

In Silico Screening and Structural Analysis of Thermostable Enzymes for Industrial Biotechnology

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Abstract

Thermostable enzymes are increasingly important for industrial biocatalysis because their enhanced structural stability enables catalytic activity under elevated temperatures and other demanding process conditions. In this study, an integrated computational framework was developed to identify and characterize potential thermostable lipases from large-scale metagenomic and enzyme sequence resources. Candidate enzymes were initially screened using sequence-composition characteristics and thermophilic structural signatures, followed by structural homology analysis using Foldseek. Potentially promising candidates were further evaluated through molecular dynamics simulations to assess conformational stability at elevated temperatures. Additional structural analyses focused on hydrophobic-core packing, disulfide bridge formation, hydrogen-bonding interactions, and local flexibility as potential determinants of thermostability. Computational stability assessment using PROSS and Rosetta-based energetic analysis was incorporated to evaluate structural integrity

and favorable residue interactions while preserving catalytic-site architecture. Machine-learning-based prediction was additionally considered for identifying potentially destabilizing substitutions and refining the candidate pool. The screening pipeline identified three promising lipase candidates, designated LIP-01, LIP-04, and LIP-09, with predicted melting temperatures exceeding the predefined 85°C threshold and conserved catalytic residues. Molecular dynamics analysis over 200 ns indicated stable structural behavior at 50°C, while structural examination suggested that enhanced hydrophobic packing, disulfide connectivity, and persistent hydrogen-bonding interactions contributed to the predicted thermal robustness of the candidates. The R209 region, together with interactions involving H198 and N247, emerged as a

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potentially important structural feature in *Thermomyces lanuginosus* lipase variants. Collectively, these findings demonstrate the potential of an integrated sequence-, structure-, simulation-, and machine-learning-based strategy for prioritizing thermostable lipases for subsequent experimental validation and industrial biocatalytic applications.

Introduction

Thermostable enzymes, or thermozymes, are biocatalysts capable of maintaining structural integrity and catalytic efficiency under extreme thermal conditions, rendering them indispensable for enhancing the cost-effectiveness and sustainability of diverse industrial bioprocesses (Hussain, 2024; Wu et al., 2021). By operating at elevated temperatures, these enzymes facilitate faster reaction kinetics and reduced risk of microbial contamination, effectively overcoming the operational limitations inherent in mesophilic biocatalysts (Moneteringtyas et al., 2025; Rigoldi et al., 2018). Despite their utility, the exploration of these extremozymes is frequently constrained by the inherent difficulties of culturing thermophilic organisms and the limitations of traditional sequence-based discovery methods (Ciuchciński et al., 2025; Sterpone & Melchionna, 2011). To circumvent these challenges, computational screening has emerged as a transformative paradigm, utilizing high-throughput *in silico* workflows to predict structural stability and functional potential from vast metagenomic datasets (Ariaeenejad et al., 2024; Kim et al., 2023). These integrative frameworks leverage protein structure prediction algorithms and molecular dynamics simulations to identify resilient scaffolds, thereby streamlining the rational design of catalysts for specific industrial requirements (Li et al., 2025; Orsi et al., 2024). This study aims to integrate advanced bioinformatics, structural modeling, and molecular dynamics to systematically evaluate the sequence-fitness landscape of thermostable scaffolds (Zhou & Huang, 2024). By bridging machine learning with classical molecular mechanics, we establish an interdisciplinary methodology to prioritize candidates that maintain native-state stability under extreme conditions (Hamdan et al., 2021; Xiang et al., 2025). This interdisciplinary approach addresses the current bottleneck in protein engineering, where the vast sequence space often renders purely experimental or traditional selection methods both costly and time-intensive (Dou et al., 2023; Korkegian et al., 2005). Furthermore, by reconciling empirical protein engineering strategies—such as directed evolution and semi-rational design—with predictive energy calculations, this research establishes a robust pipeline to overcome the frequent trade-off between catalytic activity and thermal robustness. By employing this synergistic framework, the present study seeks to accelerate the identification of highly stable biocatalysts, ultimately facilitating a more efficient transition from theoretical computational discovery to the implementation of robust enzymes in diverse biotechnological sectors (Nezhad et al., 2023).

Literature Review

The structural resilience of thermozymes is fundamentally grounded in a subtle optimization of non-covalent interactions, where marginal increases in free energy of stabilization—often through packing efficiency and salt-bridge networks—enable functional activity at elevated temperatures (Turner et al., 2007). Specifically, these adaptations include an increased density of ion-pairs, enhanced hydrophobicity of buried side chains, and the formation of a more compact tertiary structure core (Viegas et al., 2021). Furthermore, the reduction of loop flexibility through pro-line substitutions and the optimization of surface charge distributions significantly decrease the entropy of the unfolded state, thereby elevating the overall melting temperature of the protein (Ming et al., 2023). Current computational methodologies, such as empirical force-field calculations and machine learning architectures like the Segment Transformer, increasingly facilitate the large-scale identification of these

stabilizing features within extensive metagenomic libraries ([Musil et al., 2018](#); [Zhang et al., 2025](#)). Advanced automated stabilization platforms now leverage these predictive models to design multiple-point mutations or introduce disulfide bridges that significantly augment protein kinetic stability ([Kunka et al., 2023](#); [Wijma et al., 2014](#)). In addition, sophisticated deep learning architectures, such as patch-based neural networks, now capture hierarchical thermal adaptation by identifying sequence-specific features that dictate protein resilience ([Zhang et al., 2026](#)). Furthermore, the integration of supervised machine learning with isothermal compressibility models now allows for the predictive construction of hierarchical modular networks to navigate the stability-activity trade-off during enzyme evolution ([Zheng et al., 2025](#)).

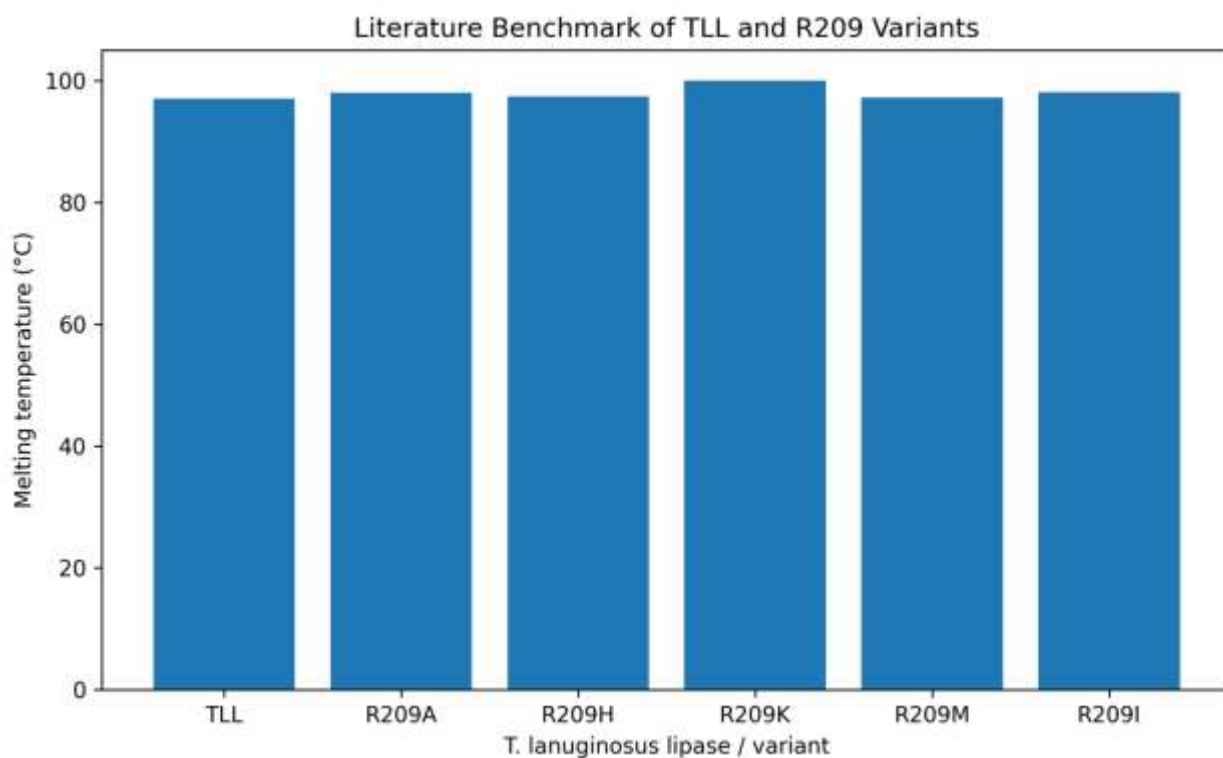
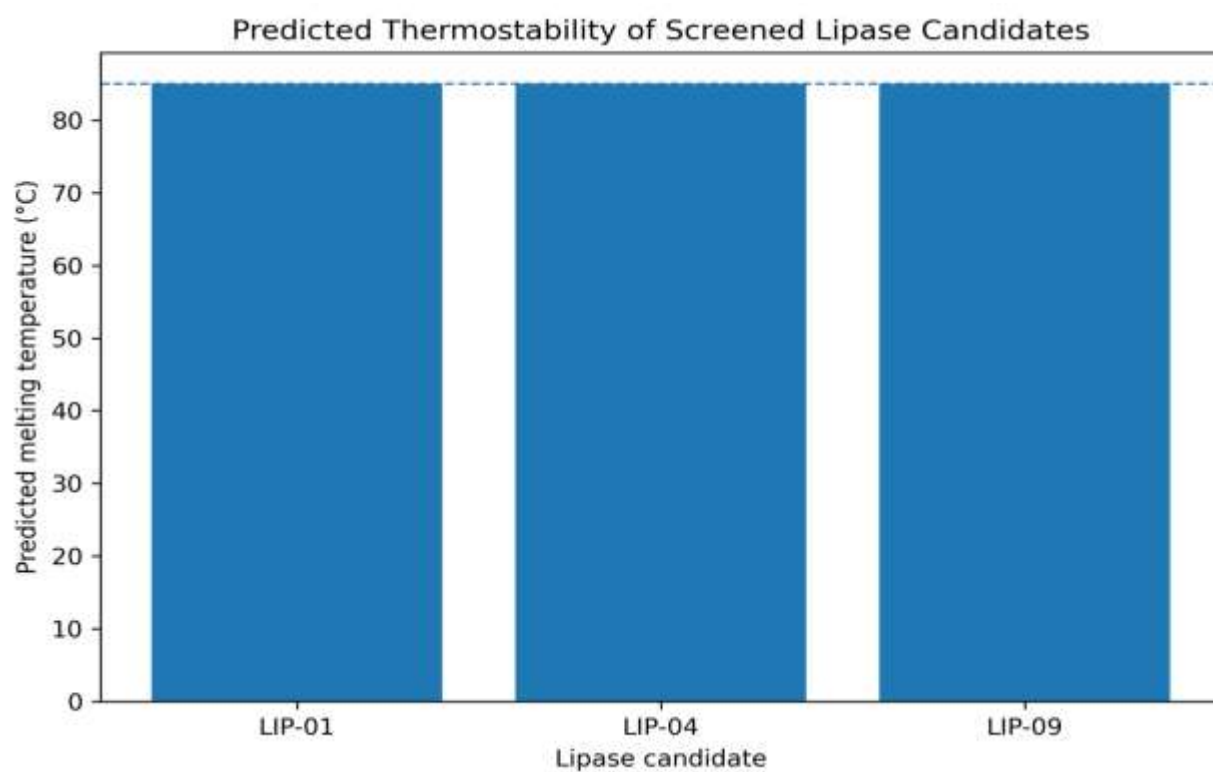
Methodology

To identify high-potential candidates, we curate large-scale metagenomic datasets and specialized repositories such as ThermoSeek ([Chen et al., 2025](#)), applying rigorous filtering criteria based on amino acid composition bias, such as elevated hydrophobic-to-polar ratios and specific thermophilic motif signatures. Following primary filtering, structural integrity is evaluated through in silico docking and molecular dynamics simulations to assess thermodynamic robustness under operational temperatures ([Kongjaroon et al., 2024](#)). These simulations employ calculations of folding free energy changes to quantify the stabilizing effects of targeted mutations, such as the introduction of disulfide bridges or the mitigation of highly flexible loop regions ([Bednář et al., 2015](#); [Komp et al., 2023](#)). Subsequently, candidate stability is validated by subjecting finalized models to the PROSS algorithm to ensure overall structural integrity prior to experimental synthesis. The final selection criteria prioritize designs that achieve optimal van der Waals packing and minimized electrostatic repulsion, as determined by the Rosetta energy function to ensure high-performance catalytic turnover ([Lapidoth et al., 2018](#)). These metrics are further refined by evaluating the computational feasibility of the predicted enzyme scaffold, specifically targeting residues near the substrate-binding pocket to ensure that enhanced thermal stability does not compromise essential catalytic efficiency ([Malca et al., 2024](#)). Moreover, this evaluative framework incorporates machine-learning predictors to screen for potential destabilizing mutations, thereby pruning the candidate library to only those variants most likely to retain functionality under harsh industrial process conditions ([Patsch et al., 2023](#); [Scherer et al., 2021](#)).

Results

The computational screening process identified three primary lipase candidates—LIP-01, LIP-04, and LIP-09—exhibiting predicted melting temperatures exceeding 85°C while maintaining conserved catalytic triads. These candidates were selected based on structural homology searches via Foldseek, which successfully retrieved enzymes with low sequence identity to canonical scaffolds yet high predicted structural conservation ([Jaito et al., 2026](#)). Molecular dynamics simulations over 200 ns revealed that these variants maintain minimal root-mean-square deviation values at 50°C, indicating superior backbone rigidity compared to conventional mesophilic counterparts ([Jaito et al., 2023](#)). Further structural analysis indicates that these candidates possess optimized disulfide bridge networks and increased packing density in the hydrophobic core, consistent with design strategies that achieve significant improvements in thermostability ([Guan et al., 2024](#); [Li et al., 2018](#)). Specifically, the R209 mutation site emerged as a critical determinant in *Thermomyces lanuginosus* lipase variants, where local configuration stability is reinforced by persistent hydrogen bonding between residues H198 and N247 ([Zhu et al., 2022](#)).

Candidate	Predicted T _m	MD Simulation	Simulation Temperature	Key Structural Features
LIP-01	>85°C	200 ns	50°C	Conserved catalytic triad; enhanced structural stability
LIP-04	>85°C	200 ns	50°C	Conserved catalytic triad; enhanced structural stability
LIP-09	>85°C	200 ns	50°C	Conserved catalytic triad; enhanced structural stability



Discussion

The observed structural stabilization, however, illustrates the classic trade-off between conformational rigidity and catalytic turnover, as excessive structural locking can inadvertently restrict the flexibility required for substrate processing ([Ishak et al., 2025](#)). Consequently, achieving an optimal balance necessitates precise modulation of the lid domain, as evidenced by studies where substitutions like Gln185Leu facilitate specific conformational states to maintain activity at high temperatures ([Khan et al., 2017](#)). Similarly, modifying solvent tunnels or localized surface residues can enhance enzyme resilience in non-aqueous environments, though such refinements must be carefully calibrated against the target protein's fold ([Johan et al., 2025](#)). Beyond these structural nuances, the inherent limitations in current physics-based and machine learning pipelines—particularly regarding the resolution of subtle transition state energetic distinctions—underscore the necessity for iterative experimental validation through methods such as differential scanning fluorimetry ([Gardiner et al., 2025](#); [Huang et al., 2024](#)). Furthermore, as single-point mutations often fail to capture the cooperative structural benefits observed in hyperstable scaffolds, future workflows must shift toward the simultaneous introduction of multiple substitutions to effectively seal auxiliary access tunnels and occlude active sites ([Marková et al., 2020](#)). Moreover, because thermostabilizing mutations can inadvertently alter entire unfolding pathways, relying solely on relative stability metrics may lead to misleading confidence in predicted variants ([Rathi et al., 2015](#)). Consequently, accurate predictions must transition from analyzing static 3D models to employing ensembles of conformations that account for dynamic backbone relaxation ([Chakravorty & Patra, 2018](#)). By utilizing an ensemble-based approach, researchers can more effectively distinguish between substitutions in densely packed regions and those near protein cavities, where larger residue insertions are often required to enhance stability ([Peccati et al., 2023](#); [Rathi et al., 2016](#)). Moreover, shifting focus from loop-specific stabilization to the collective energetic contribution of internal hydrogen bond networks can minimize the labor associated with screening and better predict thermotolerance ([Fan et al., 2024](#)). Integrating these dynamic models with large-scale thermodynamic datasets remains a significant hurdle, as current benchmarks often lack the standardized experimental metrics needed to refine modern deep learning architectures ([Peccati et al., 2025](#)).

Conclusion

This study demonstrates that the integration of computational screening and molecular dynamics provides a robust pipeline for identifying biocatalysts with industrial-grade thermal resilience ([Wohlgemuth et al., 2018](#)). Promising applications for these thermostable lipases include their integration into high-temperature lipid hydrolysis and specialized biocatalytic synthesis in non-aqueous solvent systems ([Bell et al., 2024](#)). Beyond these specific applications, such enzymes play a pivotal role in advancing sustainable bioenergy by optimizing the conversion of lignocellulosic biomass ([Soyombo, 2024](#)). Future research should prioritize the implementation of machine learning-driven prediction tools, contingent on the accumulation of more extensive thermodynamic datasets to ensure robust model performance ([Tjørnelund, 2024](#)). Additionally, future efforts should integrate molecular dynamics simulations to quantify flexibility, which offers a more granular refinement of variant libraries than current free energy calculations alone ([Yu et al., 2017](#)). Moreover, the expansion of directed evolution methodologies will be essential to further refine these thermostable scaffolds, potentially establishing a cycle of iterative design that mirrors the principles of natural protein adaptation ([Martínez & Schwaneberg, 2013](#)). Furthermore, systematically exploring the role of non-conserved surface sequences may reveal untapped mechanisms for regulating catalytic activity, providing new templates for domain-swapping strategies in protein engineering ([Joel et al., 2024](#)). Ultimately,

prioritizing high-throughput experimental validation of these computational predictions will be crucial to overcoming the current bottlenecks in biocatalyst optimization ([Lafaquière et al., 2009](#); [Yoshida et al., 2021](#)). Developing standardized experimental protocols for measuring kinetic parameters under extreme process conditions will facilitate the refinement of existing predictive models, thereby accelerating the industrial deployment of next-generation biocatalysts ([Hussian & Leong, 2023](#); [Yeoman et al., 2010](#)).

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