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Isolation, characterization, and optimization of protease-producing actinomycetes from Rhizosphere soil

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Abstract
Proteases are industrially significant enzymes with diverse applications in food processing, pharmaceuticals, leather treatment, and waste management. In this study, three Actinomycetes isolates were obtained from rhizosphere soil samples using starch casein agar. Screening on skim milk agar revealed clear proteolytic zones, indicating extracellular protease production. Morphological examination confirmed Gram-positive characteristics, and biochemical profiling demonstrated positive results for methyl red, Voges–Proskauer, catalase, starch hydrolysis, and multiple carbohydrate fermentations. Optimization studies revealed that maximum enzyme activity was achieved at 40 °C (137.22 U/L), pH 7.5 (140.20 U/L), and in the presence of $CuSO_4$ (157.22 U/L). Protein estimation using the Lowry method determined the concentration of crude enzyme extracts, and SDS–PAGE analysis indicated a molecular weight of approximately 42 kDa for the purified protease. These findings highlight the potential of Actinomycetes as a source of industrial proteases and provide optimized culture conditions to enhance enzyme yield for possible commercial applications.

Keywords: Protease-producing actinomycetes, rhizosphere soil, protease activity optimization, extracellular protease production, sds–page analysis

Introduction

Proteases are one of the most widely utilized enzymes in industries such as food processing, pharmaceuticals, leather, detergents, and waste management due to their ability to hydrolyze peptide bonds and breakdown proteins into peptides and amino acids. Microbial proteases, particularly those derived from Actinomycetes, are highly valued for their robustness and adaptability to extreme conditions, making them ideal for industrial applications PMCjournalajb2t.com.

Actinomycetes, a group of filamentous, Gram-positive bacteria, are known to secrete a wide array of extracellular enzymes, including proteases. Previous studies on thermophilic Actinomycetes, such as *Thermomonospora fusca* and *Thermoactinomyces vulgaris*, revealed proteases with optimal activities at elevated temperatures (60–70 °C) and alkaline pH (8.0–9.0), with observed molecular weights of 21.5 kDa and 23.8 kDa PMC. Moreover, high-molecular-mass multicatalytic protease complexes have been identified in *Frankia* species, with components ranging between ~19 kDa and 40 kDa after-denaturation PMC.

Comparative data from diverse microbial sources offers perspective: proteases from various *Bacillus* species typically range between 27–68 kDa and operate optimally at alkaline pH (8.0–11.0) and moderate to high temperatures (40–70 °C) PMC. For instance, a *Bacillus circulans* protease (~39.5 kDa) exhibits thermal and detergent stability, while *Pseudomonas* species produce ~43 kDa proteases stable in solvents PMC. These benchmarks indicate that Actinomycetes-derived proteases can have molecular weights and functional properties comparable to those from other industrially significant bacteria.

Metal ions are also known to influence protease production. Studies show that the presence of $CuSO_4$ can enhance protease yield in filamentous fungi; for example, metal ions like $CuSO_4$ promoted maximum enzyme production in one system, outperforming other salts like $CaCl_2$ or $ZnSO_4$ ispub.com. Nevertheless, the specific effect of $CuSO_4$ on Actinomycetes-derived proteases remains underexplored.

Research Gap and Study Objective

Despite the known potential of Actinomycetes in protease production, there is limited literature on proteases around 42 kDa derived from rhizosphere Actinomycetes optimized under moderate conditions. Likewise, the specific enhancement effect of $CuSO_4$ on Actinomycetes protease yield has not been thoroughly investigated.

To address this gap, the present study isolates and characterizes protease-producing Actinomycetes from rhizosphere soil. The aims are to:

1. Identify isolates with significant proteolytic activity.
2. Biochemically profile and optimize culture conditions—including temperature, pH, and metal ion effects (especially $CuSO_4$).
3. Quantify enzyme activity using the Lowry method.
4. Determine the protease molecular weight via SDS–PAGE.

This work not only contributes to the understanding of Actinomycetes-derived proteases but also sets a foundation for their potential application in industrial bioprocessing.

Materials and Methods

1. Sample Collection and Isolation of Protease-Producing Microorganisms

Soil samples were collected from various rhizosphere sites in sterile containers and transported to the laboratory for analysis. Samples (1 g) were suspended in 9 mL sterile distilled water and serially diluted up to 10^{-6}. Appropriate dilutions were spread-plated onto starch casein agar (SCA)

and skim milk agar plates. Plates were incubated at 30 °C for 24 h. Colonies exhibiting zones of clearance on skim milk agar were considered positive for protease production and subcultured to obtain pure isolates.

2. Morphological and Microscopic Identification

Morphological characteristics (colony shape, pigmentation, and texture) were recorded. Gram staining was performed on 16 ± 2 h-old cultures grown at 37 ℃, following the method described by Cappuccino & Sherman (2002) [4]. Microscopic examination confirmed the Gram reaction and cell morphology.

3. Biochemical Characterization

Biochemical tests were carried out as per standard protocols to identify enzymatic and metabolic capabilities:

- **Indole Test:** Inoculated into indole medium; development of a red ring after adding Kovac's reagent indicated a positive result.
- **Methyl Red Test:** Cultures grown in MR-VP broth; red coloration after methyl red addition indicated a positive result.
- **Voges–Proskauer (VP) Test:** MR-VP broth cultures treated with α-naphthol and 40% KOH; rose coloration indicated a positive result.
- **Citrate Utilization Test:** Simmons' citrate agar slants incubated at 37 °C for 48 h; growth and blue coloration indicated a positive result.
- **Carbohydrate Utilization:** Tested with lactose, glucose, sucrose, maltose, and mannitol; acid/gas production recorded.
- **Catalase Test:** Fresh culture mixed with 3% H_2O_2; effervescence indicated a positive result.
- **Starch Hydrolysis:** Cultures streaked on LB agar containing 1% starch; clear zones after Gram's iodine addition indicated starch degradation.

4. Optimization of Culture Conditions for Protease Production

Optimization was performed using the **one-factor-at-a-time** approach.

- **Temperature:** Tested range: 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C.
- **pH:** Adjusted to 6.5, 7.0, 7.5, 8.0, and 8.5 using phosphate or Tris buffers.
- **Metal Ion Supplementation:** Broths supplemented with 1% (w/v) salts — $MgSO_4$, $ZnSO_4$, $MnSO_4$, $FeCl_2$, and $CuSO_4$.

Enzyme activity was measured at each condition, and the highest values were used to determine optimum parameters.

5. Enzyme Extraction and Protein Estimation

Cultures were incubated under optimized conditions. Samples were collected at 24 h intervals, and optical density (OD) was measured at 600 nm. The cultures were centrifuged at 8,000 rpm for 10 min, and supernatants were used as crude enzyme extracts. Protein concentration was determined using the Lowry method with Bovine Serum Albumin (BSA) as standard (Lowry *et al*., 1951) [6].

6. SDS–PAGE for Molecular Weight Determination

The molecular weight of the partially purified protease was determined by Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) using 10% acrylamide gels.

- **Separating Gel Preparation:** 4.0 mL distilled water, 3.3 mL acrylamide, 2.5 mL separating buffer (pH 8.8), 100 µL 10% SDS, 100 µL 10% ammonium persulfate, 10 µL TEMED.
- **Stacking Gel Preparation:** Similar procedure with pH 6.8 stacking buffer.
- **Sample Loading:** 20–40 µL protein samples mixed with loading dye and boiled for 5 min.
- **Electrophoresis:** Run at constant voltage until the dye front reached the bottom.
- **Staining:** Coomassie Brilliant Blue R-250 used for protein visualization.
  The molecular weight was estimated by comparing migration distance to a standard protein marker ladder.

Results

Isolation and Screening of Protease-Producing Actinomycetes

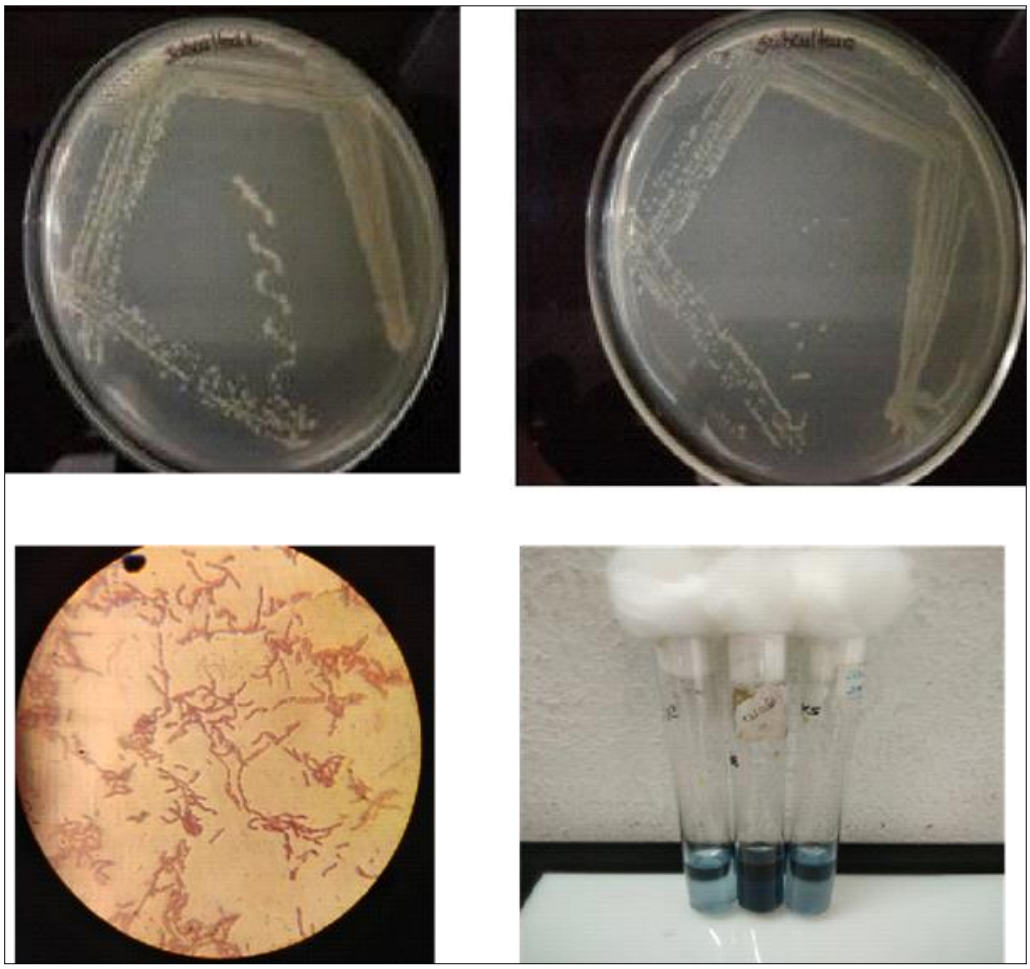
Three morphologically distinct Actinomycetes isolates (designated GS, KS, and SS) were recovered from rhizosphere soil samples using starch casein agar (SCA) plates. Screening on skim milk agar revealed distinct zones of clearance, indicating extracellular protease production. Gram staining confirmed that all isolates were Gram-positive filamentous bacteria.

Biochemical Characterization

The biochemical test results for the most potent isolate are summarized in Table 1. The isolate was positive for methyl red, Voges–Proskauer, catalase, starch hydrolysis, and multiple carbohydrate fermentations (lactose, glucose, sucrose, maltose, mannitol), and negative for indole and citrate utilization.

Table 1: Biochemical test results of the selected Actinomycetes isolate.

Test	Result
Indole	–
Methyl Red	+
Voges–Proskauer	+
Citrate Utilization	–
Lactose	+
Glucose	+
Sucrose	+
Maltose	+
Mannitol	+
Catalase	+
Starch Hydrolysis	+



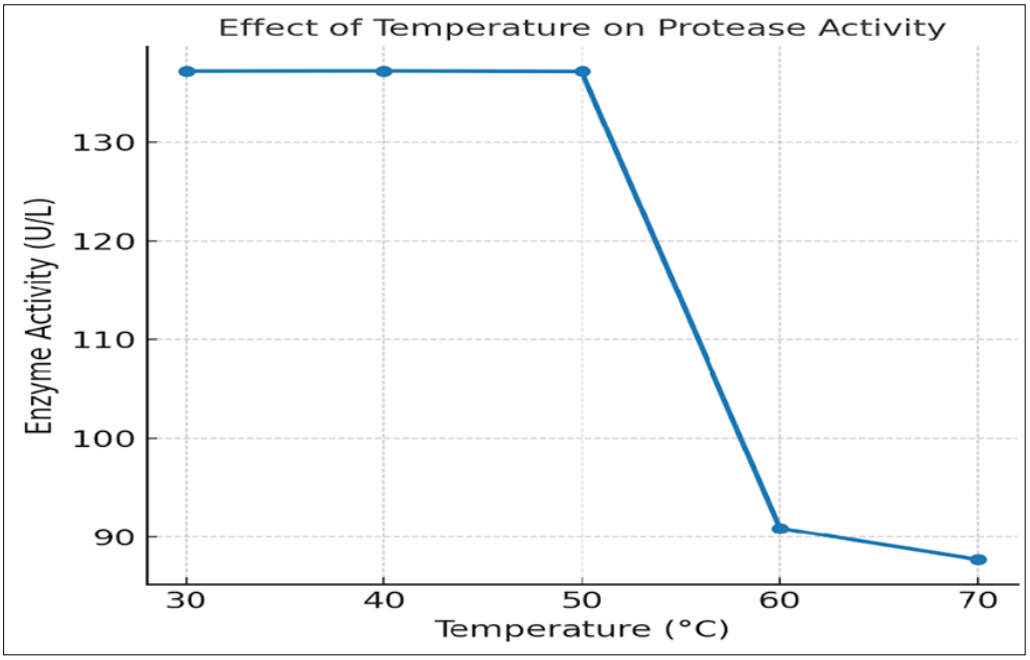
Gram Staining Estimation of protease ninhydrin test

Microscopic observation and confirmation

Optimization of Culture Conditions for Protease Production

1. Effect of Temperature

Protease activity was influenced by incubation temperature (Figure 1). Maximum activity (137.22 U/L) was recorded at 40 °C. Activity declined significantly at higher temperatures, reaching 87.68 U/L at 70 °C.

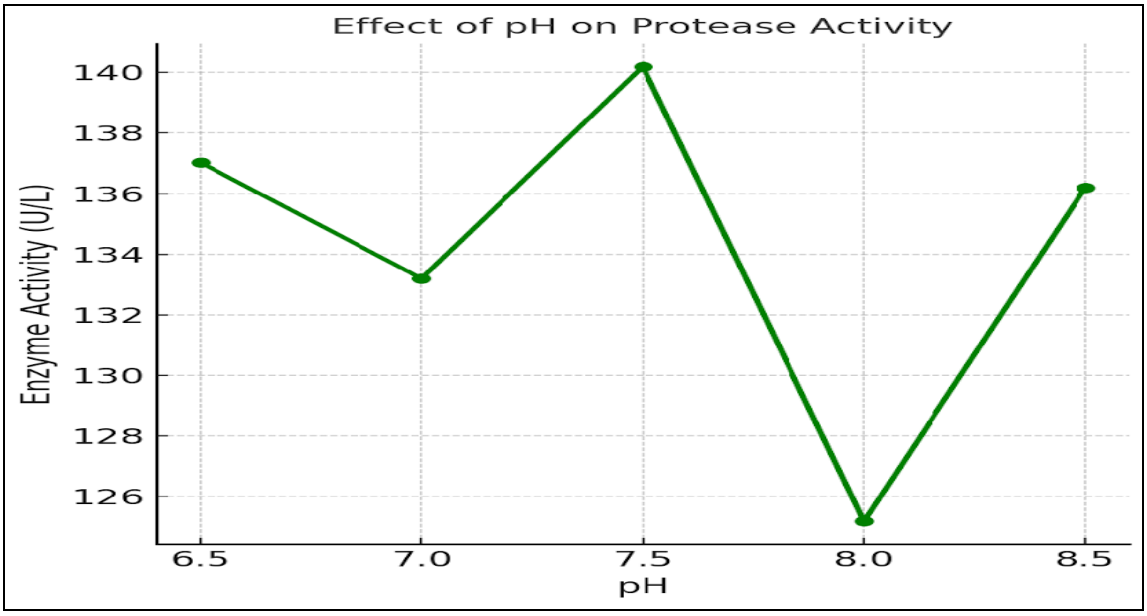


(Values represent the mean of triplicate experiments. Error bars indicate ±SD.)

Fig 1: Effect of incubation temperature on protease activity.

2. Effect of pH

The effect of pH on enzyme activity is shown in Figure 2. The optimum pH for protease production was 7.5 (140.20 U/L), with lower activity observed at both acidic (pH 6.5) and alkaline (pH 8.5) conditions.

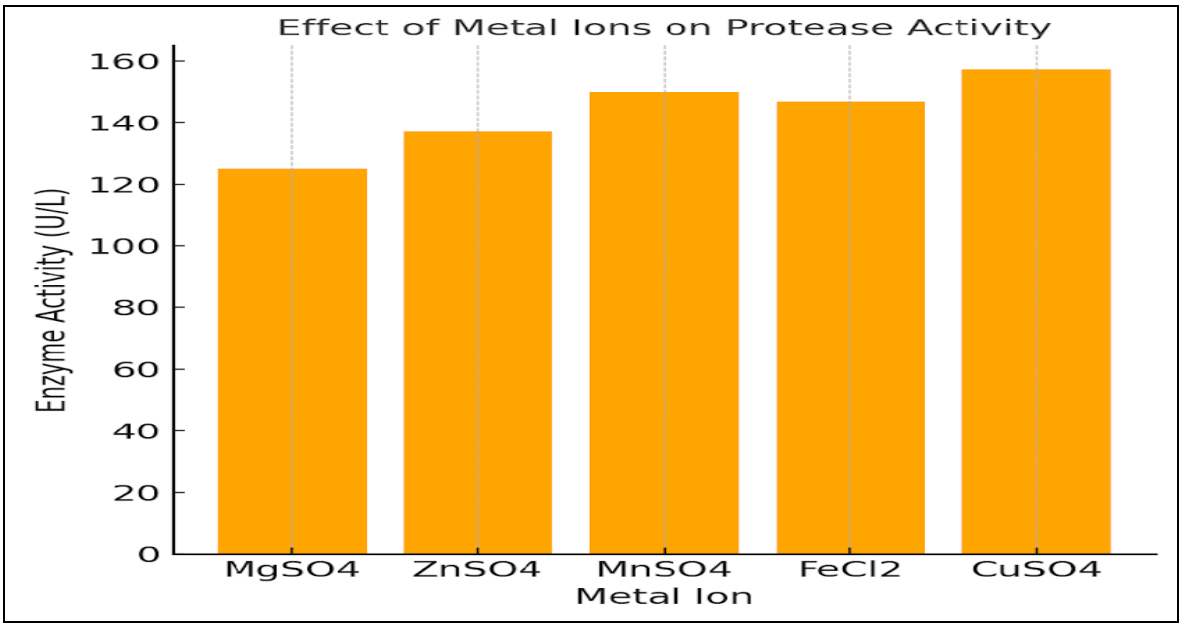


(Values represent the mean of triplicate experiments. Error bars indicate ±SD.)

Fig 2: Effect of pH on protease activity.

3. Effect of Metal Ions

Metal ion supplementation enhanced protease activity in varying degrees (Figure 3). Among the tested salts, $CuSO_4$ yielded the highest activity (157.22 U/L), followed by $MnSO_4$ (150.00 U/L) and $FeCl_2$ (146.90 U/L). $MgSO_4$ produced the lowest activity (125.12 U/L).\



(Values represent the mean of triplicate experiments. Error bars indicate ±SD.)

Fig 3: Effect of metal ions on protease activity.

4. Protein Estimation

Protein concentration of crude enzyme extracts, determined using the Lowry method with BSA as standard, indicated increasing protein yield with prolonged incubation up to 100 µL sample volume. The crude extract corresponding to 100 µL contained the highest protein concentration (2.135 OD).

5. Molecular Weight Determination by SDS–PAGE

SDS–PAGE analysis of the partially purified protease revealed a distinct single band corresponding to a molecular weight of approximately 42 kDa when compared with standard protein markers. This indicates the production of a relatively pure enzyme preparation suitable for further biochemical characterization.

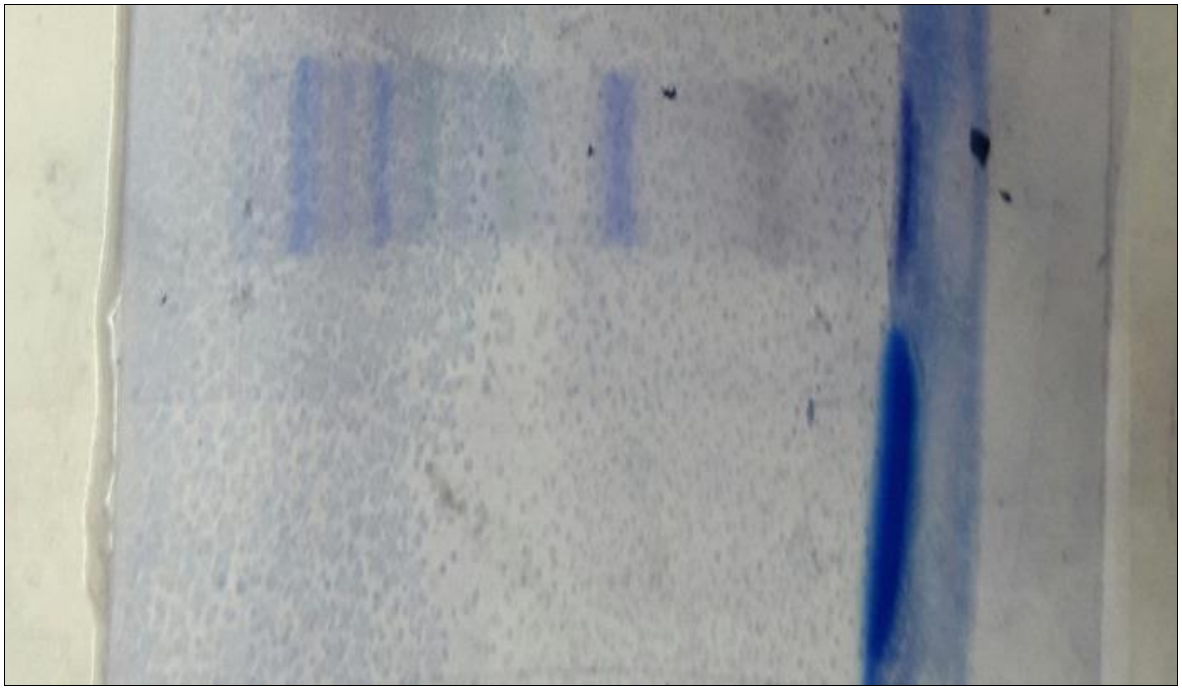


Fig: SDS page gel

Discussion

In the present study, three Actinomycetes isolates were successfully obtained from rhizosphere soil and screened for extracellular protease production. The most potent isolate demonstrated distinct proteolytic zones on skim milk agar and a broad range of positive biochemical activities, confirming its potential as a protease producer.

Comparison with Previous Studies

The optimal temperature for protease production in our isolate was 40 °C, which is comparable to findings by Patel *et al*. (2005) [8], who reported maximum protease activity from *Streptomyces sp.* at 37–45 °C. In contrast, thermophilic Actinomycetes such as *Thermomonospora fusca* and *Thermoactinomyces vulgaris* exhibit optimal protease activity at 60–70 °C (Vishwanatha *et al*., 2009) [10], suggesting that our isolate may be better suited for applications requiring moderate temperature stability.

The optimum pH of 7.5 aligns with reports for several mesophilic *Streptomyces* proteases (Abdallah *et al*., 2013) [1] but differs from alkaline proteases from *Bacillus licheniformis*, which often have optima above pH 9.0 (Rao *et al*., 1998) [9]. This pH preference suggests potential suitability in industries where near-neutral conditions are desirable, such as certain food processing or pharmaceutical applications.

Interestingly, $CuSO_4$ supplementation significantly enhanced protease activity, yielding 157.22 U/L — a result that is rarely documented for Actinomycetes proteases. While manganese and calcium ions are more commonly associated with protease activation (Anwar & Saleemuddin, 1998) [2], Cu^{2+} ions have been reported to improve protease yield in filamentous fungi (Ertan *et al*., 2006) [5]. The observed enhancement here may be due to Cu^{2+} acting as a structural stabilizer for the enzyme or as a cofactor for catalytic site conformation. This finding could be explored for industrial-scale fermentation optimization.

Protein Concentration and Purity

Protein estimation by the Lowry method showed a steady increase in yield with sample concentration, consistent with efficient enzyme secretion during incubation. SDS–PAGE analysis revealed a single prominent band at ~42 kDa, comparable to proteases from *Bacillus circulans* (~39.5 kDa) (Banerjee *et al*., 1999) [3] and *Pseudomonas aeruginosa* (~43 kDa) (Morales *et al*., 2004) [7]. This similarity in molecular weight suggests that the enzyme may belong to the subtilisin-like serine protease family, though further sequencing is required for confirmation.

Industrial Relevance

The characteristics of this protease — activity at moderate temperature, near-neutral pH, and enhancement by Cu^{2+} — make it a promising candidate for:

- Food industry applications where enzyme stability in mild conditions is essential.
- Detergent formulations that require moderate temperature washing efficiency.
- Leather processing, where controlled proteolysis is needed at near-neutral pH.

Moreover, the relatively low molecular weight (~42 kDa) could facilitate efficient secretion in recombinant expression systems, allowing for large-scale production.

Novelty of the Study

While Actinomycetes-derived proteases have been extensively studied, reports of Cu^{2+}-enhanced protease activity in rhizosphere-derived strains remain limited. The combination of optimum parameters identified in this study — particularly $CuSO_4$ supplementation — provides a new approach to increasing protease yield, potentially improving industrial enzyme production economics.

Conclusion

This study successfully isolated and characterized protease-producing Actinomycetes from rhizosphere soil, identifying

optimal culture conditions for maximum enzyme production. The most potent isolate exhibited Gram-positive morphology and strong proteolytic activity, with biochemical characteristics aligning with typical Actinomycetes profiles.

Optimization experiments revealed that maximum protease activity occurred at 40 °C, pH 7.5, and in the presence of $CuSO_4$, which yielded a substantial enhancement over other metal ions. SDS–PAGE analysis indicated a molecular weight of approximately 42 kDa, comparable to several industrially significant proteases.

These results suggest that the identified Actinomycetes strain holds potential for applications in industries requiring moderate temperature and near-neutral pH activity, such as food processing, detergent formulation, and leather treatment. The novel observation of Cu^{2+}-enhanced protease yield highlights a promising avenue for industrial fermentation optimization. Further research should focus on enzyme purification, kinetic characterization, and gene sequencing to fully elucidate its biotechnological potential.

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