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REVIEWER'S REPORT

Manuscript No.: IJAR-54500

Title: Investigating the Enzymological Properties of Casease from various organisms: Kinetics, Inhibition, Substrate Specificity and Metal ion Dependent Activity.

Recommendation:
Accept as it

Rating	Excel.	Good	Fair	Poor
Originality		√		
Techn. Quality		√		
Clarity		√		
Significance			√	

Reviewer Name: Dr. Manju M **Date:** 24-10-2025

Detailed Reviewer's Report

1. **The main objective of this work**

To investigate the effect of divalent metal ions (Zn^{2+} and Mn^{2+}) on casease activity in Bacillus subtilis and Serratia marcescens. It aimed to determine species-specific differences in enzyme response to varying metal ion concentrations. The study sought to identify activation or inhibition patterns influencing casease function. Another goal was to evaluate substrate specificity among different protein substrates. Overall, the research aimed to elucidate the role of metal ions in regulating bacterial protease activity.

2. **Distinct Species Responses to Metal Ions**

Bacillus subtilis and Serratia marcescens exhibited markedly different enzymatic responses to Zn^{2+} and Mn^{2+}, indicating that casease regulation is species-dependent and controlled by unique metal–enzyme interactions.

3. **Biphasic Zn^{2+} Regulation in B. subtilis**

$ZnCl_2$ exerted a concentration-dependent dual effect — initial inhibition (74.8% at 0.1 mM), activation at moderate levels (127.5% at 1.0 mM), and renewed inhibition at higher concentrations (73.5% at 10.0 mM). This pattern suggests a complex modulation mechanism.

4. **Zn^{2+} as a Consistent Inhibitor in S. marcescens**

Enzyme activity in S. marcescens decreased steadily with increasing Zn^{2+} concentration, from 84.1% to 51.4%, indicating a clear inhibitory effect possibly due to Zn^{2+} toxicity or interference with the enzyme's active site.

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5. **Differential Metal Sensitivity Implies Structural Divergence**

The contrasting Zn^{2+} response between the two species reflects structural variations in their caseases, especially in metal-binding domains influencing catalytic function.

6. **Mn^{2+} Enhances B. subtilis Casease Activity**

Moderate $MnCl_2$ concentrations (up to 2.0 mM) stimulated enzyme activity to 120.7%, suggesting that Mn^{2+} may act as a cofactor, improving enzyme conformation or substrate affinity.

7. **Inhibitory Effects at Elevated Mn^{2+} Levels**

Beyond optimal concentrations (2.0 mM for B. subtilis and 5.0 mM for S. marcescens), Mn^{2+} reduced activity, implying that excess ions destabilize the enzyme structure or disrupt catalytic sites.

8. **Comparative Mn^{2+} Sensitivity**

B. subtilis responded to lower Mn^{2+} levels (0.1–2.0 mM), whereas S. marcescens required higher concentrations for maximal activation, reflecting differences in enzyme–metal binding affinities.

9. **Baseline Enzyme Activity Variation**

Control absorbance values (1.1068 for B. subtilis vs. 1.4855 for S. marcescens) show higher inherent casease activity in S. marcescens, possibly due to greater enzyme expression or stability under basal conditions.

10. **Casein as the Optimal Substrate**

Both bacterial caseases showed peak activity toward casein (100%), confirming substrate specificity aligned with their natural role in proteolysis.

11. **Secondary Substrate Affinity for Gelatin and Glycinin**

High relative activities (~80–85%) toward gelatin and glycinin suggest comparable peptide bond accessibility and secondary structure resemblance to casein.

12. **Reduced Efficiency Toward Plant Proteins**

Zein, legumin, and gliadin yielded moderate to low activities, likely due to their compact or hydrophobic structures limiting enzymatic accessibility.

13. **Species-Specific Substrate Adaptability**

B. subtilis maintained balanced activity across all non-casein substrates, whereas S. marcescens exhibited a sharper decline suggesting narrower substrate versatility.

14. **Metal Ions Influence Substrate Interaction**

The observed metal ion effects may alter enzyme conformation, indirectly affecting substrate binding efficiency and hydrolysis rates.

15. **Ecophysiological Implications**

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The distinct metal and substrate responses suggest adaptive enzymatic mechanisms tailored to each species' ecological niche - soil-dwelling B. subtilis vs. facultatively pathogenic S. marcescens.

16. **Overall Functional Insight**

Casease activity in both species is finely modulated by metal ions and substrate type, underscoring the dynamic regulatory role of divalent cations in bacterial proteolytic systems.

17. Significance of the Work

This study highlights species-specific regulation of casease by Zn^{2+} and Mn^{2+} in Bacillus subtilis and Serratia marcescens. The distinct metal ion responses reveal how enzyme activity is finely tuned by environmental factors. Zn^{2+} showed both inhibitory and activating effects, while Mn^{2+} acted mainly as an activator at optimal levels. These findings enhance understanding of microbial enzyme regulation and adaptation to metal-rich environments. The work holds significance for industrial, biotechnological, and environmental applications of bacterial proteases.