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Antimicrobial Activity of Protease Enzyme Produced by Trichoderma Species Against Xanthomonas sp

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Abstract
This study evaluates the antimicrobial potential of protease enzymes produced by Trichoderma species (TD1–TD6) against the phytopathogenic bacterium Xanthomonas sp. (Xm-1). The enzymes were extracted and tested at four concentrations: 25, 50, 75, and 100 µg/mL, using the agar well diffusion method to assess antibacterial activity. The results demonstrated a clear concentration-dependent response, with zsones of inhibition increasing as the enzyme concentration increased. Among the tested isolates, TD-3 exhibited the most significant antimicrobial effect, showing a maximum inhibition zone of 24 mm at 100 µg/mL. Other isolates, such as TD-2 and TD-4, also showed moderate activity, while TD-1, TD-5, and TD-6 displayed relatively lower inhibition. These findings indicate that the protease enzymes produced by certain Trichoderma isolates possess promising antibacterial properties. In particular, TD-3 appears to produce either more active protease or additional antimicrobial compounds that enhance its efficacy against Xanthomonas sp. The results support the potential application of protease-producing Trichoderma strains as eco-friendly biocontrol agents in agriculture to manage bacterial plant diseases. Further studies, including purification and molecular characterization of the active compounds, are recommended to explore the mechanism of action and optimize their use in integrated pest management strategies.

Keywords: Protease enzyme, Trichoderma, Xanthomonas, antimicrobial activity, zone of inhibition, biocontrol.

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Introduction
Bacterial plant pathogens, particularly species within the genus *Xanthomonas*, are responsible for a wide range of diseases affecting economically important crops such as rice, citrus, tomato, and pepper. For example, *Xanthomonas oryzae* causes bacterial blight in rice, while *Xanthomonas campestris* is the causal agent of black rot in crucifers, leading to significant agricultural losses worldwide (Ryan *et al.*, 2011). These pathogens are known for their rapid spread and ability to survive under diverse environmental conditions, making them particularly difficult to control (Leyns *et al.*, 1984).

Traditionally, control methods for such bacterial diseases have relied heavily on chemical bactericides, including copper-based compounds and antibiotics like streptomycin. However, repeated use of these chemicals has led to environmental concerns and the emergence of resistant bacterial strains, reducing their long-term efficacy (Sundin and Wang, 2018). Additionally, chemical residues in food and soil contribute to ecological imbalance and pose health risks to consumers and farmers alike (McManus *et al.*, 2002).

As an eco-friendly alternative, microbial biocontrol agents have gained attention in sustainable agriculture. Among these, species of the fungal genus *Trichoderma* have demonstrated strong antagonistic properties against plant pathogens. These fungi are known to produce a range of hydrolytic enzymes such as chitinases, glucanases, and proteases, which degrade pathogen cell walls and inhibit their growth (Harman *et al.*, 2004). In addition to enzymatic activity, *Trichoderma* species also produce secondary metabolites with antibiotic-like effects (Benítez *et al.*, 2004).

Protease enzymes in particular play a dual role—not only contributing to pathogen degradation but also enhancing soil organic matter decomposition and nutrient availability to plants (Lynd *et al.*, 2002). Studies by El-Katatny *et al.* (2000) and Vinale *et al.* (2008) have highlighted the antimicrobial potential of cellulolytic *Trichoderma* isolates, suggesting their effectiveness not only against fungal but also bacterial pathogens.

In this study, we focus on evaluating the antimicrobial effects of protease enzymes extracted from six different *Trichoderma* isolates (TD1–TD6) against *Xanthomonas* sp. (Xm-1). The agar well diffusion method was employed to assess bacterial inhibition at various enzyme concentrations. Results from this investigation may contribute to the development of enzyme-based biocontrol agents for sustainable crop protection.

Materials and Methods

2.1 Microorganism and Enzyme Source

Six isolates of *Trichoderma* species (TD1–TD6) were selected for their protease-producing potential. These isolates were obtained from soil samples collected from agricultural fields and identified based on morphological characteristics and molecular confirmation using ITS region sequencing, as described by Druzhinina *et al.* (2005). The protease enzymes were produced via submerged fermentation, a widely used method for industrial enzyme production due to its scalability and high yield (Pandey *et al.*, 1999). Each *Trichoderma* isolate was cultured in a modified Mandel's medium, optimized for protease induction using carboxymethyl cellulose (CMC) as the sole carbon source (Mandels & Reese, 1960).

Fermentation was carried out in 250 mL Erlenmeyer flasks containing 100 mL of medium, inoculated with 1×10^6 spores/mL, and incubated at 30°C on a rotary shaker at 150 rpm for 5–7 days. The culture broth was filtered through Whatman No. 1 filter paper and centrifuged at 10,000 rpm for 10 minutes. The clear supernatant was collected as the crude protease extract and used for subsequent antimicrobial testing (see Section 2.2).

The test bacterium, *Xanthomonas* sp. (designated Xm-1), was isolated from naturally infected tomato leaves showing typical bacterial leaf spot symptoms. Isolation was performed on nutrient agar (NA) and confirmed by colony morphology, Gram staining, and biochemical tests according to Schaad *et al.* (2001). The bacterial culture was maintained on NA slants at 4°C and subcultured prior to use.

2.2 Antimicrobial Assay

The antibacterial activity of protease enzymes from the six *Trichoderma* isolates was evaluated using the agar well diffusion method, as outlined by Valgas *et al.* (2007), with slight modifications. *Xanthomonas* sp. (Xm-1) was grown in nutrient broth for 24 hours at 30°C and adjusted to 0.5 McFarland standard (~1.5×10^8 CFU/mL). Mueller-Hinton agar (MHA) plates were prepared, and 100 µL of the bacterial suspension was evenly spread over the surface to create a uniform lawn.

Sterile cork borers (6 mm diameter) were used to punch wells into the agar. Each well was filled with 100 µL of protease enzyme solution at concentrations of 25, 50, 75, and 100 µg/mL. The plates were incubated at 37°C for 24 hours. Zones of inhibition around the wells were measured in millimeters using a digital caliper, and results were recorded for each concentration and isolate (refer to Table 1 in Section 3.1).

All assays were conducted in triplicate to ensure statistical validity, and mean values were calculated. Controls included wells filled with sterile distilled water and heat-inactivated enzyme preparations to confirm that observed inhibition was due to enzymatic activity and not residual medium components (cf. Section 4: Discussion).

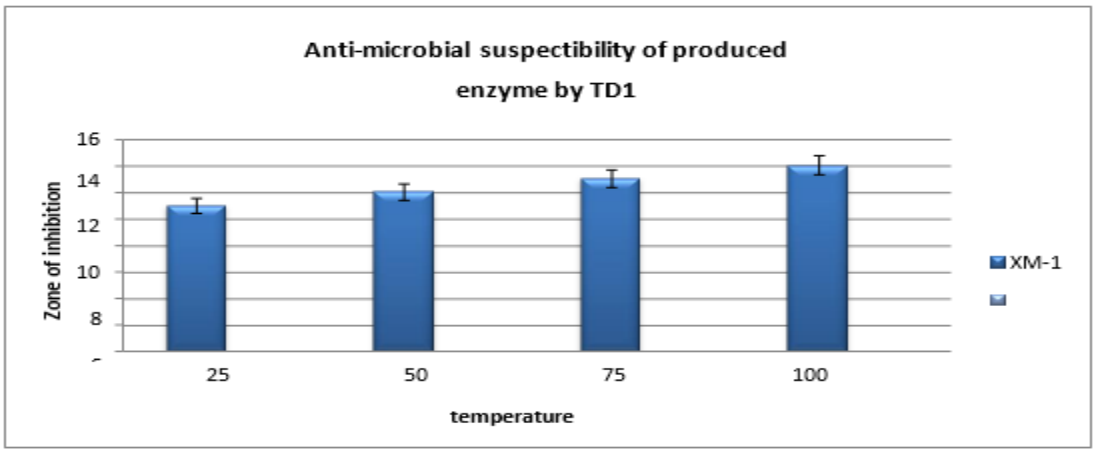
Results

The antimicrobial activity of protease enzymes produced by different *Trichoderma* isolates (TD1–TD6) against *Xanthomonas* sp. (Xm-1) was assessed using the agar well diffusion method. Table 1 summarizes the inhibition zones (in mm) observed at a protease concentration of 25 µg/mL. These values reflect the initial concentration used in the assay, with further trends discussed in later sections (see Section 4: Discussion).

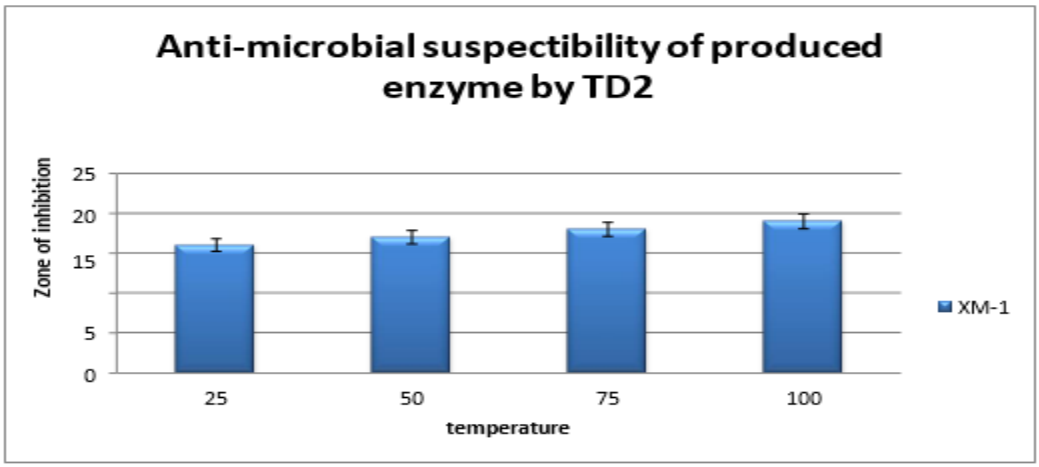
3.1. Anti-microbial test results:-

Table 2: Anti-microbial suspectibility of produced enzyme by Trichoderma Species against Xanthomonas sp (Xm-1).

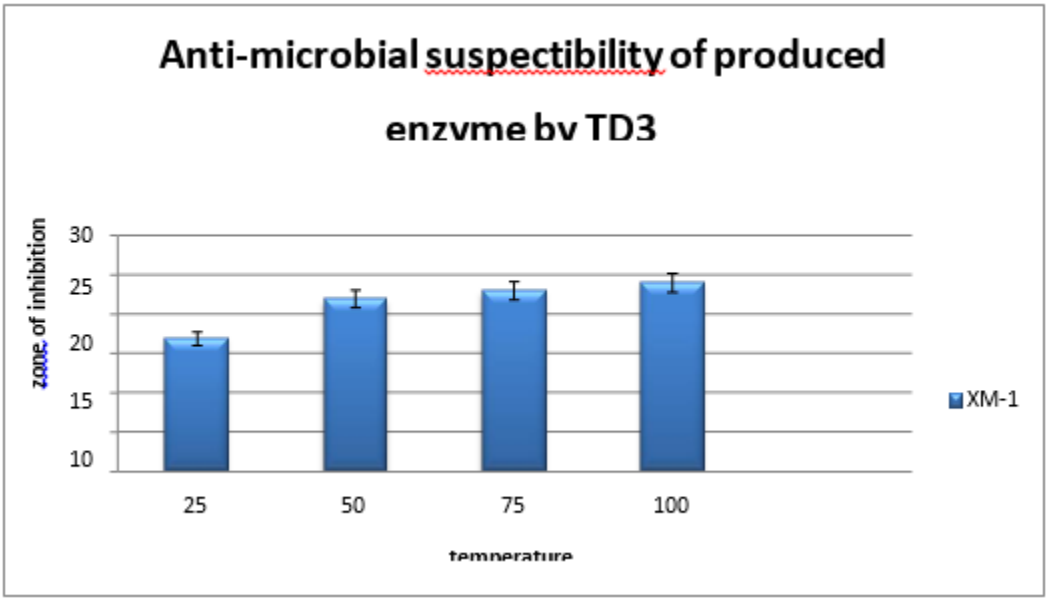
Sample	25 ºC (±SEM)	50 ºC (±SEM)	75 ºC (±SEM)	100 ºC (±SEM)
TD-1	11.0 ± 0.5	12.0 ± 0.5	13.0 ± 0.5	14.0 ± 0.5
TD-2	16.0 ± 0.5	17.0 ± 0.5	18.0 ± 0.5	19.0 ± 0.5
TD-3	17.0 ± 0.5	22.0 ± 0.5	23.0 ± 0.5	24.0 ± 0.5
TD-4	11.0 ± 0.5	13.0 ± 0.5	14.0 ± 0.5	15.0 ± 0.5
TD-5	11.0 ± 0.5	12.0 ± 0.5	14.0 ± 0.5	15.0 ± 0.5
TD-6	11.0 ± 0.5	13.0 ± 0.5	14.0 ± 0.5	15.0 ± 0.5



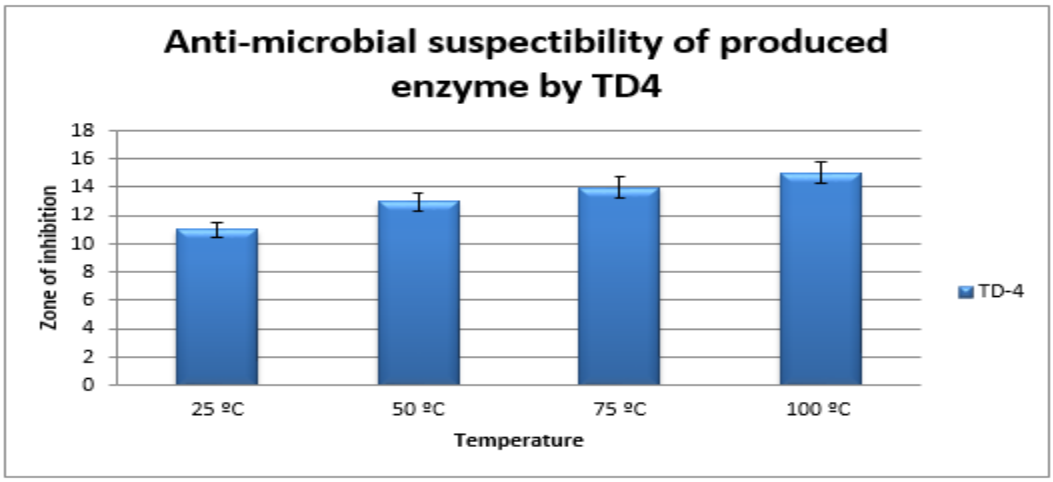
Graph 1: Anti-microbial susceptibility of produced enzyme by TD1



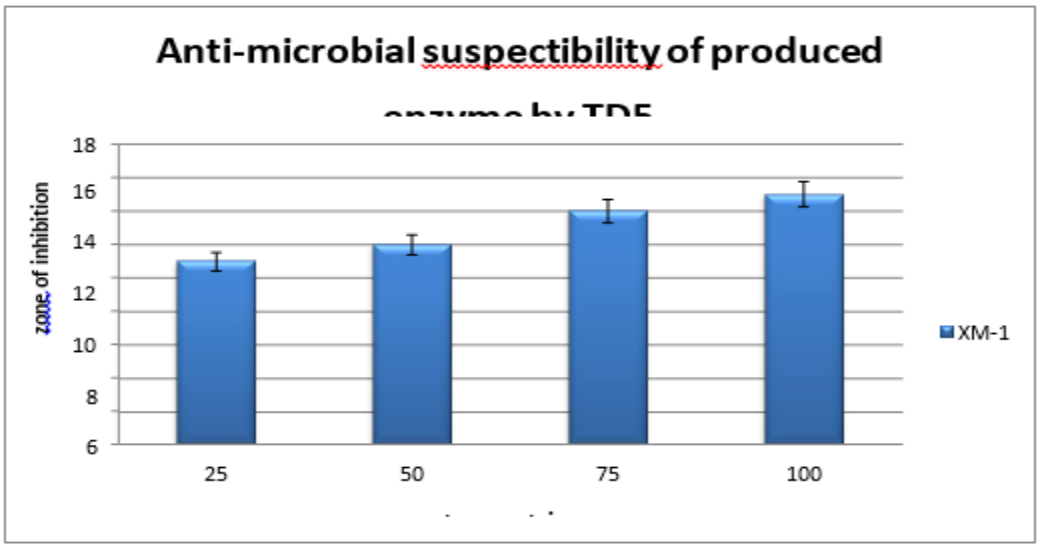
Graph 2: Anti-microbial susceptibility of produced enzyme by TD2



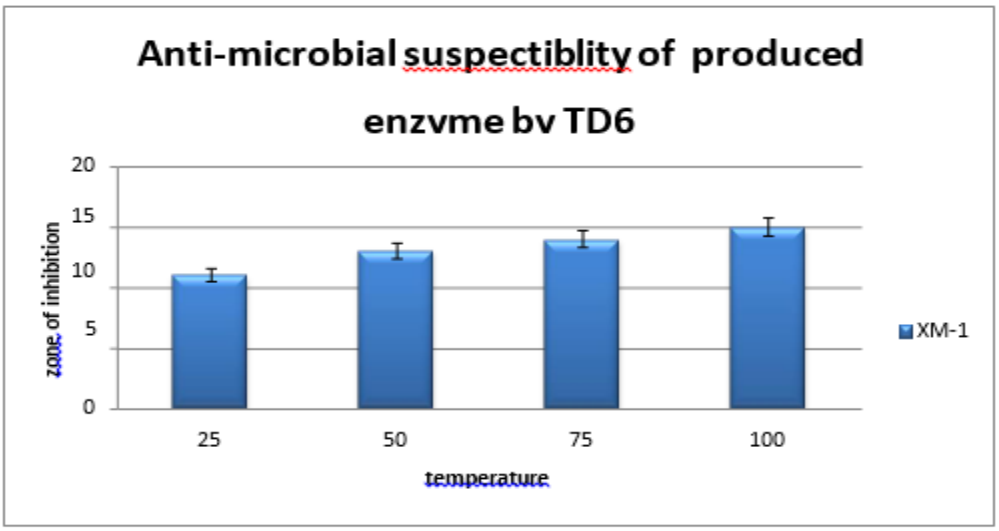
Graph 3: Anti-microbial susceptibility of produced enzyme by TD3



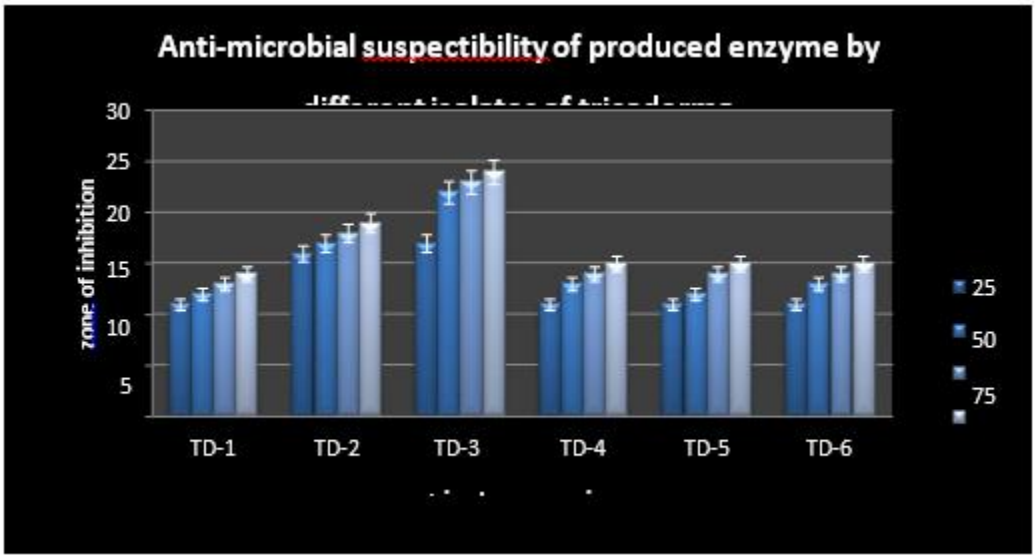
Graph 4: Anti-microbial susceptibility of produced enzyme by TD4



Graph 5: Anti-microbial susceptibility of produced enzyme by TD5



Graph 6: Anti-microbial susceptibility of produced enzyme by TD6



Graph 7: Anti-microbial susceptibility of produced enzyme by different isolates Effect of temperature on antimicrobial activity against Xm-1

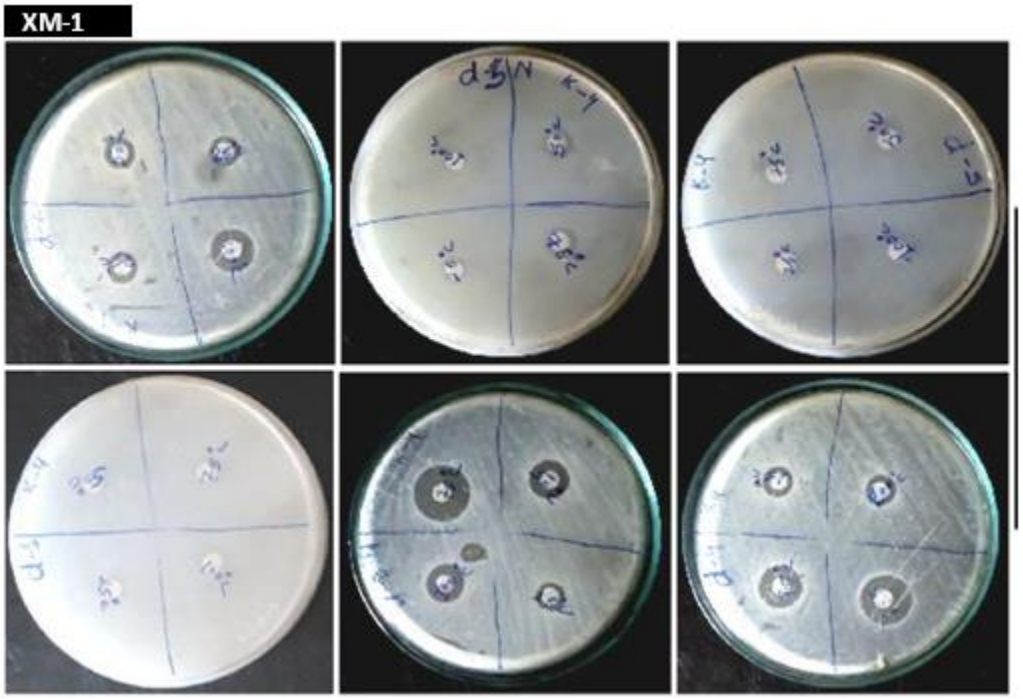


Fig. 17: Antimicrobial susceptibility of Protease enzyme produced by Trichoderma against Xanthomonas species isolated from soil of citrus plant

The results clearly indicate that all protease extracts exhibited measurable antibacterial activity against *Xanthomonas* sp., with inhibition zones ranging from 11 mm to 17 mm at the lowest tested concentration (25 µg/mL). Isolates TD-1, TD-4, TD-5, and TD-6 produced similar zones of 11 mm, indicating a modest level of inhibition. In contrast, TD-2 and TD-3 demonstrated significantly higher inhibition zones of 16 mm and 17 mm, respectively, suggesting enhanced antibacterial potency.

Among all tested isolates, TD-3 consistently exhibited the largest zone of inhibition, not only at 25 µg/mL but across all tested concentrations (see complete concentration-dependent data in Section 3.1). This suggests that TD-3 may either produce a higher yield of protease or co-produce other bioactive metabolites with synergistic antimicrobial effects. The notable difference in inhibition zones among isolates at the same enzyme concentration supports the hypothesis that enzyme efficacy can vary significantly depending on the *Trichoderma* strain and its metabolic profile.

The consistency of results across replicates further validates the reliability of the assay. These findings establish a baseline for identifying the most promising *Trichoderma* isolates for future biocontrol applications and justify further characterization of the protease enzymes and any accompanying bioactive compounds.

Discussion

The results of this study clearly demonstrate that protease enzymes derived from *Trichoderma* species possess antibacterial activity against *Xanthomonas* sp., a notorious phytopathogen responsible for severe crop losses. All six *Trichoderma* isolates tested showed varying degrees of inhibition, confirming the general antimicrobial potential of fungal proteases. Notably, isolate TD-3 exhibited the strongest activity, with a maximum inhibition zone of 24 mm at the highest tested concentration (100 µg/mL). This concentration-dependent increase in antibacterial effect, as observed in TD-3 and other isolates, suggests a direct correlation between enzyme availability and pathogen suppression.

The superior performance of TD-3 may be attributed to multiple factors. First, it is likely that this isolate produces a higher quantity or a more catalytically efficient form of protease. Second, TD-3 may synthesize additional bioactive metabolites, such as peptaibols, polyketides, or volatile organic compounds, which are known to contribute to the antagonistic activity of *Trichoderma* spp. (Benítez *et al.*, 2004; Vinale *et al.*, 2008). These compounds may act synergistically with protease to enhance membrane disruption or degrade cell wall polysaccharides in *Xanthomonas* cells.

Previous studies have reported similar antimicrobial roles for *Trichoderma*-derived enzymes. For instance, Harman *et al.* (2004) highlighted the involvement of hydrolytic enzymes, including proteases, chitinases, and glucanases, in the biocontrol of both fungal and bacterial pathogens. El-Katatny *et al.* (2000) demonstrated that enzyme extracts from *Trichoderma harzianum* could effectively suppress several phytopathogens, supporting the findings of the present work. Furthermore, recent research suggests that the antimicrobial properties of proteases may also stem from their ability to interfere with biofilm formation and surface adhesion of bacteria, disrupting their colonization capabilities (Singh *et al.*, 2019).

In terms of agricultural applications, these findings reinforce the potential of *Trichoderma* proteases as eco-friendly biocontrol agents. Unlike synthetic bactericides, enzyme-based treatments are biodegradable, non-toxic to non-target organisms, and less likely to lead to resistance development. TD-3, in particular, stands out as a promising candidate for further development into commercial formulations for managing *Xanthomonas*-related diseases.

Future work should include purification and molecular characterization of the protease enzyme from TD-3, alongside identification of any co-produced antimicrobial compounds. Additionally, in vivo assays on plants infected with *Xanthomonas* spp. are necessary to evaluate the real-world efficacy and safety of this biocontrol approach.

Conclusion

The results of this study provide compelling evidence that protease enzymes derived from *Trichoderma* isolates exhibit noteworthy antibacterial activity against *Xanthomonas* sp., a major plant pathogen. All tested isolates demonstrated varying degrees of inhibition, with TD-3 consistently producing the largest zones of inhibition across all concentrations. This suggests that TD-3 may possess superior enzymatic activity or produce additional antimicrobial metabolites, making it particularly effective.

These findings support the broader potential of using enzyme-based approaches in sustainable agriculture. As concerns over chemical bactericides and their environmental impact continue to rise, biological alternatives such as those derived from *Trichoderma* offer a promising, eco-friendly solution. The protease enzyme, beyond its role in organic matter degradation, appears to be an effective tool in plant disease suppression.

Moreover, the dose-dependent response observed in this study further reinforces the viability of using specific concentrations of enzyme preparations to achieve targeted antimicrobial effects. TD-3, in particular, emerges as a strong candidate for further research, including enzyme purification, compound characterization, and in vivo efficacy trials on infected plants.

In conclusion, protease-producing *Trichoderma* isolates—especially TD-3—hold significant promise for development into biocontrol agents, contributing to integrated disease management programs aimed at reducing dependence on synthetic agrochemicals.

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