activity occurred at pH 10 (72.17 U/mL), with maintained functionality across alkaline conditions (Figure 9), confirming classifi- cation as an alkaline protease.

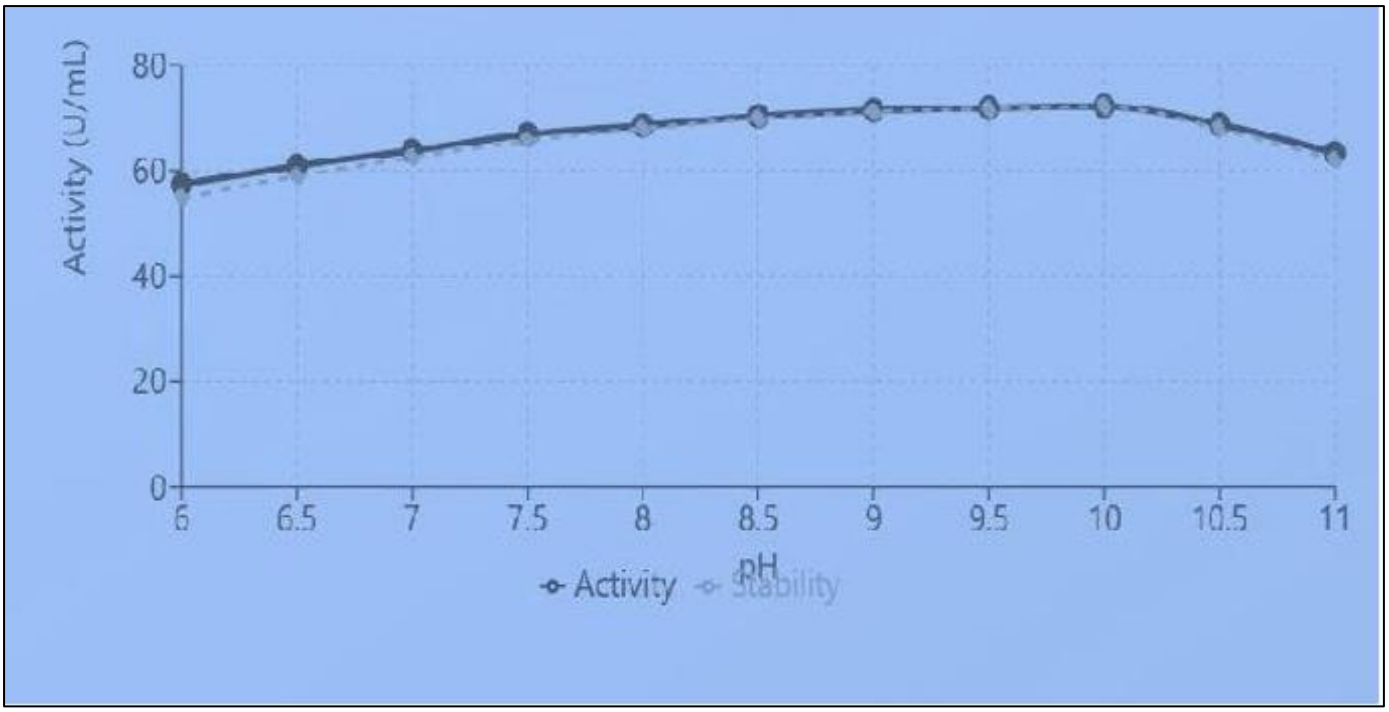

Fig 9 pH-Dependent Activity and Stability Characteristics of the Protease

➢ *Large-Scale Enzyme Production*

Scale-up fermentation in 500 mL and 1000 mL vessels successfully produced sufficient enzyme quantities for subsequent broiler feeding trials and industrial application testing.

➢ *Enzyme Purification*

- *Salting-Out Precipitation*

Ammonium sulfate fractionation (10-85% saturation) achieved maximal enzyme recovery (71.09 U/mL) at 60% saturation, yielding 0.57 mg/mL protein with specific activity of 124.72 U/mg (Figure 3.10).

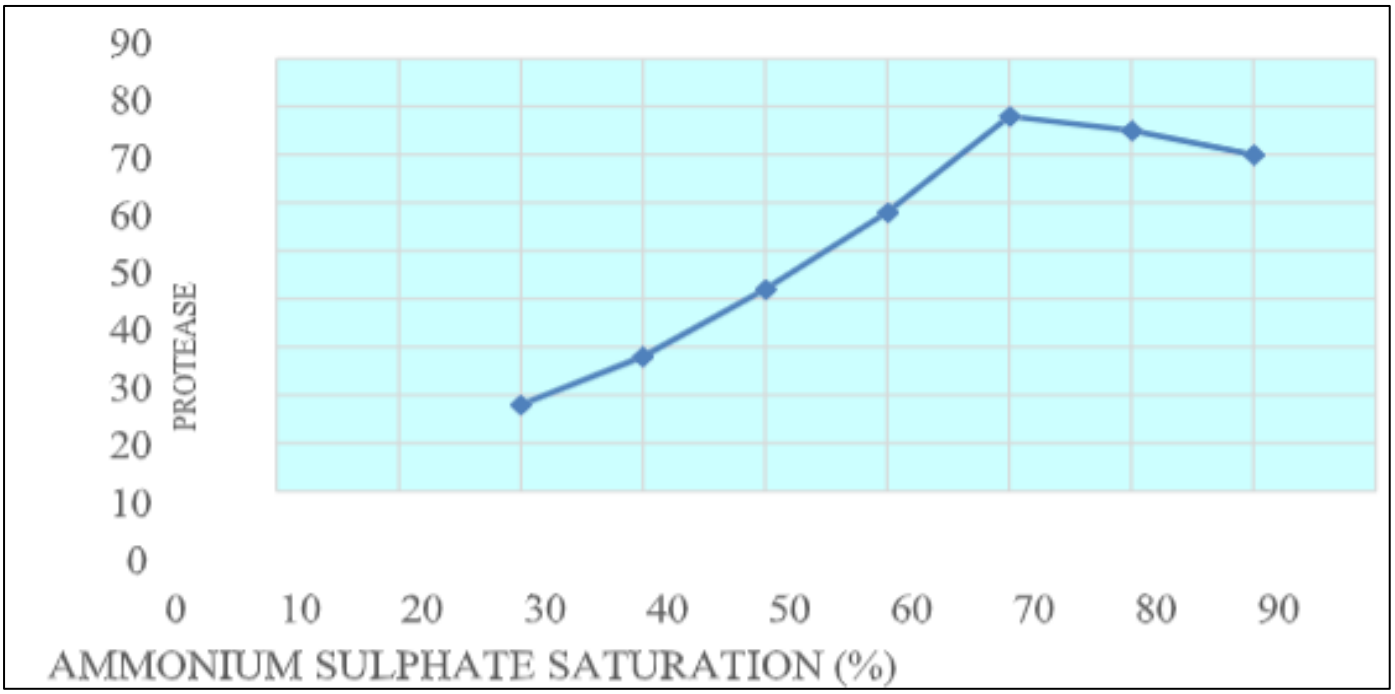

Fig 10 Protease Precipitation Profile using Ammonium Sulfate Fractionation

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- *Conflict of Interest*

  Authors declare no conflict of interests.

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