Response to Reviewer 3 Comments

I appreciate the authors' effort in addressing the topic of improvement new tannase with an entitled “Enhancing the Thermostability of a New Tannase Through Rational Design and Site-Directed Mutagenesis: A Quality Improvement Strategy for Green Tea Infusion”; however, I recommend clarifying many aspects in Section (Introduction, Material and Method, and Results &Discussion) to ensure the manuscript is transparent and clear.

Authors’ response:

We would like to thank you very much for taking the time to review this manuscript. We appreciated all the comments and suggestions. Please find the detailed responses below.

Comments 1: L 17, The aim of the study given in abstract is lack and insufficient. It should be expanded to include the exact purpose and full context of the study's purpose.

Response 1:

Thank you for your kind recommendation. We agree with this comment. The abstract has been expanded, please refer to the content highlighted in red (line 15–25).

Comments 2: L 66, Is there another treatment to reduce tannin levels except from enzymatic treatments even on a small scale? If so, authors should give the other treatment procedures in the text.

Response 2:

Thank you for your kind recommendation. We agree with this comment. A chemical detannification method has been added in the text and compared with the enzymatic strategy. Please refer to the content highlighted in red (line 75–81, pp. 2).

Comments 3: L 90, Currently, Is a commercial tannese use in tea industry? Authors should mention about the commercial types of this enyzme used in food industry.

Response 3:

Thank you for your kind recommendation. We agree with this comment. The relevant substance has been added, and this part has been re-written. Please refer to the content highlighted in red (line 88–115, pp. 2–3).

Comments 4: L 153, why was the His60 Ni purification type chosen? Is it food-grade method in terms of contaning Ni residue?

Response 4:

Thank you for pointing this out. With your permission, I'd like to provide clarification as follows:

Nickel-affinity chromatography achieves recombinant protein purification through specific binding between immobilized nickel ions (Ni^{2+}) and hexahistidine tags (His-tag) fused to the C-/N-terminus of target proteins. This technique offers various advantages, such as user-friendly operation, high binding capacity, mild conditions preserving enzyme

activity, and exceptional purification purity, etc. In the stationary phase, Ni^{2+} form stable coordination structures with free carboxyl groups in the support matrices of Sepharose or cross-linked polymers. Crucially, the mild elution conditions (e.g., imidazole gradients) prevent Ni^{2+} leaching, thereby eliminating risks of enzyme contamination and nickel residue. Consequently, this method is extensively adopted for purifying food-grade enzymes.

Comments 5: L 109, *Yarrowia lipolytica* is explained as food-grade from GRAS or FDA? It should be clarified in the text.

Response 5:

Thank you for your kind recommendation. We agree with this comment. This information has been added and mentioned in the **Section 3.2** of **Results and Discussion**. Please refer to the content highlighted in red (line 341–347, pp. 8–9).

Comments 6: L 127, Why was *Escherichia coli* DH5α chosen as the vector? What ensures expression of tannase with *Escherichia coli* DH5α?

Response 6:

Thank you for pointing this out. We sincerely apologize for the lack of clarity in the article that led to misunderstanding. Please allow me to clarify:

The expression vector used was pINA1312, not *E. coli* DH5α which was used for vector preservation. After ligating the *tanPL1* gene into the pINA1312 vector, it was transformed into *E. coli* DH5α strain for vector storage. For subsequent enzyme expression, the vector was extracted from *E. coli* cells and transformed into *Y. lipolytica* URA^- strain for tannase production.

The detailed explanations have been supplemented into the **Section 2.1 and 2.3** of **Materials and Methods**, please refer to the content highlighted in red (line 153, 174–179, pp. 4).

Comments 7: L 150, *Escherichia coli* DH5α primarily produces proteins intracellularly."After 84 h ..... the best extracellular tannase 150 activity,....." It was written as an extracellular tannase, can the authors explain this difference?

Response 7:

Thank you for pointing this out. This issue pertains to the same conceptual point as the previous inquiry in **Comments 6** and similarly stems from insufficient clarification in the article, which led to misunderstanding. The *E. coli* DH5α strain was not used for expression but for the expression vector storage. However, the vector was then transformed into *Y. lipolytica* URA^- strain for tannase expression. Due to the fusion of the XPR2 signal peptide, tannase was secreted extracellularly in this yeast expression system. The detailed explanations have been supplemented into the **Section 2.3** of **Materials and Methods**, please refer to the content highlighted in red (line 174–179, pp. 4).

Comments 8: L 288, On what basis were these fungal organisms selected? Which organism was chosen as the reference? In addition, What is the similarity rate of these sequences?

Response 8:

Thank you for pointing this out. To clarify our methodology: No specific reference species were predetermined during sequence alignment. The tannase sequences for alignment were selected based on highest sequence similarity to TanPL1 retrieved from NCBI BLAST analysis, after which their source organisms were identified. Since TanPL1 originates from *Penicillium longicatenatum* SM102, the tannase sequences exhibiting the highest sequence identity with TanPL1 predominantly derive from fungal genera such as *Penicillium* and *Aspergillus*. Crucially, tannase sequences were chosen for alignment before their source strains were found, with sequence identities to TanPL1 ranging between 37.09% and 57.59%. Additionally, the sequence alignment was performed to validate TanPL1 as a tannase based on sequence homology, and confirm its active sites and conserved domains, so as to exclude these critical regions from consideration for site-directed mutagenesis in the subsequent experiments.

Comments 9: L 491, why was the title of "Treatment of Green Tea Infusion with Tannase MuTanPL1" explained in Results & Discussion chapter? If it is given in this chapter, the authors should support it by comparing different studies for the application of green tea treatment?

Response 9:

Thank you for your kind recommendation. However, heading 3.6 serves as the parent section title, under which subsections 3.6.1 and 3.6.2 are nested, and a lot of substances were described and discussed. Collectively, both subsections constitute integral components of the unified **Section 3.6**. More reasonable titles of **Section 3.6**, **3.6.1**, **3.6.2** in **Results and Discussion** have been adopted, and the paragraph under the heading of **Section 3.6** has been revised, please refer to the content highlighted in red (line 527–537, pp. 14; and line 627, pp. 16).

Comments 10: L 597, For the sensory analysis, some taste attributes have been examined however, is there any change in the color of tea because of this treatment? It has not been clarified in the text.

Response 10:

Thank you for pointing this out. In our experiments, tannase treatment did not induce detectable changes in tea infusion color. Moreover, in our opinion, color attributes should be excluded from this study as we focus on quality improvement conferred by enzymatic processing, whereas color alterations do not intrinsically signify quality enhancement. Variations in infusion color arising from diverse tea sources and processing methods cannot objectively indicate superiority, nor do specific color shifts correlate with improved quality. Crucially, the visual sensory attribute improvement should center on turbidity reduction – tannase treatment yielded significantly clearer infusions with lower turbidity values – representing the more significant quality enhancement results presented in this work.