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# The Characterization and Application of Enzymes in Bioprocesses

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Edited by  
Loredana Marcolongo

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# **The Characterization and Application of Enzymes in Bioprocesses**



# **The Characterization and Application of Enzymes in Bioprocesses**

Guest Editor

**Loredana Marcolongo**



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# About the Editor

## **Loredana Marcolongo**

Loredana Marcolongo is a researcher at the Naples Unit of the Research Institute on Terrestrial Ecosystems (IRET) of the National Research Council of Italy (CNR). She received her Master's degree in Chemistry and a PhD in Applied Biology at the University of Naples "Federico II".

Her main research interests are the study and development of eco-sustainable processes for the valorization of plant biomass and agro-industrial waste, with a focus on the extraction and application of bioactive molecules and biopolymers through green processes, with a view to minimizing environmental impact.

This involves the isolation, identification and characterization of proteins, enzymes and biologically active compounds from natural sources and also from extremophilic microorganisms. Her work has led to the identification of various biocatalysts and new compounds derived from natural sources, with a particular focus on their potential for practical applications.

She has published her research outcomes extensively in peer-reviewed scientific articles, review papers, and book chapters and currently serves as an editorial board member for journals regarding biocatalyst and bioactive molecules.



# Preface

Enzymes represent the most sophisticated biological catalysts found in nature, driving essential biochemical reactions with unparalleled specificity and efficiency, and are present in all domains of life. By reducing the activation energy of chemical transformations, these biocatalysts enable life to exist within precise homeostatic boundaries. In the contemporary era of biotechnology, there has been a shift in focus towards utilizing these natural tools for industrial, pharmaceutical and environmental applications, resulting in the development of a bio-based economy.

This Reprint of the Special Issue “The Characterization and Application of Enzymes in Bioprocesses” provides a thorough examination of the multidisciplinary strategies combining biochemistry, structural biology, and genetic engineering, necessary to adapt these biological molecules for the rigors of industrial implementation.

The main motivations behind this scientific collection span the following: the urgent need for sustainable and cost-effective alternatives to traditional chemical catalysts; the fundamental role of enzymes in environmental remediation, the agri-food industry and food processing, as well as in the pharmaceutical and medical sectors; and a deeper understanding of the molecular and kinetic mechanisms of action of these biological molecules. This Reprint brings together a series of original research articles and review papers that highlight the integration of the fundamental molecular characterization of biocatalysts, the cornerstones of modern biotechnology, with their potential and effective applications across a wide range of processes.

This Special Issue aims to encourage reflection on how to realize the enormous potential of biocatalysts in line with societal priorities, scientific capabilities and broader environmental protection objectives. This will lead to decisive green transitions in future industrial processes. Furthermore, interdisciplinary collaboration must be fostered, integrated methodological approaches adopted, and constant dialogue between researchers and policymakers encouraged. The circular bioeconomy approach undoubtedly represents the way forward.

Through the collective insights of its contributors, this issue aims to bridge the gap between fundamental molecular characterization and the practical demands of industrial scalability. We would like to express our gratitude to all the authors whose innovative work has made this collection a significant contribution to the field of modern enzymology.

I would also like to thank the editorial and administrative staff for their constant support throughout the production of this Special Issue, particularly Ms. Amor Lv, Section Managing Editor, whose professionalism and support were instrumental in bringing it to fruition.

**Loredana Marcolongo**  
*Guest Editor*





Editorial

# Special Issue “The Characterization and Application of Enzymes in Bioprocesses”

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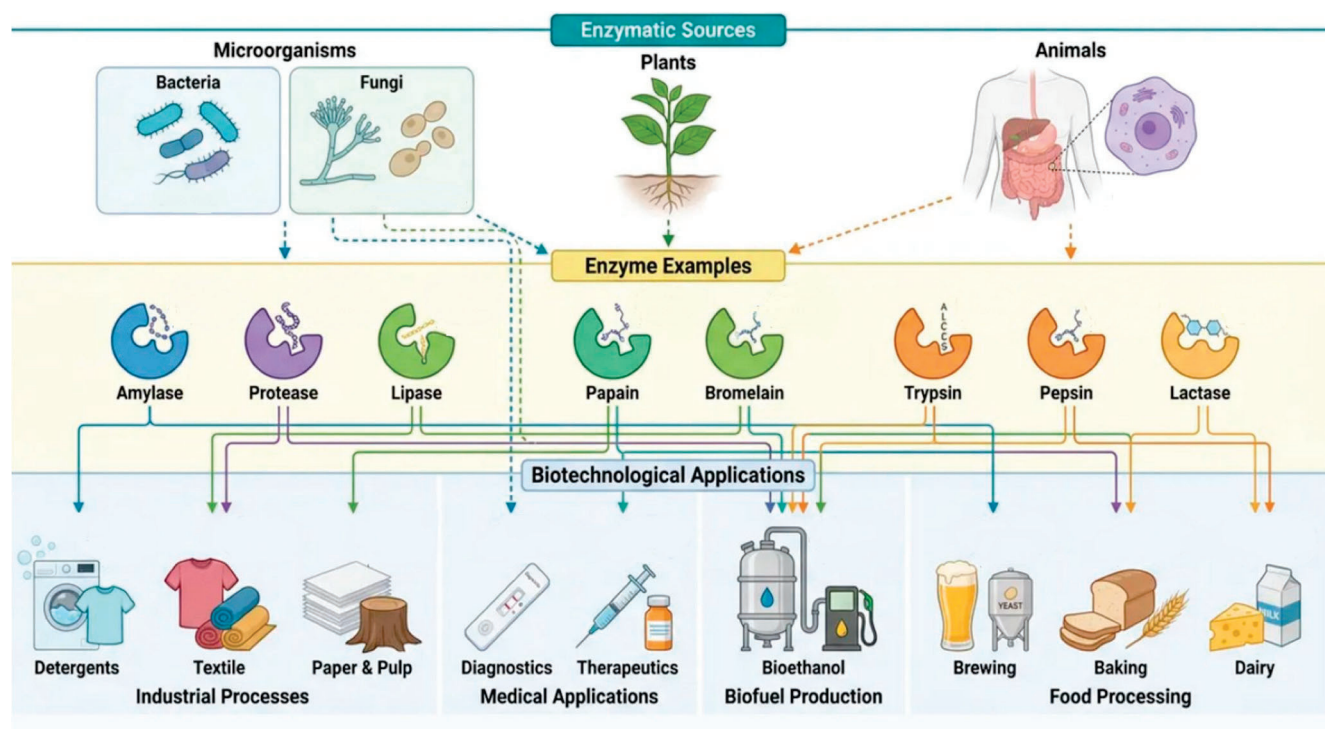
Enzymes are highly efficient biological catalysts that accelerate biochemical reactions in animals, plants and microorganisms without being consumed in the process. The unique feature of these biocatalysts is that they act with a high degree of specificity and efficiency, typically converting a single type of substrate (or at most a range of similar types) into product molecules by reducing the activation energy required for the reaction [1]. Cellular metabolism synthesises macromolecules through sequential enzymatic reactions via various distinct metabolic pathways. Each pathway comprises several stages, with specialised enzymes catalysing individual transformations of substrates (S) into products. Enzymatic specificity arises from evolutionarily refined active sites that bind and orient specific substrates, enabling highly efficient catalysis with minimal errors. The absence or limitation of a specific enzyme halts the flow of the pathway, often proving fatal to the cell [2].

The term ‘enzyme’ was coined in 1877 by Wilhelm Kühne, describing the ability of yeast to hydrolyse starch into glucose, and it is derived from the Greek words *en* (meaning ‘within’) and *zyme* (meaning ‘yeast’). Almost a century of further research was needed to confirm that enzymes are proteins. In recent years, it has been shown that certain chemical reactions can be carried out by RNA molecules, known as ribozymes [3]. Victor Henri was the first to formalise enzyme kinetics, proposing the reversible formation of an enzyme–substrate (E-S) complex prior to catalysis, with the maximum reaction rate denoted as  $V(\max)$ . This was in response to the need to describe enzymatic activity in standard units that could be measured, so that different enzymes could be compared based on their rate and ability to bind to their substrates. This conceptual framework forms the basis of modern enzymology, emphasising substrate specificity, catalytic efficiency, and the interdependence of metabolic pathways in cellular homeostasis [4].

Thanks to their exceptional properties, enzymes play a crucial role in numerous bioprocesses. This is a key sector of biotechnology which uses living organisms and their enzymes to obtain a variety of products. They are essential for industry; unlike traditional chemical catalysts, biocatalysts significantly accelerate reactions under several conditions, demonstrate excellent substrate specificity to minimise by-products, and improve product quality. Biocatalysts also remain unchanged after the reaction, minimising energy consumption and making them sustainable and environmentally friendly alternatives [5].

In recent decades, there has been a surge in enzyme research, driven by advances in genomics, protein engineering, and synthetic biology. Enzymes are versatile and are used in many sectors (Figure 1), including agro-industry, detergents and textiles, medicine, pharmaceuticals, diagnostics and analytical devices, and the environmental sector for the production of biofuels, biomaterials, and bioremediation [6]. However, practical applications require ‘enhanced’ enzymes that are more stable at high temperatures, tolerant

of organic solvents, and scalable to industrial volumes, in order to withstand with extreme process conditions.



**Figure 1.** Examples of enzymatic sources and their potential applications in biotechnology. This figure was created with Motifolio ([www.motifolio.com](http://www.motifolio.com)).

To realise their full potential, enzymes undergo in-depth characterisation using tools from physical chemistry, structural biology and computational modelling. Multidisciplinary approaches combine these tools with genetic engineering, directed evolution or rational design to optimise enzyme activity, stability and specificity. Numerous studies are also aimed at improving immobilisation techniques and researching nanozymes—a new class of nanomaterials with characteristics similar to enzymes—which offer greater stability and lower costs for specific applications [7].

The Special Issue “The Characterisation and Application of Enzymes in Bioprocesses” in the *International Journal of Molecular Science* comprises two review articles and seven original research papers that provide significant insights into the field of enzymes and biocatalysts, as well as the progress made in their study and application.

In the first contribution, Salas-Garrucho et al. (Contribution 1) characterised a novel histidine ammonia-lyase (HAL) from the thermophilic bacterium *Geobacillus kaustophilus* (GkHAL), alongside eight active site mutants. HAL is an enzyme essential for the non-oxidative deamination of L-histidine to produce trans-urocanic acid, which plays a crucial role in amino acid metabolism. Mutational analysis has enabled the establishment of the structure–function relationship within GkHAL, identifying the residues essential for catalytic activity. These findings contribute to highlight their potential for industrial applications, particularly in environments requiring high thermal stability.

In their work, Monterrey et al. (Contribution 2) investigated *Burkholderia cenocepacia*, an opportunistic pathogen associated with cystic fibrosis, that exhibits elevated intracellular polyphosphate (polyP) accumulation, suggesting polyP metabolism as a target for novel biocatalysts. They characterised the polyphosphate kinase 2-III (BcPPK2-III) and the recombinant enzyme was structurally validated. Biochemical assays demonstrated that BcPPK2-III catalyses ATP synthesis from AMP using polyP. The enzyme exhibits

broad divalent cation dependence, and kinetic analysis confirmed a stepwise sequential diphosphorylation mechanism, with ADP as an intermediate, also showing high thermo and pH stability. These characteristics make BcPPK2-III a promising candidate for ATP regeneration in biocatalytic processes, enabling cost-effective and scalable applications to be carried out under particularly challenging industrial conditions.

The development of improved cellulase-based bioprocesses, with enhanced enzyme production efficiency in industrial applications, was investigated in the original paper of Myeong et al. (Contribution 3). In this study, they optimised carbon source composition, agitation speed, and turbulence for enhanced cellulase production by *Trichoderma* sp. KMF006 in submerged fermentation (SmF), evaluating flask-scale parameters and bioreactor scale-up feasibility. The results provide a foundation for hydrodynamic optimisation in industrial SmF, highlighting the importance of balanced aeration, substrate accessibility and shear stress. A cellulolytic enzyme was also studied by Zuo et al. (Contribution 4), who enhanced the catalytic activity and stability of *Oenococcus oeni*  $\beta$ -glucosidase via site-directed mutagenesis targeting key residues in the catalytic pocket, yielding dominant mutants III and IV. Compared to the wild-type, mutants exhibited higher activity, improved thermal stability, and increased substrate affinity, with greater tolerance to low pH. Molecular docking revealed that hydrogen bonding and  $\pi$ - $\pi$  interactions were the primary drivers of the enhanced binding of the enzyme to its substrate. These modifications expand the industrial applications of *O. oeni*  $\beta$ -glucosidase, particularly in enhancing the flavour of food and wine via glycoside hydrolysis, providing a foundation for enzyme engineering.

Gavrilova et al. (Contribution 5) explored the molecular functions of organelle-localised RNase H1. R-loops are three-stranded nucleic acid structures implicated in genome regulation/stabilisation and RNase H1 (RNH1) resolves R-loops and maintains genome stability in eukaryotic cells. RNH1C is essential for chloroplast development in *Arabidopsis thaliana* (AtRNH1C). The study characterised recombinant AtRNH1C in relation to R-loops of various structures using synthetic substrates, demonstrating that AtRNH1C cuts RNA within DNA/RNA hybrids, elucidating the molecular mechanism of a plant organelle-specific RNase H1. The work reveals the kinetic/mechanistic principles underlying the degradation of R-loops by RNase H1 enzymes, highlighting AtRNH1C's specific adaptation to maintain homeostasis in the chloroplast environment. Another original paper by Bodakowska-Boczniewicz and Garncarek (Contribution 6) focuses on the characterisation of an enzyme complex. The study investigated the effect of immobilising naringinase on the optimal activity parameters of its subunits. Naringinase from *Aspergillus niger* KMS was adsorbed onto a carob gum support activated with polyethyleneimine. The two naringinase subunits,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, were characterised in various forms: free; immobilised on a magnetic polysaccharide carrier; and cross-linked with dextran aldehyde. The characterisation of the immobilisation of the two subunits is essential for their application, as it enables the targeted deglycosylation of flavonoids. This increases their bioavailability and antioxidant capacity, which are useful properties for food processing applications.

An interesting study by Tarara et al. (Contribution 7) demonstrates the biodegradation of UV-treated low-density polyethylene (LDPE) by a soil-derived bacterial consortium comprising *Pseudomonas* and *Bacillus* species. Over 30 days, the consortium achieved 7% mass loss for PE film and 13% for PE powder when these were used as the sole carbon sources. The analysis revealed surface oxidation and degradation products while metagenomic analysis identified an upregulation of a cytochrome P450 reductase from the *Bacillus thuringiensis* 9.1 strain, and the purified enzyme was found to modify the PE surface via oxidation. These findings elucidate the mechanisms by which the consortium synergistically degrades PE, highlighting bioremediation strategies for recalcitrant plastic

waste. In the field of plastic waste degradation, the review by Ogunlusi et al. (Contribution 8) summarises the latest developments in the discovery, engineering and heterologous expression of *Ideonella sakaiensis* PETase and its homologues for polyethylene terephthalate (PET) biodegradation. This addresses the environmental crisis posed by persistent PET plastics. Key innovations include in silico/AI-driven enzyme screening, high-throughput assays and optimisation strategies for expression to boost secretion, solubility and yield across various microbial hosts. While laboratory successes demonstrate enhanced PET hydrolysis, persistent challenges include limitations of industrial-scale fermentation, stability of the enzymes under process conditions and integration into waste infrastructures. Future research will overcome these challenges in order to enable cost-effective and scalable PET recycling and bioremediation.

Leontakianakou et al. (Contribution 9) explored the potential of ferulic acid esterases (FAEs). These serine hydrolases act as debranching enzymes and cleave the ester bonds between ferulic acid and polysaccharides, facilitating biomass degradation via synergy with other CAZymes and enabling conversion of agricultural residues to fermentable sugars for biofuels and chemicals. The review provided an interesting and detailed account of the natural occurrence, forms and cross-linking mechanisms of FAEs, their classification systems and substrate specificities, their detection methods, and their versatile applications, including antioxidant, anti-inflammatory, anti-tumour and photoprotective properties in pharmaceutical and cosmetic products; use as vanillin precursors in food products; and synthesis of biopolymers. The biosynthetic potential of FAEs in non-aqueous (trans)esterification processes highlights opportunities for industrial exploitation and underscores the need for further advanced studies on the structure–function relationship.

This Special Issue features a diverse collection of articles that enhance our understanding of the potential of enzymatic biocatalysts across various sectors, including pharmaceuticals, food production, agriculture, environmental remediation, and bio-based manufacturing. Consequently, detailed characterisation and application of enzymes are essential to fully harness their potential as sustainable and versatile catalysts in modern bioprocesses.

**Acknowledgments:** I would like to thank all of the contributing authors of this Special Issue on the Characterisation and Application of Enzymes in Bioprocesses.

**Conflicts of Interest:** The author declares no conflicts of interest.

#### List of Contributions:

1. Salas-Garrucho, F.M.; Carrillo-Moreno, A.; Contreras, L.M.; Rodríguez-Vico, F.; Clemente-Jiménez, J.M.; Las Heras-Vázquez, F.J. Exploring the Kinetics and Thermodynamics of a Novel Histidine Ammonia-Lyase from *Geobacillus kaustophilus*. *Int. J. Mol. Sci.* **2024**, *25*, 10163. <https://doi.org/10.3390/ijms251810163>.
2. Monterrey, D.T.; Azcona, L.; Revuelta, J.; Sánchez-Moreno, I.; García-Junceda, E. Polyphosphate Kinase from *Burkholderia cenocepacia*, One Enzyme Catalyzing a Two-Step Cascade Reaction to Synthesize ATP from AMP. *Int. J. Mol. Sci.* **2024**, *25*, 12995. <https://doi.org/10.3390/ijms252312995>.
3. Myeong, S.; Lee, Y.-Y.; Yun, J. Optimization and Bioreactor Scale-Up of Cellulase Production in *Trichoderma* sp. KMF006 for Higher Yield and Performance. *Int. J. Mol. Sci.* **2025**, *26*, 3731. <https://doi.org/10.3390/ijms26083731>.
4. Zuo, J.; Zhang, J.; Ma, H.; Zhang, Y.; Li, P.; Wu, Y.; Tian, P.; Fan, Q.; Cao, L.; Sun, J.; et al. Site-Directed Mutagenesis Increased the Catalytic Activity and Stability of *Oenococcus oeni*  $\beta$ -Glucosidase: Characterization of Enzymatic Properties and Exploration of Mechanisms. *Int. J. Mol. Sci.* **2025**, *26*, 3983. <https://doi.org/10.3390/ijms26093983>.
5. Gavrilova, A.A.; Kuznetsova, A.A.; Novopashina, D.S.; Zheng, C.; Sun, Q.; Kuznetsov, N.A. Biochemical Characterization of R-Loop Degradation by Chloroplast-Localized RNase H1 from *Arabidopsis thaliana*. *Int. J. Mol. Sci.* **2025**, *26*, 11125. <https://doi.org/10.3390/ijms262211125>.

6. Bodakowska-Boczniewicz, J.; Garncarek, Z. Characterization of  $\alpha$ -L-Rhamnosidase and  $\beta$ -D-Glucosidase Subunits of Naringinase Immobilized on a Magnetic Polysaccharide Carrier. *Int. J. Mol. Sci.* **2025**, *26*, 9813. <https://doi.org/10.3390/ijms26199813>.
7. Tarara, M.; Ahuja, S.; Mellies, J.L. A Cytochrome P450 Facilitates Polyethylene Metabolism in a Microbial Community. *Int. J. Mol. Sci.* **2025**, *26*, 8775. <https://doi.org/10.3390/ijms26188775>.
8. Ogunlusi, T.S.; Ikoyo, S.S.; Dadashpour, M.; Gao, H. Engineering IsPETase and Its Homologues: Advances in Enzyme Discovery and Host Optimisation. *Int. J. Mol. Sci.* **2025**, *26*, 6797. <https://doi.org/10.3390/ijms26146797>.
9. Leontakianakou, S.; Adlercreutz, P.; Nordberg Karlsson, E. Fantastic Ferulic Acid Esterases and Their Functions. *Int. J. Mol. Sci.* **2025**, *26*, 7474. <https://doi.org/10.3390/ijms26157474>.

## References

1. Narváez, A.; Domínguez, E. Enzymes | overview. In *Encyclopedia of Analytical Science*, 2nd ed.; Worsfold, P., Townshend, A., Poole, C., Eds.; Elsevier: Oxford, UK, 2005; pp. 508–523. [CrossRef]
2. Peracchi, A. The limits of enzyme specificity and the evolution of metabolism. *Trends Biochem. Sci.* **2018**, *43*, 984–996. [CrossRef]
3. Heckmann, C.M.; Paradisi, F. Looking back: A short history of the discovery of enzymes and how they became powerful chemical tools. *Chem. Cat. Chem.* **2020**, *12*, 6082–6102. [CrossRef] [PubMed]
4. Traut, T.W. Conditions necessary for life: Take minerals, water, oxygen, and stir. In *Enzymes: The Worker Bees of The Cell*; Traut, T.W., Ed.; Springer Nature: Cham, Switzerland, 2025; pp. 1–16, Correction in *Enzymes*; Springer: Cham, Switzerland, 2025; pp. C1–C2. [CrossRef]
5. Farhan, M.; Hasani, I.W.; Khafaga, D.S.R.; Ragab, W.M.; Ahmed Kazi, R.N.; Aatif, M.; Muteeb, G.; Fahim, Y.A. Enzymes as Catalysts in Industrial Biocatalysis: Advances in Engineering, Applications, and Sustainable Integration. *Catalysts* **2025**, *15*, 891. [CrossRef]
6. Ndochinwa, O.G.; Wang, Q.Y.; Amadi, O.C.; Nwagu, T.N.; Nnamchi, C.I.; Okeke, E.S.; Moneke, A.N. Current status and emerging frontiers in enzyme engineering: An industrial perspective. *Heliyon* **2024**, *10*, e32673. [CrossRef] [PubMed]
7. Tan, W.Y.; Gopinath, S.C.B.; Anbu, P.; Yaakub, A.R.W.; Subramaniam, S.; Chen, Y.; Sasidharan, S. Bio-Enzyme Hybrid with Nanomaterials: A Potential Cargo as Sustainable Biocatalyst. *Sustainability* **2023**, *15*, 7511. [CrossRef]

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Article

# Exploring the Kinetics and Thermodynamics of a Novel Histidine Ammonia-Lyase from *Geobacillus kaustophilus*

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**Abstract:** Histidine ammonia-lyase (HAL) plays a pivotal role in the non-oxidative deamination of L-histidine to produce *trans*-urocanic, a crucial process in amino acid metabolism. This study examines the cloning, purification, and biochemical characterization of a novel HAL from *Geobacillus kaustophilus* (GkHAL) and eight active site mutants to assess their effects on substrate binding, catalysis, thermostability, and secondary structure. The GkHAL enzyme was successfully overexpressed and purified to homogeneity. Its primary sequence displayed 40.7% to 43.7% similarity with other known HALs and shared the same oligomeric structure in solution. Kinetic assays showed that GkHAL has optimal activity at 85 °C and pH 8.5, with high thermal stability even after preincubation at high temperatures. Mutations at Y52, H82, N194, and E411 resulted in a complete loss of catalytic activity, underscoring their essential role in enzyme function, while mutations at residues Q274, R280, and F325 did not abolish activity but did reduce catalytic efficiency. Notably, mutants R280K and F325Y displayed novel activity with L-histidinamide, expanding the substrate specificity of HAL enzymes. Circular dichroism (CD) analysis showed minor secondary structure changes in the mutants but no significant effect on global GkHAL folding. These findings suggest that GkHAL could be a promising candidate for potential biotechnological applications.

**Keywords:** histidine ammonia-lyase; enzymatic kinetics; thermostability; thermal inactivation; circular dichroism

## 1. Introduction

The ammonia-lyase family encompasses a group of homologous enzymes in the degradation of amino acids via deamination. In contrast to the usual oxidative deamination catabolic route, ammonia-lyase enzymes carry out a non-oxidative deamination of the  $\alpha$ -amino group via elimination of a non-acidic  $\beta$  hydrogen, giving the correspondent acids [1,2]. Within the family, some of the most representative and studied enzymes are phenylalanine ammonia-lyases (PALs; EC 4.3.1.5), histidine ammonia-lyases (HALs; EC 4.3.1.3), aspartate ammonia-lyases (AALs; EC 4.3.1.2), and tyrosine ammonia-lyases (TALs; EC 4.3.1.23) [3,4]. Over the years, it was thought that this group was characterized by a catalytical essential dehydroalanine site [5–7]. However, more recent studies demonstrate a common characteristic active site containing 3,5-dihydro-5-methylene-4H-imidazole-4-one, known as the MIO catalytic cofactor [8–10]. This highly electrophilic moiety is formed post-translationally and autocatalytically from the Ala-Ser-Gly sequence [2,4].

The enzymes characterized in this study are the wild-type HAL and three mutant variants. HAL, considered a universal enzyme, is supposed to be the first enzyme and principal regulator of the non-oxidative degradation pathway of L-histidine amino acid [11,12]. HAL carries the degradation of L-histidine to form the  $\alpha,\beta$ -unsaturated *trans*-urocanate and releases ammonia [3,4,8,12]. The reaction mechanism is proposed to be following a

Friedel-Crafts attack [2,3,13,14]. In this mechanism, the electron pair from the imidazole of (S)-His attacks MIO, leading to the formation of a cation on this imidazole ring, which in turn acidifies the hydrogen atom at the  $\beta$ -position of (S)-His. Afterwards, the enzyme's basic group abstracts the  $\beta$ -proton, resulting in the formation of urocanate and the elimination of ammonia, thereby regenerating the electrophilic group. Other analogous enzyme mechanisms have been extensively studied. For example, PAL and AAL demonstrate a comparable non-oxidative reaction involving their respective substrates, leading to the formation of trans unsaturated carboxylic acid through a shared Friedel–Crafts mechanism [10,13,15,16]. Regarding the substrate specificity of aromatic ammonia-lyases, it has been demonstrated that it is limited to the individual aromatic amino acids: L-histidine, L-phenylalanine, and L-tyrosine. Despite a single, unconfirmed report of a tryptophan ammonia-lyase [17], naturally occurring MIO-dependent enzymes with alternative specificities remain undiscovered.

Even though HAL shares the same catalytic moiety as other enzymes of the family (PAL, TAL, and AAL), it is only active within a narrow substrate range, specifically 2 and 4-fluoro-L-histidine [18], 4-nitro-L-histidine [19], L-histidine methyl ester [20], L-histidine [13,19,20], and N $\tau$ -methylhistidine [21]. It was observed that zinc increases the activity of HAL from *Pseudomonas putida* (PpuHAL) during L-histidine degradation [20,22]. Such metal activation could be explained by assuming its interaction with H83 and the substrate histidine [23]. However, in the X-ray crystal structure of PpuHAL, no Zn<sup>2+</sup> ion is observed [24].

HAL is fairly ubiquitous in nature, being present in a wide range of organisms, from bacteria to humans, spanning through plant and animal species. HAL has been isolated from several biological sources, and the sequence and functional overexpression of the gene *hutH* have been reported [9,20,24–29]. The crystal and active site structures of these enzymes have been previously studied [24–29]. Based on the eight reported crystal structures, the HAL structures are homotetramers with four identical active sites formed by amino acids from three distinct monomers.

Histidine is one of the nine essential amino acids crucial for the metabolism of most animals, necessitating its acquisition through the diet. In mammals, a lack or defect of HAL can lead to diseases such as histidinemia, a genetic metabolic disorder characterized by elevated levels of the amino acid histidine in the human body, and low levels of urocanic acid, which serves as a UV protector in the skin [30]. However, in most bacteria, it only blocks the histidine utilization route, which is not lethal. The pathway of histidine catabolism is highly conserved among bacteria, enabling cells to utilize this amino acid as a source of carbon, energy, and nitrogen [12]. HAL enzymes have been extensively studied in several bacterial genera, including *Pseudomonas*, *Bacillus*, *Serratia*, and *Achromobacter*, among others [3,8,12,13,24–27], as well as in some extremophiles [29]. HALs have promising industrial applications, such as their reported role in producing optically pure D-histidine [31]. More recently, it has been shown that bacterial HALs possess an antineoplastic effect and can selectively inhibit cell proliferation, highlighting their potential for therapeutic use [32–34]. Additionally, HAL from *Kurthia* sp. (KxHAL) has been used to produce *cis*-urocanic acid, a stereoisomer with immunosuppressant properties. Moreover, in vitro studies indicate that KxHAL can reduce viral proliferation [34].

In our endeavor to obtain a new HAL with superior robustness for industrial applications compared to those previously described, our laboratory began screening thermophilic microorganisms. In this context, thermostable enzymes derived from thermophilic and extremophilic microorganisms emerge as appealing options for industrial processes operating in harsh conditions [35]. Thermozyms have been demonstrated to offer numerous advantages, such as increased tolerance and high reaction rates at elevated temperatures. The genus *Geobacillus* mainly comprises Gram-positive thermophilic bacteria. The NCBI taxonomy browser currently lists 182 complete genome sequences for *Geobacillus* species (<https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi> (accessed on 30 August 2024)). One of these is the genome of *Geobacillus kaustophilus*, a bacterium with a maxi-

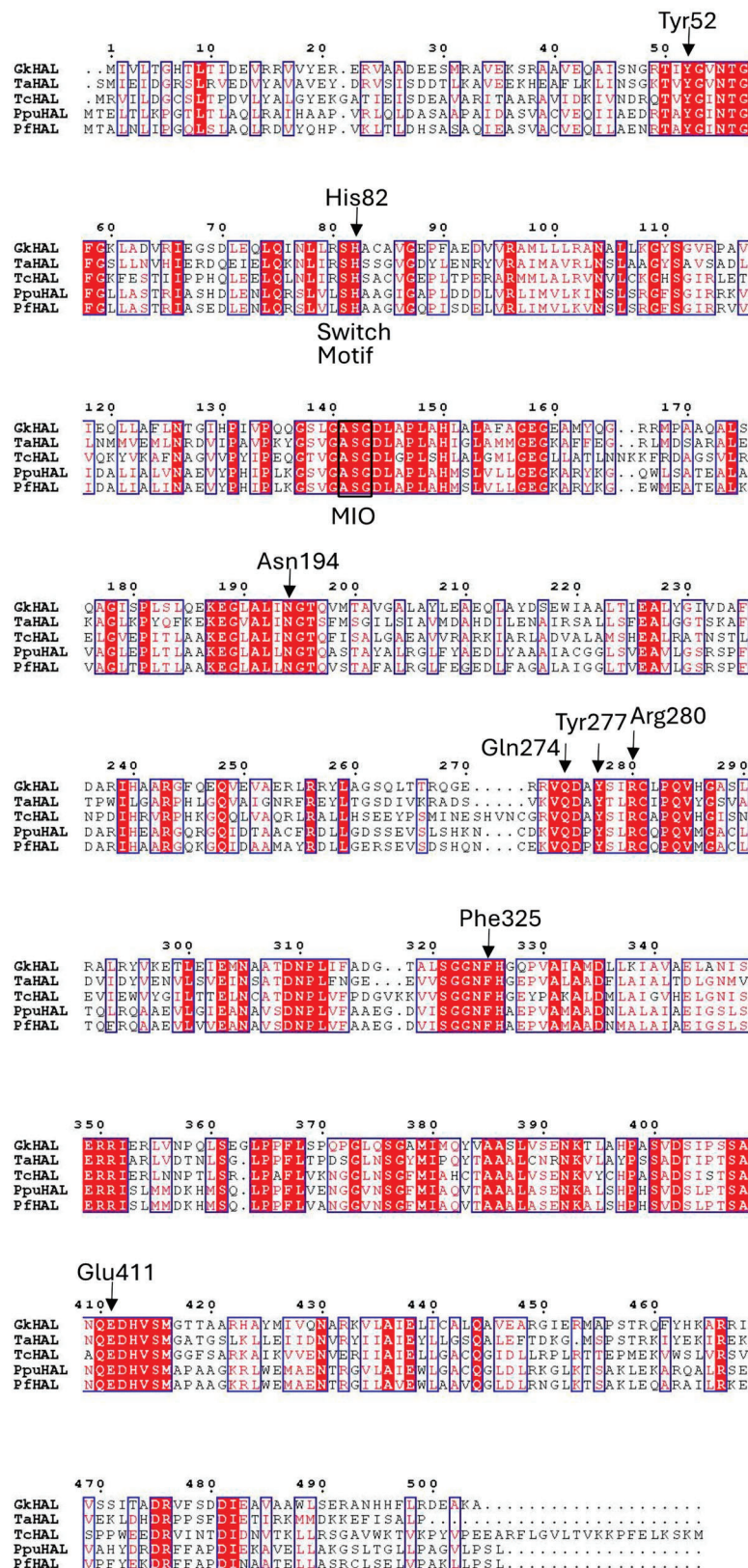
mum growth temperature limit of 74 °C and an optimal growth temperature of 60 °C. The genome of *G. kaustophilus* consists of a chromosome of 3.54 Mb and a single plasmid of 47.9 kb [36]. In their study, the authors assigned biological roles to 1914 CDSs, one of which corresponds to a putative HAL. Therefore, the capability of *G. kaustophilus* to thrive in harsh conditions makes it a valuable source of thermostable enzymes.

In this work, we describe the cloning, purification, and biochemical characterization of a *GkHAL* enzyme, along with eight other active site mutants. These HAL mutants were produced to evaluate their importance in substrate binding and catalysis through enzyme kinetic behavior. We tested enzyme activity with various substrates and discovered that L-histidinamide is a new substrate for two out of the eight HAL mutants. The kinetic and thermal characteristics of both the wild-type *GkHAL* and its mutants are also examined and discussed. Our results show that *GkHAL* exhibits kinetic thermostability, achieving maximum activity at 85 °C. Kinetic and thermodynamic studies allowed us to test enzyme efficiency with each substrate. Circular dichroism (CD) spectra and thermal denaturation were performed to understand the influence of such mutations on the secondary structure composition of *GkHAL* and its thermostability features.

## 2. Results

### 2.1. Sequence Analysis of Histidine Ammonia-Lyase from *Geobacillus kaustophilus* CECT4264

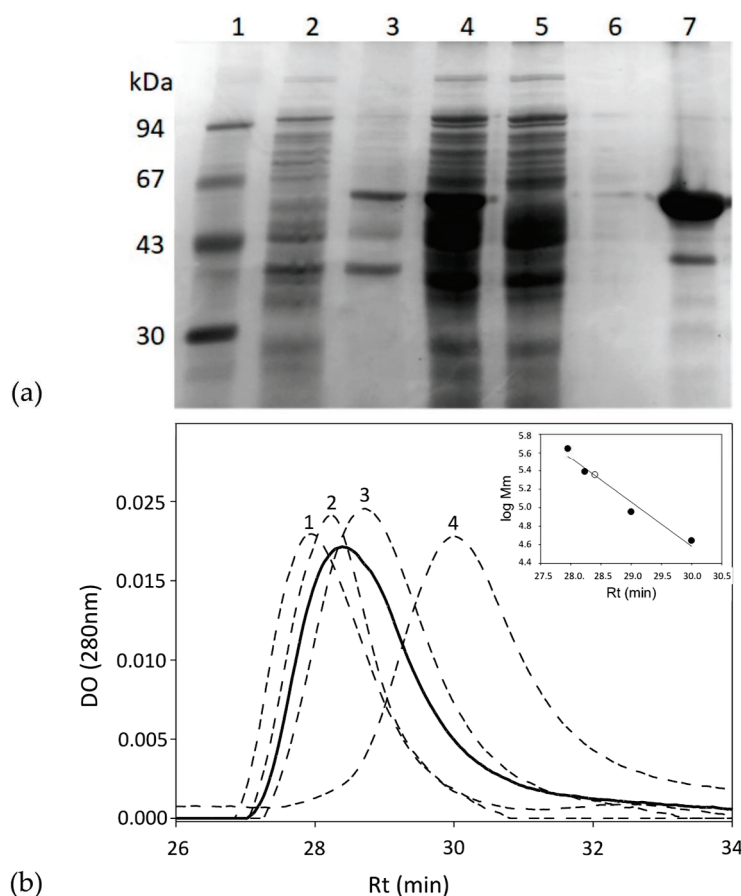
*G. kaustophilus* CECT4264 showed a distinct fragment for the *hutH* gene (*gkhutH*). The GenBank accession number of *GkhutH* is BAD74670.1. The gene encompassing the region 430274–431788 encodes a polypeptide of 504 amino acids with a calculated molecular mass of 54.7 kDa. The amino acid sequence deduced for *GkHAL* was compared to HAL sequences with demonstrated activity or solved structures (Figure 1). The highest similarity in amino acid sequence (43.7%) was found with HAL from *Thermoplasma acidophilum* [29], whereas the lowest identity percentage (40.7%) was observed when comparing the *G. kaustophilus* sequence with HAL from *Trypanosoma cruzi* [28]. When contrasting with sequences from the *Pseudomonas* genus, the identity regarding *P. putida* (*Ppu*) was 42.3% [24], whereas in comparison with *P. fluorescens* (*Pf*), the identity decreased to 41.9% [27]. All of these HAL sequences feature a conserved amino acid triad, Ala-Ser-Gly, capable of forming the 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO) prosthetic group, which is found in most of the aromatic ammonia-lyases identified to date [8,37]. The *GkHAL* sequence also reveals the presence of the characteristic substrate switch motif, Ser/His, for ammonia-lyase activity [27], identified at positions 81–82. Furthermore, as depicted in Figure 1, this dyad is indeed present within the aromatic binding region of the other HALs. Additionally, the multiple alignments showed that *GkHAL* contains conserved catalytic active sites, which are similar to those found in other homologous HAL proteins. For instance, the amino acid residues Y and E, which are predicted to coordinate the carboxylic and amino groups of the substrate L-histidine, were conserved in *GkHAL* (Tyr52 and Glu411). Moreover, the amino acid residues N, Q, and R, which participate in hydrogen bonding to the ammonium function of the substrate [25] and in anchoring the carboxylate moiety of the substrate or the cationic intermediate, are highly conserved in *GkHAL* (Asn194, Gln274, and Arg280). On the other hand, the active site residue Y277 in *GkHAL* is possibly involved in assisting Glu411 in its key catalytic role, while the residue Phe325 may stabilize the cationic intermediate, as shown previously in *PpuHAL* [25]. These findings suggest that the *gkhutH* gene encodes a functional HAL protein.



**Figure 1.** Sequence alignment for histidine ammonia-lyases of contrasted activity. GkHAL, *Geobacillus kaustophilus* CECT4264, Genbank acc. No. PQ065632; TaHAL, *Thermoplasma acidophilum*, Uniprot Q9HIL6; TcHAL, *Trypanosoma cruzi*, Uniprot Q4E133; PpuHAL, *Pseudomonas putida*, Uniprot P21310; PfHAL, *Pseudomonas fluorescens*, NCBI Reference Sequence No. WP\_003220818.1.

## 2.2. Expression, Production, and Purification of GkHAL and Its Active Site Mutants

The *gkhutH* gene was inserted into the pDANI3 expression vector with *Nde*I and *Bam*HI restriction enzymes to produce the C-terminal His6-tagged GkHAL. The GkHAL enzymes were functionally expressed in *E. coli* BL21 and purified in one step using immobilized cobalt affinity by chromatography [38]. SDS-PAGE analysis of the purified wild-type GkHAL enzyme revealed a band with 95% purity and an apparent molecular mass of approximately 55 kDa (Figure 2a), which is consistent with the deduced molecular mass from the amino acid sequence (56.5 kDa). The protein GkHAL was eluted at pH 8.5 at 28.4 min in the Biosep-SEC-S2000 column (HPLC), which yields an apparent molecular mass of 220 kDa (Figure 2b), suggesting a tetrameric structure for the protein in solution.



**Figure 2.** (a) SDS-PAGE analysis of each purification step of the GkHAL wild-type from *E. coli* BL21 harboring the pJMC93 plasmid. Lane 1: low molecular weight marker; lane 2: non-induced precipitate; lanes 3 and 4: pellet and supernatant of the resuspended crude extract after cell sonication; lane 5: flow-through after adding the sonicated supernatant to the metal affinity column; lane 6: eluate after washing the metal affinity column with buffer; lane 7: purified enzyme. (b) Size-exclusion chromatography of the purified GkHAL wild-type (black line) and molecular weight markers (dashed line). The molecular weight markers consisted of (1) apoferritin from equine spleen (443 kDa), (2) dihydropyrimidinase from *Sinorhizobium meliloti* CECT4114 (*Smel*Dhp) (245 kDa) [39], (3) L-carbamoylase from *Geobacillus stearothermophilus* CECT43 (*BsLcar*) (44 kDa) [40], and (4) chicken ovalbumin (44 kDa). The inset represents the fit of the retention time (Rt) versus the logarithm of the molecular masses (Mm) of the protein standards (black circles) and that obtained for GkHAL (white circle).

The purity and monomeric molecular weight of the purified mutated enzymes were also analyzed using SDS-PAGE gel electrophoresis (Figures S1–S7). Enzymes were obtained at yields ranging from 26 to 74 mg of pure protein per liter of cell culture. In addition, among

the mutated enzymes analyzed, GkHAL Y277F was the only one that could not be expressed or purified, suggesting that this residue has an essential role in conformational stability.

### 2.3. Protein Characterization of GkHAL and Its Mutants Followed by Activity Measurements

The activities of both the GkHAL wild-type and its mutants were compared by measuring their ability to convert histidine into *trans*-urocanic acid under standard assay conditions. Mutations at residues Y52F, H82L, N194D, and E411D resulted in enzyme forms that failed to produce *trans*-urocanic acid, indicating that these mutations hindered the enzymes' capability to catalyze the reaction. The Q274N, R280K, and F325Y mutated enzymes were able to produce the reaction but with less activity compared to the GkHAL wild-type. Mutants with analogous changes in these residues in PpuHAL also showed a lesser reduction in activity loss [25], pointing to the conservative nature of the active site in histidine ammonia-lyases.

We investigated the effect of pH (2–12) on the activity of GkHAL and its mutants (Q274N, R280K, and F325Y) at 65 °C using L-histidine as the substrate. The pH range of the purified proteins is illustrated in Figure S8. All proteins displayed maximum activity between pH 7.5 and 8.5, with activity declining below pH 7 and above pH 9. The maximum and optimum pH of 8.5 for GkHAL enzymes align with data from HALs of other bacterial strains such as *P. putida* [20], *P. fluorescens* R124 [27], and *T. acidophilum* [29].

The effect of temperature on enzyme activity of GkHAL and its mutants (Q274N, R280K, and F325Y) was assayed at different temperatures ranging from 20 to 98 °C, at pH 8.5. Both the wild-type enzyme and the Q274N and R280K mutants showed maximum activity at 85 °C, while the F325Y mutant exhibited maximum activity at 80 °C (Figure S9). As thermal stability studies on the activity of wild-type GkHAL revealed a gradual loss of activity after preincubation for 15 min at temperatures above 70 °C (Figure S10), the reactions were conducted at 65 °C.

To explore the substrate specificity of the enzymes, their activity was tested with various substrates. We began by measuring the activity of the other proteinogenic amino acids as possible substrates, but none of them were substrates for GkHAL or its mutants, confirming its stricter substrate specificity [9,13,19,27]. Among the L-histidine homologous substrates tested, all proteins showed activity with L-histidine methyl ester. However, this behavior differs because the highest rate of urocanate methyl ester formation was detected for the wild-type GkHAL and the mutant Q274N, whereas the mutants R280K and F325Y exhibited a slow activity rate under identical assay conditions (Table 1). Previous investigations have shown that L-histidine methyl ester can serve either as a substrate [20] or as a competitive inhibitor [41]. Furthermore, the mutated enzymes R280K and F325Y exhibited activity with L-histidinamide (Table 1). However, the mutant R280K showed a 13-fold higher rate of urocanamide production compared to F325Y. Neither the wild-type nor the Q274N-mutated GkHAL catalyzed the reaction with L-histidinamide.

**Table 1.** Kinetic constants and velocity rates for GkHAL enzymes.

| Enzyme      | Substrate                | $K_m$ | $k_{cat}$       | $V_{max}$                               | $k_{cat}/K_m$ (rel. Decrease)    |
|-------------|--------------------------|-------|-----------------|-----------------------------------------|----------------------------------|
|             |                          | mM    | s <sup>−1</sup> | ×10 <sup>−7</sup> mol min <sup>−1</sup> | mM <sup>−1</sup> s <sup>−1</sup> |
| WT GkHAL    | L-histidine              | 4.83  | 12.239          | 4.243                                   | 2.5367 (1)                       |
|             | L-histidine methyl ester | 8.77  | 13.243          | 4.591                                   | 1.5101 (1)                       |
| Q274N GkHAL | L-histidine              | 12.58 | 1.925           | 0.628                                   | 0.1530 (17)                      |
|             | L-histidine methyl ester | 47.21 | 2.428           | 0.793                                   | 0.0514 (29)                      |
| R280K GkHAL | L-histidine              | 7.99  | 0.096           | 0.231                                   | 0.0120 (211)                     |
|             | L-histidine methyl ester | 3.43  | 0.090           | 0.021                                   | 0.0262 (58)                      |
|             | L-histidinamide          | 5.39  | 0.220           | 0.052                                   | 0.0408                           |
| F325Y GkHAL | L-histidine              | 3.38  | 0.547           | 0.273                                   | 0.1617 (16)                      |
|             | L-histidine methyl ester | 22.98 | 0.454           | 0.227                                   | 0.0197 (77)                      |
|             | L-histidinamide          | 5.40  | 0.008           | 0.004                                   | 0.0015                           |

#### 2.4. Kinetic and Thermal Characterization of Enzymes

Kinetic parameters of GkHAL and its mutants were measured at varying substrate concentrations between 0.25 and 50 mM.  $K_m$  values were calculated for L-histidine and L-histidine methyl ester for all enzymes and for L-histidinamide for those that showed to carry the reaction. Maximum velocity  $V_{max}$ ,  $K_m$ ,  $k_{cat}$ , and catalytic efficiency were obtained for each protein and substrate combination (Table 1).

A  $K_m$  value comparison indicates that all mutants have similar affinities for L-histidine as a natural substrate. However, the GkHAL mutant Q274N showed a higher  $K_m$  value (12.58 mM) than the other enzymes (3.38–7.99 mM). Using L-histidine methyl ester as a substrate resulted in a similar trend, with higher  $K_m$  values observed for the mutants Q274N and F325Y. Considering that the R280K mutation resulted in a low  $K_m$  (3.43 mM), it can be inferred that this mutant exhibits a higher binding affinity with L-histidine methyl ester compared to L-histidine itself. On the other hand, mutant enzymes capable of catalyzing the reaction with L-histidinamide displayed comparable  $K_m$  values to those of L-histidine, reflecting similar affinities for both substrates.

Although GkHAL mutants exhibited similar  $K_m$  values when L-histidine was used as a substrate, their  $k_{cat}$  values were 6 to 127 times lower than that of wild-type GkHAL, indicating that they are less active. The  $k_{cat}$  values determined for L-histidine methyl ester followed the same trend as those observed for L-histidine, suggesting that the mutant enzymes react with it at a significantly lower rate. The effect of the R280K mutation is somewhat controversial, as it shows a low  $K_m$  with L-histidine methyl ester, yet the resulting  $k_{cat}$  is low ( $0.090 \text{ s}^{-1}$ ), indicating strong binding between the enzyme and substrate but poor catalytic function.

Catalytic efficiency is defined as the relationship between catalytic function and substrate binding, represented by the ratio  $k_{cat}/K_m$ . As expected, the wild-type GkHAL exhibited the highest efficiency with its standard substrate, L-histidine ( $2.5367 \text{ mM}^{-1} \text{ s}^{-1}$ ). Furthermore, the catalytic efficiency values were also highest with the wild-type enzyme for L-histidine methyl ester. In contrast, mutated enzymes show 16 to 211 times less efficiency. In relation to L-histidinamide, the R280K mutant demonstrated the highest catalytic efficiency. However, this efficiency is 62 times lower than that of the wild-type enzyme with L-histidine.

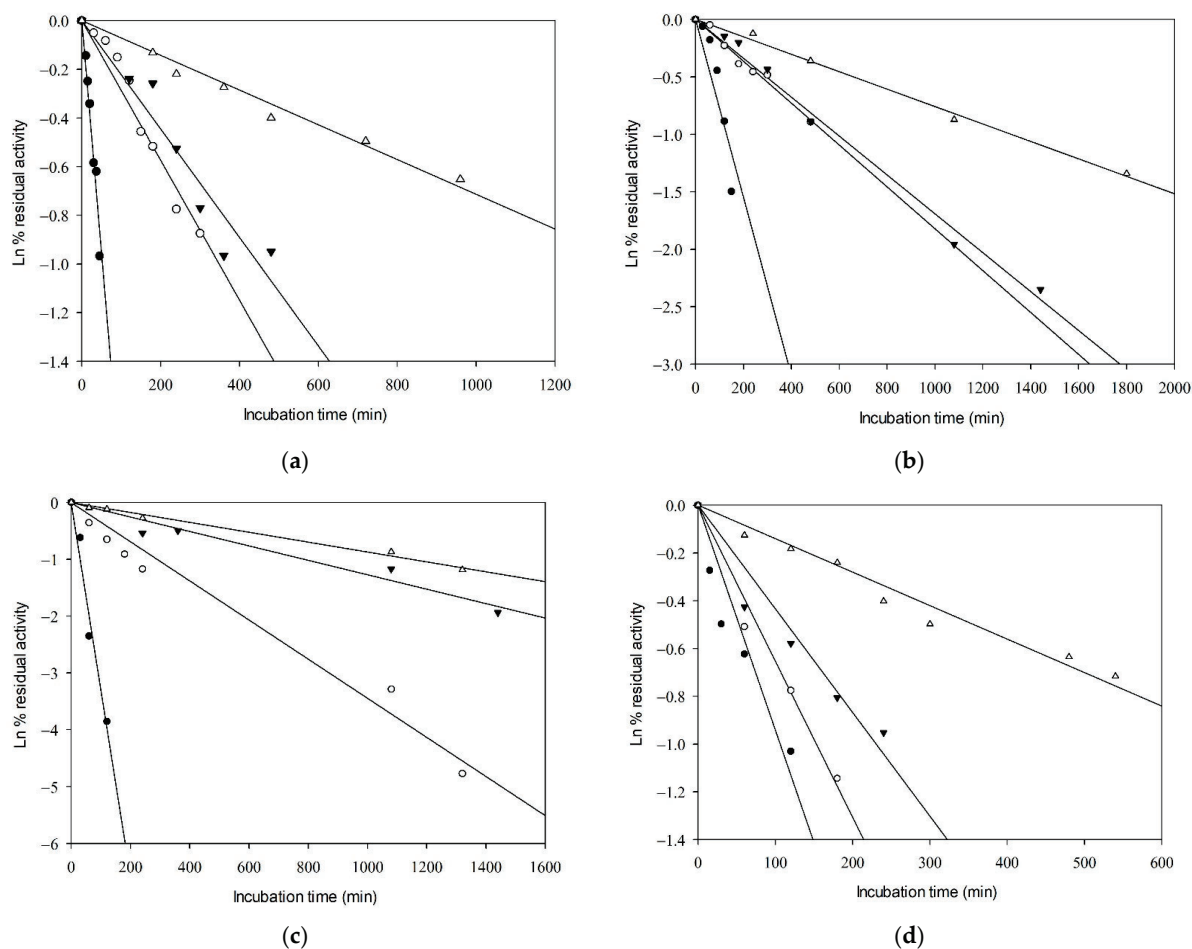
The thermal inactivation parameters of GkHAL proteins were studied at temperatures of 65, 70, 75, and 80 °C in 100 mM DEA buffer (pH 8.5) (Table 2). The exponential decay was analyzed using semi-logarithmic plots. Linear Arrhenius plots were obtained, indicating first-order inactivation of the enzymes in all cases (Figure 3). Steeper slopes indicate faster inactivation rates. As expected, higher temperatures resulted in shorter times for enzyme inactivation. The inactivation rate constant ( $k_d$ ) was derived from the slopes of the Arrhenius plots, with higher values indicating more rapid inactivation. The half-life times ( $t_{1/2}$ ) of the active enzymes were determined from the inactivation rates. The thermal inactivation parameters, including free energy ( $\Delta G^*$ ), enthalpy ( $\Delta H^*$ ), and entropy ( $\Delta S^*$ ) of the active enzymes (Table 2), were calculated as described in references [42,43].

**Table 2.** Thermodynamic parameters for thermal inactivation of GkHAL enzymes.

| Incubation Temperature                                  | Half-Life | Inactivation Rate Constant $k_d$ | $\Delta G^*$           | $\Delta H^*$           | $\Delta S^*$                         |
|---------------------------------------------------------|-----------|----------------------------------|------------------------|------------------------|--------------------------------------|
| K                                                       | min       | $\text{min}^{-1}$                | $\text{kcal mol}^{-1}$ | $\text{kcal mol}^{-1}$ | $\text{kcal K}^{-1} \text{mol}^{-1}$ |
| Wild-type GkHAL ( $E_a = 49.42 \text{ kcal mol}^{-1}$ ) |           |                                  |                        |                        |                                      |
| 353                                                     | 34        | 0.0206                           | 26.38                  | 48.72                  | 0.063                                |
| 348                                                     | 224       | 0.0031                           | 27.30                  | 48.73                  | 0.062                                |
| 343                                                     | 301       | 0.0023                           | 27.10                  | 48.74                  | 0.063                                |
| 338                                                     | 990       | 0.0007                           | 27.50                  | 48.75                  | 0.063                                |

Table 2. Cont.

| Incubation Temperature                              | Half-Life | Inactivation Rate Constant $k_d$ | $\Delta G^*$           | $\Delta H^*$           | $\Delta S^*$                         |
|-----------------------------------------------------|-----------|----------------------------------|------------------------|------------------------|--------------------------------------|
| K                                                   | min       | $\text{min}^{-1}$                | $\text{kcal mol}^{-1}$ | $\text{kcal mol}^{-1}$ | $\text{kcal K}^{-1} \text{mol}^{-1}$ |
| Q274N GkHAL ( $E_a = 35.64 \text{ kcal mol}^{-1}$ ) |           |                                  |                        |                        |                                      |
| 353                                                 | 71        | 0.0097                           | 26.91                  | 34.93                  | 0.023                                |
| 348                                                 | 385       | 0.0018                           | 27.68                  | 34.94                  | 0.021                                |
| 343                                                 | 408       | 0.0017                           | 27.31                  | 34.95                  | 0.022                                |
| 338                                                 | 866       | 0.0008                           | 27.41                  | 34.96                  | 0.022                                |
| R280K GkHAL ( $E_a = 44.41 \text{ kcal mol}^{-1}$ ) |           |                                  |                        |                        |                                      |
| 353                                                 | 21        | 0.0335                           | 26.04                  | 43.71                  | 0.050                                |
| 348                                                 | 187       | 0.0037                           | 27.18                  | 43.72                  | 0.048                                |
| 343                                                 | 533       | 0.0013                           | 27.49                  | 43.73                  | 0.047                                |
| 338                                                 | 770       | 0.0009                           | 27.33                  | 43.74                  | 0.049                                |
| F325Y GkHAL ( $E_a = 25.11 \text{ kcal mol}^{-1}$ ) |           |                                  |                        |                        |                                      |
| 353                                                 | 88        | 0.0079                           | 27.05                  | 24.42                  | -0.007                               |
| 348                                                 | 112       | 0.0062                           | 26.82                  | 24.43                  | -0.007                               |
| 343                                                 | 182       | 0.0038                           | 26.76                  | 24.44                  | -0.007                               |
| 338                                                 | 433       | 0.0016                           | 26.94                  | 24.45                  | -0.007                               |



**Figure 3.** Kinetics of thermal inactivation of the catalytic activity of (a) wild-type GkHAL, (b) Q274N GkHAL, (c) R280K GkHAL, and (d) F325Y GkHAL. Incubation temperatures shown are 80 °C (close circles), 75 °C (open circles), 70 °C (close inverted triangles), and 65 °C (open triangles).

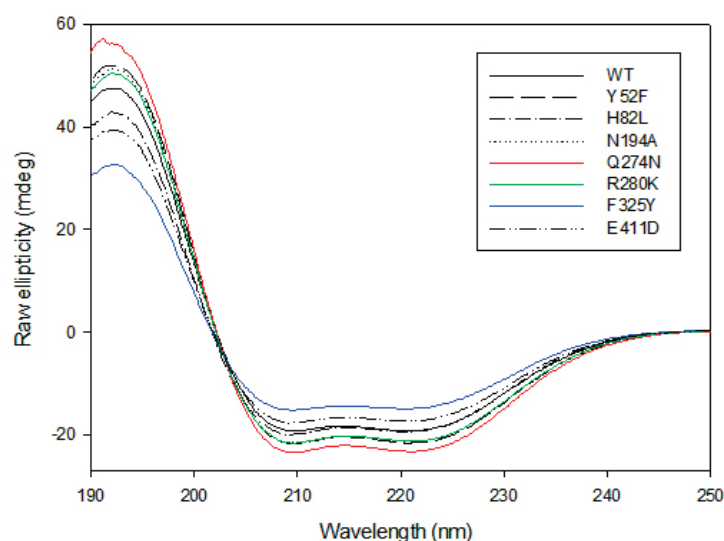
As depicted in Table 2, an increase in temperature led to a decrease in  $t_{1/2}$  values and an increase in the first-order heat inactivation rate constants ( $k_d$ ) for both the *GkHAL* wild-type and its mutants. At 65 °C, the wild-type *GkHAL* exhibited the highest half-life, indicating greater stability compared to the mutated enzymes. At 80 °C, the trend reversed, with the wild-type enzyme showing a shorter half-life compared to the mutated enzymes, except for the R280K mutation. Therefore, mutated enzymes appear to exhibit better stability at high temperatures. The activation energy for deactivation ( $E_a$ ) represents the amount of energy molecules need to absorb to begin enzyme inactivation and attain the height of the barrier, or transition state, for inactivation. Regarding  $E_a$ , the Q274N, R280K, and F325Y mutations exhibited lower values of 35.64, 44.41, and 25.11 kcal/mol, respectively, compared to the wild-type enzyme, which had 49.42 kcal/mol.

Thermodynamic parameters provide valuable insights into protein stability, revealing stabilization or destabilization effects that may not be evident from half-life times alone. The  $\Delta G^*$  value is directly related to protein stability; thus, a higher  $\Delta G^*$  indicates greater enzyme stability [44]. Free energy decreased with the rise in temperature in wild-type *GkHAL* and mutants Q274N and R280K, suggesting the destabilization of the enzymes, which is consistent with the half-life and  $k_d$  results. This trend is also observed for the F325Y mutant in the temperature range of 338 to 348 K. The  $\Delta G^*$  values obtained for the different proteins were similar, indicating no significant stability differences. Table 2 also shows that  $\Delta H^*$  slightly decreases with rising temperatures in both *GkHAL* and its mutated forms, indicating that less energy is required to denature the enzymes at higher temperatures. Additionally, the positive enthalpy values suggest that the inactivation process is endothermic [45].

Given that the activation energy for deactivation and the thermodynamic parameters of inactivation are typically higher for wild-type *GkHAL* compared to the Q274N, R280K, and F325Y mutations, we can suggest that these mutants may be less stable against thermal inactivation.

## 2.5. Circular Dichroism of Enzymes

The CD spectra of the wild-type *GkHAL* and its mutants have been investigated experimentally (Figure 4). The CD spectrum of *GKHAL* at pH 8.5 exhibits two negative peaks at 210 and 222 nm and a positive peak at 192 nm. This is characteristic of a protein with an  $\alpha$ -helical or turn-like conformation content, as well as a fraction of  $\beta$ -sheet structure [43], suggesting that the purified protein adopts a well-defined folded structure. The loss of ellipticity at 222 nm is attributed to  $\alpha$ -helical content, while the loss at 210 nm indicates the presence of  $\beta$ -sheets in the structure of the protein. As shown in Figure 4, all mutants clearly show structural helicity, indicating that they keep their internal chirality. However, except for the H82L mutant, whose spectrum completely overlaps with that of *GkHAL*, all other mutants show slight differences from the wild-type *GkHAL*. Since the mutants Y52F, H82L, N194D, and E411D lacked activity on L-histidine, we did not analyze their CD spectra. To assess the slight variations in the secondary structures of mutants Q274N, R280K, and F325Y in comparison to the wild-type *GkHAL*, we utilized the k2d algorithm available on DichroWeb [46]. This tool facilitated the determination of the percentages of  $\alpha$ -helices,  $\beta$ -sheets, and random structures for each enzyme. Table 3 presents the deconvolution results, indicating the relative percentages of each secondary structure. Based on the CD data, the wild-type *GkHAL* exhibits a conformation comprising 47%  $\alpha$ -helix structure, 15%  $\beta$ -sheet structure, and 38% random coil elements. For the structure of the R280K mutant, the amount of  $\alpha$ -helix structure is considerably higher, while the amount of  $\beta$ -sheet structure is distinctly lower compared to the wild-type enzyme. In contrast, the  $\alpha$ -helical content in the Q274N and F325Y mutants decreases significantly, from 47% to 39% and from 47% to 35%, respectively, while the  $\beta$ -sheet content increases compared to the wild-type *GkHAL*. Nonetheless, it is still difficult to ascertain whether these minor structural changes are connected to the newly observed activity in the R280K and F325Y mutants.



**Figure 4.** Far-UV CD spectra for *GkHAL* enzymes recorded at 25 °C using a J-815 spectrophotometer. Six spectra were averaged for each curve.

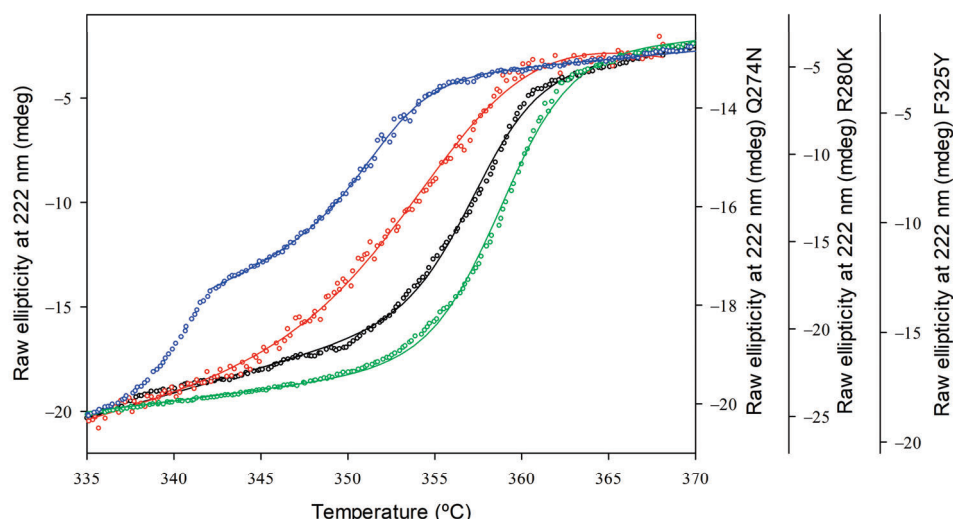
**Table 3.** Estimated secondary structure composition (%) of wild-type *GkHAL* and its mutants Q274, R280K, and F325Y determined using a k2d program, and their apparent melting values.

| Enzyme             | $\alpha$ -Helices (%) | $\beta$ -Sheets (%) | Random Coil (%) | $T_{m(app)}^a$ (K) | $\Delta T_{m(app)}^b$ (K) |
|--------------------|-----------------------|---------------------|-----------------|--------------------|---------------------------|
| WT <i>GkHAL</i>    | 47                    | 15                  | 38              | $357.36 \pm 0.06$  | -                         |
| Q274N <i>GkHAL</i> | 39                    | 20                  | 41              | $355.20 \pm 0.28$  | -2.16                     |
| R280K <i>GkHAL</i> | 55                    | 9                   | 36              | $358.80 \pm 0.04$  | 1.40                      |
| F325Y <i>GkHAL</i> | 35                    | 18                  | 47              | $351.85 \pm 0.14$  | -5.51                     |

<sup>a</sup>  $T_{m(app)}$  is the temperature at which 50% of the protein is unfolded. <sup>b</sup> Change in  $T_{m(app)}$  relative to the wild-type protein.

The thermostability of *GkHAL* variants was evaluated by observing temperature-induced unfolding using CD spectroscopy. Structural changes in protein folding were studied at a 4  $\mu$ M protein concentration with a temperature interval of 62 and 95 °C, measuring the loss of ellipticity at 222 nm. Figure 5 presents the melting profiles of the variants, along with the associated non-linear least squares fit using the Gibbs–Helmholtz equation to determine the  $T_m$  values for each protein. For the wild-type *GkHAL*, a single sigmoidal transition was observed with an apparent  $T_m$  ( $T_{m(app)}$ ) value of  $357.36 \pm 0.06$  K, indicating that the thermal denaturation is a two-state process [47]. Compared to wild-type *GkHAL*, the R280K mutant not only displays an increase in helical content but also shows a modest improvement in thermal stability, with a  $T_{m(app)}$  value of  $358.80 \pm 0.04$  K. In contrast, mutants like Q274N and F325Y, which have reduced helical content, exhibited a melting transition with a decrease in apparent melting temperature by 2.16 K and 5.51 K, respectively, compared to the wild-type (Table 3).

We conducted a second CD spectrum to determine if the structure recovered after thermal denaturation. None of the proteins exhibited a spectrum similar to those in Figure 4. Furthermore, all the proteins showed aggregation after heating, suggesting that they unfolded into intermediates that were prone to aggregation. As a result, *GkHAL* and its mutants cannot completely refold because partially unfolded species accumulate and aggregate, rendering the unfolding process irreversible and precluding thermodynamic analysis [48].



**Figure 5.** Thermal unfolding profiles of *GkHAL* enzymes monitored by far-UV CD. Wild-type *GkHAL* (black open circles) and *GkHAL* mutants Q274N (red open squares), R280K (green open diamonds), and F325Y (blue open hexagons). All profiles were measured with a scan rate of 1 K/min.

### 3. Discussion

#### 3.1. Cloning, Expression, and Characterization of Histidine Ammonia-Lyase from *Geobacillus kaustophilus*

*G. kaustophilus*, a thermophilic bacterium capable of growing across a broad temperature range from 42 °C to a maximum of 74 °C, can serve as a valuable source for discovering highly thermoresistant enzymes [36,49,50]. In this study, we identified a putative *hutH* gene in *G. kaustophilus* HTA426 through genome analysis. To investigate the function of this gene product, the *hutH* gene was amplified from the genome of *G. kaustophilus* CECT4264 using PCR, enabling the construction of the recombinant plasmid pJMC93. The *GkHAL* recombinant protein was then successfully expressed as a soluble protein, allowing us to further investigate its characteristics and functions.

Based on the multiple alignment of the deduced amino acid sequence of the cloned *GkHAL*, we found more than 40% identity with the major HAL enzymes characterized to date [24,27–29]. Similar to other HAL enzymes, *GkHAL* contains the amino acids that form the MIO internal cofactor, which acts as an electrophilic group essential for histidine deamination [4,8,37]. The recognition of histidine by HALs has been attributed to the presence of the SH motif in the aromatic binding region [51]. Other residues in this region may also be involved in substrate interaction, such as leucine at position 76 in *PpuHAL* [52] and at position 80 in *PfHAL* [27,52], alanine and serine at position 85 in *PpuHAL* or *TcHAL* [28], and methionine at position 380, which is conserved across all bacterial HALs described so far [28]. Given that these residues are fully conserved in *GkHAL*, it is reasonable to infer a strict substrate specificity, aligning with previous reports for other HALs. Additionally, the sequence similarity reveals that *GkHAL* shares the same active site residues as other HALs. In this context, the residues Tyr52 and Glu414, which are crucial for catalysis, are fully conserved. Furthermore, we identified Asn194, Gln274, and Arg280 in *GkHAL* as equivalent residues that are thought to play crucial roles in substrate positioning and maintaining the active center [9,13,25]. The sum of the results indicates that the *hutH* gene of *Geobacillus kaustophilus* encodes a functional HAL protein. On the other hand, the similarity of the substrate binding and catalytic motifs of *GkHAL* to those in other bacterial HALs suggests that it would exhibit similar catalytic properties.

Recombinant *GkHAL* was successfully expressed and purified, enabling us to determine its enzyme activity and biochemical properties. It has been reported that *PpuHAL* activity is stimulated by divalent metal ions such as  $Zn^{2+}$  [22], likely because the ion might coordinate the interaction between L-histidine and the imidazole group of His83, as previously suggested [23,25]. More recently, Csuka et al. reported that  $Zn^{2+}$  ions did

not result in any further increase in the enzyme activity of PfHAL [27]. In this context, we examined the effect of 10  $\mu\text{M}$   $\text{Zn}^{2+}$  on the reaction catalyzed by GkHAL. The velocity ratio values of GkHAL in the absence or presence of the metal ion were nearly identical ( $20.4 \pm 1.4$  nmol/min and  $20.8 \pm 0.9$  nmol/min, respectively). Additionally, the enzyme activity was unaffected by 10 mM EDTA ( $21.0 \pm 0.5$  nmol/min). From the findings, it can be suggested that  $\text{Zn}^{2+}$  is not required for the enzyme activity of *G. kaustophilus* CECT4264 HAL. While Miranda et al. [28] measured TcHAL activity in the presence of  $\text{Mn}^{2+}$ , they did not address the role of these ions in the enzyme activity. Finally, there is no available information about the requirement for divalent metal ions in the most recently characterized HAL [29].

The monomeric size determined for the GkHAL subunit is consistent with that observed in all recombinant histidine ammonia-lyase enzymes studied to date [20,21,27–29]. Our findings also suggest that the functional structure of GkHAL is a tetramer. Notably, the crystal structures of PpuHAL [24], TcHAL [28], and TaHAL [29] have revealed a homotetrameric organization with four active centers.

### 3.2. The Kinetics of GkHAL Enzymes: Comparison with Other HALs

Based on sequence analysis, Tyr52, His82, Asn194, Gln274, Tyr277, Arg280, Phe325, and Glu411 were identified as potentially important for catalysis, substrate positioning, and active center maintenance. These residues were therefore selected for site-directed mutagenesis to verify their functions. Comparisons of the activities of wild-type and mutant GkHAL enzymes revealed new characteristics arising from these mutations. The GkHAL mutations Y52F, H82L, N194D, and E411D showed no activity on L-histidine as the natural substrate. Thus, the amino acids Tyr52, His82, Asn194, and Glu411 must be essential for the active site structure and the formation of the enzyme-substrate complex. Changes in the composition of the specified sites led to the inactivation of the GkHAL. Although similar mutants in *Pseudomonas putida* HAL showed activity on L-histidine, their  $k_{\text{cat}}$  values were 1,000 to 20,930 times lower than the wild-type enzyme [25]. On the other hand, the mutants Q274N, R280K, and F325Y were able to catalyze the elimination of ammonia from L-histidine, although at a lower rate than the wild-type GkHAL enzyme. Comparison of the kinetic constants with those reported for similar mutants of PpuHAL revealed that they exhibit the same trends, regardless of the reaction temperature (25 °C for PpuHAL [25] and 65 °C for GkHAL (this work)). Thus, it can be concluded that the activity of these active mutant enzymes mirrors the results observed in *P. putida* [9,13,25,53].

In addition to L-histidine, GkHAL was tested with various homologous substrates to L-histidine to study its promiscuity or substrate scope. All enzymes that were active with the natural substrate could also react with L-histidine methyl ester. However, only the R280K and F325Y mutants were able to react with L-histidinamide. Previous reports indicate that L-histidine methyl ester serves as a substrate for PpuHAL [20] and acts as an inhibitor of *Achromobacter liquidum* HAL [41]. Moreover, substrates such as 4-nitro L-histidine [19] and N $\tau$ -methylhistidine [21] have also been found to react with HAL. To the best of our knowledge, this is the first report demonstrating that HAL mutants of the active center are active with a substrate other than L-histidine. While ammonia-lyase enzymes are notably characterized by their strong L-enantioselectivity, there is increasing interest in their potential applications for kinetic resolution and deracemization of amino acids [4,31,32]. A key challenge in enhancing the industrial applicability of HAL is to widen its limited substrate range. The site-active mutants under study were engineered to assess their role in hydrolyzing L-histidine as a preferred substrate and to determine if these modifications could expand the substrate scope. Our results show that, despite alterations in its active site, HAL generally retains a restricted substrate scope.

The  $K_{\text{m}}$  value of L-histidine for GkHAL is comparable to those of the closely related PpuHAL (3.9 mM) [25] and PfHAL (2.7 mM) [27]. *Pseudomonas putida* HAL with mutations similar to those studied here also displayed a  $K_{\text{m}}$  range comparable to the values we obtained [9,13,24]. Reactions with other substrates show  $K_{\text{m}}$  values that are somewhat

comparable to those for L-histidine across all enzymes, suggesting that the binding to these homologous substrates is similar in each case.

Regardless of the substrate, the wild-type enzyme exhibits significantly higher turnover values, indicating superior catalytic activity compared to the mutant enzymes. Dramatic losses of activity were observed in the R280K and F325Y mutated enzymes, with these losses varying depending on the substrate. Overall, the catalytic efficiencies of the mutant enzymes were found to be lower than those of the wild-type enzymes. The catalytic efficiencies of the Q274N mutant are one to two orders lower due to high  $K_m$  values, which implies an added difficulty in enzyme–substrate binding. This could be because this residue plays a role in maintaining the correct geometry of R280 and Tyr52, as previously suggested for *PpuHAL* [9,25]. On the other hand, the F325Y mutation showed the opposite behavior. Although its  $K_m$  values are similar to the wild-type HAL, the low catalytic activity resulted in an efficiency rate comparable to the Q274N mutation, at least with the natural substrate. In *PpuHAL*, the impact of mutating this phenylalanine residue to alanine has shown differential behavior, with activity decreasing by 100 to 2200-fold [25,53]. Moreover, the F329G mutant was inactive with L-histidine due to its failure to form the MIO group [53]. Nonetheless, it is clear that these findings collectively underscore the importance of the phenyl ring in HAL enzyme catalysis. The R280K mutation shows the lowest catalytic function, with its efficiency falling to two orders of magnitude below that of the wild-type enzyme. Surprisingly, the  $k_{cat}$  value for R280K *GkHAL* with L-histidinamide is nearly 2.5 times higher than that for the native substrate. This indicates that the mutation allows the enzyme to be more effective with the homologous substrate than with L-histidine. Interestingly, new pathways emerged, as both the R280K and F325Y mutations enabled the enzyme to catalyze the reaction with L-histidinamide, a substrate with which the wild-type *GkHAL* is inactive.

### 3.3. The Kinetics of Thermal Inactivation of *GkHAL* Mutants: Relationship with Thermostability

The maximum activity of *GkHAL* was observed at 85 °C, aligning with the thermophilic nature of its gene donor organism. This high-temperature performance underscores the importance of studying its thermostability and evaluating whether mutations can enhance or diminish this characteristic. The efficiency of the enzymes over time is represented by the inactivation rate constant ( $k_d$ ) and half-life times ( $t_{1/2}$ ). At lower temperatures, the wild-type *GkHAL* exhibited the greatest stability over time, while the F325Y mutant enzyme showed superior kinetic stability at higher temperatures. Half-life times found for PAL from *Anabaena variabilis* [54] and *Lactuca sativa* L. [55] at temperatures of 67 and 60 °C were 8 h and 2.8 h, respectively. In comparison, *GkHAL* seems to better maintain its stability at high temperatures than other aromatic ammonia-lyase enzymes.

The linear behavior of the thermal inactivation plots suggests that HAL inactivation occurs through a mechanism solely influenced by temperature [56,57]. The activation energy for the process resulted in similar range energies but was significantly lower for Q274N and F325Y, indicating easier inactivation consistent with their shorter half-life times. High  $E_a$  for *GkHAL* enzymes indicates significant stability at elevated temperatures, consistent with their thermophilic nature [36,58]. Thermodynamic parameters for the enzyme inactivation process were derived from the activation energy.  $\Delta G^*$  values for *GkHAL* enzymes ranged from 26.04 and 27.68 kcal mol<sup>−1</sup>, which are similar to those reported for other thermophilic enzymes, such as  $\alpha$ -amylases from *Bacillus amyloliquefaciens* (22.90 kcal mol<sup>−1</sup>) [42] or  $\beta$ -xylosidase from *Geobacillus stearothermophilus* (27.60 kcal mol<sup>−1</sup>) [43]. However,  $\Delta H^*$  and  $\Delta S^*$  values displayed a higher difference between the wild-type enzyme and the mutated ones. Protein inactivation is an endothermic denaturalization process, as energy is required to alter its structure.  $\Delta H^*$  is a measure of the number of bonds broken during inactivation [59].  $\Delta H^*$  values for the R280K mutation and the wild-type were higher compared to those for the Q274N and F325Y mutations, suggesting that the latter two mutant enzymes have lower heat resistance. Entropy generally augments with the denaturalization of the protein.  $\Delta S^*$  values are known to provide important insights into solvation levels, protein

compactness [59], and aggregation [60]. The positive  $\Delta S^*$  values observed for wild-type GkHAL and the mutants Q274N and R280K indicate an increase in disorder or randomness within these enzymes or the surrounding solvent system during denaturation [45]. In contrast, the negative  $\Delta S^*$  values observed for the F325Y mutant suggest that the partially unfolded state is more ordered compared to the native state structure and may also point to the occurrence of an aggregation process [60]. Finally, the enthalpy and entropy values for wild-type GkHAL are lower than those of other thermophilic enzymes, such as  $\alpha$ -amylase [42] and  $\beta$ -xylosidase [43], which may indicate slight differences in the stability mechanisms among these enzymes.

#### 3.4. Conformational Stability and Irreversible Thermal Denaturalization

Far-UV CD spectra were employed to assess the secondary structure of GkHAL and to monitor any structural changes and correct folding in its active site mutants [61]. GkHAL enzymes showed secondary structures with  $\alpha$ -helices accounting for a significant portion, ranging from 35% to 55% of the total. A previous study demonstrated that PpuHAL exhibits a helical conformation at a higher percentage of 75% [62]. The opposite case can be found in  $\beta$ -sheets, appearing at low percentages for GkHAL and at 0% for *P. putida* [62]. Random conformation was also found to be more present in *G. kaustophilus*. Since it is possible to determine the secondary structure from a PDB structure, we used the BestSel server to analyze the crystal structure (PDB 1B8F) reported for PpuHAL [63]. The analysis revealed an  $\alpha$ -helix content of 55%, a very low  $\beta$ -sheet content of 5.7%, and 39.2% turns and other structures (Figure S11). These estimations are consistent with the results obtained for wild-type GkHAL.

The effect of temperature on GkHAL and its mutants, initially investigated through activity measurements, was further assessed by evaluating thermal denaturation using CD spectroscopy. All four proteins displayed sigmoidal curves, indicating that they have well-folded structures. The thermal denaturation of GkHAL and its mutants was found to be irreversible, which precluded the estimation of the thermodynamic parameters governing thermal unfolding [43,47]. Despite this limitation, we were able to determine the apparent melting temperature. This approach aligns with methods used for other proteins that exhibit irreversible thermal denaturation, allowing us to gain insights into their thermal stability. The high  $T_{m(app)}$  value for wild-type GkHAL confirms its significant thermostability, which is consistent with the kinetic inactivation results obtained. Only the F325Y mutation resulted in a significantly lower melting temperature, suggesting the importance of this residue for proper folding. In PpuHAL, the analogous residue (F329) is proposed to stabilize the  $\sigma$ -complex-like conformation [9,13,25]. These findings are consistent with the thermodynamic data on enzymatic thermal inactivation, which demonstrate that the F325Y mutant requires the lowest energy for denaturation. As a result, the F325Y mutant appears to be the most unstable of the three mutants at elevated temperatures.

## 4. Materials and Methods

### 4.1. Materials

All chemicals were of analytical grade and were used without further purification. Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostic S.L. (Indianapolis, IN, USA). Kapa-Hifi polymerase was from KapaBiosystems (Cultek S.L.U., San Fernando de Henares, Spain). Primers were from Integrated DNA Technologies, IDT (Coralville, IA, USA). TALON TM metal affinity resin was purchased from Clontech Laboratories, Inc. (Takara Scientific Chemical, Waltham, MA, USA). L- and D-histidine were from Alfa Aesar GmbH & Co. K.G. (Thermo Fischer Scientific, Waltham, MA, USA), and *trans*-urocanic acid was from Acros Organics (Thermo Fischer Scientific, Waltham, MA, USA). L-histidinamide, 1-methyl-L-histidine, 3-methyl-L-histidine, and 2-mercapto-L-histidine were from Sigma Aldrich (St. Louis, MO, USA), benzyl-L-histidine and  $\beta$ -alanyl-L-histidine were from TCI Europe (Zwijndrecht, Belgium), L-histidine methyl ester was purchased from Acros Organics (Thermo Fischer Scientific, Waltham, MA, USA), and diamine L-

histidine was from Bachem (Bubendorf, Switzerland). Diethanolamine was purchased from Panreac (Castellar del Vallés, Spain), acetic acid, formic acid, monobasic sodium phosphate, and dibasic potassium phosphate were from Merck (Darmstadt, Germany), and sodium carbonate was from Riedel de Haën (Honeywell, Charlotte, NC, USA).

#### 4.2. Microbes and Culture Conditions

*G. kaustophilus* CECT4264, acquired from the Spanish Type Culture Collection (CECT), was used as a possible donor of the histidine ammonia-lyase gene (*hutH*). The strain was cultivated at 55 °C for 20 h in Luria–Bertani medium (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2). *E. coli* DH5α was used to clone the gene *hutH*, and *E. coli* BL21 was used to overexpress the recombinant protein.

#### 4.3. Cloning of the Histidine Ammonia-Lyase (*hutH*) Gene

A single-colony isolate of *G. kaustophilus* CECT4264 was chosen for DNA isolation using a boiling procedure [49]. A supernatant aliquot of 5 µL containing the genomic DNA of the strain was used to amplify the gene encoding for putative *hutH* by PCR. The primers used to amplify the gene from *G. kaustophilus* CECT4264 (*gkhutH*) were designed based on GenBank sequence accession number NC\_006510.1. These were GkHAL5 (5'-GGAATTCCATATGATCGTATTGA-CCGGGC-3') and GkHAL3 (5'-CGGGATCCAGCTTTCGCTTCATCGCGG-3'), including *Nde* I and *Bam*H I restriction sites, respectively (in italics in the primer sequence).

The PCR product was purified from an agarose gel using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA), treated with *Nde* I and *Bam*H I enzymes (New England Biolabs, Ipswich, MA, USA), and then ligated into the pDANI3 plasmid, which was cut with the same enzymes to create the plasmid pJMC93. The plasmid pDANI3 was made in our laboratory [64] and derived from the rhamnose-inducible expression vector, including a C-terminal His6-tag (pJOE4036.1) [65] and including the TEV (Tobacco Etch Virus) recognition site, ENLYFQS [66]. The resulting construct allows the production of recombinant GkHAL fused at the C terminus to a polyhistidine tag (His6 tag), allowing its removal by TEV treatment. The cloned sequence was confirmed by sequencing using an Applied Biosystems 3500 Series Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) at the Nucleic Acids Analysis Service of the University of Almeria. The sequence was then aligned and compared as previously described [49].

Mutagenesis of GkHAL was performed using the QuikChange II Site-directed mutagenesis kit from Stratagene (San Diego, CA, USA) following the manufacturer's protocol, using the plasmid pJMC93 as the template and the primers described in Table S1. Mutations (Tyr52Phe, His82Leu, Asn194Asp, Gln274Asn, Tyr277Phe, Arg280Lys, Phe325Tyr, and Glu411Asp) were also confirmed by DNA sequencing.

#### 4.4. Expression and Purification of the GkHAL Enzyme and Mutants

The protocol was similar to that described in [61]. *E. coli* BL21/pJMC93 and the mutated strains were grown in an LB medium supplemented with ampicillin. For induction and expression of the genes, L-rhamnose was added to the culture and incubated at 30 °C overnight. The cells were collected by centrifugation (Beckman J-26 XPI centrifuge) (6500× *g*, 4 °C, 25 min) (Beckman Coulter, Brea, CA, USA) and stored at −20 °C until use.

The cell wall disruption was performed by sonication as described before [47] after resuspension in the wash buffer. The enzyme purification was performed by metal affinity chromatography using cobalt resin [38]. Protein purity was determined at different stages of the purification by SDS-PAGE electrophoresis. Additional gel filtration chromatography was carried out to eliminate any DNA or protein co-eluting with the protein of interest [61]. The purified enzymes were concentrated using Vivaspinn concentrators (Sartorius, Göttingen, Germany), dialyzed against 100 mM diethanolamine (DEA) buffer, pH 8.5, and stored at 4 °C. Protein concentrations were determined from the absorbance of the extinction coefficient of tyrosine residues [67].

#### 4.5. Molecular Mass Analysis

Size-exclusion chromatography–high-pressure liquid chromatography (SEC-HPLC) analysis was conducted to determine the molecular mass of both the wild-type and mutated GkHAL enzymes in their native states. The HPLC System (Breeze HPLC System, Waters, Barcelona, Spain) used a Biosep-SEC-S2000 column (Phenomenex, Madrid, Spain) that was calibrated using a non-denatured protein molecular weight marker kit (Bio-Rad, Madrid, Spain) [38,47]. The molecular mass of the monomeric form of both the wild-type and mutated GkHAL enzymes was determined using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a low molecular weight marker kit (Cytiva, Marlborough, MA, USA).

#### 4.6. Enzyme Assay and Protein Determination

The GkHAL activity assay was based on the transformation of L-histidine to give *trans*-urocanic acid. The activity was measured with the spectrophotometer ThermoScientific NanoDrop 2000 (Waltham, MA, USA) at 280 nm. Standard enzymatic assay was carried out by adapting the method described in [68] with the purified GkHAL and mutated enzymes (at final concentrations from 0.4 to 4.3  $\mu\text{M}$ ), along with L-histidine as the substrate (1 mM) dissolved in 100 mM DEA buffer (pH 8.5), in a reaction volume of 500  $\mu\text{L}$  at 65 °C. The protein was added to 485  $\mu\text{L}$  of DEA buffer, and the reaction was started with the addition of 5  $\mu\text{L}$  of a 0.1 M L-histidine solution. The activities of both wild-type and mutant enzymes were assessed. More active enzymes were measured at intervals of 1 to 5 min, while less active ones were measured at intervals of 5 to 30 min. The activity was determined utilizing a molar extinction coefficient for *trans*-urocanic acid of  $18,800 \text{ M}^{-1} \text{ cm}^{-1}$ , as outlined in [69].

The optimal pH of purified GkHAL wild-type and its mutants was determined by conducting the standard assay in the appropriate buffers ranging from 1.92 to 11.9: phosphoric buffer (pH 2–3), formic buffer (pH 3–4), acetate buffer (pH 4–5.5), phosphate buffer (pH 6–7.5), Tris-HCl buffer (pH 8 and pH 9), carbonate buffer (pH 9.5–11), and sodium phosphate buffer (pH 11.5–12). The effect of the temperature on the GkHAL wild-type and its mutant forms was studied by performing the standard enzymatic assay within the temperature range of 4 to 99 °C. Thermal stability of GkHAL wild-type was determined after 15 min of preincubation at different temperatures from 4 to 90 °C in 100 mM DEA buffer pH 8.5, followed by the standard activity assay.

GkHAL activity on other substrates was studied with the standard assay, only varying L-histidine to a different reactive. In addition to the proteinogenic amino acids, GkHAL activity was tested on D-histidine, L-histidinamide, L-histidine methyl ester, benzyl-L-histidine,  $\beta$ -alanyl-L-histidine, 1-methyl-L-histidine, 3-methyl-L-histidine,  $\beta$ -mercapto-L-histidine, and diamine-L-histidine, with a substrate concentration in reaction of 1 mM.

Determination of  $K_m$  and  $V_{max}$  for L-histidine, L-histidine methyl ester, and L-histidinamide was performed by varying substrate concentrations from 0.25 to 50 mM. Kinetic parameters ( $K_m$ ,  $V_{max}$ ) were calculated with non-linear square hyperbole plots following the Michaelis–Menten equation [56]. The fitting was made using SigmaPlot® (version 12.0, Systat Software, Inc., San Jose, CA, USA). The turnover number ( $k_{cat}$ ) was calculated by dividing  $V_{max}$  by the enzyme concentration. The catalytic efficiency was determined through the ratio of  $k_{cat}$  to  $K_m$  ( $k_{cat}/K_m$ ).

Thermal inactivation kinetics were determined by performing the standard assay with previous incubations of the enzymes at 65, 70, 75, and 80 °C. Pre-incubation times vary from 5 min to 60 min for higher temperatures and from 1 to 24 h for lower temperatures. The inactivation rate constant ( $k_d$ ) of the enzymes was determined using relative residual activities fitted to Arrhenius plots. The half-life value ( $t_{1/2}$ ) of the enzymes at a particular temperature was then determined from each  $k_d$ . Activation parameters, including activation energy for denaturation and changes in enthalpy, free energy, and entropy for each incubation temperature, were determined as outlined in references [42,44,45,57].

#### 4.7. Circular Dichroism

Circular dichroism spectra were recorded using a Jasco J815 spectropolarimeter equipped with a thermostated cell holder and interfaced with a Peltier accessory.

Far-UV spectra were performed to assess the secondary structure of wild-type and mutant GkHAL enzymes. The protein concentration was 4  $\mu$ M (in protomer units) in 50 mM DEA buffer, pH 8.5. CD spectra were acquired at a scan speed of 50 nm/min with a response time of 4 s at 25 °C and averaged over six scans spanning from 190 to 260 nm. Spectra were corrected by subtracting the baseline scan (buffer). The secondary protein structure was decomposed and analyzed using the k2d algorithm online on the DichroWeb site [46].

Thermal denaturalization of GkHAL and its mutants was performed with a total protein concentration of 4  $\mu$ M in an interval of temperatures of 25 to 95 °C with an increase of 60 °C per hour. Data were collected every 0.2 °C at 222 nm with an 8 s response and 1 nm of bandwidth. Precipitation occurred in all cases upon heating.

The thermal transitions of wild-type and GkHAL mutants were analyzed using a two-state model [47]. The spectral parameters were directly fitted to the following equation through non-linear least squares analysis:

$$S_{\text{obs}} = \frac{S_N + S_U \exp\left(-\frac{\Delta H_{\text{VH}}}{R} \left(\frac{1}{T} - \frac{1}{T_m}\right)\right)}{1 + \exp\left(-\frac{\Delta H_{\text{VH}}}{R} \left(\frac{1}{T} - \frac{1}{T_m}\right)\right)} \quad (1)$$

where  $S_{\text{obs}}$  represents the ellipticity at 222 nm, and  $S_N = A_N + B_N T$  and  $S_U = A_U + B_U T$  refer to the linear dependence of the native (N) and unfolded (U) states, which have the slopes  $B_N$  and  $B_U$  and the intercepts  $A_N$  and  $A_U$ , respectively.  $\Delta H_{\text{VH}}$  corresponds to the apparent change in van't Hoff enthalpy,  $R$  is the universal gas constant,  $T$  is the temperature in Kelvin, and  $T_m$  is the transition midpoint at which 50% of the protein is unfolded. Data fitting was carried out with Kaleidagraph (<https://www.synergy.com/>).

#### 4.8. Nucleotide Sequence Accession Number

The nucleotide sequence of GkHAL has been deposited in the GenBank database under accession No. PQ065632.

### 5. Conclusions

This study successfully characterized a novel histidine ammonia-lyase from *Geobacillus kaustophilus*, highlighting its high thermal stability and catalytic efficiency. The mutational analysis provided critical insights into the structure–function relationships within the enzyme, revealing essential residues for catalytic activity and identifying mutations that expand substrate specificity. These results not only contribute to our understanding of HAL enzymes but also underscore their potential for industrial applications, particularly in environments demanding high thermal resilience. Future work could investigate the immobilization of these enzymes to further enhance their thermostability, with the aim of evaluating their potential use as industrial biocatalysts.

**Supplementary Materials:** The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms251810163/s1>.

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## References

1. Xue, Y.P.; Cao, C.H.; Zheng, Y.G. Enzymatic asymmetric synthesis of chiral amino acids. *Chem. Soc. Rev.* **2018**, *47*, 1516. [CrossRef] [PubMed]
2. Turner, N.J. Ammonia lyases and aminomutases as biocatalysts for the synthesis of  $\alpha$ -amino and  $\beta$ -amino acids. *Curr. Opin. Chem. Biol.* **2011**, *15*, 234–240. [CrossRef] [PubMed]
3. Asano, Y.; Kato, Y.; Levy, C.; Baker, P.; Rice, D. Structure and function of amino acid ammonia-lyases. *Biocatal. Biotransform.* **2004**, *22*, 131–138. [CrossRef]
4. Heberling, M.M.; Wu, B.; Bartsch, S.; Janssen, D.B. Priming ammonia lyases and aminomutases for industrial and therapeutic applications. *Curr. Opin. Chem. Biol.* **2013**, *17*, 250–260. [CrossRef] [PubMed]
5. Hermes, J.D.; Weiss, P.M.; Cleland, W.W. Use of nitrogen-15 and deuterium isotope effects to determine the chemical mechanism of phenylalanine ammonia-lyase. *Biochemistry* **1985**, *24*, 2959–2967. [CrossRef] [PubMed]
6. Hodgins, D.S. Yeast phenylalanine ammonia-lyase. *J. Biol. Chem.* **1971**, *246*, 2977–2985. [CrossRef]
7. Wickner, R.B. Dehydroalanine in histidine ammonia lyase. *J. Biol. Chem.* **1969**, *244*, 6550–6552. [CrossRef]
8. Peng, F.; Engel, U.; Aliyu, H.; Rudat, J. Origin and evolution of enzymes with MIO prosthetic group: Microbial coevolution after the mass extinction event. *Front. Genet.* **2022**, *13*, 851738. [CrossRef]
9. Langer, B.; Langer, M.; Rétey, J. Methylidene-imidazolone (MIO) from histidine and phenylalanine ammonia-lyase. *Adv. Protein Chem.* **2001**, *58*, 175–188. [CrossRef]
10. Cooke, H.A.; Christianson, C.V.; Bruner, S.D. Structure and chemistry of 4-methylideneimidazole-5-one containing enzymes. *Curr. Opin. Chem. Biol.* **2009**, *13*, 460–468. [CrossRef]
11. Brosnan, M.E.; Brosnan, J.T. Histidine metabolism and function. *J. Nutr.* **2020**, *150*, 2570S–2575S. [CrossRef] [PubMed]
12. Bender, R.A. Regulation of the histidine utilization (Hut) system in bacteria. *Microbiol. Mol. Biol. Rev.* **2012**, *76*, 565–584. [CrossRef] [PubMed]
13. Poppe, L.; Rétey, J. Friedel-Crafts-type mechanism for the enzymatic elimination of ammonia from histidine and phenylalanine. *Angew. Chem. Int. Ed.* **2005**, *44*, 3668–3688. [CrossRef] [PubMed]
14. Furuta, T.; Takahashi, H.; Kasuya, Y. Reversible reaction via a carbanion intermediate in the elimination of ammonia from L-histidine catalysed by histidine ammonia-lyase. *J. Chromatog. B Biomed. Sci. Appl.* **1991**, *562*, 363–368. [CrossRef] [PubMed]
15. Fujii, T.; Sakai, H.; Kawata, Y.; Hata, Y. Crystal structure of thermostable aspartase from *Bacillus* sp. YM55-1: Structure-based exploration of functional sites in the aspartase family. *J. Mol. Biol.* **2003**, *328*, 635–654. [CrossRef]
16. Chesters, C.; Wilding, M.; Goodall, M.; Micklefield, J. Thermal bifunctionality of bacterial phenylalanine aminomutase and ammonia lyase enzymes. *Angew. Chem. Int. Ed.* **2012**, *51*, 4344–4348. [CrossRef]
17. Kumavath, R.N.; Ramana, C.V.; Sasikala, C.; Barh, D.; Kumar, A.P.; Azevedo, V. Isolation and characterization of L-tryptophan ammonia lyase from *Rubrivivax benzoatilyticus* strain JA2. *Curr. Protein Pept. Sci.* **2015**, *16*, 775–781. [CrossRef]
18. Klee, C.B.; Kirk, K.L.; Cohen, L.A.; McPhie, P. Histidine ammonia-lyase. The use of 4-fluorohistidine in identification of the rate-determining step. *J. Biol. Chem.* **1975**, *250*, 5033–5040. [CrossRef]
19. Klee, C.B.; Kirk, K.L.; Cohen, L.A. 4-Nitro-L-histidine as a substrate for histidine ammonia-lyase: The role of  $\beta$ -hydrogen acidity in the rate-limiting step. *Biochem. Biophys. Res. Commun.* **1979**, *87*, 343–348. [CrossRef]
20. Hernandez, D.; Phillips, A.T. Purification and characterization of *Pseudomonas putida* histidine ammonia-lyase expressed in *Escherichia coli*. *Protein Expr. Purif.* **1993**, *4*, 473–478. [CrossRef]
21. Beliaeva, M.A.; Atac, R.; Seebeck, F.P. Bacterial Degradation of N $\tau$ -Methylhistidine. *ACS Chem. Biol.* **2022**, *17*, 1989–1995. [CrossRef] [PubMed]
22. Klee, C.B. Metal activation of histidine ammonia-lyase. Metal ion-sulfhydryl group relationship. *J. Biol. Chem.* **1972**, *247*, 1398–1406. [CrossRef] [PubMed]

23. Seff, A.L.; Pilbák, S.; Silaghi-Dumitrescu, I.; Poppe, L. Computational investigation of the histidine ammonia-lyase reaction: A modified loop conformation and the role of the Zinc(II) ion. *J. Mol. Model.* **2011**, *17*, 1551–1563. [CrossRef]
24. Schwede, T.F.; Rétey, J.; Schulz, G.E. Crystal structure of histidine ammonia-lyase revealing a novel polypeptide modification as the catalytic electrophile. *Biochemistry* **1999**, *38*, 5355–5361. [CrossRef]
25. Röther, D.; Poppe, L.; Viergutz, S.; Langer, B.; Rétey, J. Characterization of the active site of histidine ammonia-lyase from *Pseudomonas putida*. *Eur. J. Biochem.* **2001**, *268*, 6011–6019. [CrossRef]
26. Baedeker, M.; Schulz, G.E. Autocatalytic peptide cyclization during chain folding of histidine ammonia-lyase. *Structure* **2002**, *10*, 61–67. [CrossRef]
27. Csuka, P.; Juhász, V.; Kohári, S.; Filip, A.; Varga, A.; Sátorhelyi, P.; Bencze, L.C.; Barton, H.; Paizs, C.; Poppe, L. *Pseudomonas fluorescens* strain R124 encodes three different MIO enzymes. *ChemBioChem* **2018**, *19*, 411–418. [CrossRef] [PubMed]
28. Miranda, R.R.; Silva, M.; Barisón, M.J.; Silber, A.M.; Lulek, J. Crystal structure of histidine ammonia-lyase from *Trypanosoma cruzi*. *Biochimie* **2020**, *175*, 181–188. [CrossRef]
29. Ade, C.; Marcelino, T.F.; Dulchavsky, M.; Wu, K.; Bardwell, J.C.; Städler, B. Microreactor equipped with naturally acid-resistant histidine ammonia lyase from an extremophile. *Mater. Adv.* **2022**, *3*, 3649–3662. [CrossRef]
30. Moro, J.; Tomé, D.; Schmidely, P.; Demersay, T.C.; Azzout-Marniche, D. Histidine: A systematic review on metabolism and physiological effects in human and different animal species. *Nutrients* **2020**, *12*, 1414. [CrossRef]
31. Shibatani, T.; Nishimura, N.; Nabe, K.; Kakimoto, T.; Chibata, I. Enzymatic production of urocanic acid by *Achromobacter liquidum*. *Appl. Microbiol.* **1974**, *27*, 688–694. [CrossRef] [PubMed]
32. Parmeggiani, F.; Weise, N.J.; Ahmed, S.T.; Turner, N.J. Synthetic and therapeutic applications of ammonia-lyases and aminomutases. *Chem. Rev.* **2018**, *118*, 73–118. [CrossRef] [PubMed]
33. Jack, G.; Wiblin, C.; McMahon, P. The effect of histidine ammonia lyase on some murine tumours. *Leuk. Res.* **1983**, *7*, 421–429. [CrossRef]
34. Sethuraman, N.; Roberts, J.; Macallister, T. Cloning, Overexpression and Therapeutic Use of Bioactive Histidine Ammonia Lyase. U.S. Patent 6939541, 6 September 2005.
35. Burkhardt, C.; Baruth, L.; Meyer-Heydecke, N.; Klippel, B.; Margaryan, A.; Paloyan, A.; Panosyan, H.H.; Antranikian, G. Mining thermophiles for biotechnologically relevant enzymes: Evaluating the potential of European and Caucasian hot springs. *Extremophiles* **2024**, *28*, 5. [CrossRef]
36. Takami, H.; Takaki, Y.; Chee, G.J.; Nishi, S.; Shimamura, S.; Suzuki, H.; Matsui, S.; Uchiyama, I. Thermoadaptation trait revealed by the genome sequence of thermophilic *Geobacillus kaustophilus*. *Nucleic Acids Res.* **2004**, *32*, 6292–6303. [CrossRef]
37. Brack, Y.; Sun, C.; Yi, D.; Bornscheuer, U.T. Systematic analysis of the MIO-forming residues of aromatic ammonia lyases. *ChemBioChem* **2024**, *25*, e202400016. [CrossRef]
38. Martínez-Rodríguez, S.; Clemente-Jiménez, J.M.; Rodríguez-Vico, F.; las Heras-Vázquez, F.J. Molecular Cloning and Biochemical Characterization of L-N-Carbamoylase from *Sinorhizobium meliloti* CECT4114. *Microb. Physiol.* **2005**, *9*, 16–25. [CrossRef] [PubMed]
39. Martínez-Rodríguez, S.; Encinar, J.A.; Hurtado-Gómez, E.; Prieto, J.; Clemente-Jiménez, J.M.; Las Heras-Vázquez, F.J.; Rodríguez-Vico, F.; Neira, J.L. Metal-triggered changes in the stability and secondary structure of a tetrameric dihydropyrimidinase: A biophysical characterization. *Biophys. Chem.* **2009**, *139*, 42–52. [CrossRef] [PubMed]
40. Martínez-Rodríguez, S.; García-Pino, A.; Las Heras-Vázquez, F.J.; Clemente-Jiménez, J.M.; Rodríguez-Vico, F.; Loris, R.; García-Ruiz, J.M.; Gavira, J.A. Crystallization and preliminary crystallographic studies of the recombinant L-N-carbamoylase from *Geobacillus stearothermophilus* CECT43. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **2008**, *64 Pt 12*, 1135–1138. [CrossRef]
41. Shibatani, T.; Kakimoto, T.; Chibata, I. Crystalline L-histidine ammonia-lyase of *Achromobacter liquidum*. Crystallization and enzymic properties. *Eur. J. Biochem.* **1975**, *55*, 263–269. [CrossRef]
42. D’Amico, S.; Marx, J.C.; Gerday, C.; Feller, G. Activity-Stability Relationships in Extremophilic Enzymes. *J. Biol. Chem.* **2003**, *278*, 7891–7896. [CrossRef] [PubMed]
43. Contreras, L.M.; Gómez, J.; Prieto, J.; Clemente-Jiménez, J.M.; las Heras-Vázquez, F.J.; Rodríguez-Vico, F.; Blanco, F.J.; Neira, J.L. The family 52  $\beta$ -xylosidase from *Geobacillus stearothermophilus* is a dimer: Structural and biophysical characterization of a glycoside hydrolase. *Biochim. Biophys. Acta Proteins Proteom.* **2008**, *1784*, 1924–1934. [CrossRef] [PubMed]
44. Gouda, M.D.; Singh, S.A.; Rao, A.G.A.; Thakur, M.S.; Karanth, N.G. Thermal inactivation of glucose oxidase: Mechanism and stabilization using additives. *J. Biol. Chem.* **2003**, *278*, 24324–24333. [CrossRef] [PubMed]
45. Saqib, A.A.N.; Siddiqui, K.S. How to calculate thermostability of enzymes using a simple approach. *Biochem. Mol. Biol. Educ.* **2018**, *46*, 398–402. [CrossRef] [PubMed]
46. Miles, A.J.; Ramalli, S.G.; Wallace, B.A. DichroWeb, a website for calculating protein secondary structure from circular dichroism spectroscopic data. *Protein Sci.* **2021**, *31*, 37–46. [CrossRef]
47. Martínez-Gómez, A.I.; Soriano-Maldonado, P.; Andújar-Sánchez, M.; Clemente-Jiménez, J.M.; Rodríguez-Vico, F.; Neira, J.L.; Las Heras-Vázquez, F.J.; Martínez-Rodríguez, S. Biochemical and mutational studies of allantoinase from *Bacillus licheniformis* CECT 20T. *Biochimie* **2014**, *99*, 178–188. [CrossRef]
48. Benjwal, S.; Verma, S.; Röhm, K.H.; Gursky, O. Monitoring protein aggregation during thermal unfolding in circular dichroism experiments. *Protein Sci.* **2006**, *15*, 635–639. [CrossRef]

49. Pozo-Dengra, J.; Martínez-Gómez, A.I.; Martínez-Rodríguez, S.; Clemente-Jiménez, J.M.; Rodríguez-Vico, F.; las Heras-Vázquez, F.J. Racemization study on different *N*-acetylamino acids by a recombinant *N*-succinylamino acid racemase from *Geobacillus kaustophilus* CECT4264. *Process Biochem.* **2009**, *44*, 835–841. [CrossRef]
50. Suzuki, H.; Kobayashi, J.; Wada, K.; Furukawa, M.; Doi, K. Thermoadaptation-directed enzyme evolution in an error-prone thermophile derived from *Geobacillus kaustophilus* HTA426. *Appl. Environ. Microbiol.* **2015**, *81*, 149–158. [CrossRef]
51. Brack, Y.; Sun, C.; Yi, D.; Bornscheuer, U.T. Exploring the substrate switch motif of aromatic ammonia lyases. *ChemBioChem* **2023**, *24*, e202300584. [CrossRef]
52. Varga, A.; Csuka, P.; Sonesouphap, O.; Bánóczy, G.; Tosa, M.I.; Katona, G.; Molnár, Z.; Bencze, L.C.; Poppe, L.; Paizs, C. A novel phenylalanine ammonia-lyase from *Pseudozyma antarctica* for stereoselective biotransformations of unnatural amino acids. *Catal. Today* **2021**, *366*, 185–194. [CrossRef]
53. Baedeker, M.; Schulz, G.E. Structures of two histidine ammonia-lyase modifications and implications for the catalytic mechanism. *Eur. J. Biochem.* **2002**, *269*, 1790–1797. [CrossRef] [PubMed]
54. Jaliani, H.Z.; Farajnia, S.; Mohammadi, S.A.; Barzegar, A.; Talebi, S. Engineering and kinetic stabilization of the therapeutic enzyme *Anabeana variabilis* phenylalanine ammonia lyase. *Appl. Biochem. Biotechnol.* **2013**, *171*, 1805–1818. [CrossRef]
55. Zhu, B.F.; Liu, Y.; Pei, X.Q.; Wu, Z.L. Characterization of phenylalanine ammonia lyases from lettuce (*Lactuca sativa* L.) as robust biocatalysts for the production of D- and L-amino acids. *J. Agric. Food Chem.* **2023**, *71*, 2935–2942. [CrossRef] [PubMed]
56. Fersht, A.R. *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*; W.H. Freeman: New York, NY, USA, 1999.
57. Atkins, P.W.; de Paula, J. *Physical Chemistry*; Oxford University Press: Oxford, NY, USA, 2006.
58. Maffucci, I.; Laage, D.; Sterpone, F.; Stirnemann, G. Thermal adaptation of enzymes: Impacts of conformational shifts on catalytic activation energy and optimum temperature. *Chemistry* **2020**, *26*, 10045–10056. [CrossRef]
59. Zaman, U.; Khan, S.U.; Alem, S.F.M.; Rehman, K.U.; Almhazia, A.A.; Naglah, A.M.; Al-Wasidi, A.S.; Refat, M.S.; Saeed, S.; Zaki, M.E.A. Purification and thermodynamic characterization of acid protease with novel properties from *Melilotus indicus* leaves. *Int. J. Biol. Macromol.* **2023**, *230*, 123217. [CrossRef]
60. Zaman, U.; Rehman, K.U.; Khan, S.U.; Refat, M.S.; Badshah, S.; Hajira, B.; Iqbal, A.; Khan, W.U.; Alsuhaibani, A.M. Identification, kinetics and thermodynamic analysis of novel  $\beta$ -galactosidase from *Convolvulus arvensis* seeds: An efficient agent for delactosed milk activity. *Int. J. Biol. Macromol.* **2022**, *220*, 1545–1555. [CrossRef]
61. Martínez-Rodríguez, S.; García-Pino, A.; Las Heras-Vázquez, F.J.; Clemente-Jiménez, J.M.; Rodríguez-Vico, F.; García-Ruiz, J.M.; Loris, R.; Gavira, J.A. Mutational and structural analysis of L-*N*-carbamoylase reveals new insights into a peptidase M20/M25/M40 family member. *J. Bacteriol.* **2012**, *194*, 5759. [CrossRef]
62. Langer, M.; Reck, G.; Rétey, J.; Reed, J. Identification of serine-143 as the most likely precursor of dehydroalanine in the active site of histidine ammonia-lyase. A study of the overexpressed enzyme by site-directed mutagenesis. *Biochemistry* **1994**, *33*, 6462–6467. [CrossRef]
63. Micsonai, A.; Moussong, É.; Wien, F.; Boros, E.; Vadász, H.; Murvai, N.; Lee, Y.H.; Molnár, T.; Réfrégiers, M.; Goto, Y.; et al. BeStSel: Webserver for secondary structure and fold prediction for protein CD spectroscopy. *Nucleic Acids Res.* **2022**, *50*, W90–W98. [CrossRef]
64. Navarro-Jiménez, P.D.; Clemente-Jiménez, J.M.; Rodríguez-Vico, F.; Martínez-Rodríguez, S.; Las Heras Vázquez, F.J. Utilización de Técnicas Moleculares para el Rediseño de un Vector de Expresión. Master's Thesis, University of Almería, Almería, Spain, 2012.
65. Stumpp, T.; Wilms, B.; Altenbuchner, J. Ein neues, l-rhamnose-induzierbares expressionssystem für *Escherichia coli*. *Biospektrum* **2000**, *6*, 33–36.
66. Parks, T.D.; Leuther, K.K.; Howard, E.D.; Johnston, S.A.; Dougherty, W.G. Release of proteins and peptides from fusion proteins using a recombinant plant virus proteinase. *Anal. Biochem.* **1994**, *216*, 413–417. [CrossRef] [PubMed]
67. Gill, S.C.; von Hippel, P.H. Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **1989**, *182*, 319–326. [CrossRef] [PubMed]
68. Tabor, H.; Mehler, A.H. Histidase and urocanase. *Methods Enzymol.* **1955**, *2*, 228–233. [CrossRef]
69. Mehler, A.H.; Tabor, H. Deamination of histidine to form urocanic acid in liver. *J. Biol. Chem.* **1953**, *201*, 775–784. [CrossRef]

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Article

# Polyphosphate Kinase from *Burkholderia cenocepacia*, One Enzyme Catalyzing a Two-Step Cascade Reaction to Synthesize ATP from AMP

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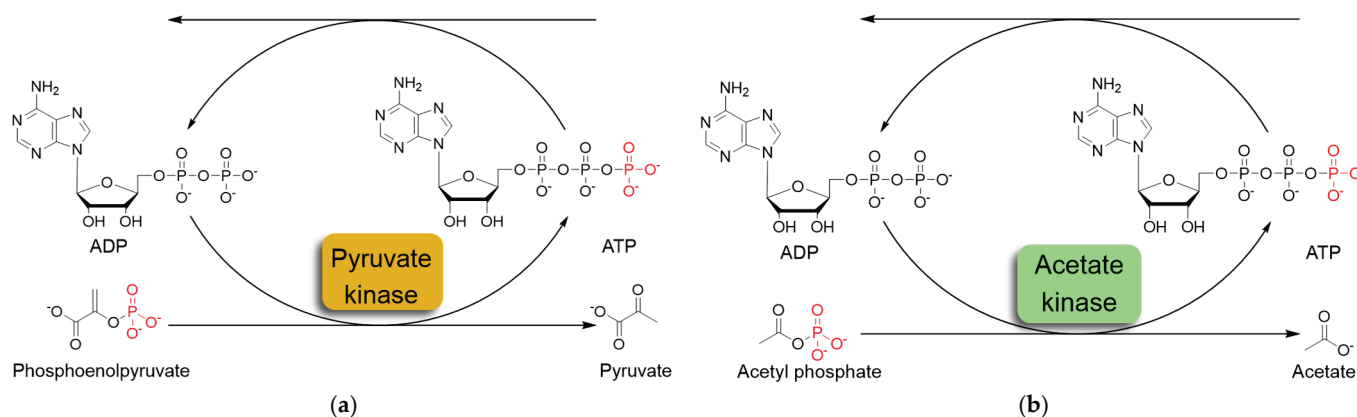
**Abstract:** This study characterizes a novel polyphosphate kinase from *Burkholderia cenocepacia* (BcPPK2-III), an enzyme with potential applications in ATP regeneration processes. Bioinformatic and structural analyses confirmed the presence of conserved motifs characteristic of PPK2 enzymes, including Walker A and B motifs, and the subclass-specific residue E137. Molecular docking simulations showed AMP had the highest binding affinity (−7.0 kcal/mol), followed by ADP (−6.5 kcal/mol), with ATP having the lowest affinity (−6.3 kcal/mol). It was overexpressed in *Escherichia coli*, after purification enzymatic activity assays revealed that BcPPK2-III needed divalent cations (Mg<sup>2+</sup>, Mn<sup>2+</sup>, Co<sup>2+</sup>) as cofactors to be active. Functional assays revealed its ability to synthesize ATP from AMP through a stepwise phosphorylation mechanism, forming ADP as an intermediate, achieving 70% ATP conversion (TTN 4354.7) after 24 h. Kinetic studies indicated cooperative behavior and substrate preference, with AMP phosphorylation to ADP being the most efficient step. The enzyme demonstrated high thermostability (T<sub>50</sub> = 62 °C) and a broad pH stability range (pH 6.0–9.0), making it suitable for diverse biocatalytic applications. The study highlights BcPPK2-III as a robust and versatile candidate for cost-effective ATP regeneration, offering advantages in industrial processes requiring stoichiometric amounts of ATP.

**Keywords:** biocatalysis; ATP regeneration; polyphosphate; polyphosphate kinase; cofactor recycling; cascade reactions; *Burkholderia cenocepacia*

## 1. Introduction

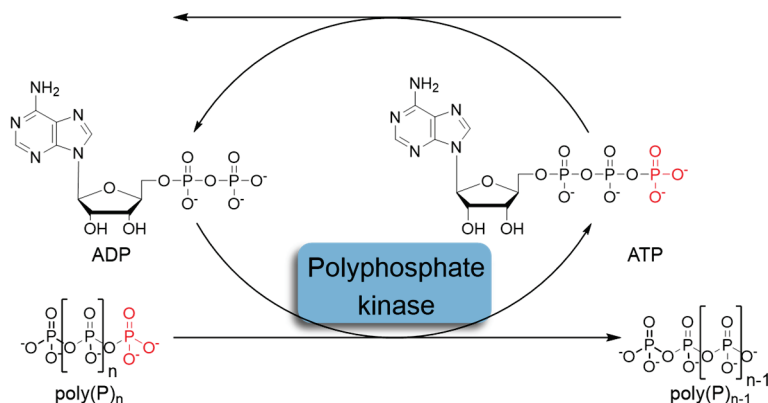
Adenosine 5′-triphosphate (ATP) is one of the most important molecules in living systems and probably the foremost phosphate compound of all. Normally known as the universal energy “currency” or “store” of the cell [1], it is also used as a building block for nucleic acid synthesis and is involved in the synthesis of key phosphorus-containing intermediates for biosyntheses and cofactors such as flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD<sup>+</sup>), S-adenosylmethionine (SAM), and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) [2–5]. ATP is a crucial molecule for many biocatalytic processes; however, its industrial application is limited by the requirement for stoichiometric amounts of this costly cofactor. With a price of approximately USD 35 per gram, depending on the supplier and grade, the high cost of ATP poses a significant challenge to the economic viability of large-scale ATP-dependent biocatalytic processes. ATP recycling systems can provide higher yields in these reactions by shifting the reaction equilibrium toward the desired product [6]. Other benefits of ATP recycling systems include reduced product inhibition by decreasing the concentration of the side product ADP, while keeping the ATP concentration high to facilitate phosphorylation reactions, simplified product purification, and cost savings by reducing the required amount of this expensive substrate [7].

The most commonly used ATP regeneration systems from ADP are based on the use of phosphoenolpyruvate as the phosphate donor (Figure 1a) in a coupled reaction catalyzed by pyruvate kinase (PK) and on the use of acetylphosphate (Figure 1b) coupled with acetate kinase (AK) [8].



**Figure 1.** Schematic representation of in situ ATP regeneration systems using pyruvate kinase (a) and acetate kinase (b).

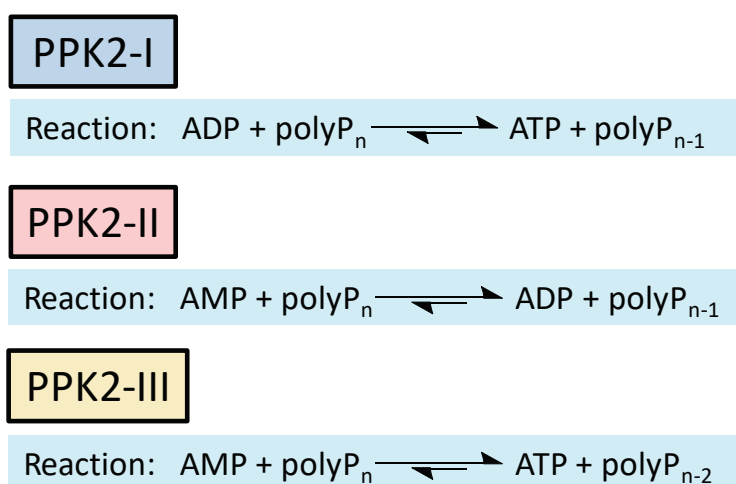
In recent decades, the regeneration system based on the use of polyphosphate kinases (PPK) that catalyze the reversible transfer of  $\gamma$ -phosphate from ATP to inorganic polyphosphate (polyP) has attracted considerable attention (Figure 2) [9,10]. Inorganic polyphosphate is a linear polymer consisting of up to hundreds of orthophosphate ( $P_i$ ) residues linked by high-energy phosphoanhydride bonds [11–13].



**Figure 2.** Schematic representation of in situ ATP regeneration systems using polyphosphate kinase and inorganic polyphosphate as the phosphoryl donor.

Regeneration systems based on the use of PPKs have several advantages that make them very attractive in terms of their application in biocatalysis. First and foremost, polyP is a low-cost phosphate group donor. A large amount of polyP is regularly produced as sodium hexametaphosphate (about 13–18 residues) for industrial purposes such as food additives, which also makes polyP inexpensive compared to other phosphoryl group donors. To get an idea of the magnitude of savings from using polyP as a phosphate donor, consider that a commercial form of polyP costing about USD 20/kg can provide ATP equivalents that would cost more than USD 35,000/kg, and that other phosphate donors commonly used in ATP regeneration, such as phosphoenolpyruvate (PEP), are even more expensive than ATP (USD 264,000/kg) [14–16]. The other major advantage of this system is that there are PPKs that allow regeneration of ATP from AMP.

Methods to regenerate ATP from AMP or adenosine are not well established, although they have great potential for biocatalysis [9,17]. There are several cases where the regeneration of ATP from AMP based on low-cost polyphosphate as the sole energy source would be of great value, such as the regeneration of acyl-CoA with integrated ATP regeneration [18] or the in situ synthesis of PAPS in reactions catalyzed by sulfotransferases [19]. PAPS is synthesized from sulfate and two ATP molecules through ATP sulfurylase, forming adenosine 5'-phosphosulfate (APS), and APS kinase, which phosphorylates APS to PAPS, consuming a second ATP molecule [20,21]. Polyphosphate kinase enzymes play an important role in regulating intracellular nucleotide concentrations in vivo by participating in the reversible transfer of phosphate groups between nucleotides and polyP [22]. To date, two major families of highly conserved PPKs have been characterized in bacteria that are neither sequentially nor structurally related. Polyphosphate kinase 1 (PPK1) is responsible for the synthesis of polyP using ATP as a donor of phosphate groups, while polyphosphate kinase 2 (PPK2) enzymes are responsible for its degradation [22]. PPK2 can be divided into three subclasses (I, II, and III) depending on the reaction they catalyze, as shown in Figure 3. They all use polyP as a phosphate group donor to catalyze the phosphorylation of the various nucleotides (AMP, ADP and ATP). The substrate preference of PPK2-II is AMP from which they can synthesize ADP. The substrate preference of PPK2-I and PPK2-III is ADP and AMP, respectively [22,23].



**Figure 3.** Reactions catalyzed by the different PPK family 2 (PPK2) subclasses.

Given the interest that class III of PPK2 represents from a biocatalysis point of view, we consider it necessary to identify and describe new members of this enzyme family in order to increase the tools available for the development of efficient ATP regeneration processes. Although several gene sequences have been found that could encode PPK2-III enzymes, most of them are putatively assigned, i.e., they lack experimental verification. In this regard, Motomura et al. (2014) [24] constructed an unrooted phylogenetic tree of PPK2 using amino acid sequences from 17 characterized and 192 putative PPK2 homologs. This phylogenetic analysis revealed 40 sequences belonging to the class III subfamily. However, only the sequence from the bacterium *Meiothermus ruber* was amplified and expressed to characterize the biochemical properties of the PPK2-III enzymes.

*Burkholderia cenocepacia* is a Gram-negative bacterium and belongs to the *Burkholderia cepacia* complex (Bcc). Although common in the environment, it can also cause disease in plants and act as an opportunistic pathogen that frequently infects patients with cystic fibrosis and chronic granulomatous diseases. Studies have shown that *B. cepacia* isolates from various sources accumulate higher levels of intracellular polyphosphate (polyP) and increase the uptake of inorganic phosphate when grown in acidic environments [25]. The importance of polyP metabolism in this bacterium suggests that *B. cenocepacia* could be

an interesting source for the overexpression and characterization of a new PPK2-III with interesting properties for its application in biocatalysis.

Herein, in this work, we addressed the characterization of the putative polyphosphate kinase from *Burkholderia cenocepacia*. A study of its secondary and tertiary structure was performed using theoretical structural analysis and 3D modeling. The enzyme was overexpressed in *E. coli*, and after its purification, its enzymatic activity, divalent cation dependence and reaction mechanism were studied. Enzyme kinetic parameters and stability were also analyzed and discussed. In addition, we have been concerned with elucidating the mechanism of action of this enzyme, as it is controversial whether it is a stepwise sequential diphosphorylation mechanism or a pyrophosphorylation mechanism and thus occurs in a single step [23].

## 2. Results and Discussion

### 2.1. Bioinformatic Analysis and Structural Modeling of BcPPK2-III

#### 2.1.1. Sequence Analysis of the Putative PPK2-III from *Burkholderia cenocepacia*

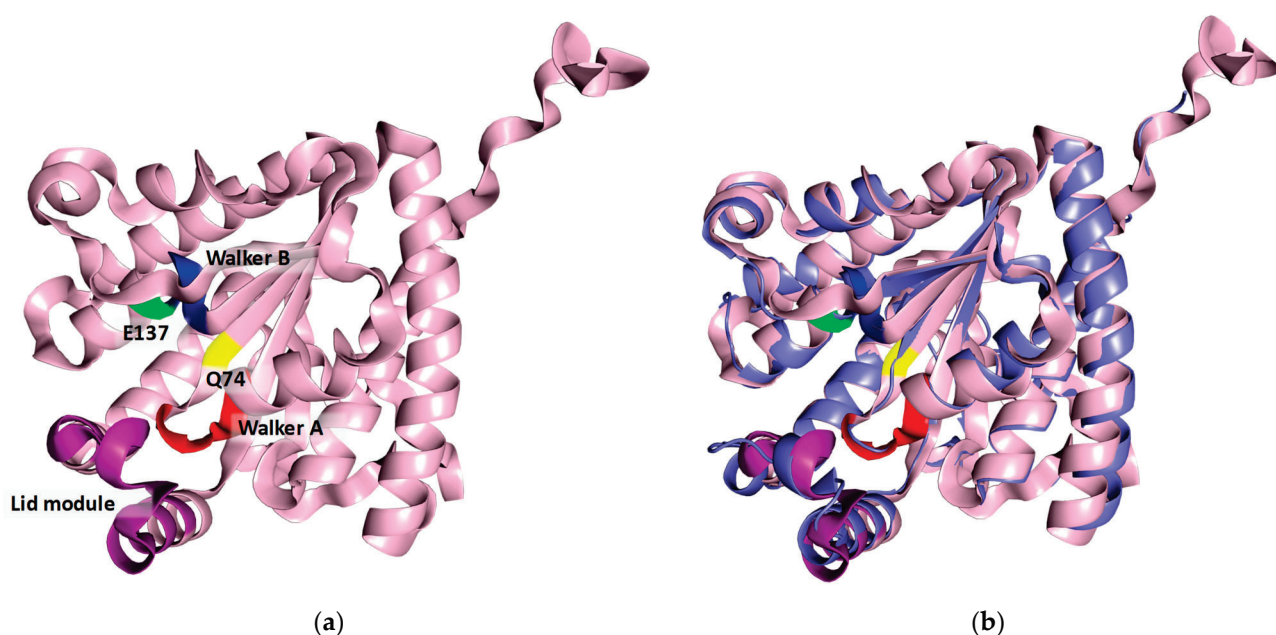
PPK2 belongs to the P-loop kinases. This protein fold is by far the most common among enzymes that bind or hydrolyze nucleoside triphosphates (NTPs) and is characterized by the presence of two conserved sequence motifs (Walker A and Walker B) and a lid module (RxxxxxxPxxxxxxD). The Walker A (DxxGK) and Walker B (RSxY) motifs are conserved in all PPK2 subclasses, whereas the amino acid following the Walker B motif appears to be subclass-specific. These amino acids are asparagine (subclass I), glycine (subclass II), and glutamic acid (subclass III) [24,26,27].

Using as query the sequence of the PPK2-III enzyme from *Meiothermus ruber* (Mrub\_2488), a sequence alignment of the putative PPK2-III from *B. cenocepacia* was performed using the BLAST® program (Figure S1) [28,29]. The sequence analysis shows 51% identity, and the *E*-value, indicating the probability of alignment occurring by chance, was  $10^{-95}$ . Structural comparison showed excellent agreement between the two proteins, with a root mean square deviation (RMSD) of 1.14 Å and a template modeling score (TM-score) of 0.97, confirming a high degree of structural similarity. Conserved motifs characteristic of PPK2 enzymes, including Walker A and B motifs and the cap module, were identified in the *B. cenocepacia* sequence (Figure S1). Additionally, the sequence contains glutamine (Q74) preceding the Walker A motif and glutamic acid (E137) downstream of the Walker B motif, the latter being the class III signature residue of PPK2 and contributing to the binding of the adenine base [27]. Mutational studies have shown that E137 plays a critical role in phosphorylation activity for AMP, ADP, and GDP, although it does not solely determine substrate specificity [30].

The sequence of BcPPK2-III was also analyzed for possible transmembrane regions using the protein topology prediction method, TMHMM, based on a hidden Markov model [31]. As no such zone was found, we can assume that the protein is free in the cytosol, which increases the chances that it will be expressed in the soluble form.

#### 2.1.2. Molecular Modeling of BcPPK2-III

The 3D structure of the protein was modeled using the ColabFold v1.5.5 open-source software [32,33] completed with 3D structure modeling. The high Model Confidence and Sequence coverage (Figure S2a and b, respectively) indicate the good quality of the predicted enzyme structure. The vast majority of the structure has been predicted with a very high (>90%) degree of confidence (Figure S2a). The obtained results were visualized using PyMOL. The three conserved motifs (Walker A motif, Walker B motif, and lid module) are highlighted in red, blue, and green, respectively (Figure 4). The spatial position and proximity of the three motifs supports the hypothesis that the active center is located below the lid module, between motifs A and B [24,27].



**Figure 4.** (a) Model of the 3D structure of the recombinant protein *BcPPK2-III*. The conserved motifs are shown in colors: Walker A is shown in red, Walker B in blue and the lid module in violet. The conserved amino acids Q74 and E137 are displayed in yellow and green, respectively. (b) Structural alignment of *BcPPK2-III* (light pink) with *MrubPPK2-III* (PDB: 5LD1) (slate gray).

### 2.1.3. Modeling of the Enzyme–Substrate Complexes

Molecular docking is a helpful tool in predicting the affinity and binding mode of a native molecule in the enzyme active site. In order to harness a more complete understanding of *BcPPK2-III* and the molecular foundation of interaction and conformation of the three main compounds within the binding pose, a docking study was performed using Maestro Schrödinger's Glide module. The results of the molecular docking are summarized in Table 1, which lists the conformer of each substrate that yields the best docking score, as well as the residues of the active site of the enzyme involved in the interaction with the substrates.

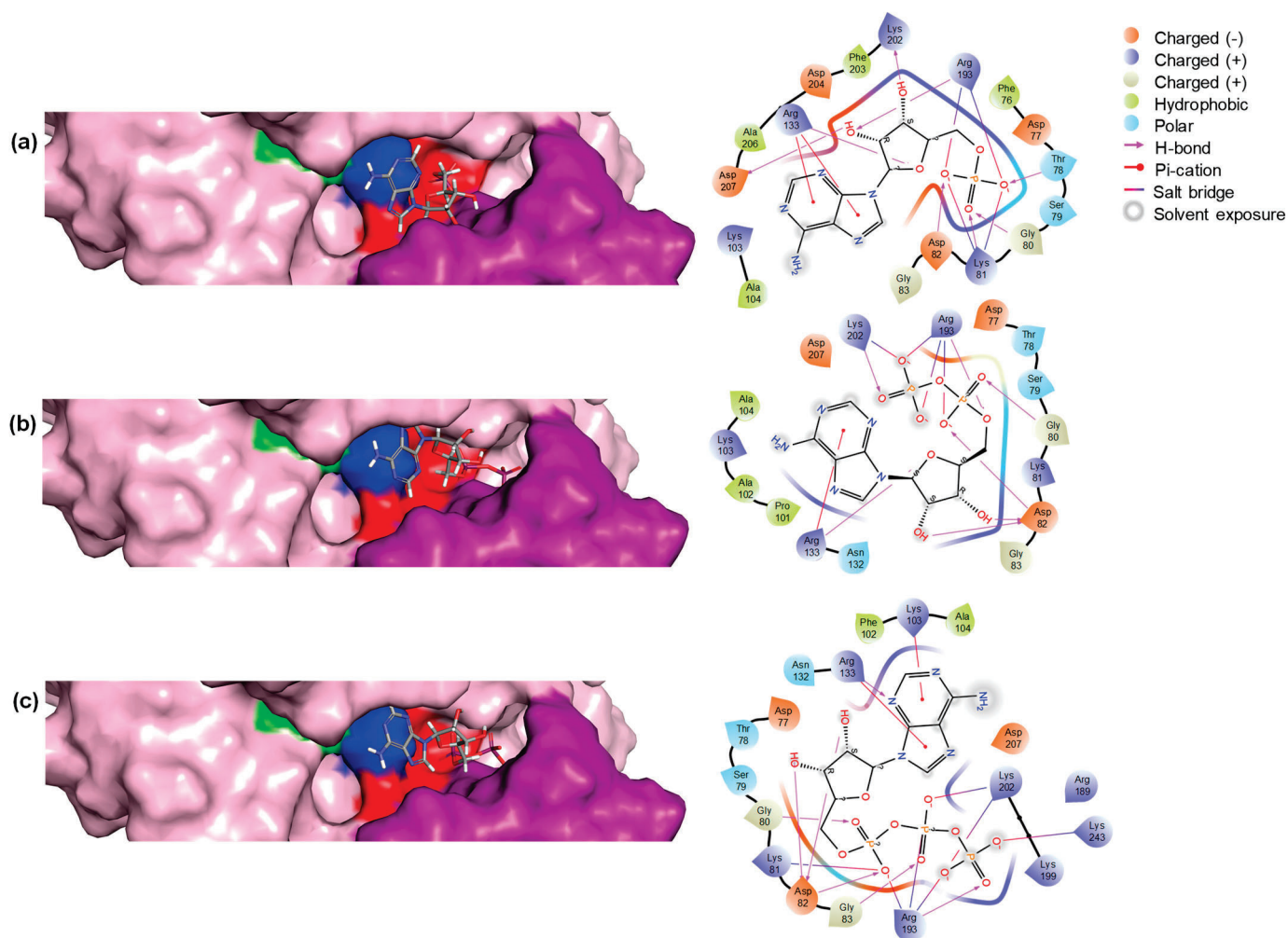
**Table 1.** Molecular docking results.

| Compound | Interacting Residues                                                | Docking Score (kcal/mol) | Glide Emodel |
|----------|---------------------------------------------------------------------|--------------------------|--------------|
|          | Thr78, Lys81, Gly80, Asp82, Arg133, Arg193, Lys202, Asp207          | −7.0                     | −100.3       |
|          | Gly80, Asp82, Arg133, Arg193, Lys202                                | −6.5                     | −110.3       |
|          | Gly80, Lys81, Asp82, Gly83, Lys 103, Arg133, Arg193, Lys202, Lys243 | −6.3                     | −136.7       |

The docking scores indicated that AMP had the highest binding affinity with a score of −7.0 kcal/mol, followed by ADP with a score of −6.5 kcal/mol, while ATP showed a relatively lower affinity with a score of −6.3 kcal/mol. The Glide Emodel results provided further insights into the stability and quality of the binding poses. ATP exhibited the most favorable Emodel score of −136.7 considering the lower docking score, suggesting a highly stable interaction within the active site. This suggests that ATP binds with more internal

strain than the other ligands. This strain could arise from conformational adjustments required to accommodate the triphosphate moiety within the active site. Such adjustments could make the binding less energetically favorable, lowering the docking score despite having strong overall stability. AMP also demonstrated a strong binding pose with an Emodel score of  $-100.3$ . ADP showed intermediate values on both parameters. These results underscore the nuanced interplay between binding affinity and pose stability, highlighting AMP as the most suitable ligand.

The binding pose and residue interaction of the three ligands are displayed in Figure 5. The results are consistent with the expected binding of the three compounds, as has been described before [34].



**Figure 5.** Structural representation of the enzyme with three different substrates bound in the active site. (a) The enzyme is bound with AMP, (b) ADP, and (c) ATP. Each panel includes a 3D structure of the enzyme–substrate complex and a 2D representation showing the residues interacting with the compounds. In the 3D structures, key residues are highlighted: Walker A motif residues in red, Walker B motif residues in blue, glutamic acid (E) in green, and the lid domain residues in violet.

The interactions between ATP, ADP, and AMP with the enzyme are mediated by several key residues that are critical for binding and catalytic activity. In particular, residues from the Walker A motif, like Asp82 and Gly80, play a critical role in anchoring the nucleotides, while Thr78 and Lys81 enhance binding stability through hydrogen bonding and electrostatic interactions. The Walker B motif residues, Arg133 and Arg193, further stabilize the interactions with the phosphate groups, enhancing binding affinity. Additionally, Lys202 from the lid motif helps maintain the structural integrity of the active

site, ensuring proper positioning of the nucleotides for optimal catalytic activity. These interactions highlight the importance of these residues in facilitating nucleotide binding and enzyme function.

AlphaFold3 modeling of BcPPK2-III with different divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Co}^{2+}$ ) and ligands (ATP, ADP, and AMP) revealed no significant structural differences among the predicted models. The enzyme's overall fold and active site architecture remained consistent regardless of the specific cation or ligand present. Detailed visualizations of the modeled structures are presented in Figure S3.

## 2.2. Heterologous Overexpression, Purification, and Identification of BcPPK2-III

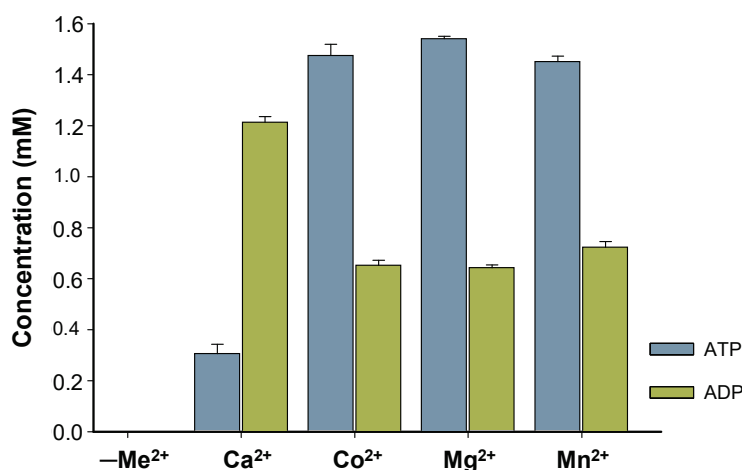
Expression of the recombinant protein was induced with 0.5 mM IPTG at an OD<sub>600</sub> nm of 0.5–0.6. The expression and purification of the BcPPK2-III enzyme was analyzed by SDS-PAGE (Figure S4). The system yielded a high degree of overexpression of the target enzyme, with 71 mg of pure protein per liter of culture. The CFE (Figure S4, lane 1) showed a majority band corresponding to the recombinant protein given its theoretical size (32 kDa). Densitometric analysis revealed that the recombinant protein accounted for 60% of the total soluble proteins in the CFE. The analysis of the IMAC purification process of the recombinant protein shows that the recombinant protein binds very specifically to the resin, as it was not detected in the flow-through after loading the CFE (Figure S4, lane 2), nor in the subsequent washes performed to remove proteins not specifically bound to the resin (Figure S4, lane 3). On the contrary, in the combined fractions obtained after elution of the recombinant protein with imidazole (Figure S4, lane 4), a single band corresponding to BcPPK2-III was observed with a purity higher than 98%. Interestingly, when the resin was analyzed after elution of the recombinant protein (Figure S4, lane 5), it was found that a considerable amount of the protein remained bound to the resin with a high degree of purity (77.5%). We did not analyze whether the protein that remained bound on the resin was functional or not. In any case, our results indicate that the protein binds very strongly and selectively to the  $\text{Co}^{2+}$ -IDA resin, providing an efficient method for simultaneous purification and immobilization of the recombinant enzyme.

The identity of the recombinant protein was confirmed by peptide mass fingerprinting (Figure S5). Seventeen different peptides were identified, corresponding to 68% of the protein sequence. This result confirmed that the sequence of the purified protein matched the sequence of BcPPK2-III.

## 2.3. Functional Characterization of BcPPK2-III

### 2.3.1. Metal Cofactor Dependence of BcPPK2-III

Characterization of BcPPK2-III initially focused on the enzyme metal cofactor determination. Although the Luria–Bertani (LB) medium used for protein expression contains trace amounts of metal ions, these were effectively removed during purification via immobilized metal affinity chromatography (IMAC). During elution, imidazole displaced any metal ions bound to the protein by competing with histidine residues for metal coordination. This process ensured that the purified BcPPK2-III was devoid of residual metal ions from the expression medium. Four different divalent cations ( $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Ca}^{2+}$ ) were tested. The enzymatic assays revealed no activity in the absence of added divalent cations (negative control, Figure 6). BcPPK2-III shows activity across all tested cations (Figure 6) and exhibits substantial and similar activity towards the production of ATP when  $\text{Co}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Mn}^{2+}$  are used as cofactors, confirming that the enzyme's catalytic function relies on the deliberate addition of metal ions post-purification. Interestingly, when the enzyme used  $\text{Ca}^{2+}$  as a cofactor, it exhibited distinct behavior wherein the production of ADP surpassed that of ATP. Determining this specificity is of great interest because it has been described that metal cofactor preference plays a crucial role in the efficiency of the enzyme towards ATP production. Generally PPK2 enzymes show dependence on  $\text{Mn}^{2+}$  [24] or  $\text{Mg}^{2+}$  [27]. In the cases of PPK2-III from *Meiothermus ruber* and PPK2-III from *Ralstonia eutropha*, both divalent cations were used by the enzyme to produce ATP [20,24].



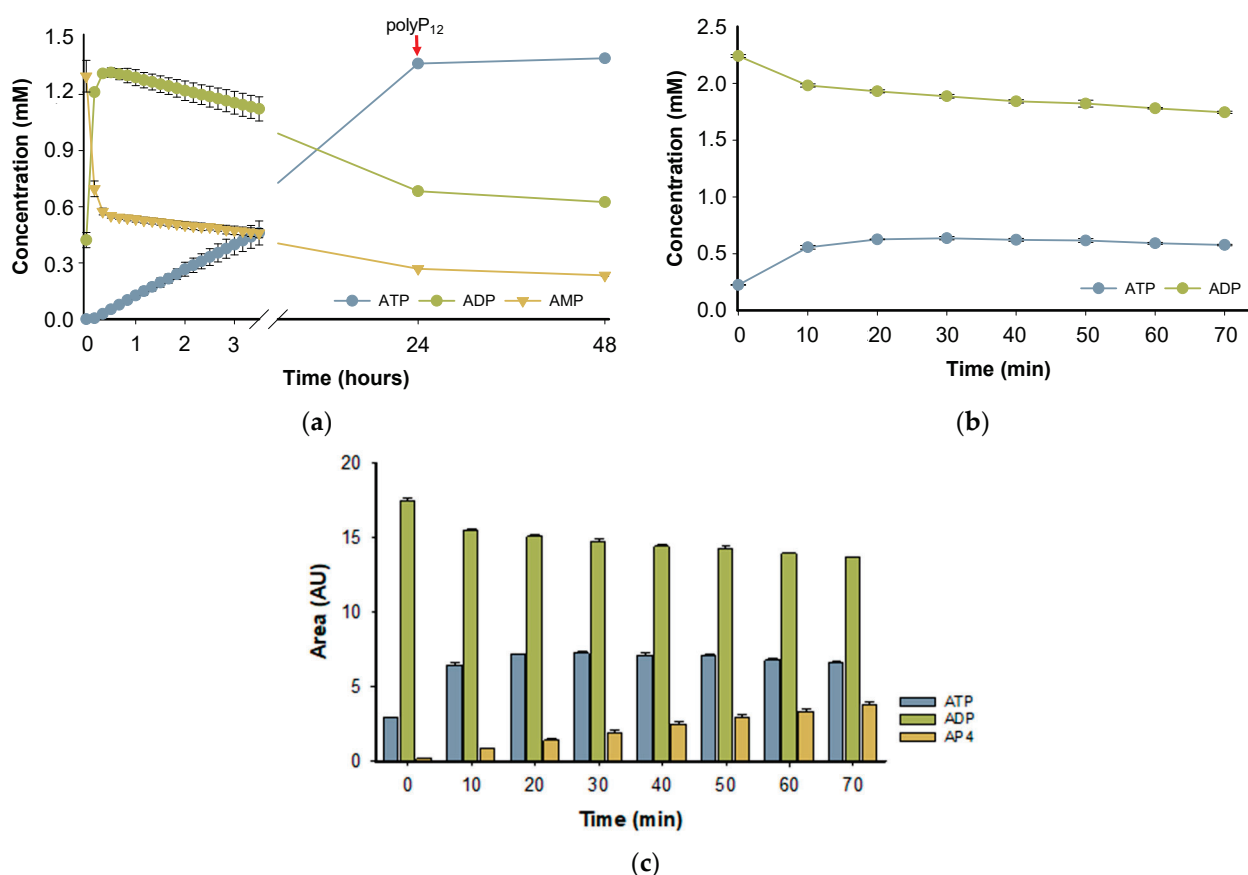
**Figure 6.** Effect of metal ions in enzyme activity. Reaction after 24 h using the same concentration of polyP<sub>12</sub> (10 mM) and same concentration of divalent cation (10 mM), for Ca<sup>2+</sup>, Co<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>. Concentration of produced ATP (blue) and ADP (green) is represented. A negative control (reaction mixture without cation, indicated as -Me<sup>2+</sup>) is included.

This is consistent with Nocek and colleagues [30] since PPK2-III was reported to exhibit high and similar activity with three different cations, illustrating the versatility of the enzyme along with its distinct Ca<sup>2+</sup>-dependent activity. Further studies should be performed to explain the different behavior found with Ca<sup>2+</sup>, considering the role of its smaller ionic radius and the solvation shell of the cation.

In addition, it is worth mentioning that the 24 h reaction using Mn<sup>2+</sup> as an enzyme cofactor resulted in a precipitate that could indicate the formation of a nanoflower-like structure [35] between the shorter polyP chains and the cation, resulting after enzyme activity (Figure S6). This could translate into lower availability of the phosphate groups, which could influence the enzymatic efficiency of the enzyme to produce ATP. In this sense, further characterization of the enzyme was carried out using Mg<sup>2+</sup> as a metal cofactor.

### 2.3.2. Reaction Mechanism and Stepwise Phosphorylation Pathway

The PPK2-III family can perform ATP formation from AMP through two main reaction mechanisms. The most common mechanism is step-by-step phosphorylation, in such a way that starting from AMP, the enzyme catalyzes the formation of ATP with an intermediate formation of ADP. On the other hand, the direct production of ATP from AMP in the so-called pyrophosphorylation mechanism has been reported, adding two phosphate groups to AMP, without forming the intermediate ADP [24,36]. To assess the enzymatic mechanism of BcPPK2-III, the progress of the reaction over 48 h was analyzed (Figure 7a). According to the time-course experiment, in the first 10 min of the reaction, the enzyme produces ADP and, in a lower rate, forms ATP, reaching a high yield (≈70%) after 24 h. PPK2-III enzymes prefer larger chains of polyP<sub>n</sub> and the shortening of the phosphate donor during the reaction is a limiting factor in enzyme activity [25,32]. Subsequently, the reaction was supplemented with polyP<sub>12</sub> and the analysis after 48 h did not show a significant increase in ATP yield, probably due to enzyme stability or inhibition.



**Figure 7.** Reaction time-course, showing the concentration of AMP (yellow triangles), ADP (green circles) and ATP (blue circles). In both reactions, the starting material concentration was 2 mM,  $Mg^{2+}$  was used as divalent cation and PolyP<sub>12</sub> (10 mM) as a phosphate donor, and the experiments were performed in triplicate. **(a)** The starting material of the reaction was AMP and the red arrow indicates the supplementation of the reaction with PolyP<sub>12</sub> (10 mM). **(b)** The reaction was carried out with ADP as the initial substrate. **(c)** The time-course of the reaction initiated with ADP as the substrate, showing the appearance of AP4.

To further support the hypothesis that the reaction mechanism proceeds by a stepwise phosphorylation process and that the observed decrease in ADP is not due to adenylate kinase side reactivity, a reaction was performed with ADP as the starting substrate instead of AMP. Figure 7b shows the reaction time-course using ADP as a substrate. When using ADP as a substrate, we observed the formation of ATP, which was confirmed by comparison with a standard in HPLC analysis. In addition to ATP, a third peak was detected, which we hypothesize to be adenosine tetraphosphate (AP4) based on its elution profile, UV spectra and retention time (Figures 7c and S7c). However, further analysis is required for confirmation. These results are in agreement with the published literature, since it has been described that PPK2 enzymes can mediate the formation of oligophosphorylated products up to nucleoside nona-phosphates [37].

These results experimentally confirm that the BcPPK2-III enzyme belongs to subclass III, as it catalyzes the stepwise phosphorylation of AMP to ADP and subsequently to ATP.

### 2.3.3. Kinetic Analysis and Catalytic Efficiency of BcPPK2-III

It is noteworthy that the PPK2-III enzyme displayed a sigmoidal curve in all three reactions studied (AMP→ATP, AMP→ADP and ADP→ATP) instead of the expected hyperbolic profile in the Michaelis–Menten kinetics (Figure S8). The nonlinear regression analysis performed on the experimental data showed a better fit to the Hill equation than to the classical Michaelis–Menten equation, which was confirmed with directed and secondary kinetics data plotting. The fit to the Hill equation model was assessed by calculating the  $R^2$  of the fit, which was 0.95 for the AMP to ATP reaction, 0.89 for the AMP to ADP reaction, and 0.96 for the ADP to ATP reaction. These values are significant and reproducible as the experiments were carried out in triplicate. In addition, the Hill coefficient ( $n$ ) was greater than 1 in all three cases—4.8 for the AMP to ATP reaction, 2.9 for the AMP to ADP reaction, and 3.7 for the ADP to ATP reaction—indicating positive cooperativity. Positive cooperativity is unusual in simple enzyme kinetics and suggests a structure in which interactions between sub-sites within the active center (Figure 5) facilitate the sequential progression of the reaction, as observed in enzymes with multiple phosphorylation sites or cascading active subunits. This cooperative behavior complicates the accurate calculation of kinetic constants, as the kinetic behavior of the enzyme is strongly influenced by the experimental conditions. Despite these challenges, we present reliable kinetic data for each catalytic step, with calculated apparent constants that align with previously reported values for comparable enzymes (<https://www.brenda-enzymes.org/enzyme.php?ecno=2.7.4.1> (accessed on 4 November 2024)).

The catalytic efficiency of the enzyme, as assessed by total turnover number (TTN), initial turnover rate (ITR) and kinetic constants (Table 2), showed that ATP production is significantly more efficient when ADP serves as a substrate, achieving a rate approximately eight times higher ( $1240 \text{ M}^{-1}\text{s}^{-1}$ ) than with AMP. Additionally, the phosphorylation efficiency of AMP to ADP is more than three times higher than that of AMP to ATP, with correspondingly higher ITR values for the conversion of AMP to ADP. This result highlights the importance of substrate choice in optimizing enzyme performance.

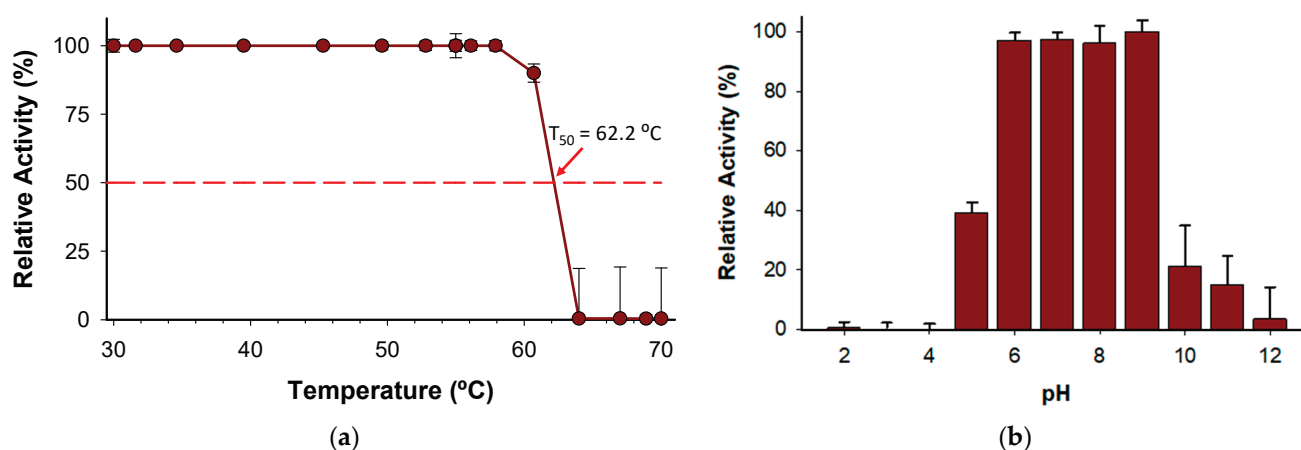
**Table 2.** Kinetic parameters of BcPPK2-III.

| Reaction   | $V_{\max}$ (U/mg) | $k_{\text{cat}}$ ( $\text{s}^{-1}$ ) | $K_M$ (mM)      | $k_{\text{cat}}/K_M$ ( $\text{M}^{-1}\text{s}^{-1}$ ) | $R^2$ | ITR *              | TTN § |
|------------|-------------------|--------------------------------------|-----------------|-------------------------------------------------------|-------|--------------------|-------|
| AMP to ATP | $0.224 \pm 0.011$ | $0.119 \pm 0.011$                    | $0.79 \pm 0.04$ | $151 \pm 16$                                          | 0.95  | $7.700 \pm 0.001$  | 4355  |
| AMP to ADP | $1.12 \pm 0.034$  | $0.594 \pm 0.033$                    | $1.14 \pm 0.33$ | $520 \pm 150$                                         | 0.89  | $25.217 \pm 0.001$ |       |
| ADP to ATP | $1.65 \pm 0.15$   | $0.88 \pm 0.15$                      | $0.71 \pm 0.06$ | $1240 \pm 230$                                        | 0.96  |                    |       |

\*  $\mu\text{mol ATP } \mu\text{mol BcPPK2-III}^{-1}\text{min}^{-1}$ . §  $\mu\text{mol ATP } \mu\text{mol BcPPK2-III}^{-1}$ .

### 2.3.4. Enzyme Stability

The  $T_{50}$  (the temperature at which the enzyme retains 50% of its activity after a ten-minute incubation) was determined to be  $62^\circ\text{C}$  (Figure 8a). Notably, the enzyme holds 100% activity from  $30$  to  $58^\circ\text{C}$ , which indicates a high degree of thermostability considering other enzymes described belonging to the same family [24]. In addition, the stability of the enzyme has been evidenced by pH profile experiments (Figure 8b) since the enzyme retains more than 97% of activity from pH 6.0 to 9.0. The wide range of pH activity exhibited by the novel BcPPK2-III is a distinctive attribute that enhances its versatility to be included in several systems as an ATP regeneration module. Enzymes from this family have been described to show activity across a wide pH range, including neutral, acidic and alkaline conditions [36,38].



**Figure 8.** (a) Kinetic thermostability ( $T_{50}$ ) of BcPPK2-III, (b) pH profile. Values are presented as the mean  $\pm$  standard deviation of three independent experiments.

### 3. Materials and Methods

#### 3.1. Materials and General Procedures

Synthesis of the gene *ppk2-III* from *Burkholderia cenocepacia*, optimization of the nucleotide sequence, and cloning into the plasmid pET-28b(+) were performed by GenScript (Piscataway, NJ, USA). Peptide mass fingerprinting was performed at the Proteomics Unit of the Complutense University of Madrid. *Escherichia coli* strain BL21 (DE3) was obtained from Promega Biotech Ibérica S.L. (Madrid, Spain). The GenElute™ Plasmid Miniprep Kit from Sigma-Aldrich (Darmstadt, Germany) was used to purify the plasmid. Promega 1kb DNA Step Ladder Molecular Weight Marker and restriction enzymes were purchased from ThermoFisher Scientific Inc. (Waltham, MA, USA). Kanamycin, DNase I, and other reagents were purchased from Sigma-Aldrich (Darmstadt, Germany). Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was purchased from AppliChem GmbH (Darmstadt, Germany). The acrylamide/bis-acrylamide 30% (29:1) solution used for protein analysis by SDS-PAGE was purchased from Bio-Rad (Hercules, CA, USA). The Low Molecular Weight Calibration Kit from GE Healthcare was used. SDS-PAGE gels were run on a MiniProtein® Tetracell cuvette from Bio-Rad (Hercules, CA, USA). Agarose gels were run in a RunOne™ Electrophoresis Cell cuvette from EmbiTec (San Diego, CA, USA) using SYBR Safe from ThermoFisher Scientific (Waltham, MA, USA). Analysis of the gels was performed using a Gene Flash Bio Imaging Photodocumenter from Syngene LTD (Bengaluru, Karnataka). Iminodiacetic acid agarose (IDA-agarose) was purchased from Agarose Bead Technologies (Miami, FL, USA). Solvents were of analytical grade. DNA manipulation was performed according to standard procedures [39].

#### 3.2. Bioinformatic and Computational Analysis

##### 3.2.1. Sequence Analysis and Structural Modeling of BcPPK2-III

Secondary structural motifs of the protein were identified by the theoretical structural analysis performed in the PSIPRED Protein Analysis Workbench [40]. Three-dimensional structure modeling without a template was performed using the ColabFold open-source software [34,35]. In addition, the structure of BcPPK2-III was modeled using AlphaFold 3, a state-of-the-art deep learning algorithm for protein structure prediction. The modeling was conducted to explore potential interactions between the enzyme and various divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Co}^{2+}$ ) as well as ligands (ATP, ADP, and AMP). The input sequence of BcPPK2-III was used without additional structural templates or constraints, and the resulting models were analyzed for cation- and ligand-binding configurations.

Pairwise structural alignment was performed using the RCSB PDB tool to compare the 3D structure of BcPPK2-III with MrubPPK2-III (PDB: 5LD1), taking into account RMSD values and TM-score [41].

### 3.2.2. Molecular Docking Modeling of the Enzyme–Substrate Complexes

The docking study was conducted using Maestro (version 13.2.128) for molecular modeling and visualization and MMshare (version 5.8.128, Release 2022-2) for molecular mechanics. The protein structure was prepared using Maestro. Ligand structures were sketched using Chemdraw and subsequently prepared using Maestro's ligand preparation tools. This preparation involved generating 3D coordinates, optimizing the ligand geometry, and assigning appropriate force field parameters. The ligand structures were energy minimized using the OPLS3e force field within Maestro to ensure accurate docking results. Docking simulations were performed using the Glide module within Maestro. The protein's active site was defined by setting a grid box around the binding pocket, and the ligands were docked into this grid to explore potential binding modes. Default Glide settings, including standard precision (SP) mode, were used for the docking simulations. Each ligand was docked in multiple poses, and the results were analyzed to identify binding modes with favorable docking scores. The top-ranked binding poses were selected based on their docking scores and interactions with the active site residues. Binding interactions and the best docking poses were visualized and analyzed using Maestro's visualization tools to assess the quality of ligand binding.

### 3.3. Expression and Purification of BcPPK2-III

#### 3.3.1. Cloning of *ppk2-III* Gene

The *ppk2-III* gene from *B. cenocepacia* was synthesized and optimized for expression in *E. coli*. This significantly improved the codon adaptation index (CAI) from 0.68 to 0.97. Considering that a CAI value of 1 occurs when the sequence is optimal for expression in the specific organism, the performed optimization significantly improves the chances of correct expression of the *ppk2-III* gene in *E. coli*. The percentage of guanines (G) and cytosines (C) could be decreased by 8%, which could significantly increase the stability of DNA and the secondary structure of the mRNA. Cloning of the optimized *ppk2-III* gene was performed in the expression plasmid pET-28b(+), which allows induction of protein overexpression with isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG). The protein is expressed fused to a 6-His tag at the N-terminus, allowing its one-step purification by ion metal affinity chromatography (IMAC) from cell-free extract (CFE). The plasmid, designated pET-28b(+)-*ppk2-III*, was then transformed into competent *E. coli* BL21 (DE3) cells. Restriction analysis of the plasmid after its purification revealed two bands (Figure S9): the band corresponding to the plasmid pET-28-b(+), with a size of 5368 bp, and a band of 279 bp corresponding to the expected size of the *ppk2-III* gene.

#### 3.3.2. Heterologous Expression and Purification of Recombinant BcPPK2-III

For expression of the BcPPK2-III protein, preinocula of recombinant colonies were prepared in 5 mL LB medium containing 5  $\mu$ L kanamycin (50  $\mu$ g/mL) and incubated overnight at 37 °C with shaking (160 rpm). The culture was extended and incubated under the same conditions until the exponential phase ( $OD_{600nm} = 0.5$ – $0.7$ ) was reached. At this point, protein expression was induced with IPTG at a final concentration of 1 mM. Orbital agitation was kept constant and the temperature was lowered to 30 °C to avoid the formation of inclusion bodies. The culture was then centrifuged at 4000 rpm for 20 min to recover the cells. The pellet was resuspended in Na<sub>2</sub>HPO<sub>4</sub> buffer (50 mM, 300 mM NaCl, pH = 8.0). Cell disruption was performed by sonication (70% amplitude, 10 s pulse and 15 s pause). The lysed cells were separated by centrifugation of the mixture at 8000 rpm for 20 min. The supernatant obtained was treated with DNaseI (10  $\mu$ g/mL cells) and MgCl<sub>2</sub> (0.95  $\mu$ g/mL cells) for 20 min on ice. Streptomycin (1% weight/volume) was then added and allowed to act for an additional 20 min. Finally, the cell-free extract (CFE) was obtained by centrifugation, which, after aliquoting, was frozen with liquid NO<sub>2</sub> and stored at  $-80$  °C.

Purification by divalent ion affinity chromatography (IMAC) was performed using a Co<sup>2+</sup>-IDA-agarose column. The resin was washed with milliQ H<sub>2</sub>O and equilibrated

with 10 mM imidazole, 50 mM Na<sub>2</sub>HPO<sub>4</sub> and 300 mM NaCl buffer, pH = 8.0. CFE was added to the resin at a ratio of 1:1 (v/v). To remove proteins non-specifically bound to the resin, the column was washed with 2 volumes of the same buffer. The recombinant protein was then eluted with 1 volume of the same buffer containing 2.0 M imidazole. The eluates were dialyzed to remove the imidazole on 30 kDa cut-off Vivaspin centricones. Finally, the enzyme was kept in Tris-hydroxymethyl-aminomethane (Tris-HCl) buffer (50 mM pH = 8.0 at 4 °C). The protein concentration in the purification fractions was quantified by Ab<sub>280nm</sub> using the theoretical size of the protein (32 kDa) and the theoretical molar extinction coefficient ( $\epsilon$ ) determined by the content of tryptophan (W), tyrosine (Y), and cysteine (C) and calculated using Equation (1):

$$\epsilon = (nW \times 5500) + (nY \times 1490) + (nC \times 125) \quad (1)$$

Expression and successive purification steps were monitored by SDS-PAGE (Figure S4) at 11% in the resolving gel. Quantification of gels and protein purity was performed by densitometric analysis (GeneTools 3.07).

### 3.4. Functional Characterization and Stability Assays

#### 3.4.1. HPLC Analysis of BcPPK2-III Activity

Analysis of the enzymatic activity of BcPPK2-III in all cases was performed by reverse high-performance liquid chromatography (RHPLC) with an Agilent 1260 Infinity II equipped with an InfinityLab Poroshell 120 EC-18 column (150 mm × 4.6 mm, 4  $\mu$ m) and photodiode array (PDA). The injections were performed by an autosampler and 3  $\mu$ L of enzyme reaction was injected. The solvent system used solvent (A): 0.1% (v/v) trifluoroacetic acid (TFA) in H<sub>2</sub>O and solvent (B): 0.05% (v/v) TFA in CH<sub>3</sub>CN, with a flow rate of 1 mL min<sup>-1</sup>, detection at 260 nm and column temperature of 30 °C. Samples were analyzed using a gradient elution of 100% A over 4 min and of 0 to 10% of B over 4 min. Commercial standards of ATP, ADP, and AMP with retention times of 2.8 min, 3.5 min and 7.3 min, respectively, were used to identify the different nucleotides present in the reaction mixture (Figure S7).

#### 3.4.2. Enzyme Activity Assay, Metal Ion Preference and Reaction Progress

Unless stated otherwise, enzyme activity assays were performed using 100 mM Tris-HCl buffer pH = 7.0, polyP (10 mM) with a chain length of 12 units as the donor of phosphate groups, AMP or ADP as substrates with a concentration of 2 mM, and MgSO<sub>4</sub> (10 mM) as the metal ion cofactor. Reactions were initiated by the addition of BcPPK2-III (10  $\mu$ g/mL) and maintained at 30 °C with orbital shaking (700 rpm). BcPPK2-III reaction progress was monitored by RHPLC every 10 min for the first 6 h and after 24 h. Additionally, 10 mM of polyP was supplemented to the same reaction and measured after 48 h.

A study of the enzymatic activity of BcPPK2-III was performed using the standard conditions and introducing various metal ions (Ca<sup>2+</sup>, Co<sup>2+</sup>, Mg<sup>2+</sup> and Mn<sup>2+</sup>) with a final concentration of 10 mM in the reaction.

#### 3.4.3. Kinetic Constants Determination

The kinetic constants determination was performed with the following conditions: 100 mM Tris-HCl buffer pH = 7.0, polyP (10 mM), AMP or ADP as substrates with a concentration from 0.5 to 2 mM, and MgSO<sub>4</sub> (10 mM) as the metal ion cofactor. Reactions were initiated by the addition of BcPPK2-III (20  $\mu$ g/mL). The reaction rate was determined at early reaction times, ensuring that less than 10% of the substrate was converted. This approach guarantees that measurements were taken within the reaction's "linear phase", well before reaching equilibrium and at the initial step of the AMP→ADP or ADP→ATP reaction sequence.

Initial velocities (V, in U/mg) were fitted to the Hill equation, and kinetic constants (V<sub>max</sub> and K<sub>M</sub>) were calculated using the built-in nonlinear regression tools in SigmaPlot 14.0 (Figure S8).

#### 3.4.4. Stability Assays of BcPPK2-III

Thermostability and pH profile assays were performed with a final enzyme concentration of 10 µg/mL. The temperature gradient scale ranging from 30 to 70 °C was established as follows: 30.0, 31.6, 34.6, 39.5, 45.3, 49.6, 52.8, 55, 56.1, 57.9, 60.7, 64, 67, 68.9, and 70 °C. This gradient profile was achieved using a thermocycler (Bio-rad T100 Thermal Cycler). After 10 min of incubation, BcPPK2-III samples were removed, chilled on ice for 10 min and incubated further at room temperature for 10 min. Finally, samples of 20 µL were added to 180 µL volume of reaction mixture containing 100 mM Tris-HCl buffer, pH = 7.0, polyP (10 mM), AMP (2 mM) and MgSO<sub>4</sub> (10 mM).

The optimum pH activity was determined using 100mM Britton-Robinson buffer at different pH values (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0) containing the reaction mixture with the aforementioned concentrations of AMP, polyP and MgSO<sub>4</sub>.

#### 3.5. Scanning Transmission Electron Microscopy

Transmission electron microscopy images were recorded with a field emission scanning electron microscope (FESEM), Hitachi SU-8000, operated at 30 kV in transmitted electron imaging mode (sTEM) equipped with a charge-coupled device (CCD) camera. Briefly, for sample preparation, one drop of reaction solution in water (~5–10 µL) was left to dry over ultrathin carbon type-A film supported on a 400-mesh copper grid (3 mm in diameter) (from Ted Pella, Inc. Redding, Redding, CA, USA).

### 4. Conclusions

It was experimentally demonstrated that the enzyme encoded by the *ppk2-III* gene is a polyphosphate kinase of family 2, subclass III, which is capable of producing ATP from AMP. Sequence analysis confirmed the presence of conserved motifs, including the Walker A and B motifs, characteristic of PPK2 enzymes. Structural characterization through 3D modeling, molecular docking and peptide mass fingerprinting verified the identity and integrity of the recombinant protein.

The biochemical characterization of BcPPK2-III revealed that the enzyme is active with several divalent cations, exhibiting substantial activity with Mg<sup>2+</sup>, Mn<sup>2+</sup> and Co<sup>2+</sup> and a distinct ADP production preference with Ca<sup>2+</sup>. The enzyme achieved a 70% conversion of ATP from AMP after 24 h, with a total turnover number of 4355. The reaction mechanism was determined to proceed through a stepwise phosphorylation process, forming ADP as an intermediate before generating ATP. In addition, the enzyme could be producing AP<sub>4</sub> as a final product, when the first substrate of the reaction is ADP. According to the determined kinetic constants, the phosphorylation of AMP to ADP is the most efficient step.

Enzyme stability tests showed that BcPPK2-III retains full activity at temperatures between 30 and 58 °C, with a T<sub>50</sub> of 62 °C, indicating high thermostability. The enzyme also maintained more than 97% of its activity across a pH range of 6.0–9.0, with optimal activity at pH 9.0. These two properties are very valuable from a biocatalysis point of view, as in many processes—such as ATP regeneration—it is necessary to combine several enzymes with different activity optima or to work under very demanding conditions in order to adapt the process to an industrial scale.

These findings suggest that BcPPK2-III is a robust and versatile enzyme with significant potential for use in ATP regeneration, reducing costs and improving the efficiency of biocatalytic processes that require stoichiometric amounts of ATP. The enzyme's ability to function with multiple metal cofactors and its stability under various conditions make it a valuable candidate for industrial applications.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms252312995/s1>.

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## References

1. Hanson, R.W. The Role of ATP in Metabolism. *Biochem. Educ.* **1989**, *17*, 86–92. [CrossRef]
2. Richter, M. Functional Diversity of Organic Molecule Enzyme Cofactors. *Nat. Prod. Rep.* **2013**, *30*, 1324. [CrossRef] [PubMed]
3. Fischer, J.D.; Holliday, G.L.; Rahman, S.A.; Thornton, J.M. The Structures and Physicochemical Properties of Organic Cofactors in Biocatalysis. *J. Mol. Biol.* **2010**, *403*, 803–824. [CrossRef]
4. Wohlgemuth, R.; Liese, A.; Streit, W. Biocatalytic Phosphorylations of Metabolites: Past, Present, and Future. *Trends Biotechnol.* **2017**, *35*, 452–465. [CrossRef]
5. Mueller, J.W.; Shafqat, N. Adenosine-5'-Phosphosulfate—A Multifaceted Modulator of Bifunctional 3'-Phospho-Adenosine-5'-Phosphosulfate Synthases and Related Enzymes. *FEBS J.* **2013**, *280*, 3050–3057. [CrossRef]
6. Abu, R.; Woodley, J.M. Application of Enzyme Coupling Reactions to Shift Thermodynamically Limited Biocatalytic Reactions. *ChemCatChem* **2015**, *7*, 3094–3105. [CrossRef]
7. Fehlau, M.; Kaspar, F.; Hellendahl, K.F.; Schollmeyer, J.; Neubauer, P.; Wagner, A. Modular Enzymatic Cascade Synthesis of Nucleotides Using a (d)ATP Regeneration System. *Front. Bioeng. Biotechnol.* **2020**, *8*, 1–10. [CrossRef]
8. Iturrate, L.; García-Junceda, E. DHAP-Dependent Aldolases in the Core of Multistep Processes. In *Multi-Step Enzyme Catalysis: Biotransformations and Chemoenzymatic Synthesis*; García-Junceda, E., Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2008; pp. 61–81.
9. Andexer, J.N.; Richter, M. Emerging Enzymes for ATP Regeneration in Biocatalytic Processes. *ChemBioChem* **2015**, *16*, 380–386. [CrossRef] [PubMed]
10. Zhao, H.; van der Donk, W.A. Regeneration of Cofactors for Use in Biocatalysis. *Curr. Opin. Biotechnol.* **2003**, *14*, 583–589. [CrossRef]
11. Achbergerová, L.; Nahálka, J. Polyphosphate—An Ancient Energy Source and Active Metabolic Regulator. *Microb. Cell Fact.* **2011**, *10*, 63. [CrossRef]
12. Kornberg, A.; Rao, N.N.; Ault-Riché, D. Inorganic Polyphosphate: A Molecule of Many Functions. *Annu. Rev. Biochem.* **1999**, *68*, 89–125. [CrossRef]
13. Rao, N.N.; Gómez-García, M.R.; Kornberg, A. Inorganic Polyphosphate: Essential for Growth and Survival. *Annu. Rev. Biochem.* **2009**, *78*, 605–647. [CrossRef]
14. Bordes, I.; García-Junceda, E.; Sánchez-Moreno, I.; Castillo, R.; Moliner, V. Computational Study of the Phosphoryl Donor Activity of Dihydroxyacetone Kinase from ATP to Inorganic Polyphosphate. *Int. J. Quantum Chem.* **2018**, *118*, e25520. [CrossRef]
15. Iwamoto, S.; Motomura, K.; Shinoda, Y.; Urata, M.; Kato, J.; Takiguchi, N.; Ohtake, H.; Hirota, R.; Kuroda, A. Use of an Escherichia Coli Recombinant Producing Thermostable Polyphosphate Kinase as an ATP Regenerator to Produce Fructose 1,6-Diphosphate. *Appl. Environ. Microbiol.* **2007**, *73*, 5676–5678. [CrossRef]
16. Sánchez-Moreno, I.; Bordes, I.; Castillo, R.; Ruiz-Pernía, J.; Moliner, V.; García-Junceda, E. Tuning the Phosphoryl Donor Specificity of Dihydroxyacetone Kinase from ATP to Inorganic Polyphosphate. An Insight from Computational Studies. *Int. J. Mol. Sci.* **2015**, *16*, 27835–27849. [CrossRef]
17. Tavanti, M.; Hosford, J.; Lloyd, R.C.; Brown, M.J.B. ATP Regeneration by a Single Polyphosphate Kinase Powers Multigram-Scale Aldehyde Synthesis in Vitro. *Green Chem.* **2021**, *23*, 828–837. [CrossRef]
18. Mordhorst, S.; Maurer, A.; Popadić, D.; Brech, J.; Andexer, J.N. A Flexible Polyphosphate-Driven Regeneration System for Coenzyme A Dependent Catalysis. *ChemCatChem* **2017**, *9*, 4164–4168. [CrossRef]
19. Xu, R.; Wang, Y.; Huang, H.; Jin, X.; Li, J.; Du, G.; Kang, Z. Closed-Loop System Driven by ADP Phosphorylation from Pyrophosphate Affords Equimolar Transformation of ATP to 3'-Phosphoadenosine-5'-Phosphosulfate. *ACS Catal.* **2021**, *11*, 10405–10415. [CrossRef]

20. Monterrey, D.T.; Benito-Arenas, R.; Revuelta, J.; García-Junceda, E. Design of a Biocatalytic Cascade for the Enzymatic Sulfation of Unsulfated Chondroitin with in Situ Generation of PAPS. *Front. Bioeng. Biotechnol.* **2023**, *11*, 1–10. [CrossRef] [PubMed]
21. Datta, P.; Fu, L.; He, W.; Koffas, M.A.G.; Dordick, J.S.; Linhardt, R.J. Expression of Enzymes for 3'-Phosphoadenosine-5'-Phosphosulfate (PAPS) Biosynthesis and Their Preparation for PAPS Synthesis and Regeneration. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 7067–7078. [CrossRef] [PubMed]
22. Mordhorst, S.; Singh, J.; Mohr, M.K.F.; Hinkelmann, R.; Keppler, M.; Jessen, H.J.; Andexer, J.N. Several Polyphosphate Kinase 2 Enzymes Catalyse the Production of Adenosine 5'-Polyphosphates. *ChemBioChem* **2019**, *20*, 1019–1022. [CrossRef]
23. Ogawa, M.; Uyeda, A.; Harada, K.; Sato, Y.; Kato, Y.; Watanabe, H.; Honda, K.; Matsuura, T. Class III Polyphosphate Kinase 2 Enzymes Catalyze the Pyrophosphorylation of Adenosine-5'-Monophosphate. *ChemBioChem* **2019**, *20*, 2961–2967. [CrossRef]
24. Motomura, K.; Hirota, R.; Okada, M.; Ikeda, T.; Ishida, T.; Kuroda, A. A New Subfamily of Polyphosphate Kinase 2 (Class III PPK2) Catalyzes Both Nucleoside Monophosphate Phosphorylation and Nucleoside Diphosphate Phosphorylation. *Appl. Environ. Microbiol.* **2014**, *80*, 2602–2608. [CrossRef] [PubMed]
25. Moriarty, T.F.; Mullan, A.; McGrath, J.W.; Quinn, J.P.; Elborn, J.S.; Tunney, M.M. Effect of Reduced PH on Inorganic Polyphosphate Accumulation by Burkholderia Cepacia Complex Isolates. *Lett. Appl. Microbiol.* **2006**, *42*, 617–623. [CrossRef]
26. Leipe, D.D.; Koonin, E.V.; Aravind, L. Evolution and Classification of P-Loop Kinases and Related Proteins. *J. Mol. Biol.* **2003**, *333*, 781–815. [CrossRef]
27. Nocek, B.; Kochinyan, S.; Proudfoot, M.; Brown, G.; Evdokimova, E.; Osipiuk, J.; Edwards, A.M.; Savchenko, A.; Joachimiak, A.; Yakunin, A.F. Polyphosphate-Dependent Synthesis of ATP and ADP by the Family-2 Polyphosphate Kinases in Bacteria. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 17730–17735. [CrossRef]
28. Altschul, S.; Madden, T.L.; Schäffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402. [CrossRef]
29. Altschul, S.F.; Wootton, J.C.; Gertz, E.M.; Agarwala, R.; Morgulis, A.; Schäffer, A.A.; Yu, Y. Protein Database Searches Using Compositionally Adjusted Substitution Matrices. *FEBS J.* **2005**, *272*, 5101–5109. [CrossRef] [PubMed]
30. Nocek, B.P.; Khusnutdinova, A.N.; Ruzskowski, M.; Flick, R.; Burda, M.; Batyrova, K.; Brown, G.; Mucha, A.; Joachimiak, A.; Berlicki, L.; et al. Structural Insights into Substrate Selectivity and Activity of Bacterial Polyphosphate Kinases. *ACS Catal.* **2018**, *8*, 10746–10760. [CrossRef]
31. Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E.L. Predicting Transmembrane Protein Topology with a Hidden Markov Model: Application to Complete Genomes. *J. Mol. Biol.* **2001**, *305*, 567–580. [CrossRef]
32. Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Židek, A.; Potapenko, A.; et al. Highly Accurate Protein Structure Prediction with AlphaFold. *Nature* **2021**, *596*, 583–589. [CrossRef] [PubMed]
33. Mirdita, M.; Schütze, K.; Moriwaki, Y.; Heo, L.; Ovchinnikov, S.; Steinegger, M. ColabFold: Making Protein Folding Accessible to All. *Nat. Methods* **2022**, *19*, 679–682. [CrossRef] [PubMed]
34. Parnell, A.E.; Mordhorst, S.; Kemper, F.; Giurrandino, M.; Prince, J.P.; Schwarzer, N.J.; Hofer, A.; Wohlwend, D.; Jessen, H.J.; Gerhardt, S.; et al. Substrate Recognition and Mechanism Revealed by Ligand-Bound Polyphosphate Kinase 2 Structures. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 3350–3355. [CrossRef] [PubMed]
35. Lee, S.J.; Jang, H.; Lee, D.N. Recent Advances in Nanoflowers: Compositional and Structural Diversification for Potential Applications. *Nanoscale Adv.* **2023**, *5*, 5165–5213. [CrossRef]
36. Zhang, X.; Cui, X.; Li, Z. Characterization of Two Polyphosphate Kinase 2 Enzymes Used for ATP Synthesis. *Appl. Biochem. Biotechnol.* **2020**, *191*, 881–892. [CrossRef]
37. Frank, C.; Teleki, A.; Jendrossek, D. Characterization of Agrobacterium Tumefaciens PPKs Reveals the Formation of Oligophosphorylated Products up to Nucleoside Nona-Phosphates. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 9683–9692. [CrossRef] [PubMed]
38. Batten, L.E.; Parnell, A.E.; Wells, N.J.; Murch, A.L.; Oyston, P.C.F.; Roach, P.L. Biochemical and Structural Characterization of Polyphosphate Kinase 2 from the Intracellular Pathogen Francisella Tularensis. *Biosci. Rep.* **2016**, *36*. [CrossRef]
39. Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbour: New York, NY, USA, 1989; ISBN 0-87969-309-6.
40. Buchan, D.W.A.; Jones, D.T. The PSIPRED Protein Analysis Workbench: 20 Years On. *Nucleic Acids Res.* **2019**, *47*, W402–W407. [CrossRef]
41. Bittrich, S.; Segura, J.; Duarte, J.M.; Burley, S.K.; Rose, Y. RCSB Protein Data Bank: Exploring Protein 3D Similarities via Comprehensive Structural Alignments. *Bioinformatics* **2024**, *40*, btac370. [CrossRef]

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Article

# Optimization and Bioreactor Scale-Up of Cellulase Production in *Trichoderma* sp. KMF006 for Higher Yield and Performance

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**Abstract:** This study optimized operating parameters to enhance cellulase production and evaluated scale-up feasibility in submerged fermentation (SmF) using *Trichoderma* sp. KMF006. Flask-scale experiments assessed the effects of Avicel:cellulose ratios (4:0–0:4), agitation speeds (150–210 rpm), and turbulence (baffled vs. non-baffled flasks), with optimized conditions applied to a 10 L bioreactor. A 3:1 Avicel:cellulose ratio (A3C1) significantly accelerated cellulase production, reaching peak activity 6 days earlier than Avicel alone. An agitation speed of 180 rpm was optimal, balancing enzyme activity and energy efficiency. Turbulence enhanced cellulase yields, with baffled flasks increasing EG, BGL, and CBH activities 19.9-, 6.2-, and 8.9-fold, respectively, compared to the control. Biochar further improved cellulase production but only under turbulent conditions, demonstrating a synergistic effect. At the bioreactor scale, the A3-180\_Imp (A3C1, 180 rpm, impeller-induced turbulence) achieved the highest enzymatic activity (33.60 U/mL EG, 3.46 U/mL BGL, and 0.63 U/mL CBH). The filter paper unit (FPU) was 84 FPU/mL, a two-fold increase compared to the control. However, excessive turbulence at 210 rpm reduced enzyme stability, emphasizing the importance of balancing shear stress. These findings provide a systematic framework for optimizing SmF conditions, highlighting the significance of balancing hydrodynamic conditions for efficient cellulase production at an industrial scale.

**Keywords:** cellulase production; submerged fermentation (SmF); carbon source composition; hydrodynamic conditions; bioreactor scale-up

## 1. Introduction

Over the past few centuries, the global population has grown rapidly, leading to a significant increase in the use of petrochemical products such as plastics and fossil fuels. This excessive consumption has caused climate change and environmental pollution to become critical international issues [1,2]. In response, lignocellulosic biomass (LCB) has garnered attention as a promising alternative resource [3]. LCB, one of the most abundant and sustainable resources available globally, is categorized into three generations: first generation (agricultural crops), second generation (forestry and agricultural residues), and third generation (microalgae and bacteria) [4,5]. The use of LCB in biorefinery processes to produce bioproducts as substitutes for fossil-based resources has been actively studied [6,7]. Prominent bioproducts include biofuels and bioplastics, both of which are projected to experience steady market growth [8,9]. Germany, France, the United States, and 30 other countries have adopted the Renewable Fuel Standard (RFS) as defined by the REN21 report, blending bioethanol and biodiesel into gasoline and diesel, respectively, for transportation fuel [10]. Additionally, the European Union introduced the Single-Use Plastic Directive

(EU 2019/904) as part of its Circular Economy Action Plan, mandating the use of recycled materials and promoting bioplastics [11].

LCB consists of cellulose, hemicellulose, and lignin, which are tightly bound in a rigid structure [12]. This composition makes pretreatment and saccharification essential steps for the production of bioproducts [13,14]. Saccharification is typically performed using either acid hydrolysis or cellulase enzymes. While acid hydrolysis is fast and can produce high concentrations of sugars, it requires harsh temperature conditions (200–240 °C) and generates inhibitory byproducts such as furfural, hydroxymethylfurfural (HMF), vanillin, and formic acid. In contrast, enzymatic saccharification using cellulase is gaining attention as an environmentally friendly alternative [15,16]. Cellulase is a complex enzyme system composed of endoglucanase (EG, EC 3.2.1.4),  $\beta$ -glucosidase (BGL, EC 3.2.1.21), and cellobiohydrolase (CBH, EC 3.2.1.91) [17]. These enzymes are secreted by microorganisms, with fungi such as *Trichoderma* sp., *Aspergillus* sp., and *Penicillium* sp. being the primary sources for industrial production [17]. However, the high cost of cellulase production remains a significant barrier to the practical production of biofuels, highlighting the need for cost-effective production processes [9,13,18].

For efficient cellulase production, two fermentation processes are commonly employed: solid-state fermentation (SSF) and submerged fermentation (SmF) [19,20]. SSF allows for high enzyme yields as it provides environmental conditions similar to those in nature, promoting microbial growth [21]. Although SmF generally results in lower enzyme activity than SSF, particularly with lignocellulosic substrates, it remains the preferred method for industrial application due to its ease of enzyme recovery, process control, and scalability [17]. To improve cellulase productivity in SmF, previous studies have focused on optimizing parameters such as nutrient composition, pH, temperature, incubation period, and aeration [22–25]. Moreover, innovative strategies, including the integration of SSF and SmF [26,27] or the use of activity-enhancing supplements [28,29], have been explored to overcome the limitations of conventional SmF systems.

This study was built upon prior research that demonstrated the potential of cellulase production by incorporating biochar during *Trichoderma* sp. cultivation in SmF [30]. Biochar, a high-carbon material with high porosity and surface area, is known to enhance microbial growth and cellulase activity by serving as a microbial habitat [31–36]. While core cultivation parameters such as temperature and pH were previously optimized for this strain [37], the present study focused on evaluating physical and structural factors including carbon source composition, agitation speed, and turbulence efficiency, as these directly affect oxygen transfer and substrate accessibility in SmF systems. Flask-scale experiments were conducted to evaluate the effects of three factors—carbon source, agitation speed, and turbulence efficiency—on cellulase production. Protein concentration and cellulase activity were measured, and the most effective conditions were identified using ANOVA-based statistical comparisons. These conditions were then applied to a bioreactor to assess the potential for scale-up, with final enzymatic activity evaluated using the Filter Paper Unit (FPU) assay.

## 2. Results and Discussion

### 2.1. Effect of Carbon Source Composition on Cellulase Activity

The time profiles of cellulase activity under different carbon source compositions are presented in Figure S1, while the maximum enzyme activities are summarized in Table 1. EG activity did not exhibit a consistent trend across different carbon compositions. In the control condition (A4C0), EG activity reached its maximum activity at 18 days (24.19 U/mL), whereas the experimental groups exhibited faster enzyme production, with the maximum activities observed between 12 and 15 days. Among these, A3C1 exhibited the highest EG

activity, reaching 27.84 U/mL at 12 days. BGL and CBH exhibited more consistent patterns regardless of carbon composition. BGL activity reached its peak between 15 and 18 days, while CBH reached its maximum value between 12 and 15 days. Although no statistically significant differences were observed ( $p > 0.05$ ), A3C1 exhibited the highest activity across all three enzymes, with the maximum values of 27.84 U/mL (EG), 0.87 U/mL (BGL), and 0.25 U/mL (CBH). Protein concentration followed a similar trend, peaking between 12 and 15 days within the range of 0.58 to 0.73 mg/mL, with no significant differences among experimental groups ( $p > 0.05$ ).

**Table 1.** The maximum enzymatic activities and protein concentration under different flask-level experimental conditions.

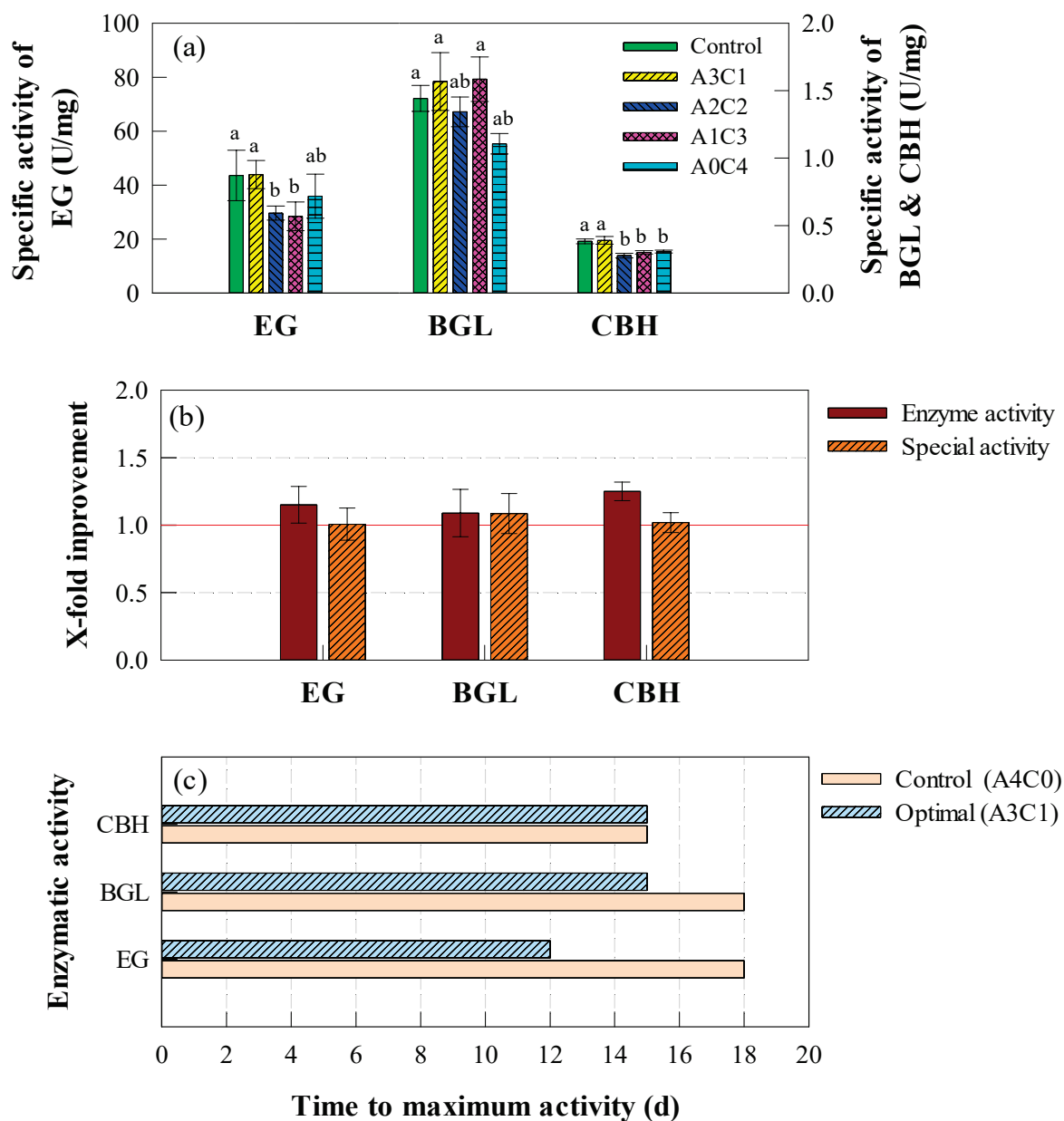
| C Source Comp. <sup>(1)</sup>   | EG (U/mL)                  | BGL (U/mL)                | CBH (U/mL)               | Protein (mg/mL)          |
|---------------------------------|----------------------------|---------------------------|--------------------------|--------------------------|
| Control (A4C0)                  | 24.19 ± 5.18 <sup>ab</sup> | 0.80 ± 0.05 <sup>ab</sup> | 0.20 ± 0.01 <sup>b</sup> | 0.58 ± 0.09 <sup>a</sup> |
| A3C1                            | 27.84 ± 3.29 <sup>ab</sup> | 0.87 ± 0.14 <sup>a</sup>  | 0.25 ± 0.01 <sup>a</sup> | 0.68 ± 0.19 <sup>a</sup> |
| A2C2                            | 17.93 ± 5.55 <sup>b</sup>  | 0.72 ± 0.05 <sup>ab</sup> | 0.18 ± 0.03 <sup>b</sup> | 0.73 ± 0.06 <sup>a</sup> |
| A1C3                            | 16.75 ± 3.13 <sup>b</sup>  | 0.70 ± 0.07 <sup>b</sup>  | 0.17 ± 0.02 <sup>b</sup> | 0.60 ± 0.07 <sup>a</sup> |
| A0C4                            | 21.38 ± 4.88 <sup>ab</sup> | 0.66 ± 0.05 <sup>b</sup>  | 0.20 ± 0.01 <sup>b</sup> | 0.66 ± 0.06 <sup>a</sup> |
| Agitation speed <sup>(2)</sup>  | EG (U/mL)                  | BGL (U/mL)                | CBH (U/mL)               | Protein (mg/mL)          |
| 150 rpm                         | 16.90 ± 2.12 <sup>b</sup>  | 1.77 ± 0.42 <sup>b</sup>  | 0.33 ± 0.06 <sup>b</sup> | 0.71 ± 0.08 <sup>b</sup> |
| 180 rpm                         | 32.04 ± 3.82 <sup>a</sup>  | 3.60 ± 0.69 <sup>a</sup>  | 0.53 ± 0.10 <sup>a</sup> | 1.05 ± 0.07 <sup>a</sup> |
| 210 rpm                         | 32.35 ± 4.57 <sup>a</sup>  | 3.83 ± 0.19 <sup>a</sup>  | 0.59 ± 0.06 <sup>a</sup> | 1.10 ± 0.05 <sup>a</sup> |
| Turbulence effi. <sup>(3)</sup> | EG (U/mL)                  | BGL (U/mL)                | CBH (U/mL)               | Protein (mg/mL)          |
| Baf-w/o BC                      | 27.59 ± 3.03 <sup>b</sup>  | 1.15 ± 0.07 <sup>b</sup>  | 0.30 ± 0.01 <sup>b</sup> | 0.69 ± 0.04 <sup>b</sup> |
| Baf-1% BC                       | 34.41 ± 3.02 <sup>a</sup>  | 1.46 ± 0.15 <sup>a</sup>  | 0.33 ± 0.02 <sup>a</sup> | 0.97 ± 0.03 <sup>a</sup> |
| Rou-w/o BC                      | 10.18 ± 1.14 <sup>c</sup>  | 0.63 ± 0.09 <sup>c</sup>  | 0.14 ± 0.02 <sup>c</sup> | 0.43 ± 0.05 <sup>c</sup> |
| Rou-1% BC                       | 1.73 ± 1.05 <sup>d</sup>   | 0.24 ± 0.02 <sup>d</sup>  | 0.04 ± 0.00 <sup>d</sup> | 0.42 ± 0.07 <sup>c</sup> |

<sup>(1)</sup> C source composition: Avicel (A) and cellulose (C) were mixed at different ratios ( $w/v$ ), where the number represents the respective proportions. <sup>(2)</sup> Agitation speed: Fixed carbon source ratio (A3C1) was used at agitation speeds of 150, 180, and 210 rpm. <sup>(3)</sup> Turbulence efficiency: Fixed carbon source ratio (A3C1) at 180 rpm was tested in baffled and round flasks, with and without biochar (1%,  $w/v$ ). Significant differences among the experimental conditions are represented by different letters ( $p < 0.05$ ). All data are expressed as mean ± standard deviation (SD) from six measurements ( $n = 6$ ).

To further evaluate enzyme production efficiency, the maximum specific activities of EG, BGL, and CBH were analyzed (Figure 1a). Specific activity is defined as enzymatic activity normalized to protein concentration (U/mg), which allows for an evaluation of enzymatic efficiency regardless of total protein content [30]. The results showed that EG exhibited the highest specific activity in both A3C1 (43.94 U/mg) and the control (43.62 U/mg), with no significant difference between them ( $p > 0.05$ ). The specific activities of BGL in A3C1 (1.57 U/mg) and A1C3 (1.59 U/mg) were slightly higher than those in other groups, though the differences were not significant ( $p > 0.05$ ). In contrast, CBH activity was significantly higher in A3C1 (0.39 U/mg) and the control (0.38 U/mg) than in other groups ( $p < 0.05$ ).

To assess the relative improvement in cellulase activity under A3C1 compared to the control, Figure 1b presents the fold increase in enzymatic activity and specific activity. Enzymatic activity in A3C1 was 1.09–1.25 times higher than in the control, while specific activity showed a 1.01–1.09-fold increase. Although these differences were relatively minor, the time required to reach the maximum activity was significantly shorter in A3C1 than in the control (Figure 1c). EG activity peaked at 12 days in A3C1, compared to 18 days in

the control, while BGL activity peaked at 15 days in A3C1 and 18 days in the control. CBH showed no difference in peak timing between the two conditions.



**Figure 1.** Comparison of enzymatic activities and performance under different carbon source compositions in flask-scale experiments. **(a)** Maximum specific activities (U/mg) of EG, BGL, and CBH across all experimental conditions. **(b)** X-fold improvement in enzymatic activities of cellulase under optimal (A3C1) compared to control (A4C0). X-fold values were calculated separately for enzyme activity (EA, U/mL) and specific activity (SA, U/mL). **(c)** Time to maximum enzymatic activities (days) of cellulase under control (A4C0) and optimal (A3C1) conditions. Error bars represent standard deviation (SD) from six measurements ( $n = 6$ ). Statistical significance was determined by one-way ANOVA followed by Tukey's HSD test ( $p < 0.05$ ).

These results suggest that, while the overall improvement in enzymatic activity was moderate, the A3C1 condition significantly reduced the cultivation time required to achieve the maximum cellulase activity. Optimizing the carbon source ratio to Avicel:cellulose = 3:1 (A3C1) was effective in accelerating enzyme production, making it the most suitable composition for cellulase production.

Avicel is widely recognized as a primary carbon source for inducing cellulase production in *Trichoderma* species [38]. As a form of microcrystalline cellulose, Avicel is relatively more hydrolysable than other insoluble cellulose substrates [38]. Several studies have demonstrated its effectiveness in cellulase production across various fungal species and fermentation systems. For instance, Silva et al. (2020) compared the cellulase activity of *Trichoderma reesei* RP698 using 12 different carbon sources, including Avicel, filter paper, and carboxymethyl cellulose (CMC), with Avicel resulting in the highest cellulase activity [39]. SmF using Avicel as the primary carbon source has successfully supported cellulase production in *Orpinomyces* sp. [40]. Similarly, *Daldinia* sp. cultivated in SmF with Avicel as the carbon source exhibited significant cellulase activity [41]. In contrast, the present study demonstrates that a mixed carbon source composed of Avicel and cellulose at a 3:1 ratio (A3C1) significantly shortened the time required to reach the maximum cellulase activity *Trichoderma* sp. KMF006, compared to Avicel alone (A4C0). This suggests that mixed substrates may facilitate a more rapid onset of enzymatic activity by influencing the induction process for cellulase production. While studies on mixed carbon sources remain limited, similar effects have been observed in previous research. Singhanian et al. (2015) reported that combining wheat bran and Avicel in a 4:1 ratio enhanced cellulase production [42]. Similarly, Saini et al. (2015) demonstrated through response surface methodology (RSM) that a mixture of Avicel (0.5%, *w/v*), wheat bran (2.5%, *w/v*), and ammonium sulfate (0.53%, *w/v*) yielded an estimated maximum cellulase activity of 1.26 FPU/mL [43]. These findings collectively indicate that optimizing carbon source composition can be a useful strategy for improving cellulase production efficiency.

Cellulases produced by *Trichoderma* species are classified as adaptive enzymes, the expression of which is significantly induced in the presence of specific inducers [44,45]. Previous studies have suggested that insoluble cellulose alone does not effectively induce early-stage cellulase production. Instead, intermediate metabolites, particularly cello-oligosaccharides such as cellobiose, play a crucial role in triggering cellulase synthesis [46,47]. Notably, BGL functions not only as a hydrolytic enzyme but also as a transglycosylation catalyst, synthesizing  $\beta$ -disaccharides that serve as potent inducers of cellulase biosynthesis [44,46]. Consistent with this finding, Li et al. (2016) demonstrated that cellulase activity in *T. reesei* was significantly enhanced in the presence of  $\beta$ -disaccharides [45]. In this study, the use of a mixed carbon source (A3C1) shortened the time required to reach maximum enzymatic activity. EG achieved its highest activity 6 days earlier, and BGL reached its maximum activity 4 days earlier compared to the Avicel-only condition (A4C0). The earlier activation of EG likely facilitated the production of more cello-oligosaccharides, such as cellobiose, which could have contributed to the accelerated activation of BGL. Previous studies have reported that BGL production in *T. reesei* not only hydrolyzes cello-oligosaccharides but also enhances induction signaling through transglycosylation [46,47]. However, since  $\beta$ -disaccharides formation was not directly quantified in this study, further investigation is required to validate this hypothesis.

In conclusion, the mixed carbon source (A3C1) improved cellulase production efficiency by reducing the time required for enzyme activation in *Trichoderma* sp. KMF006 compared to Avicel alone. This reduction in activation time has potential industrial relevance, as it may enhance process efficiency in cellulase production. Since  $\beta$ -disaccharide formation was not directly assessed, further research is needed to quantitatively determine the role of mixed carbon sources in the early induction of cellulase biosynthesis. Therefore, future studies should explore the mechanistic relationship between early-stage induction signals,  $\beta$ -disaccharide accumulation, and the accelerated activation of cellulase enzymes.

## 2.2. Effect of Agitation Speed on Cellulase Activity

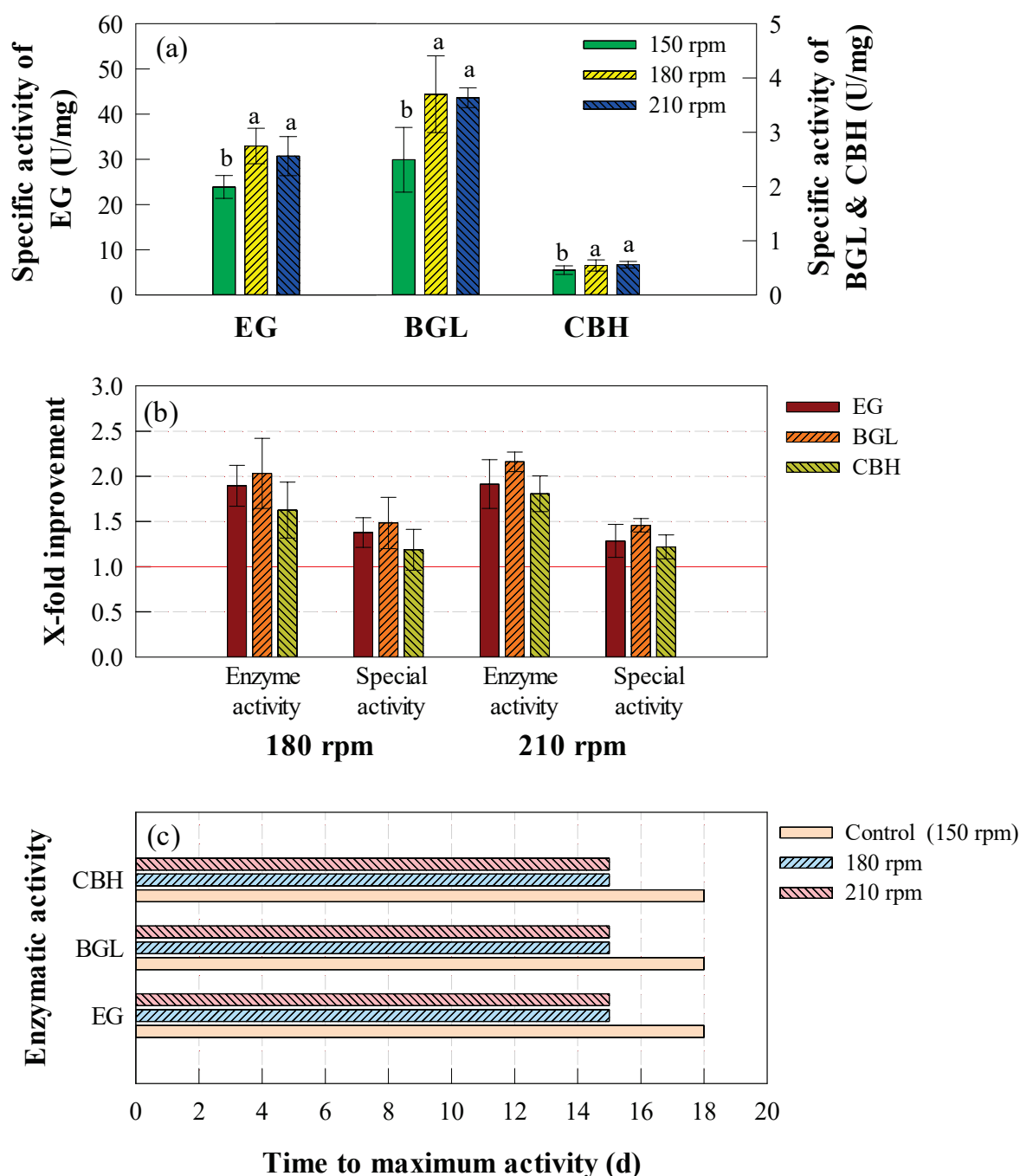
The influence of agitation speed on cellulase activity was examined by comparing enzyme production at 150 rpm (control), 180 rpm, and 210 rpm (Figure S2). The maximum enzyme activities under these conditions are summarized in Table 2. Overall, increasing agitation speed significantly enhanced both enzymatic activity and protein concentration, with 180 and 210 rpm yielding higher values than the control ( $p < 0.05$ ). The maximum activities of EG, BGL, and CBH were recorded at day 15 for 180 and 210 rpm, reaching 32.04–32.35 U/mL, 3.60–3.83 U/mL, and 0.53–0.59 U/mL, respectively. In contrast, the control exhibited significantly lower maximum activities, with EG, BGL, and CBH peaking at 16.90, 1.77, and 0.33 U/mL at day 18. A similar trend was observed for protein concentration, where all conditions exhibited maximum levels at day 18. However, the control resulted in a maximum protein concentration of only 0.71 mg/mL, which was significantly lower than the 1.05–1.10 mg/mL observed at 180 and 210 rpm ( $p < 0.05$ ). These results suggest that increasing agitation speed not only enhances enzymatic activity but also improves overall protein production efficiency.

**Table 2.** Experimental conditions for flask- and reactor-level experiments.

| Scale   | Operational parameter                | Experiment       | Experimental Condition                                                     |
|---------|--------------------------------------|------------------|----------------------------------------------------------------------------|
| Flask   | C source composition <sup>(1)</sup>  | A4C0 (Control)   | Avicel:Cellulose = 4:0 ( <i>w/v</i> ), 150 rpm                             |
|         |                                      | A3C1             | Avicel:Cellulose = 3:1 ( <i>w/v</i> ), 150 rpm                             |
|         |                                      | A2C2             | Avicel:Cellulose = 2:2 ( <i>w/v</i> ), 150 rpm                             |
|         |                                      | A1C3             | Avicel:Cellulose = 1:3 ( <i>w/v</i> ), 150 rpm                             |
|         |                                      | A0C4             | Avicel:Cellulose = 0:4 ( <i>w/v</i> ), 150 rpm                             |
|         | Agitation speed <sup>(2)</sup>       | 150 rpm          | Fixed C source ratio (A3C1), 150 rpm                                       |
|         |                                      | 180 rpm          | Fixed C source ratio (A3C1), 180 rpm                                       |
|         |                                      | 210 rpm          | Fixed C source ratio (A3C1), 210 rpm                                       |
|         | Turbulence efficiency <sup>(3)</sup> | Baf-w/o BC       | Fixed C source ratio (A3C1), 180 rpm, baffled flask, no biochar            |
|         |                                      | Baf-1% BC        | Fixed C source ratio (A3C1), 180 rpm, baffled flask, 1% biochar            |
|         |                                      | Rou-w/o BC       | Fixed C source ratio (A3C1), 180 rpm, round flask, no biochar              |
|         |                                      | Rou-1% BC        | Fixed C source ratio (A3C1), 180 rpm, round flask, 1% biochar              |
| Reactor |                                      | C0-150 (Control) | Avicel:Cellulose = 4:0 ( <i>w/v</i> , control), 150 rpm, no turbulence     |
|         |                                      | A3-180           | Fixed C source ratio (A3C1), 180 rpm, no turbulence                        |
|         |                                      | A3-180_Imp       | Fixed C source ratio (A3C1), 180 rpm, impeller adjusted (turbulence added) |
|         |                                      | A3-210_Imp       | Fixed C source ratio (A3C1), 210 rpm, impeller adjusted (turbulence added) |

<sup>(1)</sup> C source composition experiments were conducted at a fixed agitation speed of 150 rpm, with a total carbon concentration of 2% (*w/v*) and 1% (*w/v*) biochar. <sup>(2)</sup> Agitation speed experiments varied the shaking speed, while carbon source (A3C1), biochar, and other parameters were fixed. <sup>(3)</sup> Turbulence efficiency experiments were conducted using baffled and round-bottom flasks, Carbon source ration (A3C1) and agitation speed (180 rpm) were fixed. Conditions included with or without 1% (*w/v*) biochar.

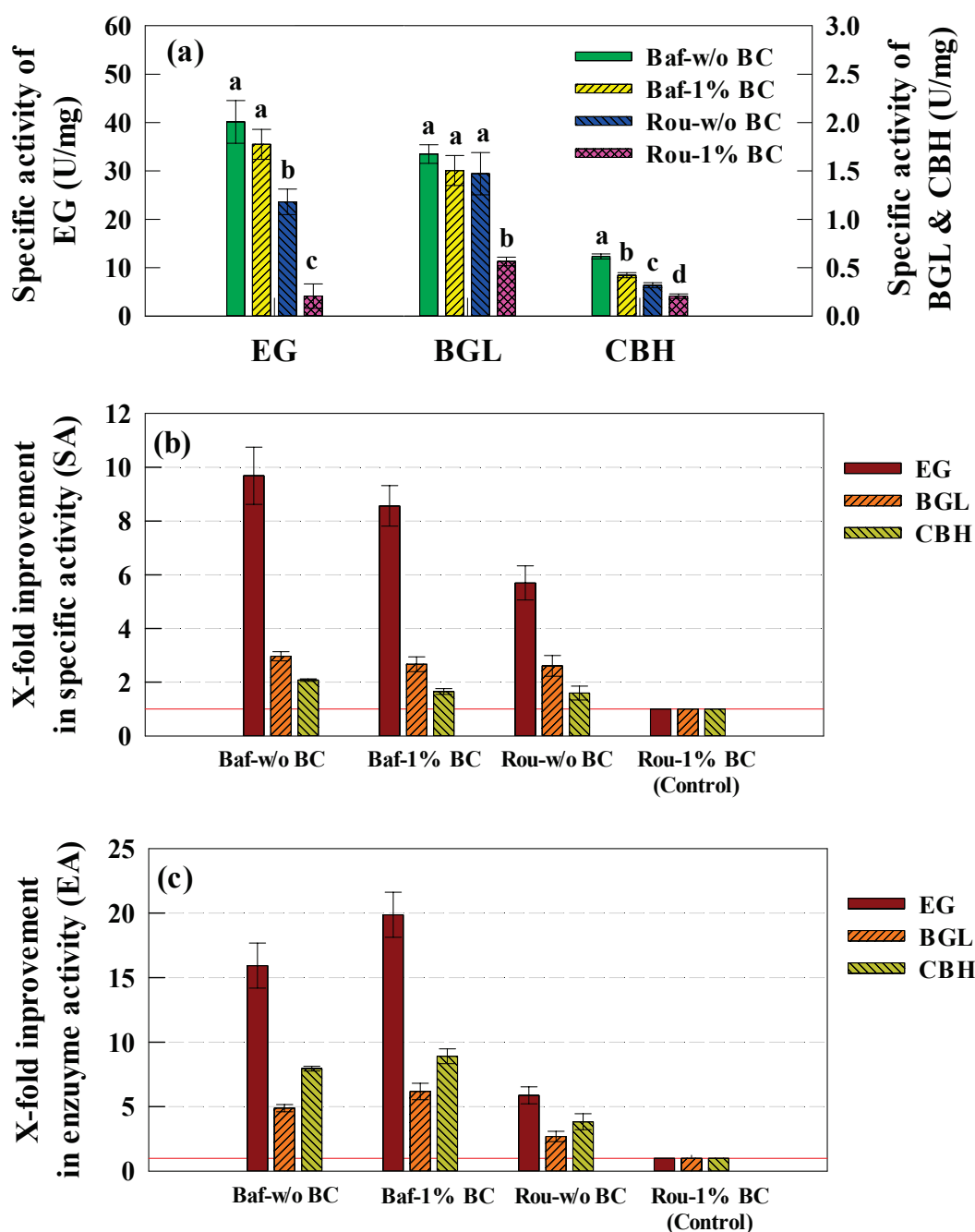
To further evaluate the impact of agitation speed, specific enzymatic activity was analyzed (Figure 2a). Both 180 and 210 rpm exhibited significantly higher specific activities compared to the control ( $p < 0.05$ ). The specific activities of EG, BGL, and CBH in the control were 23.89, 2.49, and 0.46 U/mg, respectively, whereas 180 and 210 rpm resulted in values ranging from 30.73 to 32.95 U/mg (EG), 3.64 to 3.70 U/mg (BGL), and 0.54 to 0.56 U/mg (CBH). Notably, there was no significant difference between 180 and 210 rpm ( $p > 0.05$ ), indicating that further increasing agitation speed beyond 180 rpm does not provide additional enzymatic efficiency benefits. This may suggest that the physiological limits of the strain were reached at 180 rpm, beyond which additional agitation did not further stimulate enzyme synthesis [48].



**Figure 2.** Comparison of enzymatic activities and performance under different agitation speed in flask-scale experiments. (a) Maximum specific activities (U/mg) of EG, BGL, and CBH across all experimental conditions. (b) X-fold improvement in enzymatic activities (EA, U/mL) and specific activities (SA, U/mg) of cellulase at 180 and 210 rpm compared to control (150 rpm). (c) Time to maximum enzymatic activities (days) of cellulase under control (150 rpm) and experimental conditions (180 and 210 rpm). Error bars represent standard deviation (SD) from six measurements ( $n = 6$ ). Statistical significance was determined by one-way ANOVA followed by Tukey's HSD test ( $p < 0.05$ ).

The relative enhancement of cellulase activity at higher agitation speeds compared to the control is illustrated in Figure 2b. At 180 and 210 rpm, the maximum activity increased by 1.90–1.91-fold for EG, 2.03–2.16-fold for BGL, and 1.63–1.81-fold for CBH compared to the control. A similar pattern was observed in specific activity, where EG, BGL, and CBH exhibited 1.29–1.38-, 1.46–1.49-, and 1.19–1.22-fold improvements, respectively. In addition

to enhanced activity, higher agitation speeds also accelerated enzyme production as shown in Figure 3b. While the control required 18 days to reach peak cellulase activity, cultures at 180 and 210 rpm reached their maximum enzymatic activity on day 15, demonstrating the role of agitation in expediting enzyme biosynthesis.



**Figure 3.** Comparison of enzymatic activities and x-fold improvements under different turbulence conditions in flask-scale experiments. (a) Maximum specific activities (U/mg) of EG, BGL, and CBH across four experimental conditions. (b) X-fold improvement in specific activities (SA, U/mL) of cellulase under turbulence conditions relative to Rou-1% BC as the control. (c) X-fold improvement in enzymatic activities (EA, U/mL) of cellulase under turbulence conditions relative to Rou-1% BC as the control. Error bars represent standard deviation (SD) from six measurements ( $n = 6$ ). Statistical significance was determined by one-way ANOVA followed by Tukey's HSD test ( $p < 0.05$ ).

Although no statistically significant difference was observed between 180 and 210 rpm, increasing agitation speed above 180 rpm did not result in additional enzymatic improve-

ments. In addition, considering the potential increase in energy consumption at higher agitation speeds, 180 rpm is suggested as the optimal condition for cellulase production.

Agitation speed is a key parameter influencing cellulase production by regulating oxygen transfer, shear stress, and fungal morphology. Increased agitation enhances turbulent flow, thereby improving oxygen transfer efficiency, which is crucial for microbial metabolism and enzyme secretion [48]. However, excessive shear stress at high agitation speeds may cause mechanical damage to fungal hyphae, whereas insufficient agitation can lead to pellet formation, restricting oxygen and nutrient diffusion [17,48]. Several studies have evaluated the effect of agitation speed on cellulase production. Sirohi et al. (2019) evaluated the optimal agitation speed for cellulase production in *Trichoderma* sp. using response surface methodology (RSM) [49]. Pea hulls were used as the substrate and the cultivation temperature was set at 30 °C. Agitation speed was tested within a range of 93 to 127 rpm, and RSM analysis identified 120 rpm as the optimal condition. In another study using *Pycnoporus* sp., cellulase production was assessed under varying agitation speeds [50]. The strain was cultivated at 30 °C using palm oil mill effluent and oil palm frond as substrates. The results indicated that the highest cellulase activity was achieved at 350 rpm among tested speeds ranging from 100 to 500 rpm. In bacterial systems, agitation speed also plays a crucial role. A study on *Micrococcus* sp. investigated the effect of agitation speed on cellulase production using carboxymethyl cellulose (CMC) as the substrate. The strain was incubated at 25 °C, and experimental results showed that maximum cellulase production occurred at 50 rpm when tested across a range of 0 to 200 rpm [51]. Direct comparisons among these studies are challenging due to differences in microbial strains, cultivation conditions, and experimental design. Nevertheless, these findings collectively highlight the critical role of agitation speed in optimizing cellulase production and emphasize the need for strain-specific optimization to achieve maximal enzyme yields.

### 2.3. Effect of Turbulence on Cellulase Activity

The impact of turbulence on cellulase activity was assessed by comparing baffled and non-baffled conditions with or without 1% biochar supplementation. The time profiles of enzymatic activity under these conditions are presented in Figure S3, and the maximum enzyme activities are summarized in Table 2. Overall, the presence of turbulence significantly enhanced cellulase activity, while biochar alone did not consistently improve enzyme production unless turbulence was present. The maximum enzymatic activity values confirm this trend (Table 2), with Baf-1% BC exhibiting the highest enzyme production, reaching 34.41 U/mL (EG), 1.46 U/mL (BGL), and 0.33 U/mL (CBH) at the peak point. The Baf-w/o BC condition also demonstrated increased activity, with values of 27.59, 1.15, and 0.30 U/mL, respectively. In contrast, non-baffled conditions resulted in significantly lower cellulase production, with Rou-w/o BC exhibiting higher activity than Rou-1% BC.

To further analyze enzyme production efficiency, specific enzymatic activity was evaluated by normalizing enzymatic activity to protein concentration (Figure 3a). In contrast to total enzymatic activity, specific activity was not significantly affected by biochar addition under turbulent conditions ( $p > 0.05$ ). EG exhibited high specific activity in all turbulent conditions, reaching 35.48–40.14 U/mg, irrespective of biochar supplementation. There was no significant difference between Baf-w/o BC and Baf-1% BC ( $p > 0.05$ ), but both exhibited significantly higher specific activity than non-baffled conditions ( $p < 0.05$ ). For BGL, specific activity ranged from 1.50 to 1.68 U/mg, with no significant differences between Baf-w/o BC and Baf-1% BC. However, Rou-1% BC showed the lowest specific activity, significantly lower than all other conditions ( $p < 0.05$ ). For CBH, all experimental groups exhibited significant differences ( $p < 0.05$ ), with Baf-w/o BC showing the highest specific activity at 0.62 U/mg, followed by Baf-1% BC (0.42 U/mg), Rou-w/o BC (0.32 U/mg), and Rou-1%

BC (0.21 U/mg). These results indicate that turbulence significantly improved specific enzymatic activity for EG and CBH, whereas biochar supplementation had a greater impact on BGL-specific activity.

To quantify the improvement in specific activity relative to the control, Rou-1% BC was set as the baseline condition (Figure 3b). Baf-w/o exhibited the highest fold-change improvement in specific activity, increasing EG, BGL, and CBH activity by 9.7-, 3.0-, and 2.1-fold, respectively. In comparison, Baf-1% BC showed slightly lower increases (8.6-, 2.7-, and 1.7-fold, respectively), while Rou-w/o BC exhibited moderate improvements (5.7-, 2.6-, and 1.6-fold). These findings indicate that turbulence alone plays a crucial role in enhancing specific cellulase activity, whereas biochar supplementation does not significantly alter specific enzyme efficiency.

However, specific activity alone does not provide a complete picture of enzyme production efficiency. Therefore, total enzymatic activity was analyzed to evaluate the overall cellulase production yield (Figure 3c). Compared to the control (Rou-1% BC), Baf-1% BC exhibited the highest fold-change improvement in total enzymatic activity, with EG, BGL, and CBH increasing by 19.9-, 6.2-, and 8.9-fold, respectively. Baf-w/o BC also demonstrated substantial increases (15.9-, 4.9-, and 8.0-fold), confirming that turbulence alone significantly enhances enzyme production. Interestingly, in non-baffled conditions, Rou-w/o BC showed moderate improvements (5.9-, 2.7-, and 3.8-fold), whereas Rou-1% BC (control) exhibited the lowest values.

When biochar was introduced under turbulent conditions, Baf-1% BC exhibited the highest total enzyme yield despite having slightly lower specific activity than Baf-w/o BC. This discrepancy is attributed to the higher protein concentration observed in Baf-1% BC, which resulted in an overall increase in cellulase production. Thus, although Baf-w/o BC showed the highest specific activity, Baf-1% BC was the optimal condition for cellulase production, as it maximized both enzymatic activity and total yield. These results highlight the synergistic effect of turbulence and biochar, where biochar supplementation enhances cellulase production only when combined with adequate turbulence.

Biochar has been reported to enhance microbial growth and enzyme activity due to its highly porous structure, which offers a favorable microenvironment and serves as a source of readily available carbon and nitrogen [30,36]. Our previous study investigated the effect of biochar supplementation on cellulase activity using the same *Trichoderma* sp. KMF006 strain under baffled flask conditions [30]. In that study, biochar addition led to a 12.1-, 5.8-, and 7.2-fold increase in EG, BGL, and CBH activity, respectively. The current study, which employed the same strain and baffled flasks to promote turbulence, demonstrated a similar positive trend (Table 1). Enzyme activities were significantly higher in biochar-supplemented groups, reaffirming its stimulatory effect on cellulase production. However, this enhancement was observed only under turbulent conditions. In non-baffled flasks, biochar supplementation did not improve cellulase activity and even led to lower values in some cases. This indicates that the effectiveness of biochar may depend on specific hydrodynamic conditions, particularly the intensity of turbulence. Since biochar's stimulatory effect is closely related to its interaction with fungal growth and reproduction [36], sufficient turbulence may help maintain particle suspension and enhance microbial contact. In contrast, low turbulence may lead to particle sedimentation or aggregation, thereby reducing its effectiveness. Although the exact mechanism remains unclear, further studies are needed to clarify how hydrodynamic conditions influence biochar behavior in submerged systems.

Although agitation can introduce turbulence, its effect becomes increasingly limited when scaling up to larger bioreactors, where maintaining efficient oxygen transfer and mixing is more challenging [52]. Thus, optimizing turbulence conditions is crucial for

improving cellulase production beyond what agitation alone can achieve. The strong influence of turbulence has been demonstrated in several studies, where optimized turbulent flow increased cellulase production [53,54]. The effect of turbulence on cellulase production was evaluated in *Pichia pastoris* by comparing baffled and non-baffled flasks [54]. In baffled flasks, turbulence-induced aeration enhanced protein synthesis and enzyme activity. Mitra et al. (2011) demonstrated that turbulence improved oxygen and nutrient distribution, leading to a significant increase in FPase activity in *Chaetomium crispatum* [53]. The turbulence effect can be attributed to enhanced oxygen transfer and improved mixing dynamics, both of which are critical for cellulase synthesis in filamentous fungal cultures, such as *Trichoderma* species [55]. In submerged fermentation, filamentous fungal growth increases broth viscosity, which can exacerbate oxygen transfer limitations and hinder enzyme production. Turbulence mitigates this issue by generating shear stress, which reduces viscosity and decreases bubble size, thereby increasing the gas–liquid interfacial area and enhancing oxygen solubility [53]. This is particularly important for *Trichoderma* sp., as its cellulase production is highly sensitive to oxygen availability [52]. Beyond oxygen transfer, turbulence also plays a key role in maintaining a stable microenvironment within the fermentation broth. Efficient mixing prevents the accumulation of extracellular metabolic byproducts, which could otherwise inhibit enzyme production [56]. Baffles, in particular, generate strong shear forces that improve the homogeneity of nutrient and oxygen distribution, creating favorable conditions for cellulase synthesis [55]. However, *Trichoderma* cellulase activity is highly sensitive to shear stress, making it essential to balance turbulence levels. While moderate turbulence enhances enzyme production, excessive shear stress can damage fungal hyphae, leading to reduced cellulase productivity [55]. This highlights the importance of optimizing turbulence conditions to maximize cellulase production while minimizing the potential negative effects on fungal integrity.

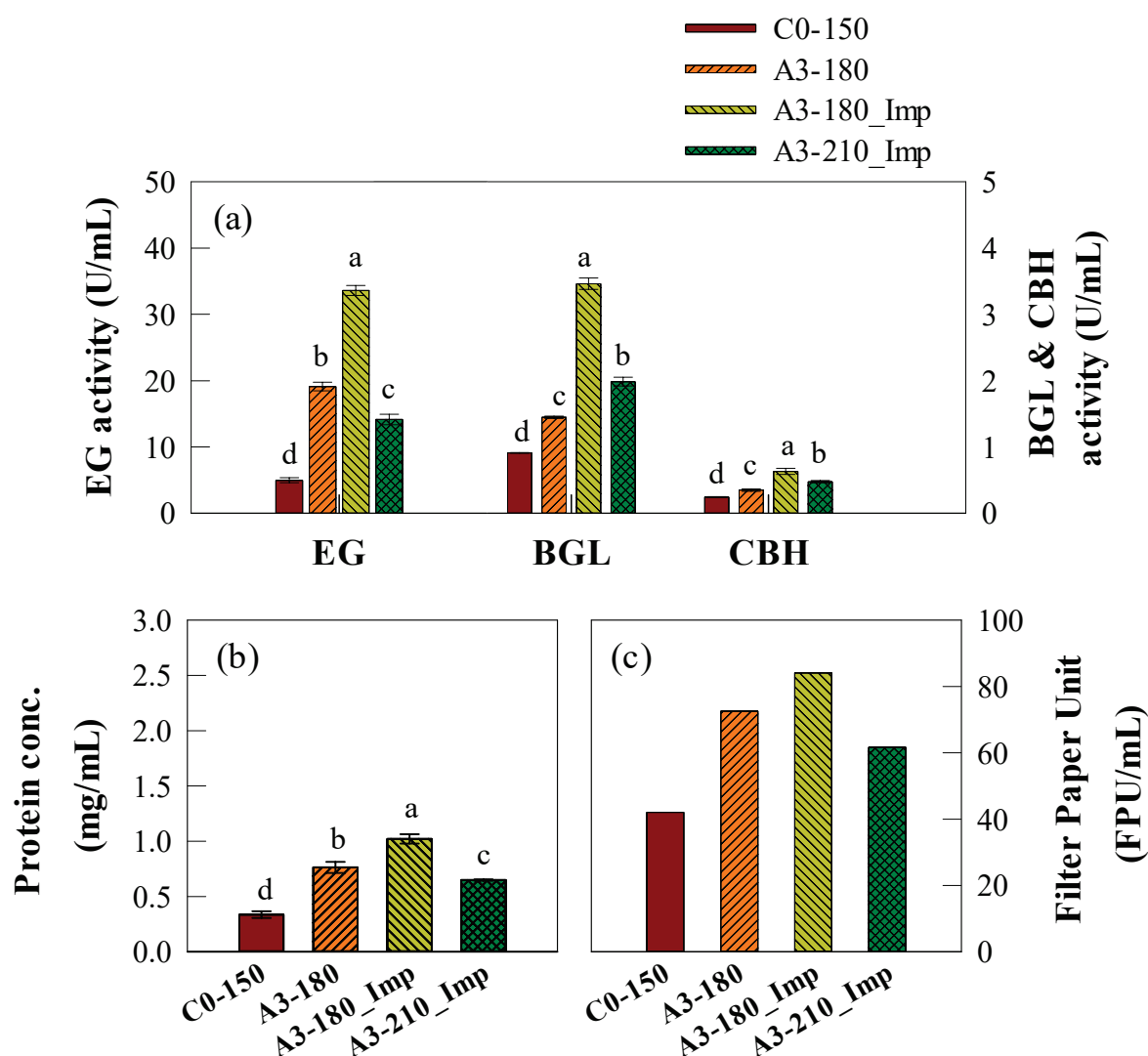
#### 2.4. Scale-Up of Cellulase Production in a SmF Bioreactor

The SmF reactor experiment was conducted to evaluate the effects of scaling up the optimal flask-level culture conditions. The basic control was set as C0-150 (Avicel:Cellulose = 4:0, 150 rpm), and the optimal conditions from the flask experiments were sequentially applied, including carbon source composition (A3C1), agitation speed (180 and 210 rpm), and turbulence formation using an impeller.

Figure S4 presents the time profiles of enzymatic activity and protein concentration in the SmF reactor, which were monitored for up to 12 days. In contrast to the flask-scale experiments, where batch aeration allowed prolonged enzyme production for up to 18 days, the continuous aeration system in the reactor led to a shorter enzyme activity maintenance period. Furthermore, although our previous study monitored enzymatic activities for 18 days, it decreased after day 12 in the reactor system. Additionally, considering the constraints of subsequent saccharification processes, prolonged monitoring beyond 12 days was deemed impractical. Excluding the control, enzymatic activity and protein concentration continuously increased until day 12 across all experimental groups. Among them, A3-180\_Imp exhibited the highest final enzymatic activities. In contrast, C0-150 displayed the lowest enzymatic activities, and its protein concentration did not significantly increase after day 9.

The maximum enzymatic activities at the final time point are shown in Figure 4a. The control reactor (C0-150), operating under the most basic conditions, exhibited the lowest enzymatic activities for all cellulase components, with EG, BGL, and CBH values of 4.99, 0.91, and 0.27 U/mL, respectively. When the optimal carbon source composition (A3C1) and agitation speed (180 rpm) were applied, these significantly increased to 19.12, 1.45, and 0.35 U/mL, respectively, in the A3-180 reactor. Further enhancement was observed

when turbulence was introduced using an impeller (A3-180\_Imp), leading to the highest activities. Among all tested conditions, A3-180\_Imp exhibited the highest enzyme activities, reaching 33.60, 3.46, and 0.63 U/mL for EG, BGL, and CBH, respectively. In contrast, when the agitation speed was increased to 210 rpm (A3-210\_Imp), the synergistic effect with turbulence was not observed. Instead, enzymatic activity decreased to 14.15, 1.99, and 0.48 U/mL, showing lower values than A3-180 despite the presence of turbulence. Particularly for EG, enzymatic activity in A3-210\_Imp was significantly lower than in A3-180, indicating that excessive agitation speed negatively affected enzyme production.



**Figure 4.** Final enzymatic activities (EG, BGL, and CBH) and filter paper unit (FPU) measurements of KMF006 cultured in a SmF reactor. (a) Enzymatic activities of EG, BGL, and CBH. (b) Protein concentrations of each experimental group. (c) FPU measurements representing overall cellulase activity. Experimental conditions are represented as follows: C0-150, Control condition (A4C0, 150 rpm); A3-180, A3C1, 180 rpm; A3-180\_Imp, A3C1, 180 rpm with impeller adjustment; A3-210\_Imp, A3C1, 210 rpm with impeller adjustment. Error bars represent standard deviation (SD) from three biological replicates measured in duplicate ( $n = 6$ ). Statistical significance was determined by one-way ANOVA followed by Tukey's HSD test ( $p < 0.05$ ).

Protein concentration trends exhibited a similar pattern to enzymatic activities, with the highest values observed in A3-180\_Imp (1.02 mg/mL), followed by A3-180 (0.76 mg/mL), A3-210\_Imp (0.65 mg/mL), and C0-150 (0.34 mg/mL). These results suggest that, at the reactor scale, an agitation speed of 180 rpm was optimal for cellulase production,

and excessive agitation and turbulence may have had a detrimental effect on enzyme stability or microbial growth, potentially leading to reduced enzyme secretion.

Figure 4c illustrates the filter paper unit (FPU) per mL, which represents overall cellulase activity. FPU trends aligned with those of enzymatic activity and protein concentration. The highest FPU was observed in A3-180\_Imp (84 FPU/mL), followed by A3-180 (72 FPU/mL) and A3-210\_Imp (61 FPU/mL). The control reactor (C0-150) exhibited the lowest FPU value at 42 FPU/mL. Since the FPU reflects the total cellulase activity available for saccharification, these findings indicate that optimization at the reactor scale can have a direct impact on the efficiency of subsequent hydrolysis processes.

SmF, a liquid-phase cultivation system, provides a uniform growth environment and allows precise process control, making it widely utilized for the industrial production of cellulase [57]. The efficiency of cellulase production in SmF is influenced by various operating parameters (such as temperature, pH, carbon source composition, aeration, and turbulence), and optimizing these variables has been a key strategy for enhancing enzyme production [57,58]. Turbulence, in particular, plays a crucial role in improving oxygen transfer and mass distribution, ultimately affecting fungal metabolism and enzyme secretion [59]. Accordingly, various bioreactor designs have been introduced and evaluated to enhance turbulence effects and optimize process performance. Of these, stirred tank reactors (STRs) generate turbulence through mechanical agitation to enhance oxygen transfer and mixing. They also allow precise control over key process parameters, such as temperature and pH, making them widely used in industrial applications [57,58]. Airlift reactors, on the other hand, utilize natural gas flow to induce turbulence and optimize oxygen transfer while minimizing shear stress, thereby preventing hyphal damage and simultaneously enhancing enzyme productivity [58]. The impact of turbulence control on cellulase production has been experimentally validated in several studies. In STR, an optimized agitation speed of 220 rpm and an aeration rate of 0.6 vvm led to high cellulase activity when cultivating *Penicillium funiculosum* [60]. A comparative study between STRs and airlift reactors demonstrated that STRs achieved superior cellulase activity [61]. Meanwhile, applying mechanical agitation in a 35 L airlift bioreactor successfully promoted fungal growth and cellulase production [62]. Further supporting this, turbulence generated through mechanical agitation was found to significantly improve oxygen transfer efficiency in airlift bioreactors [59].

To further investigate the role of turbulence control in SmF, this study systematically examined how turbulence affects cellulase activity by sequentially applying optimized conditions from flask experiments to a reactor system. Among the tested conditions, the highest cellulase activity was observed under the A3-180\_Imp condition (A3C1, 180 rpm, and impeller-attached for turbulence). In contrast, increasing agitation speed to 210 rpm led to a decrease in cellulase activity (Figure 4). These findings suggest that while turbulence enhances oxygen transfer and nutrient distribution, excessive agitation may generate shear stress that negatively impacts fungal morphology and enzyme stability [55]. Previous studies have demonstrated that high agitation rates reduce cellulase yield, likely due to shear stress affecting fungal structure and enzymatic stability [63,64]. Consistent with previous studies, this study experimentally verified that turbulence regulation plays a critical role in cellulase production in SmF. Since optimal conditions may vary depending on the experimental environment and fungal strain used, direct comparisons with previous studies remain challenging. Nevertheless, these findings emphasize that the precise control of agitation speed and turbulence levels in SmF systems can be an effective strategy for maximizing cellulase production efficiency.

### 3. Materials and Methods

#### 3.1. Microorganism and Pre-Culture Conditions

A cellulase-producing strain, *Trichoderma* sp. KMF006, which was previously isolated [37], was used in this study. The strain was cultivated on malt extract agar (MEA), which consisted of 20 g·L<sup>-1</sup> of malt extract, 20 g·L<sup>-1</sup> of glucose, 1 g·L<sup>-1</sup> of peptone, and 20 g·L<sup>-1</sup> of agar. The plates were incubated at 26 °C until the surface was densely covered with green conidia [30]. The cultured strain was stored at 4 °C until use in pre-culture.

For pre-culture, strain KMF006 was grown in 250 mL baffled flasks containing 100 mL of potato dextrose broth (PDB). Circular plugs (1 cm in diameter) were cut from the MEA plates, and 10 plugs were inoculated into the PDB medium. The flasks were incubated at 26 °C with shaking at 150 rpm for 5 days, after which the pre-culture was used as the inoculum [30].

#### 3.2. Assessment of Carbon Source Composition on Cellulase Production

To evaluate the optimal cellulase activity based on carbon source composition, Avicel and cellulose (Daejung Chemicals & Metals Co., Ltd., Gyeonggi-do, Republic of Korea) were used as carbon sources in various ratios (Table 2). The total carbon source concentration was fixed at 2% (*w/v*) in all experimental groups. The control group (A4C0) consisted of 2% (*w/v*) Avicel, while the experimental groups (A3C1, A2C2, A1C3, and A0C4) contained Avicel and cellulose mixtures at ratios of 3:1, 1:1, 1:3, and 0:4 (*w/v*). The cellulose used in this study had an average particle size of 20–100 µm.

Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD test. See Section 3.6.4 for details.

The basal medium composition was adapted from a previous study [30] and included yeast extract (10 g·L<sup>-1</sup>), H<sub>2</sub>PO<sub>4</sub> (5 g·L<sup>-1</sup>), K<sub>2</sub>HPO<sub>4</sub> (5 g·L<sup>-1</sup>), and MgSO<sub>4</sub>·7H<sub>2</sub>O (3 g·L<sup>-1</sup>), with the initial pH adjusted to 5.03. Pre-culture inoculum (5%, *v/v*) was added to each flask, and cultures were incubated at 31.3 °C with shaking at 150 rpm.

All experiments were conducted in 150 mL flasks with a working volume of 100 mL. Based on previous findings, 1% (*w/v*) biochar was uniformly added to all experimental groups to enhance cellulase activity [30]. The biochar was sterilized at 121 °C for 30 min prior to use. To monitor cellulase activity, 1 mL of culture medium was sampled at 3-day intervals and centrifuged at 10,000 rpm for 10 min to obtain the supernatant. All experiments were performed in triplicate to ensure reproducibility.

#### 3.3. Assessment of Agitation Speed on Cellulase Production

To investigate the effect of agitation speed on cellulase activity, experiments were conducted at agitation speeds of 150 rpm (control), 180 rpm, and 210 rpm (experimental groups) as shown in Table 1. The carbon source composition was fixed at Avicel:cellulose = 3:1 (*w/v*), as determined in previous experiments, with a total carbon source concentration of 2% (*w/v*). The medium composition, culture conditions, working volume, biochar addition, and sampling procedure were identical to those described in Section 2.2. All experiments were performed in triplicate to ensure reproducibility.

#### 3.4. Assessment of Turbulence Efficiency on Cellulase Production

To evaluate the effect of turbulence on cellulase activity, experiments were conducted using 250 mL baffled-bottom flasks and round-bottom flasks. Baffled-bottom flasks (Baf) were used to enhance turbulence, whereas round-bottom flasks (Rou) served as a control with reduced turbulence. Based on the results obtained from cellulase activity experiments with different carbon source compositions and agitation speeds, the carbon source ratio

was fixed at A3C1 (Avicel:cellulose = 3:1, *w/v*), and the agitation speed was set at 180 rpm (Table 1). Each flask contained 100 mL of culture medium as the working volume.

To investigate whether the effect of turbulence on cellulase activity varied depending on biochar addition, the experimental groups were divided into four conditions (Table 1). The Baf-w/o BC group was incubated in baffled-bottom flasks without biochar, and the Baf-1% BC group was cultured in baffled-bottom flasks with 1% (*w/v*) biochar. In contrast, the Rou-w/o BC group was cultured in round-bottom flasks without biochar, while the Rou-1% BC group was cultured in the same type of flask but with 1% (*w/v*) biochar. The medium composition, culture conditions, and sampling procedures were identical to those described in Section 2.2. All experiments were performed in triplicate to ensure reproducibility, and cellulase activity was monitored at 3-day intervals.

### 3.5. SmF Bioreactor Conditions for High Activity Cellulase Production

To evaluate cellulase activity in a bioreactor system, a 10 L SmF bioreactor was employed with a working volume of 6 L. The medium composition consisted of yeast extract ( $10 \text{ g}\cdot\text{L}^{-1}$ ),  $\text{H}_2\text{PO}_4$  ( $5 \text{ g}\cdot\text{L}^{-1}$ ),  $\text{KH}_2\text{PO}_4$  ( $5 \text{ g}\cdot\text{L}^{-1}$ ), and  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  ( $3 \text{ g}\cdot\text{L}^{-1}$ ), with the initial pH adjusted to 5.03. After inoculating 5% (*v/v*) pre-culture, aeration was maintained at a flow rate of 2 L/min, and the bioreactor was operated at 31.3 °C. The optimal culture conditions for carbon source composition, agitation speed, and turbulence, which were determined from flask-scale experiments, were sequentially applied to the SmF bioreactor.

Bioreactor experiments were conducted under four different operational conditions to analyze their effect on cellulase activity (Table 1). The control group (C0-150) was wetted with Avicel (2%, *w/v*) as the sole carbon source, agitated at 150 rpm, with no turbulence. Experimental group 1 (A3-180) applied the optimal carbon source composition (Avicel:cellulose = 3:1, *w/v*) and agitation speed (180 rpm) without turbulence. Experimental group 2 (A3-180\_Imp) followed the same conditions as experimental group 1 but included turbulence, which was generated by attaching an impeller. Lastly, experimental group 3 (A3-210\_Imp) applied a higher agitation speed (210 rpm) with turbulence to assess the combined effects of high agitation and turbulence.

Samples were collected at 3-day intervals during the culture period to monitor cellulase activity and protein concentration. After cultivation, the culture broth was subjected to filtration and concentration processes to evaluate cellulase production yield. The medium was centrifuged at 13,000 rpm for 10 min at 4 °C to separate the supernatant, which was then filtered using filter paper (Whatman No. 10, Cytiva, UK). The filtered supernatant was concentrated to 1/60 of its initial volume using an Amicon® Stirred Cell (UFSC40001, Merck Millipore, Darmstadt, Germany) equipped with a 10 kDa cutoff polyethersulfone membrane (PG 10, Merck Millipore, Germany). The concentrated cellulase was evaluated for enzymatic activity using the Filter Paper Unit (FPU) assay. For each bioreactor condition, a single run was performed. During cultivation, three biological samples were collected at 3-day intervals to monitor enzymatic activity. Each sample was analyzed in duplicate, and the results are expressed as mean  $\pm$  standard deviation (SD).

### 3.6. Enzymatic Activity and Protein Analysis

#### 3.6.1. Enzymatic Activity Analysis

EG activity was measured using the Somogyi–Nelson method [64]. The substrate used was 2% carboxymethylcellulose (CMC, Sigma-Aldrich, Burlington, MA, USA) dissolved in a 0.1 M sodium citrate buffer (pH 5.0). A reaction mixture consisting of 45  $\mu\text{L}$  of CMC solution and 5  $\mu\text{L}$  of enzyme sample was incubated at 60 °C for 30 min. After the reaction, 50  $\mu\text{L}$  of Nelson reagent and 850  $\mu\text{L}$  of distilled water were added to the mixture to analyze the amount of reducing sugars produced. Absorbance was measured at 650 nm, and one

unit (U) of enzymatic activity (EG) was defined as the amount of enzyme required to release 1  $\mu\text{mol}$  of glucose per minute under the assay conditions.

BGL and CBH activities were evaluated using 0.1 M p-nitrophenyl- $\beta$ -D-glycopyranoside (pNPG, Sigma-Aldrich, USA) and 0.1 M p-nitrophenyl- $\beta$ -D-cellobioside (pNPC, Sigma-Aldrich, USA) as substrates, respectively. The substrates were prepared by dissolving in a 0.1 M sodium citrate buffer (pH 5.0). A reaction mixture of 20  $\mu\text{L}$  of substrate and 20  $\mu\text{L}$  of enzyme sample was incubated at 65  $^{\circ}\text{C}$  for 15 min. The reaction was terminated by adding 50  $\mu\text{L}$  of 2 M  $\text{Na}_2\text{CO}_3$ , and the amount of 4-nitrophenol produced was quantified by measuring absorbance at 405 nm. One unit (U) of enzymatic activity (BGL and CBH) was defined as the amount of enzyme required to release 1  $\mu\text{mol}$  of 4-nitrophenol per minute under the assay conditions [65].

All experiments were performed in duplicate, and the results were calculated as the mean values. Absorbance was measured using a UV/Vis spectrophotometer (Synergy LX multi-mode reader, Biotek, Agilent Technologies, Winooski, VT, USA).

### 3.6.2. Protein Concentration Analysis

Protein concentration was analyzed to monitor the growth of *Trichoderma* sp. KMF006 using a protein assay kit (Bio-rad, Hercules, CA, USA) based on the Bradford's method [66]. The assay was conducted by mixing 400  $\mu\text{L}$  of distilled water, 100  $\mu\text{L}$  of protein assay reagent, and 10  $\mu\text{L}$  of enzyme sample, followed by incubation at room temperature for 5 min. After the reaction, the supernatant was collected, and the absorbance was measured at 595 nm using a UV/Vis spectrophotometer (Agilent Technologies, USA). Protein concentration (mg/mL) in the solution was determined using a standard curve prepared with bovine serum albumin (BSA) as the standard protein. All experiments were performed in triplicate.

### 3.6.3. Filter Paper Unit (FPU) Analysis

FPU was measured following the NREL/TP-510-42628 (2008) protocol [67,68]. A 1 cm  $\times$  6 cm Whatman No. 1 filter paper (50 mg) was used as the substrate, which was placed in a 15 mL test tube. Subsequently, 1 mL of 0.1 M sodium citrate buffer (pH 5.0) and 0.5 mL of enzyme sample were added. The reaction mixture was incubated at 50  $^{\circ}\text{C}$  for 1 h. To terminate the reaction, 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added, and the tube was heated in boiling water for 5 min, followed by cooling at room temperature for 10 min. The cooled sample was centrifuged at 13,000 rpm for 10 min at 4  $^{\circ}\text{C}$  to separate the supernatant. A 40  $\mu\text{L}$  aliquot of the supernatant was mixed with 500  $\mu\text{L}$  of distilled water, and the absorbance was measured at 540 nm using a UV/Vis spectrophotometer (Agilent Technologies, USA).

### 3.6.4. Statistical Analysis

For flask-scale experiments, three independent cultures (biological replicates) were prepared under each condition. Each sample was measured in duplicate (technical replicates), and the results were expressed as mean  $\pm$  standard deviation (SD), with a total of  $n = 6$  per condition. T-test and one-way ANOVA followed by Tukey's HSD test for multiple comparisons were conducted using R software (v. 4.4.1). Statistical significance was considered at  $p < 0.05$ .

## 4. Conclusions

This study systematically evaluated the effects of carbon source composition, agitation speed, and turbulence on cellulase production by *Trichoderma* sp. KMF006 in both flask-scale and bioreactor-scale submerged fermentation (SmF) systems. The findings highlight key process parameters that influence enzyme activity and production efficiency.

Optimizing the carbon source composition to a 3:1 ratio of Avicel to cellulose (A3C1) significantly enhanced cellulase production, particularly by accelerating the time to peak

enzymatic activity compared to Avicel alone. Agitation speed was also found to be a critical factor, with both 180 rpm and 210 rpm yielding similar enzymatic activity and protein concentrations. However, considering energy consumption and process efficiency, 180 rpm was determined to be the optimal condition for cellulase production. Turbulence played a significant role in enhancing cellulase production by improving oxygen transfer and nutrient distribution. In flask-scale experiments, baffled flasks resulted in higher enzymatic activity compared to non-baffled conditions, confirming the positive effect of turbulence. Additionally, biochar supplementation further enhanced enzyme production, but only in the presence of turbulence, indicating a synergistic effect.

At the reactor scale, turbulence was introduced via an impeller, which further improved cellulase production under optimized conditions. The A3-180\_Imp condition (A3C1, 180 rpm, impeller-induced turbulence) yielded the highest enzymatic efficiency and protein concentration, demonstrating that precise control over agitation and turbulence is essential for large-scale cellulase production. These findings emphasize the need to balance aeration and shear stress to maximize enzyme yields in industrial SmF systems.

While this study systematically evaluated the effects of carbon source composition, agitation speed, and turbulence on cellulase production, certain aspects require further investigation. The direct quantification of intermediates involved in cellulase induction was not performed. Additionally, although turbulence was found to enhance cellulase production, its interaction with other process parameters, such as aeration rate and shear stress distribution, remains to be fully elucidated. Future studies should focus on elucidating the role of intermediates in cellulase induction, investigating the interactions between turbulence and key process parameters, and developing strategies for large-scale process optimization.

Nevertheless, this study demonstrated the significant impact of carbon source composition, agitation speed, and turbulence control in SmF, providing a comprehensive framework for optimizing cellulase production. The findings contribute to advancing cellulase bioprocessing strategies and offer practical insights for improving enzyme production efficiency in industrial applications.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms26083731/s1>.

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## References

1. Razzak, S.A.; Hossain, M.M.; Lucky, R.A.; Bassi, A.S.; De Lasa, H. Integrated CO<sub>2</sub> capture, wastewater treatment and biofuel production by microalgae culturing—A review. *Renew. Sust. Energy Rev.* **2013**, *27*, 622–653. [CrossRef]
2. Ibrahim, N.I.; Shahar, F.S.; Hameed Sultan, M.T.; Md Shah, A.U.; Azrie Safri, S.N.; Mat Yazik, M.H. Overview of bioplastic introduction and its applications in product packaging. *Coatings* **2021**, *11*, 1423. [CrossRef]

3. Rodionova, M.V.; Bozieva, A.M.; Zharmukhamedov, S.K.; Leong, Y.K.; Chi-Wei Lan, J.; Veziroglu, A.; Veziroglu, T.N.; Tomo, T.; Chang, J.S.; Allakhverdiev, S.I. A comprehensive review on lignocellulosic biomass biorefinery for sustainable biofuel production. *Int. J. Hydrogen Energy* **2022**, *47*, 1481–1498. [CrossRef]
4. Mangal, M.; Rao, C.V.; Banerjee, T. Bioplastic: An eco-friendly alternative to non-biodegradable plastic. *Polym. Int.* **2023**, *72*, 984–996. [CrossRef]
5. Kudakasseril Kurian, J.; Raveendran Nair, G.; Hussain, A.; Vijaya Raghavan, G.S. Feedstocks, logistics and pre-treatment processes for sustainable lignocellulosic biorefineries: A comprehensive review. *Renew. Sust. Energy Rev.* **2013**, *25*, 205–219. [CrossRef]
6. Rodionova, M.V.; Poudyal, R.S.; Tiwari, I.; Voloshin, R.A.; Zharmukhamedov, S.K.; Nam, H.G.; Zayadan, B.K.; Bruce, B.D.; Hou, H.J.M.; Allakhverdiev, S.I. Biofuel production: Challenges and opportunities. *Int. J. Hydrogen Energy* **2017**, *42*, 8450–8461. [CrossRef]
7. Yu, D.; Guo, J.; Meng, J.; Sun, T. Biofuel production by hydro-thermal liquefaction of municipal solid waste: Process characterization and optimization. *Chemosphere* **2023**, *328*, 138606. [CrossRef]
8. Shah, M.; Rajhans, M.; Pandya, H.A.; Mankad, A.U. Mankad bioplastic for future: A review Then and Now. *World J. Adv. Res. Rev.* **2021**, *9*, 056–067. [CrossRef]
9. Siqueira, J.G.W.; Rodrigues, C.; Vandenberghe, L.P.d.S.; Woiciechowski, A.L.; Soccol, C.R. Current advances in on-site cellulase production and application on lignocellulosic biomass conversion to biofuels: A review. *Biomass Bioenergy* **2020**, *132*, 105419. [CrossRef]
10. REN21 Secretariat. *Renewable 2018 Global Status Report*; REN21: Paris, France, 2018. Available online: <https://www.ren21.net/wp-content/uploads/2019/08/Full-Report-2018.pdf> (accessed on 10 March 2025).
11. Eckert, S.; Karassin, O.; Steinebach, Y. A policy portfolio approach to plastics throughout their life cycle: Supranational and national regulation in the European Union. *Environ. Policy Gov.* **2024**, *34*, 427–441. [CrossRef]
12. Van Dyk, J.S.; Pletschke, B.I. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy. *Biotechnol. Adv.* **2012**, *30*, 1458–1480. [CrossRef]
13. Woo, W.X.; Tan, J.P.; Wu, T.Y.; Yeap, S.K.; Luthfi, A.A.I.; Manaf, S.F.A.; Jamali, N.S.; Hui, Y.W. An overview on the factors affecting enzymatic saccharification of lignocellulosic biomass into fermentable sugars. *Rev. Chem. Eng.* **2024**, *40*, 279–303. [CrossRef]
14. Abo, B.O.; Gao, M.; Wang, Y.; Wu, C.; Ma, H.; Wang, Q. Lignocellulosic biomass for bioethanol: An overview on pretreatment, hydrolysis and fermentation processes. *Rev. Environ. Health* **2019**, *34*, 57–68. [CrossRef]
15. El-Zawawy, W.K.; Ibrahim, M.M.; Abdel-Fattah, Y.R.; Soliman, N.A.; Mahmoud, M.M. Acid and enzyme hydrolysis to convert pretreated lignocellulosic materials into glucose for ethanol production. *Carbohydr. Polym.* **2011**, *84*, 865–871. [CrossRef]
16. Sukumaran, R.K.; Singhanian, R.R.; Mathew, G.M.; Pandey, A. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. *Renew. Energy* **2009**, *34*, 421–424. [CrossRef]
17. Singh, A.; Bajar, S.; Devi, A.; Pant, D. An overview on the recent developments in fungal cellulase production and their industrial applications. *Bioresour. Technol. Rep.* **2021**, *14*, 100652. [CrossRef]
18. Klein-Marcuschamer, D.; Oleskowicz-Popiel, P.; Simmons, B.A.; Blanch, H.W. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol. Bioeng.* **2012**, *109*, 1083–1087. [CrossRef]
19. Singh, A.; Bajar, S.; Devi, A.; Bishnoi, N.R. Evaluation of cellulase production from *Aspergillus niger* and *Aspergillus heteromorphus* under submerged and solid-state fermentation. *Environ. Sustain.* **2021**, *4*, 437–442. [CrossRef]
20. Korsa, G.; Konwarh, R.; Masi, C.; Ayele, A.; Haile, S. Microbial cellulase production and its potential application for textile industries. *Ann. Microbiol.* **2023**, *73*, 13. [CrossRef]
21. Srivastava, N.; Srivastava, M.; Mishra, P.K.; Singh, P.; Ramteke, P.W. Application of cellulases in biofuels industries: An overview. *J. Biofuels Bioenergy* **2015**, *1*, 55–63. [CrossRef]
22. Infanzón-Rodríguez, M.I.; Ragazzo-Sánchez, J.A.; del Moral, S.; Calderón-Santoyo, M.; Gutiérrez-Rivera, B.; Aguilar-Uscanga, M.G. Optimization of cellulase production by *Aspergillus niger* ITV 02 from sweet sorghum bagasse in submerged culture using a Box–Behnken design. *Sugar Tech.* **2020**, *22*, 266–273. [CrossRef]
23. Matkar, K.; Chapla, D.; Divecha, J.; Nighojkar, A.; Madamwar, D. Production of cellulase by a newly isolated strain of *Aspergillus sydowii* and its optimization under submerged fermentation. *Int. Biodeterior. Biodegrad.* **2013**, *78*, 24–33. [CrossRef]
24. Ramanathan, G.; Banupriya, S.; Abirami, D. Production and optimization of cellulase from *Fusarium oxysporum* by submerged fermentation. *Bioprocess. Biosyst. Eng.* **2010**, *69*, 454–459.
25. Reihani, S.F.S.; Khosravi-Darani, K. Influencing factors on single-cell protein production by submerged fermentation: A review. *Electron. J. Biotechnol.* **2019**, *37*, 34–40. [CrossRef]
26. Cunha, F.M.; Esperança, M.N.; Zangirolami, T.C.; Badino, A.C.; Farinas, C.S. Sequential solid-state and submerged cultivation of *Aspergillus niger* on sugarcane bagasse for the production of cellulase. *Bioresour. Technol.* **2012**, *112*, 270–274. [CrossRef]

27. Intasit, R.; Cheirsilp, B.; Suyotha, W.; Boonsawang, P. Synergistic production of highly active enzymatic cocktails from lignocellulosic palm wastes by sequential solid-state submerged fermentation and co-cultivation of different filamentous fungi. *Biochem. Eng. J.* **2021**, *173*, 108086. [CrossRef]
28. da Silva Delabona, P.; Lima, D.J.; Robl, D.; Rabelo, S.C.; Farinas, C.S.; da Cruz Pradella, J.G. Enhanced cellulase production by *Trichoderma harzianum* by cultivation on glycerol followed by induction on cellulosic substrates. *J. Ind. Microbiol. Biotechnol.* **2016**, *43*, 617–626. [CrossRef]
29. Lan, T.Q.; Wei, D.; Yang, S.T.; Liu, X. Enhanced cellulase production by *Trichoderma viride* in a rotating fibrous bed bioreactor. *Bioresour. Technol.* **2013**, *133*, 175–182. [CrossRef]
30. Myeong, S.; Yun, J. Culture of *Trichoderma* sp. with biochar to produce high-activity cellulase in a laboratory. *Bioresources* **2024**, *19*, 2029–2044. [CrossRef]
31. Du, J.; Zhang, Y.; Qu, M.; Yin, Y.; Fan, K.; Hu, B.; Zhang, H.; Wei, M.; Ma, C. Effects of biochar on the microbial activity and community structure during sewage sludge composting. *Bioresour. Technol.* **2019**, *272*, 171–179. [CrossRef] [PubMed]
32. Awad, Y.M.; Lee, S.S.; Kim, K.H.; Ok, Y.S.; Kuzyakov, Y. Carbon and nitrogen mineralization and enzyme activities in soil aggregate-size classes: Effects of biochar, oyster shells, and polymers. *Chemosphere* **2018**, *198*, 40–48. [CrossRef] [PubMed]
33. Yin, Y.; Li, M.; Tao, X.; Yang, C.; Zhang, W.; Li, H.; Zheng, Y.; Wang, X.; Chen, R. Biochar enhanced organic matter transformation during pig manure composting: Roles of the cellulase activity and fungal community. *J. Environ. Manag.* **2023**, *333*, 117464. [CrossRef]
34. Mohanty, S.K.; Valenca, R.; Berger, A.W.; Yu, I.K.M.; Xiong, X.; Saunders, T.M.; Tsang, D.C.W. Plenty of room for carbon on the ground: Potential applications of biochar for stormwater treatment. *Sci. Total Environ.* **2018**, *625*, 1644–1658. [CrossRef]
35. Wang, J.; Wang, S. Preparation, modification and environmental application of biochar: A review. *J. Clean. Prod.* **2019**, *227*, 1002–1022. [CrossRef]
36. Zhang, Y.; Wang, J.; Feng, Y. The effects of biochar addition on soil physicochemical properties: A review. *Catena* **2021**, *202*, 105284. [CrossRef]
37. Kim, Y.; Park, S.; Kim, Y. Novel *Trichoderma* sp. KMF006 Strain Producing Cellulase with High Activity. Korean Patent No. 10-2155046, 11 September 2020.
38. Kogo, T.; Yoshida, Y.; Koganei, K.; Matsumoto, H.; Watanabe, T.; Ogihara, J.; Kasumi, T. Production of rice straw hydrolysis enzymes by the fungi *Trichoderma reesei* and *Humicola insolens* using rice straw as a carbon source. *Bioresour. Technol.* **2017**, *233*, 67–73. [CrossRef]
39. Silva, J.C.R.; Salgado, J.C.S.; Vici, A.C.; Ward, R.J.; Polizeli, M.L.T.M.; Guimarães, L.H.S.; Furriel, R.P.M.; Jorge, J.A. A novel *Trichoderma reesei* mutant RP698 with enhanced cellulase production. *Braz. J. Microbiol.* **2020**, *51*, 537–545. [CrossRef]
40. Hodrová, B.; Kopečný, J.; Káš, J. Cellulolytic enzymes of rumen anaerobic fungi *Orpinomyces joyonii* and *Caecomyces communis*. *Res. Microbiol.* **1998**, *149*, 417–427. [CrossRef]
41. Karnchanatat, A.; Petsom, A.; Sangvanich, P.; Piapukiew, J.; Whalley, A.J.S.; Reynolds, C.D.; Gadd, G.M.; Sihanonth, P. A novel thermostable endoglucanase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. *Enzyme Microb. Technol.* **2008**, *42*, 404–413. [CrossRef]
42. Singhanian, R.R.; Saini, R.; Adsul, M.; Saini, J.K.; Mathur, A.; Tuli, D. An integrative process for bio-ethanol production employing SSF produced cellulase without extraction. *Biochem. Eng. J.* **2015**, *102*, 45–48. [CrossRef]
43. Saini, R.; Saini, J.K.; Adsul, M.; Patel, A.K.; Mathur, A.; Tuli, D.; Singhanian, R.R. Enhanced cellulase production by *Penicillium oxalicum* for bio-ethanol application. *Bioresour. Technol.* **2015**, *188*, 240–246. [CrossRef] [PubMed]
44. Gao, J.; Qian, Y.; Wang, Y.; Qu, Y.; Zhong, Y. Production of the versatile cellulase for cellulose bioconversion and cellulase inducer synthesis by genetic improvement of *Trichoderma reesei*. *Biotechnol. Biofuels* **2017**, *10*, 272. [CrossRef]
45. Li, Y.; Liu, C.; Bai, F.; Zhao, X. Overproduction of cellulase by *Trichoderma reesei* RUT C30 through batch-feeding of synthesized low-cost sugar mixture. *Bioresour. Technol.* **2016**, *216*, 503–510. [CrossRef]
46. Suto, M.; Tomita, F. Induction and catabolite repression mechanisms of cellulase in fungi. *J. Biosci. Bioeng.* **2001**, *92*, 305–308. [CrossRef]
47. Kubicek, C.P.; Mikus, M.; Schuster, A.; Schmoll, M.; Seiboth, B. Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. *Biotechnol. Biofuels* **2009**, *2*, 19. [CrossRef]
48. Yu, L.; Chao, Y.; Wensel, P.; Chen, S. Hydrodynamic and kinetic study of cellulase production by *Trichoderma reesei* with pellet morphology. *Biotechnol. Bioeng.* **2012**, *109*, 1755–1768. [CrossRef]
49. Sirohi, R.; Singh, A.; Tarafdar, A.; Shahi, N.C.; Verma, A.K.; Kushwaha, A. Cellulase production from pre-treated pea hulls using *Trichoderma reesei* under submerged fermentation. *Waste Biomass Valorization* **2019**, *10*, 2651–2659. [CrossRef]
50. Teoh, Y.; Don, M.M.; Fadzilah, K. Optimization of cellulase production by *Pycnoporus sanguineus* in 5 L stirred tank bioreactor and enhanced fermentation by employing external loop. *Chiang Mai J. Sci.* **2017**, *44*, 774–787.
51. Mmango-Kaseke, Z.; Okaiyeto, K.; Nwodo, U.U.; Mabinya, L.V.; Okoh, A.I. Optimization of cellulase and xylanase production by *Micrococcus* species under submerged fermentation. *Sustainability* **2016**, *8*, 1168. [CrossRef]

52. Park, J.K.; Hyun, S.H.; Jung, J.Y. Conversion of *G. hansenii* PJK into non-cellulose-producing mutants according to the culture condition. *Biotechnol. Bioprocess. Eng.* **2004**, *9*, 383–388. [CrossRef]
53. Mitra, S.; Banerjee, P.; Gachhui, R.; Mukherjee, J. Cellulase and xylanase activity in relation to biofilm formation by two intertidal filamentous fungi in a novel polymethylmethacrylate conico-cylindrical flask. *Bioprocess. Biosyst. Eng.* **2011**, *34*, 1087–1101. [CrossRef]
54. Batra, J.; Beri, D.; Mishra, S. Response surface methodology-based optimization of  $\beta$ -glucosidase production from *Pichia pastoris*. *Appl. Biochem. Biotechnol.* **2014**, *172*, 380–393. [CrossRef]
55. Weber, J.; Agblevor, F.A. Microbubble fermentation of *Trichoderma reesei* for cellulase production. *Process Biochem.* **2005**, *40*, 669–676. [CrossRef]
56. Ma, H.; Liu, Y.; Li, Z. The synergistic hydrogen production of bicellular fermentation systems and fluid dynamics simulation in reactor under stirring. *Bioresour. Technol. Rep.* **2023**, *22*, 101473. [CrossRef]
57. Dey, P.; Rangarajan, V.; Singh, J.; Nayak, J.; Dilip, K.J. Current perspective on improved fermentative production and purification of fungal cellulases for successful biorefinery applications: A brief review. *Biomass Convers. Biorefin.* **2022**, *12*, 967–995. [CrossRef]
58. Shokrkar, H.; Ebrahimi, S.; Zamani, M. A review of bioreactor technology used for enzymatic hydrolysis of cellulosic materials. *Cellulose* **2018**, *25*, 6279–6304. [CrossRef]
59. Chisti, Y.; Jauregui-Haza, U.J. Oxygen transfer and mixing in mechanically agitated airlift bioreactors. *Bioprocess. Eng.* **2002**, *10*, 143–153. [CrossRef]
60. de Albuquerque de Carvalho, M.L.; Carvalho, D.F.; de Barros Gomes, E.; Maeda, R.N.; Anna, L.M.M.S.; de Castro, A.M.; Pereira, N., Jr. Optimisation of cellulase production by *Penicillium funiculosum* in a stirred tank bioreactor using multivariate response surface analysis. *Enzyme Res.* **2014**, *2014*, 703291. [CrossRef]
61. Kim, S.W.; Kang, S.W.; Lee, J.S. Cellulase and xylanase production by *Aspergillus niger* KKS in various bioreactors. *Bioresour. Technol.* **1997**, *59*, 63–67. [CrossRef]
62. Ahamed, A.; Vermette, P. Culture-based strategies to enhance cellulase enzyme production from *Trichoderma reesei* RUT-C30 in bioreactor culture conditions. *Biochem. Eng. J.* **2008**, *40*, 399–407. [CrossRef]
63. Lejeune, R.; Baron, G.V. Effect of agitation on growth and enzyme production of *Trichoderma reesei* in batch fermentation. *Biotechnol. Bioeng.* **1995**, *43*, 249–258. [CrossRef]
64. Hayward, T.K.; Hamilton, J.; Tholudur, A.; McMillan, J.D. Improvements in titer, productivity, and yield using Solka-Floc for cellulase production. In Proceedings of the Twenty-First Symposium on Biotechnology for Fuels and Chemicals, Fort Collins, CO, USA, 2–6 May 1999; Humana Press: Totowa, NJ, USA, 2000; pp. 859–874.
65. Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **1944**, *153*, 375–380. [CrossRef]
66. Joo, A.R.; Jeya, M.; Lee, K.M.; Sim, W.I.; Kim, J.S.; Kim, I.W.; Kim, Y.S.; Oh, D.K.; Gunasekaran, P.; Lee, J.K. Purification and characterization of a  $\beta$ -1,4-glucosidase from a newly isolated strain of *Fomitopsis pinicola*. *Appl. Microbiol. Biotechnol.* **2009**, *83*, 285–294. [CrossRef] [PubMed]
67. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
68. NREL. Measurement of cellulase activities: Laboratory analytical procedure (LAP). In *NREL Technical Report (NREL/TP-510-42628)*; 2008. Available online: <https://www.nrel.gov/docs/gen/fy08/42628.pdf> (accessed on 12 April 2025).

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Article

# Site-Directed Mutagenesis Increased the Catalytic Activity and Stability of *Oenococcus oeni* $\beta$ -Glucosidase: Characterization of Enzymatic Properties and Exploration of Mechanisms

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**Abstract:** *Oenococcus oeni*  $\beta$ -glucosidase can significantly improve food flavor, but its catalytic activity and stability performance need to be enhanced. In this study, the two dominant mutants III and IV were obtained by site-directed mutation of key amino acid residues in the catalytic pocket of *Oenococcus oeni*  $\beta$ -glucosidase. Compared with the wild-type enzyme, the activities of mutants III and IV were increased by 2.81 and 3.18 times, respectively. Their thermal stability was also significantly improved. Mutants III and IV showed a significant increase in affinity for the standard substrate *p*-NPG, with the  $K_m$  value decreasing by 18.2% and 33.3%, respectively. Molecular docking analysis indicated that hydrogen bonding and  $\pi$ - $\pi$  aromatic interactions were the primary factors influencing the changes in enzyme properties, with F133 and N181 identified as key amino acids affecting catalytic activity and stability. This research is of great significance for enhancing food flavor and expands the potential industrial applications of *Oenococcus oeni*  $\beta$ -glucosidase.

**Keywords:**  $\beta$ -glucosidase; catalytic activity; thermal stability; site-directed mutation; molecular docking

## 1. Introduction

$\beta$ -glucosidase (BGL, EC3.2.1.21) is also known as  $\beta$ -D-glucoside glucose hydrolase, gentiobiase, cellobiase and amygdalinase. It is an important component of the cellulase system. The main function of BGLs is to catalyze the hydrolysis of  $\beta$ -1,4-glycosidic bonds at the non-reducing ends of alkyl and aryl groups and different oligosaccharides, releasing  $\beta$ -D-glucose and its corresponding ligands [1]. In addition, BGLs also have a certain hydrolysis activity on  $\beta$ -1,1,  $\beta$ -1,2,  $\beta$ -1,3 and  $\beta$ -1,6 glycosidic bonds. BGLs can also weakly hydrolyze  $\beta$ -D-cellulosic glycosides,  $\beta$ -D-galactoside,  $\beta$ -D-hydroxyglucoside and  $\alpha$ -D-glucoside bonds, releasing a variety of ligands [2]. The sources of BGLs are diverse and can be generally divided into plant sources, animal sources and microbial sources [3]. In recent years, due to the advantages of their high yield, low price and high catalytic efficiency, the current research has focused on BGLs derived from microbiology. Among them, BGLs derived from fungi such as *Aspergillus niger* and yeast were the research hotspots, while the BGLs from *Oenococcus oeni* were rarely reported [4].

BGLs are widely used in the bioenergy, chemical and food industries due to a variety of functions such as their glycoside hydrolysis ability and substrate specificity [5]. In the food industry,  $\beta$ -glucosidase can hydrolyze hundreds of  $\beta$ -glucoside flavor precursors, which can significantly improve food flavor and quality [6]. For example, soybean contains 12 different isoflavones. Endogenous  $\beta$ -glucosidase can hydrolyze isoflavones to obtain  $\beta$ -glucoside ligands, such as phenolic aglycones [7]. Many other related studies have shown that  $\beta$ -glucosidases can improve the aroma quality of tea by increasing the contents of aromatic alcohols and their oxides, such as nerol, geraniol and phenylethanol. They also have a positive effect on the contents of some aliphatic compounds, terpenes and aromatic compounds [8,9]. In the wine industry, adding  $\beta$ -glucosidase can significantly enhance wine aroma, directly manifested as increasing the contents of terpenes in wine [10]. In addition, it can also reduce the degradation rate of anthocyanins in wine, which is conducive to wine fermentation [11,12].

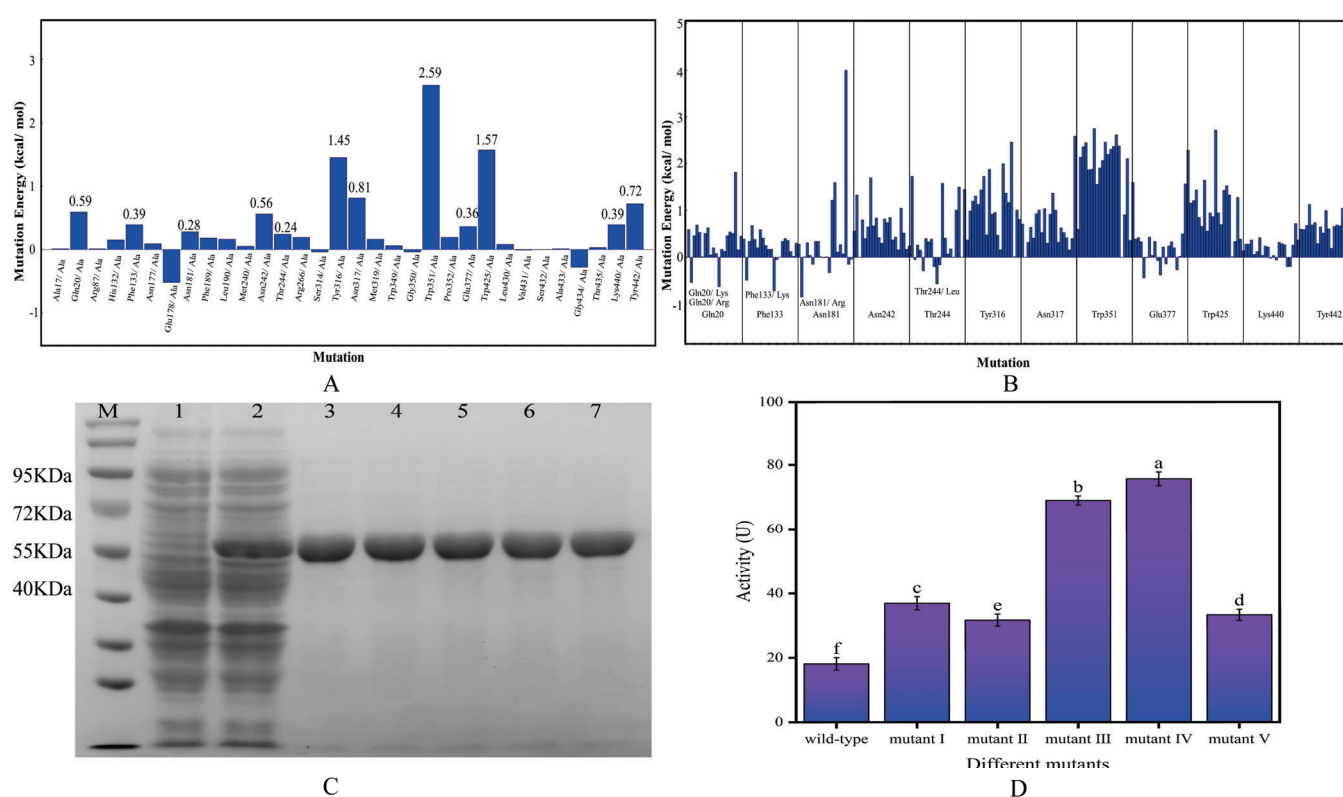
Due to the high industrial application value of BGLs, designing and producing BGLs with higher catalytic activity and stability through various methods has always been a hot research direction. Many studies have also been conducted to improve the enzymatic properties of existing BGLs. Molecular docking technology has been widely used to construct enzyme–substrate complex models for predicting binding modes and catalytic mechanisms [13]. Xia et al. obtained a BGL3A mutant with increased catalytic activity through molecular docking and saturation mutagenesis [14]. Binding energy scanning is also a commonly used method to guide the molecular modification of BGLs. It can predict the changes of binding energy in advance, making the mutation selection more reasonable, and successfully realize the activity modification of various industrial enzymes [13]. Teze et al. obtained a mutant with an increased yield, from 36% to 90%, by mutating the  $-1$  subsite of BGLs in *Thermus thermophilus* by binding energy scanning. It was concluded that decreasing the interaction of the glycosyl subsite decreases the stability of the transition state in the reaction. This has a greater effect on the hydrolysis reaction than on the transglycosylation reaction, thus increasing the transglycosylation/hydrolysis ratio [15]. Hassan et al. also modified the  $\beta$ -glucosidase in *Halothermothrix orenii* by rational design. They constructed five mutants in the vicinity of the  $-1$  and  $+1$  subsites. One of the mutants near the  $-1$  subsite synthesized oligogalactose in an increased yield of 57.4% [16]. Yang et al. also demonstrated similar results using  $\beta$ -glucosidase from *Thermotoga naphthophila*, with the most effective mutant exhibiting a significant increase in hydrophilicity of the  $-1$  subunit [17]. Lee et al. improved the thermal stability of BGLs derived from *Trichoderma reesei* by altering the amino acids that are external to the active site [18]. The study by Ranaei et al. showed an increase in the enzyme's affinity to the substrate by decreasing the glycosylation site, increasing the catalytic efficiency to 143% of the wild-type enzyme [19].

*Oenococcus oeni* is one of the commonly used bacterial strains in wine fermentation processes. The  $\beta$ -glucosidase produced by *Oenococcus oeni* can effectively act on the glycosidically bound aroma precursors in the wine matrix, promoting the cleavage of the bound aroma molecules and releasing a large amount of free volatile aroma compounds, thereby achieving a dual enhancement in both the complexity and intensity of the aroma in the wine fermentation system. Consequently, the aim of this study was to enhance the catalytic performance of *Oenococcus oeni*  $\beta$ -glucosidase through molecular modification techniques. Based on the significant application value and our previous systematic research on *Oenococcus oeni*  $\beta$ -glucosidase, in this study, we used a site-directed mutagenesis strategy to modify the *Oenococcus oeni*  $\beta$ -glucosidase to obtain mutants with high enzyme activity and good stability. Additionally, further expression, purification and enzymatic characterization were carried out on the mutants. This study is expected to provide theoretical guidance for the modification of BGLs to broaden their application fields.

## 2. Results and Discussion

### 2.1. Screening of Mutation Sites and Mutants

The results of alanine scanning and single mutation binding energy changes showed that 12 residues (Trp351, Trp425, Tyr316, Asn317, Tyr442, Gln20, Asn242, Phe133, Lys440, Glu377, Asn181 and Thr244) had a mutation energy greater than 0.2 kcal/mol, indicating that these residues were key residues in the structure of  $\beta$ -glucosidase (Figure 1A). Four of the 12 key residues can form hydrophobic interactions with small molecules, and two can form hydrogen bonds, which also proves the rationality of alanine scanning. Single mutations were performed on the above 12 residues. The mutation results are shown in Figure 1B. A single point mutation was identified for those with a binding energy change of less than  $-0.5$  kcal/mol, leading to the identification of the mutation sites Q20K, Q20R, F133K, N181R and T244L, which were designated as mutant I, mutant II, mutant III, mutant IV and mutant V, respectively.



**Figure 1.** (A) Results of alanine scan; (B) results of single mutation binding energy change; (C) SDS-PAGE analysis (M: marker; 1: empty vector control; 2: bacteriophage containing the expression plasmid; 3–7: target protein purification of mutants I to V); (D) determination of enzyme activity (Lowercase letters indicate significant differences in catalytic activity between different mutants and wild type,  $p < 0.05$ ).

It can be seen in Figure 1C that the expression of the bacterium's own proteins were high when the target gene was not introduced (lane 1), and after the insertion of the target gene (lane 2), there was a clear band at 55 KDa with a higher expression level, indicating the successful expression of the target protein. Lanes 3–7 were the results of protein purification of the five mutants, which had only a single and cleared band at 55 KDa, and there is no other heterozygous band, indicating that the target proteins have been well purified, and the molecular weight of 55 KDa was consistent with the prediction. It can be seen from Figure 1D that the enzyme activities of mutants I–V were all greater than that of the wild-type enzyme, among which the enzyme activity of mutant III and mutant IV increased

more significantly, reaching 3.81 and 4.18 times that of the wild-type enzyme, respectively. Consequently, these two mutants were chosen for further experiments in this study.

## 2.2. Characterization of Enzymatic Properties

### 2.2.1. Effects of Temperature on Enzyme Activity

Temperature is one of the main factors affecting enzyme activity, and the thermal stability of enzymes directly reflects their adaptability and application potential to various environmental conditions [20]. As shown in Figure 2A, the optimal catalytic temperature for both the wild-type enzyme and mutant III was 50 °C, while that for mutant IV was 45 °C. For the wild-type enzyme, the catalytic activity was maintained at a high level when the temperature was between 35 and 55 °C, with the relative activity exceeding 80%. However, its relative activity decreased sharply to below 50% when the temperature exceeded 65 °C. For mutants III/IV, their relative activity remained above 80% at 65 °C, demonstrating stronger heat resistance. Zhang et al. mutated *Leuconostoc mesenteroides* dextranucrase, in which the optimal temperature of mutants L838C–V887C and A948C–A1013C were increased by 10 °C compared to the wild-type enzyme [21]. Additionally, the mutants III/IV also demonstrated more excellent thermal stability. As shown in Figure 2B, when the wild-type enzyme was incubated below 50 °C, the enzyme activity could be maintained at a high level, but when the temperature exceeded 50 °C, its activity decreased significantly with the increase of incubation time. In contrast, the catalytic activities of mutants III/IV remained above 80% after incubation at 70 °C for six hours (Figure 2C,D), indicating a significant improvement in the thermal stability of the enzyme after mutation, which provided a possibility for its application under high temperature conditions. Dawei et al. applied combinatorial targeted mutagenesis to inulosucrase from *Lactobacillus casei* UMB7800, resulting in three mutants, G531P–S344D, G531P–H434P and G531P–E256D, with significantly enhancement in thermal stability [22]. Additionally, the half-life of the wild-type enzyme and mutants at their optimal temperatures are shown in Table S2. The half-life of the wild-type enzyme was 23 h, and the half-lives of mutant III and mutant IV were prolonged compared with those of the wild-type enzyme by 3 h and 8 h, respectively. The prolongation of the half-life will well broaden the application field of mutants.

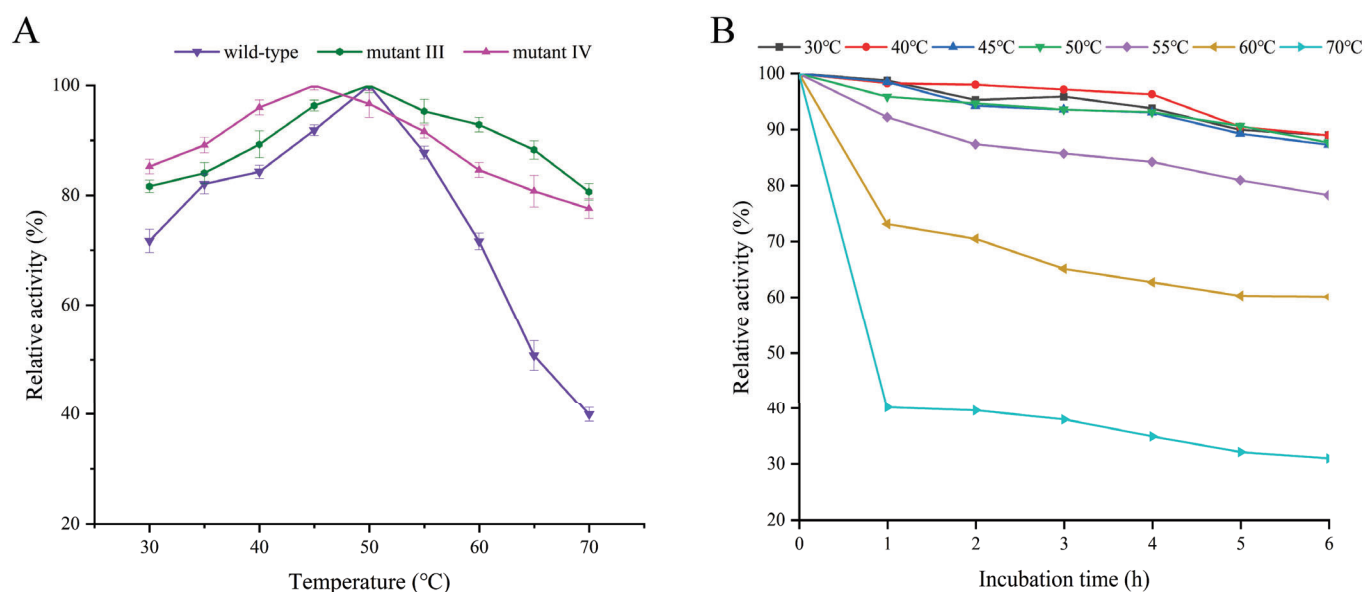
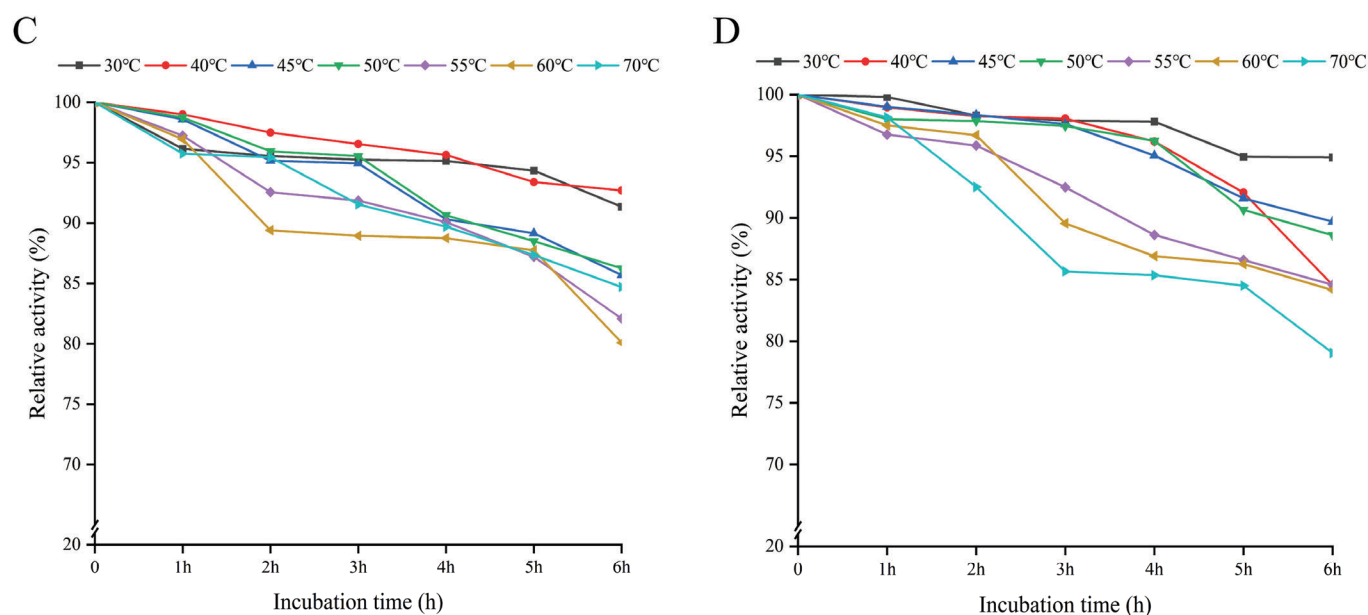


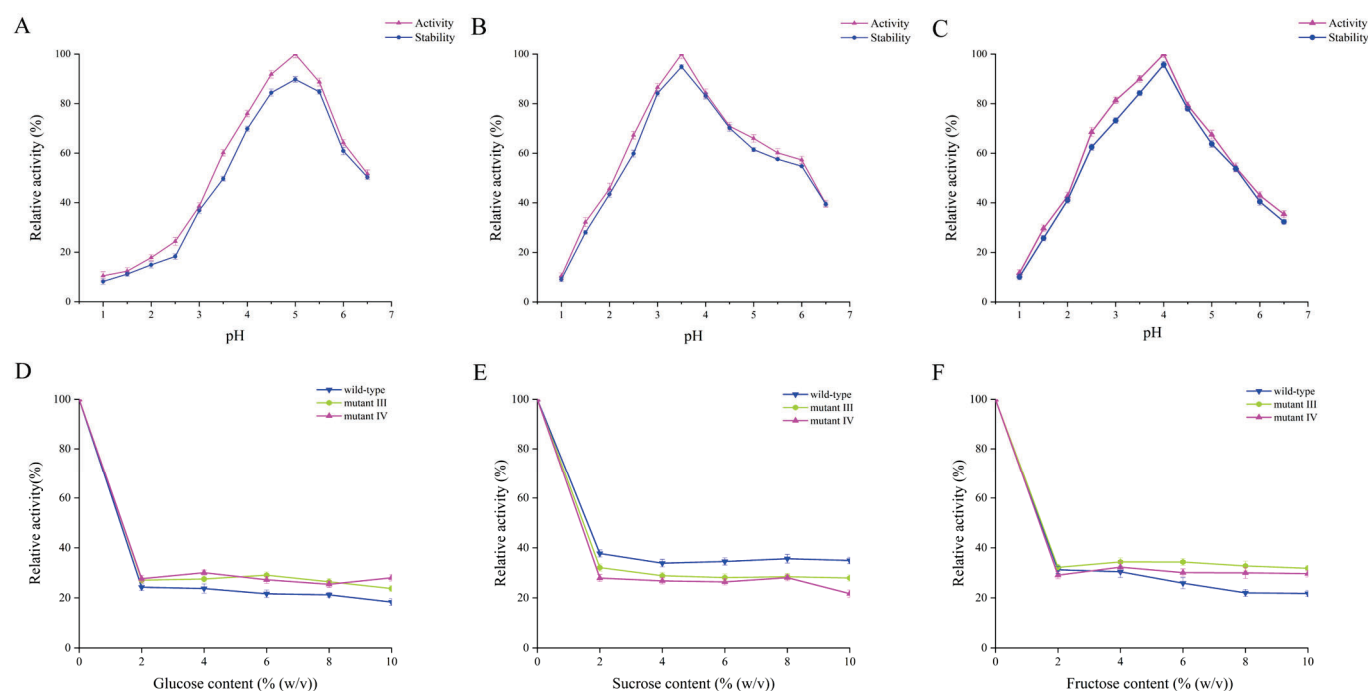
Figure 2. Cont.



**Figure 2.** The optimum temperature and thermal stability of the wild-type enzyme and mutants III/IV. (A) Optimum temperature of wild-type enzyme and mutants III/IV; (B) thermal stability of the wild-type enzyme; (C) thermal stability of mutant III; (D) thermal stability of mutant IV. The optimum temperature was obtained by controlling the same conditions of pH.

### 2.2.2. Effects of pH on Enzyme Activity

The optimal reaction pH and acid stability of the wild-type enzyme and mutants III/IV were determined. The results are shown in Figure 3A–C. The optimal pHs for the wild-type enzyme and mutants III/IV were 5.0, 3.5 and 4.0, respectively, which indicated that the mutants had higher catalytic activity than the wild-type enzyme under more acidic conditions. It is worth mentioning that when the pH was 3.0, the relative activity of the wild-type enzyme was only 38.54%, while the relative activity of the mutant enzymes exceeded 60%, especially mutant III, whose relative enzyme activity even reached 86.54%. Nielsen et al. replaced phenylalanine (Phe) with alanine (Ala) at position 290 of  $\alpha$ -glucosidase from *Chimeric Bacillus* Ba2. Since the side chain of Phe has a larger size and a different charge distribution compared to that of Ala, this structural change may affect the electrostatic interactions near the enzyme's active center, which in turn changes the enzyme's pH activity distribution [23]. Shi et al. performed a targeted mutation of GadB1 in *Lactobacillus brevis* Lb85 and obtained mutant T17I-D294G-Q346H and mutant E312S, and the combination of these two mutations altered the optimal pH of mutant T17I-D294G-Q346H-E312S compared to the wild-type enzyme [24]. Additionally, the trends of the curves in Figure 3A–C were largely similar. After being incubated in various pH buffers for 12 h, the catalytic activities of the three BGLs remained close to their initial values, indicating that all three enzymes exhibited good acid stability.



**Figure 3.** (A) The optimum pH and acid stability of the wild-type enzyme; (B) the optimum pH and acid stability of mutant III; (C) the optimum pH and acid stability of mutant IV; (D) effect of glucose on enzyme activity; (E) effect of sucrose on enzyme activity; (F) effect of fructose on enzyme activity. The optimum pH was obtained at the optimum temperature for both the wild-type enzyme and mutants. The results of the effects of different sugars on enzyme activity were obtained under the optimum temperature and optimum pH for both the wild-type enzyme and mutants.

### 2.2.3. Effects of Different Sugars on Enzyme Activity

As the enzymatic products of BGLs, glucose and its analogues can inhibit the activity of BGLs to varying degrees [25]. Consequently, the impact of three sugars (glucose, sucrose and fructose) on enzyme activity was examined. As shown in Figure 3D–F, all three sugars had inhibitory effects on enzyme activity. Specifically, the relative activities of mutants III/IV at different glucose concentrations were essentially the same, whereas the relative activity of the wild-type enzyme was lower than that of the mutants. With the increase of glucose concentration, the activity of all three enzymes significantly decreased. When the glucose concentration was 10%, the enzyme activities of the wild-type and mutants III/IV were maintained at 18.35%, 23.75% and 28.03%, respectively. It has been reported that the relative binding position of glucose within the channels of the catalytically active center of BGL is a key reason for the regulation of glucose tolerance. When glucose is present in the reaction environment, glucose competes with substrate molecules for the catalytic activity center of BGL, resulting in a decrease in the enzymatic activity of BGL. Compared with the wild-type, the mutants have a deeper and narrower substrate-binding channel that prevents glucose from entering the catalytically active center of the enzyme [26], so the mutants III/IV have higher enzyme activity and higher glucose tolerance than the wild-type enzyme. It has also been reported that the hydrophobicity and spatial position of the channel entrance at the active site also play an important role in glucose tolerance [27]. Sun et al. mutated the phenylalanine residue at position 171 in BGLA to a tryptophan residue, resulting in BGLA (F171W) exhibiting higher glucose tolerance than wild-type BGLA [28]. When sucrose was introduced into the reaction environment, the relative activity of the three enzymes decreased significantly with the increase of sucrose concentration. When the sucrose concentration was increased to 10%, the enzyme activity of the wild-type enzyme and mutants III/IV remained at 35%, 30% and 21.71%, respectively. When the fructose

concentration increased from 0 to 10%, the relative activity of the wild-type enzyme showed a downward trend, and the relative activity decreased to 28.75%. However, the relative activity of mutants III/IV firstly showed a slight increase when the fructose concentration rose from 2% to 4%, and then decreased. When the fructose concentration was at 10%, the relative enzyme activity of mutants III/IV (31.82% and 29.68%, respectively) was still higher than that of the wild-type enzyme. Although glucose, sucrose and fructose belong to different types of sugar categories, their structures exhibit certain similarities. Like glucose, when sucrose and fructose are present in the environment, they can compete with substrate molecules for the active site of  $\beta$ -glucosidase, thereby exerting an inhibitory effect on the catalytic activity of  $\beta$ -glucosidase. In summary, the wild-type enzyme demonstrated better tolerance to sucrose compared to mutants III/IV, while mutants III/IV exhibited greater tolerance to glucose and fructose than the wild-type enzyme.

#### 2.2.4. Effects of Metal Ions and Some Additives on Enzyme Activity

In the application process of BGLs, the presence of metal ions and some additives will greatly affect the catalytic activity of the enzyme. These metal ions and additives have a wide range of sources, such as soil, water and fertilizers [29]. Figure 4 and Table 1 showed the effects of different metal ions and additives on enzyme activity, and their semi-inhibitory concentration ( $IC_{50}$ ). As depicted in Figure 4, at a concentration of 5 mM,  $K^+$  enhanced the enzyme activity of both the wild-type enzyme and mutant III, but strongly inhibited mutant IV, with an inhibition rate of 85.02%.  $Na^+$  increased the activity of the wild-type enzyme and mutant IV, while inhibiting mutant III at a rate of 97.45%.  $Mn^{2+}$  inhibited the wild-type enzyme's activity by 76.51%, but promoted the activity of mutants III and IV. Additionally,  $Li^+$  and  $Ba^{2+}$  were the only metal ions that enhanced the activity of both the wild-type enzyme and mutants III and IV. As for other metal ions, such as  $Ag^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Fe^{2+}$  and  $Fe^{3+}$ , they all had inhibitory effects on the enzyme activity of the three BGLs. Various additives such as EDTA, SDS, DTT, Triton X-100 and Tween-80 also inhibited catalytic activity to different extents, with Tween-80 exhibiting the strongest inhibition and SDS the weakest. The SDS molecule contains  $ROSO_3^-$ , which has high electronegativity and can interact with the hydrophobic cavity formed by amino acid residues, creating competitive antagonism with the substrate and significantly reducing BGL catalytic activity. In contrast, Triton X-100 has a  $-(CH_2-CH_2-O)_n$ -hydrophobic chain that can also bind to the hydrophobic cavities and compete with the substrate, leading to decreased catalytic activity. Tween-80 and DTT, as a non-ionic surfactant and metal ion protease inhibitor, can effectively inhibit the formation of disulfide bonds on protein surfaces, thereby reducing enzyme activity. Furthermore, Tween-80 can disrupt intramolecular and intermolecular hydrogen bonds, causing protein unfolding and altering its secondary and tertiary structures, ultimately inhibiting enzyme activity.

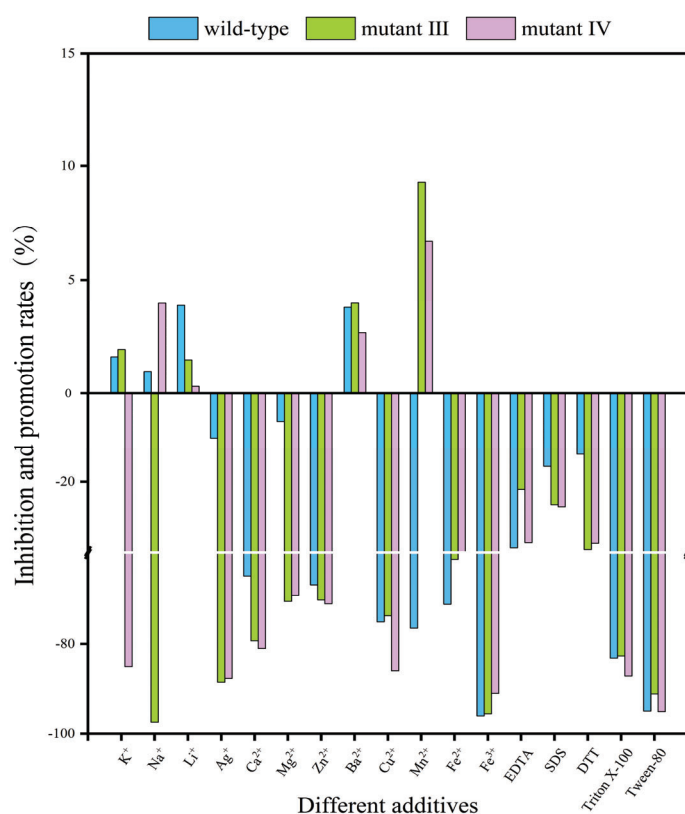
**Table 1.**  $IC_{50}$  of different metal ions and additives on the wild-type enzyme and mutants.

| Additives | Concentration (mM) | Wild-Type Enzyme | Mutant III     | Mutant IV      |
|-----------|--------------------|------------------|----------------|----------------|
|           |                    | $IC_{50}$ (mM)   | $IC_{50}$ (mM) | $IC_{50}$ (mM) |
| $K^+$     | 5                  | Activator        | Activator      | 2.42           |
| $Na^+$    | 5                  | Activator        | 0.929          | Activator      |
| $Li^+$    | 5                  | Activator        | Activator      | Activator      |
| $Ag^+$    | 5                  | 7.43             | 3.03           | 2.93           |
| $Ca^{2+}$ | 5                  | 4.09             | 3.38           | 3.28           |
| $Mg^{2+}$ | 5                  | 8.92             | 3.37           | 5.35           |
| $Zn^{2+}$ | 5                  | 3.87             | 3.19           | 2.94           |

Table 1. Cont.

| Additives        | Concentration (mM) | Wild-Type Enzyme      | Mutant III            | Mutant IV             |
|------------------|--------------------|-----------------------|-----------------------|-----------------------|
|                  |                    | IC <sub>50</sub> (mM) | IC <sub>50</sub> (mM) | IC <sub>50</sub> (mM) |
| Ba <sup>2+</sup> | 5                  | Activator             | Activator             | Activator             |
| Cu <sup>2+</sup> | 5                  | 3.12                  | 3.48                  | 1.58                  |
| Mn <sup>2+</sup> | 5                  | 3.76                  | Activator             | Activator             |
| Fe <sup>2+</sup> | 5                  | 2.83                  | 3.96                  | 4.43                  |
| Fe <sup>3+</sup> | 5                  | 0.31                  | 2.81                  | 2.42                  |
| EDTA             | 5                  | 6.41                  | 6.89                  | 7.16                  |
| SDS              | 5                  | 7.89                  | 6.72                  | 6.29                  |
| DTT              | 5                  | 7.93                  | 5.49                  | 6.17                  |
| Triton-X100      | 0.2%               | -                     | -                     | -                     |
| Tween-80         | 0.2%               | -                     | -                     | -                     |

IC<sub>50</sub> represents the half inhibitory concentration. ("—" represents no enzymatic activity).



**Figure 4.** The effects of metal ions and some additives on the activity of the wild-type enzyme and mutants III/IV. The results were obtained under the conditions of the optimum temperature and optimum pH for both the wild-type enzyme and mutants.

#### 2.2.5. Analysis of Enzymatic Reaction Kinetic Constants and Substrate Specificity

The kinetic parameters of the wild-type enzyme and its mutants catalyzing on four kinds of substrates are shown in Table 2. Overall, all three BGLs were able to catalyze these substrates, but the kinetic parameters of the reactions were obviously different. The parameter  $V_{\max}$  reflects the maximum reaction rate when the enzyme catalyzes the substrate, and the  $V_{\max}$  of *p*NP- $\beta$ -glu catalyzed by the three BGLs was greater than those of the other three kinds of substrates. The  $V_{\max}$  of the mutants III/IV in catalyzing three substrates (*p*NP- $\beta$ -glu, *p*NP- $\beta$ -gal and *p*NP- $\beta$ -xyl) were all larger than that of the wild-type enzyme, indicating that the mutants had a higher catalytic efficiency.  $K_m$  represents the affinity

between enzymes and substrates, i.e., the smaller the  $K_m$  value, the greater the affinity. In this study, the three enzymes had the highest affinity with substrate *p*NP- $\beta$ -glu, with  $K_m$  values all less than  $0.33 \pm 0.03$  mM. Additionally, the three enzymes had the lowest affinity with substrate *p*NP- $\beta$ -xyl, with the highest  $K_m$  value reaching  $1.31 \pm 0.22$  mM. In addition, mutations increased the affinity of the enzyme to the substrates *p*NP- $\beta$ -glu and *p*NP- $\beta$ -xyl, while decreasing the affinity to substrate *p*NP- $\alpha$ -glu. Similar findings were reported by Tonin et al., who noted that targeted mutations in hydroxysteroid dehydrogenases led to decreased substrate affinity in some mutants [30], aligning with our results. The specificity constant,  $K_{cat}/K_m$ , helps identify the most suitable substrate when an enzyme can catalyze multiple substrates. As shown in Table 2, the  $K_{cat}/K_m$  value for *p*NP- $\beta$ -glu was the highest for both the wild-type enzyme and mutants III/IV, indicating that *p*NP- $\beta$ -glu is the most suitable substrate for all three enzymes.

**Table 2.** Kinetic parameters of the wild-type enzyme and mutants catalyzing four kinds of substrates.

| Substrates                 | Enzyme           | $V_{max}$<br>( $\mu$ M/min/mg) | $K_m$ (mM)        | $K_{cat}$ ( $S^{-1}$ ) | $K_{cat}/K_m$<br>( $S^{-1} \cdot mM^{-1}$ ) |
|----------------------------|------------------|--------------------------------|-------------------|------------------------|---------------------------------------------|
| <i>p</i> NP- $\beta$ -glu  | wild-type enzyme | $363.46 \pm 4.13^c$            | $0.33 \pm 0.03^c$ | $333.99 \pm 3.80^c$    | $1012.09^c$                                 |
|                            | mutant III       | $532.82 \pm 6.51^b$            | $0.27 \pm 0.05^b$ | $489.77 \pm 5.98^b$    | $1813.96^b$                                 |
|                            | mutant IV        | $841.65 \pm 3.82^a$            | $0.22 \pm 0.03^a$ | $773.64 \pm 3.52^a$    | $3516.54^a$                                 |
| <i>p</i> NP- $\beta$ -gal  | wild-type enzyme | $230.97 \pm 5.71^c$            | $0.81 \pm 0.16^b$ | $212.24 \pm 5.25^c$    | $262.02^c$                                  |
|                            | mutant III       | $349.18 \pm 4.83^a$            | $0.52 \pm 0.17^a$ | $320.97 \pm 4.44^a$    | $617.25^a$                                  |
|                            | mutant IV        | $257.38 \pm 5.47^b$            | $0.87 \pm 0.24^c$ | $236.85 \pm 5.03^b$    | $272.24^b$                                  |
| <i>p</i> NP- $\beta$ -xyl  | wild-type enzyme | $307.72 \pm 3.64^c$            | $1.31 \pm 0.22^c$ | $282.77 \pm 3.34^c$    | $215.85^c$                                  |
|                            | mutant III       | $337.28 \pm 4.61^b$            | $1.24 \pm 0.13^b$ | $310.03 \pm 4.24^b$    | $250.02^b$                                  |
|                            | mutant IV        | $358.83 \pm 3.88^a$            | $1.17 \pm 0.16^a$ | $330.21 \pm 3.57^a$    | $282.23^a$                                  |
| <i>p</i> NP- $\alpha$ -glu | wild-type enzyme | $29.61 \pm 1.83^a$             | $1.07 \pm 0.20^a$ | $27.21 \pm 1.68^a$     | $25.43^a$                                   |
|                            | mutant III       | $25.81 \pm 2.06^b$             | $1.19 \pm 0.23^b$ | $23.73 \pm 1.89^b$     | $19.94^b$                                   |
|                            | mutant IV        | $19.93 \pm 1.98^c$             | $1.27 \pm 0.19^c$ | $18.35 \pm 1.82^c$     | $14.45^c$                                   |

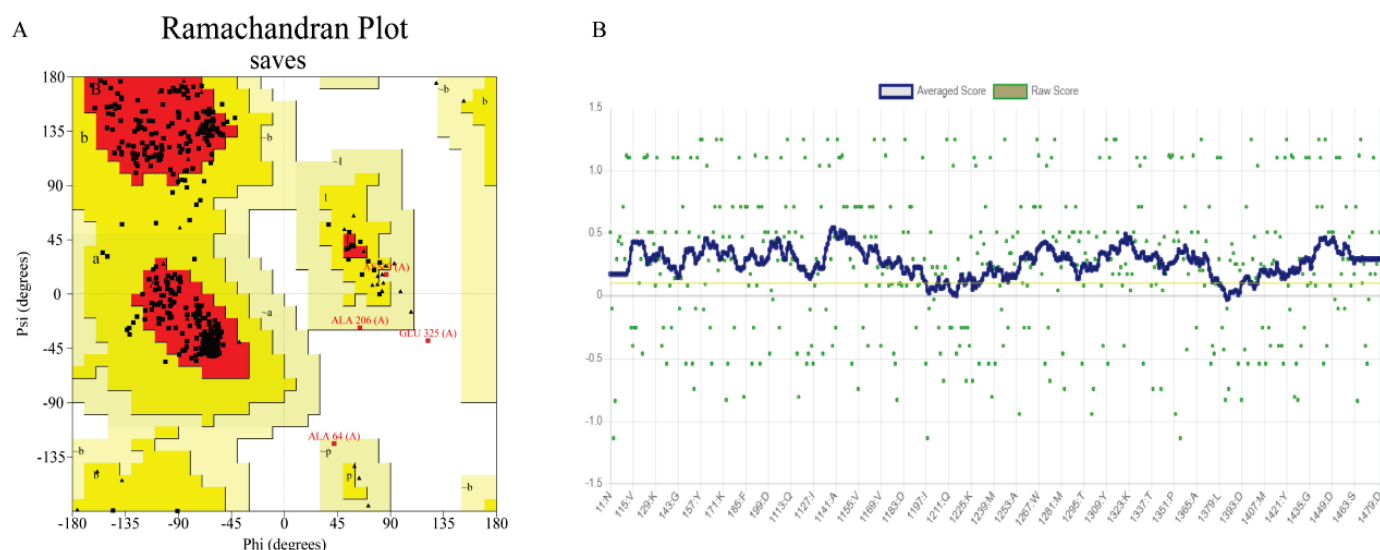
In columns, for the same substrate, means with the different lowercase superscripted letters indicate a significant difference ( $p < 0.05$ ). Data are presented as mean  $\pm$  standard deviation ( $n = 3$ ).

### 2.3. Mechanism Analysis of Enzymatic Property Change

Some special sites in the amino acid structure of the enzyme are the key to its high catalytic activity, such as hydrogen bonding [31], hydrophobic bonding [32], salt bridges [33], aromatic–aromatic, cation–aromatic [34] and other non-covalent and disulfide bridges connecting peptide chains [35]. It is essential to deeply analyze the connection between enzyme activity and structure to optimize enzyme function. In this research, we initially predicted the isoelectric points, molecular weights, instability coefficients and other characteristics of the three BGLs. The findings (Table S3) indicated that there were minor differences in the pI and molecular weight among the three BGLs. All three were identified as stable proteins (with stability factors below 40), with mutant IV exhibiting the highest stability, followed by mutant III. In contrast, the wild-type enzyme demonstrated the lowest stability compared to the mutants.

A search of the PDB database revealed that the crystal structure of  $\beta$ -glucosidase from *Lactobacillus plantarum* (ID: 3QOM) showed a high degree of sequence identity (51.00%) with the wild-type enzyme. Therefore, the crystal structure of 3QOM is a good template for constructing a 3D structural model of the wild-type enzyme. The results of the homology modeling and the reasonableness assessment of the model are shown in the form of a “Ramachandran plot” (Figure 5A). The amino acid residues located in the stereochemically permissive region reached 99.80%, indicating that 99.80% of the amino acid residues in the

three-dimensional structure of the wild-type enzyme had a spatial conformation within a reasonable range, which was in accordance with the energy law of stereochemistry. The compatibility between the 3D structural model of the wild-type enzyme and its own amino acid sequence was assessed by the Verify 3D program (<https://www.doe-mbi.ucla.edu/verify3d/>, (accessed on 3 November 2024)). As shown in Figure 5B, the percentage of amino acid residues with scores above 0.1 was 92.07%, which was in line with the requirements of the evaluation program. Taken together, the 3D structural model of the wild-type enzyme was highly reliable and stable, and can be used in the subsequent molecular docking process.



**Figure 5.** Quality evaluation of the 3D model of the wild-type enzyme. (A) Ramachandran plot (Black represents amino acid residues, red represents the core region, yellow represents the allowed region, and white represents the not allowed region. Residues in most favoured regions (A, B, L), Residues in additional allowed regions (a, b, l, p), Residues in generously allowed regions (~a, ~b, ~l, ~p)); (B) Verify 3D score (Yellow lines represent 0.1 standard lines).

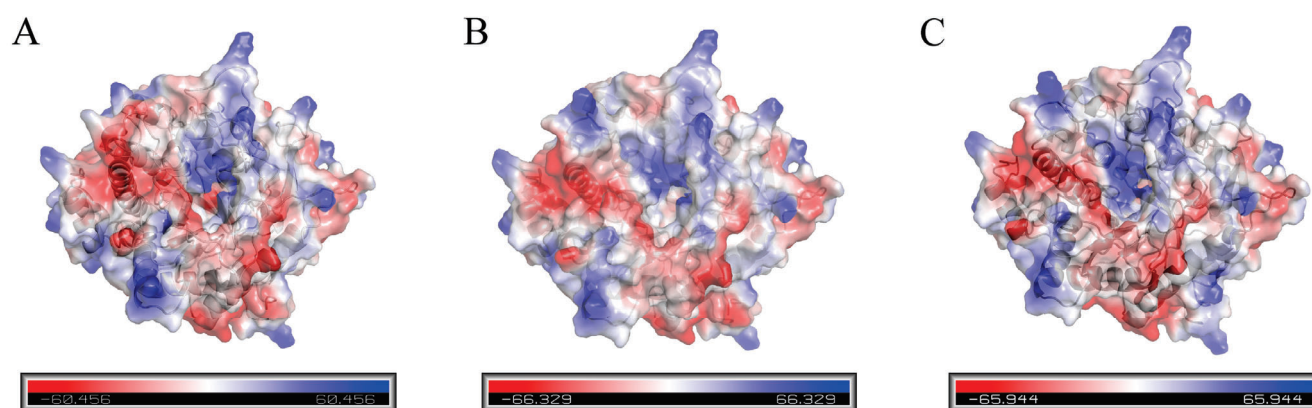
The number of salt bridges between the wild-type enzyme and mutants before and after binding to the substrate were analyzed using Protein Tools ([www.proteintools.uni-bayreuth.de](http://www.proteintools.uni-bayreuth.de), (accessed on 3 April 2025)), and the results are shown in Table 3. The number of salt bridges of the wild-type enzyme and mutant III/IV were 22, 18 and 18, respectively, and the number of salt bridges after binding to the substrate *p*-NPG remained the same, but mutant III/IV had one salt bridge connected to the substrate *p*-NPG, which may make mutant III/IV have an increased affinity with the substrate *p*-NPG, thus enhancing their catalytic activity [33].

**Table 3.** Comparison of the number of salt bridges between the wild-type enzyme and mutants.

| Different Enzyme          | Salt Bridge | Salt Bridge Connected to <i>p</i> -NPG |
|---------------------------|-------------|----------------------------------------|
| wild-type                 | 22          | -                                      |
| mutant III                | 18          | -                                      |
| mutant IV                 | 18          | -                                      |
| wild-type- <i>p</i> -NPG  | 22          | 0                                      |
| mutant III- <i>p</i> -NPG | 18          | 1                                      |
| mutant IV- <i>p</i> -NPG  | 18          | 1                                      |

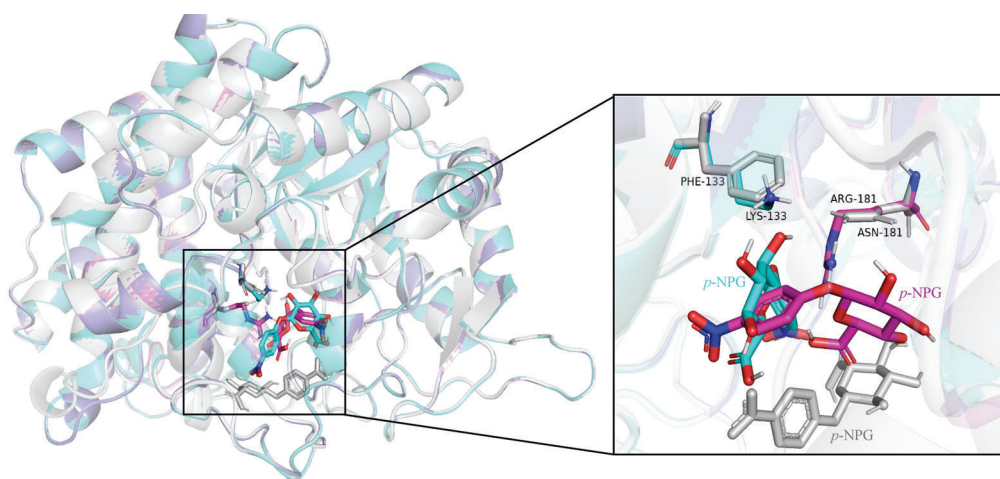
The surface electrostatic potential of the wild-type enzyme and mutants were analyzed by PyMOL, and the results are shown in Figure 6. The Phe133 and Asn181 of the wild-type

enzyme were replaced by the high-potential amino acids Lys133 and Arg181, respectively. The surface electrostatic potential of mutant III/IV changed and was significantly higher than that of the wild-type. Studies have shown that the change of the electrostatic potential on the surface of the protease will change the stability of the enzyme [36], and the mutation leads to the increase of the electrostatic charge on the surface of the  $\beta$ -glucosidase, which makes the surface of the  $\beta$ -glucosidase protein easier to form a hydrated film and increases the solubility of the  $\beta$ -glucosidase. The rigidity of the C-terminal and N-terminal structures of the mutants has also been significantly enhanced, thereby delaying the expansion of the flexible region and improving the stability of the mutants III/IV [37].



**Figure 6.** Analysis of the surface electrostatic potential of the wild-type enzyme and mutants. ((A) Wild-type enzyme; (B) mutant III; (C) mutant IV).

It can be seen from Figure 7 that, compared with the wild-type enzyme, after the mutant III/IV binds to the substrate *p*-NPG, *p*-NPG can penetrate deeper into the center of the active pocket and enhance the affinity of the mutants with the substrate.

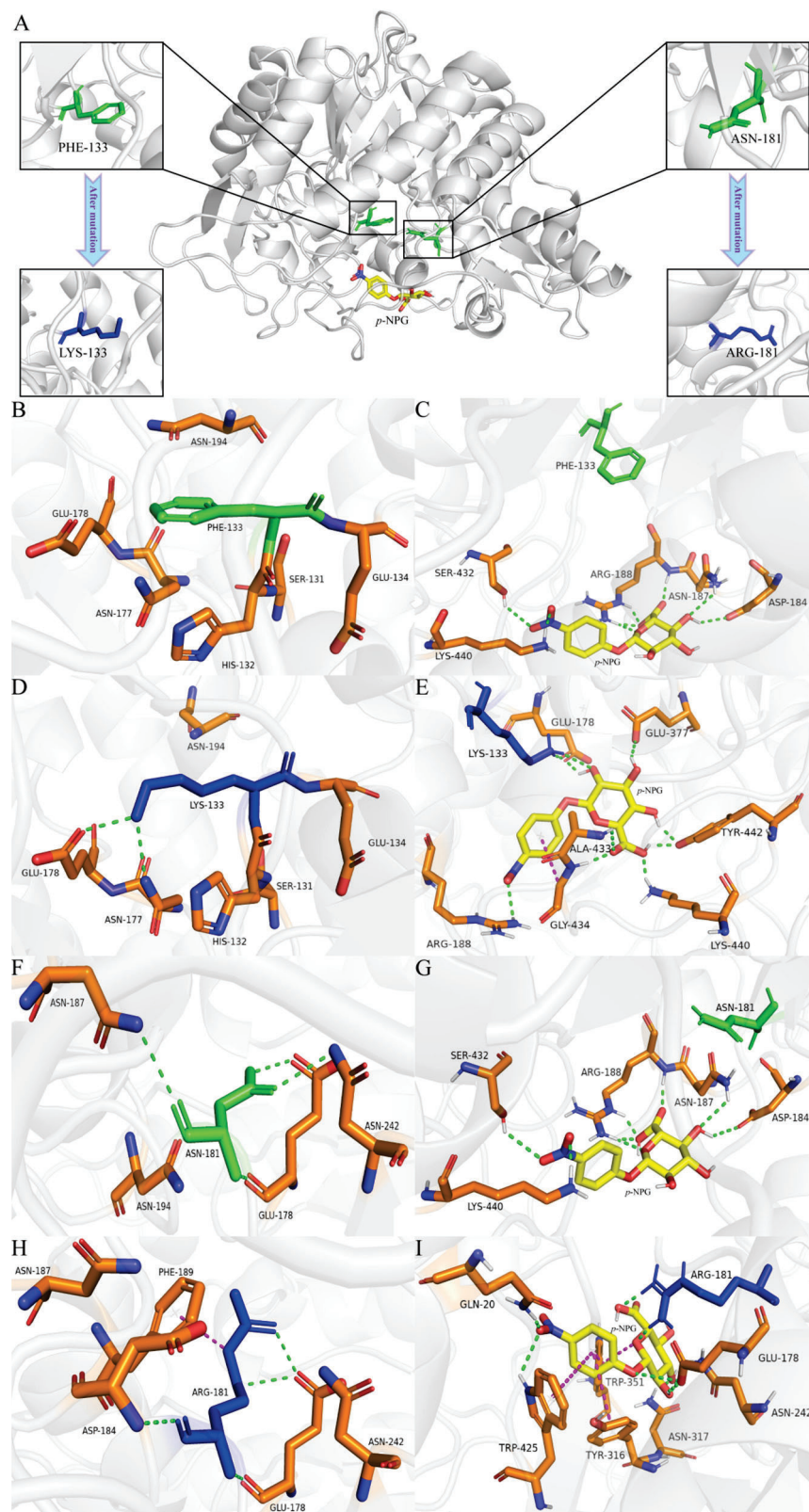


**Figure 7.** Comparative effect of superposition of substrate binding sites in the wild-type enzyme and mutant active sites (gray: wild-type enzyme; cyan: mutant III; purple: mutant IV).

To thoroughly investigate the mechanisms behind the enhancement of enzyme activity and stability, we analyzed the alterations in the microenvironment surrounding the mutation site through molecular simulations. The results are shown in Figure 8. The distances of the mutated amino acid residues from the surrounding critical amino acid residues of the wild-type and mutants were measured using PyMOL. For the wild-type enzyme, the distances of F133 from the critical amino acids S131, H132, E134, N177, E178 and N194 are 6.3 Å, 5.0 Å, 4.4 Å, 7.3 Å, 8.9 Å and 5.8 Å, respectively, and the distances between N181

and the key amino acids E178, N187, N194 and N242 are 5.6 Å, 7.4 Å, 5.8 Å and 5.4 Å, respectively. The distances between K133 of mutant III and the key amino acids S131, H132, E134, N177, E178 and N194 are 5.0 Å, 5.0 Å, 4.4 Å, 7.3 Å and 8.9 Å, respectively, and the distances of R181 of mutant IV from the key amino acids E178, D184, N187, F189 and N242 are 3.6 Å, 5.1 Å, 7.2 Å, 3.9 Å and 4.5 Å, respectively. Compared to the wild-type enzyme, the mutated LYS133 and ARG181 showed reduced distances to key amino acids. It can be seen from Figure 8A that F133 and N181 of the wild-type enzyme are located in the center of the catalytic active pocket, indicating that the two amino acids are important for the wild-type enzyme to exert catalytic activity. The substrate molecule *p*-NPG is located in the edge region of the active pocket. The F133 of the wild-type BGL (Figure 8B) and its surrounding amino acids rely on van der Waals forces to maintain the spatial structure without hydrogen bonding. The F133 contains a benzene ring, which may lead to steric hindrance and exclude *p*-NPG from the catalytic activity pocket, thereby affecting the activity of BGL [14]. Mutations may lead to changes in the ionization state and charge state of amino acid residues, which in turn affect the interaction with other amino acid residues and the substrate, thereby affecting the conformational stability, structural stability and catalytic activity [38]. When the substrate *p*-NPG was present (Figure 8C), it formed eight hydrogen bonds with D184, N187, R188, S432 and K440 in the edge region of the catalytically active pocket, and did not form hydrogen bonds with F133. After the non-polar and hydrophobic F133 was mutated to the polar and hydrophilic K133 (Figure 8D), K133 formed a hydrogen bond with its surrounding amino acid residue E178. This change in polarity and hydrophobicity enhances the hydrophilicity at the center of the catalytic pocket, facilitating substrate binding and product release [39]. The increased polarity of the amino acids boosts the affinity between BGL and *p*-NPG, allowing the substrate to penetrate further into the catalytic pocket and achieve better geometric compatibility with BGL. Additionally, the loss of the benzene ring reduces steric hindrance and increases flexibility, further enhancing enzyme catalytic activity [40]. After the mutation, *p*-NPG forms two hydrogen bonds with the crucial amino acid K133 (Figure 8E) and establishes eight hydrogen bonds along with one  $\pi$ - $\pi$  aromatic interaction with the surrounding residues E178, R188, E377, A433, G434, K440 and Y442. Compared to the wild-type, this results in two additional lattice hydrogen bonds and one  $\pi$ - $\pi$  aromatic interaction between the enzyme and substrate, which promotes their binding and improves enzyme activity [14].

The N181 of the wild-type enzyme formed four hydrogen bonds with its surrounding amino acid residues E178, N187 and N242 (Figure 8F). In the presence of the substrate *p*-NPG (Figure 8G), it established hydrogen bonds with amino acids in the catalytic activity pocket of the enzyme, but not with N181. After N181 was mutated to R181 (Figure 8H), the side chain of the amino acid became longer, resulting in the formation of four hydrogen bonds with E178 and D184, as well as a  $\pi$ - $\pi$  aromatic interaction with F189. The *p*-NPG could enter the center of the catalytic activity pocket more deeply, and formed two hydrogen bonds with R181 of the mutant BGL, and formed six hydrogen bonds and four  $\pi$ - $\pi$  aromatic interactions with Q20, E178, N242, Y316, N317, W351 and W425 around it (Figure 8I). After mutation, the number of  $\pi$ - $\pi$  aromatic interactions increased by three compared to the wild-type enzyme, while the number of hydrogen bonds remained the same. However, two hydrogen bonds were now directly linked to the crucial amino acid R181 of the mutant BGL, which strengthened the binding affinity of the mutant BGL for the substrate *p*-NPG and improved its catalytic stability [38].



**Figure 8.** (A) The 3D protein model and mutation site information of the wild-type enzyme; (B) hydrogen bonding interactions between the F133 site and its surrounding amino acids in the wild-type enzyme; (C) hydrogen bonding interactions between *p*-NPG and the surrounding amino acid residues after molecular docking between the wild-type enzyme and *p*-NPG; (D) hydrogen bonding interaction between the K133 site and the surrounding amino acids of mutant III; (E) hydrogen bonding and  $\pi$ - $\pi$  interactions between *p*-NPG and the surrounding amino acid residues after molecular docking between mutant III and *p*-NPG; (F) hydrogen bonding interaction between the N181

site and the surrounding amino acids of the wild-type enzyme; (G) hydrogen bonding interactions between *p*-NPG and the surrounding amino acid residues after molecular docking between the wild-type enzyme and *p*-NPG; (H) hydrogen bonding and  $\pi$ - $\pi$  interaction between the R181 site and the surrounding amino acids of mutant IV; (I) hydrogen bonding and  $\pi$ - $\pi$  interactions between *p*-NPG and the surrounding amino acid residues after molecular docking between mutant IV and *p*-NPG. (The green dashed line represents a hydrogen bond, and the purple dashed line represents a  $\pi$ - $\pi$  interaction).

### 3. Materials and Methods

#### 3.1. Bacterial Strains and Materials

*Oenococcus oeni* SD-2a was preserved at the college of food and bioengineering, Henan University of Science and Technology. Competent *E. coli* DH5 $\alpha$  was purchased from Sangon Biotech (Shanghai, China). *Lactococcus lactis* MG1363 was purchased from Changsha Fenghui Biotechnology (Changsha, China). The pMG36e plasmid was purchased from HonorGene (Changsha, China), and the SpeedyCut enzyme, DNA polymerase and Ni-IDA 6FF Sefinose (TM) Resin Kit were purchased from Sangon Biotech (Shanghai, China).

*p*-Nitrophenyl- $\beta$ -D-glucopyranoside (*p*-NPG/*p*NP- $\beta$ -glu), *p*-Nitrophenyl- $\beta$ -D-galactoside (*p*NP- $\beta$ -gal), *p*-Nitrophenyl- $\beta$ -D-xylopyranoside (*p*NP- $\beta$ -xyl) and *p*-Nitrophenyl- $\alpha$ -D-glucopyranoside (*p*NP- $\alpha$ -glu) were purchased from Yuanye Bio-Technology (Shanghai, China), and M17 broth medium was purchased from Aobox Bio-tech (Beijing, China). Chemicals with purity  $\geq 98\%$  were purchased from Solarbio Life-Science (Beijing, China), mainly including sodium chloride (NaCl), sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), peptone, glucose, potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), sodium acetate (NaAc), magnesium sulfate heptahydrate (MgSO<sub>4</sub>·7H<sub>2</sub>O), disodium hydrogen phosphate and anhydrous ethanol.

#### 3.2. Prediction of Mutation Sites

$\beta$ -glucosidase BGL0224 (MT330371.1) from *Oenococcus oeni* SD-2a obtained in our previous research was used as the wild-type enzyme [3]. The protein sequence of the wild-type enzyme was entered into the PDB protein database for comparison. Normally, when the sequence identity between the crystal structure and the target protein exceeds 30%, the known crystal structure can be used as a template to construct a 3D structure model of the target protein. Homology modeling was performed using SWISS-MODEL (<https://swissmodel.expasy.org/interactive>, (accessed on 11 October 2024)), and the quality of the model was evaluated and characterized using a Ramachandran plot (<https://saves.mbi.ucla.edu/>, (accessed on 4 November 2024)) and the Verify 3D program (<https://www.doe-mbi.ucla.edu/verify3d/>, (accessed on 3 November 2024)) for subsequent molecular docking. The 3D structure of wild-type BGL was obtained as the receptor structure of molecular docking. The structure of the substrate was constructed and hydrogenated. The structure was optimized using the MOPAC 2016 program to calculate the atomic charge of PM3. Finally, Autodock Tools 1.5.6 was used to deal with the structure of the ligand and receptor, and the docking box was used to wrap the active site. The number of grid points in the XYZ direction was set to 60  $\times$  60  $\times$  60, the grid spacing was 0.375 Å, the number of dockings was set to 100, and the remaining parameters were used as default values. The Calculate Mutation Energy (Binding) module in the Discovery Studio 2019 software was used to scan all the residues of 5 Å near the *p*-NPG binding pocket.

#### 3.3. Construction of Recombinant Plasmid pMG36e-0224 and Its Mutants

The gene of BGL0224 was amplified by PCR using the genomic DNA of *Oenococcus oeni* SD-2a as the template. The purified PCR product and linearized plasmid vector were ligated by T4 DNA ligase to connect the BGL0224 gene fragment to the pMG36e linearized

plasmid vector [41]. The recombinant plasmid with the correct sequence was named pMG36e-0224. Based on the rapid site-directed mutagenesis strategy, the recombinant plasmid pMG36e-0224 was used as a template for whole plasmid PCR to construct different mutants. The mutation primers were designed by Primer X ([https://www.bioinformatics.org/primerx/cgi-bin/DNA\\_1.cgi](https://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi), (accessed on 10 August 2024)). The primer sequences are shown in Table S1. The PCR procedure was as follows: pre-denaturation at 95 °C for 10 min, 97 °C denaturation for 10 s, 56–69 °C annealing for 15 s, 70 °C extension for 60 s, for 35 cycles, and finally, 4 °C heat preservation. The accuracy of the mutation results were verified by sequencing. Next, the wild-type and mutant plasmids were electro-transformed into *Lactococcus lactis* MG1363 for expression and purification [42]. The purified wild-type and mutant target proteins were detected by SDS-PAGE.

#### 3.4. Determination of Enzyme Activity

The determination of enzyme activity was conducted according to Dong et al., with slight modifications [43]. First, 10 µL BGLs (10 mg/mL) were added to 490 µL 25 mM *p*-Nitrophenyl-β-D-glucopyranoside (*p*-NPG) prepared with 20 mM sodium phosphate buffer (pH 5.0). After incubation at 37 °C for 30 min, 500 µL 1 M Na<sub>2</sub>CO<sub>3</sub> solution were added to terminate the reaction. The absorbance was measured at 420 nm, and the *p*-Nitrophenol (*p*-NP) released from the system was determined. The concentration of *p*-NP was calculated according to the standard curve. Under the experimental conditions, the amount of 1 µM *p*-NP produced per minute was defined as a unit of BGL enzyme activity (U).

#### 3.5. Determination of Optimal Temperature and Thermal Stability

The optimum temperature and temperature stability of the enzyme were determined with reference to Liu et al., with slight modifications [44]. The lyophilized powders of the wild-type and mutant BGLs were dissolved in 20 mM sodium phosphate buffer (pH 5.0) to give a final concentration of 10 mg/mL. Then, 10 µL of enzyme solution were added to 490 µL of 25 mM *p*-NPG substrate solution configured with sodium phosphate buffer and mixed well. The enzyme reaction was carried out at a temperature range of 30–70 °C (with a gradient of 5 °C) for 30 min. After the reaction was completed, 500 µL of 1 M Na<sub>2</sub>CO<sub>3</sub> solution were added to develop the color, and the absorbance value of the mixture was measured at 420 nm, and the activity of BGL was calculated according to the standard curve. Thermal stability was determined by reacting the above 500 µL of enzyme–substrate mixture at a temperature range of 30–70 °C for 6 h. Then, the activity of BGL was measured at 1 h, 2 h, 3 h, 4 h, 5 h and 6 h, and the rest of the conditions remained unchanged.

#### 3.6. Determination of Optimal pH and Acid Stability

The optimum pH and pH stability of the enzyme were determined with reference to Capaldo et al., with slight modifications [4]. The lyophilized powders of the wild-type and mutant BGLs were dissolved in 20 mM sodium phosphate buffer (pH range 1.0–6.5, gradient 0.5) at different pHs to give a final concentration of 10 mg/mL. Then, 10 µL of enzyme solution were added to 490 µL of 25 mM *p*-NPG substrate solution configured with sodium phosphate buffer (pH range 1.0–6.5 with a gradient of 0.5) and mixed well. The enzyme reaction was carried out at the optimal temperature for 30 min, 500 µL of 1 M Na<sub>2</sub>CO<sub>3</sub> solution were added at the end of the reaction, and the absorbance value of the mixture was measured at 420 nm, and the activity of BGL was calculated according to the standard curve. Acid stability was determined by reacting the above 500 µL of enzyme–substrate mixture for 12 h at 4 °C, and then the activity of BGL was measured. The rest of the conditions were unchanged.

### 3.7. Determination of Different Sugars on Enzyme Activity

The method for determining the effect of different sugars on enzyme activity referred to Zhao et al., with slight modifications [2]. To determine the effects of different types and concentrations of sugars on BGL enzyme activity, glucose, sucrose and fructose were configured into solutions with concentrations of 0–10% (on a 2% gradient) using 20 mM sodium phosphate buffer. The lyophilized powder of the wild-type and mutant BGLs was dissolved in the prepared sodium phosphate buffer with different concentrations of the three sugar solutions, and the final concentration was 10 mg/mL. A *p*-NPG substrate solution (25 mM) was also prepared with the prepared sodium phosphate buffer with different concentrations of the three sugar solutions. Then, 10  $\mu$ L of enzyme solution were added to 490  $\mu$ L of substrate solution and mixed evenly. The reaction lasted for 30 min under the optimal reaction conditions of the BGLs. Then, 500  $\mu$ L 1 M of  $\text{Na}_2\text{CO}_3$  solution were added to terminate the reaction. Next, the absorbance value of the reaction system at 420 nm was measured, and the activity of the BGLs was calculated based on the standard curve.

### 3.8. Determination of Metal Ions and Some Additives on Enzyme Activity

The method for the determination of the effects of different metal ions and additives on enzyme activity was referred to Zhou et al., with slight modifications [45]. To determine the effects of metal ions and some additives on the BGLs' activity, lyophilized powders of the wild-type and mutant BGLs were formulated into solutions with a final concentration of 10 mg/mL using 20 mM sodium phosphate buffer as well as 25 mM of *p*-NPG substrate solution and different concentrations (0.1 mM, 0.5 mM, 1.0 mM, 5.0 mM and 10 mM) of metal ion solutions ( $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Li}^+$ ,  $\text{Ag}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ ) and additive solutions (SDS, DTT, EDTA, TritonX-100 and Tween-80). The enzyme solutions (10  $\mu$ L), *p*-NPG solution (490  $\mu$ L) and different concentrations of additive solutions (500  $\mu$ L) were mixed well. The reaction lasted for 30 min under the optimal reaction conditions of the enzyme. Then, 1 mL 1 M  $\text{Na}_2\text{CO}_3$  was added to terminate the reaction. The absorbance value of the reaction system was measured at 420 nm and the activity of the BGLs was calculated according to the standard curve. The  $\text{IC}_{50}$  (half of the maximum inhibitory concentration) of the reagents on BGL activity was calculated using a logistic function with Growth/Sigmoidal-type nonlinear fitting in Origin Pro 8.0.

### 3.9. Enzymatic Reaction Kinetics and Substrate Specificity

The determination of enzymatic reaction kinetics was conducted according to Zhang et al., with slight modifications [3]. Different concentrations (0.1 mM, 0.2 mM, 0.5 mM, 1.0 mM, 2.0 mM, 5.0 mM, 8.0 mM, 10 mM, 12 mM, 15 mM, 20 mM and 25 mM) of *p*-NPG substrate solutions were prepared with 20 mM sodium phosphate buffer. Then, 100  $\mu$ g of enzyme lyophilized powder were added to 500  $\mu$ L of *p*-NPG solutions at different concentrations. Next, this was reacted for 0 min, 5 min, 10 min, 15 min, 20 min, 25 min and 30 min under optimal reaction conditions, and at the end of each set of reactions, 500  $\mu$ L 1 M of  $\text{Na}_2\text{CO}_3$  solution were added to terminate the reaction. The absorbance value of each reaction system was measured at 420 nm and the amount of *p*-NP corresponding to different reaction times at specific substrate concentrations was calculated from the standard curve. The kinetic constants ( $V_{\max}$  and  $K_m$ ) of each reaction system were determined by fitting using the Hill function in the class of Growth/Inverse Curve for nonlinear fitting in the Origin Pro 8.0 software.

### 3.10. Mechanism Analysis

ExPASy (<https://web.expasy.org/protparam/>, accessed on 22 July 2024) was used to analyze physical and chemical indicators such as the molecular weight and isoelectric point (pI). Homology modeling of the mutant and wild-type BGLs was performed by the online prediction platform SWISS-MODEL (<https://swissmodel.expasy.org/interactive>, accessed on 11 October 2024). Autodock Tools 1.5.6 was used to dock the wild-type and mutant BGLs with the substrate *p*-NPG, and the parameter settings for the docking process referred to Section 2.2. The PyMOL 3.1.0 software was used for visual analysis.

### 3.11. Statistical Analysis

All experiments were set up in three parallels. The activities of the wild-type enzyme and five mutants were analyzed by ANOVA at the significance level of  $p < 0.05$  using the SPSS 26.0 software, and multiple comparisons of the means were performed using Duncan's method. The kinetic parameters of the enzymatic reactions for the wild-type enzyme and mutants catalyzing four substrates were subjected to independent sample *t*-tests at a significance level of  $p < 0.05$ , and the data were expressed as mean  $\pm$  standard deviation. The experimental results were plotted using the Origin 2021 software.

## 4. Conclusions

In this research, the critical amino acids in the catalytic activity pocket of BGL0224 were identified by binding energy prediction, and the dominant mutants III and IV were further obtained by site-directed mutation. Compared with the wild-type enzyme, the enzyme activities of the mutants were significantly increased under optimal reaction conditions, and the affinity for the substrate *p*-NPG was also enhanced. The mutants were more tolerant to low pH environments. In addition, when glucose and fructose, as well as certain metal ions, were present in the environment, the activities of both the wild-type enzyme and the mutants were inhibited. Molecular simulation results showed that the change of enzyme properties was due to the change of enzyme structure after mutation, mainly manifested as the introduction of new hydrogen bonds and  $\pi$ - $\pi$  bonds at the active site. This allows the substrate *p*-NPG to function deeper in the catalytic activity pocket, thus enhancing the binding ability between the enzyme and substrate, resulting in higher catalytic activity and stability exhibited by the mutant. Overall, this study expands the industrial applicability of *Oenococcus oeni*  $\beta$ -glucosidase.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms26093983/s1>.

**Author Contributions:** Methodology, Y.W.; software, P.T.; validation, Q.F.; investigation, H.M. and L.C.; data curation, Y.Z., P.L. and J.S.; writing—original draft, J.Z. (Junpeng Zuo); writing—review & editing, J.Z. (Jie Zhang) and S.G. All authors have read and agreed to the published version of the manuscript.

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## References

- Mallerman, J.; Papinutti, V.L.; Levin, N. Characterization of  $\beta$ -glucosidase produced by the white rot fungus *Flammulina velutipes*. *J. Microbiol. Biotech.* **2014**, *25*, 57–65. [CrossRef] [PubMed]
- Zhao, Y.; Zhang, B.; Gu, H.; Xu, T.; Chen, Q.; Li, J.; Zhou, P.; Guan, X.; He, L.; Liang, Y. A mutant GH3 family  $\beta$ -glucosidase from *Oenococcus oeni* exhibits superior adaptation to wine stresses and potential for improving wine aroma and phenolic profiles. *Food Microbiol.* **2024**, *119*, 104458. [CrossRef] [PubMed]
- Zhang, J.; Zhao, N.; Xu, J.; Qi, Y.; Wei, X.; Fan, M. Homology analysis of 35  $\beta$ -glucosidases in *Oenococcus oeni* and biochemical characterization of a novel  $\beta$ -glucosidase BGL0224. *Food Chem.* **2021**, *334*, 127593. [CrossRef]
- Capaldo, A.; Walker, M.; Ford, C.; Jiranek, V.  $\beta$ -Glucoside metabolism in *Oenococcus oeni*: Cloning and characterisation of the phospho- $\beta$ -glucosidase bglD. *Food Chem.* **2011**, *125*, 476–482. [CrossRef]
- Florindo, R.N.; Souza, V.P.; Manzone, L.R.; Camilo, C.M.; Marana, S.R.; Polikarpov, I.I.; Nascimento, A.S. Structural and biochemical characterization of a GH3  $\beta$ -glucosidase from the probiotic bacteria *Bifidobacterium adolescentis*. *Biochimie* **2018**, *148*, 107–115. [CrossRef]
- Bogati, K.; Walczak, M. The impact of drought stress on soil microbial community, enzyme activities and plants. *Agronomy* **2022**, *12*, 189. [CrossRef]
- De Lima, F.S.; Kurozawa, L.E.; Ida, E.I. The effects of soybean soaking on grain properties and isoflavones loss. *LWT-Food Sci. Technol.* **2014**, *59*, 1274–1282. [CrossRef]
- Şener, A. Extraction, partial purification and determination of some biochemical properties of  $\beta$ -glucosidase from Tea Leaves (*Camellia sinensis* L.). *J. Food Sci. Tech.* **2015**, *52*, 8322–8328. [CrossRef]
- Tong, D.-P.; Zhu, K.-X.; Guo, X.-N.; Peng, W.; Zhou, H.-M. The enhanced inhibition of water extract of black tea under baking treatment on  $\alpha$ -amylase and  $\alpha$ -glucosidase. *Int. J. Biol. Macromol.* **2018**, *107*, 129–136. [CrossRef]
- Zhang, J.; Wang, T.; Zhao, N.; Xu, J.; Qi, Y.; Wei, X.; Fan, M. Performance of a novel  $\beta$ -glucosidase BGL0224 for aroma enhancement of Cabernet Sauvignon wines. *LWT-Food Sci. Technol.* **2021**, *144*, 111244. [CrossRef]
- Wang, Y.; Zhang, C.; Li, J.; Xu, J. Different influences of  $\beta$ -glucosidases on volatile compounds and anthocyanins of Cabernet Gernischt and possible reason. *Food Chem.* **2013**, *140*, 245–254. [CrossRef]
- Zhang, Y.; Wang, X.; Wang, S.; Zhang, Q.; Peng, S.; Li, X.; Zhang, Y.; Qiao, Y. In silico investigation of the association of the TRPM8 ion channel with the pungent flavor of Chinese herbs. *J. Tradit. Chin. Med.* **2016**, *3*, 248–255. [CrossRef]
- Mushtaq, A.; Azam, U.; Mehreen, S.; Naseer, M.M. Synthetic  $\alpha$ -glucosidase inhibitors as promising anti-diabetic agents: Recent developments and future challenges. *Eur. J. Med. Chem.* **2023**, *249*, 115119. [CrossRef] [PubMed]
- Xia, W.; Bai, Y.; Shi, P. Improving the substrate affinity and catalytic efficiency of  $\beta$ -glucosidase Bgl3A from *Talaromyces leycettanus* JCM12802 by rational design. *Biomolecules* **2021**, *11*, 1882. [CrossRef] [PubMed]
- Teze, D.; Hendrickx, J.; Czjzek, M.; Ropartz, D.; Sanejouand, Y.-H.; Tran, V.; Tellier, C.; Dion, M. Semi-rational approach for converting a GH1  $\beta$ -glycosidase into a  $\beta$ -transglycosidase. *Protein Eng. Des. Sel.* **2014**, *27*, 13–19. [CrossRef]
- Hassan, N.; Geiger, B.; Gandini, B.; Patel, B.K.; Kittl, R.; Haltrich, D.; Nguyen, T.-H.; Divne, C.; Tan, T.C. Engineering a thermostable *Halothermothrix orenii*  $\beta$ -glucosidase for improved galacto-oligosaccharide synthesis. *Appl. Microbiol. Biot.* **2016**, *100*, 3533–3543. [CrossRef]
- Yang, J.; Wang, Q.; Zhou, Y.; Li, J.; Gao, J.; Guo, Z. Engineering *T. naphthophila*  $\beta$ -glucosidase for enhanced synthesis of galactooligosaccharides by site-directed mutagenesis. *Biochem. Eng. J.* **2017**, *127*, 1–8. [CrossRef]
- Lee, H.-L.; Chang, C.-K.; Jeng, W.-Y.; Wang, A.H.-J.; Liang, P.-H. Mutations in the substrate entrance region of  $\beta$ -glucosidase from *Trichoderma reesei* improve enzyme activity and thermostability. *Protein Eng. Des. Sel.* **2012**, *25*, 733–740. [CrossRef]
- Ranaei Siadat, S.O.; Mollasalehi, H.; Heydarzadeh, N. Substrate affinity and catalytic efficiency are improved by decreasing glycosylation sites in *Trichoderma reesei* cellobiohydrolase I expressed in *Pichia pastoris*. *Biotechnol. Lett.* **2016**, *38*, 483–488. [CrossRef]
- Kambiré, M.S.; Gnanwa, J.M.; Boa, D.; Kouadio, E.J.P.; Kouamé, L.P. Modeling of enzymatic activity of free  $\beta$ -glucosidase from Palm weevil, *Rhynchophorus palmarum* Linn. (Coleoptera: Curculionidae) larvae: Effects of pH and temperature. *Biophys. Chem.* **2021**, *276*, 106611. [CrossRef]
- Zhang, Y.; Yang, J.; Yu, X.; Hu, X.; Zhang, H. Engineering *Leuconostoc mesenteroides* dextranase by inserting disulfide bridges for enhanced thermotolerance. *Enzym. Microb. Tech.* **2020**, *139*, 109603. [CrossRef]

22. Ni, D.; Zhang, S.; Huang, Z.; Liu, X.; Xu, W.; Zhang, W.; Mu, W. Multistrategy engineering of an inulosucrase to enhance the activity and thermostability for efficient production of microbial inulin. *J. Agric. Food Chem.* **2024**, *72*, 18100–18109. [CrossRef] [PubMed]
23. Nielsen, J.E.; Borchert, T.V.; Vriend, G. The determinants of  $\alpha$ -amylase pH-activity profiles. *Protein Eng. Des. Sel.* **2001**, *14*, 05–512. [CrossRef] [PubMed]
24. Shi, F.; Xie, Y.; Jiang, J.; Wang, N.; Li, Y.; Wang, X. Directed evolution and mutagenesis of glutamate decarboxylase from *Lactobacillus brevis* Lb85 to broaden the range of its activity toward a near-neutral pH. *Enzym. Microb. Tech.* **2014**, *61*–62, 35–43. [CrossRef]
25. Zhang, P.; Zhang, R.; Sirisena, S.; Gan, R.; Fang, R. Beta-glucosidase activity of wine yeasts and its impacts on wine volatiles and phenolics: A mini-review. *Food Microbiol.* **2021**, *100*, 103859. [CrossRef] [PubMed]
26. Li, S.; Cao, L.; Yang, X.; Wu, X.; Xu, S.; Liu, Y. Simultaneously optimizing multiple properties of  $\beta$ -glucosidase Bgl6 using combined (semi-) rational design strategies and investigation of the underlying mechanisms. *Bioresour. Technol.* **2023**, *374*, 128792. [CrossRef]
27. Gutierrez-Gutierrez, D.A.; Fuentes-Garibay, J.A.; Viader-Salvadó, J.M.; Guerrero-Olazarán, M. Biochemical characterization of the  $\beta$ -glucosidase Glu1B from *Coptotermes formosanus* produced in *Pichia pastoris*. *Enzym. Microb. Tech.* **2023**, *163*, 110155. [CrossRef]
28. Sun, J.; Wang, W.; Ying, Y.; Hao, J. A novel glucose-tolerant GH1  $\beta$ -glucosidase and improvement of its glucose tolerance using site-directed mutation. *Appl. Biochem. Biotech.* **2020**, *192*, 999–1015. [CrossRef]
29. Fabjanowicz, M.; Płotka-Wasyłka, J. Metals and metal-binding ligands in wine: Analytical challenges in identification. *Trends Food Sci. Tech.* **2021**, *112*, 382–390. [CrossRef]
30. Tonin, F.; Otten, L.G.; Arends, I.W. NAD<sup>+</sup>-Dependent Enzymatic Route for the Epimerization of Hydroxysteroids. *ChemSusChem* **2019**, *12*, 3192–3203. [CrossRef]
31. Carlsson, A.C.C.; Scholfield, M.R.; Rowe, R.K.; Ford, M.C.; Alexander, A.T.; Mehl, R.A.; Ho, P.S. Increasing enzyme stability and activity through hydrogen bond-enhanced halogen bonds. *Biochemistry* **2018**, *57*, 4135–4147. [CrossRef] [PubMed]
32. Song, S.; Liu, Q.; Chai, W.-M.; Xia, S.-S.; Yu, Z.-Y.; Wei, Q.-M. Inhibitory potential of 4-hexylresorcinol against  $\alpha$ -glucosidase and non-enzymatic glycation: Activity and mechanism. *J. Biosci. Bioeng.* **2021**, *131*, 241–249. [CrossRef] [PubMed]
33. Gumpena, R.; Kishor, C.; Ganji, R.J.; Jain, N.; Addlagatta, A. Glu121-Lys319 salt bridge between catalytic and N-terminal domains is pivotal for the activity and stability of *Escherichia coli* aminopeptidase N. *Protein Sci.* **2012**, *21*, 727–736. [CrossRef]
34. Kim, J.Y.; Kinoshita, M.; Kume, S.; Gt, H.; Sugiki, T.; Ladbury, J.E.; Kojima, C.; Ikegami, T.; Kurisu, G.; Goto, Y. Non-covalent forces tune the electron transfer complex between ferredoxin and sulfite reductase to optimize enzymatic activity. *Biochem. J.* **2016**, *473*, 3837–3854. [CrossRef]
35. Tiwari, M.K.; Häggglund, P.M.; Möller, I.M.; Davies, M.J.; Bjerrum, M.J. Copper ion/H<sub>2</sub>O<sub>2</sub> oxidation of Cu/Zn-Superoxide dismutase: Implications for enzymatic activity and antioxidant action. *Redox Biol.* **2019**, *26*, 101262. [CrossRef]
36. Der, B.S.; Kluwe, C.; Miklos, A.E. Alternative computational protocols for supercharging protein surfaces for reversible unfolding and retention of stability. *PLoS ONE* **2013**, *8*, e64363. [CrossRef] [PubMed]
37. Guérin, F.; Barbe, S.; Pizzut-Serin, S. Structural investigation of the thermostability and product specificity of amylosucrase from the bacterium *Deinococcus geothermalis*. *J. Biol. Chem.* **2012**, *287*, 6642–6654. [CrossRef]
38. Flannelly, D.F.; Aoki, T.G.; Aristilde, L. Short-time dynamics of pH-dependent conformation and substrate binding in the active site of beta-glucosidases: A computational study. *J. Struct. Biol.* **2015**, *191*, 352–364. [CrossRef]
39. Kulkarni, T.S.; Khan, S.; Villagomez, R.; Mahmood, T.; Lindahl, S.; Logan, D.T.; Linares-Pastén, J.A.; Nordberg Karlsson, E. Crystal structure of  $\beta$ -glucosidase 1A from *Thermotoga neapolitana* and comparison of active site mutants for hydrolysis of flavonoid glucosides. *Proteins* **2017**, *85*, 872–884. [CrossRef]
40. Song, W.; Li, Y.; Tong, Y.; Tao, J.; Rao, S.; Li, J.; Zhou, J.; Liu, S. Improving the catalytic efficiency of *Aspergillus fumigatus* glucoamylase toward raw starch by engineering its N-glycosylation sites and saturation mutation. *J. Agric. Food Chem.* **2022**, *70*, 12672–12680. [CrossRef]
41. Troy, C. The Pandemic Threat of Emerging H5 and H7 Avian Influenza Viruses. *Viruses* **2018**, *10*, 461. [CrossRef] [PubMed]
42. Samy, A.; Naguib, M. Avian respiratory coinfection and impact on avian influenza pathogenicity in domestic poultry: Field and experimental findings. *Vet. Sci.* **2018**, *5*, 23. [CrossRef] [PubMed]
43. Dong, M.; Fan, M.; Zhang, Z.; Xu, Y.; Li, A.; Wang, P.; Yang, P. Purification and characterization of  $\beta$ -glucosidase from *Oenococcus oeni* 31MBR. *Eur. Food Res. Technol.* **2014**, *239*, 995–1001. [CrossRef]
44. Liu, J.; Zhang, X.; Fang, Z. The 184th residue of  $\beta$ -glucosidase Bgl1B plays an important role in glucose tolerance. *J. Biosci. Bioeng.* **2011**, *112*, 447–450. [CrossRef]
45. Zhou, C.; Dong, A.; Wang, Q.; Yu, Y.; Fan, X.; Cao, Y.; Li, T. Effect of common metal ions and anions on laccase catalysis of guaiacol and lignocellulosic fiber. *BioResources* **2017**, *12*, 5102–5117. [CrossRef]

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Review

# Engineering IsPETase and Its Homologues: Advances in Enzyme Discovery and Host Optimisation

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## Abstract

Polyethylene terephthalate (PET) pollution represents a significant environmental challenge due to its widespread use and recalcitrant nature. PET-degrading enzymes, particularly *Ideonella sakaiensis* PETases (IsPETase), have emerged as promising biocatalysts for mitigating this problem. This review provides a comprehensive overview of recent advancements in the discovery and heterologous expression of IsPETase and closely related enzymes. We highlight innovative approaches, such as in silico and AI-based enzyme screening and advanced screening assays. Strategies to enhance enzyme secretion and solubility, such as using signal peptides, fusion tags, chaperone co-expression, cell surface display systems, and membrane permeability modulation, are critically evaluated. Despite considerable progress, challenges remain in achieving industrial-scale production and application. Future research must focus on integrating cutting-edge molecular biology techniques with host-specific optimisation to achieve sustainable and cost-effective solutions for PET biodegradation and recycling. This review aims to provide a foundation for further exploration and innovation in the field of enzymatic plastic degradation.

**Keywords:** polyethylene terephthalate; PETase; heterologous expression; in silico screening; enzyme assays; host optimisation

## 1. Introduction

One of the greatest innovations of the 20th century, plastics have become indispensable to human existence because of their advantageous qualities. Since 2000, global plastic production has doubled from approximately 200 million tonnes (Mt) to ~400 Mt in 2019 [1]. Unfortunately, because plastics are not biodegradable, plastic trash builds up in landfills and the ocean, which causes major environmental issues [2].

On a global scale, the recycling rate for plastic garbage stands at a modest 18%, with a slightly higher proportion of 24% being submitted to burning. The other 58% of plastic waste is either deposited in landfills or released into the natural environment, resulting in the long-term buildup and persistence of plastics [3]. Plastics can be classified into two distinct groups: biodegradable plastics, which can come from either natural or synthetic sources and possess the ability to decompose through the action of microorganisms, and nondegradable plastics, which are derived from petrochemicals and exhibit a higher molecular weight due to the repetitive arrangement of small monomer units, commonly known as synthetic polymers. Illustrations of the latter classification include polyethylene (PE),

polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyethylene terephthalate (PET), and other plastics [4].

### 1.1. PET

With an annual output of around 50 Mt, PET has become one of the most used synthetic polymers since its invention in the 1940s and has emerged as an important contributor to the growing problem of plastic waste. The primary cause of its propensity for degradation is largely attributed to the existence of hydrolysable ester linkages in its composition [5].

Physical and chemical approaches have been implemented to degrade plastic waste, such as ultraviolet (UV) treatment, physical stress, hydrolysis, and ammonolysis [6]. However, these approaches all have some limitations. For example, physical treatments degrade plastics into smaller fragments, thus will not significantly reduce the plastic entering the landfill flow, and the additives in the plastic will also be degraded to release substances that may affect the environment [7]. Chemical treatment requires the use of solvents that are not environmentally friendly and always result in the release of byproducts such as carbon monoxide (CO), carbon dioxide (CO<sub>2</sub>), terephthalic acid, anhydrides, carboxylic acids, and esters [8].

To confront the imminent peril of plastic accumulation in marine and terrestrial environments, it is crucial to implement efficient biodegradative methods that alleviate the burden of plastic in the environment.

### 1.2. PETase from *Ideonella sakaiensis*

PET does not biodegrade well and instead accumulates as garbage. It was recently discovered that bacteria and fungi possess thermophilic hydrolases, which are best suited for catalysing PET hydrolysis at elevated temperatures. Remarkably, enzymes derived from *Ideonella sakaiensis* were reported to digest PET well at room temperature [9].

PETase, derived from *I. sakaiensis*, also identified as *IsPETase*, is an enzyme that has similarities to cutinase. It has attracted attention as a potentially effective and innovative approach to completely break down the polymer into its fundamental constituent units. *IsPETase* exhibits increased activity at ambient temperatures and acts specifically on highly crystalline PET [10], while the high-resolution (0.92 Å) X-Ray crystal structure of *IsPETase* provided more information on the enzyme's working mechanism and suggested that it is not fully optimised for crystalline PET degradation [11].

There are concerns about the structural and sequence characteristics that enable *IsPETase* to outperform its homologues at lower temperatures, since *IsPETase* can break down PET films at ambient temperature, whereas other hydrolases require a high temperature to promote PET hydrolysis [9]. Although with these advantages, the practical implementation of *IsPETase* in the biodegradation of PET is hindered by its low solubility [12], highlighting the need to screen more similar enzymes to make *IsPETase* a more feasible target to be engineered for PET's biodegradation.

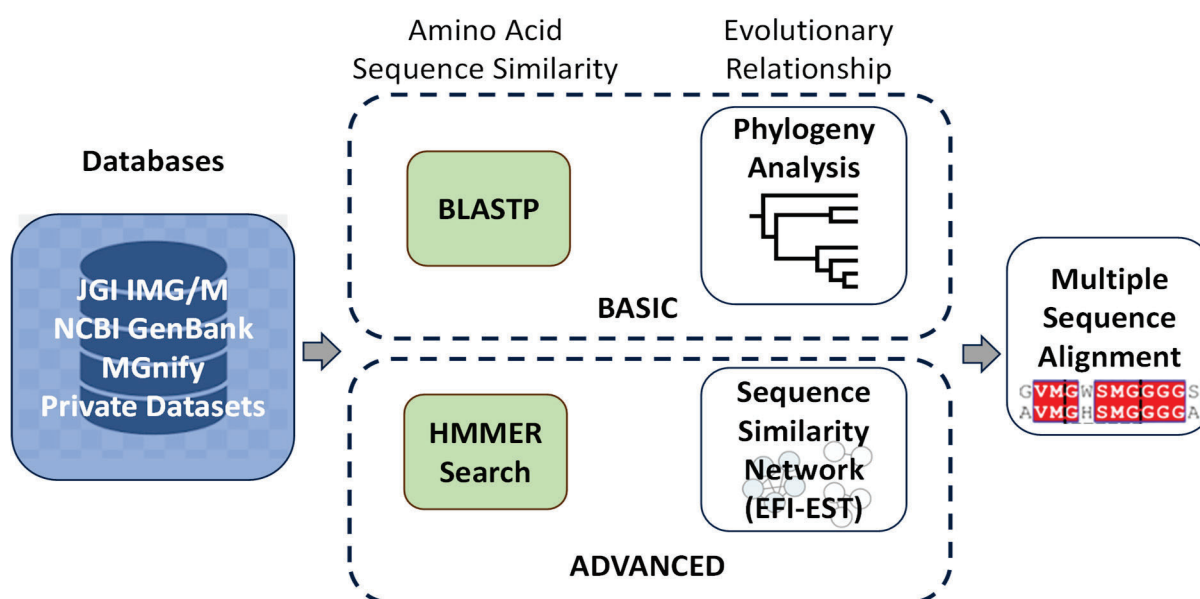
This review provides a focused synthesis of recent advances in the discovery and engineering of PET-degrading enzymes, particularly homologues of *IsPETase*. We explore innovative in silico and AI-driven screening strategies, evaluate diverse enzymatic activity assays, and critically assess recombinant expression systems across bacterial, yeast, and algal hosts. Emphasis is placed on enhancing enzyme solubility, secretion, and activity through molecular and host-specific engineering approaches. By highlighting both the progress and persistent challenges in PETase development, this review aims to guide future research toward scalable, sustainable solutions for enzymatic PET biodegradation and recycling.

## 2. Screening *IsPETase* Homologues

### 2.1. *In Silico* Screening

#### 2.1.1. Amino Acid Sequence-Based Screening

Several research teams have employed *in silico*-based methodologies to screen enzymes exhibiting a high level of similarity to *IsPETase*, sourced from a diverse range of microorganisms or environmental samples. The objective of these efforts is to identify potential candidates for further genetic engineering. Researchers hope to accelerate the degradation process of PET [13–15]. These screening approaches, as outlined in Figure 1, offer a promising pathway towards the development of more effective biocatalysts for environmental applications in plastic waste management.



**Figure 1.** Strategies to search novel *IsPETase* homologues. This schematic illustrates the main computational approaches used to identify potential PET-degrading enzymes from genomic and metagenomic databases. The strategies are broadly categorised into amino acid sequence-based screening, which includes tools such as BLASTP to identify homologous sequences based on similarity to *IsPETase*. Hidden Markov Models (HMMs) are also employed to detect conserved motifs across large datasets.

Studies employing the strategies outlined in Figure 1 have been published. However, only a tiny fraction of these investigations resulted in confirmed enzymatic activity of the newly identified candidates, leaving further validation still required [14,16–19].

Many studies have utilised the NCBI (National Center for Biotechnology Information; <http://ncbi.nlm.nih.gov>) database for enzyme screening, employing a variety of *in silico* strategies. These range from simpler methods such as BLASTP [15] to more advanced techniques like PSI-BLAST [19,20]. These approaches are particularly appealing to researchers without extensive bioinformatic expertise, as they are relatively straightforward to implement. Remarkably, they have proven effective in identifying promising enzyme candidates, including novel enzymes whose activities have been experimentally validated.

For instance, a more comprehensive strategy was adopted by searching across multiple databases, including NCBI, UniProt, and the JGI Integrated Microbial Genomes and Microbiomes (JGI/IMG) database [14]. The researchers employed an *IsPETase*-specific Hidden Markov Model (HMM)-based search algorithm, which enabled the discovery of novel *IsPETase* homologues. Similarly, the MGnify database, another resource for microbiome data, was explored using an HMM-based approach [17]. Both studies successfully

identified enzymes that exhibited activity against PET, highlighting the efficiency of this search technique in uncovering enzymes with plastic-degrading capabilities.

In contrast to the broad database-wide searches, other researchers have focused their efforts on specific groups of microorganisms within databases. For example, the basic BLASTP strategy was applied to 52 *Streptomyces* genome sequences obtained from NCBI, which included 23 terrestrial and 29 marine isolates. The work targeted approach led to the discovery of an *IsPETase*-like enzyme, whose activity was confirmed through experimental assays [16]. On the other hand, BLAST searches were conducted across 7375 terrestrial metagenomic samples in the JGI/IMG database [13] (<https://img.jgi.doe.gov/>). While homologues closely related to *IsPETase* were identified, the activity of these candidates was not tested, leaving their potential for PET degradation unverified.

These diverse strategies demonstrate the adaptability and potential of *in silico* screening in identifying promising enzymes for further study in PET biodegradation research.

### 2.1.2. Artificial Intelligence (AI)-Driven and Structure-Based Screening

In addition to engineering *IsPETase*, AI tools have also been employed for PETase screening. However, the limited number of experimentally confirmed PETases presents a challenge for effective model training. To address this limitation, existing studies have devised various strategies to enhance training datasets, thereby mitigating the need for additional PETase sequences.

One machine learning (ML) model, employing the support vector machine method, was trained using a dataset of PETase homologues derived from thermal metagenomes. The homologues were collected through *in silico* screening based on amino acid sequences [21], and the ML-assisted screening resulted in the identification of 74 candidate enzymes. Of these, 51 were successfully expressed in *Escherichia coli*, and 37 exhibited detectable PET hydrolytic activity, including 23 enzymes that had not been previously reported.

In a separate study, a Generative Adversarial Network (GAN) was utilised for data augmentation. By employing a pre-trained deep Evolutionary Scale Language Model (ESM), potential PET-degrading enzymes were identified from a custom-built metagenomic library. Ultimately, 21 novel potential PET-degrading enzymes were identified through structure analysis, though enzymatic activity assays were not conducted to confirm their functionality [22].

To enhance the accuracy of *in silico* screening strategies, structural features were considered during the screening phase, rather than being assessed solely at the confirmation stage. In one study, novel PETases were identified from the AlphaFold protein structure database. Candidate enzymes were selected based on the presence of key *IsPETase* features, including an aspartic acid residue at positions 43–49 and a histidine residue at positions 71–81, following the Ser-Met-Gly-Gly-Gly motif. These candidates were further refined using the catalytic triad C- $\alpha$  mutual distance criterion, yielding an initial selection of 4594 putative PET hydrolases. Subsequent analysis, including sequence similarity network construction and multiple sequence alignment, narrowed this to a final pool of 107 candidate sequences. Although the most promising candidate, LSPET4, did not surpass *IsPETase* in activity, mutated *IsPETase* based on the structure feature of LSPET4 demonstrated a 9.32-fold increase in *IsPETase* hydrolytic activity at 45 °C [23].

Another study developed a novel PETase discovery pipeline by integrating protein language models with a structural representation tree. The most promising enzyme identified, *KbPETase*, exhibited twice the activity of *IsPETase* at 30 °C. Notably, when compared with FastPETase, the most active *IsPETase* mutant with the optimal catalytic performance at 50 °C, *KbPETase* still displayed a catalytic efficiency 1.3 times greater than that of FastPETase [24].

## 2.2. Assays to Screen PETase

Researchers typically combine *in silico* approaches with PET-degrading activity assays to screen novel PETases. This dual approach allows them to identify enzymes that are structurally similar to *IsPETases* and capable of degrading PET. The most commonly used assays for measuring PET-degrading activity are depicted in Figure 2. These screening techniques vary in scale from low to ultra-high throughput, depending on the context. High and ultra-high throughput assays are generally employed when screening engineered enzymes resulting from mutation or directed evolution [25–29]. However, when it comes to screening *IsPETase* homologues from environmental samples or databases like NCBI, medium or even low-throughput assays are often more suitable due to the low number of hits from *in silico* searching. These lower throughput methods strike a balance between efficiency and affordability, making them a more practical choice for such exploratory studies, where fewer samples may be initially tested [14,30].

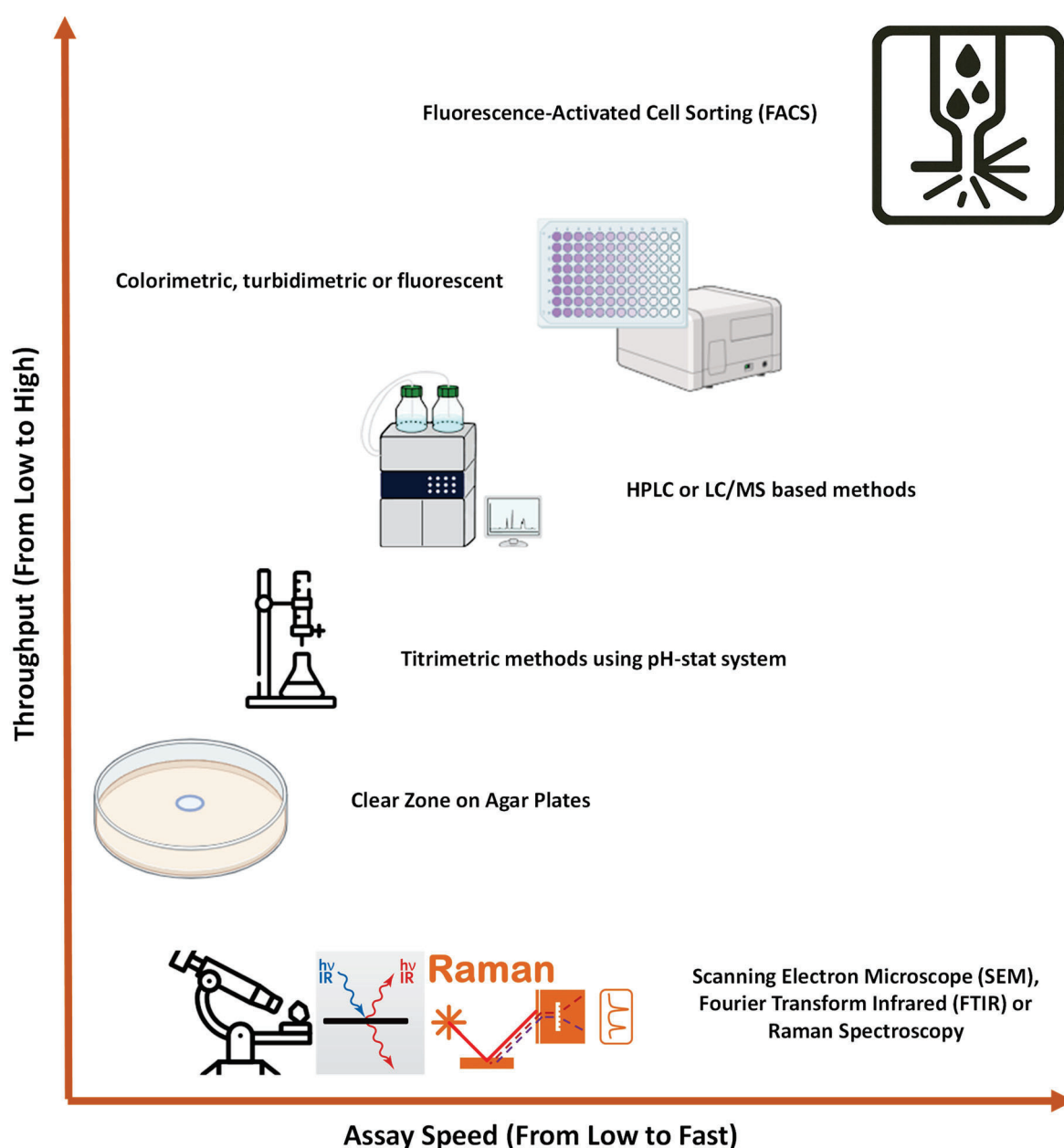


Figure 2. Assays to screen PET-degrading enzymes.

Building on this framework, researchers tailor their choice of assay based on the nature of the PETase candidates and the experimental constraints. Agar plate-based methods, for example, provide a straightforward and visual means of assessing polymer degradation but are limited by their low throughput [14,30]. Titrimetric methods, including the use of pH-stat systems, provide a moderate-throughput alternative for quantifying acid release during PET hydrolysis [31,32]. HPLC or LC/MS-based assays offer higher specificity and sensitivity, enabling detailed quantification of degradation products, though they require more time and instrumentation [20,33,34].

For high-throughput applications, colorimetric, turbidimetric, or fluorescent assays are widely used due to their compatibility with microplate formats and automation [25,28,35]. At the ultra-high-throughput level, Fluorescence-Activated Cell Sorting (FACS) allows for rapid screening of vast enzyme libraries, particularly in yeast surface display systems [29].

While not typically used for routine screening, scanning electron microscope (SEM), Fourier Transform Infrared spectroscopy (FTIR) and Raman spectroscopy serve as valuable tools in specialised contexts [36–38]. These methods are slower, more costly, and lower in throughput, but they provide unique insights into enzyme–substrate interactions and the degradation of crystalline PET. As such, they are often employed to complement biochemical assays in mechanistic or structural studies.

### 3. Heterologous Expression Platforms to Screen PETases

Beyond screening for novel homologues, *IsPETase* itself has been engineered to enhance its properties. Challenges such as inefficient enzyme secretion and cytoplasmic stress have led to the development of secretion expression and surface display platforms in diverse hosts [39]. These advanced platforms facilitate the rapid screening of enzyme variants, eliminating the need for cell lysis and avoiding the formation of inclusion bodies, thereby streamlining the screening process [12,40,41].

The successful expression of *IsPETase* and its homologues in heterologous systems is critical for both fundamental research and industrial applications. Various microbial hosts, including bacteria, yeasts, and microalgae, have been explored for this purpose, each offering distinct advantages and limitations. For example, *E. coli* is widely used due to its rapid growth and well-established genetic tools, while *Bacillus subtilis* offers efficient secretion capabilities. Yeasts such as *Pichia pastoris* and *Saccharomyces cerevisiae* provide eukaryotic post-translational modifications, and microalgae offer environmentally sustainable platforms for enzyme production. In the following subsections, we examine host-specific strategies that have been developed to enhance PETase expression, solubility, and secretion in these systems.

#### 3.1. Bacteria

##### 3.1.1. *E. coli*

Most studies that have expressed *IsPETase* or its homologues in *E. coli* have utilised pET-series vectors and BL21 (DE3) or derived strains, such as BL21 (DE3)-T1R [42] and BL21-Gold (DE3) [26], with IPTG added to induce expression.

As an alternative to the widely used *E. coli* BL21 (DE3), *E. coli* SHuffle T7 was engineered for the intracellular expression of *IsPETase*<sup>Mut</sup>, an active mutant of *IsPETase*. This alternative was chosen due to the propensity of proteins with disulfide linkages to misfold and accumulate as inclusion bodies during overexpression, a phenomenon initially observed with the expression vectors pBAD and pET30a [43,44]. This was corroborated by a separate study in which *IsPETase* was expressed in multiple *E. coli* hosts, with SHuffle T7 Express demonstrating the highest enzymatic activity. However, this advantage was not observed for FastPETase (N233K/R224Q/S121E/D186H/R280A) [45] and Hot-PETase [46],

two variants of *IsPETase* [47]. An additional noteworthy finding indicated that the formation of inclusion bodies during the overexpression of *IsPETase*<sup>Mut</sup> in *E. coli* was primarily attributed to protein aggregation driven by interactions among hydrophobic regions, rather than the presence of disulfide bonds [12]. Consequently, the precise role of SHuffle T7 as an expression host requires further elucidation.

A novel, growth-decoupled *E. coli* strain, enGenes Biotech's e<sup>x</sup>-press V2, was employed in one study to express the PET-degrading enzyme polyester hydrolyse Leipzig 7 (PHL7) [48]. In this system, induction with arabinose inhibits *E. coli* host cell RNA polymerase, thereby arresting cell growth and reallocating cellular resources to enhance recombinant protein production [49]. PET-degrading activity was detected in the unpurified fermentation supernatant; however, enGenes e<sup>x</sup>-press V2 was not benchmarked against established *E. coli* host strains [48].

### Enhanced Secretion System

A frequently encountered challenge in expressing *IsPETase* in *E. coli* is its low solubility. Current research has explored various strategies to address this limitation.

The Sec-dependent pathway is associated with post- and co-translational translocation of pre-folded polypeptides across the inner membrane [26,42]. Signal peptides employing the Sec-dependent pathway are frequently utilised, including PelB, PhoA, OmpC, OmpF, OmpA, and MalE [50]. Among these, PelB has found widespread commercial use in pET vector expression systems, such as pET20b, 22b, and 26b [50], making it a convenient starting point for enhancing *IsPETase* secretion.

Employing this approach, *IsPETase* was cloned into pET22b(+), which contains the DNA sequence for PelB, and subsequently generated a library via standard error-prone PCR (epPCR) to induce mutations in PelB. Using an assay to evaluate the ability to degrade 4-*p*NPA (*para*-nitrophenyl acetate), they identified mutants *IsPETase* with significantly enhanced activity. The increased PETase concentration in the supernatant correlated with improved activity, exhibiting at least 1.7-fold enhancement across various assays [26].

In another study, multiple Sec-dependent signal peptides, including MalE, LamB, and SurA, were tested and demonstrated that LamB, a maltoporin involved in maltose transportation, facilitated the production of significantly higher levels of extracellular *IsPETase* in *E. coli* [42].

Further advancements in the heterologous expression of *IsPETase* in *E. coli* were achieved by modifying signal peptides with N-terminal enhancers. These enhancers, derived from the endogenous signal peptide of  $\beta$ -fructofuranosidase ( $\beta$ -FFase) originating from *Arthrobacter arilaitensis* NJEM01, proved effective in achieving extracellular synthesis of *IsPETase* with increased enzymatic activity [51].

An intriguing finding in this area arises from the use of native signal peptides. When an *IsPETase* homologue from *Streptomyces* sp. SM14 was expressed in *E. coli* with its native signal peptide; the enzyme was successfully exported, as confirmed by the polycaprolactone (PCL) plate clearing assay [16]. This result underscores the potential of leveraging native signal peptides for effective extracellular expression and secretion in heterologous systems.

### Chaperone Co-Expression and Fusion Tags

*IsPETase*<sup>Mut</sup> was co-expressed with three chaperone systems: GroEL/ES, DnaK/DnaJ/GrpE, and TF. The findings revealed that *IsPETase*<sup>Mut</sup> co-expressed with GroEL/ES exhibited a 12.5-fold increase in expression levels [12]. These results align with previous studies indicating that co-expression with chaperones enhances protein solubility in *E. coli* [52].

An additional approach to avoiding inclusion body formation in *E. coli* involves fusing highly soluble tags to recombinant proteins, such as maltose-binding protein (MBP),

transcription termination/anti-termination factor (NusA), thioredoxin (TrxA), and small ubiquitin-related modifier (SUMO) [53]. Previous studies have demonstrated that NusA offers superior soluble expression of cold-adapted *Vibrio* proteins in *E. coli* compared to other fusion tags [54]. Similarly, substantial improvements in the solubility of *IsPETase*<sup>Mut</sup> were achieved through co-expression with the chaperone GroEL/ES and the use of fusion expression with NusA [12].

In a separate study, a novel fusion tag comprising carbohydrate-binding module 66 (CBM66) was fused with *IsPETase*. This approach resulted in significantly increased expression levels of soluble *IsPETase*, achieving yields of approximately 370 mg/L [55].

More recently, the two strategies were combined to enhance *IsPETase* production. A vesicle nucleating peptide (VNp) tag-based secretion system was utilised in conjunction with the co-expression of the DnaJ-DnaK chaperone team. This combined approach resulted in a marked improvement in the production of FastPETase, an *IsPETase* mutant with enhanced hydrolytic activity [56].

### Cell Surface Display (CSD) System

Microbial cell surface display systems enable peptides and proteins to be presented on the surface of microbial cells by fusing them with anchoring motifs [57]. The outer membrane-bound fatty acid transporter (FadL) from *E. coli* was previously demonstrated to be effective for an esterase enzyme display on the surface of *E. coli* [58]. When FadL was co-expressed with the CSD system, *IsPETase* exhibited activity in the tributyrin plate clearing assay and demonstrated enhanced thermostability [40].

Building on the advancements in CSD systems, the PgsA protein from *B. subtilis* was employed as an anchoring protein for the heterologous expression of *IsPETase* in *E. coli* [59]. PgsA is part of the enzyme complex that synthesises poly- $\gamma$ -glutamic acid (PGA) in *B. subtilis* and has been fused with lipase B from *Candida antarctica* (CalB) to display the enzyme in an active form on the cell surface of *E. coli* [60]. The fused protein was expressed in *E. coli*, and it was confirmed that the active form of the *IsPETase* was present on the cell surface of *E. coli* [59].

### Increasing Membrane Permeability

Colicin families offer the functional diversity of unique cell-killing mechanisms that allow the simultaneous release of recombinant enzymes from cells. Of over 20 colicins, the lysis gene from Colicin E7 was effectively reported to enhance the secretion of intracellular recombinant enzymes from *E. coli* [61]. An *IsPETase* homologue screened from metagenome analysis, F148, was co-expressed with Colicin E7 in *E. coli* BL21 (DE3). Compared with the control strain only expressing F148, during the same period, nearly 3-fold degradation products could be observed, since Colicin E7 enabled protein release simultaneously by obstructing the outer membrane through phospholipase activation [62].

In another study, a comparable strategy was adopted by fusing *IsPETase* with *Thermobifida fusca* cutinase, an *IsPETase* homologue previously reported to degrade phospholipids and thereby increase cell membrane permeability [63]. This approach successfully enhanced enzyme secretion, providing another example of facilitating secretion through the modulation of cell membrane permeability [64].

#### 3.1.2. *B. subtilis*

*B. subtilis* is widely recognised as a robust biotechnological workhorse for the heterologous expression of enzymes. Its non-pathogenic nature and designation as a GRAS (generally regarded as safe) microorganism render it fully compliant with regulatory requirements for industrial applications [65,66]. Similar strategies to those employed in *E. coli* have been adapted for use in *B. subtilis* to enhance the extracellular expression of *IsPETases*.

### Enhanced Secretion System

The use of five *Bacillus*-derived signal peptides for expressing *IsPETase* was investigated in *B. subtilis* WB600, a strain derived from *B. subtilis* 168 with six protease genes knocked out [67]. The signal peptides tested were amy from *Bacillus amyloliquefaciens*; Sac and AprE, both from *B. subtilis* 168; apr and BprA, both from *Bacillus licheniformis* WX-02. Among these, the signal peptide amy (SPamy) demonstrated the most significant effect, increasing enzyme activity in the cell culture supernatant nearly four-fold [68].

In contrast to studies on *IsPETase* expression in *E. coli*, which consistently reported that the Sec-dependent secretion pathway enhances *IsPETase* secretion, research on *B. subtilis* has yielded conflicting results. In a separate study, the expression of *IsPETase* in *B. subtilis* 168 was examined using three Sec signal peptides (AmyE, Bpr, and SacB) and two Tat signal peptides (PhoD and YwbN), which direct proteins through the twin-arginine translocation (Tat) pathway. However, none of these signal peptides improved *IsPETase* secretion compared to its native signal peptide [69]. Thus further research is required to elucidate the discrepancies observed in the findings of these studies.

### Chaperone Overexpression

In *B. subtilis* CBS2, the *hrcA* gene, a negative regulator of the *dnaK* and *groESL* operons [70,71], was inactivated. However, the protein titre of *IsPETase* was not improved in the chaperone-overexpressing strain. In contrast, a PET hydrolase from the bacterium HR29 (*BhrPETase*) exhibited an approximately 20-fold increase in production [72].

## 3.2. Yeast

### 3.2.1. *S. cerevisiae*

Surface display systems have been adopted to enhance the extracellular concentration of *IsPETase*. The anchoring protein GCW51 was successfully utilised to display *IsPETase* on the surface of yeast [73]. In a more recent study, a novel surface display platform was developed based on *S. cerevisiae* spores to display FastPETase [41].

Unlike the cell wall of vegetative *S. cerevisiae* cells, which consists of a two-layer structure comprising  $\beta$ -glucan and mannan, the spore wall features a four-layer structure composed of mannan,  $\beta$ -glucan, chitosan, and dityrosine. Displaying proteins on the surface of *S. cerevisiae* spores offers distinct advantages over traditional cell surface display, as it eliminates the need for transmembrane transport. Furthermore, the unique structure of the spore wall imparts resistance to environmental stresses, as demonstrated by a study, where the displayed FastPETase exhibited significantly improved thermostability [45].

Yeast surface display systems provide a promising approach to producing *IsPETase*, offering the advantages of bypassing enzyme purification, resulting in substantial time and cost savings, and facilitating the reuse of the biocatalyst.

### 3.2.2. *Yarrowia lipolytica*

*Y. lipolytica* has been identified as a potential host for the expression of *IsPETase*, owing to its ability to assimilate atypical carbon sources, allowing for the assimilation of degradation products from PET [74]. Following the successful expression of *IsPETase* in *Y. lipolytica*, the same group optimised its expression by testing various pre-treatment methods [75]. However, in comparison to other host systems, research in *Y. lipolytica* has yet to progress towards a deeper understanding and optimisation at the molecular biology level.

### 3.2.3. *P. pastoris*

The methylotrophic yeast *P. pastoris* is widely recognised as a versatile workhorse for the production of heterologous proteins in industrial applications [76,77]. This expression system has been shown to support efficient secretory expression, and its post-translational glycosylation further enhances expression yields by increasing the resistance of glycoproteins to proteolytic degradation [78,79]. In another study, *IsPETase* was successfully expressed in *P. pastoris*, demonstrating significant improvements in the enzyme's specific activity and thermostability both for native *IsPETase* and FastPETase [80].

In another study, a chaperone co-expression strategy was employed in *P. pastoris* to enhance the production of the FastPETase variant, FastPETase-212/277. A panel of chaperones was evaluated, including protein disulphide isomerase (PDI), thiol oxidase (Ero1), the bZIP transcription factor HAC1, and proprotein convertase Kex2. Among these, Ero1 was identified as the most effective in boosting expression [81].

### 3.3. Microalgae

Microalgae present a compelling option for the synthesis of recombinant plastic-degrading enzymes and various environmental applications, owing to their ubiquitous presence in aquatic systems, minimal production of endotoxins, and ability to grow without the need for supplemental organic carbon [82]. In a study, the photosynthetic microalga *Phaeodactylum tricornutum* was employed to secrete a modified PETase, PETaseR280A, into the surrounding culture medium for PET degradation. The gene encoding PETaseR280A was co-expressed with the signal peptide of *P. tricornutum* alkaline phosphatase. The secreted enzyme demonstrated PET-degrading activity, confirming successful expression and secretion [83]. However, the application of *P. tricornutum* is constrained by its specific growth requirements, including low temperatures, silica as a nutrient, and high salinity.

To overcome these limitations, *Chlamydomonas reinhardtii*, a GRAS-designated freshwater microalga, was employed in a subsequent study for the expression of wild-type *IsPETase*. *C. reinhardtii* was selected due to its faster growth rate and freshwater habitat [84]. While the enzyme's activity was confirmed, the authors did not specify whether a signal peptide was employed to assist the secretion, and enzyme activity was assessed using cell lysates.

In another study, *C. reinhardtii* was employed as the host organism, with the original signal peptide retained to target the recombinant protein to the thylakoid lumen. This study differed by integrating the *IsPETase* gene into the plastome at a specific site, restoring a photosynthesis-essential gene. This restoration allowed antibiotic-free selection following gene integration [85].

In summary, these findings underscore the potential of bioengineered microalgae for producing *IsPETase* and homologues, offering promising applications in bioremediation strategies for PET-polluted aquatic environments.

## 4. Microbial Expression Systems for *IsPETases* and Homologues

The exploration of microbial platforms for *IsPETase* expression and the advancement of in silico analyses, screening assays, and novel enzyme discovery strategies underscore the rapid progress in this field.

### 4.1. Prokaryotic Hosts

Microbial hosts, particularly *E. coli* (Table 1), remain the backbone for heterologous expression, offering high yields and a broad toolkit for genetic manipulation. Sec-dependent secretion pathways, fusion tags, and chaperone co-expression strategies have been instrumental in improving the solubility, stability, and extracellular secretion of *IsPETase*.

Recent integrative approaches, such as combining vesicle nucleating peptides with chaperone systems, have further advanced production levels. However, limitations such as protein misfolding and inclusion body formation necessitate continued optimisation and host-specific engineering.

**Table 1.** Strategies employed to optimise the extracellular production of *IsPETases* or homologues using microbial expression systems.

| Microbe                          | Bacteria       |                    | Yeast                |                    | Microalgae            |
|----------------------------------|----------------|--------------------|----------------------|--------------------|-----------------------|
|                                  | <i>E. coli</i> | <i>B. subtilis</i> | <i>S. cerevisiae</i> | <i>P. pastoris</i> | <i>P. tricornutum</i> |
| Enhancing secretion system       | ✓              | ✓                  |                      |                    | ✓                     |
| Chaperone co-expression          | ✓              | ✓                  |                      | ✓                  |                       |
| Fusion tags                      | ✓              |                    |                      |                    |                       |
| CSD system                       | ✓              |                    | ✓                    |                    |                       |
| Increasing membrane permeability | ✓              |                    |                      |                    |                       |

Note: ✓ indicates that the corresponding strategy has been applied to the microbial host for enhancing *IsPETase* expression or secretion.

CSD systems represent a complementary approach, allowing *IsPETase* localisation on the cell membrane and bypassing purification steps. The use of anchor proteins such as FadL and PgsA in *E. coli* has resulted in enhanced enzyme activity and thermostability. While promising, further investigation into the stability, scalability, and cost-effectiveness of CSD systems is essential to translate these findings into industrial applications.

In *B. subtilis*, signal peptide optimisation, such as the use of amy from *B. amyloliquefaciens*, has demonstrated success in increasing extracellular *IsPETase* production. However, conflicting results regarding Sec- and Tat-dependent pathways underscore the complexity of secretion mechanisms in this host. Comparative molecular studies are needed to elucidate these discrepancies and fully harness *B. subtilis* as an efficient expression host with a secretion system. Only the strategies of enhancing the secretion system with signal peptides and co-expressing chaperones have been explored in *B. subtilis* to date (Table 1). Several fusion tags have been developed to facilitate the secretion of target proteins in *B. subtilis*, including cellulase [86], SUMO3 [87], Spy [88], and lysyl tRNA synthetase [89]. Further studies are anticipated to improve *IsPETase* secretion and extracellular production in this expression host.

As *B. subtilis* spores can be readily engineered to display heterologous antigens or proteins on their surface, the CSD system has been adapted for spore-surface display in *B. subtilis* [90]. Regarding the final strategy, which has been successfully employed in *E. coli*, thereby increasing membrane permeability to enhance protein secretion, there are also successful examples in *B. subtilis*. For instance, it has been demonstrated that the addition of Triton X-100 increased the secretion level of  $\gamma$ -cyclodextrin glycosyltransferase by 21.5%, an effect attributed to enhanced cell membrane permeability [91].

Thus, future research could focus on advancing *B. subtilis* as an expression host for *IsPETases* through the development of fusion tag technology, spore-surface display systems, and strategies for modulating cell membrane permeability.

#### 4.2. Eukaryotic Hosts

Eukaryotic hosts, including yeasts and microalgae, offer unique advantages due to their advanced post-translational modification systems and regulatory compliance for

industrial applications. *P. pastoris* has shown significant potential, with glycosylation improving enzyme stability and chaperone co-expression strategies enhancing yields [81]. Yeast surface display systems, particularly those employing *S. cerevisiae* spores, have innovatively addressed the challenges of purification and environmental stress resistance [92]. Microalgae, such as *P. tricornutum* and *C. reinhardtii*, have emerged as promising platforms for IsPETase production in aquatic environments [93,94]. However, challenges related to scalability, nutrient requirements, and environmental conditions must be addressed to maximise the potential of these hosts. Furthermore, only a limited number of strategies have been tested in these systems to enhance IsPETase secretion, leaving considerable potential for the exploration and implementation of alternative approaches.

#### 4.3. Expression and Production of IsPETase and Homologues in Scaled-Up Systems

The industrial-scale application of IsPETase and homologues hinges on the development of robust, high-yield expression systems capable of producing active enzymes under fermentor conditions. While significant strides have been made in engineering IsPETase variants with enhanced activity and thermostability, translating these advances into scalable bioprocesses remains a key challenge.

Among the various microbial hosts explored, *E. coli* remains the most widely used due to its rapid growth, well-characterised genetics, and ease of manipulation. However, *P. pastoris* has demonstrated superior capabilities in protein secretion and post-translational modifications. Notably, co-expression of the chaperone Ero1 in *P. pastoris*, combined with a high-cell-density fed-batch fermentation strategy in a 30-L bioreactor, enabled the production of up to 3.23 g/L of secreted FastPETase [81]. This represents one of the highest reported yields for the expression of IsPETase and homologues in a eukaryotic system. Although this system demonstrated excellent productivity, it required complex media and extended cultivation times to achieve optimal results.

In *E. coli*, the highest yield to date was also achieved for FastPETase, using a synergistic strategy that combined co-expression of cognate chaperones (DnaK/DnaJ from *I. sakaiensis*) with fusion of VNp6. This approach enabled the secretion of over 2 g/L FastPETase in a 5 L fed-batch fermenter [56].

Despite these promising results, several limitations persist. One of the most significant challenges is the lack of standardisation in reporting outcomes. Many studies use enzyme activity or PET degradation efficiency as proxies for expression levels, which allows researchers to avoid time-consuming purification procedures [95]. While this approach is practical, it complicates direct comparison across studies. This inconsistency in metrics, combined with variations in substrate type, reaction conditions, and quantification methods, hampers reproducibility and comparative assessment.

## 5. Conclusions

In summary, the combination of advanced expression systems, in silico methods, screening assays, and extracellular production strategies is accelerating the development of IsPETase for PET degradation. However, the field still faces challenges, including the scalability of production systems, discrepancies in secretion efficiency across hosts, and the need for standardised screening protocols. Interdisciplinary approaches that integrate synthetic biology, computational modelling, and environmental microbiology hold the key to overcoming these challenges and advancing the application of IsPETase for sustainable bioremediation. Additionally, exploring the potential of diverse microbial systems could provide novel insights and enhance the robustness of PET degradation strategies. Continued investment in these areas will be crucial for developing effective and scalable solutions to address plastic pollution.

Furthermore, while laboratory-scale advancements in PETase engineering are promising, their translation to industrial-scale applications presents significant challenges. These include the need for cost-effective and high-yield fermentation systems, robust enzyme stability under process conditions, and integration into existing waste management infrastructures. Although various microbial hosts have been explored for the expression of IsPETase and its homologues, including *E. coli*, *B. subtilis*, yeasts, and microalgae, comparative studies remain limited, and each system presents distinct advantages and constraints. *E. coli* is the most widely used host due to its rapid growth and genetic tractability, but issues such as inclusion body formation and limited secretion efficiency persist. *B. subtilis* offers better secretion potential but yields inconsistent results across signal peptide systems. Yeasts like *P. pastoris* provide post-translational modifications and improved enzyme stability, while microalgae offer sustainable platforms but are still in early development stages. Despite these advances, the state-of-the-art in IsPETase expression under fermentor conditions is still emerging. Limitations such as low solubility, inefficient folding, and sub-optimal secretion continue to hinder large-scale application. Regulatory hurdles such as the approval of genetically modified organisms (GMOs) for environmental use, biosafety concerns, and compliance with international standards also pose barriers to implementation. Addressing these issues will require interdisciplinary collaboration among biotechnologists, process engineers, and policymakers to ensure that PETase-based biodegradation strategies are not only scientifically viable but also scalable, safe, and regulatory-compliant.

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## Abbreviations

The following abbreviations are used in this manuscript:

|                 |                                         |
|-----------------|-----------------------------------------|
| 4-pNA           | <i>Para</i> -nitrophenyl acetate        |
| β-FFase         | β-fructofuranosidase                    |
| AI              | Artificial intelligence                 |
| BHET            | Bis(2-hydroxyethyl) terephthalate       |
| CBM66           | Carbohydrate-binding module 66          |
| CO              | Carbon monoxide                         |
| CO <sub>2</sub> | Carbon dioxide                          |
| CSD             | Cell surface display                    |
| ESM             | Evolutionary scale language model       |
| epPCR           | Error-prone PCR                         |
| FACS            | Fluorescence-activated cell sorting     |
| FTIR            | Fourier transform infrared spectroscopy |
| GAN             | Generative adversarial network          |
| GMO             | Genetically modified organism           |
| GRAS            | Generally regarded as safe              |
| HMM             | Hidden Markov model                     |

|         |                                                  |
|---------|--------------------------------------------------|
| HPLC    | High-performance liquid chromatography           |
| JGI/IMG | JGI integrated microbial genomes and microbiomes |
| LC      | Liquid chromatography                            |
| ML      | Machine learning                                 |
| Mt      | Million tonnes                                   |
| NCBI    | National Center for Biotechnology Information    |
| PCL     | Polycaprolactone                                 |
| PDI     | Protein disulphide isomerase                     |
| PE      | Polyethylene                                     |
| PET     | Polyethylene terephthalate                       |
| PGA     | poly- $\gamma$ -glutamic acid                    |
| PP      | Polypropylene                                    |
| PS      | Polystyrene                                      |
| PVC     | Polyvinyl chloride                               |
| SEM     | Scanning electron microscope                     |
| SUMO    | Small ubiquitin-related modifier                 |
| Tat     | Twin-arginine translocation                      |
| UV      | Ultraviolet                                      |
| VNp     | Vesicle nucleating peptide                       |

## References

1. Cowger, W.; Willis, K.A.; Bullock, S.; Conlon, K.; Emmanuel, J.; Erdle, L.M.; Eriksen, M.; Farrelly, T.A.; Hardesty, B.D.; Kerge, K.; et al. Global Producer Responsibility for Plastic Pollution. *Sci. Adv.* **2024**, *10*, eadj8275. [CrossRef]
2. Barnes, D.K.A.; Galgani, F.; Thompson, R.C.; Barlaz, M. Accumulation and Fragmentation of Plastic Debris in Global Environments. *Philos. Trans. R. Soc. B Biol. Sci.* **2009**, *364*, 1985–1998. [CrossRef]
3. Chamas, A.; Moon, H.; Zheng, J.; Qiu, Y.; Tabassum, T.; Jang, J.H.; Abu-Omar, M.; Scott, S.L.; Suh, S. Degradation Rates of Plastics in the Environment. *ACS Sustain. Chem. Eng.* **2020**, *8*, 3494–3511. [CrossRef]
4. Zeenat; Elahi, A.; Bukhari, D.A.; Shamim, S.; Rehman, A. Plastic Degradation by Microbes: A Sustainable Approach. *J. King Saud Univ. Sci.* **2021**, *33*, 101538. [CrossRef]
5. Bronscheuer, U.T. Feeding on Plastic. *Science* **2016**, *351*, 1154–1155. [CrossRef]
6. Zaini, N.; Kasmuri, N.; Mojiri, A.; Kindaichi, T.; Nayono, S.E. Plastic Pollution and Degradation Pathways: A Review on the Treatment Technologies. *Heliyon* **2024**, *10*, e28849. [CrossRef] [PubMed]
7. Grewert, B.; Plassmann, M.M.; MacLeod, M. Pathways for Degradation of Plastic Polymers Floating in the Marine Environment. *Environ. Sci. Process. Impacts* **2015**, *17*, 1513–1521. [CrossRef]
8. Al-Sabagh, A.M.; Yehia, F.Z.; Eshaq, G.; Rabie, A.M.; ElMetwally, A.E. Greener Routes for Recycling of Polyethylene Terephthalate. *Egypt. J. Pet.* **2016**, *1*, 53–64. [CrossRef]
9. Yoshida, S.; Hiraga, K.; Takehana, T.; Taniguchi, I.; Yamaji, H.; Maeda, Y.; Toyohara, K.; Miyamoto, K.; Kimura, Y.; Oda, K. A Bacterium that Degrades and Assimilates Poly(ethylene terephthalate). *Science* **2016**, *351*, 1196–1199. [CrossRef]
10. Palm, G.J.; Reisky, L.; Bottcher, D.; Muller, H.; Michels, E.A.P.; Walczak, M.C.; Berndt, L.; Weiss, M.S.; Bornscheuer, U.T.; Weber, G. Structures of the Plastic-Degrading *Ideonella sakaiensis* MHETase Bound to a Substrate. *Nat. Commun.* **2019**, *10*, 1717. [CrossRef]
11. Austin, H.P.; Allen, M.D.; Donohoe, B.S.; Rorrer, N.A.; Kearns, F.L.; Silveira, R.L.; Pollard, B.C.; Dominick, G.; Duman, R.; Omari, K.E.; et al. Characterization and Engineering of a Plastic-Degrading Aromatic Polyesterase. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E4350–E4357. [CrossRef]
12. Aer, L.; Jiang, Q.; Gul, I.; Qi, Z.; Feng, J.; Tang, L. Overexpression and Kinetic Analysis of *Ideonella sakaiensis* PETase for Polyethylene Terephthalate (PET) Degradation. *Environ. Res.* **2022**, *212 Pt D*, 113472. [CrossRef]
13. Karunatillaka, I.; Jaroszewski, L.; Godzik, A. Novel Putative Polyethylene Terephthalate (PET) Plastic Degrading Enzymes from the Environmental Metagenome. *Proteins Struct. Funct. Bioinf.* **2022**, *90*, 504–511. [CrossRef]
14. Zhang, H.; Perez-Garcia, P.; Dierkes, R.F.; Applegate, V.; Schumacher, J.; Chibani, C.M.; Sternagel, S.; Preuss, L.; Weigert, S.; Schmeisser, C.; et al. The Bacterioidetes *Aequorivita* sp. and *Kaistella jeonii* Produce Promiscuous Esterases with PET-Hydrolyzing Activity. *Front. Microbiol.* **2022**, *12*, 803896. [CrossRef] [PubMed]
15. Saini, P.; Grewall, A.; Hooda, S. In silico Approach for Identification of Polyethylene Terephthalate Hydrolase (PETase)-Like Enzymes. *Bioremediation J.* **2023**, *27*, 311–323. [CrossRef]

16. Almeida, E.L.; Rincon, A.F.C.; Jackson, S.A.; Dobson, A.D.W. In silico Screening and Heterologous Expression of a Polyethylene Terephthalate Hydrolase (PETase)-Like Enzyme (SM14est) with Polycaprolactone (PCL)-Degrading Activity, from the Marine Sponge-Derived Strain *Streptomyces* sp. SM14. *Front. Microbiol.* **2019**, *10*, 2187. [CrossRef] [PubMed]
17. Eiamthong, B.; Meesawat, P.; Wongsatit, T.; Jitdee, J.; Sangsri, R.; Patchsung, M.; Aphicho, K.; Suraritdechachai, S.; Huguenin-Dezot, N.; Tang, S.; et al. Discovery and Genetic Code Expansion of a Polyethylene Terephthalate (PET) Hydrolase from the Human Saliva Metagenome for the Degradation and Bio-Functionalization of PET. *Angew. Chem. Int. Ed. Engl.* **2022**, *134*, e202203061. [CrossRef]
18. Ho, N.H.E.; Effendi, S.S.W.; Ting, W.-W.; Yi, Y.-C.; Yu, J.-Y.; Chang, J.-S.; Ng, I.-S. Heterologous Expression and Characterization of *Aquabacterium parvum* Lipase, a Close Relative of *Ideonella sakaiensis* PETase in *Escherichia coli*. *Biochem. Eng. J.* **2023**, *197*, 108985. [CrossRef]
19. Liu, X.; Jin, J.; Sun, H.; Li, S.; Zhang, F.; Yu, X.; Cao, Q.; Song, Y.; Li, N.; Lu, Z.; et al. Perspectives on the Microorganisms with the Potentials of PET-Degradation. *Front. Microbiol.* **2025**, *16*, 134532. [CrossRef]
20. Joo, S.; Cho, I.J.; Seo, H.; Son, H.F.; Sagong, H.-Y.; Shin, T.J.; Choi, S.Y.; Lee, S.Y.; Kim, K.-J. Structural Insight into Molecular Mechanism of Poly(ethylene terephthalate) Degradation. *Nat. Commun.* **2018**, *9*, 382. [CrossRef]
21. Erickson, E.; Gado, J.E.; Avilan, L.; Bratti, F.; Brizendine, R.K.; Cox, P.A.; Gill, R.; Graham, R.; Kim, D.-J.; Konig, G.; et al. Sourcing Thermotolerant Poly(ethylene terephthalate) Hydrolase Scaffolds from Natural Diversity. *Nat. Commun.* **2022**, *13*, 7850. [CrossRef]
22. Jahanshahi, D.A.; Barzani, M.R.R.; Bahram, M.; Ariaeenejad, S.; Kavousi, K. Metagenomic Exploration and Computational Prediction of Novel Enzymes for Polyethylene Terephthalate Degradation. *Ecotoxicol. Environ. Saf.* **2025**, *289*, 117640. [CrossRef] [PubMed]
23. Liu, W.; Li, C.; Li, B.; Zhu, L.; Ming, D.; Jiang, L. Structure-Guided Discovery and Rational Design of a New Poly(ethylene terephthalate) Hydrolase from AlphaFold Protein Structure Database. *J. Hazard. Mater.* **2024**, *480*, 136389. [CrossRef] [PubMed]
24. Wu, B.; Zhong, B.; Zheng, L.; Huang, R.; Jiang, S.; Li, M.; Tan, P.; Hong, L. Harnessing Protein Language Model for Structure-Based Discovery of Highly Efficient and Robust PET Hydrolases. *Nat. Commun.* **2025**, *16*, 6211. [CrossRef] [PubMed]
25. Meng, X.; Yang, L.; Liu, H.; Li, Q.; Xu, G.; Zhang, Y.; Guan, F.; Zhang, Y.; Zhang, W.; Wu, N.; et al. Protein Engineering of a Stable IsPETase for PET Plastic Degradation by Premuse. *Int. J. Biol. Macromol.* **2021**, *180*, 667–676. [CrossRef]
26. Shi, L.; Liu, H.; Gao, S.; Weng, Y.; Zhu, L. Enhanced Extracellular Production of IsPETase in *Escherichia coli* via Engineering of the pelB Signal Peptide. *J. Agric. Food Chem.* **2021**, *69*, 2245–2252. [CrossRef]
27. Zurier, H.S.; Goddard, J.M. A High-Throughput Expression and Screening Platform for Applications-Driven PETase Engineering. *Biotechnol. Bioeng.* **2023**, *120*, 1000–1014. [CrossRef]
28. Liu, K.; Xu, Z.; Zhao, Z.; Chen, Y.; Chai, Y.; Ma, L.; Li, S. A Dual Fluorescence Assay Enables High-Throughput Screening for Poly(ethylene terephthalate) Hydrolases. *ChemSusChem* **2023**, *16*, e202202019. [CrossRef]
29. Cribari, M.A.; Unger, M.J.; Unarta, I.C.; Ogorek, A.N.; Huang, X.; Martell, J.D. Ultrahigh-Throughput Directed Evolution of Polymer-Degrading Enzymes Using Yeast Display. *J. Am. Chem. Soc.* **2023**, *145*, 27380–27389. [CrossRef]
30. Brott, S.; Pfaff, L.; Schuricht, J.; Schwarz, J.-N.; Bottcher, D.; Badenhorst, C.P.S.; Wei, R.; Bornscheuer, U.T. Engineering and Evaluation of Thermostable IsPETase variants for PET Degradation. *Eng. Life Sci.* **2022**, *22*, 192–203. [CrossRef]
31. Herzog, K.; Muller, R.-J.; Deckwer, W.-D. Mechanism and Kinetics of the Enzymatic Hydrolysis of Polyester Nanoparticles by Lipases. *Polym. Degrad. Stab.* **2006**, *91*, 2486–2498. [CrossRef]
32. Ronkvist, A.M.; Xie, W.; Lu, W.; Gross, R.A. Cutinase-Catalyzed Hydrolysis of Poly(ethylene terephthalate). *Macromolecules* **2009**, *42*, 5128–5138. [CrossRef]
33. Sulaiman, S.; Yamato, S.; Kanaya, E.; Kim, J.-J.; Koga, Y.; Takano, K.; Kanaya, S. Isolation of a Novel Cutinase Homolog with Polyethylene Terephthalate-Degrading Activity from Leaf-Branch Compost by Using a Metagenomic Approach. *Appl. Environ. Microbiol.* **2012**, *78*, 1556–1562. [CrossRef]
34. Carniel, A.; Valoni, E.; Nicomedes, J., Jr.; Gomes, A.; Castro, A. Lipase from *Candida antarctica* (CALB) and Cutinase from *Humicola insolens* Act Synergistically for PET Hydrolysis to Terephthalic Acid. *Process Biochem.* **2017**, *59*, 84–90. [CrossRef]
35. Wei, R.; Oeser, T.; Barth, M.; Weigl, N.; Lubs, A.; Schulz-Siegmund, M.; Hacker, M.C.; Zimmermann, W. Turbidimetric Analysis of the Enzymatic Hydrolysis of Polyethylene Terephthalate Nanoparticles. *J. Mol. Catal. B Enzym.* **2014**, *103*, 72–78. [CrossRef]
36. De Brito, A.L.C.P.; Mattioni, J.V.; Ramos, G.R.; Nakamura, M.; Toma, H.E. Direct Monitoring of the Enzymatically Sequestering and Degrading of PET Microplastics using Hyperspectral Raman Microscopy. *Micron* **2024**, *187*, 103722. [CrossRef]
37. Srivastava, P.; Singh, S.; Soni, M.; Pratap, J.V.; Subramanian, S.; Manickam, N. Enzymatic Degradation of PET by Hydrolase from *Brucella intermedia* IITR130 and Its Genomic Insights. *Biodegradation* **2025**, *36*, 45. [CrossRef] [PubMed]
38. Cavus, A.N.; Cifer, A.B.; Akdogan, K.; Caloglu, B.; Kerimak-Oner, M.N.; Yildirim, D.; Binay, B. A Novel Thermostable PETase from *Kibdelosporangium aridum*: Heterologous Expression, Immobilization and Poly(ethylene terephthalate) Decomposition Applications. *J. Clean. Prod.* **2025**, *510*, 145624. [CrossRef]
39. Satta, A.; Zampieri, G.; Loprete, G.; Campanaro, S.; Treu, L.; Bergantino, E. Metabolic and Enzymatic Engineering Strategies for Polyethylene Terephthalate Degradation and Valorization. *Rev. Environ. Sci. Biotechnol.* **2024**, *23*, 351–383. [CrossRef]

40. Jia, Y.; Samak, N.A.; Hao, X.; Chen, Z.; Wen, Q.; Xing, J. Hydrophobic Cell Surface Display System of PETase as a Sustainable Biocatalyst for PET Degradation. *Front. Microbiol.* **2022**, *13*, 1005480. [CrossRef]
41. Bai, J.; Wang, Y.; Li, W.; Si, C.; Lan, Z.; Liu, Y.; Nakanishi, H.; Li, Z. Development of a Self-Assembled Dual-Enzyme Co-Display Platform on the Surface of the Natural “Chitosan Beads” of Yeast Spores. *Int. J. Biol. Macromol.* **2025**, *286*, 138308. [CrossRef]
42. Seo, H.; Kim, S.; Son, H.F.; Sagong, H.-Y.; Joo, S.; Kim, K.-J. Production of Extracellular PETase from *Ideonella sakaiensis* using Sec-Dependent Signal Peptide in *E. coli*. *Biochem. Biophys. Res. Commun.* **2019**, *508*, 250–255. [CrossRef] [PubMed]
43. Lobstein, J.; Emrich, C.A.; Jeans, C.; Faulkner, M.; Riggs, P.; Berkmen, M. SHuffle, a Novel *Escherichia coli* Protein Expression Strain Capable of Correctly Folding Disulfide Bonded Proteins in its Cytoplasm. *Microb. Cell Fact.* **2012**, *11*, 753. [CrossRef] [PubMed]
44. Castillo-Corujo, A.; Uchida, Y.; Saaranen, M.J.; Ruddock, L.W. *Escherichia coli* Cytoplasmic Expression of Disulfide-Bonded Proteins: Side-by-Side Comparison between Two Competing Strategies. *J. Microbiol. Biotechnol.* **2024**, *34*, 1126–1134. [CrossRef] [PubMed]
45. Lu, H.; Diaz, D.J.; Czarnecki, N.J.; Zhu, C.; Kim, W.; Shroff, R.; Acosta, D.J.; Alexander, B.R.; Cole, H.O.; Zhang, Y.; et al. Machine Learning-Aided Engineering of Hydrolases for PET Depolymerization. *Nature* **2022**, *604*, 662–667. [CrossRef]
46. Bell, E.L.; Smithson, R.; Kilbride, S.; Foster, J.; Hardy, F.J.; Ramachandran, S.; Tedstone, A.A.; Haigh, S.J.; Garforth, A.A.; Day, P.J.R.; et al. Directed Evolution of an Efficient and Thermostable PET Depolymerase. *Nat. Catal.* **2022**, *5*, 673–681. [CrossRef]
47. Carter, L.M.; MacFarlane, C.E.; Karlock, S.P.; Sen, T.; Kaar, J.L.; Berberich, J.A.; Boock, J.T. Increased Cytoplasmic Expression of PETase Enzymes in *E. coli*. *Microb. Cell Fact.* **2024**, *23*, 319. [CrossRef]
48. Fohler, L.; Leibetseder, L.; Cserjan-Puschmann, M.; Striedner, G. Manufacturing of the Highly Active Thermophile PETases PHL7 and PHL7mut3 using *Escherichia coli*. *Microb. Cell Fact.* **2024**, *23*, 272. [CrossRef]
49. Stargardt, P.; Feuchtenhofer, L.; Cserjan-Puschmann, M.; Striedner, G.; Mairhofer, J. Bacteriophage Inspired Growth-Decoupled Recombinant Protein Production in *Escherichia coli*. *ACS Synth. Biol.* **2020**, *9*, 1336–1348. [CrossRef]
50. Zhou, Y.; Lu, Z.; Wang, X.; Selvaraj, J.N.; Zhang, G. Genetic Engineering Modification and Fermentation Optimization for Extracellular Production of Recombinant Proteins using *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 1545–1556. [CrossRef]
51. Cui, L.; Qiu, Y.; Liang, Y.; Du, C.; Dong, W.; Cheng, C.; He, B. Excretory Expression of IsPETase in *E. coli* by an Enhancer of Signal Peptides and Enhanced PET Hydrolysis. *Int. J. Biol. Macromol.* **2021**, *188*, 568–575. [CrossRef]
52. Fatima, K.; Naqvi, F.; Younas, H. A Review: Molecular Chaperone-Mediated Folding, Unfolding and Disaggregation of Expressed Recombinant Proteins. *Cell Biochem. Biophys.* **2021**, *79*, 153–174. [CrossRef]
53. Kaur, J.; Kumar, A.; Kaur, J. Strategies for Optimization of Heterologous Protein Expression in *E. coli*: Roadblocks and Reinforcement. *Int. J. Biol. Macromol.* **2018**, *106*, 803–822. [CrossRef]
54. Niiranen, L.; Espelid, S.; Karlsen, C.R.; Mustonen, M.; Paulsen, S.M.; Heikinheimo, P.; Willassen, N.P. Comparative Expression Study to Increase the Solubility of Cold Adapted *Vibrio* Proteins in *Escherichia coli*. *Protein Expr. Purif.* **2007**, *52*, 210–218. [CrossRef] [PubMed]
55. Ko, H.; Kang, M.; Kim, M.-J.; Yi, J.; Kang, J.; Bae, J.-H.; Sohn, J.-H.; Sung, B.H. A Novel Protein Fusion Partner, Carbohydrate-Binding Module Family 66, to Enhance Heterologous Protein Expression in *Escherichia coli*. *Microb. Cell Fact.* **2021**, *20*, 232. [CrossRef] [PubMed]
56. Wu, T.; Sun, H.; Wang, W.; Xie, B.; Wang, Z.; Lu, J.; Xu, A.; Dong, W.; Zhou, J.; Jiang, M. Boosting Extracellular FastPETase Production in *E. coli*: A Combined Approach of Cognate Chaperones Co-Expression and Vesicle Nucleating Peptide Tag Fusion. *Int. J. Biol. Macromol.* **2024**, *283*, 137857. [CrossRef]
57. Lee, S.Y.; Choi, J.H.; Xu, Z. Microbial Cell-Surface Display. *Trends Biotechnol.* **2003**, *21*, 45–52. [CrossRef] [PubMed]
58. Lee, S.H.; Choi, J.-I.; Park, S.J.; Lee, S.Y.; Park, B.C. Display of Bacterial Lipase on the *Escherichia coli* Cell Surface by Using FadL as an Anchoring Motif and Use of the Enzyme in Enantioselective Biocatalysis. *Appl. Environ. Microbiol.* **2004**, *70*, 5074–5080. [CrossRef]
59. Yamashita, T.; Matsumoto, T.; Yamada, R.; Ogino, H. Display of PETase on the Cell Surface of *Escherichia coli* Using the Anchor Protein PgsA. *Appl. Biochem. Biotechnol.* **2024**, *196*, 5471–5483. [CrossRef]
60. Narita, J.; Okano, K.; Tateno, T.; Tanino, T.; Sewaki, T.; Sung, M.-H.; Fukuda, H.; Kondo, A. Display of Active Enzymes on the Cell Surface of *Escherichia coli* Using PgsA Anchor Protein and Their Application to Bioconversion. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 564–572. [CrossRef]
61. Wang, N.; Guo, X.; Ng, I. Simultaneous Release of Recombinant Cellulases Introduced by Coexpressing Colicin E7 Lysis in *Escherichia coli*. *Biotechnol. Bioprocess Eng.* **2016**, *21*, 491–501. [CrossRef]
62. Effendi, S.S.W.; Hu, R.-E.; Hsiang, C.-C.; Ting, W.-W.; Huang, C.-L.; Ng, I.-S. Exploring PETase-Like Enzyme from Shotgun Metagenome and Co-Expressing Colicin E7 in *Escherichia coli* for Effective PET Degradation. *Process Biochem.* **2024**, *140*, 78–87. [CrossRef]
63. Su, L.; Woodard, R.W.; Chen, J.; Wu, J. Extracellular Location of *Thermobifida fusca* Cutinase Expressed in *Escherichia coli* BL21(DE3) without Mediation of a Signal Peptide. *Appl. Environ. Microbiol.* **2013**, *79*, 4192–4198. [CrossRef]

64. Aer, L.; Qin, H.; Wo, P.; Feng, J.; Tang, L. Signal Peptide Independent Secretion of Bifunctional Dual-Hydrolase to Enhance the Bio-Depolymerization of Polyethylene Terephthalate. *Bioresour. Technol.* **2024**, *391*, 129884. [CrossRef]
65. Schallmeyer, M.; Singh, A.; Ward, O.P. Developments in the Use of *Bacillus* Species for Industrial Production. *Can. J. Microbiol.* **2004**, *50*, 1–17. [CrossRef] [PubMed]
66. Cui, W.; Han, L.; Suo, F.; Liu, Z.; Zhou, L.; Zhou, Z. Exploitation of *Bacillus subtilis* as a Robust Workhorse for Production of Heterologous Proteins and Beyond. *World J. Microbiol. Biotechnol.* **2018**, *34*, 145. [CrossRef] [PubMed]
67. Wu, X.C.; Lee, W.; Tran, L.; Wong, S.L. Engineering a *Bacillus subtilis* Expression-Secretion System with a Strain Deficient in Six Extracellular Proteases. *J. Bacteriol.* **1991**, *173*, 4952–4958. [CrossRef]
68. Wang, N.; Guan, F.; Lv, X.; Han, D.; Zhang, Y.; Wu, N.; Xia, X.; Tian, J. Enhancing Secretion of Polyethylene Terephthalate Hydrolase PETase in *Bacillus subtilis* WB600 Mediated by the SP<sub>amy</sub> Signal Peptide. *Let. Appl. Microbiol.* **2020**, *71*, 235–241. [CrossRef]
69. Huang, X.; Cao, L.; Qin, Z.; Li, S.; Kong, W.; Liu, Y. Tat-Independent Secretion of Polyethylene Terephthalate Hydrolase PETase in *Bacillus subtilis* 168 Mediated by Its Native Signal Peptide. *J. Agric. Food Chem.* **2018**, *66*, 13217–13227. [CrossRef]
70. Yuan, G.; Wong, S. Isolation and Characterization of *Bacillus subtilis* groE Regulatory Mutants: Evidence for orf39 in the dnaK Operon as a Repressor Gene in Regulating the Expression of Both groE and dnaK. *J. Bacteriol.* **1995**, *177*, 6462–6468. [CrossRef]
71. Yuan, G.; Wong, S. Regulation of groE Expression in *Bacillus subtilis*: The Involvement of the Sigma A-Like Promoter and the Roles of the Inverted Repeat Sequence (CIRCE). *J. Bacteriol.* **1995**, *177*, 5427–5433. [CrossRef]
72. Xi, X.; Ni, K.; Hao, H.; Shang, Y.; Zhao, B.; Qian, Z. Secretory Expression in *Bacillus subtilis* and Biochemical Characterization of a Highly Thermostable Polyethylene Terephthalate Hydrolase from Bacterium HR29. *Enzyme Microb. Technol.* **2021**, *143*, 109715. [CrossRef] [PubMed]
73. Chen, Z.; Duan, R.; Xiao, Y.; Wei, Y.; Zhang, H.; Sun, X.; Wang, S.; Cheng, Y.; Wang, X.; Tong, S.; et al. Biodegradation of Highly Crystallized Poly(ethylene terephthalate) Through Cell Surface Codisplay of Bacterial PETase and Hydrophobin. *Nat. Commun.* **2022**, *13*, 7138. [CrossRef] [PubMed]
74. Kosiorowska, K.E.; Moreno, A.D.; Iglesias, R.; Leluk, K.; Mironczuk, A.M. Production of PETase by Engineered *Yarrowia lipolytica* for Efficient Poly(ethylene terephthalate) Biodegradation. *Sci. Total Environ.* **2022**, *846*, 157358. [CrossRef] [PubMed]
75. Kosiorowska, K.E.; Moreno, A.D.; Iglesias, R.; Biniarz, P.; Mironczuk, A.M. Streamlining Biological Recycling of Poly(ethylene terephthalate) via Pre-Treatment Methods. *Int. Biodeterior. Biodegrad.* **2024**, *193*, 105842. [CrossRef]
76. Cereghino, G.P.; Cregg, J.M. Applications of Yeast in Biotechnology: Protein Production and Genetic Analysis. *Curr. Opin. Biotechnol.* **1999**, *10*, 422–427. [CrossRef]
77. Cereghino, G.P.L.; Cereghino, J.L.; Ilgen, C.; Cregg, J.M. Production of Recombinant Proteins in Fermenter Cultures of the Yeast *Pichia pastoris*. *Curr. Opin. Biotechnol.* **2022**, *13*, 329–332. [CrossRef]
78. Gemmill, T.R.; Trimble, R.B. Overview of N- and O-Linked Oligosaccharide Structures Found in Various Yeast Species. *Biochim. Biophys. Acta Gen. Subj.* **1999**, *1426*, 227–237. [CrossRef]
79. Yang, Z.; Zhang, Z. Engineering Strategies for Enhanced Production of Protein and Bio-Products in *Pichia pastoris*: A Review. *Biotechnol. Adv.* **2018**, *36*, 182–195. [CrossRef]
80. Deng, B.; Yue, Y.; Yang, J.; Yang, M.; Xing, Q.; Peng, H.; Wang, F.; Li, M.; Ma, L.; Zhai, C. Improving the Activity and Thermostability of PETase from *Ideonella sakaiensis* through Modulating its Post-Translational Glycan Modification. *Commun. Biol.* **2023**, *6*, 39. [CrossRef]
81. Chen, C.-C.; Li, X.; Min, J.; Zeng, Z.; Ning, Z.; He, H.; Long, X.; Niu, D.; Peng, R.; Liu, X.; et al. Complete Decomposition of Poly(ethylene terephthalate) by Crude PET Hydrolytic Enzyme Produced in *Pichia pastoris*. *Chem. Eng. J.* **2024**, *481*, 148418. [CrossRef]
82. Yan, N.; Fan, C.; Chen, Y.; Hu, Z. The Potential for Microalgae as Bioreactors to Produce Pharmaceuticals. *Int. J. Mol. Sci.* **2016**, *17*, 962. [CrossRef] [PubMed]
83. Moog, D.; Schmitt, J.; Senger, J.; Zarzycki, J.; Rexer, K.-H.; Linne, U.; Erb, T.; Maier, U.G. Using a Marine Microalga as a Chassis for Polyethylene Terephthalate (PET) Degradation. *Microb. Cell Fact.* **2019**, *18*, 171. [CrossRef] [PubMed]
84. Kim, J.W.; Park, S.-B.; Tran, Q.-G.; Cho, D.-H.; Choi, D.-Y.; Lee, Y.J.; Kim, H.-S. Functional Expression of Polyethylene Terephthalate-Degrading Enzyme (PETase) in Green Microalgae. *Microb. Cell Fact.* **2020**, *19*, 97. [CrossRef]
85. Rocco, G.D.; Taunt, H.N.; Berto, M.; Jackson, H.O.; Piccinini, D.; Carletti, A.; Scurani, G.; Braidi, N.; Purton, S. A PETase Enzyme Synthesised in the Chloroplast of the Microalga *Chlamydomonas reinhardtii* is Active Against Post-Consumer Plastics. *Sci. Rep.* **2023**, *13*, 10028. [CrossRef]
86. Vogtentanz, G.; Collier, K.D.; Bodo, M.; Chang, J.H.; Day, A.G.; Estell, D.A.; Falcon, B.C.; Ganshaw, G.; Jarnagin, A.S.; Kellis, J.T., Jr.; et al. A *Bacillus subtilis* Fusion Protein System to Produce Soybean Bowman-Birk Protease Inhibitor. *Protein Expr. Purif.* **2007**, *55*, 40–52. [CrossRef]
87. Heinrich, J.; Drewniok, C.; Neugebauer, E.; Kellner, H.; Wiegert, T. The YoaW Signal Peptide Directs Efficient Secretion of Different Heterologous Proteins Fused to a StrepII-SUMO Tag in *Bacillus subtilis*. *Microb. Cell Fact.* **2019**, *18*, 31. [CrossRef]

88. Wu, P.; Tao, Q.; Liu, Y.; Zeng, C.; Li, Y.; Yan, X. Efficient Secretion of Mussel Adhesion Proteins Using a Chaperone Protein Spy as Fusion Tag in *Bacillus subtilis*. *Biotechnol. J.* **2023**, *18*, 2200582. [CrossRef]
89. Le, N.T.P.; Phan, T.T.P.; Truong, T.T.T.; Schumann, W.; Nguyen, H.D. N-terminal LysSN-His-Tag Improves the Production of Intracellular Recombinant Protein in *Bacillus subtilis*. *Cell Biochem. Funct.* **2023**, *41*, 823–832. [CrossRef]
90. Wang, H.; Wang, Y.; Yang, R. Recent Progress in *Bacillus subtilis* Spore-Surface Display: Concept, Progress, and Future. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 933–949. [CrossRef]
91. Jiang, Z.; Xie, X.; Li, Z.; Ban, X.; Gu, Z.; Tang, X.; Hong, Y.; Cheng, L.; Li, C. Regulation of Cell Membrane Permeability Enhanced the Non-Classical Secretion of  $\gamma$ -Cyclodextrin Glycosyltransferase in *Bacillus subtilis*. *J. Agric. Food Chem.* **2022**, *70*, 16307–16315. [CrossRef]
92. O’Riordan, N.M.; Juric, V.; O’Neill, S.K.; Roche, A.P.; Young, P.W. A Yeast Modular Cloning (MoClo) Toolkit Expansion for Optimization of Heterologous Protein Secretion and Surface Display in *Saccharomyces cerevisiae*. *ACS Synth. Biol.* **2024**, *13*, 1246–1258. [CrossRef]
93. Molino, J.V.D.; Saucedo, B.; Kang, K.; Walsh, C.; Diaz, C.J.; Tessman, M.; Mayfield, S. Efficient Secretion of a Plastic Degrading Enzyme from the Green Algae *Chlamydomonas reinhardtii*. *Sci. Rep.* **2025**, *15*, 24690. [CrossRef]
94. Webster, L.J.; Villa-Gomez, D.; Brown, R.; Clarke, W.; Schenk, P.M. A Synthetic Biology Approach for the Treatment of Pollutants with Microalgae. *Front. Bioeng. Biotechnol.* **2024**, *12*, 1379301. [CrossRef]
95. Lee, S.H.; Seo, H.; Hong, H.; Park, J.; Ki, D.; Kim, M.; Kim, H.-J.; Kim, K.-J. Three-Directional Engineering of IsPETase with Enhanced Protein Yield, Activity, and Durability. *J. Hazard. Mater.* **2023**, *459*, 132297. [CrossRef]

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Review

# Fantastic Ferulic Acid Esterases and Their Functions

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## Abstract

Ferulic acid (FA) is one of the most abundant hydroxycinnamic acids found in plant cell walls. Its dehydrodimers play an important role in maintaining the structural rigidity of the plant cell wall. Ferulic acid esterases (FAEs) act as debranching enzymes, cleaving the ester bond between FA and the substituted carbohydrate moieties in FA-containing polysaccharides in the plant cell wall. This enzymatic reaction facilitates the degradation of lignocellulosic materials and is crucial for the efficient utilization of biomass resources. This review focuses on the occurrence of ferulic acid in nature and its different forms and outlines the various classification systems of FAEs, their substrate specificity, and the synergistic interactions of these enzymes with other CAZymes. Additionally, it highlights the various methods that have been developed for detecting hydroxycinnamic acids and estimating the enzyme activity, as well as the versatile applications of ferulic acid.

**Keywords:** hydroxycinnamic acids; catalysis; bioconversion

## 1. Introduction

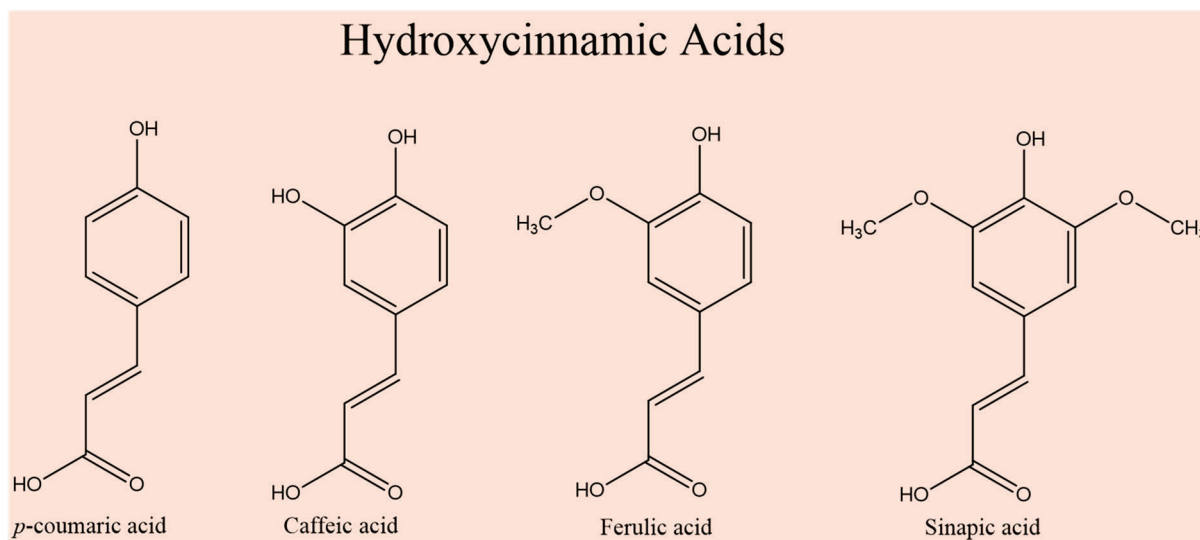
Agricultural residues derived from plant cell walls can be converted to fermentable sugars that can be used in production of biofuels and chemicals. Today's concern with developing alternatives from biobased sources has enhanced the interest in such materials and increased the amount of research in that field [1]. Lignin and hemicellulose together account for 40–70% of plant biomass by weight [2]. However, the complete degradation of lignocellulosic material is challenging due to its complex composition and the strong interactions between its components, which make it resistant to hydrolysis. Ferulic acid (FA) further contributes to this recalcitrance by acting as a structural component, substituting carbohydrate moieties in the xylan chain, the primary constituent of hemicellulose.

Ferulic acid, or its IUPAC name 3-methoxy-4-hydroxycinnamic acid, is covalently bound to polysaccharides such as xylan, galactoglucomannan, and pectin. It plays a crucial role in cross-linking polysaccharide chains and linking hemicellulose to lignin through radical coupling with monolignols in ferulated xylan [2].

Feruloyl esterases (E.C. 3.1.1.73), also known as ferulic acid esterases (FAEs), cinnamoyl esterases, cinnamoyl ester hydrolases, and *p*-coumaroyl esterases, belong to the carboxyl esterase family. These enzymes break the bond between FA (or other hydroxycinnamic acids) and polysaccharides. They play a key role in biomass degradation by working synergistically with other enzymes to release sugar monomers. Due to their catalytic abilities, FAEs have widespread applications in industries such as pharmaceuticals, agriculture, and the biofuels industry.

## 2. Origin and Occurrence of Different Ferulic Acid Forms

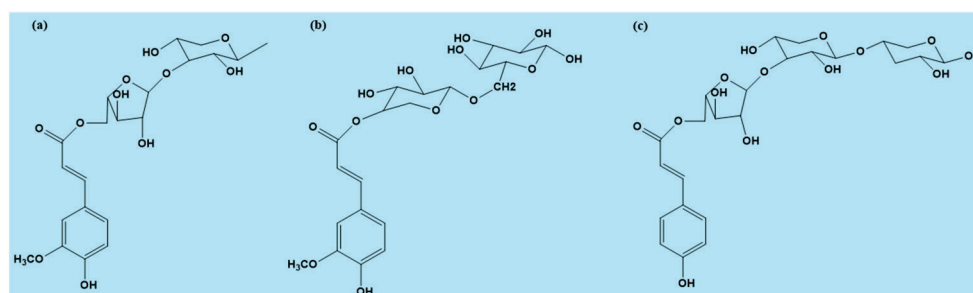
FA belongs to the broader category of hydroxycinnamic acids, alongside *p*-coumaric acid, caffeic acid, and sinapic acid (see Figure 1). These compounds form the largest class of phenolic compounds, which are extensively studied for their antioxidant activity and their role in preventing diseases, including cancer, cardiovascular disorders, and neurodegenerative conditions [3,4].



**Figure 1.** Illustration of the four main hydroxycinnamic acids, *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid, highlighting their structural similarities and different functional groups on the phenyl ring.

Ferulic acid is often found linked to polysaccharides, primarily glucuronarabinoxylan (GAX) and, more frequently, arabinoxylan (AX), as glucuronic acid is not widely found in cereal grains. GAX consists of  $\beta$ -(1 $\rightarrow$ 4)-linked xylose units that are branched via glycosidic bonds to glucuronic or methyl glucuronic acid at the C(O)2 position, and  $\alpha$ -L-arabinosyl at the C(O)2 and/or C(O)3 positions. In AX, glucuronic acid is absent, and the structure primarily comprises  $\beta$ -(1 $\rightarrow$ 4)-linked xylopyranosyl units with  $\alpha$ -L-arabinosyl substitutions at the C(O)2 and/or C(O)3 positions.

In monocotyledons, particularly in the Poaceae family (e.g., wheat, maize, barley, and rice), ferulic acid has been extensively studied [5]. Here, ferulic acid is linked through its carboxyl group to the C(O)5 of arabinose, forming 3-O-(5-O-trans-feruloyl- $\alpha$ -L-arabinofuranosyl)-(1 $\rightarrow$ 3)-D-xylose (Figure 2a). In bamboo shoots, ferulic acid connects to the xylose backbone, forming O-(4-O-trans-feruloyl- $\alpha$ -D-xylopyranosyl)-(1 $\rightarrow$ 6)-D-glucopyranose [6] (Figure 2b).



**Figure 2.** Hydroxycinnamic acids bound to a polysaccharide chain: (a) ferulic acid (FA) attached to arabinose, which is linked to xylose; (b) FA directly bound to xylose, which is connected to glucose; and (c) *p*-Coumaric acid attached to arabinose, which is linked to xylose.

Aside from ferulic acid, *p*-coumaric acid is also present in plant cell walls in smaller amounts. Interestingly, in bamboo shoots, *p*-coumaric acid is linked to arabinose via an ester bond, forming 3-O-[5-O-(trans-*p*-coumaroyl)- $\alpha$ -L-arabinofuranosyl]-(1 $\rightarrow$ 3)-O- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose (Figure 2c). Both ferulic and *p*-coumaric acids exist predominantly in their trans-isomeric forms. Cis-ferulic acid has been reported in significantly smaller amounts, comprising approximately 2–18% of the total ferulic acid content in maize roots [7]. However, it remains understudied, primarily due to challenges in separating the cis and trans isomers [8].

In dicotyledons, ferulic acid is found in smaller amounts compared to monocotyledons. It serves as a side chain of arabinan and galactan in sugar beet pulp [9] and spinach. The latter can also be esterified with a *p*-coumaric acid in the primary cell wall [10]. In galactan, ferulic acid forms an ester bond at the C(O)6 position, while in arabinan, it forms ester bonds at the C(O)2 or C(O)3 positions [11]. Evidence of its presence have been found in Chinese water chestnut, carrots, glass wort, and pine hypocotyl cell walls [12–15].

### 3. Ferulate Dimers and Cross-Links

Ferulic acid has a bifunctional role in plant cell walls. Ferulate can form dimers either through the aromatic ring or the aliphatic chain. These dimers result in cross-links between polysaccharide–polysaccharide chains or polysaccharide–lignin chains, influencing the accessibility and digestibility of the cell wall [16].

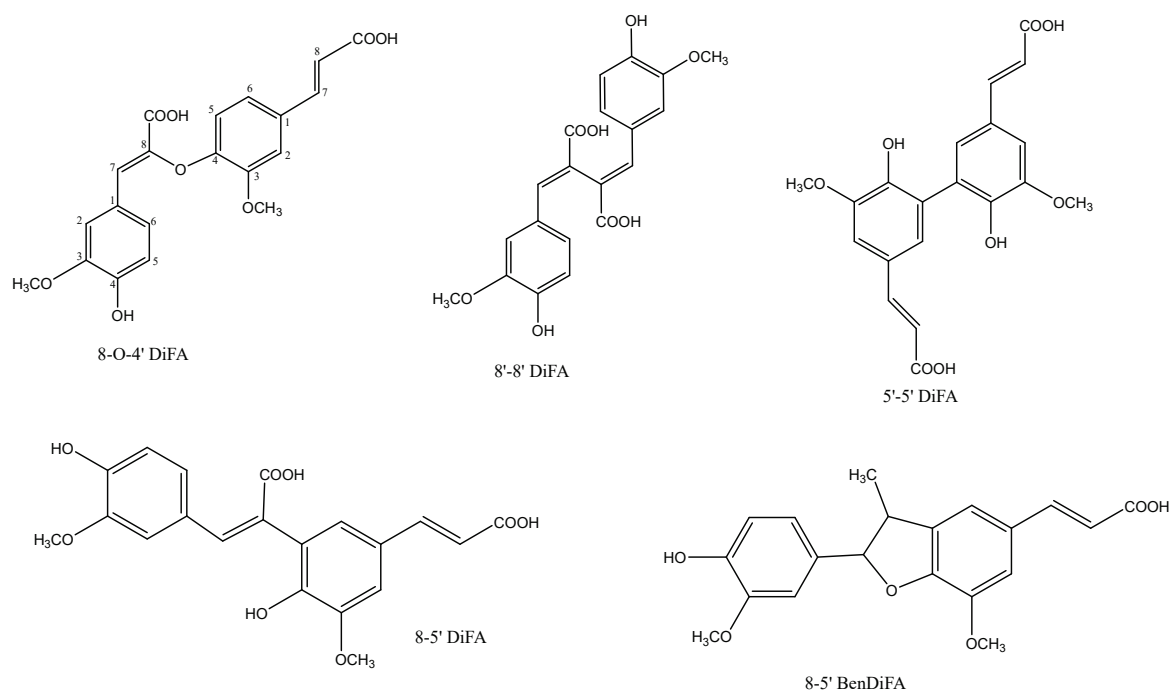
The general structure of ferulate consists of the ferulic acid aromatic ring which is linked via an ether bond to lignin, while the carboxyl group forms an ester bond with the polysaccharide chain [17]. Interestingly, ferulates act as initiation sites for the lignification process through direct covalent binding to monolignols such as coniferyl and sinapyl alcohols [18].

The formation of ferulate dimers in polysaccharides can occur through two pathways: photochemically [19] and via peroxidase-catalyzed oxidative coupling. The first method produces cyclodimers [20,21], whereas radical-mediated dimerization results in a wide range of dehydrodimers [18].

The five main type of dehydrodimers (Figure 3) found in nature are [18]:

- 8-O-4'-DiFA:(Z)- $\beta$ -{4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxy-cinnamic acid;
- 8-5' DiFA: (E,E)-4,4'-dihydroxy-3,5'-dimethoxy- $\beta$ ,3'-bicinnamic acid;
- 8-5'-BenDiFA: in the benzofuran form;
- 8-8'-DiFA: (E,E)-4,4'-dihydroxy-3,3'-dimethoxy- $\beta$ , $\beta$ '-bicinnamic acid;
- 5-5' DiFA: (E,E)-4,4'-dihydroxy-5,5'-dimethoxy- $\beta$ ,3'-bicinnamic acid.

The exact type of dehydrodimers in a sample can be determined after their isolation (using enzymatic degradation or chemical saponification) and depends on the species that are being treated. Diferulates are present in a wide range of cereals such as maize, wheat, spelt, rice, wild rice, barley, rye, oats, and millet. For example, Bartolome et al. reported that in alkali- or enzyme-treated spent barley grain, 5'-5' DiFA was the predominant diferulate [22]. It is estimated that the diferulate content is 8–39 times higher in the insoluble dietary fiber fraction than in the soluble one. The proportions of various dimers are different in the two fractions, with 8-5' DiFA being predominant in the insoluble fraction and 8-8' DiFA in the soluble [23]. Hatfield reported that model substrates of feruloylated arabinose mainly resulted in formation of 8-5' DiFA and 8-5' DiFA benzofuran [18].

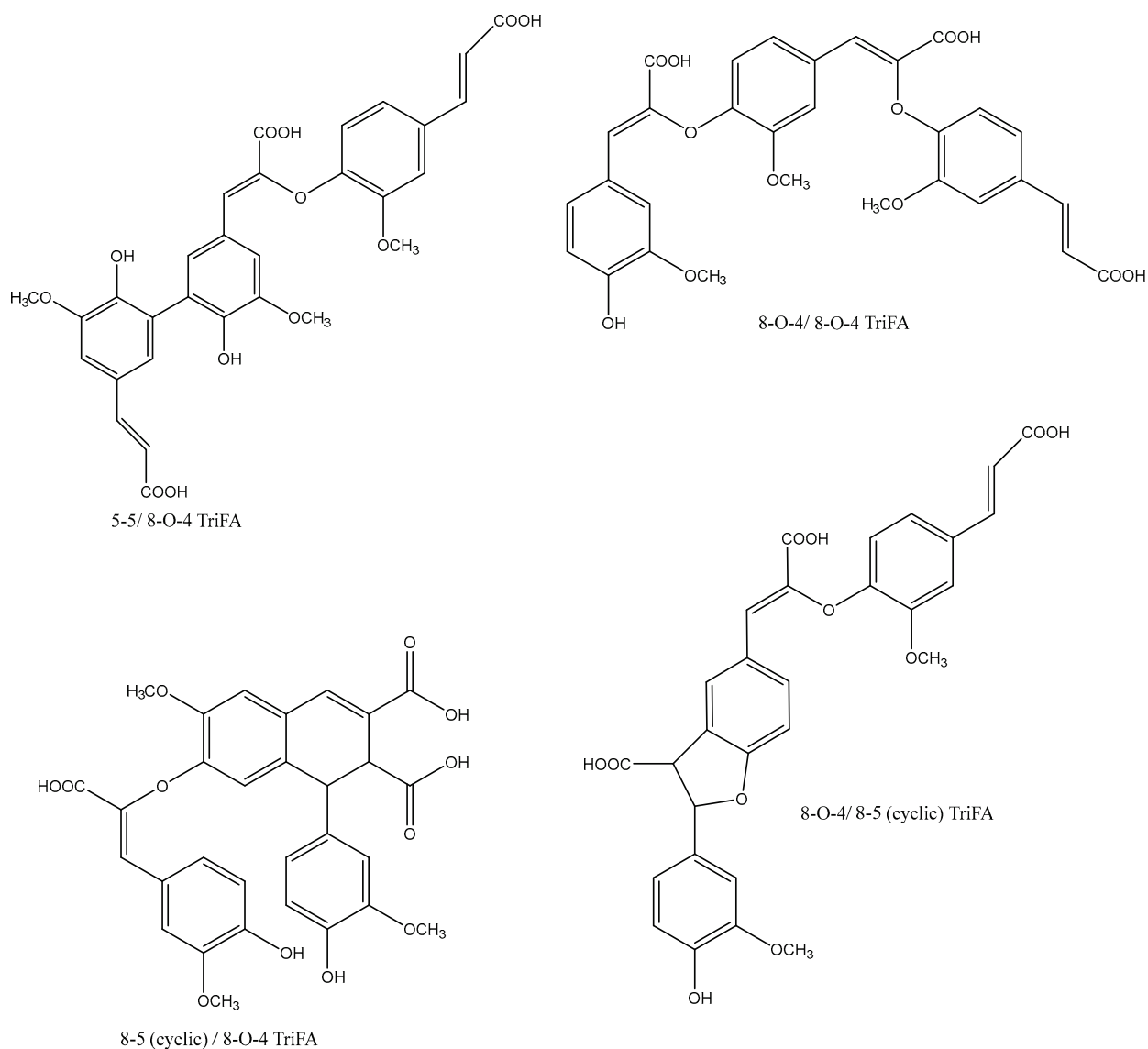


**Figure 3.** Structures of FA dehydrodimers.

Oxidative coupling is not only limited to the formation of dehydrodimers. Several findings support the presence of both dehydrotrimers (Figure 4) and dehydrotetramers in maize bran [24–29]. These structures include the 5-5/8-O-4-coupled dehydrotrimer [27], which is hypothesized to be the prevailing trimer in corn bran, the 8-O-4/8-O-4-dehydrotrimer, and the 8-8 (cyclic)/8-O-4-dehydrotrimer [24]. The isolation of 8-O-4/8-O-4- and 8-8(cyclic)/8-O-4-coupled dehydrotrimers suggests that radical-coupled ferulate dehydrotrimers could potentially lead to cross-linking of three polysaccharide chains [24].

The two tetramers that have been isolated today are probably formed by 4-O-5 coupling of two DiFA 8-5' (cyclic), and by 5-5' DiFA–DiFA coupling [29].

Hydroxycinnamic acids were also widely hypothesized to covalently cross-link with Tyr and Cys residues in the cell wall proteins [30]. Proteins have multiple roles in the cell wall, such as transport, inhibitory functions, and structural purposes. Their secretion influences their solubility state, and these insoluble proteins are hypothesized to have ionic or salt interactions with polysaccharides [30]. Although no evidence that these linkages exist in nature has been reported yet, *in vitro* experiments have been conducted, and covalent coupling between proteins and polysaccharides is expected to result in new macromolecules with functional properties. FA could act as a substrate for the formation of FA oligomers in the presence of horseradish peroxidase (HRP) and  $H_2O_2$ , and this degree of oligomerization increases when the FA is bound to Gly-Tyr-Gly tripeptide [16,31]. In 2004, Boeriu et al. achieved cross-linking between protein and an FA polysaccharide. In the presence of HRP and  $H_2O_2$ , they enzymatically conjugated arabinoxylan to  $\beta$ -casein, which contains Tyr residues [32].



**Figure 4.** Structures of FA trimers.

#### 4. Feruloyl (Ferulic Acid) Esterases and Their Classification

FAEs are an important subclass of carboxylic esterases (E.C. 3.1.1), since they facilitate the degradation of plant cell walls via hydrolysis of the ester bond. The release of the ferulate ester groups can result in breaking of the cross-link between hemicellulose chains or between hemicellulose and lignin. FAEs usually exist in a monomeric form with a single catalytic site, and in some cases contain a carbohydrate-binding module (CBM). The presence of a CBM in FAEs has been shown to improve the catalytic efficiency of the protein [33–38]. Multiple attempts have been made to systematically classify these enzymes, and different systems have been developed over the years when trying to address their diversity. Below we summarize the main aspects, key features, and limitations of these systems (Table 1).

**Table 1.** FAE classification systems.

| Classification System  | Description                                                                                                    | Main Features                                                                                      | Limitations                                                                    |
|------------------------|----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| CAZy (CE1)             | Groups FAEs into the CE1 based on sequence similarity and enzymatic catalytic mechanism.                       | Standardized database for carbohydrate-active enzymes.                                             | Does not consider the functional diversity of FAEs.                            |
| Crepin (2004) [39]     | Categorizes FAEs into types A, B, C, D, and putative E based on sequence similarity and substrate specificity. | Correlations based on sequence similarities and specificity on synthetic methyl esters.            | Based on limited data; it can cluster unrelated enzymes within the same group. |
| Benoit (2008) [40]     | Seven fungal FAE subfamilies based on phylogenetic analysis of fungal genomes.                                 | Identifies evolutionary relationships within fungal species.                                       | Limited to fungal genomic data. Excludes bacterial FAEs.                       |
| Udatha (2011) [41]     | Twelve FAE families based on sequence and structure from fungi, bacteria, and plants.                          | Validation via computational and experimental methods.                                             | Lacks functional correlation for some enzymes.                                 |
| Dilokpimol (2016) [42] | Thirteen subfamilies of fungal FAEs based on phylogenetics and substrate specificity.                          | Divides fungal FAEs into subfamilies within CE1 based on biochemical data from new fungal genomes. | Error-prone in grouping bacterial and fungal FAEs together.                    |

FAEs as carbohydrate-active enzymes are initially classified under the Carbohydrate Esterase Family 1 (CE1) in the CAZy database. This classification, with the criteria of sequence similarity and being within the same CE family, includes trehalose 6-O-mycosyltransferase (EC 2.3.1.122), diacylglycerol O-acyltransferase (EC 2.3.1.20) carboxylic ester hydrolase (EC 3.1.1), and acetyl xylan esterase (EC 3.1.1.72) [43]. Based on the CAZy database, additionally to CE1, one FAE is classified in CE15 as a 4-O-methyl-glucuronoyl esterase/feruloyl esterase (FI6\_CE15).

A widely used FAE classification system is the one proposed by Crepin et al. in 2004 [39]. The criteria used for clustering were primary sequence identity and activity against synthetic substrates and diferulate. Based on these criteria, four subclasses were proposed: A, B, C, and D.

Four model substrates were used to estimate activity and were the methyl esters of the acids. These types of hydroxycinnamic acids are methyl ferulate (MFA), methyl caffeate (MCA), methyl sinapate (MSA), and methyl *p*-coumarate (MpCA). All enzymes from types C and D were active on all synthetic substrates, whereas type A was not active on MCA, and type B was not active on MSA. Additionally, type B and C enzymes were unable to release ferulic acid dehydromers from model and complex substrates [39].

A phylogenetic tree was constructed based on the available sequences, highlighting their similarity to other enzyme families. Type A enzymes resemble lipases, type B is similar to acetyl xylan esterases, type C is related to chlorogenate esterase and tannase, and type D shares similarities with xylanases. In addition, six sequences clustered outside this subclassification scheme, suggesting the existence of a fifth class, E.

The system, however, has certain limitations, which are acknowledged by the authors. These include a lack of comparable activity data, a reliance on primary sequence identity, and gaps in the knowledge regarding the type of biomass that triggers the gene expression of these proteins [39].

In 2008, the presence of FAEs in fungal genomes was extensively investigated by Benoit et al [40]. Starting with a strict threshold  $1E^{-70}$  of BlastP and continuing with

phylogenetic analysis, multiple alignment (MUSCLE), and tree reconstruction methods, they proposed seven FAE subfamilies. Biochemically characterized members were included in three of these subfamilies (SF). The biochemical properties of these enzymes appeared to be different, suggesting that the SF described different classes of FAEs [40]. However, the number of FAE genes varies significantly, even among closely related species. This suggests that FAEs evolved from different esterase families and do not necessarily have a common ancestor. Some of these diverse esterase families that FAEs descent from have been classified in specific SFs, such as SF1–4 from tannases, SF6 from acetyl xylan esterase, and SF7 from lipases. However, in comparison to Crepin’s system, Type D FAEs are missing in Benoit’s analysis.

In parallel, in 2011, Udatha et al. [41] conducted a descriptor-based computational analysis that led to a broader classification of FAEs from fungi, bacteria, and plants. This system categorized FAEs into 12 families, with further subclassification within each family to distinguish substrate specificities [41].

Benoit’s work on fungal genes was later expanded by Dilokpimol et al. in 2016 [42]. They investigated the evolutionary relationships between the fungal FAE families and other related enzyme families such as acetyl xylan esterases, lipases, and tannases. Their phylogenetic tree analysis resulted in 13 FAE subfamilies. Some subfamilies, such as SF1–SF3 and SF5–SF7, remained consistent with earlier classifications, while SF8–SF13 represented newly identified evolutionary groups, highlighting the diversity within FAEs [42]. This work managed to provide a complete classification of fungal FAEs and illustrated that despite the catalytic triad Ser-His-Asp being conserved, FAEs do not have a common ancestor.

Moreover, this updated phylogenetic analysis demonstrated that Crepin’s ABCD system does not align well with the evolutionary relationships of FAEs. Some subfamilies are predominantly associated with a single type (A, B, C, or D), while others include multiple types, indicating greater complexity than previously understood.

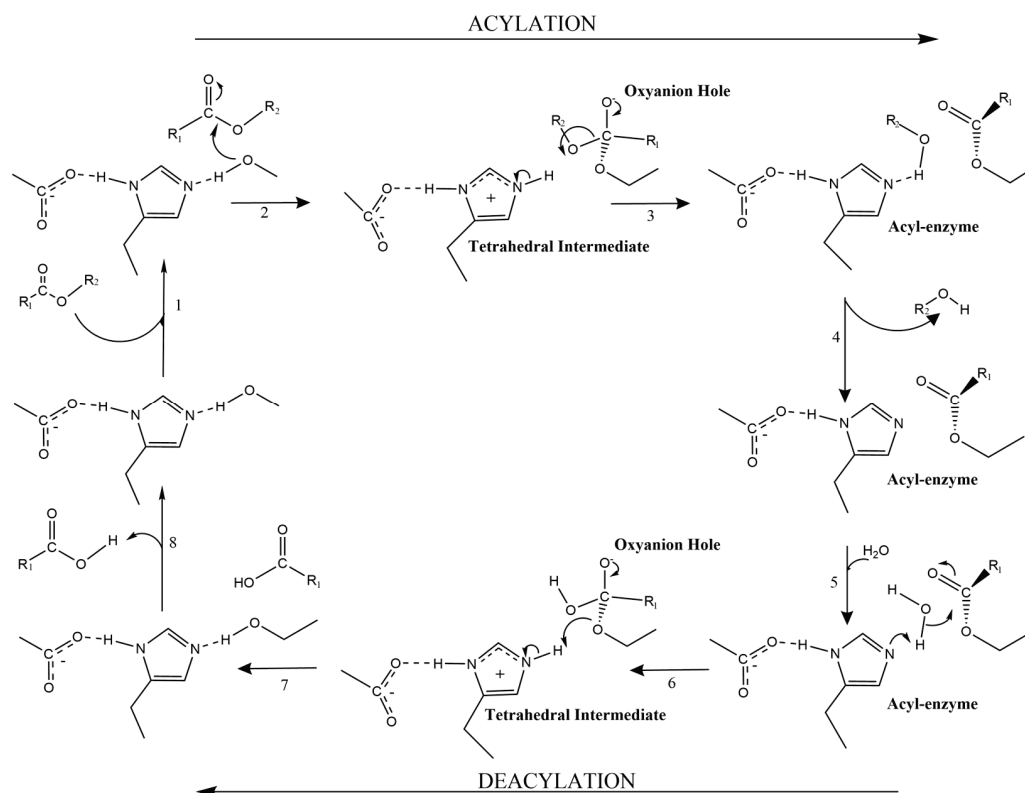
## 5. Catalytic Mechanism and Well-Characterized FAEs

The catalytic mechanism of FAEs has been widely studied and is similar to that adopted by lipases, following the mechanism of serine hydrolases (Figure 5). The catalytic triad consists of Ser-His-Asp/Glu, and the consensus sequence GX SXG is surrounding the Ser. In brief, the hydroxyl group of serine (nucleophile) attacks the carbonyl carbon of the ester. This results in the formation of a tetrahedral intermediate, which then collapses to generate an acyl-enzyme intermediate, with the acyl moiety being covalently bound to the enzyme.

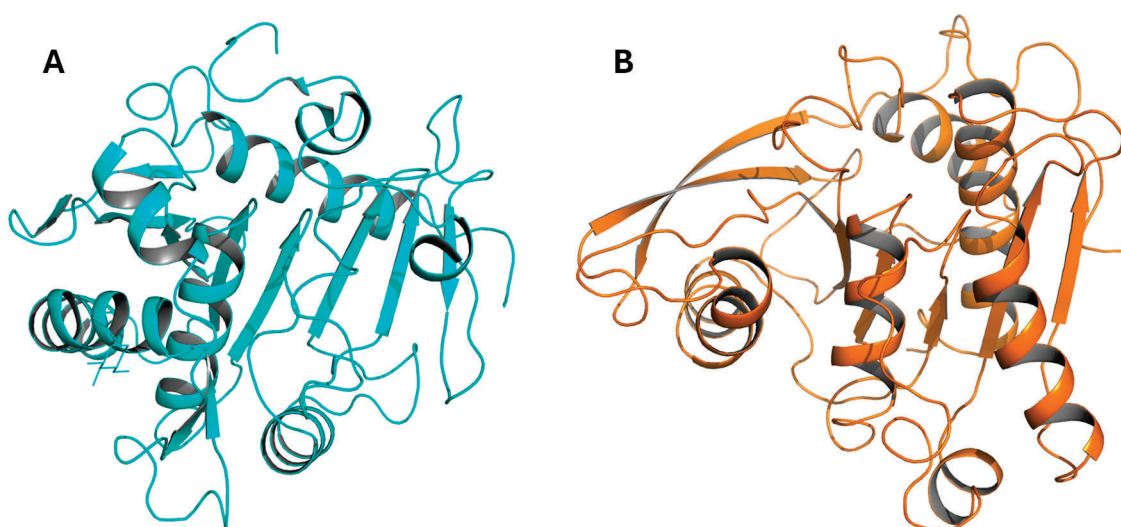
In the second step, a water molecule acts as a nucleophile, hydrolyzing the acyl-enzyme intermediate. Along with the protonation of the ester oxygen by His, this leads to the release of the acid in the deacylation step. In both steps, tetrahedral intermediates are formed and are energetically stabilized by the oxyanion hole, where two NH groups form hydrogen bonds with the carbonyl oxygen [44]. Specifically, for FAEs, the catalytic acid can interact directly with the para-hydroxy group, forming a hydrogen bond. Upon mutation, the esterase activity is maintained, but the recognition on hydroxycinnamate acids is abolished [45]. In some cases the same hydrogen bond is hypothesized to restrict the activity only on monomeric FA and prevent the recognition of dimers [46].

Currently, in the BRENDA database ([brenda-enzyme.info](http://brenda-enzyme.info)) on functional and molecular information of enzymes, only 13 PDB entries and structure links are available, and all these enzymes have an  $\alpha/\beta$  fold. Only few detailed studies on FAEs are available and originate from *Aspergillus niger* [47–52] and *Clostridium thermocellum* [53–55] (Figure 6). For example, AnFAE from *A. niger* is a  $\alpha/\beta$  hydrolase, with the catalytic triad being described

above [48,49,51]. This protein has a significant sequence similarity of 37% to a lipase from *Rhizomucor miehi* but does not exhibit any lipase activity [51,52]. Another detailed study on FAEs concerns enzymes designated XynY and XynZ [53–55], originating from *Clostridium thermocellum*. They exhibit a canonical  $\alpha/\beta$ -hydrolase fold based on an eight-stranded  $\beta$ -sheet surrounded by  $\alpha$ -helices. Despite this, they exhibit a poor structural homology with AnFAE, although the catalytic site of all three enzymes is well conserved.



**Figure 5.** Schematic view of the serine hydrolases mechanism. The figure illustrates all the steps for carbohydrate hydrolysis and the formation of a tetrahedral intermediate, which is stabilized by the oxyanion hole and then collapses from the formation of the acyl enzyme complex during acylation. The addition of water initiates the reverse process of deacylation.



**Figure 6.** Cartoon diagrams of AnFAE, PDB 1UZA (A), and XynZ, PDB 1JJF (B), highlighting the low overall structural homology between the two enzymes, which are both classified as FAEs.

A number of different FAEs are characterized to varying degrees (see Table 2) and occur either as monomers or dimers. Some FAEs have interesting and distinct features, including activity in the presence of various metal ions or activity under more extreme conditions (pH and/or temperature).

**Table 2.** Summary of well-characterized FAEs.

| Enzyme    | Organism                               | Oligomeric State | pH Opt. | Temp Opt.       | Interesting Characteristics                                                                                                                  |
|-----------|----------------------------------------|------------------|---------|-----------------|----------------------------------------------------------------------------------------------------------------------------------------------|
| AnFAEA    | <i>Aspergillus niger</i>               | Monomer          | 6.0 *   | 37 °C *         | Tyr80 and Trp260 mutations to accommodate bigger substrates [46] (PDB 2BJH).                                                                 |
| AoFAEB    | <i>Aspergillus oryzae</i>              | Dimer            | 7.0 *   | 37 °C *         | C residues neighboring the catalytic S and H form a disulfide bond (motif found in tannases), which is crucial for activity [56] (PDB 3WMT). |
| XynZ      | <i>Clostridium thermocellum</i>        | Not mentioned    | 6.0     | 60 °C           | CBM containing FAEs that are active with $\Delta$ CBM truncation but inactive when unknown (non-catalytic domain) is omitted [55].           |
| StFaeC    | <i>Sporotrichum thermophile</i>        | Dimer            | 6.0     | 55 °C           | Type C FAE, active on all methyl hydroxycinnamic acids [57].                                                                                 |
| PpFAE     | <i>Penicillium piceum</i>              | Not mentioned    | 3.0     | 70 °C           | FAE type C with broad pH activity range 2.0–8.0 [58].                                                                                        |
| LhFAE     | <i>Lactarius hatsudake</i>             | Monomer          | 4.0     | 30 °C           | Metal ion-tolerant: retains 80% of activity in 5 mM metal ions; $Mn^{2+}$ boosts activity to 114% [59].                                      |
| LbFAE     | <i>Lentilactobacillus buchneri</i>     | Dimer            | 6.5     | 40 °C           | Internally facing active site; inactive on dehydrodimers [45].                                                                               |
| LJ0536    | <i>Lactobacillus johnsonii</i> N6.2    | Dimer            | -       | -               | Open canal catalytic site; solvent exposed [60].                                                                                             |
| GthFAE    | <i>Geobacillus thermoglucosidasius</i> | Dimer            | 8.5     | 50 °C           | Mutagenesis increased T <sub>m</sub> , but activity decreased in variants [61].                                                              |
| LaFae     | <i>Lactobacillus acidophilus</i>       | Dimer            | 8.0     | 25–37 °C        | Phe→Ala mutation near catalytic site increased activity; cap domain flexibility [62].                                                        |
| AmCE1     | <i>Anaeromyces mucronatus</i>          | Monomer          | 7.2 *   | 25 °C *         | Structurally based loop domain “β-clamp” responsible for exolytic activity [63] (PDB 5CXU).                                                  |
| FAE-II    | <i>Fusarium oxysporum</i>              | Not mentioned    | 7.0     | 45 °C           | Synergistic interaction with xylanase; active in a ternary solvent system [64].                                                              |
| AocFaeC   | <i>Aspergillus ochraceus</i>           | Monomer          | 6.5     | 40 °C           | Butanol-specific biocatalyst, with 5x higher butyl caffeate synthesis rate compared to type B FAE from <i>A. niger</i> [65].                 |
| Fae125    | <i>Talaromyces wortmannii</i>          | Not mentioned    | 4.7/4.7 | 24.5 °C/38.9 °C | The optimal values of pH and temperature correspond to the synthesis of PFA and AFA, respectively [66] **.                                   |
| XynII-Fae | <i>Prevotella ruminicola</i> 23        | Not mentioned    | 7.0     | 40 °C           | Bifunctional xylanase/feruloyl esterase, with each activity originating from a distinct domain [67].                                         |

\* Optimizing conditions in these cases were not part of the published work, and the values mentioned in the table correspond to the ones used to determine the enzymatic activity. \*\* PFA: prenyl ferulate; AFA: L-arabinose ferulate.

A metal ion-tolerant, 55kDa monomeric FAE from the mushroom *Lactarius hatsudake* (LhFAE) was characterized in 2016. LhFAE maintained 80% of the initial enzyme activity in the 5.0 mM presence of  $K^+$ ,  $Al^{3+}$ ,  $Pb^{2+}$ ,  $Cd^{2+}$ ,  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $Zn^{2+}$ , and  $Ca^{2+}$  ions [59].

Stability at extreme pH values has also been targeted. In 2005, Topakas et al. reported an FAE, designated StFaeC, from *Sporotrichum thermophile*, which was active on all methyl hydroxycinnamic acids and classified as type C. StFaeC was a dimer in solution and had an optimal pH at 6.0, but still retained 50% of its activity at pH 10 [57]. A low-pH-tolerant FAE, PpFAE, was isolated from *Penicillium piceum*. PpFAE exhibited activity at a broad pH range of 2.0–8.0 with an optimum at 3.0, while the optimum temperature was 70 °C [58]. As most FAEs exhibit optima under less harsh conditions (often pH 6–7 and temperature up to 50 °C), the high acidic thermal stability suggests wider application fields for this enzyme.

Further work includes the engineering of FAEs. In 2022, Yang et al. reported a new FAE from *Geobacillus thermoglucosidasius* and performed site-directed mutagenesis to enhance thermostability. Although the mutations increased the  $T_m$ , the enzyme activity decreased for all variants. The most favorable outcome (highest  $T_m$ ) was observed in a double mutant. In this variant, an arginine substitution led to salt bridge formation and reduced flexibility in the cap region, while an asparagine substitution improved the hydrophilicity of the protein surface [61]. This indicates that the flexibility of the catalytic region is of importance for the activity, which was corroborated for a novel FAE from *Lactobacillus acidophilus*, designated as LaFae [62]. In this dimeric protein, a phenylalanine-to-alanine mutation near the catalytic site increased the activity. This variant probably exhibits enhanced flexibility of the cap domain, facilitating substrate binding [62].

Accessibility to the catalytic site may be another factor of importance. In 2022, Kasmaei et al. reported a new type of FAE homodimer in LbFAE from *Lentilactobacillus buchneri*. This enzyme exhibits a unique dimerization profile, and the active site of each monomer is located internally, facing the other protomer. This enzyme can only accommodate a single FA and is not active on dehydrodimers [45]. This contrasts with the findings of Lai et al. (2011), in whose study the characterized dimeric protein from *Lactobacillus johnsonii* N6.2 (LJ0536) demonstrated a solvent-exposed, open canal, large catalytic site [60].

These diverse structural and functional studies emphasize the versatility of FAEs across different microbial sources. The number of structurally resolved enzymes is still limited. However recent advances in protein engineering and characterization highlight FAEs' potential for industrial and biotechnological applications.

## 6. FAEs in (Trans)Esterification

Various studies have demonstrated the versatility of fungal FAEs in a wide variety of reaction media containing mainly non-aqueous solvents, enabling efficient esterification and transesterification of phenolic acids with simple alcohols and with carbohydrates. For some applications, free ferulic acid is the most useful substance, but in other cases, derivatives of ferulic acid have more promising properties. Therefore, derivatization of ferulic acid, for example by esterification, is of great interest. Some of these cases are highlighted in the following section.

Giuliani et al. used an FAE to esterify ferulic acid with 1-pentanol in a microemulsion containing n-hexane, water, and the surfactant cetyltrimethylammoniumbromide [68]. A high yield was obtained (up to 60%), but due to the low solubility of ferulic acid in the system, the reactions were carried out at concentrations below 1 mM.

In 2003, Topakas et al. purified and characterized an FAE-II from *Fusarium oxysporum* that retained its catalytic activity in a surfactantless microemulsion containing n-hexane, 1-propanol, and water, in which the solubility of cinnamic acid derivatives was up to 100 times higher. This type of microemulsion is also called a ternary solvent system and has been used in several subsequent studies of FAE-catalyzed synthesis reactions. The *F. oxysporum* enzyme catalyzed the esterification of various phenolic acids but not cinnamic acid, indicating the necessity of a C-4 substitution on the phenyl ring [64].

In 2005, the same group reported the first enzymatic feruloylation of a carbohydrate, L-arabinose, with *Sporotrichum thermophile* FAE type C (StFaeC), in another ternary system consisting of n-hexane/t-butanol/water [57]. Other ternary systems were used for the feruloylation of di- and oligo-saccharides (n-hexane, 2-butanone, and MES–NaOH buffer), using an FAE from a commercial multi-enzyme preparation as a catalyst [69]. This composition was slightly altered for the synthesis of feruloylated galactobiose, where 2-butanone was exchanged with 1,4-dioxane [69].

Optimization of butyl hydroxycinnamate esters by transesterification of methyl esters catalyzed by a novel type C FAE from *Aspergillus ochraceus* was carried out in a ternary system containing isooctane/butanol/water [65]. This enzyme was quite specific for butanol, while other alcohols were converted at much lower rates. Synthesis of feruloyl glycerol esters was achieved in a solvent-free system of just ferulic acid in a mixture of glycerol and water using re-AoFaeA as a catalyst, achieving a yield of 60.3% [70].

Successful FAE-catalyzed transesterification reactions in aqueous/organic two-phase systems were carried out using *Talaromyces wortmannii* FAEs as catalysts [66]. In the reaction between vinyl ferulate and prenol, just n-hexane with a small addition of water was used as the reaction medium, and in the reaction between vinyl ferulate and L-arabinose, a small amount of DMSO was added as well. The reactions were optimized, giving yields of 87.5% for prenol and 56.2% for L-arabinose. In the case of prenyl ferulate synthesis, the aqueous phase was recovered after the reaction, making it possible to reuse the enzyme, a procedure which was repeated for six consecutive cycles.

## 7. Synergistic Degradation and Bifunctional Esterases

Given the complexity of the plant cell wall, it is natural to assume that different types of enzymes are necessary for its efficient degradation. The increased activity of enzymes when combined with others from different classes is referred to as *synergy*. There are multiple reports of FAEs working synergistically with various carbohydrate-degrading enzymes to break down different biomass side streams. FAEs have demonstrated synergistic effects with xylanases [47,57,59,70], cellulases [58], and laccases. The combination of AnXyn11A and AnFaeA from *Aspergillus niger* BE-2 increased the initial release of ferulic acid (FA) from destarched wheat bran from 16.8% to 61% when added simultaneously [71]. In addition, an engineered bifunctional xylanase/feruloyl esterase (XynII-Fae) from *Prevotella ruminicola* 23 showed synergistic effects with commercial cellulase for the delignification of corn stover [67]. In this engineered bifunctional protein, each reaction was catalyzed by separate domains, with a TIM barrel and  $\alpha/\beta$  hydrolase folding, respectively. A recent study from Schmitz et al., conducted in 2022 [72], examined the degree of synergy for multiple commercial carbohydrate enzymes in different biomass types, including corn bran, soluble oat bran, insoluble oat bran, and oat hull. The study found varying levels of synergy depending on the substrate [72]. In this work, FAEs showed synergy with laccase and xylanases from GH families 5, 10, and 11. However, not all side streams exhibited this effect, suggesting that the composition and nature of the biomass play a determining role in the degree of degradation [72].

The chemical complexity of plant biomass has directed evolution towards enzymes with dual functionality. This dual activity can occur within the same type of bond, such as esters, or span across different bond types, like ester and glycosidic bonds. For example, Dilokpimol et al. (2022) reported three novel feruloyl/acetyl xylan esterases (FXEs) that are capable of efficiently releasing both ferulic acid and acetic acid from wheat arabinoxylan [73]. Earlier, in 2000, Blum et al. demonstrated that XynY and XynZ, components of the *Clostridium thermocellum* cellulosome, exhibited FAE activity originating from their C-terminal and N-terminal domains, respectively, in addition to their established xylanase

activity. The xylanase function in these proteins is associated with GH family 10, while the feruloyl activity originates from distinct catalytic domains [55]. Here again, the enzyme is not a single fold carrying out both activities, it is a multi-domain structure with dual functionality. More recently, Schmitz et al. (2023) reported a bifunctional arabinoxylanase/feruloyl esterase belonging to GH family 5, in which both activities share the same active site. In that case, the recognition of the arabinose substitution is specifically required for xylanase activity [74].

## 8. Estimation of Enzymatic Activity and Detection of FA

The catalytic activity of FAEs can be studied using either complex natural substrates or synthetic model compounds. When complex substrates are used, the released FA is monitored. High-Performance Liquid Chromatography (HPLC) with a C-18 reverse-phase column is commonly employed for quantitative analysis, typically using UV detection at 320 nm [75]. Gas Chromatography–Mass Spectrometry (GC-MS) is also suitable for the detection, quantification, and separation of geometrical isomers of FA [76]. Kvasnička et al. separated 12 phenolic acids including FA, with capillary zone electrophoresis (CZE) using HPLC with UV detection [77]. There are multiply reports of Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) being used for the identification and quantification of FA in a diverse range of substrates, such as Chinese herbal medicine [78], rat plasma [79], and milk [80].

Qualitative detection can be achieved with thin-layer chromatography TLC [81] and high-performance thin-layer chromatography (HPTLC), which have been proven effective for the separation and detection of ferulic acid and vanillin [82].

In terms of synthetic substrates, methyl esters of hydroxycinnamic acids are frequently used, particularly for enzyme classification into types A, B, C, and D. Methyl ferulate (MFA), methyl caffeate (MCA), methyl sinapate (MSA), and methyl *p*-coumarate (MpCA) are incubated with the enzyme, and the hydrolysis products are analyzed via HPLC, providing a detailed estimation of the enzyme activity with these different substrates [39,48,83]. Another widely used method involves the use of *p*-nitrophenyl (*p*NP) esters, such as *p*NP-ferulate or *p*NP-acetate, in spectrophotometric assays [83]. These allow for real-time monitoring of enzyme activity by measuring the release of *p*-nitrophenol at 410 nm.

Frequently, FAEs exhibit dual activity as feruloyl esterases and acetyl xylan esterases. It is therefore of interest to test the enzymatic activity and preference in both *p*NP-ferulate and *p*NP-acetate [73,74,84]. These synthetic substrate assays are advantageous for enzyme screenings, since they are compatible with high-throughput methods and kinetic analysis and can facilitate FAE classification. Nevertheless, it is important to note that they do not reflect the complexity found in nature. Therefore, high activity on synthetic substrates does not necessarily translate to efficient activity on plant cell wall materials.

## 9. Applications of FAEs and FA

FAEs' applications can be viewed in two ways. One focuses on their enzymatic effect on biomass, where degrading the side chain enhances the accessibility of hydrolytic enzymes to cellulosic and hemicellulosic substrates and lignin–polysaccharide chains (described in the Section 7). The other considers FA as a valuable product with diverse applications in pharmaceuticals, cosmetics, food, feed, food packaging, and biogels [85].

In pharmaceuticals and cosmetics, FA is widely used for its antioxidant, anti-inflammatory, anti-aging, and photoprotective properties [85,86]. It is an important ingredient in sunscreens and skin-whitening products due to its ability to inhibit melanin accumulation, protect against UV radiation, and prevent skin photoaging [86–88].

Additionally, FA exhibits anti-cancer properties by inhibiting reactive oxygen species (ROS), protecting cellular components from oxidative damage, and regulating intracellular signaling related to proliferation, apoptosis, and metastasis. FA and its derivatives also offer antithrombotic, immune-regulating, and neuroprotective benefits, aiding in the treatment of cardiovascular and cerebrovascular diseases [89]. Studies further suggest wound-healing properties, demonstrated in experiments on diabetic rats [90,91]. These benefits derive from FA's free radical scavenging ability and reduction in oxidative damage [85]. FA can be used as substrate in enzymatic decarboxylation reactions producing styrene derivatives, which can be converted further to various products, such as biobased polymers [92–94].

In the food industry, FA is a precursor for vanillin production [95], a flavoring compound that is also used in drug delivery systems and as battery energy storage through vanillin-cross-linked chitosan electrodes [96].

FA is also incorporated into fibrous films for fruit preservation, where its core–sheath-structured fibers provide both immediate and sustained antioxidant release, helping to prevent oxidation, reduce water loss, and extend shelf life [97]. Despite its broad potential across various fields, FA's practical applications remain limited due to its low water solubility [85].

## 10. Conclusions

This review aims to provide a comprehensive overview of FAEs and ferulic acid (FA). It explores the various natural forms and cross-links of ferulic acid, emphasizing the compound's structural complexity. All FAEs are serine hydrolases, playing an important role in the effective valorization of biomass, alone or in synergy with other enzymes. The released ferulic acid has broad applications across the pharmaceutical, food, and chemical industries. FAEs are also used as biosynthetic catalysts in (trans)esterification reactions involving non-aqueous solvents. By evaluating the current classification systems and the structural diversity of these enzymes, this review highlights the need for further research to fully elucidate their structure–function relationships and to deepen our understanding of the complex patterns of nature to which they correspond.

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## Abbreviations

The following abbreviations are used in this manuscript:

|         |                                    |
|---------|------------------------------------|
| FA      | Ferulic Acid                       |
| FAE     | Ferulic Acid Esterase              |
| CAZymes | Carbohydrate-Active Enzymes        |
| GAX     | Glucuronoarabinoxylan              |
| AX      | Arabinoxylan                       |
| DiFA    | Di-Ferulic Acid                    |
| BenDiFA | Benzofuran Form of Di-Ferulic Acid |
| HRP     | Horseradish Peroxidase             |
| CBM     | Carbohydrate-Binding Module        |
| CE1     | Carbohydrate Esterase Family 1     |

|             |                                                |
|-------------|------------------------------------------------|
| MFA         | Methyl Ferulate                                |
| MCA         | Methyl Caffate                                 |
| MSA         | Methyl Sinapate                                |
| MpCA        | Methyl <i>p</i> -Coumarate                     |
| GH          | Glycoside Hydrolase Family                     |
| HPLC        | High-Performance Liquid Chromatography         |
| GC-MS       | Gas Chromatography–Mass Spectrometry           |
| CZE         | Capillary Zone Electrophoresis                 |
| LC-MS/MS    | Liquid Chromatography–Tandem Mass Spectrometry |
| TLC         | Thin-Layer Chromatography                      |
| HPTLC       | High-Performance Thin-Layer Chromatography     |
| <i>p</i> NP | Para Nitrophenol                               |
| ROS         | Reactive Oxygen Species                        |

## References

1. Saha, B.C. Hemicellulose bioconversion. *J. Ind. Microbiol. Biotechnol.* **2003**, *30*, 279–291. [CrossRef]
2. Terrett, O.M.; Dupree, P. Covalent interactions between lignin and hemicelluloses in plant secondary cell walls. *Curr. Opin. Biotechnol.* **2019**, *56*, 97–104. [CrossRef]
3. Natella, F.; Nardini, M.; Giannetti, I.; Dattilo, C.; Scaccini, C. Coffee drinking influences plasma antioxidant capacity in humans. *J. Agric. Food Chem.* **2002**, *50*, 6211–6216. [CrossRef]
4. Johnston, K.L.; Clifford, M.N.; Morgan, L.M. Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: Glycemic effects of chlorogenic acid and caffeine. *Am. J. Clin. Nutr.* **2003**, *78*, 728–733. [CrossRef]
5. Scheller, H.V.; Ulvskov, P. Hemicelluloses. *Annu. Rev. Plant Biol.* **2010**, *61*, 263–289. [CrossRef] [PubMed]
6. Ishii, T.; Hiroi, T. Linkage of phenolic acids to cell-wall polysaccharides of bamboo shoot. *Carbohydr. Res.* **1990**, *206*, 297–310. [CrossRef] [PubMed]
7. Locher, R.; Martin, H.V.; Grison, R.; Pilet, P.E. Cell wall-bound trans- and cis-ferulic acids in growing maize roots. *Physiol. Plant.* **1994**, *90*, 734–738. [CrossRef]
8. Tang, Y.; Hao, J.; Fan, C.; Cao, X. Preparative separation of high-purity trans- and cis-ferulic acid from wheat bran by pH-zone-refining counter-current chromatography. *J. Chromatogr. A* **2021**, *1636*, 461772. [CrossRef]
9. Rombouts, F.M.; Thibault, J.-F. Feruloylated pectic substances from sugar-beet pulp. *Carbohydr. Res.* **1986**, *154*, 177–187. [CrossRef]
10. Fry, S.C. Feruloylated pectins from the primary cell wall: Their structure and possible functions. *Planta* **1983**, *157*, 111–123. [CrossRef]
11. Colquhoun, I.J.; Ralet, M.-C.; Thibault, J.-F.; Faulds, C.B.; Williamson, G. Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. *Carbohydr. Res.* **1994**, *263*, 243–256. [CrossRef]
12. Parr, A.J.; Waldron, K.W.; Ng, A.; Parker, M.L. The wall-bound phenolics of Chinese water chestnut (*Eleocharis dulcis*). *J. Sci. Food Agric.* **1996**, *71*, 501–507. [CrossRef]
13. Massiot, P.; Rouau, X.; Thibault, J.-F. Characterisation of the extractable pectins and hemicelluloses of the cell wall of carrot. *Carbohydr. Res.* **1988**, *172*, 229–242. [CrossRef]
14. Renard, C.; Champenois, Y.; Thibault, J.-F. Characterisation of the extractable pectins and hemicelluloses of the cell wall of glasswort, *Salicornia ramosissima*. *Carbohydr. Polym.* **1993**, *22*, 239–245. [CrossRef]
15. Sanchez, M.; Pena, M.J.; Revilla, G.; Zarra, I. Changes in dehydrodiferulic acids and peroxidase activity against ferulic acid associated with cell walls during growth of *Pinus pinaster* hypocotyl. *Plant Physiol.* **1996**, *111*, 941–946. [CrossRef]
16. Ward, G.; Hadar, Y.; Bilkis, I.; Konstantinovskiy, L.; Dosoretz, C.G. Initial steps of ferulic acid polymerization by lignin peroxidase. *J. Biol. Chem.* **2001**, *276*, 18734–18741. [CrossRef]
17. Nordkvist, E.; Salomonsson, A.C.; Åman, P. Distribution of insoluble bound phenolic acids in barley grain. *J. Sci. Food Agric.* **1984**, *35*, 657–661. [CrossRef]
18. Hatfield, R.D.; Ralph, J.; Grabber, J.H. Cell wall cross-linking by ferulates and diferulates in grasses. *J. Sci. Food Agric.* **1999**, *79*, 403–407. [CrossRef]
19. Ford, C.W.; Hartley, R.D. GC/MS characterisation of cyclodimers from *p*-coumaric and ferulic acids by photodimerisation—A possible factor influencing cell wall biodegradability. *J. Sci. Food Agric.* **1989**, *46*, 301–310. [CrossRef]
20. Ford, C.W.; Hartley, R.D. Cyclodimers of *p*-coumaric and ferulic acids in the cell walls of tropical grasses. *J. Sci. Food Agric.* **1990**, *50*, 29–43. [CrossRef]
21. Hartley, R.D.; Whatley, F.R.; Harris, P.J. 4, 4'-Dihydroxytruxillic acid as a component of cell walls of *Lolium multiflorum*. *Phytochemistry* **1988**, *27*, 349–351. [CrossRef]

22. Bartolome, B.; Faulds, C.B.; Kroon, P.A.; Waldron, K.; Gilbert, H.J.; Hazlewood, G.; Williamson, G. An *Aspergillus niger* esterase (ferulic acid esterase III) and a recombinant *Pseudomonas fluorescens* subsp. *cellulosa* esterase (Xy1D) release a 5-5' ferulic dehydrodimer (diferulic acid) from barley and wheat cell walls. *Appl. Environ. Microbiol.* **1997**, *63*, 208–212. [CrossRef] [PubMed]
23. Bunzel, M.; Ralph, J.; Marita, J.M.; Hatfield, R.D.; Steinhart, H. Diferulates as structural components in soluble and insoluble cereal dietary fibre. *J. Sci. Food Agric.* **2001**, *81*, 653–660. [CrossRef]
24. Funk, C.; Ralph, J.; Steinhart, H.; Bunzel, M. Isolation and structural characterisation of 8-O-4/8-O-4 and 8-8/8-O-4-coupled dehydrotriferulic acids from maize bran. *Phytochemistry* **2005**, *66*, 363–371. [CrossRef]
25. Bunzel, M.; Ralph, J.; Funk, C.; Steinhart, H. Structural elucidation of new ferulic acid-containing phenolic dimers and trimers isolated from maize bran. *Tetrahedron Lett.* **2005**, *46*, 5845–5850. [CrossRef]
26. Rouau, X.; Cheynier, V.; Surget, A.; Gloux, D.; Barron, C.; Meudec, E.; Louis-Montero, J.; Criton, M. A dehydrotrimer of ferulic acid from maize bran. *Phytochemistry* **2003**, *63*, 899–903. [CrossRef]
27. Bunzel, M.; Ralph, J.; Funk, C.; Steinhart, H. Isolation and identification of a ferulic acid dehydrotrimer from saponified maize bran insoluble fiber. *Eur. Food Res. Technol.* **2003**, *217*, 128–133. [CrossRef]
28. Bunzel, M.; Ralph, J.; Brüning, P.; Steinhart, H. Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. *J. Agric. Food Chem.* **2006**, *54*, 6409–6418. [CrossRef]
29. Bunzel, M.; Heuermann, B.; Kim, H.; Ralph, J. Peroxidase-catalyzed oligomerization of ferulic acid esters. *J. Agric. Food Chem.* **2008**, *56*, 10368–10375. [CrossRef] [PubMed]
30. Iiyama, K.; Lam, T.B.-T.; Stone, B.A. Covalent cross-links in the cell wall. *Plant Physiol.* **1994**, *104*, 315. [CrossRef] [PubMed]
31. Oudgenoeg, G.; Dirksen, E.; Ingemann, S.; Hilhorst, R.; Gruppen, H.; Boeriu, C.G.; Piersma, S.R.; van Berkel, W.J.; Laane, C.; Voragen, A.G. Horseradish peroxidase-catalyzed oligomerization of ferulic acid on a template of a tyrosine-containing tripeptide. *J. Biol. Chem.* **2002**, *277*, 21332–21340. [CrossRef] [PubMed]
32. Boeriu, C.G.; Oudgenoeg, G.; Spekking, W.T.J.; Berendsen, L.B.; Vancon, L.; Boumans, H.; Gruppen, H.; van Berkel, W.J.; Laane, C.; Voragen, A.G. Horseradish peroxidase-catalyzed cross-linking of feruloylated arabinoxylans with  $\beta$ -casein. *J. Agric. Food Chem.* **2004**, *52*, 6633–6639. [CrossRef]
33. Ferreira, L.; Wood, T.M.; Williamson, G.; Faulds, C.; Hazlewood, G.P.; Black, G.W.; Gilbert, H.J. A modular esterase from *Pseudomonas fluorescens* subsp. *cellulosa* contains a non-catalytic cellulose-binding domain. *Biochem. J.* **1993**, *294*, 349–355. [CrossRef]
34. Kroon, P.A.; Williamson, G.; Fish, N.M.; Archer, D.B.; Belshaw, N.J. A modular esterase from *Penicillium funiculosum* which releases ferulic acid from plant cell walls and binds crystalline cellulose contains a carbohydrate binding module. *Eur. J. Biochem.* **2000**, *267*, 6740–6752. [CrossRef]
35. Holck, J.; Fredslund, F.; Møller, M.S.; Brask, J.; Krogh, K.B.; Lange, L.; Welner, D.H.; Svensson, B.; Meyer, A.S.; Wilkens, C. A carbohydrate-binding family 48 module enables feruloyl esterase action on polymeric arabinoxylan. *J. Biol. Chem.* **2019**, *294*, 17339–17353. [CrossRef]
36. Mamiya, A.; Sakka, M.; Kosugi, A.; Katsuzaki, H.; Tanaka, A.; Kunitake, E.; Kimura, T.; Sakka, K. Significance of a family-6 carbohydrate-binding module in a modular feruloyl esterase for removing ferulic acid from insoluble wheat arabinoxylan. *Enzym. Microb. Technol.* **2020**, *138*, 109546. [CrossRef] [PubMed]
37. Holck, J.; Djajadi, D.T.; Brask, J.; Pilgaard, B.; Krogh, K.B.; Meyer, A.S.; Lange, L.; Wilkens, C. Novel xylanolytic triple domain enzyme targeted at feruloylated arabinoxylan degradation. *Enzym. Microb. Technol.* **2019**, *129*, 109353. [CrossRef]
38. Lin, S.; Hunt, C.J.; Holck, J.; Brask, J.; Krogh, K.B.; Meyer, A.S.; Wilkens, C.; Agger, J.W. Fungal feruloyl esterases can catalyze release of diferulic acids from complex arabinoxylan. *Int. J. Biol. Macromol.* **2023**, *232*, 123365. [CrossRef]
39. Crepin, V.F.; Faulds, C.B.; Connerton, I. Functional classification of the microbial feruloyl esterases. *Appl. Microbiol. Biotechnol.* **2004**, *63*, 647–652. [CrossRef]
40. Benoit, I.; Danchin, E.G.; Bleichrodt, R.-J.; de Vries, R.P. Biotechnological applications and potential of fungal feruloyl esterases based on prevalence, classification and biochemical diversity. *Biotechnol. Lett.* **2008**, *30*, 387–396. [CrossRef] [PubMed]
41. Udatha, G. Descriptor-based computational analysis reveals a novel classification scheme for feruloyl esterase enzyme families. In Proceedings of the International Conference on Bioinformatics, Kuala Lumpur, Malaysia, 30 November 2011–2 December 2011.
42. Dilokpimol, A.; Mäkelä, M.R.; Aguilar-Pontes, M.V.; Benoit-Gelber, I.; Hildén, K.S.; de Vries, R.P. Diversity of fungal feruloyl esterases: Updated phylogenetic classification, properties, and industrial applications. *Biotechnol. Biofuels* **2016**, *9*, 231. [CrossRef]
43. Lombard, V.; Henrissat, B.; Garron, M.-L. CAZac: An activity descriptor for carbohydrate-active enzymes. *Nucleic Acids Res.* **2025**, *53*, D625–D633. [CrossRef]
44. Faber, K.; Faber, K. *Biotransformations in Organic Chemistry: A Textbook*; Springer: Heidelberg, Germany, 2004; Volume 6.
45. Kasmaei, K.M.; Kalyani, D.C.; Reichenbach, T.; Jiménez-Quero, A.; Vilaplana, F.; Divne, C. Crystal structure of the feruloyl esterase from *Lentilactobacillus buchneri* reveals a novel homodimeric state. *Front. Microbiol.* **2022**, *13*, 1050160. [CrossRef]
46. Faulds, C.B.; Molina, R.; Gonzalez, R.; Husband, F.; Juge, N.; Sanz-Aparicio, J.; Hermoso, J.A. Probing the determinants of substrate specificity of a feruloyl esterase, AnFaeA, from *Aspergillus niger*. *FEBS J.* **2005**, *272*, 4362–4371. [CrossRef]

47. Dilokpimol, A.; Mäkelä, M.R.; Mansouri, S.; Belova, O.; Waterstraat, M.; Bunzel, M.; de Vries, R.P.; Hildén, K.S. Expanding the feruloyl esterase gene family of *Aspergillus niger* by characterization of a feruloyl esterase, FaeC. *New Biotechnol.* **2017**, *37*, 200–209. [CrossRef] [PubMed]
48. Faulds, C.B.; Williamson, G. Purification and characterization of a ferulic acid esterase (FAE-III) from *Aspergillus niger*: Specificity for the phenolic moiety and binding to microcrystalline cellulose. *Microbiology* **1994**, *140*, 779–787. [CrossRef]
49. De Vries, R.P.; Michelsen, B.; Poulsen, C.; Kroon, P.; Van Den Heuvel, R.; Faulds, C.; Williamson, G.; Van Den Hombergh, J.; Visser, J. The faeA genes from *Aspergillus niger* and *Aspergillus tubingensis* encode ferulic acid esterases involved in degradation of complex cell wall polysaccharides. *Appl. Environ. Microbiol.* **1997**, *63*, 4638–4644. [CrossRef]
50. de Vries, R.P.; vanKuyk, P.A.; Kester, H.C.; Visser, J. The *Aspergillus niger* faeB gene encodes a second feruloyl esterase involved in pectin and xylan degradation and is specifically induced in the presence of aromatic compounds. *Biochem. J.* **2002**, *363*, 377–386. [CrossRef]
51. Hermoso, J.A.; Sanz-Aparicio, J.; Molina, R.; Juge, N.; González, R.; Faulds, C.B. The crystal structure of feruloyl esterase A from *Aspergillus niger* suggests evolutive functional convergence in feruloyl esterase family. *J. Mol. Biol.* **2004**, *338*, 495–506. [CrossRef] [PubMed]
52. Aliwan, F.O.; Kroon, P.A.; Faulds, C.B.; Pickersgill, R.; Williamson, G. Ferulic acid esterase-III from *Aspergillus niger* does not exhibit lipase activity. *J. Sci. Food Agric.* **1999**, *79*, 457–459. [CrossRef]
53. Prates, J.A.; Tarbouriech, N.; Charnock, S.J.; Fontes, C.M.; Ferreira, L.S.M.; Davies, G.J. The structure of the feruloyl esterase module of xylanase 10B from *Clostridium thermocellum* provides insights into substrate recognition. *Structure* **2001**, *9*, 1183–1190. [CrossRef]
54. Schubot, F.D.; Kataeva, I.A.; Blum, D.L.; Shah, A.K.; Ljungdahl, L.G.; Rose, J.P.; Wang, B.-C. Structural basis for the substrate specificity of the feruloyl esterase domain of the cellulosomal xylanase Z from *Clostridium thermocellum*. *Biochemistry* **2001**, *40*, 12524–12532. [CrossRef]
55. Blum, D.L.; Kataeva, I.A.; Li, X.-L.; Ljungdahl, L.G. Feruloyl esterase activity of the *Clostridium thermocellum* cellulosome can be attributed to previously unknown domains of XynY and XynZ. *J. Bacteriol.* **2000**, *182*, 1346–1351. [CrossRef]
56. Suzuki, K.; Hori, A.; Kawamoto, K.; Thangudu, R.R.; Ishida, T.; Igarashi, K.; Samejima, M.; Yamada, C.; Arakawa, T.; Wakagi, T. Crystal structure of a feruloyl esterase belonging to the tannase family: A disulfide bond near a catalytic triad. *Proteins Struct. Funct. Bioinform.* **2014**, *82*, 2857–2867. [CrossRef] [PubMed]
57. Topakas, E.; Vafiadi, C.; Stamatis, H.; Christakopoulos, P. Sporotrichum thermophile type C feruloyl esterase (StFaeC): Purification, characterization, and its use for phenolic acid (sugar) ester synthesis. *Enzym. Microb. Technol.* **2005**, *36*, 729–736. [CrossRef]
58. Gao, L.; Wang, M.; Chen, S.; Zhang, D. Biochemical characterization of a novel feruloyl esterase from *Penicillium piceum* and its application in biomass bioconversion. *J. Mol. Catal. B Enzym.* **2016**, *133*, S388–S394. [CrossRef]
59. Wang, L.; Li, Z.; Zhu, M.; Meng, L.; Wang, H.; Ng, T.B. An acidic feruloyl esterase from the mushroom *Lactarius hatsudake*: A potential animal feed supplement. *Int. J. Biol. Macromol.* **2016**, *93*, 290–295. [CrossRef]
60. Lai, K.-K.; Stogios, P.J.; Vu, C.; Xu, X.; Cui, H.; Molloy, S.; Savchenko, A.; Yakunin, A.; Gonzalez, C.F. An inserted  $\alpha/\beta$  subdomain shapes the catalytic pocket of *Lactobacillus johnsonii* cinnamoyl esterase. *PLoS ONE* **2011**, *6*, e23269. [CrossRef]
61. Yang, W.; Sun, L.; Dong, P.; Chen, Y.; Zhang, H.; Huang, X.; Wu, L.; Chen, L.; Jing, D.; Wu, Y. Structure-guided rational design of the *Geobacillus thermoglucosidasius* feruloyl esterase GthFAE to improve its thermostability. *Biochem. Biophys. Res. Commun.* **2022**, *600*, 117–122. [CrossRef] [PubMed]
62. Jeon, S.; Hwang, J.; Do, H.; Le, L.T.H.L.; Lee, C.W.; Yoo, W.; Lee, M.J.; Shin, S.C.; Kim, K.K.; Kim, H.-W. Feruloyl Esterase (La Fae) from *Lactobacillus acidophilus*: Structural Insights and Functional Characterization for Application in Ferulic Acid Production. *Int. J. Mol. Sci.* **2023**, *24*, 11170. [CrossRef]
63. Gruninger, R.J.; Cote, C.; McAllister, T.A.; Abbott, D.W. Contributions of a unique  $\beta$ -clamp to substrate recognition illuminates the molecular basis of exolysis in ferulic acid esterases. *Biochem. J.* **2016**, *473*, 839–849. [CrossRef]
64. Topakas, E.; Stamatis, H.; Biely, P.; Kekos, D.; Macris, B.; Christakopoulos, P. Purification and characterization of a feruloyl esterase from *Fusarium oxysporum* catalyzing esterification of phenolic acids in ternary water–organic solvent mixtures. *J. Biotechnol.* **2003**, *102*, 33–44. [CrossRef]
65. Romero-Borbón, E.; Grajales-Hernández, D.; Armendáriz-Ruiz, M.; Ramírez-Velasco, L.; Rodríguez-González, J.A.; Cira-Chávez, L.A.; Estrada-Alvarado, M.I.; Mateos-Díaz, J.C. Type C feruloyl esterase from *Aspergillus ochraceus*: A butanol specific biocatalyst for the synthesis of hydroxycinnamates in a ternary solvent system. *Electron. J. Biotechnol.* **2018**, *35*, 1–9. [CrossRef]
66. Antonopoulou, I.; Iancu, L.; Jütten, P.; Piechot, A.; Rova, U.; Christakopoulos, P. Screening of novel feruloyl esterases from *Talaromyces wortmannii* for the development of efficient and sustainable syntheses of feruloyl derivatives. *Enzym. Microb. Technol.* **2019**, *120*, 124–135. [CrossRef] [PubMed]
67. Wang, H.; Qi, X.; Gao, S.; Zhang, Y.; An, Y. Biochemical characterization of an engineered bifunctional xylanase/feruloyl esterase and its synergistic effects with cellulase on lignocellulose hydrolysis. *Bioresour. Technol.* **2022**, *355*, 127244. [CrossRef]

68. Giuliani, S.; Piana, C.; Setti, L.; Hochkoeppler, A.; Pifferi, P.; Williamson, G.; Faulds, C. Synthesis of pentylferulate by a feruloyl esterase from *Aspergillus niger* using water-in-oil microemulsions. *Biotechnol. Lett.* **2001**, *23*, 325–330. [CrossRef]
69. Couto, J.; St-Louis, R.; Karboune, S. Optimization of feruloyl esterase-catalyzed synthesis of feruloylated oligosaccharides by response surface methodology. *J. Mol. Catal. B Enzym.* **2011**, *73*, 53–62. [CrossRef]
70. Zeng, Y.; Yin, X.; Wu, M.-C.; Yu, T.; Feng, F.; Zhu, T.-D.; Pang, Q.-F. Expression of a novel feruloyl esterase from *Aspergillus oryzae* in *Pichia pastoris* with esterification activity. *J. Mol. Catal. B Enzym.* **2014**, *110*, 140–146. [CrossRef]
71. Wu, H.; Li, H.; Xue, Y.; Luo, G.; Gan, L.; Liu, J.; Mao, L.; Long, M. High efficiency co-production of ferulic acid and xylooligosaccharides from wheat bran by recombinant xylanase and feruloyl esterase. *Biochem. Eng. J.* **2017**, *120*, 41–48. [CrossRef]
72. Schmitz, E.; Leontakianakou, S.; Norlander, S.; Karlsson, E.N.; Adlercreutz, P. Lignocellulose degradation for the bioeconomy: The potential of enzyme synergies between xylanases, ferulic acid esterase and laccase for the production of arabinoxylooligosaccharides. *Bioresour. Technol.* **2022**, *343*, 126114. [CrossRef]
73. Dilokpimol, A.; Verkerk, B.; Li, X.; Bellemare, A.; Lavallee, M.; Frommhagen, M.; Underlin, E.N.; Kabel, M.A.; Powlowski, J.; Tsang, A. Screening of novel fungal Carbohydrate Esterase family 1 enzymes identifies three novel dual feruloyl/acetyl xylan esterases. *FEBS Lett.* **2022**, *596*, 1932–1943. [CrossRef]
74. Schmitz, E.; Leontakianakou, S.; Adlercreutz, P.; Nordberg Karlsson, E.; Linares-Pastén, J.A. Novel function of CtXyn5A from *Acetivibrio thermocellus*: Dual arabinoxylanase and feruloyl esterase activity in the same active site. *ChemBioChem* **2023**, *24*, e202200667. [CrossRef]
75. Pussayanawin, V.; Wetzel, D.L. High-performance liquid chromatographi determination of ferulic acid in wheat milling fractions as a measure of bran contamination. *J. Chromatogr. A* **1987**, *391*, 243–255. [CrossRef] [PubMed]
76. Razboršek, M.I.; Ivanović, M.; Kolar, M. Validated stability-indicating GC-MS method for characterization of forced degradation products of trans-caffeic acid and trans-ferulic acid. *Molecules* **2021**, *26*, 2475. [CrossRef] [PubMed]
77. Kvasnička, F.; Čopíková, J.; Ševčík, R.; Krátká, J.; Syntytsia, A.; Voldřich, M. Determination of phenolic acids by capillary zone electrophoresis and HPLC. *Cent. Eur. J. Chem.* **2008**, *6*, 410–418. [CrossRef]
78. Jong, T.-T.; Lee, M.-R.; Chiang, Y.-C.; Chiang, S.-T. Using LC/MS/MS to determine matrine, oxymatrine, ferulic acid, mangiferin, and glycyrrhizin in the Chinese medicinal preparations Shiao-feng-saan and Dang-guei-nian-tong-tang. *J. Pharm. Biomed. Anal.* **2006**, *40*, 472–477. [CrossRef]
79. Li, N.; Liu, C.; Mi, S.; Wang, N.; Zheng, X.; Li, Y.; Huang, X.; He, S.; Chen, H.; Xu, X. Simultaneous determination of oleanolic acid, p-coumaric acid, ferulic acid, kaemperol and quercetin in rat plasma by LC–MS–MS and application to a pharmacokinetic study of Oldenlandia diffusa extract in rats. *J. Chromatogr. Sci.* **2012**, *50*, 885–892. [CrossRef] [PubMed]
80. Waterstraat, M.; Hildebrand, A.; Rosler, M.; Bunzel, M. Development of a QuEChERS-based stable-isotope dilution LC-MS/MS method to quantitate ferulic acid and its main microbial and hepatic metabolites in milk. *J. Agric. Food Chem.* **2016**, *64*, 8667–8677. [CrossRef]
81. Sharma, O.P.; Bhat, T.K.; Singh, B. Thin-layer chromatography of gallic acid, methyl gallate, pyrogallol, phloroglucinol, catechol, resorcinol, hydroquinone, catechin, epicatechin, cinnamic acid, p-coumaric acid, ferulic acid and tannic acid. *J. Chromatogr. A* **1998**, *822*, 167–171. [CrossRef]
82. Hingse, S.S.; Digole, S.B.; Annapure, U.S. Method development for simultaneous detection of ferulic acid and vanillin using high-performance thin layer chromatography. *J. Anal. Sci. Technol.* **2014**, *5*, 21. [CrossRef]
83. Hashimoto, K.; Kaneko, S.; Yoshida, M. Extracellular carbohydrate esterase from the basidiomycete *Coprinopsis cinerea* released ferulic and acetic acids from xylan. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 1722–1724. [CrossRef]
84. Shi, Q.; Ma, J.; Abdel-Hamid, A.M.; Li, Y.; Zhong, P.; Wang, D.; Sun, Z.; Tu, T.; Zhu, W.; Cheng, Y. Mining of latent feruloyl esterase resources in rumen and insight into dual-functional feruloyl esterase-xylanase from *Pecoramyces ruminantium* F1. *Bioresour. Technol.* **2025**, *418*, 131854. [CrossRef]
85. Zheng, M.; Liu, Y.; Zhang, G.; Yang, Z.; Xu, W.; Chen, Q. The antioxidant properties, metabolism, application and mechanism of ferulic acid in medicine, food, cosmetics, livestock and poultry. *Antioxidants* **2024**, *13*, 853. [CrossRef]
86. Janus, E.; Pinheiro, L.R.; Nowak, A.; Kucharska, E.; Świątek, E.; Podolak, N.; Perużyńska, M.; Piotrowska, K.; Duchnik, W.; Kucharski, Ł. New ferulic acid and amino acid derivatives with increased cosmeceutical and pharmaceutical potential. *Pharmaceutics* **2022**, *15*, 117. [CrossRef]
87. Neopane, D.; Ansari, V.A.; Singh, A. Ferulic acid: Signaling pathways in aging. *Drug Res.* **2023**, *73*, 318–324. [CrossRef] [PubMed]
88. Lin, F.-H.; Lin, J.-Y.; Gupta, R.D.; Tournas, J.A.; Burch, J.A.; Selim, M.A.; Monteiro-Riviere, N.A.; Grichnik, J.M.; Zielinski, J.; Pinnell, S.R. Ferulic acid stabilizes a solution of vitamins C and E and doubles its photoprotection of skin. *J. Investig. Dermatol.* **2005**, *125*, 826–832. [CrossRef] [PubMed]
89. Zhang, X.-X.; Zhao, D.-S.; Wang, J.; Zhou, H.; Wang, L.; Mao, J.-L.; He, J.-X. The treatment of cardiovascular diseases: A review of ferulic acid and its derivatives. *Die Pharm. -Int. J. Pharm. Sci.* **2021**, *76*, 55–60.
90. Dwivedi, S.; Singh, D.; Deshmukh, P.T.; Soni, R.; Trivedi, R. Healing potential of ferulic acid on dermal wound in diabetic animals. *Asian J. Mol. Model* **2015**, *1*, 1–16.

91. Ghaisas, M.M.; Kshirsagar, S.B.; Sahane, R.S. Evaluation of wound healing activity of ferulic acid in diabetic rats. *Int. Wound J.* **2014**, *11*, 523–532. [CrossRef]
92. Myrtollari, K.; Calderini, E.; Kracher, D.; Schöngaßner, T.; Galusic, S.; Slavica, A.; Taden, A.; Mokos, D.; Schrüfer, A.; Wirnsberger, G. Stability Increase of Phenolic Acid Decarboxylase by a Combination of Protein and Solvent Engineering Unlocks Applications at Elevated Temperatures. *ACS Sustain. Chem. Eng.* **2024**, *12*, 3575–3584. [CrossRef]
93. Petermeier, P.; Bittner, J.P.; Jonsson, T.; de María, P.D.; Byström, E.; Kara, S. Integrated preservation of water activity as key to intensified chemoenzymatic synthesis of bio-based styrene derivatives. *Commun. Chem.* **2024**, *7*, 57. [CrossRef]
94. Myrtollari, K.; Chánique, A.M.; Kracher, D.; Herrera, D.P.; Gutierrez-Benavente, J.; Schüller, A.; Kourist, R. Engineering Substrate Acceptance of Resveratrol O-Methyltransferase from *Vitis vinifera* for the Selective Synthesis of O-Methyl Protected Biobased Hydroxystyrenes. *ChemCatChem* **2025**, *17*, e202402027. [CrossRef]
95. Kaur, B.; Chakraborty, D.; Kumar, B. Phenolic biotransformations during conversion of ferulic acid to vanillin by lactic acid bacteria. *BioMed Res. Int.* **2013**, *2013*, 590359. [CrossRef] [PubMed]
96. Liaqat, F.; Xu, L.; Khazi, M.I.; Ali, S.; Rahman, M.U.; Zhu, D. Extraction, purification, and applications of vanillin: A review of recent advances and challenges. *Ind. Crops Prod.* **2023**, *204*, 117372. [CrossRef]
97. Huang, X.; Jiang, W.; Zhou, J.; Yu, D.-G.; Liu, H. The applications of ferulic-acid-loaded fibrous films for fruit preservation. *Polymers* **2022**, *14*, 4947. [CrossRef] [PubMed]

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Article

# A Cytochrome P450 Facilitates Polyethylene Metabolism in a Microbial Community

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## Abstract

The synthetic polymer low-density polyethylene (LDPE) is a pervasive pollutant that poses serious environmental concerns and health hazards. PE plastic is rarely recycled, and therefore, biodegradation is a novel approach for managing PE plastic waste. However, few enzymes and organisms that degrade PE plastic have been identified to date. Herein, we demonstrate that a consortium of soil bacteria containing *Pseudomonas* and *Bacillus* species can grow on and degrade UV-treated PE film and powder as a sole carbon source, reducing its net mass by 7% and 13%, respectively, in 30 days. Changes in surface functional groups associated with chemical modification of PE were observed via ATR-FTIR analysis, and byproducts associated with PE biodegradation and alkane and carboxylic acid metabolism were observed via GC/MS. Using previously characterized PEases, we identified a gene, CYP102 A5, found in *Bacillus thuringiensis* strain 9.1, which encodes a cytochrome P450 reductase, whose expression was increased when grown on PE as a sole carbon source. Purified CYP102 A5 protein altered PE surface functional groups, determined by ATR-FTIR, giving evidence of PE oxidation. In sum, we identified a cytochrome P450 reductase that explains, in part, how a consortium of soil bacteria can grow on and degrade PE plastic.

**Keywords:** low-density polyethylene; PE plastic; biocatalyst; depolymerization; biodegradation; cytochrome P450 reductase; CYP; bacterial consortium

## 1. Introduction

Plastic pollution is a growing crisis facing our planet. A large contributor to our global waste problem is low-density polyethylene (LDPE), a specific type of polyethylene (PE) plastic waste. PE plastics have meager recycling rates; in the U.S, 8.59 million tons of LDPE/linear low-density polyethylene (LLDPE) were produced in 2018, yet only 4.3% of this plastic was recycled in municipal waste streams in that year [1]. On a global scale, the production of LDPE is estimated to be around 17.4% of the total international production market due to its widespread use in agriculture, packaging, and construction [2–4].

PE pollution has detrimental effects on our ecosystems, particularly within marine environments—it is estimated that out of the total 460 million tons of plastic produced every year, 9–14 million tons end up in our oceans [5,6]. The same chemical properties that make plastics attractive for commercial use, i.e., non-conductive and chemically inert, enable plastics to persist in the environment, contributing to long-lasting, negative environmental impacts. Namely, in aquatic environments, plastics can break down into microplastics (5 mm–1 nm in size) and eventually into smaller nanoplastics (1 nm–1 µm in size). PE microplastics are formed when these products are exposed to solar radiation

and to physical and mechanical forces. PE microplastic exposure is implicated in negative effects on multiple animals. For example, they affect gene expression, and on the larger scale, cognition and behaviors of aquatic organisms, like the planktivorous Indo-Pacific pelagic fish *Decapterus muroadsi*, which was found to eat microplastics that resemble their prey, weakening already fragile ecosystems [7]. Polyethylene exposure increases mortality and decreases locomotion in *Collembola* hexapods, which contribute to ecosystems by shaping soil microbial communities, and *Apis mellifera* bees, the most prevalent honey bee species, which pollinate crops and various other plants [8,9]. Studies are beginning to elucidate the negative health impacts of PE microplastics in both mouse models and in human cells, including developmental/reproductive toxicity, cytotoxicity, and chromosomal instabilities. Microplastics can cause particle toxicity, which has been shown to induce immune responses, as well as chemical toxicity from incorporated additives and absorbed environmental pollutants [10–13].

The properties of PE limit recycling due to the strong C-C and C-H bonds, which are difficult to break [14,15]. The carbon backbone has no additional functional groups and is therefore inert and resistant to degradation [16]. Besides conventional recycling, other methods, including thermal and chemical recycling, are known to be hazardous to the environment. Incineration of PE can cause air pollution via emissions of greenhouse gases and produce furans, carbon monoxide, dioxins, and other toxic compounds which cause negative downstream effects on native flora and fauna [17,18].

Given the cost to environmental and human health associated with other recycling methods, biodegradation of PE is gaining interest. A variety of microbes grow on PE, including *Pseudomonas aeruginosa*, *Rhodococcus ruber*, *Bacillus cereus*, and *Bacillus thuringiensis* [19–24]. Microorganisms that can degrade PE often have slow growth and degradation rates, ranging from 5–20% weight loss of PE after 60 days of incubation [19,25–27]. The high crystallinity, hydrophobicity, and high molecular weight of PE limit the efficiency of microbial biodegradation [28,29]. An improvement on the strategy of using single microbial isolates is to instead use microbial consortia, or groups of bacteria, that work synergistically, which may improve degradation rates due to greater metabolic versatility [30].

The ability for bacteria to utilize LDPE as a carbon source has been described, but only four enzymes have been cataloged on the open-source enzyme database “Plastics DB” to date [31]. However, a diverse array of enzymes, including lignin peroxidases, manganese peroxidases, and laccases, degrade oxidizable C-C bonds [32,33]. Additionally, alkane hydrolases and cytochrome P450 s degrade LDPE via terminal or subterminal carbon oxidation [34]. Researchers have suggested that PE is depolymerized by extracellular enzymes, and then the byproducts are brought into the cell [35], where mineralization can occur. Due to the diversity of enzymes present in hydrocarbon metabolism, it is vital to fully characterize the mechanisms necessary to degrade PE. Characterization of enzymes will enable the development of a model mechanism for LDPE degradation, and in parallel, provide insights into how to engineer the process for better efficiency, by upregulation of key conversion steps and regulatory elements, incorporating more efficient engineered enzymes or introducing enzymes from other species [35].

We previously isolated a consortium of three *Pseudomonas* and two *Bacillus* strains that degrade PET plastic [36]. Analyzing the metagenomes, we found 250 genes encoding enzymes associated with the degradation of synthetic plastics, including PE, biopolymers, and plasticizers [37]. In this report, we demonstrate that the five-member consortium of bacteria isolated from petroleum-polluted soil can grow on and synergistically degrade PE plastic, up to 13% weight reduction after a 30-day incubation. Surface modification of the PE was observed by ATR-FTIR, and degradation byproducts were identified by GC/MS. We used bioinformatics and RT-qPCR to identify putative PEases with differential

expression when grown on PE. The gene encoding CYP102 A5 [24], a cytochrome P450 reductase of *Bacillus thuringiensis*, showed the greatest increased transcription when grown on PE, and the purified enzyme altered surface functional groups, indicating oxidation of the material. Thus, we observed the growth of a bacterial consortium on PE plastic and identified a P450 enzyme that most likely initiates biodegradation.

## 2. Results

### 2.1. Bacterial Consortium Uses PE as a Sole Carbon Source

We grew the bacteria on PE as a sole carbon source in LCFBM to determine whether the full consortium can degrade PE plastic. Growth curves are plotted in Figure S1. Cultures enriched with UV-treated PE plastic and supplementary yeast extract, to initiate growth, were compared to cultures without bacteria to ensure no contamination occurred and to control for any spontaneous PE degradation that might occur within the sterile media. The growth curve was monitored over 30 days to evaluate bacterial colonization and growth.

CFU/mL values indicated that the bacterial consortium grew on both PE plastic sources (0.2% *w/v*) over the 30-day culture time (Figure 1A). The powdered PE culture grew to a CFU/mL of  $2.6 \times 10^8$ , whereas the PE film culture grew to a CFU/mL of  $3.58 \times 10^7$ . This agrees with other studies showing that a higher surface area to volume ratio for the PE powder promotes better biofilm formation and therefore higher capacity for growth [38]. The control had no bacterial growth for both plastic types. These results are consistent with the observation that both bacterial-treated plastic substrates had a statistically significant decrease in pH after 30 days compared to the control ( $p \leq 0.0001$ ) (Figure 1B). The bacteria-treated powder PE exhibited a greater decrease in pH compared to the culture with PE film ( $p \leq 0.0001$ ). Bacterial growth and pH changes, indicating metabolic activity producing acidic byproducts, suggested that the bacteria were metabolizing the PE plastic. The control had only a slight decrease in pH change from the initial 7.4 compared to media containing bacteria (Figure 1B). Figure 1C shows that the bacteria-treated PE powder and film had an average percent weight loss of ~16% and ~10%, respectively, when normalized against natural hydrolysis in the control ( $p < 0.0001$ ). Due to the increase in bacterial CFU/mL in media containing polyethylene, the change in pH values, and plastic weight loss over time, we concluded that the bacterial consortium can utilize PE plastic as a carbon source.

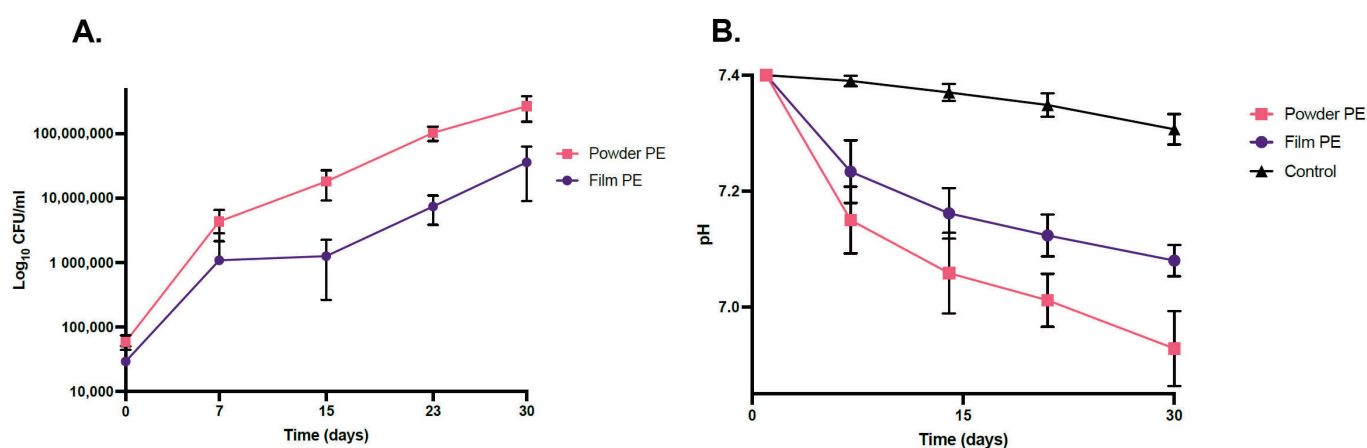
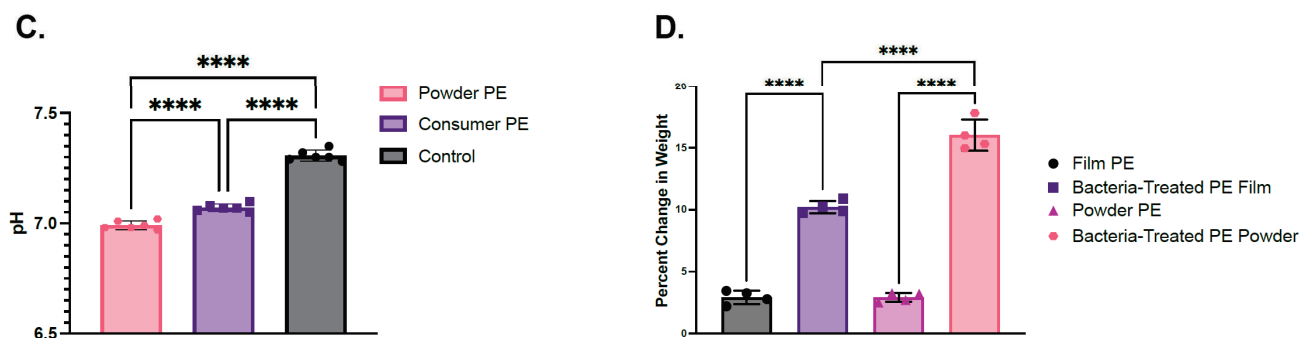


Figure 1. Cont.

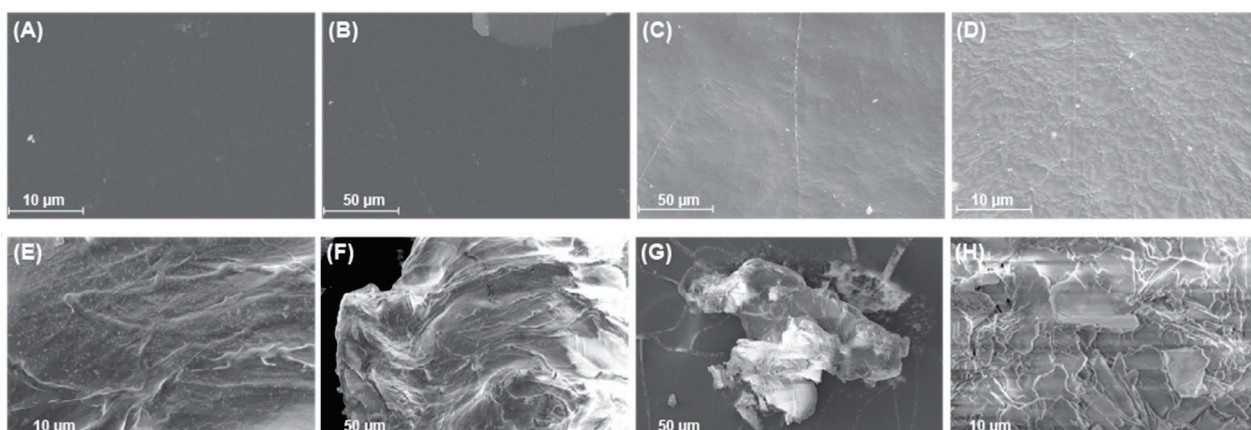


**Figure 1.** (A) Growth curves of the bacterial full consortium in LCFBM in two PE plastic sources: PE powder and PE film for 30 days. (B) pH change over time of the LCFBM medium inoculated by the bacterial full consortium over 30 days. (C) pH change after 30 days of the LCFBM medium inoculated by the bacterial consortium. (D) Percent change of plastic weight of pure PE film and powder and degraded PE powder cultured with the bacterial consortium. Untreated pure powdered PE is the control group (\*\*\*\*  $p \leq 0.0001$ ).

## 2.2. Surface Modifications of PE Plastics After Degradation

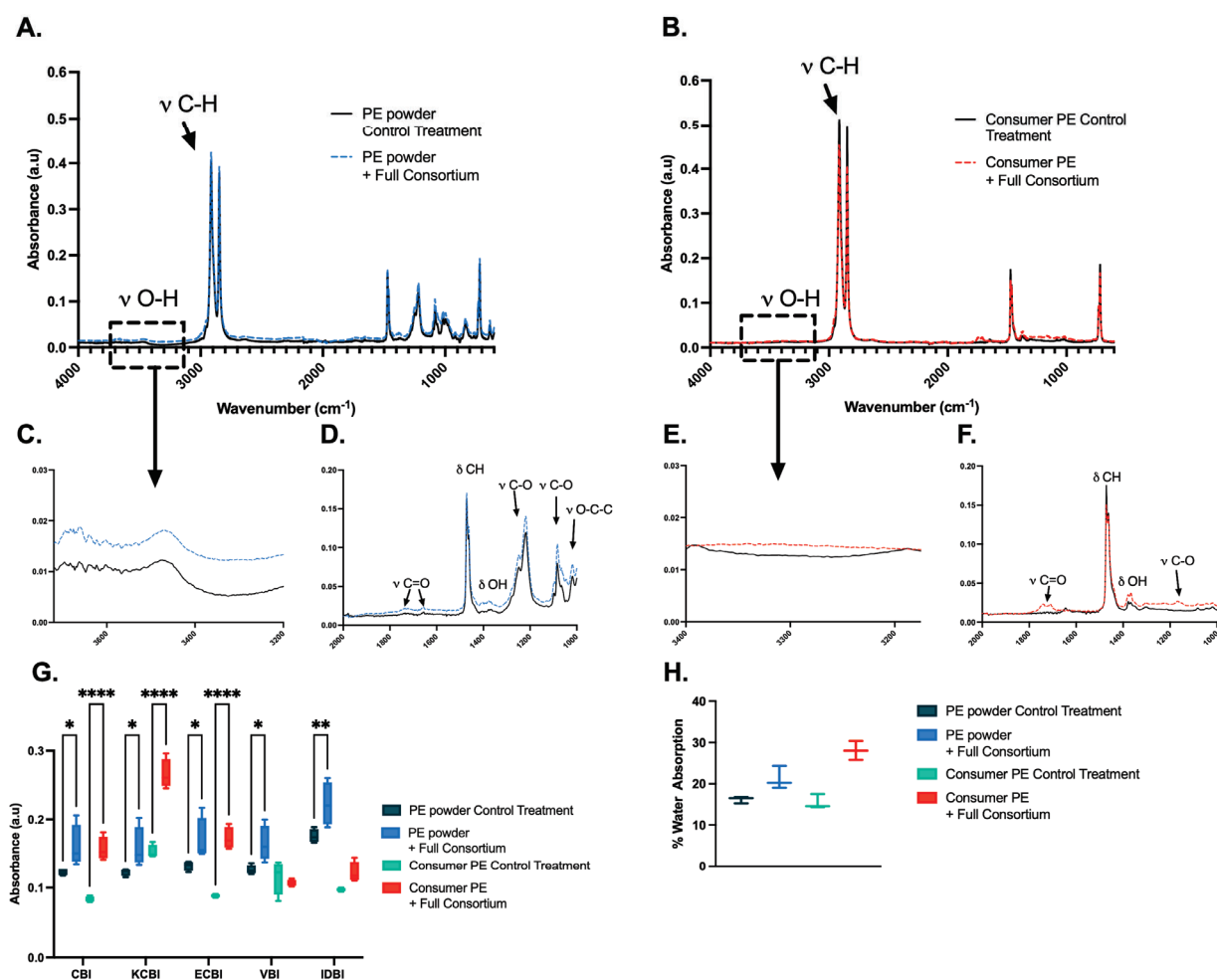
To determine whether the PE surface was being degraded, we first removed the bacteria by washing with 2% SDS and then observed the surface of amorphous PET using scanning electron microscopy (SEM). Bacteria utilize enzymes like laccase or manganese peroxidase, which often catalyze plastic biodegradation via depolymerization. Oxidation generally occurs from an edge and increases the roughness of the plastic surface [39].

The structural changes in both PE film (Figure 2A,B,E,F) and PE powder (Figure 2C,D,G,H) showed increased roughness on the surface in bacteria-treated PE samples when compared to the untreated control. The higher degree of roughness observed on the powdered PE samples versus the film samples is enforced by the greater surface area to volume ratio, thus, more efficient enzymatic degradation due to increased area that biofilms can form and degrade PE [40,41]. The difference in roughness of the surfaces, comparing the bacterial-treated PE and natural hydrolysis of the control, was attributed to bacterial growth on the plastic surface.



**Figure 2.** SEM images show the surface modification of PE film and powdered PE. LCFBM cultures containing either film PE (A,B,E,F) or PE powder (C,D,G,H) and the full consortium were grown at 30 °C for 30 days. Bacteria and biofilms were removed from the surface using 2% SDS and washed with sterile water and 70% ethanol to remove any cellular debris. Images (A,B) depict PE film untreated with bacteria, and images (D,F) show PE film treated with bacteria. Images (C,D) show powdered PE not treated with bacteria, and images (G,H) show plastic treated with the full consortium.

We examined changes in the chemical structure of the PE plastic using ATR-FTIR analysis. Both types of PE samples treated by the bacterial consortium exhibited changes in peaks associated with functional group bending and stretching. The PE film (Figure 3A) exhibited ATR-FTIR peaks at  $3315\text{ cm}^{-1}$ ,  $1366\text{ cm}^{-1}$ ,  $1709\text{ cm}^{-1}$ ,  $1418\text{ cm}^{-1}$ , and  $1220\text{ cm}^{-1}$ , corresponding to -OH stretching and bending (hydroxyl group) [42–44], C=O stretching (carboxyl groups, hydroxyl group) [45,46], and C-O stretching (ester) [47]. Additionally, characteristic peaks at  $1742\text{ cm}^{-1}$  were attributed to C=O bending (aldehyde) [48]. The PE powder (Figure 3B) exhibited similar ATR-FTIR peaks at  $3315\text{ cm}^{-1}$ ,  $1369\text{ cm}^{-1}$ ,  $1709\text{ cm}^{-1}$ ,  $1406\text{ cm}^{-1}$ , and  $1219\text{ cm}^{-1}$ . The PE powder (Figure 3B) did not have any significant differences in  $1740\text{--}1720\text{ cm}^{-1}$  compared to the control. However, changes were observed at  $670\text{ cm}^{-1}$ , indicating C=C bending (alkene) [49].



**Figure 3.** (A) ATR-FTIR spectra of average untreated pure PE powder ( $n = 3$ ) and averaged PE film ( $n = 3$ ) treated with the bacterial consortium. (B) FTIR-ATR spectra of average pure PE powder ( $n = 3$ ) and average PE powder ( $n = 3$ ) treated with the bacterial consortia. (C) Inset of OH stretching of PE powder with and without treatment of the bacterial consortia. (D) Inset of carbonyl and ester stretching of PE powder with and without treatment of the bacterial consortia. (E) Inset of OH stretching of consumer PE with and without treatment of the bacterial consortia. (F) Inset of carbonyl and ester stretching of consumer PE with and without treatment of the bacterial consortia. (G) Means of carbonyl indexes ( $n = 3$ ) of PE film and powder treated with the full consortium and associated film and powder controls (CBI—Carbonyl Bond Index; KCBI—Keto Carbonyl Bond Index; ECBI—Ester Carbonyl Index; VBI—Vinyl Bond Index; IDBI—Internal Double Bond Index; \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ ). (H) Percent Water Absorption of PE powder and film with and without treatment of the bacterial consortium.

Changes in various absorbance intensities of relevant bond wavelengths when compared to the methylene bond were observed after 30 days of incubation with the bacterial consortium for both PE powder and film (Figure 3G, Table 1). When comparing the bacteria-treated samples to the control PE plastics, most indices exhibited statistical significance. The PE film exhibited high statistical significance for the carbonyl bond index (CBI), keto carbonyl index (KCBI), and the ester carbonyl bond Index (ECBI) ( $p < 0.0001$ ). Treated PE powder also exhibited statistical significance for the CBI ( $p = 0.0266$ ), KCBI ( $p = 0.0364$ ), and the ECBI ( $p = 0.0224$ ), although to a lesser extent than the film treatment group. However, the vinyl bond index (VBI) and the internal double bond index (IDBI) varied between the powder and film, with statistical significance observed in the powder group ( $p = 0.0223$  and  $p = 0.004$ , respectively) while the film group displayed no significant difference in these same indices ( $p = 0.9009$  and  $p = 0.2397$ ).

**Table 1.** ATR-FTIR analysis of powdered and film LDPE <sup>1</sup>.

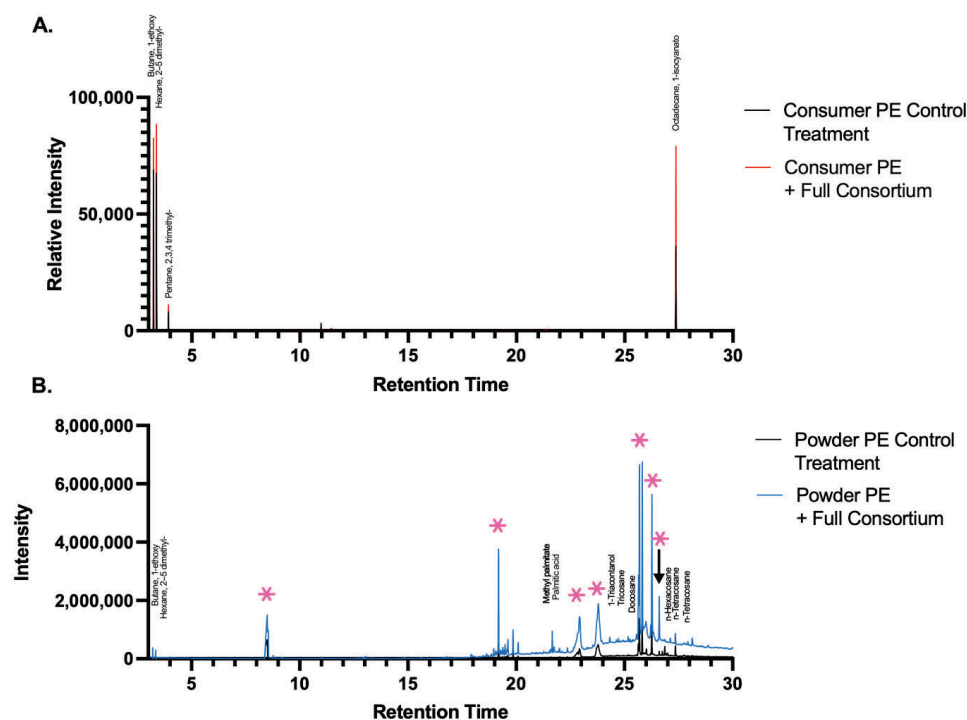
| Sample Type      | Carbonyl Bond Index (I1714/I1463) | Keto Carbonyl Bond Index (I1715/I1465) | Internal Double Bond Index (I908/I1465) | Ester Carbonyl Index (I1740/I1465) | Vinyl Bond Index (I1650/I1465) |
|------------------|-----------------------------------|----------------------------------------|-----------------------------------------|------------------------------------|--------------------------------|
| Untreated Powder | 0.12 ± 0.01                       | 0.12 ± 0.01                            | 0.18 ± 0.01                             | 0.13 ± 0.01                        | 0.13 ± 0.01                    |
| Treated Powder   | 0.16 ± 0.03                       | 0.16 ± 0.03                            | 0.22 ± 0.03                             | 0.17 ± 0.03                        | 0.16 ± 0.03                    |
| Untreated Film   | 0.08 ± 0.00                       | 0.15 ± 0.01                            | 0.10 ± 0.00                             | 0.09 ± 0.00                        | 0.12 ± 0.03                    |
| Treated Film     | 0.16 ± 0.02                       | 0.27 ± 0.02                            | 0.12 ± 0.02                             | 0.17 ± 0.02                        | 0.11 ± 0.01                    |

<sup>1</sup> Molecular index and confidence intervals were calculated by relative intensities of vibrational bands of interest from ATR-FTIR analysis of film and powdered LDPE incubated in carbon-free medium with bacteria for 4 weeks;  $n = 6$ .

The change in water absorption for PE film and PE sheets was also calculated (Figure 3H). When comparing the PE film culture to its control, it approached statistical significance ( $p = 0.0588$ ), while the PE powder was significant ( $p = 0.0004$ ) when compared to the samples not treated with bacteria. In short, we concluded that there were various new functional groups in bacteria-treated PE samples compared to controls, supporting our previous conclusions that the bacterial consortium can metabolize PE. Additionally, we concluded that the PE powder cultures exhibited greater changes in surface functional groups and total water absorption after bacterial treatment.

### 2.3. Byproducts Resulting from Bacterial Degradation of PE Plastic

After identification of functional groups on the surface of the PE plastic, we performed GC/MS analysis of bacterial culture supernatants to characterize the metabolites formed after bacterial degradation of PE. GC/MS results revealed the formation of several products: alkanes, aldehydes, and plastic additives (Figure 4A; Tables S1 and S2). The PE powder culture showed the formation of a wider range of alkanes, ethers, aldehydes, and carboxylic acid compounds during the degradation process (Figure 4B). Both plastic samples, comparing bacterial treatment with spontaneous hydrolysis, identified compounds that are common plastic additives. Our GC/MS results were consistent with the decomposition of PE being broken down to lower molecular weight monomers [24,44,50].

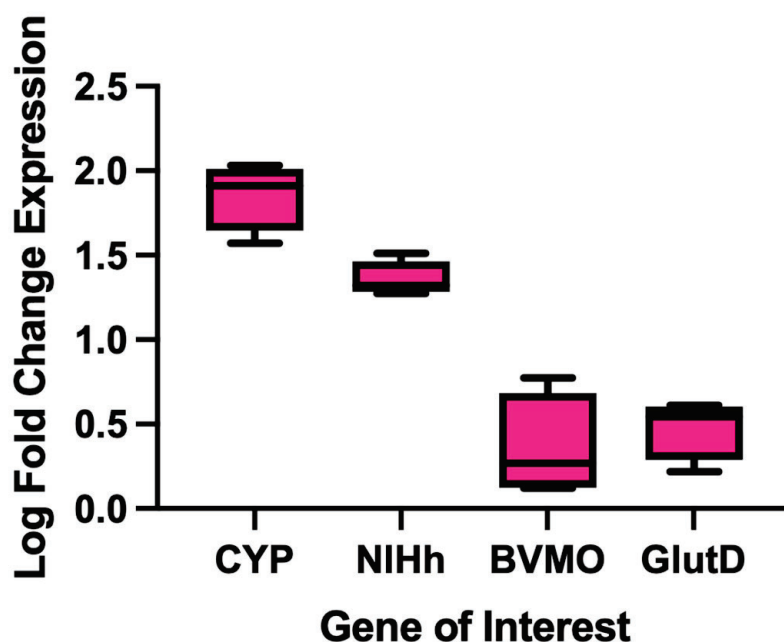


**Figure 4.** (A) GC/MS chromatogram of averaged relative intensity ( $n = 4$ ) normalized to the blank of pure PE film and degraded PE film cultured with the full consortium of metabolites found in the bacterial supernatant. Untreated film is the comparative control. (B) GC/MS chromatogram of averaged relative intensity ( $n = 4$ ) normalized to the blank of pure PE powder and degraded PE powder cultured with the full consortium. Hydrolysis of PE powder is the control group. Compounds associated with plastic are indicated via a pink asterisk, from left to right: diethylene glycol, azulene, 1,4-dimethyl-7-(1-methylethyl), two unknown phenolic compounds, 2,4-bis(1-phenylethyl)phenol, diethylene glycol dibenzoate, and diethyl phthalate. (See also Table S2).

#### 2.4. Identification of Enzymes Putatively Associated with PE Metabolism

We used a bioinformatic approach to identify enzymes involved in PE metabolism. We putatively identified four enzymes with PE-degradation capabilities, using a sequence similarity greater than 25% to known PEases: a cytochrome P450 NADPH reductase (98% similarity) from *B. thuringiensis* (9.1), a glutathione dehydrogenase (GlutD) in *Rhodococcus* sp. C-2 with 78% similarity with our two *Pseudomonas* sp. strains (10 and 13.2). A Baeyer-Villager monooxygenase (BVMO) isolated from *Alcanivorax* sp. 24 was 25.64% similar to *Pseudomonas* B10 (9.2), and the carboxylesterase NlhH identified in *Alcanivorax* sp. 24, had a 38.79% similarity to *Pseudomonas* strains 10 and 13.2, and was 32% similar to that in strain 9.2. After identification of these genes, we utilized reverse-transcription quantitative PCR (RT-qPCR) (Table S3) to test for induced expression when grown in culture with PE plastic.

RT-qPCR analysis was performed using the 16 s rRNA gene as the internal control. We measured transcriptional activity for the putative PE-degrading genes: *cyp* (strain 9.1), BVMO (strain 9.2), *glutD* (strain 10), and *nlhH* (strain 13.2). Expression of the BVMO and *glutD* genes was not significantly different under the conditions tested ( $p = 0.7504$  and  $0.3077$ ). The *nlhH* gene was slightly upregulated in the presence of PE plastic (1.356-log fold;  $p = 0.002$ ). CYP expression was greater than that of *nlhH*, a 1.857 log fold change ( $p < 0.0001$ ) (Figure 5). We thus hypothesized that the enzymes NlhH and CYP may be associated with PE metabolism. It is important to note, however, that the genomes of the five axenic strains are diverse and encode many more hydroxylases, dehydrogenases, hydrolases, and monooxygenases [37] that may participate in PE metabolism, but were not examined using RT-qPCR.



**Figure 5.** RT-qPCR expression data of genes of interest for individual strains when grown on PE plastic. Log fold change expression determined by two-step quantitative real-time PCR (RT-qPCR) of genes putatively involved in PE biodegradation. Fold change expression was calculated ( $n = 4$ ) using the Pfaffl method for bacterial isolates. Isolate associated with each tested gene, and the associated primer sequences are described in Supplementary Table S3.

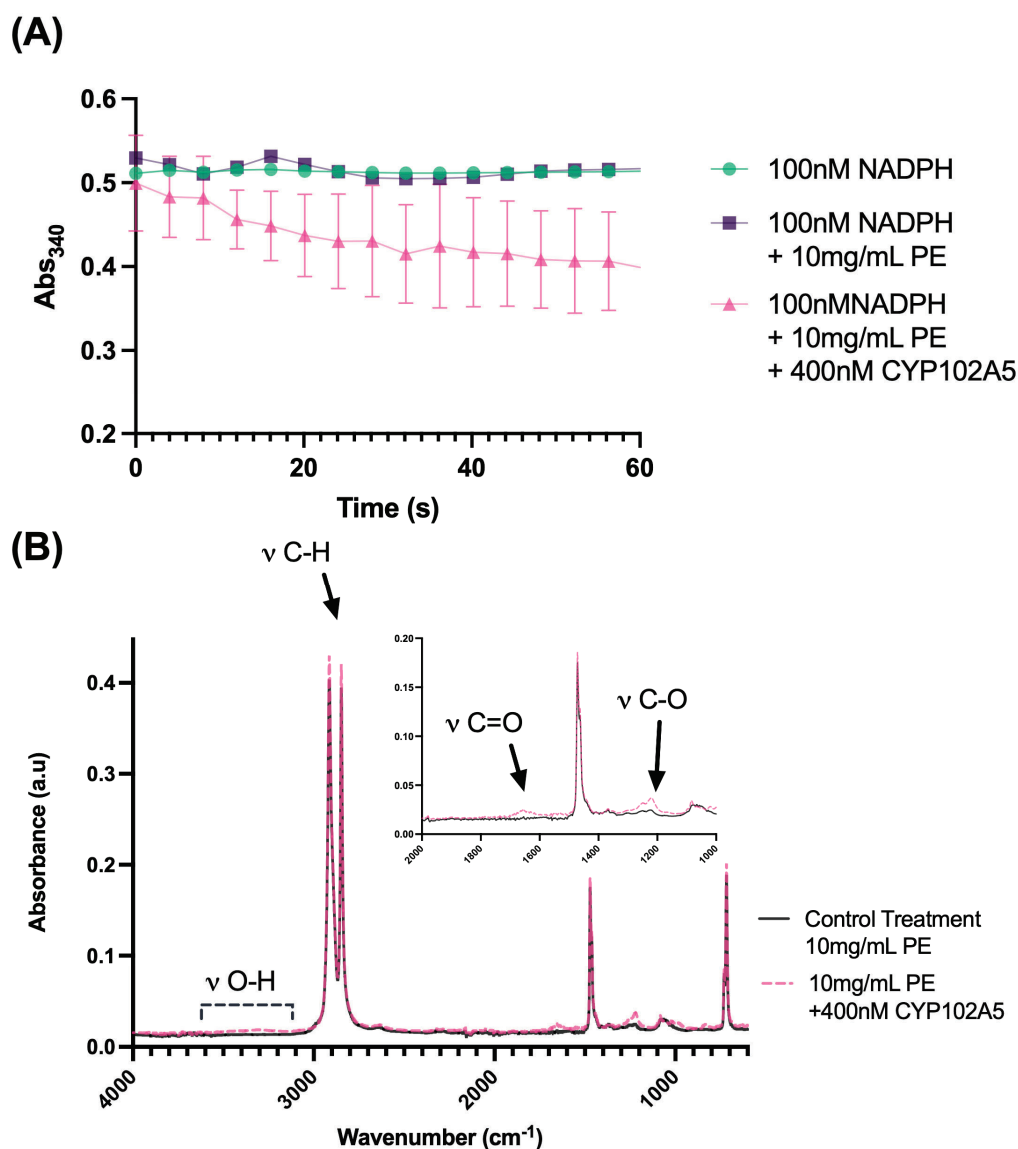
#### 2.5. Purified CYP102 A5 Cytochrome P450 Reductase Enzymatic Activity in the Presence of PE Plastic

To determine whether increased mRNA expression levels correlated with CYP102 A5 modifying PE, we measured the catalytic activity of CYP102 A5 using PE as a substrate. We observed an oxidation rate of  $23.55 \text{ nmol NADP}^+ \text{ min}^{-1}$  ( $\text{nmol of P450}^{-1}$ ) (Figure 6A). New peaks were found in the enzyme-treated samples by ATR-FTIR (Figure 6B) the presence of OH stretching in the polymer between  $3350 \text{ cm}^{-1}$  and  $3200 \text{ cm}^{-1}$ , a new C=O stretching peak was observed between  $1702 \text{ cm}^{-1}$ , and two new C-O stretching peaks were identified at  $1252 \text{ cm}^{-1}$  and  $1090 \text{ cm}^{-1}$  (Table 2).

**Table 2.** Molecular index and confidence intervals calculated by relative intensities of vibrational bands of interest from ATR-FTIR analysis of powdered LDPE incubated with CYP102 A5 and a NADPH regenerating system for 18 h <sup>1</sup>.

| Sample Type            | Carbonyl Bond Index<br>( $I_{1714}/I_{1463}$ ) | Keto Carbonyl Bond Index<br>( $I_{1715}/I_{1465}$ ) | Internal Double Bond Index<br>( $I_{908}/I_{1465}$ ) | Ester Carbonyl Index<br>( $I_{1740}/I_{1465}$ ) | Vinyl Bond Index<br>( $I_{1650}/I_{1465}$ ) |
|------------------------|------------------------------------------------|-----------------------------------------------------|------------------------------------------------------|-------------------------------------------------|---------------------------------------------|
| Control powdered PE    | $0.10 \pm 0.02$                                | $0.12 \pm 0.02$                                     | $0.17 \pm 0.02$                                      | $0.15 \pm 0.02$                                 | $0.18 \pm 0.01$                             |
| CYP102 A5, powdered PE | $0.14 \pm 0.01$                                | $0.15 \pm 0.01$                                     | $0.18 \pm 0.01$                                      | $0.16 \pm 0.01$                                 | $0.19 \pm 0.01$                             |

<sup>1</sup>  $n = 6$ .



**Figure 6.** (A) NADPH consumption by CYP102 A5 in the presence of 10 mg/mL powdered PE plastic over 60 s ( $n = 6$ ) with 95% CI intervals plotted. Representative controls of NADPH in buffer ( $n = 4$ ) and NADPH and 10 mg/mL PE ( $n = 4$ ) are also presented. (B) Averaged ATR-FTIR data of PE powder either with or without treatment with CYP102 A5 for 18 h ( $n = 8$ ).

### 3. Discussion

In this study, we demonstrate that a bacterial consortium consisting of three *Pseudomonas* and two *Bacillus* strains isolated from petroleum-polluted soil grows on PE plastic. The bacteria degrade the PE relatively efficiently; we observed a 16% weight loss for PE powder and a 10% weight loss for PE film after 30 days. Increased bacterial populations combined with a decrease in pH of the medium suggested depolymerization and fragmentation of PE into lower molecular weight compounds, which was demonstrated by ATR-FTIR [51,52]. These results together demonstrate that the bacterial consortium grows on PE as a sole carbon source. The description of microbial consortia degrading PE plastic has been limited to date.

Bacteria form biofilms on hydrophobic plastic surfaces, which are crucial for PE degradation [53]. Biofilm matrices enable plastic degradation through the secretion of exopolysaccharides for adhesion, and enzymes, such as laccases, hydrolases, and esterases, which facilitate the breakdown of the polymer [54–57]. As polymer biodegradation pro-

ceeds, the surface characteristics are altered, which can be observed via ATR-FTIR. We observed distinct changes in carbonyl indices for both PE powder and film, suggesting that the bacterial community oxidizes the surface of PE, while the appearance of new peaks, such as alcohols, ethers, and alkanes (Tables S1 and S2) indicates further degradation mediated by other secreted enzymes during depolymerization [58]. Higher water absorption (Figure 3D) means the polymer is becoming more hydrophilic, indicating that the bacterial consortia make the surface more hydrophilic.

We identified several metabolic byproducts by GC/MS after a 30-day degradation period, primarily alkanes, but also alkanes with carboxylic acid, aldehyde, and alcohol functional groups (Tables S1 and S2). These compounds are consistent with what is currently known of PE degradation and the enzymatic diversity of the degradative process [59,60]. The degradative byproducts often resemble fatty acids, which are eventually imported into the bacterium and catabolized via  $\beta$ -oxidation. Small molecules are converted into acetyl-CoA and enter the tricarboxylic acid (TCA) cycle and are finally mineralized via respiration. Identifying the metabolic byproducts was a focus of this work because it would suggest the types of enzymatic reactions occurring during degradation. Overall, the byproducts identified in the GC/MS analysis, combined with the changes in surface functional groups observed through ATR-FTIR, corroborate our previous findings that the bacteria are biodegrading PE and assimilating PE byproducts.

Four enzymes with >25% identity to previously characterized PE enzymes were evaluated via RT-qPCR. Of these four enzymes, a bi-functional cytochrome P450 NADPH reductase, encoded in *Bacillus thuringiensis* strain 9.1, was upregulated to 1.73 log fold when grown on PE in isolation and increased to 2.76 log fold when grown in combination with the full consortium on PE (Figure S2). This cytochrome P450 shares 99% sequence similarity with CYP102 A5.v1, which was solved to 0.93 Å and was previously shown to degrade PE plastic [24], and was thus named CYP102 A5 (Supplemental Text S2). CYP102 A5.v2 possesses the following mutations: S229 N, D231 N, P468 S, T673 I, K822 R, and M941 I (Supplemental Text S3 and Figure S5), several of which were previously targeted for engineering the closely related CYP102 A1/BM3 enzyme to improve degradation of n-alkanes [61–64]. CYP102 A5 is natively involved in combating polyunsaturated fatty acid toxicity and catalyzes rapid substrate oxidation (compared to turnover rates from other P450 monooxygenases) at various  $\omega$  hydroxylation positions [65]. Therefore, increased water absorption/hydrophilicity of the PE caused by CYP102 A5.v2 was predicted to occur by enzymatic biodegradation via  $\omega$  hydroxylation and enzymatic activity through the consumption of NADPH (Figure 6). When using PE as a substrate, we measured the catalytic activity of CYP102 A5.v2 and found a rate of 23.55 nmol NADP<sup>+</sup> min<sup>−1</sup> (nmol of P450)<sup>−1</sup>, while CYP102 A5.v1 for NADPH oxidation was 8.588 nmol of NADP<sup>+</sup> min<sup>−1</sup> (nmol of P450)<sup>−1</sup>. These data, paired with the observed mutations, bolster the evidence that CYP102 A5.v2 is directly involved in PE oxidation.

However, the specific mechanism of CYP102 A5.v2-mediated PE degradation in *Bacillus thuringiensis* requires further investigation. CYP102 A5.v2 is a relatively large enzyme, with the predicted dimer being ~240 kDa (Figure S3), and it lacks a signal sequence for secretion, which can be predicted using SignalP 6.0 software. Thus, the enzyme is likely not secreted from *B. thuringiensis* via conventional secretion systems and necessitates more research [66].

The cytochrome P450 reductase (CYP) facilitates terminal, subterminal, and in-chain hydroxylation of alkanes [35]. Baeyer–Villiger Monooxygenases (BVMOs) convert in-chain or subterminal ketones to esters and oxidize aldehydes [67,68]. This might explain the presence of palmitic acid, which contains a formate group (Figure S4). Glutathione dehydrogenase was identified as an alcohol dehydrogenase [69], which converts alcohols

into ketones, regardless of position on the alkane chain [70]. While we confirmed one enzyme, CYP102 A5.v2, that hydroxylates PE plastic, evidence exists indicating that other enzymes that oxidize PE are found in the consortium pangenome [36,37], and the complete degradation pathway(s) remain to be determined.

Microbial consortia are advantageous for breaking down PE and other types of plastics for multiple reasons. They are more stable than purified enzymes, which require highly purified conditions, and are more stable than single microbial isolates under metabolic stress conditions. They adapt to new environments better and exhibit greater metabolic diversity [71,72]. We have also observed synergistic growth in previous studies on PET plastic, where the full consortium utilized here can reach higher CFU/mL and OD<sub>600</sub> values, or culture yield, than individual isolates, and thus, are adapting quicker to nutrient-poor plastic environments [73]. With multiple isolates or species, synergy occurs, often through cross-feeding. PE biodegradation can be optimized by identifying additional consortium enzymes that oxidize PE and gaining a comprehensive understanding of the degradation pathways.

Bacterial consortia, like those used in this study, have the potential to significantly reduce the environmental impact of PE plastic waste. Additionally, the metabolic diversity of microbial consortia can lead to valorization, or the identification of valuable chemicals that can be harvested during the biodegradation of PE. Often, artificial intelligence (AI) is used in such efforts because of the complexity of synthetic systems. The waste stream can be reduced through biodegradation. Further, the extraction of compounds like palmitic acid, which is used in the cosmetic industry, methyl palmitate, a major component of biodiesel, or 2,5-dimethyl-hexane, which is used for aviation fuel (Tables S1 and S2), can be targeted. With improved degradation rates and the requisite technology to capture valuable byproducts, recycling of PE would become feasible, a vast improvement over the current handling of the waste, often landfill being the only option. In sum, the sheer mass of PE plastic ending up in landfills could be reduced using environmentally friendly, biological technologies.

## 4. Materials and Methods

### 4.1. Bacterial Strains and Plasmids Utilized in This Study

The five bacterial strains comprising the consortium used for PE plastic degradation in this study were previously obtained from petroleum-polluted soils in Galveston Bay, Texas [36]. Isolated species are *Pseudomonas* sp. SWI36, NRRL B-67630, strain 10; *Bacillus albus* strain PFYN0, NRRL B-67631, strain 13.1; *Bacillus thuringiensis* strain C15, NRRL B-67632, strain 9.1; *Pseudomonas* sp. strain B10, NRRL B-67633, strain 9.2; and *Pseudomonas* sp. SWI36, NRRL B-67634, strain 13.2. Plasmid pMTBN28 cyp was created using the pET28 b(+) expression vector.

For protein overexpression, three commercial strains were used: *E. coli* 10-β (New England Biolabs, Ipswich, MA, USA) for Gibson Assembly, and *E. coli* BL21 DE3 (New England Biolabs, Ipswich, MA, USA) for expression testing, and C41 (DE3) OverExpress™ for expression and purification (Sigma Aldrich, Saint Louis, MO, USA).

### 4.2. Bacterial Growth

#### 4.2.1. Pretreatment of PE Plastic

Polyethylene powder (Sigma Aldrich, Saint Louis, MO, USA) and PE one-sided cling machine film 0.55 mm (Sigma Summit, Belleville, ON, CA) were treated with UV radiation at 365 nm on a UV2 UV Transilluminator (Analytik Jena, Jena, Germany) for 4 h to simulate natural abiotic weathering. Plastic was subsequently weighed and sterilized with 70% ethanol, and then washed with sterile water and set aside in a sterile hood until dry.

#### 4.2.2. Growth of PE-Degrading Bacteria

Consortium strains were grown on low-salt lysogeny broth (LB) agar plates (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar, pH 7.0) at 30 °C. Strains were then grown in liquid LB to mid-exponential phase and normalized to an OD<sub>600</sub> value of 1.000 to standardize bacterial counts using a BioSpec mini–1240 UV-Vis Spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). After normalization, to monitor growth on PE consortia bacteria were spun down to a pellet and washed twice with a modified Liquid Carbon Free Basal Medium (LCFBM) adapted from the American Society for Testing and Materials (ASTM) standard for studying the bacteria resistance to plastics (ASTM G22–76) (per liter: 0.05% (wt/vol) yeast extract, 0.2% (wt/vol) (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, and 1% (vol/vol) trace elements (0.1% [wt/vol] FeSO<sub>4</sub>·7 H<sub>2</sub>O, 0.1% [wt/vol] MgSO<sub>4</sub>·7 H<sub>2</sub>O, 0.01% [wt/vol], CuSO<sub>4</sub>·5 H<sub>2</sub>O, 0.01% [wt/vol] MnSO<sub>4</sub>·5 H<sub>2</sub>O, and 0.01% [wt/vol] ZnSO<sub>4</sub>·7 H<sub>2</sub>O) in 10 mM pH 7.4 sodium phosphate buffer.). Cultures were inoculated by the addition of 100 µL of each rinsed isolate to 50 mL of 0.2% *w/v* PE-supplemented LCFBM in a 250 mL culture flask. Samples were incubated statically to facilitate biofilm formation [38] at 30 °C for 30 days.

#### 4.2.3. Growth Conditions of *E. coli* Strains for Gibson Assembly, Plasmid Isolation, and Protein Overexpression

*E. coli* DH5α bacteria were grown at 37 °C. Overnight cultures of these strains were grown in LB media, shaking at 180 rpm. During transformation of the pMTBN28 cyp vector (Supplemental Text S1), room temperature super optimal broth with a proprietary (NEB, Ipswich, MA, USA) catabolite repression (SOC) media (2% vegetable peptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose, pH 7.0) was used to recover *E. coli* cells after Gibson Assembly. *E. coli* transformants were grown on and selected from using LB agar plates supplemented with 50 µg/mL kanamycin at 37 °C. For protein overexpression, *E. coli* BL21 (DE3) and C41 (DE3) cell lines containing the pMTBN28 cyp plasmid were grown shaking in Terrific Broth (TB) media (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH<sub>2</sub> PO<sub>4</sub>, 0.072 M K<sub>2</sub> HPO<sub>4</sub>, 50 µg/mL kanamycin), 0.5 mM δ-aminolaevulinic acid (5-ALA) and 0.4 mM IPTG were added to induce protein expression before purification of CYP102 A5 protein.

#### 4.3. Measurement of Bacterial Growth for Evaluating PE Degradation

After microbial growth in LCFBM cultures, aliquots of culture were serially diluted in triplicate to obtain the CFU/mL every 5 days. CFU/mL values were averaged across three separate flasks. CFU mL values for the control cultures (LCFBM only samples) were collected to ensure no bacterial contamination occurred. The culture pH values were measured using a pH probe and verified with pH strips having a range of 5.1–7.2 (VWR, Tualatin, OR, USA).

#### 4.4. Weight Loss of PE Plastic and Water Absorbance Changes in Bacterial Culture

Following 30 days of bacterial biodegradation, the weight of PE powder and film was measured using the method described in Hadar, Y., and A. Sivan., 2004 [38]. Plastic was separated from the media using vacuum filtration with Watman #6 filter paper to ensure efficient recovery. The plastic film and powder were washed for 4 h using 2% SDS to remove bacteria from the plastic surface [74,75]. The surfactant and plastic were separated using gravity filtration and subsequently washed with sterile distilled water and 70% ethanol to remove any cell debris or residual bacteria colonized on the plastic surface. The plastic was then dried at 50 °C in a hot air oven to evaporate any remaining water.

The percent weight reduction was calculated using the formula described by Kyaw et al., 2014 [76].

$$\% \text{ weight reduction} = \frac{(\text{initial weight} - \text{final weight})}{\text{initial weight}} \times 100 \quad (1)$$

For water absorbance calculations, the initial mass ( $M_1$ ) was analyzed after 30 days of incubation with the bacterial consortium alongside a comparative control of plastic inoculated in LCFBM for 30 days without bacteria following the sterilization and cleaning of the remaining plastic film and particles, ensuring complete removal of surfactant that may interfere with water absorbance. 50 mL of sterile distilled water was then added to each culture bottle. All flasks were then incubated at 30 °C for 5 days, and the average mass ( $M_2$ ) was determined after 120 h. The percentage of water absorption coefficient (a%) was calculated using the formula:

$$a\% = \frac{(M_2 - M_1)}{M_1} \times 100 \quad (2)$$

#### 4.5. SEM Analysis of Bacteria-Degraded PE Plastic

After 30 days of culture growth, PE sheets and powder were analyzed using SEM imaging. After weight loss measurements, the surface landscape of PE films and PE powder was examined using a scanning electron microscope (SEM). Both PE powder and PE film samples were dried before imaging and were handled identically, ensuring that any observed surface alterations could be attributed to biological activity rather than sample processing. A 4 nm carbon coating was added to both samples, and images were taken with a 1  $\mu$ s dwell time. SEM was performed at the Multi-Scale Microscopy Core (MMC) with technical support from the Oregon Health and Science University (OHSU)/FEI Living Lab and the OHSU Center for Spatial Systems Biomedicine (OCSSB).

#### 4.6. FTIR Analysis of Bacteria-Degraded PE Plastic

ATR-FTIR analysis was used to analyze the effects of biodegradation. Molecular changes in the PE plastic sheets before and after biodegradation by the bacterial consortium were measured using a Thermo iS5 FTIR Spectrometer with iD7 ATR accessory (Thermo-Fischer, Waltham, MA, USA). Samples were scanned at a resolution of 4  $\text{cm}^{-1}$  between 600  $\text{cm}^{-1}$  and 4000  $\text{cm}^{-1}$ . The relative absorbance intensities of the carbonyl index, ester carbonyl bond, keto carbonyl bond, terminal double bond (vinyl), and the internal double bond to that of the methylene bond were calculated using the following formulas, described below. I refers to a particular wavenumber of interest ( $\text{cm}^{-1}$ ) for PE polymers [77,78]. Molecular indexes (I) were determined as a ratio to the control wavenumber ( $\text{cm}^{-1}$ ) 1462 for the following: Keto-Carbonyl Index (KBI) (1715); Ester Carbonyl Bond Index (ECBI) (1740); Vinyl Bond Index (VBI) (1650); Internal Double Bond Index (IDBI) (908); and Keto-Carbonyl Index (KBI) (1714).

#### 4.7. Detection of PE Degradation Products

After 30 days of biodegradation of PE by the full consortium of bacteria in LCFBM, the culture medium was collected and centrifuged for 15 min at 10,000 rpm at 4 °C to collect the supernatant. The supernatants from this culture and an identical sample containing LCFBM, PE, and no bacteria as the control were extracted with an equal volume of ethyl acetate (Sigma Aldrich, St. Louis, MO, USA) and subsequently concentrated utilizing a rotovap. The product was resuspended in 1 mL of ethyl acetate and filtered with a 0.22  $\mu$ m filter. Products were analyzed using a 7890 A GC and a 5975 C MSD for gas chromatography–mass spectroscopy on electron impact ionization mode (Agilent, Santa Clara, CA, USA). The metabolites were separated on a Restek Rxi–5 Sil MS column

(30 m × 0.25 mm × 0.25 µm, Restek, Center County, PA, USA). The oven temperature was initially set at 40 °C for 3 min, then increased to 280 °C at 10 °C/min, and was maintained at 280 °C for 4 min. For the detector, an electron ionization mode was set at 230 °C. The temperature of the detector and the injector was set to 150 °C. Sample volumes of 1 µm were taken per injection with a split ratio of 1:10. Samples were scanned at a range of 34–500 amu. Peak assignment was based on a comparison with the mass spectral database, W8 N08.L (Hewlett-Packard, Palo Alto, CA, USA).

#### 4.8. Gene of Interest Identification and Expression in PE Plastic Conditions

Genes associated with PE degradation were identified using the Plastic Biodegradation Database—PlasticDB [31]. Genes to investigate by RT-qPCR were selected based on calculated sequence identity according to the NCBI Protein BLAST algorithm and Jalview Clustal Omega Version 2 [79] multiple sequence alignment. Primers were designed utilizing the Primer-BLAST tool and are listed in Table S3.

Cultures were harvested after the log phase was reached for PE cultures and the controls, respectively. Whole cellular cultures were first spun and lysed to isolate RNA using the RNeasy kit (Qiagen, Germantown, MD, USA) according to manufacturer instructions. Reverse transcription of cDNA was performed according to manufacturer recommendations using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA). Pure cDNA, along with designed primers (Thermo Fisher, Waltham, MA, USA) with the iTaq Universal SYBR Green supermix (Bio Rad, Hercules, CA, USA), was amplified on a CFX thermal cycler (BioRad, Hercules, CA, USA). All 16 S ribosomal genes of each isolate were used as housekeeping genes in both individual isolate and full consortium cDNA samples. Controls with no template DNA added ( $n = 3$ ), along with a negative control of pure water, were included in each run. After assembly of PCR samples in triplicate, the samples were run according to the manufacturer's instructions. Mean cycle threshold values ( $C_t$ ) were estimated along with melting curve analysis to confirm PCR primer and product specificity. Each cDNA sample was run against a standard curve of concentrations, and the primer efficiency was determined after generation of a 5-point standard curve. Changes in gene expression were calculated by the Pfaffl method, where GOI refers to the Gene of Interest and HKG refers to the Housekeeping Gene.

$$\frac{(E_{GOI})^{\Delta C_t GOI}}{(E_{HKG})^{\Delta C_t HKG}} \quad (3)$$

#### 4.9. Expression and Purification of CYP102 A5

Gibson Assembly was performed (Supplementary Information S1) to generate the pMT28 BNcyp plasmid encoding the CYP102 A5 protein sequence using primers in Table S4. For expression of CYP102 A5, sterile LB (60 mL) containing 50 µg/mL of kanamycin was inoculated with *E.coli* Overexpress™ C41 (DE3) containing the plasmid and grown overnight at 37 °C, shaking at 200 rpm. Saturated overnight cultures were diluted 1:100 in 1 L of TB containing 50 µg/mL kanamycin. Cultures continued shaking under the same conditions until the OD<sub>600</sub> was 0.4, and 0.5 mM δ aminolaevulinic acid was added immediately to the culture. Cultures continued shaking until an OD<sub>600</sub> of 0.8 was reached, and 0.4 mM IPTG was added to chilled flasks. The cultures were left to grow and overexpress for 18 h at 30 °C and shaking at 100 rpm. Bacterial cultures were harvested by centrifugation at 5000 × *g* for 25 min at 4 °C, and cell pellets were stored at −80 °C after being flash frozen in LN<sub>2</sub>.

For purification, pellets were resuspended in 10 mL lysis buffer per gram of pellet (50 mM KCl, 100 mM potassium phosphate buffer, pH 7.4) with the following additions: 0.5 mM PMSF, 1 mM Benzamidine, 1 µg/mL Leupeptin, 0.7 µg/mL Pepstatin, 0.01 mg/mL

DNase, 1 mM MgCl<sub>2</sub>, 0.2 mg/mL lysozyme, and 1 mM TCEP. Pellets were gently homogenized and then lysed on a stir plate for 1 h at 4 °C. Lysed cells were sonicated for three total rounds of 6 repeats of 20 sec on (~70% power), 20 sec off, followed by 1 min rest between the individual rounds. The sonicated lysate was subsequently centrifuged (40,000 × g, 35 min, 4 °C) to remove the cell debris. Ni-NTA resin equilibrated in lysis buffer containing 5 mM imidazole (Takara Bio, San Jose, CA, USA) was left to batch bind with the clarified supernatant while rotating for 1 h at 4 °C. Afterwards, the resin was spun down at 900 × g at 4 °C for 5 min and then packed in a column under gravity. After collecting the flow through, the Ni-NTA resin was washed with a stepwise imidazole gradient (5–40 mM imidazole) and then CYP102 A5 was eluted with 250 mM imidazole buffer using a peristaltic pump. Wash and elution fractions were analyzed using a Nanodrop ND–1000 Spectrophotometer (Thermo Fisher, Waltham, MA, USA) and by separation using SDS-PAGE. Elutions were concentrated to ~2–3 mg/mL in an Amicon Ultra 15 50 kDa cutoff concentrator (Sigma Aldrich, Waltham, MA, USA), and the resulting concentrate (~3 mL) was left to dialyze overnight using 12,000–14,000 MWCO tubing (Spectra Labs, Rancho Dominguez, CA, USA). Samples were concentrated further to approximately 10 mg/mL and filtered through 0.45 µm centrifugal filter, spun at 8000 rpm for 1 min at 4 °C. The filtered sample was manually injected onto an AKTA Pure 150 FPLC system (Cytiva, Marlborough, MA, USA) equipped with a size exclusion chromatographic (SEC) Superdex 200 10/300 (Cytiva, Marlborough, MA, USA) column. SEC fractions were resolved on an SDS-PAGE gel. Fractions containing pure CYP102 A5 were combined and concentrated using a 50 kDa concentrator to 3 mg/mL and flash frozen in liquid nitrogen before storing at –80 °C.

#### 4.10. NADPH Consumption Assays of CYP102 A5

NADPH consumption was used to determine if CYP102 A5 is active in the presence of substrate. A baseline test for one of many native substrates of CYP102 A5, palmitic acid, was used to establish activity (Supplementary Information S2). In brief, in a 1 mL reaction volume, 50 mM of a 5 mM stock of palmitic acid in ethanol, 55 nM of purified CYP102 A5, and 200 nM of NADPH were added in a 1 mL total reaction volume suspended in buffer (50 mM pH 7.4 potassium phosphate, 100 mM potassium chloride). Compounds were added in order listed, and there was 30 s between the addition of each component. After the 200 nM of NADPH was added to the quartz cuvette, the cuvette was immediately added to an Agilent Cary 60 UV-Vis (Agilent, Santa Clara, CA, USA). The absorbance at 340 nm was collected every single second for 1 min. The beam mode was set to dual beam, and the data interval was 2.00 nm. To establish the activity of CYP102 A5 in the presence of PE powder, 10 mg/mL of PE powder was pre-added to a quartz cuvette. The total 1 mL reaction volume consists of 400 nM CYP102 A5, 100 nM NADPH, and the same buffer as listed above. NADPH was homogenized in buffer before addition to the quartz cuvette. Careful effort was focused on ensuring no plastic was stuck above the buffer line. Subsequently, 400 nM of CYP102 A5 was spiked into the sample and left to equilibrate for 10 s before collecting UV-Vis spectra. After the 10 s had elapsed, recording was started on an Agilent Cary 60 UV-Vis (Agilent, Santa Clara, CA, USA). The absorbance at 340 nm was collected every second for 60 s in total. The beam mode was set to dual beam, and the data interval was 2.00 nm.

#### 4.11. Long-Term NADPH-Regeneration Assay and FTIR Analysis of PE Plastic After Treatment with CYP102 A5

Briefly, 10 mg/mL of PE plastic, 10 mM glucose 6-phosphate, 1 unit/mL of glucose 6-phosphate dehydrogenase, 0.5 mM NADPH, and 400 nM of CYP102 A5 (or without CYP102 A5 for the hydrolysis control) were left shaking for 18 hrs at 37 °C in buffer (50 mM potassium phosphate, 100 mM KCl). After 18 h had elapsed, plastic was filtered

from the buffer via gravity using Whatman #1 filter paper. PE was washed  $3\times$  with a 10:1 H<sub>2</sub>O: methanol solution and then washed 3 more times with 2% SDS. The plastic was left to dry overnight in a 60 °C oven before ATR-FTIR analysis. Molecular changes in the PE plastic sheets before and after biodegradation by the CYP102 A5 were measured using a Thermo iS5 FTIR Spectrometer with iD7 ATR accessory (Thermo-Fischer, Waltham, MA, USA). Samples were scanned at a resolution of 4 cm<sup>−1</sup> between 600 cm<sup>−1</sup> and 4000 cm<sup>−1</sup>.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms26188775/s1>. Reference [80] has been mentioned in the text.

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## Abbreviations

The following abbreviations are used in this manuscript:

|          |                                                                      |
|----------|----------------------------------------------------------------------|
| PE       | polyethylene                                                         |
| LDPE     | low-density polyethylene                                             |
| ATR-FTIR | attenuated total reflectance–Fourier transform infrared spectroscopy |
| GC/MS    | gas chromatography–mass spectrometry                                 |
| LCFBM    | liquid carbon free basal medium                                      |
| CFU      | colony forming units                                                 |

## References

1. United States Environmental Protection Agency. *Advancing Sustainable Materials Management: 2018 Tables and Figures 2020*; United States Environmental Protection Agency: Washington, DC, USA, 2020.
2. PlasticsEurope. *Plastics-the Facts 2021: An Analysis of European Plastics Production, Demand, and Waste Data*; PlasticsEurope: Brussels, Belgium, 2021.
3. Di, J.; Reck, B.K.; Miatto, A.; Graedel, T.E. United States plastics: Large flows, short lifetimes, and negligible recycling. *Resour. Conserv. Recycl.* **2021**, *167*, 105440. [CrossRef]
4. Hutley, T.J.; Ouederni, M. Polyolefins—The History and Economic Impact. In *Polyolefin Compounds and Materials: Fundamentals and Industrial Applications*; Springer Series on Polymer and Composite Materials; Al-Ali AlMa'adeed, M., Krupa, I., Eds.; Springer Series on Polymer and Composite Materials; Springer International Publishing: Cham, Switzerland, 2016; pp. 13–50, ISBN 978-3-319-25982-6.
5. Lau, W.W.Y.; Shiran, Y.; Bailey, R.M.; Cook, E.; Stuchtey, M.R.; Koskella, J.; Velis, C.A.; Godfrey, L.; Boucher, J.; Murphy, M.B.; et al. Evaluating scenarios toward zero plastic pollution. *Science* **2020**, *369*, 1455–1461. [CrossRef]

6. OECD. *Global Plastics Outlook*; OECD: Paris, France, 2022.
7. Ory, N.C.; Sobral, P.; Ferreira, J.L.; Thiel, M. Amberstripe scad *Decapterus muroadsi* (Carangidae) fish ingest blue microplastics resembling their copepod prey along the coast of Rapa Nui (Easter Island) in the South Pacific subtropical gyre. *Sci. Total. Environ.* **2017**, *586*, 430–437. [CrossRef] [PubMed]
8. Balzani, P.; Galeotti, G.; Scheggi, S.; Masoni, A.; Santini, G.; Baracchi, D. Acute and chronic ingestion of polyethylene (PE) microplastics has mild effects on honey bee health and cognition. *Environ. Pollut.* **2022**, *305*, 119318. [CrossRef]
9. Kim, S.W.; An, Y.-J. Edible Size of Polyethylene Microplastics and Their Effects on Springtail Behavior. *Environ. Pollut.* **2020**, *266*, 115255. [CrossRef]
10. Park, E.-J.; Han, J.-S.; Seong, E.; Lee, G.-H.; Kim, D.-W.; Son, H.-Y.; Han, H.-Y.; Lee, B.-S. Repeated-oral dose toxicity of polyethylene microplastics and the possible implications on reproduction and development of the next generation. *Toxicol. Lett.* **2020**, *324*, 75–85. [CrossRef]
11. Çobanoğlu, H.; Belivermiş, M.; Sıkdokur, E.; Kılıç, Ö.; Çayır, A. Genotoxic and cytotoxic effects of polyethylene microplastics on human peripheral blood lymphocytes. *Chemosphere* **2021**, *272*, 129805. [CrossRef]
12. Gautam, R.; Jo, J.; Acharya, M.; Maharjan, A.; Lee, D.; Kc, P.B.; Kim, C.; Kim, K.; Kim, H.; Heo, Y. Evaluation of potential toxicity of polyethylene microplastics on human derived cell lines. *Sci. Total Environ.* **2022**, *838*, 156089. [CrossRef] [PubMed]
13. Rodrigues, M.O.; Abrantes, N.; Gonçalves, F.; Nogueira, H.S.; Marques, J.; Gonçalves, A.M. Impacts of plastic products used in daily life on the environment and human health: What is known? *Environ. Toxicol. Pharmacol.* **2019**, *72*, 103239. [CrossRef]
14. Bäckström, E.; Odelius, K.; Hakkarainen, M. Trash to Treasure: Microwave-Assisted Conversion of Polyethylene to Functional Chemicals. *Ind. Eng. Chem. Res.* **2017**, *56*, 14814–14821. [CrossRef]
15. Oliveira, J.; Belchior, A.; da Silva, V.D.; Rotter, A.; Petrovski, Ž.; Almeida, P.L.; Lourenço, N.D.; Gaudêncio, S.P. Marine Environmental Plastic Pollution: Mitigation by Microorganism Degradation and Recycling Valorization. *Front. Mar. Sci.* **2020**, *7*, 567126. [CrossRef]
16. Chamas, A.; Moon, H.; Zheng, J.; Qiu, Y.; Tabassum, T.; Jang, J.H.; Abu-Omar, M.; Scott, S.L.; Suh, S. Degradation Rates of Plastics in the Environment. *ACS Sustain. Chem. Eng.* **2020**, *8*, 3494–3511. [CrossRef]
17. Yao, Z.; Seong, H.J.; Jang, Y.-S. Environmental Toxicity and Decomposition of Polyethylene. *Ecotoxicol. Environ. Saf.* **2022**, *242*, 113933. [CrossRef]
18. Verma, R.; Vinoda, K.S.; Papireddy, M.; Gowda, A.N.S. Toxic Pollutants from Plastic Waste—A Review. *Procedia Environ. Sci.* **2016**, *35*, 701–708. [CrossRef]
19. Sen, S.K.; Raut, S. Microbial degradation of low density polyethylene (LDPE): A review. *J. Environ. Chem. Eng.* **2015**, *3*, 462–473. [CrossRef]
20. Nowak, B.; Pająk, J.; Drozd-Bratkowicz, M.; Rymarz, G. Microorganisms participating in the biodegradation of modified polyethylene films in different soils under laboratory conditions. *Int. Biodeterior. Biodegrad.* **2011**, *65*, 757–767. [CrossRef]
21. Santo, M.; Weitsman, R.; Sivan, A. The role of the copper-binding enzyme—Laccase—In the biodegradation of polyethylene by the actinomycete *Rhodococcus ruber*. *Int. Biodeterior. Biodegrad.* **2013**, *84*, 204–210. [CrossRef]
22. Rajandas, H.; Parimannan, S.; Sathasivam, K.; Ravichandran, M.; Yin, L.S. A novel FTIR-ATR spectroscopy based technique for the estimation of low-density polyethylene biodegradation. *Polym. Test.* **2012**, *31*, 1094–1099. [CrossRef]
23. Tribedi, P.; Sil, A.K. Low-Density Polyethylene Degradation by *Pseudomonas* Sp. AKS2 Biofilm. *Environ. Sci. Pollut. Res.* **2013**, *20*, 4146–4153. [CrossRef] [PubMed]
24. Yun, S.-D.; Lee, C.O.; Kim, H.-W.; An, S.J.; Kim, S.; Seo, M.-J.; Park, C.; Yun, C.-H.; Chi, W.S.; Yeom, S.-J. Exploring a New Biocatalyst from *Bacillus Thuringiensis* JNU01 for Polyethylene Biodegradation. *Environ. Sci. Technol. Lett.* **2023**, *10*, 485–492. [CrossRef]
25. Ghatge, S.; Yang, Y.; Ahn, J.-H.; Hur, H.-G. Biodegradation of polyethylene: A brief review. *Appl. Biol. Chem.* **2020**, *63*, 27. [CrossRef]
26. Bardají, D.K.R.; Moretto, J.A.S.; Furlan, J.P.R.; Stehling, E.G. A mini-review: Current advances in polyethylene biodegradation. *World J. Microbiol. Biotechnol.* **2020**, *36*, 32. [CrossRef]
27. Zeenat; Elahi, A.; Bukhari, D.A.; Shamim, S.; Rehman, A. Plastics degradation by microbes: A sustainable approach. *J. King Saud Univ. Sci.* **2021**, *33*, 101538. [CrossRef]
28. Yuan, Z.; Xu, X.-R. Chapter Six—Surface Characteristics and Biototoxicity of Airborne Microplastics. In *Comprehensive Analytical Chemistry*; Wang, J., Ed.; Airborne Microplastics: Analysis, Fate and Human Health Effects; Elsevier: Amsterdam, The Netherlands, 2023; Volume 100, pp. 117–164.
29. Mohanan, N.; Montazer, Z.; Sharma, P.K.; Levin, D.B. Microbial and Enzymatic Degradation of Synthetic Plastics. *Front. Microbiol.* **2020**, *11*, 580709. [CrossRef]
30. Peixoto, J.; Silva, L.P.; Krüger, R.H. Brazilian Cerrado soil reveals an untapped microbial potential for unpretreated polyethylene biodegradation. *J. Hazard. Mater.* **2017**, *324*, 634–644. [CrossRef] [PubMed]

31. Gambarini, V.; Pantos, O.; Kingsbury, J.M.; Weaver, L.; Handley, K.M.; Lear, G. PlasticDB: A Database of Microorganisms and Proteins Linked to Plastic Biodegradation. *Database* **2022**, 2022, baac008. Available online: <https://academic.oup.com/database/article/doi/10.1093/database/baac008/6546196> (accessed on 13 February 2024). [PubMed]
32. Restrepo-Flórez, J.-M.; Bassi, A.; Thompson, M.R. Microbial degradation and deterioration of polyethylene—A review. *Int. Biodeterior. Biodegradation* **2014**, *88*, 83–90. [CrossRef]
33. Wei, R.; Zimmermann, W. Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: How far are we? *Microb. Biotechnol.* **2017**, *10*, 1308–1322. [CrossRef]
34. Rojo, F. Enzymes for Aerobic Degradation of Alkanes. In *Handbook of Hydrocarbon and Lipid Microbiology*; Timmis, K.N., Ed.; Springer: Berlin/Heidelberg, Germany, 2010; pp. 781–797, ISBN 978-3-540-77584-3.
35. Yeom, S.-J.; Le, T.-K.; Yun, C.-H. P450-driven plastic-degrading synthetic bacteria. *Trends Biotechnol.* **2022**, *40*, 166–179. [CrossRef]
36. León-Zayas, R.; Roberts, C.; Vague, M.; Mellies, J.L.; Newton, I.L.G. Draft Genome Sequences of Five Environmental Bacterial Isolates That Degrade Polyethylene Terephthalate Plastic. *Genome Announc.* **2019**, *8*, e00237-19. [CrossRef]
37. Edwards, S.; León-Zayas, R.; Ditter, R.; Laster, H.; Sheehan, G.; Anderson, O.; Beattie, T.; Mellies, J.L. Microbial Consortia and Mixed Plastic Waste: Pangenomic Analysis Reveals Potential for Degradation of Multiple Plastic Types via Previously Identified PET Degrading Bacteria. *Int. J. Mol. Sci.* **2022**, *23*, 5612. [CrossRef] [PubMed]
38. Gilan, I.; Hadar, Y.; Sivan, A. Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 97–104. [CrossRef] [PubMed]
39. Jeon, J.-M.; Park, S.-J.; Choi, T.-R.; Park, J.-H.; Yang, Y.-H.; Yoon, J.-J. Biodegradation of polyethylene and polypropylene by *Lysinibacillus* species JY0216 isolated from soil grove. *Polym. Degrad. Stab.* **2021**, *191*, 109662. [CrossRef]
40. Gerritse, J.; Leslie, H.A.; de Tender, C.A.; Devriese, L.I.; Vethaak, A.D. Fragmentation of plastic objects in a laboratory seawater microcosm. *Sci. Rep.* **2020**, *10*, 1–16. [CrossRef] [PubMed]
41. Sutkar, P.R.; Gadewar, R.D.; Dhulap, V.P. Recent Trends in Degradation of Microplastics in the Environment: A State-of-the-Art Review. *J. Hazard. Mater. Adv.* **2023**, *11*, 100343. [CrossRef]
42. Park, S.Y.; Kim, C.G. Biodegradation of micro-polyethylene particles by bacterial colonization of a mixed microbial consortium isolated from a landfill site. *Chemosphere* **2019**, *222*, 527–533. [CrossRef]
43. Drnovská, H.; Lapčík, L.; Buršíková, V.; Zemek, J.; Barros-Timmons, A.M. Surface properties of polyethylene after low-temperature plasma treatment. *Colloid Polym. Sci.* **2003**, *281*, 1025–1033. [CrossRef]
44. Elsamahy, T.; Sun, J.; Elsilik, S.E.; Ali, S.S. Biodegradation of low-density polyethylene plastic waste by a constructed tri-culture yeast consortium from wood-feeding termite: Degradation mechanism and pathway. *J. Hazard. Mater.* **2023**, *448*, 130944. [CrossRef]
45. Montazer, Z.; Najafi, M.B.H.; Levin, D.B. Challenges with Verifying Microbial Degradation of Polyethylene. *Polymers* **2020**, *12*, 123. [CrossRef]
46. Balasubramanian, V.; Natarajan, K.; Hemambika, B.; Ramesh, N.; Sumathi, C.; Kottaimuthu, R.; Kannan, V.R. High-density polyethylene (HDPE)-degrading potential bacteria from marine ecosystem of Gulf of Mannar, India. *Lett. Appl. Microbiol.* **2010**, *51*, 205–211. [CrossRef]
47. Nademo, Z.M.; Shibeshi, N.T.; Gameda, M.T. Isolation and screening of low-density polyethylene (LDPE) bags degrading bacteria from Addis Ababa municipal solid waste disposal site “Koshe”. *Ann. Microbiol.* **2023**, *73*, 1–11. [CrossRef]
48. Sandt, C.; Waeytens, J.; Deniset-Besseau, A.; Nielsen-Leroux, C.; Réjasse, A. Use and misuse of FTIR spectroscopy for studying the bio-oxidation of plastics. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2021**, *258*, 119841. [CrossRef]
49. Khandare, S.D.; Agrawal, D.; Mehru, N.; Chaudhary, D.R. Marine bacterial based enzymatic degradation of low-density polyethylene (LDPE) plastic. *J. Environ. Chem. Eng.* **2022**, *10*, 107437. [CrossRef]
50. Yao, Z.; Seong, H.J.; Jang, Y.-S. Degradation of low density polyethylene by *Bacillus* species. *Appl. Biol. Chem.* **2022**, *65*, 1–9. [CrossRef]
51. Das, M.P.; Kumar, S. An Approach to Low-Density Polyethylene Biodegradation by *Bacillus Amyloliquefaciens*. *3 Biotech* **2015**, *5*, 81–86. [CrossRef]
52. Karamanlioglu, M.; Preziosi, R.; Robson, G.D. The Compostable Plastic Poly(lactic) Acid Causes a Temporal Shift in Fungal Communities in Maturing Compost. *Compos. Sci. Util.* **2017**, *25*, 211–219. [CrossRef]
53. Sivan, A. New perspectives in plastic biodegradation. *Curr. Opin. Biotechnol.* **2011**, *22*, 422–426. [CrossRef] [PubMed]
54. Wilkes, R.A.; Aristilde, L. Degradation and Metabolism of Synthetic Plastics and Associated Products by *Pseudomonas* Sp.: Capabilities and Challenges. *J. Appl. Microbiol.* **2017**, *123*, 582–593. [CrossRef] [PubMed]
55. Cai, Z.; Li, M.; Zhu, Z.; Wang, X.; Huang, Y.; Li, T.; Gong, H.; Yan, M. Biological Degradation of Plastics and Microplastics: A Recent Perspective on Associated Mechanisms and Influencing Factors. *Microorganisms* **2023**, *11*, 1661. [CrossRef]
56. Khalil, M.A.; Alorabi, J.A.; Al-Otaibi, L.M.; Ali, S.S.; Elsilik, S.E. Antibiotic Resistance and Biofilm Formation in *Enterococcus* spp. Isolated from Urinary Tract Infections. *Pathogens* **2022**, *12*, 34. [CrossRef] [PubMed]

57. Ndahebwa Muhonja, C.; Magoma, G.; Imbuga, M.; Makonde, H.M. Molecular Characterization of Low-Density Polyethylene (LDPE) Degrading Bacteria and Fungi from Dandora Dumpsite, Nairobi, Kenya. *Int. J. Microbiol.* **2018**, *2018*, 4167845. [CrossRef]
58. Woo, S.; Song, I.; Cha, H.J.; Johnson, K.N. Fast and Facile Biodegradation of Polystyrene by the Gut Microbial Flora of *Plesiophthalmus davidis* Larvae. *Appl. Environ. Microbiol.* **2020**, *86*, e01361–20. [CrossRef]
59. Liu, F.; Zhang, N.; Shang, Y.; Yao, M.; Ding, M.; Yuan, Y. Construction of *Yarrowia lipolytica* for degradation of low-density polyethylene. *Process. Saf. Environ. Prot.* **2025**, *195*, 106818. [CrossRef]
60. Uddin, M.; Venkatesan, S.K.; Pal, S.K.; Vinu, R.; Sekar, K.; Kandasamy, R. Accelerating biodegradation efficiency of low-density polyethylene and its hazardous dissolved organic matter using unexplored polyolefin-respiring bacteria: New insights on degradation characterization, biomolecule influence and biotransformation pathways. *J. Hazard. Mater.* **2025**, *492*, 138144. [CrossRef]
61. Aschenbrenner, J.C.; Ebrecht, A.C.; Tolmie, C.; Smit, M.S.; Opperman, D.J. Structure of the fungal hydroxylase, CYP505A30, and rational transfer of mutation data from CYP102A1 to alter regioselectivity. *Catal. Sci. Technol.* **2021**, *11*, 7359–7367. [CrossRef]
62. Chen, M.M.; Snow, C.D.; Vizcarra, C.L.; Mayo, S.L.; Arnold, F.H. Comparison of random mutagenesis and semi-rational designed libraries for improved cytochrome P450 BM3-catalyzed hydroxylation of small alkanes. *Protein Eng. Des. Sel.* **2012**, *25*, 171–178. [CrossRef]
63. Meinhold, P.; Peters, M.W.; Chen, M.M.Y.; Takahashi, K.; Arnold, F.H. Direct Conversion of Ethane to Ethanol by Engineered Cytochrome P450 BM3. *ChemBioChem* **2005**, *6*, 1765–1768. [CrossRef] [PubMed]
64. Peters, M.W.; Meinhold, P.; Glieder, A.; Arnold, F.H. Regio- and Enantioselective Alkane Hydroxylation with Engineered Cytochromes P450 BM-3. *J. Am. Chem. Soc.* **2003**, *125*, 13442–13450. [CrossRef]
65. Chowdhary, P.K.; Alemseghed, M.; Haines, D.C. Cloning, expression and characterization of a fast self-sufficient P450: CYP102A5 from *Bacillus cereus*. *Arch. Biochem. Biophys.* **2007**, *468*, 32–43. [CrossRef] [PubMed]
66. Teufel, F.; Armenteros, J.J.A.; Johansen, A.R.; Gíslason, M.H.; Pihl, S.I.; Tsigirgos, K.D.; Winther, O.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nat. Biotechnol.* **2022**, *40*, 1023–1025. [CrossRef]
67. Fürst, M.J.L.J.; Gran-Scheuch, A.; Aalbers, F.S.; Fraaije, M.W. Baeyer–Villiger Monooxygenases: Tunable Oxidative Biocatalysts. *ACS Catal.* **2019**, *9*, 11207–11241. [CrossRef]
68. Fiorentini, F.; Geier, M.; Binda, C.; Winkler, M.; Faber, K.; Hall, M.; Mattevi, A. Biocatalytic Characterization of Human FMO5: Unearthing Baeyer–Villiger Reactions in Humans. *ACS Chem. Biol.* **2016**, *11*, 1039–1048. [CrossRef]
69. Zadjelovic, V.; Erni-Cassola, G.; Obrador-Viel, T.; Lester, D.; Eley, Y.; Gibson, M.I.; Dorador, C.; Golyshin, P.N.; Black, S.; Wellington, E.M.H.; et al. A Mechanistic Understanding of Polyethylene Biodegradation by the Marine Bacterium *Alcanivorax*. *J. Hazard. Mater.* **2022**, *436*, 129278. [CrossRef]
70. Kim, H.R.; Lee, C.; Shin, H.; Kim, J.; Jeong, M.; Choi, D. Isolation of a polyethylene-degrading bacterium, *Acinetobacter guillouiae*, using a novel screening method based on a redox indicator. *Heliyon* **2023**, *9*, e15731. [CrossRef]
71. Bhatia, S.K.; Bhatia, R.K.; Choi, Y.-K.; Kan, E.; Kim, Y.-G.; Yang, Y.-H. Biotechnological potential of microbial consortia and future perspectives. *Crit. Rev. Biotechnol.* **2018**, *38*, 1209–1229. [CrossRef]
72. Salinas, J.; Carpena, V.; Martínez-Gallardo, M.R.; Segado, M.; Estrella-González, M.J.; Toribio, A.J.; Jurado, M.M.; López-González, J.A.; Suárez-Estrella, F.; López, M.J. Development of plastic-degrading microbial consortia by induced selection in microcosms. *Front. Microbiol.* **2023**, *14*, 1143769. [CrossRef] [PubMed]
73. Roberts, C.; Edwards, S.; Vague, M.; León-Zayas, R.; Scheffer, H.; Chan, G.; Swartz, N.A.; Mellies, J.L.; McMahon, K. Environmental Consortium Containing *Pseudomonas* and *Bacillus* Species Synergistically Degrades Polyethylene Terephthalate Plastic. *mSphere* **2020**, *5*, e01151–20. [CrossRef]
74. Awasthi, S.; Srivastava, P.; Singh, P.; Tiwary, D.; Mishra, P.K. Biodegradation of thermally treated high-density polyethylene (HDPE) by *Klebsiella pneumoniae* CH001. *3 Biotech* **2017**, *7*, 332. [CrossRef] [PubMed]
75. Khampratueng, P.; Rice, D.; Anal, A.K. Biodegradation of low-density polyethylene by the bacterial strains isolated from the dumping site community. *Discov. Appl. Sci.* **2024**, *6*, 348. [CrossRef]
76. Kyaw, B.M.; Champakalakshmi, R.; Sakharkar, M.K.; Lim, C.S.; Sakharkar, K.R. Biodegradation of Low Density Polythene (LDPE) by *Pseudomonas* Species. *Indian J. Microbiol.* **2012**, *52*, 411–419. [CrossRef] [PubMed]
77. Gupta, K.K.; Devi, D. Characteristics investigation on biofilm formation and biodegradation activities of *Pseudomonas aeruginosa* strain ISJ14 colonizing low density polyethylene (LDPE) surface. *Heliyon* **2020**, *6*, e04398. [CrossRef]
78. Almond, J.; Sugumaar, P.; Wenzel, M.N.; Hill, G.; Wallis, C. Determination of the carbonyl index of polyethylene and polypropylene using specified area under band methodology with ATR-FTIR spectroscopy. *e-Polymers* **2020**, *20*, 369–381. [CrossRef]

79. Waterhouse, A.M.; Procter, J.B.; Martin, D.M.A.; Clamp, M.; Barton, G.J. Jalview Version 2-a Multiple Sequence Alignment Editor and Analysis Workbench. *Bioinformatics* **2009**, *25*, 1189–1191. [CrossRef] [PubMed]
80. Kunzmann, P.; Mayer, B.E.; Hamacher, K. Substitution matrix based color schemes for sequence alignment visualization. *BMC Bioinform.* **2020**, *21*, 209. [CrossRef] [PubMed]

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Article

# Characterization of $\alpha$ -L-Rhamnosidase and $\beta$ -D-Glucosidase Subunits of Naringinase Immobilized on a Magnetic Polysaccharide Carrier

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## Abstract

Naringinase consists of two enzymes:  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase. The enzyme was immobilized on a carrier prepared from carob gum activated with polyethyleneimine. Cross-linking with dextran aldehyde was used to improve the stability of the immobilization. Knowledge of the characteristics of naringinase subunits is important for developing efficient and selective enzymatic reactions involving flavonoids. This study aimed to characterize two subunits of naringinase— $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase—free, immobilized on a magnetic polysaccharide carrier and cross-linked with dextran aldehyde. The characterization of free, immobilized, and stabilized naringinase, as well as  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, included the effect of pH and temperature on enzyme activity, as well as the determination of their stability depending on the pH and temperature of the environment, and the determination of kinetic constants. Immobilization and subsequent stabilization of naringinase did not affect the optimal pH for the activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase. Immobilization caused a change in the optimal temperature for the activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase from 60 to 65°. Cross-linking of immobilized naringinase with dextran aldehyde increased the temperature stability of its subunits. Cross-linking also altered the pH stability profile of  $\beta$ -D-glucosidase. Immobilization and stabilization of naringinase slightly reduced the maximum reaction rate for  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase compared to the free enzyme. As a result of immobilization, the enzymes' affinity for substrates for both subunits decreased.

**Keywords:**  $\alpha$ -L-rhamnosidase;  $\beta$ -D-glucosidase; naringinase; immobilization; magnetic carrier

## 1. Introduction

Naringinase is an enzyme with dual activity:  $\alpha$ -L-rhamnosidase (EC 3.2.1.40) and  $\beta$ -D-glucosidase (EC 3.2.1.21) [1,2]. Some authors define naringinase as an enzymatic complex with  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase activity [3,4]. In contrast, others consider it a mixture of two separate enzymes,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase [5,6]. The exact arrangement and number of subunits may vary depending on the microbial source. Chen et al. [7] demonstrated that naringinase from *Aspergillus aculeatus* was a tetramer consisting of one  $\alpha$ -L-rhamnosidase subunit and three  $\beta$ -D-glucosidase subunits. Naringinase from *A. niger* is also a multimeric enzyme; it has two subunits [8]. Two  $\beta$ -D-glucosidases and one  $\alpha$ -L-rhamnosidases were purified from the naringinase from *A. terreus* CECT 2663 [9]. So far, only the crystal structure of  $\alpha$ -L-rhamnosidase has been

elucidated. Site-directed mutagenesis studied key residues involved in enzymatic catalysis and rhamnose recognition. Acidic residues such as Asp567, Glu572, Asp579, and Glu841 were identified as crucial for hydrolysis, and their mutation was shown to reduce enzymatic activity significantly [3]. The detailed structure of naringinase remains unclear and requires further investigation. Puri [1] suggested that crystallization of naringinase from different sources will be necessary to determine their precise structures.

This enzyme plays a key role in citrus processing by hydrolyzing naringin to remove bitterness in citrus juice [1,2,10]. It is also applied to enhance the flavor of wines, fruit juices, and various beverages [1,2,10]. Additionally, it can increase flavonoid bioavailability and antioxidant capacity, serve in the production of sweeteners, and improve the quality of soy-based products [10].

Naringinase is widely used in the biotransformation of flavonoids, compounds of significant biological and therapeutic importance. Many flavonoids occur naturally in the form of glycosides, which significantly affects their biological properties, including bioavailability and solubility [5]. The presence of saccharide groups can lead to changes in the bioavailability of the corresponding flavonoid aglycone, depending on the type of sugar, e.g., glucosides are absorbed faster than other types of glycosides, such as rhamnosides and rhamnoglucosides. Due to the difficult absorption of flavonoids, solutions aimed at increasing their bioavailability are known, e.g., through deglycosylation [5]. Thanks to the ability of naringinase to deglycosylate compounds containing  $\alpha$ -rhamnose or  $\beta$ -glucose at the ends of the molecule, many natural glycosides such as naringin, rutin, hesperidin, and diosmin can be its substrates [11,12]. For example, two enzymes,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, are necessary for the deglycosylation of naringin, the dominant grapefruit juice flavonoid. The first,  $\alpha$ -L-rhamnosidase, hydrolyses naringin to rhamnose and prunin. Then,  $\beta$ -D-glucosidase hydrolyses prunin to glucose and naringenin [13,14]. Hesperidin, present in orange juice, can be deglycosylated by  $\alpha$ -rhamnosidase to rhamnose and 7-O-glucoside hesperetin (Hes-7-G), which is then hydrolyzed by  $\beta$ -D-glucosidase to glucose and hesperetin [15–17].  $\alpha$ -L-rhamnosidase from naringinase hydrolyses rutin (3-O-rutinoside of quercetin) to rhamnose and 3-O-glucoside of quercetin (quercetin-3-O-glucoside Q-3-G or isoquercitrin).  $\beta$ -D-glucosidase, in turn, hydrolyses isoquercitrin to glucose and quercetin [11,18,19]. However, these flavonoids' hydrolysis effectiveness depends on the enzymes' stability and activity in the target environment, which is most often acidic fruit (pH 3–4).

Knowledge of the properties of individual naringinase subunits, their optimal operating conditions, and the influence of environmental conditions on their activity is crucial for targeted flavonoid hydrolysis. This knowledge enables control of the enzymatic reaction, e.g., through selective inactivation of one of the subunits to stop hydrolysis at the intermediate glycoside stage. Determination of the optimal pH and temperature conditions for the activity of enzyme subunits is essential for increasing the efficiency of deglycosylation processes involving naringinase. This approach allows for precise control of the flavonol hydrolysis process.

In the context of the practical application of naringinase, particularly in the food industry, its use in industrial conditions is also an important aspect. Using the free form of the enzyme poses several limitations, such as the inability to reuse it or the difficulty of separating the enzyme from the final product. In response to these challenges, enzyme immobilization techniques are becoming increasingly important, as they allow for reuse, improved stability, and simplification of the technological process [20,21]. Natural carriers such as proteins, porous glass, silica gel, and polysaccharides are used for enzyme immobilization. Polysaccharide-based materials, in particular, are a suitable matrix for enzyme immobilization due to many chemically active groups, a large specific surface area, and

significant sorption capacity [22]. The use of such natural carriers for the immobilization of naringinase, such as carrageenan [23], chitosan [24], alginate [25–27] and corn cob powder [28], has been described in the literature. In order to increase the efficiency of enzyme binding to the matrix, carriers are often modified, e.g., by using aldehyde groups reacting with ion polymers containing amino groups, such as polyethyleneimine, or by activating the polymer skeleton containing amino groups with glutaraldehyde. Dextran aldehyde can be an additional cross-linking agent [21,29]. Recently, researchers have been particularly interested in the immobilization of enzymes on carriers with magnetic properties [30–35]. Thanks to a magnetic field, the most significant advantage of their use is the quick and easy separation of the enzyme from the reaction medium. To date, there have been few studies on the immobilization of naringinase on magnetic carriers [21,36–38]. The immobilization of naringinase often leads to increased stability under changing pH and temperature conditions and in solvent environments. Immobilization of naringinase can significantly affect its stability at different pH of the environment, often broadening and sometimes shifting the optimal range of enzyme activity [21,39,40]. According to some authors, immobilization of naringinase often contributes to an increase in the optimal temperature of activity of this enzyme [27,41,42]. The higher optimal temperature of naringin hydrolysis by naringinase increases the possibility of using the stabilized enzyme on an industrial scale [43]. There are a few reports in the literature on the effect of naringinase immobilization on the stability and optimal conditions for the activity of its subunits. Characterizing  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, which are components of naringinase, is an important step towards designing effective and selective enzymatic processes involving flavonoids. Combining knowledge about the enzymatic characteristics of naringinase subunits with modern immobilization methods provides a solid basis for developing efficient, economical, and sustainable biotechnological processes.

Naringinase from *Aspergillus niger* KMS was adsorbed onto a carrier obtained from carob gum activated with polyethyleneimine. In order to strengthen the bond between the enzyme and the carrier, naringinase was additionally stabilized by cross-linking with dextran aldehyde [21]. This study aimed to investigate the effect of immobilization of naringinase on the optimal parameters of its subunits' activity. Two subunits of naringinase were characterized,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, occurring in various forms: free, immobilized on a magnetic polysaccharide carrier, and additionally cross-linked with dextran aldehyde. The characterization includes determining the influence of pH and temperature on enzyme activity, their stability depending on the pH and temperature of the environment, and the determination of reaction kinetic constants. The influence of environmental conditions on the enzymatic activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase is essential for their practical application. This knowledge will enable targeted deglycosylation of flavonoids, which will contribute to increasing their bioavailability.

## 2. Results and Discussion

### 2.1. Immobilization Yield

The immobilization efficiency of naringinase subunits on the magnetic polysaccharide carrier was calculated based on the total activity of this enzyme used for immobilization and the activity of the immobilized enzyme. The immobilization yield of  $\alpha$ -L-rhamnosidase on the magnetic polysaccharide carrier activated with PEI was 18.09% relative to the total activity of this enzyme used for immobilization. In the case of  $\beta$ -D-glucosidase, the immobilization yield on the same carrier was 31.27%. The results indicate that  $\alpha$ -L-rhamnosidase was immobilized with a lower yield than  $\beta$ -D-glucosidase.

This difference may result from conformational features of the two subunits, which affect their interaction with the functional groups of the PEI-activated polysaccharide

carrier. Cross-linking of the immobilized enzymes likely caused further conformational changes, stabilizing the structure of the biocatalysts and contributing to increased activity. More significant changes occurred in the case of  $\beta$ -D-glucosidase.

Kimmis et al. [38], who immobilized naringinase from *A. aculeatus* and *A. niger* on polydopamine (PDA)-coated magnetic nanoparticles, obtained a similar immobilization efficiency for  $\alpha$ -L-rhamnosidase of  $19.3 \pm 0.8\%$ , while for  $\beta$ -D-glucosidase it was only  $2.2 \pm 0.1\%$  of the total activity. Chemical amination of the enzyme significantly improved the immobilization efficiency, increasing the efficiency by more than 40% for  $\alpha$ -L-rhamnosidase and more than 10-fold for  $\beta$ -D-glucosidase [38]. Wang et al. [44] using an artificial naringinase system (constructed by co-immobilization of  $\alpha$ -L-rhamnosidase (*A. oryzae* FJ0123) and  $\beta$ -glucosidase (*Thermotoga maritima* MSB8) on magnetic silica-based chitosan microspheres) obtained a significantly higher immobilization efficiency, 61.4% for  $\alpha$ -L-rhamnosidase and 90.1% for  $\beta$ -glucosidase, respectively.

## 2.2. Activity of $\alpha$ -L-rhamnosidase and $\beta$ -D-glucosidase

Table 1 shows the enzymatic activity of two subunits of naringinase  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase in three different forms: free, immobilized by adsorption, and immobilized and additionally cross-linked.

**Table 1.** Activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase as free, immobilized, and cross-linked subunits of naringinase.

| Enzyme                   | Free                                                      | Adsorbed                                                             | Adsorbed and Crosslinked                                             |
|--------------------------|-----------------------------------------------------------|----------------------------------------------------------------------|----------------------------------------------------------------------|
|                          | $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ | $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ of Carrier | $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ of Carrier |
| $\alpha$ -L-rhamnosidase | $887.17 \pm 33$                                           | $4.82 \pm 0.12$                                                      | $5.09 \pm 0.13$                                                      |
| $\beta$ -D-glucosidase   | $273.75 \pm 13$                                           | $2.57 \pm 0.06$                                                      | $3.02 \pm 0.08$                                                      |

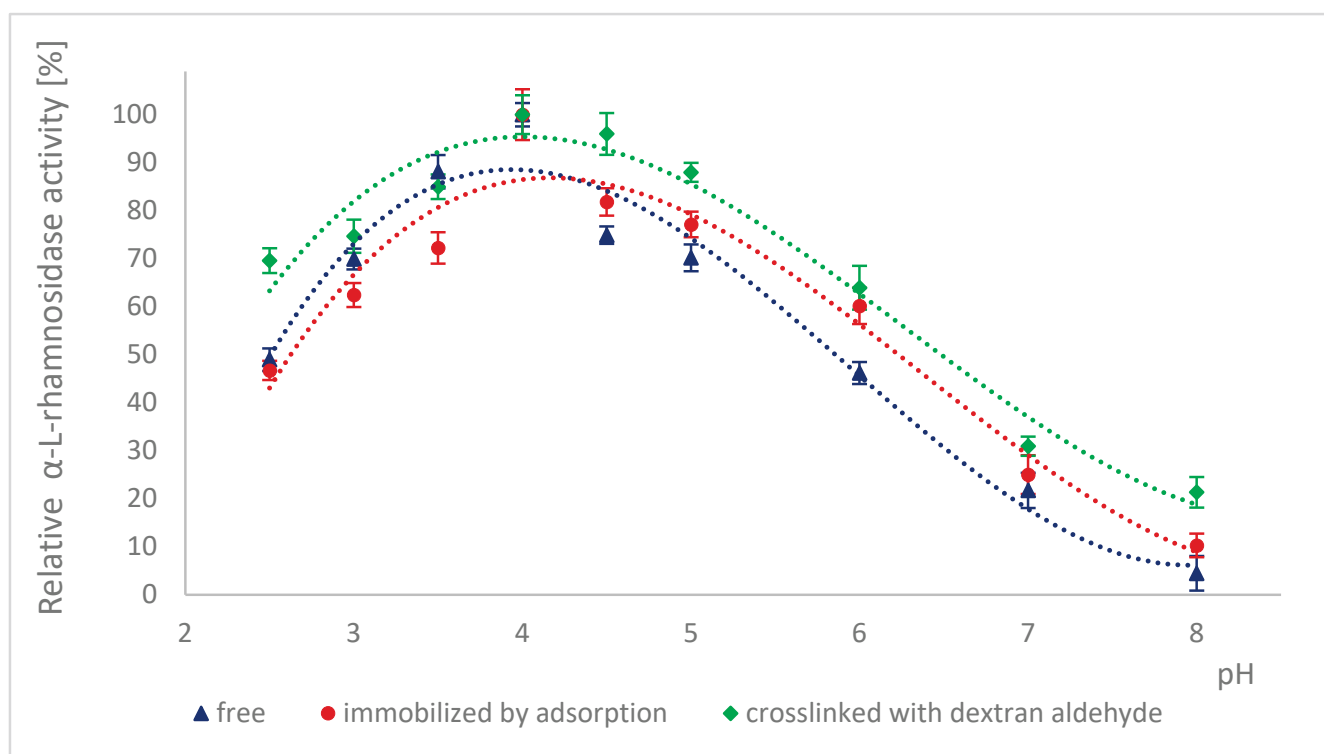
The cross-linking with dextran aldehyde did not cause activity loss, as the measured values for both subunits remained nearly identical to those obtained after simple adsorption. Additional cross-linking primarily stabilizes the immobilized enzyme preparation without reducing its catalytic potential. The activity of  $\alpha$ -L-rhamnosidase concerning  $\beta$ -D-glucosidase in the free naringinase complex was more than three times higher.

The proportional relationship between the activities of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase was not preserved after immobilization, indicating that the functional balance of the naringinase complex was altered. The activity of  $\alpha$ -L-rhamnosidase decreased relative to  $\beta$ -D-glucosidase. Immobilization may have induced greater conformational changes in the active site region of  $\alpha$ -L-rhamnosidase, resulting in a reduced subunit activity compared to  $\beta$ -D-glucosidase. This observation is also supported by the differences in the immobilization yields of the two subunits.

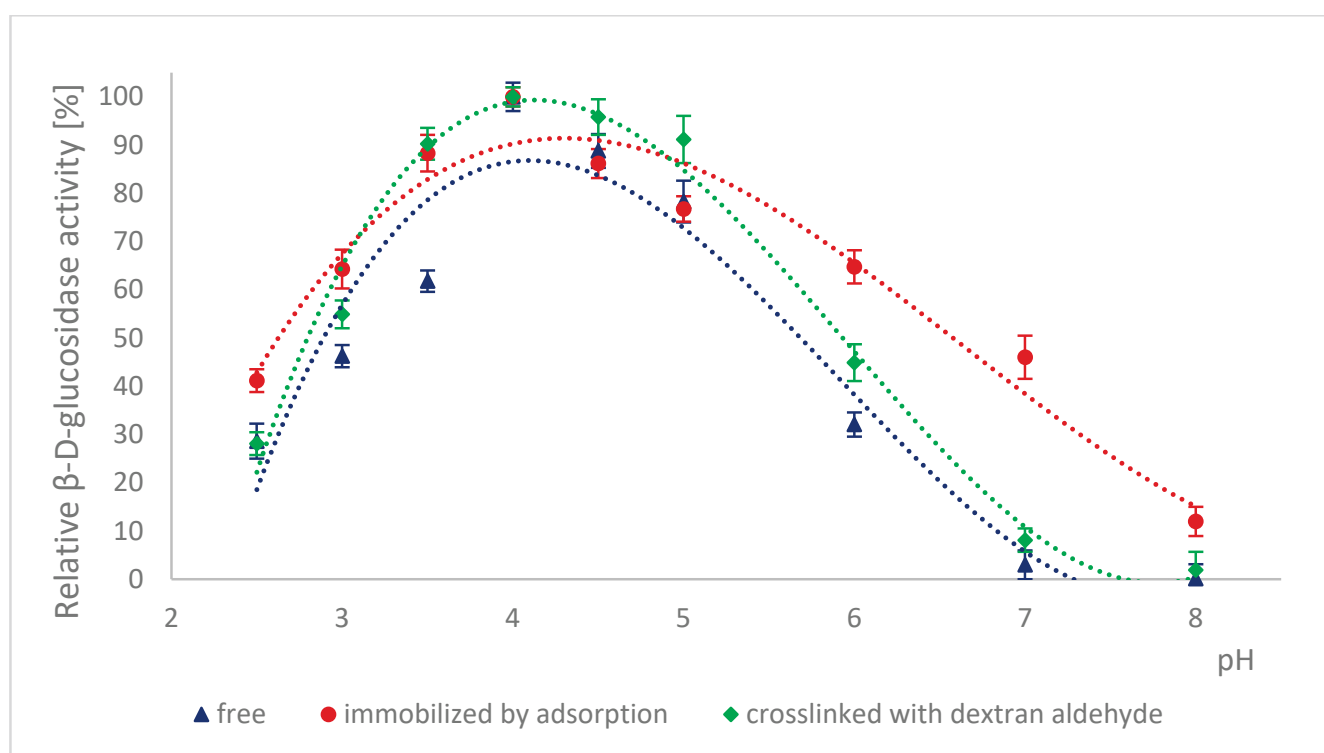
The higher activity of  $\alpha$ -L-rhamnosidase compared to  $\beta$ -D-glucosidase may be important in the selective production of compounds such as prunin, Hes-7-G or isoquercetrin [28].

## 2.3. Effect of pH on the Activity of Naringinase Subunits

Because many enzymes exhibit high activity only within a narrow pH range, it is important to describe the effect of this factor on the enzymatic activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase from free, immobilized, and stabilized naringinase. The effect of pH on the activity of free, immobilized, and stabilized naringinase subunits,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, is presented in Figures 1 and 2. Changes in enzyme activity were studied in the pH range 2.5–8.0 at a temperature of 50 °C.



**Figure 1.** Effect of pH on the activity of  $\alpha$ -L-rhamnosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS.



**Figure 2.** Effect of pH on the activity of  $\beta$ -D-glucosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS.

100% activity for free, immobilized and stabilized  $\alpha$ -L-rhamnosidase was defined as the activity at pH 4.0 which was  $887.17 \pm 33 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ,  $4.82 \pm 0.12 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  and  $5.09 \pm 0.13 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ , respectively. 100% activity for free, immobilized

and stabilized  $\beta$ -D-glucosidase was defined as the activity at pH 4.0 which was  $273.75 \pm 13 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ,  $2.57 \pm 0.06 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  and  $3.02 \pm 0.08 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ , respectively.

The activity of all tested forms of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase was highest at pH 4.0, which corresponded to the optimal pH for the activity of the free and immobilized on magnetic polysaccharide carrier naringinase from *A. niger* KMS complex [21]. Immobilization followed by stabilization of naringinase did not affect the optimal pH of its subunits,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase.

Almost across the entire pH range analyzed, i.e., from 2.5 to 8.0,  $\alpha$ -L-rhamnosidase from stabilized naringinase exhibits higher activity than the free and immobilized form of the biocatalyst under investigation. The activity of  $\beta$ -D-glucosidase from naringinase stabilized with dextran aldehyde was highest in the pH range from 3.5 to 5. Above pH 5, the highest activity was exhibited by  $\beta$ -D-glucosidase immobilized by adsorption. The high activity of the crosslinked  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase at low pH indicates the possibility of using such an immobilized enzyme in acidic medium as citrus juice.

The highest activity of both subunits at pH 4.0 is similar to the optimal pH of enzymes derived from *Aspergillus* moulds and characterized by other authors:  $\alpha$ -L-rhamnosidase [7,45–47] and  $\beta$ -D-glucosidase [7,46,48,49].  $\alpha$ -L-rhamnosidase from *A. terreus* [47], *A. kawachii* [45], *A. aculeatus* JMUdb058 [7], and *A. niger* BCC [46] showed the highest activity at pH 4.0.  $\beta$ -D-glucosidase from *A. aculeatus* JMUdb058 [7], *A. niger* BCC [46], and *Aspergillus aculeatus/Aspergillus niger* [49] showed the highest activity also at pH 4.0. Naringinase and its subunits from *A. aculeatus/A. niger* (NS 33117) also showed the highest activity at pH 4.0 [20,38].

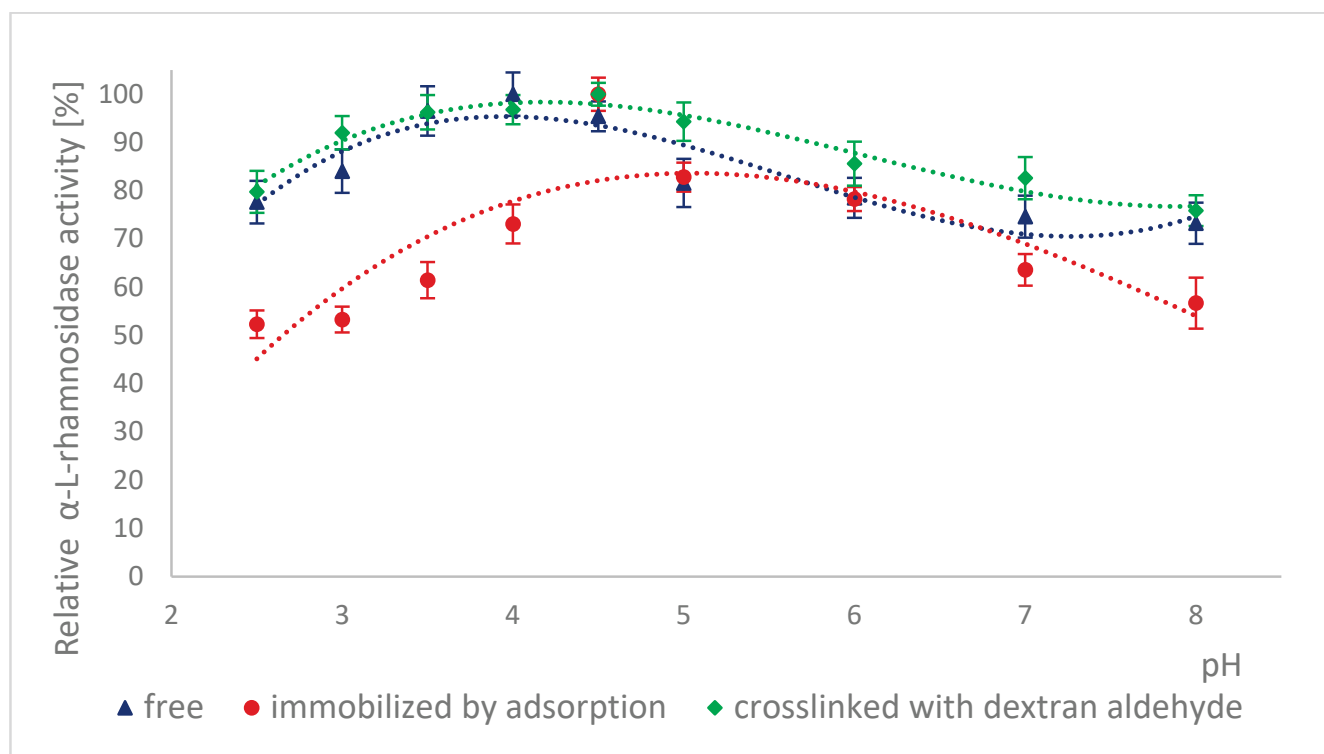
Slightly different results were obtained by other authors for  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase from *A. oryzae* 11250, whose activity dependence on the reaction pH was similar. However, the optimum was reached at pH 5.0 [50]. Roitner et al. [51] showed that the activity of  $\alpha$ -L-rhamnosidase from naringinase derived from *A. niger* was slightly dependent on pH in the range from 3 to 7, and  $\beta$ -D-glucosidase had a clear activity maximum at pH 5.

There is little information in the literature on the effect of immobilization on the activity of naringinase subunits depending on the pH of the reaction medium. Covalent binding of naringinase from *A. aculeatus/A. niger* (NS 33117) to butyl-glyoxyl agarose resulted in high activity of both subunits at pH 3–6 [49]. In contrast, in the case of immobilization on octyl-glyoxyl agarose, only  $\alpha$ -L-rhamnosidase showed high activity [49]. The immobilization on glyoxyl-agarose of the aminated naringinase complex from *A. aculeatus/A. niger* (NS 33117) modified the pH profile of the enzyme and its subunits without changing the optimal pH (4.0). Only slight differences in the pH profile were observed after naringinase amination, particularly at pH above 4.0 [38].

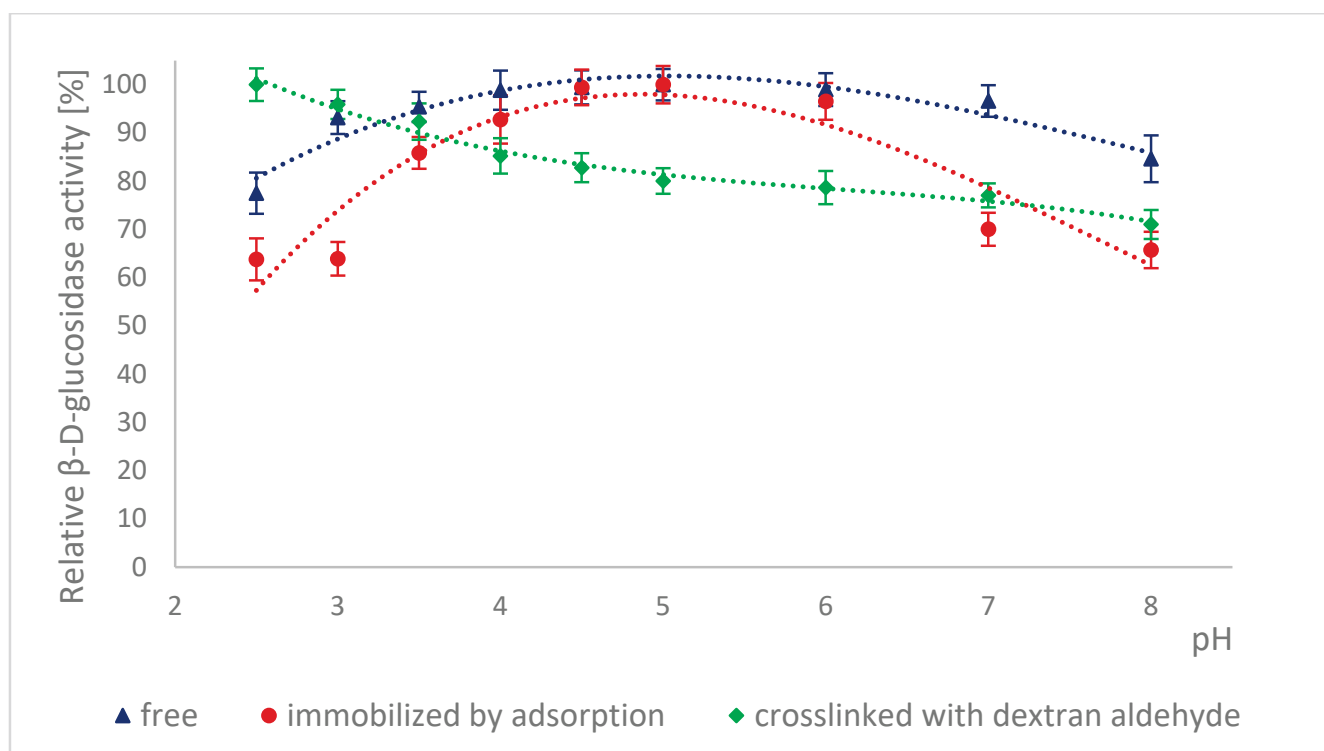
#### 2.4. Effect of Incubation in Buffers with Different pH on the Activity of Naringinase Subunits

The high stability of naringinase subunits at different pH is advantageous for biotechnological processes in the food industry. The stability of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase as subunits of free, immobilized, and stabilized naringinase was tested at different pH ranging from 2.5 to 8.0 (Figures 3 and 4).

The free and cross-linked  $\alpha$ -L-rhamnosidase pH stability profiles were similar (Figure 3). 100% activity for free  $\alpha$ -L-rhamnosidase was defined as the activity at pH 4, which was  $887.17 \pm 33 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ . 100% activity for immobilized and stabilized  $\alpha$ -L-rhamnosidase was defined as the activity at pH 4.5, which was, respectively,  $6.59 \pm 0.16 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  and  $5.26 \pm 0.06 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ .



**Figure 3.** Stability of  $\alpha$ -L-rhamnosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS after 15 h of incubation in buffers with different pH.



**Figure 4.** Stability of  $\beta$ -D-glucosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS after 15 h of incubation in buffers with different pH.

Both forms reached maximum stability at pH 4.5. The immobilized enzyme also showed the highest stability at pH 4.5, but its stability profile differed. After incubation

at pH levels lower and higher than optimal, the activity of this form of  $\alpha$ -L-rhamnosidase decreased by approximately 20% at pH 5 and by approximately 27% at pH 4.

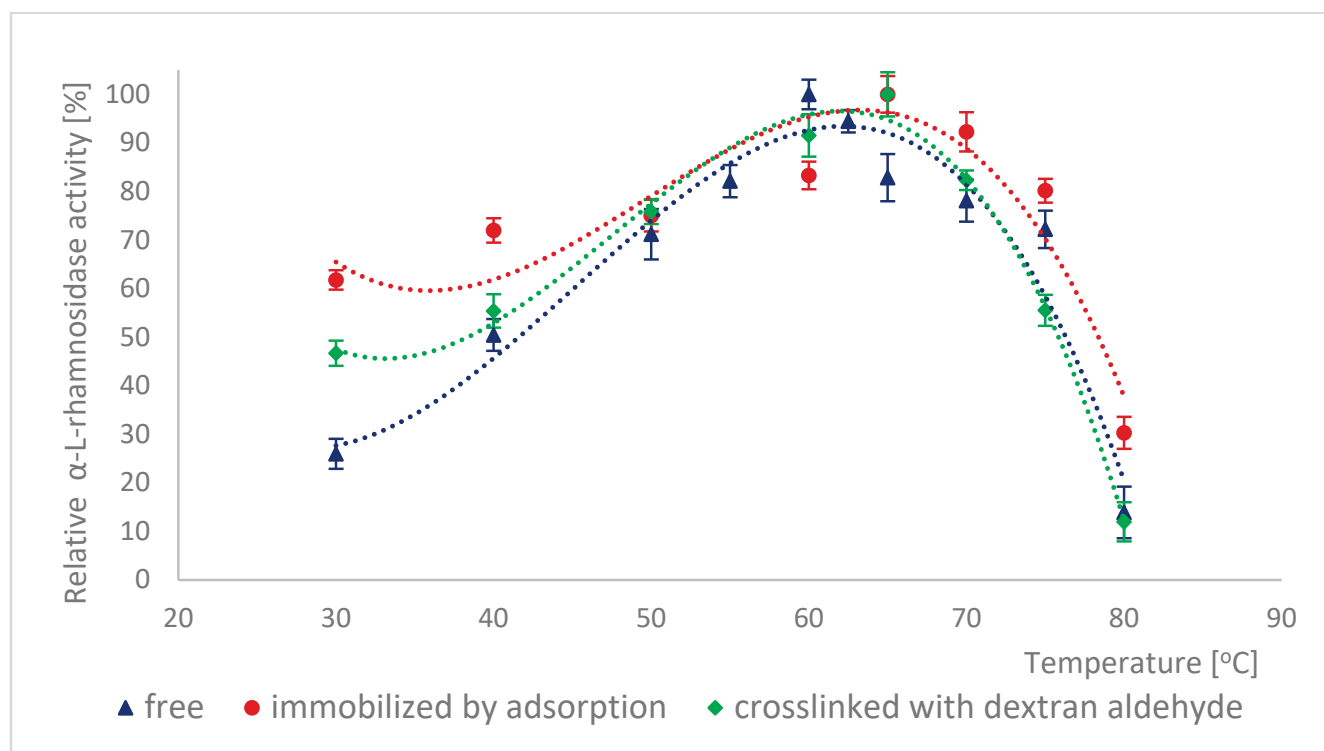
The pH stability profiles are presented in Figure 4. 100% activity for free  $\beta$ -D-glucosidase was defined as the activity at pH 5, which was  $276.84 \pm 13.15 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ . 100% activity for immobilized  $\beta$ -D-glucosidase was defined as the activity at pH 5, which was  $2.77 \pm 0.065 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ . 100% activity for stabilized  $\beta$ -D-glucosidase was defined as the activity at pH 2.5, which was  $3.54 \pm 0.094 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ . For free and immobilized  $\beta$ -D-glucosidase, the pH stability profiles were similar, although at pH below 4.0 and above 6.0, the immobilized form lost activity more rapidly (Figure 4). It could be because immobilization changed the enzyme's conformation, particularly in the active site, making it more sensitive to hydrogen and hydroxide ions. This conformational change was likely further enhanced by additional cross-links of the immobilized  $\beta$ -D-glucosidase, which led to a decrease in the activity of this form with increasing pH of the enzyme incubation environment.

Similar pH stability results were obtained by Chen et al. [7]. In this case,  $\alpha$ -L-rhamnosidase from *A. aculeatus* JMUdb058 was stable at pH 3.0 to 8.0. At the same time,  $\beta$ -D-glucosidase was stable at pH 3.0 to 6.0. Slightly different results were obtained by Gallego et al. [47], and  $\alpha$ -L-rhamnosidase from *A. terreus* was most stable at pH 4.0–6.0. However, Manzanares et al. [52] showed that  $\alpha$ -L-rhamnosidase from *A. aculeatus* was stable at pH 3.0–5.0. The differences in the pH stability profile are probably because the authors used enzymes obtained from other *Aspergillus* species.

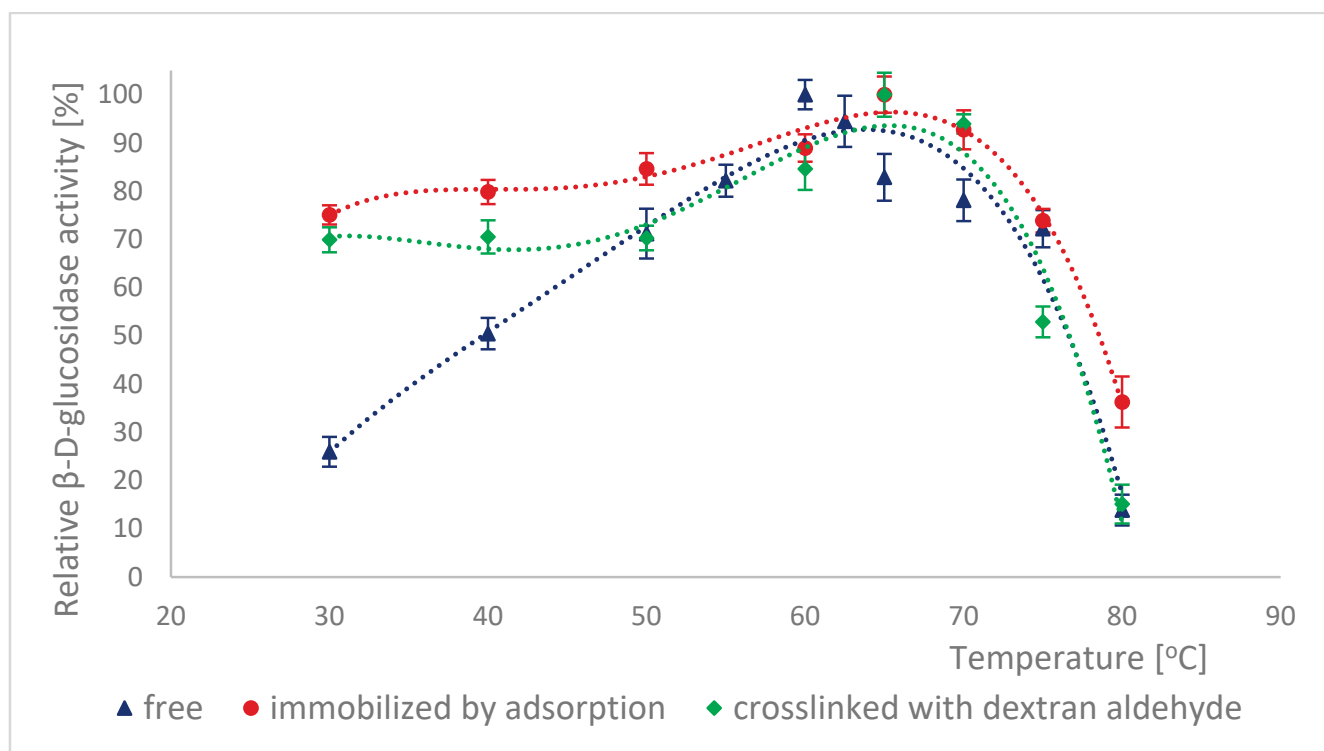
Low pH stability means that naringinase subunits, which were immobilized and cross-linked with dextran aldehyde, can be used to process acidic raw materials such as wines and citrus juices.

## 2.5. Effect of Temperature on the Activity of Naringinase Subunits

Thermal inactivation of enzymes is often a significant problem in biotechnological processes. The effect of temperature on the activity of different forms of  $\alpha$ -L-rhamnosidase is shown in Figure 5. 100% activity for free  $\alpha$ -L-rhamnosidase was defined as the activity at 60 °C, which was  $124.61 \pm 6.35 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ . 100% activity for immobilized and stabilized  $\alpha$ -L-rhamnosidase was defined as the activity at 65 °C, which was, respectively,  $6.41 \pm 0.16 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  and  $6.71 \pm 0.08 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ . For  $\beta$ -D-glucosidase, such relationships are shown in Figure 6. 100% activity for free  $\beta$ -D-glucosidase was defined as the activity at 60 °C, which was  $608.88 \pm 28.91 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ . 100% activity for immobilized and stabilized  $\alpha$ -L-rhamnosidase was defined as the activity at 65 °C, which was  $3.04 \pm 0.07 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  and  $4.30 \pm 0.11 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  respectively. In many cases, immobilization reduces the temperature sensitivity of biocatalysts. The method of immobilization and cross-linking of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase with dextran aldehyde increased the temperature at which these enzymes achieve their highest activity by 5 °C compared to their free forms (Figures 5 and 6). Furthermore, the activity of both immobilized enzyme forms also increased in the temperature range of 30–45 °C. Changes in their conformation likely caused the modification of the activity profiles of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase due to immobilization and cross-linking. This change in the optimal temperature of the enzymes increases their potential for use in biotechnological processes. The differences in activity at optimal temperatures of free and immobilized subunits were statistically significant.



**Figure 5.** Effect of temperature on the activity of  $\alpha$ -L-rhamnosidase as a subunit of free, immobilized, and stabilized naringinase from *A. niger* KMS.



**Figure 6.** Effect of temperature on the activity of  $\beta$ -D-glucosidase as a subunit of free, immobilized, and stabilized naringinase from *A. niger* KMS.

A similar optimal temperature (60 °C) in terms of activity was shown by  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase from *A. aculeatus* / *A. niger* (NS 3311) [38]. The optimal temperature remained unchanged after immobilizing the enzymes on polydopamine-

coated magnetic iron oxide nanoparticles. In turn, the free naringinase complex from *A. niger* BCC 25166 and its subunits also showed maximum activity at 60 °C [46].

For  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase from other *Aspergillus* species, researchers determined different temperatures at which these enzymes achieved their highest activity. Naringinase subunits from *A. oryzae* 11250 were most active at 45 °C [50]. In turn,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase from *A. aculeatus* JMUdb058 showed maximum activity at 50 °C [7] and from *A. terreus* also at 50 °C [53]. The optimal temperature for naringinase subunits from *A. aculeatus*/*A. niger* (NS 33117) varied depending on the substrate used, being 45 °C for p-nitrophenyl- $\beta$ -D-glucoside (pNPG) and 60 °C for p-nitrophenyl- $\alpha$ -L-rhamnopyranoside (pNPR) [20].

Other authors also observed changes in the temperature at which naringinase subunits achieved maximum activity following immobilization. Muñoz et al. [49] found that immobilization on heterofunctional supports with glyoxyl groups increased the optimal temperature for  $\alpha$ -L-rhamnosidase from *A. aculeatus*/*A. niger* (NS 33117) activity, whereas no changes were observed for  $\beta$ -D-glucosidase. A similar effect was observed by Urrutia et al. [20] for aminated naringinase from *A. aculeatus*/*A. niger* (NS 33117) bound to a glyoxyl-agarose support, where the optimal temperature for  $\alpha$ -L-rhamnosidase shifted from 40 to 60 °C.

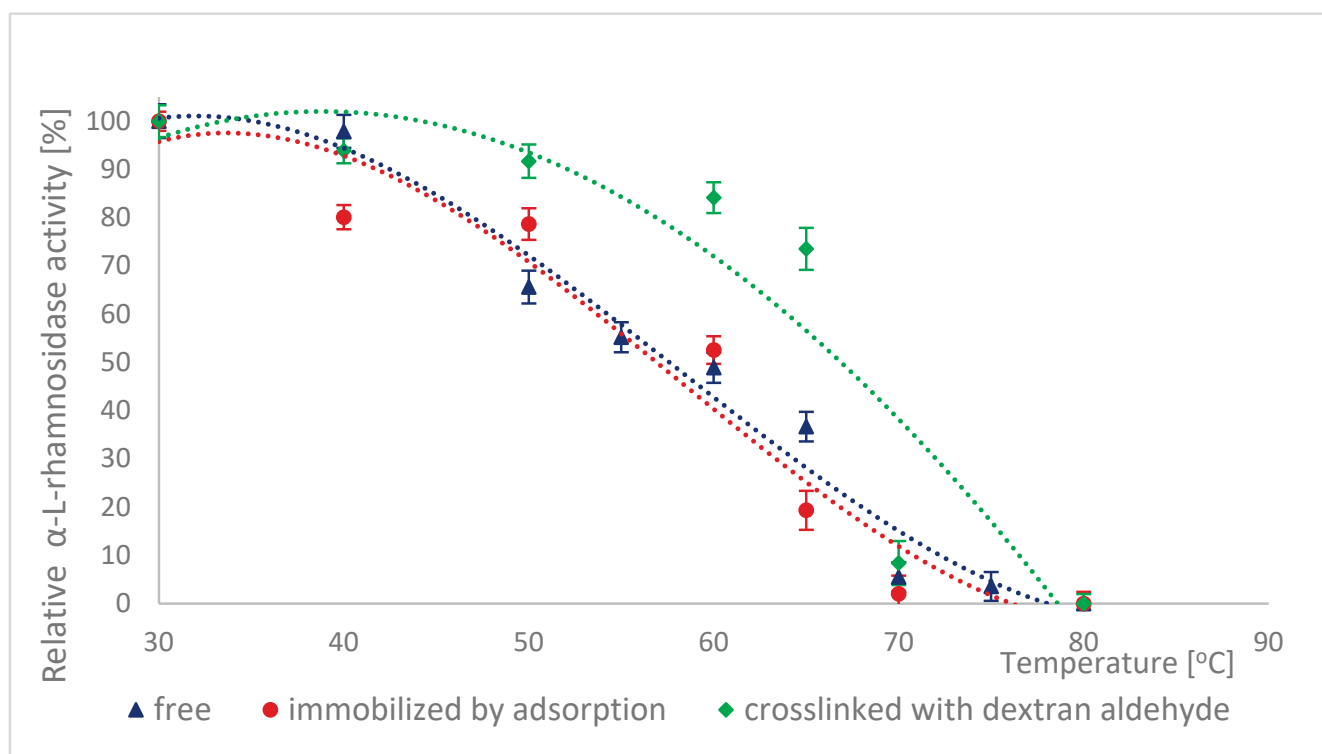
## 2.6. Thermal Stability of Naringinase Subunits

As a protein, naringinase is sensitive to temperature; hence, improving its thermal stability by immobilizing the enzyme is desirable. The thermal stability of naringinase subunits, i.e.,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, was determined by incubating naringinase in acetate buffer (pH 4.0) at temperatures ranging from 30 to 80 °C, followed by the determination of the activity of both enzymes. Figures 7 and 8 show the changes in the activity of both enzymes as a function of their incubation temperature. 100% activity for free, immobilized and stabilized  $\alpha$ -L-rhamnosidase was defined as the activity at 30 °C, which was  $130.77 \pm 36 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ,  $6.24 \pm 0.16 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  and  $5.90 \pm 0.07 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ , respectively. For  $\beta$ -D-glucosidase, 100% activity for free, immobilized, and stabilized  $\beta$ -D-glucosidase was defined as the activity at 30 °C, which was  $417.03 \pm 19.80 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ,  $3.27 \pm 0.076 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  and  $3.29 \pm 0.087 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ , respectively.

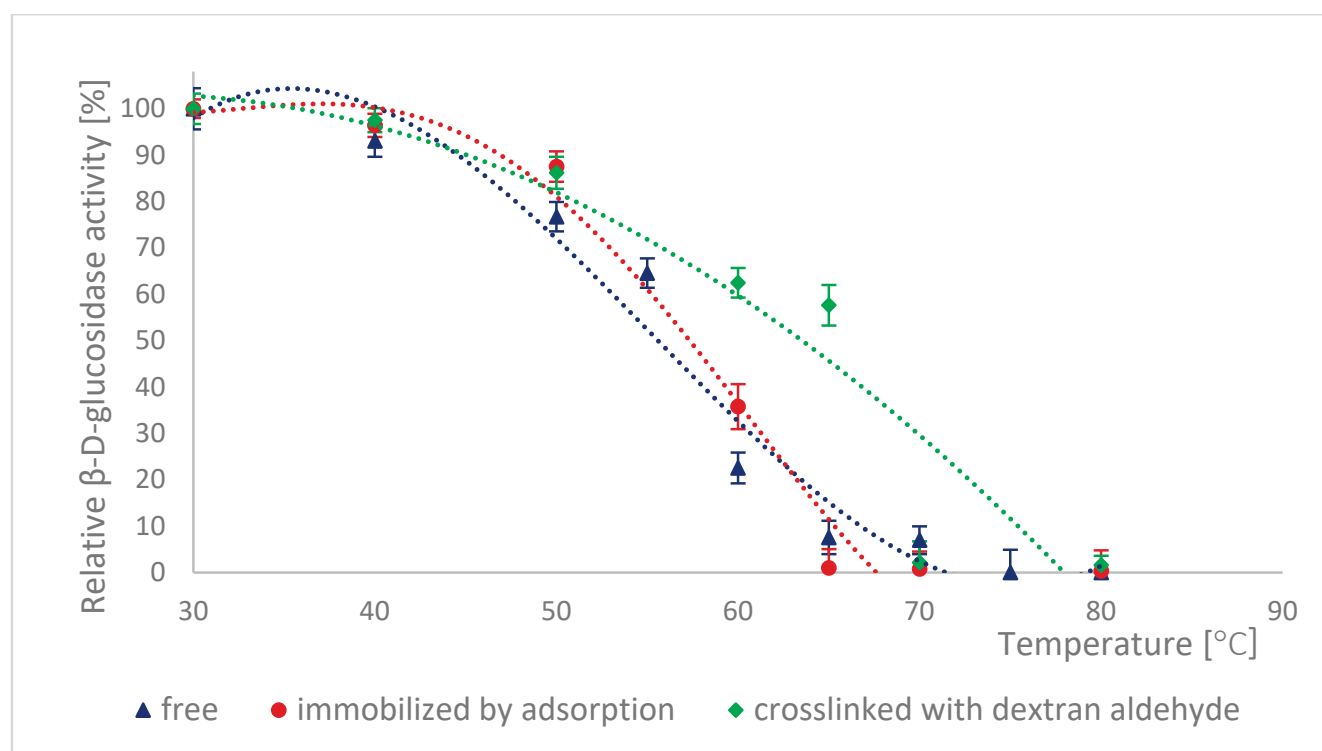
$\alpha$ -L-rhamnosidase was the most stable among the tested enzyme forms, a subunit of immobilized and dextran aldehyde-stabilized naringinase. It is worth noting that the rate of decrease in its activity increased only after exceeding a temperature of 60 °C.  $\alpha$ -L-rhamnosidase from naringinase bound with dextran aldehyde was therefore thermally stable at temperatures between 30 and 60 °C.

In the 20–50 °C temperature range, no significant changes in thermal stability were observed between the different forms of this enzyme. Dextran aldehyde cross-linking of immobilized  $\beta$ -D-glucosidase increased thermal stability above an incubation temperature of 50 °C compared to the other forms of this enzyme. Cross-linking of immobilized  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase increased the thermal stability of both subunits, likely due to stabilization of the altered spatial structure of the enzymes.

The differences in the stability of free and immobilized  $\alpha$ -L-rhamnosidase base at 30 °C were not statistically significant. For  $\beta$ -D-glucosidase, the differences were not statistically significant at 30–40 °C. In the case of free and immobilized  $\alpha$ -L-rhamnosidase, the differences in thermal stability at temperatures above 70 °C were not statistically significant. For  $\beta$ -D-glucosidase, they were not statistically significant above 65 °C.



**Figure 7.** Thermal stability of  $\alpha$ -L-rhamnosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS.



**Figure 8.** Thermal stability of  $\beta$ -D-glucosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS.

The high activity of both subunits, in free and immobilized form, at low temperatures makes the process particularly suitable for the hydrolysis of flavonoids in juices or wine,

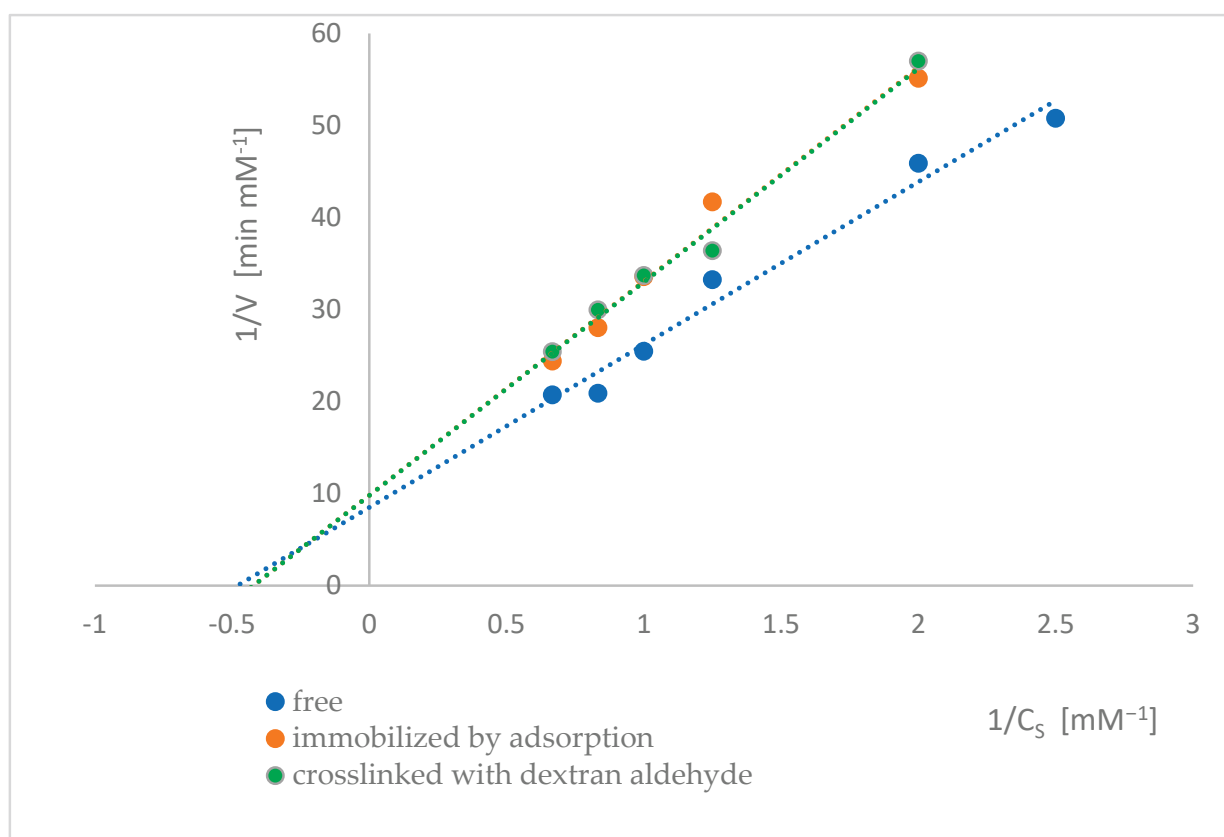
as it helps preserve thermolabile components (such as vitamin C and  $\beta$ -carotene) and maintain the desirable sensory qualities of the final product.

The stability of both naringinase subunits determines the thermal stability of the entire enzyme complex. The naringinase complex from *A. aculeatus* JMUdb058 and its subunits,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, also exhibited similar temperature stability ranges below 50 °C [7]. Kimmins et al. [38] also observed an increase in the stability of the naringinase complex from *A. aculeatus*/*A. niger* (NS 33117) on polydopamine-coated magnetic iron oxide nanoparticles at 50 °C. After 120 min of incubation, the immobilized  $\alpha$ -L-rhamnosidase retained more than 50% of its initial activity, whereas the free form showed only about 30%. In the case of  $\beta$ -D-glucosidase, immobilization allowed maintaining about 20% of the initial activity, while the free enzyme completely lost its activity.

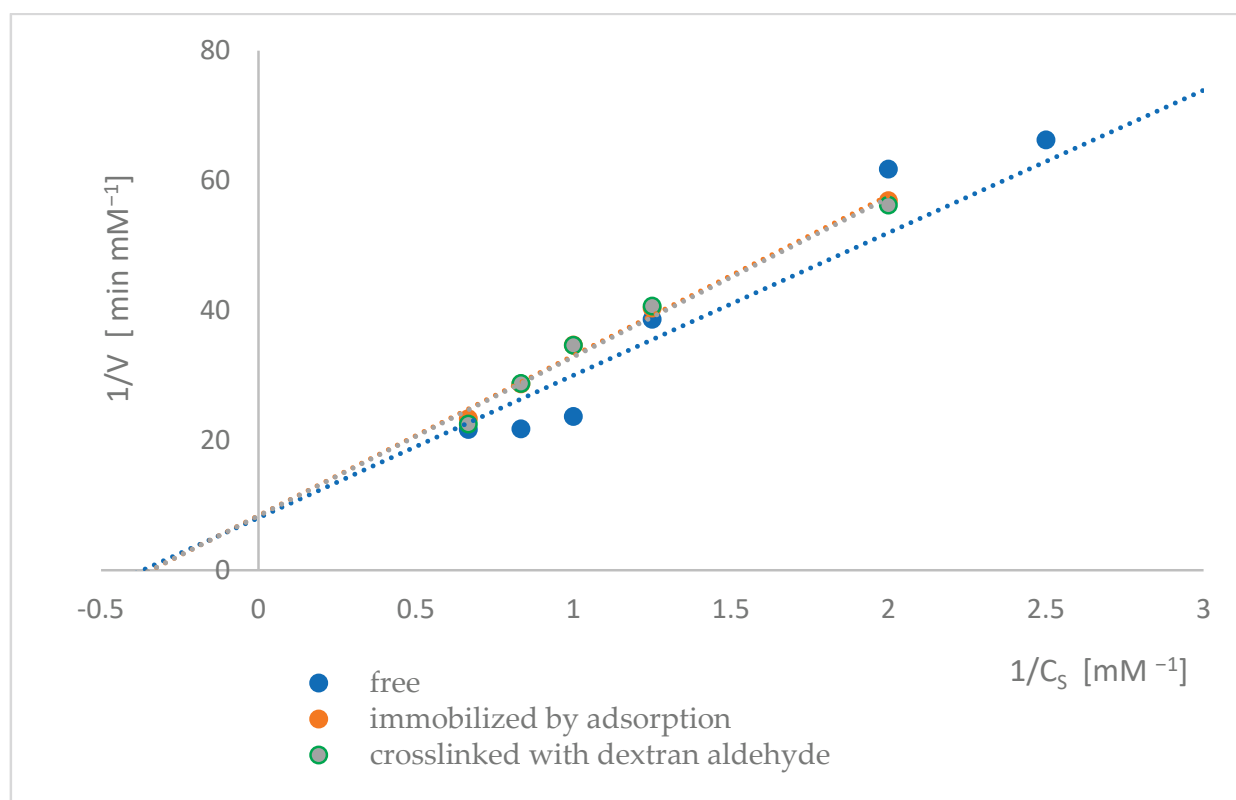
## 2.7. Determination of the Kinetic Parameters of Naringinase Subunits

Immobilization often changes the properties of the immobilized enzyme, particularly parameters such as the Michaelis-Menten constant ( $K_M$ ) and the maximum enzyme reaction rate ( $V_{max}$ ).

The kinetic parameters of naringinase subunits, i.e.,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, were determined from the dependence of the initial reaction rate on the substrate concentration. The Lineweaver-Burk method determined the  $K_M$  and  $V_{max}$  (Figures 9 and 10). The values of the kinetic parameters of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase of free, immobilized, and dextran aldehyde-bound naringinase are presented in Tables 2 and 3.



**Figure 9.** Lineweaver–Burk plots of  $\alpha$ -L-rhamnosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS.



**Figure 10.** Lineweaver–Burk plots of  $\beta$ -D-glucosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS.

**Table 2.** The values of the kinetic parameters of  $\alpha$ -L-rhamnosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS.

| Type of Enzyme                                                      | $V_{\max}$ [ $\text{mM} \cdot \text{min}^{-1}$ ] | $K_M$ [mM]                         |
|---------------------------------------------------------------------|--------------------------------------------------|------------------------------------|
| Free $\alpha$ -L-rhamnosidase                                       | $0.1176 \pm 0.0016$ <sup>(a)</sup>               | $2.0803 \pm 0.0355$ <sup>(c)</sup> |
| Adsorbed $\alpha$ -L-rhamnosidase                                   | $0.1019 \pm 0.0044$ <sup>(b)</sup>               | $2.3698 \pm 0.1288$ <sup>(d)</sup> |
| Adsorbed $\alpha$ -L-rhamnosidase crosslinked with dextran aldehyde | $0.1021 \pm 0.0053$ <sup>(b)</sup>               | $2.3678 \pm 0.1359$ <sup>(d)</sup> |

Different letters indicate statistically significant differences at the  $p < 0.05$  level.

**Table 3.** The values of the kinetic parameters of  $\beta$ -D-glucosidase as a free, immobilized, and stabilized subunit of naringinase from *A. niger* KMS.

| Type of Enzyme                                                    | $V_{\max}$ [ $\text{mM} \cdot \text{min}^{-1}$ ] | $K_M$ [mM]                         |
|-------------------------------------------------------------------|--------------------------------------------------|------------------------------------|
| Free $\beta$ -D-glucosidase                                       | $0.1240 \pm 0.0032$ <sup>(a)</sup>               | $2.7213 \pm 0.0780$ <sup>(c)</sup> |
| Adsorbed $\beta$ -D-glucosidase                                   | $0.1188 \pm 0.0026$ <sup>(b)</sup>               | $2.9280 \pm 0.0780$ <sup>(d)</sup> |
| Adsorbed $\beta$ -D-glucosidase crosslinked with dextran aldehyde | $0.1195 \pm 0.0019$ <sup>(b)</sup>               | $2.9277 \pm 0.0565$ <sup>(d)</sup> |

Different letters indicate statistically significant differences at the  $p < 0.05$  level.

The  $K_M$  of  $\alpha$ -L-rhamnosidase from free naringinase was slightly lower than that of  $\beta$ -D-glucosidase. It means that  $\alpha$ -L-rhamnosidase has a higher affinity for pNPR than  $\beta$ -D-glucosidase has for pNPG. Other authors obtained similar results for naringinase subunits, where the  $K_M$  of  $\beta$ -D-glucosidase was higher than that of  $\alpha$ -L-rhamnosidase. Ni et al. [6] found that the  $\alpha$ -L-rhamnosidase from *A. niger* DB056 has a lower  $V_{\max}$  ( $1.51 \mu\text{M} \cdot \text{mL}^{-1}$ ) than the  $\beta$ -D-glucosidase ( $9.63 \mu\text{M} \cdot \text{mL}^{-1}$ ), implying the hydrolysis of naringin to prunin was the velocity-limiting step of the naringin degradation.

The  $K_M$  of  $\alpha$ -L-rhamnosidase from *A. oryzae* 11250 toward pNPR was estimated to be 0.63 mM, which is lower than that of  $\beta$ -D-glucosidase for pNPR (2.8 mM) [50]. The  $K_M$  of  $\alpha$ -L-rhamnosidase from *A. sojae* toward pNPR was estimated to be 1.79 mM, which is lower than that of  $\beta$ -D-glucosidase for pNPR (239 mM) [54].  $\alpha$ -L-Rhamnosidase from *A. niger* DB056 had a  $K_M$  of 0.28 mM, whereas  $\beta$ -D-glucosidase had a  $K_M$  of 0.39 mM [6].  $\alpha$ -L-Rhamnosidase from *A. terreus* had a  $K_M$  of 0.52 mM, while for  $\beta$ -D-glucosidase, the  $K_M$  was 1.89 mM [53]. The differences may have resulted from species differences and the culture conditions of the microorganisms used to obtain naringinase.

The results presented in Table 2 showed that the immobilization of naringinase increased the Michaelis-Menten constant of  $\alpha$ -L-rhamnosidase compared to the free enzyme. Further stabilization of the binding of naringinase with dextran aldehyde did not significantly affect the Michaelis-Menten constant, suggesting that the affinity toward the substrate was reduced when the enzyme was immobilized. A lower maximum reaction rate was accompanied an increase in the apparent Michaelis constant (decreased enzyme affinity for the substrate). This is likely related to conformational changes in the naringinase subunits, the hydrodynamic conditions of the environment, and the electrochemical conditions of both the carrier and the substrate.

As a result of immobilization of the tested biocatalyst, the maximum reaction rate of  $\alpha$ -L-rhamnosidase decreased slightly. The binding of immobilized naringinase with dextran aldehyde did not significantly affect the maximum reaction rate of  $\alpha$ -L-rhamnosidase compared to the enzyme immobilized by adsorption.

The Michaelis-Menten constant of immobilized  $\beta$ -D-glucosidase was slightly higher than that of the free enzyme. Stabilizing immobilized naringinase with dextran aldehyde did not significantly affect the  $K_M$  compared to the enzyme immobilized by adsorption, suggesting that the affinity toward the substrate was reduced when the enzyme was immobilized.

Immobilization of naringinase on a magnetic carrier resulted in a significant reduction in the maximum reaction rate of  $\beta$ -D-glucosidase compared to the free enzyme. Further binding of naringin with dextran aldehyde resulted in a slight decrease in the maximum reaction rate.

As a result of the immobilization and stabilization of naringinase,  $\alpha$ -L-rhamnosidase showed a lower  $V_{max}$  than  $\beta$ -D-glucosidase, suggesting that  $\alpha$ -L-rhamnosidase is the main factor inhibiting the rate of naringin hydrolysis by naringinase.

As a result of immobilization, the  $V_{max}$  of both subunits decreased, indicating a lower catalytic efficiency of the immobilized enzyme. This may be caused by the limited accessibility of the enzyme to pNPR/pNPG and the diffusion of p-nitrophenol [30]. As a result of immobilization, the  $K_M$  of both subunits increased, thus decreasing the affinity of the enzymes for substrates. Nevertheless, higher  $K_M$  and lower  $V_{max}$  after immobilization are typical for immobilized biocatalysts [55]. Usually, this is due to diffusion limitations in the microenvironment and steric constraints that reduce substrate accessibility and enzyme flexibility [56].

There are no reports in the literature on the effect of naringinase immobilization on the kinetic parameters of its subunits. Only the effect of immobilization on individual enzymes,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, was studied. Similar results to ours were obtained in the study by Spagna et al. [56], where the  $K_M$  of  $\alpha$ -L-rhamnosidase from *A. niger* immobilized on chitosan activated with glutaraldehyde doubled, and the  $V_{max}$  decreased twofold compared with the free enzyme.  $\beta$ -D-glucosidase from *A. niger* immobilized in alginate showed an apparent  $K_M$  higher and  $V_{max}$  lower than the free enzyme [57].

Slightly different results were obtained by immobilizing  $\alpha$ -L-rhamnosidase from *A. niger* on a magnetic metal–organic framework. Compared with the free enzyme, immobilized  $\alpha$ -L-rhamnosidase showed a relatively minor  $V_{max}$  and a lower  $K_M$  [30]. The authors

explained this change in  $K_M$  because the active site was more accessible to the enzyme due to a conformational change after immobilization on a carrier with a high specific surface area.

In the literature, a different relationship was reported for naringinase subunits from *A. niger* DB056 [6], *A. terreus* [53], and *A. oryzae* 11250 [50]. The differences may have resulted from species differences and the culture conditions of the microorganisms used to obtain naringinase.

### 3. Materials and Methods

#### 3.1. Materials

Naringinase from *A. niger* KMS, cultured under submerged conditions, was isolated by concentrating the post-culture fluid through ultrafiltration, followed by protein precipitation using acetone, yielding a solid preparation with an activity of  $816 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ . p-nitrophenyl- $\alpha$ -L-rhamnopyranoside (pNPR) and p-nitrophenyl- $\beta$ -D-glucoside (pNPG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polysaccharides and activators were used for enzyme immobilization: carob gum obtained from *Ceratonia siliqua* (Sigma-Aldrich, St. Louis, MO, USA) and a 50% aqueous solution of polyethyleneimine (Sigma-Aldrich, St. Louis, MO, USA).

#### 3.2. Analytical Methods

##### 3.2.1. Determination of $\alpha$ -L-rhamnosidase and $\beta$ -D-glucosidase Activity from Free Naringinase

The method described by Spagna et al. assessed the activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase [56,58]. The method was based on spectrophotometric determination of the concentration of p-nitrophenol obtained in the decomposition reaction of p-nitrophenyl- $\alpha$ -rhamnopyranoside or p-nitrophenyl- $\alpha$ -glucopyranoside in reaction with  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, respectively. The absorbance of the solutions was measured at a wavelength of  $\lambda = 410 \text{ nm}$ . The absorbance was then converted to the concentration of p-nitrophenol using a regression equation characterizing the standard curve, prepared based on measurements of the absorbance of aqueous solutions of p-nitrophenol in the concentration range 0.01–0.20 mM. In the case of determining the activity of free naringinase subunits,  $0.4 \text{ cm}^3$  2 mM solution of pNPR or pNPG was dissolved in 0.1 M acetate buffer (pH 4.0), and  $0.01 \text{ cm}^3$  of naringinase solution was added to  $0.09 \text{ cm}^3$  of water. The reaction was carried out for 5 or 10 min, after which  $1 \text{ cm}^3$  1 M  $\text{Na}_2\text{CO}_3$  was added to stop the reaction.

The  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase activity is expressed in  $\mu\text{mol}$  of pNPR or pNPG hydrolyzed for 1 min by 1 g of the enzyme preparation.

##### 3.2.2. Determination of $\alpha$ -L-rhamnosidase and $\beta$ -D-glucosidase Activity from Immobilized and Stabilized Naringinase

To determine the activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase of naringinase bound to a carrier,  $0.09 \text{ cm}^3$  of water and  $0.4 \text{ cm}^3$  of 2 mM solution of pNPR or pNPG dissolved in 0.1 M acetate buffer (pH 4.0) were added to 37.5 mg of carrier with immobilized enzyme. The reaction was carried out for 5 or 10 min, after which  $1 \text{ cm}^3$  1 M  $\text{Na}_2\text{CO}_3$  was added to stop the reaction.

The  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase activity is expressed in  $\mu\text{mol}$  of pNPR or pNPG hydrolyzed for 1 min by 1 g of the immobilized preparation.

#### 3.3. Immobilization of Naringinase

Naringinase from *A. niger* KMS was adsorbed onto a magnetic carrier obtained from carob gum activated with polyethyleneimine, as described in our previous work. In order

to strengthen the bond between the enzyme and the carrier, naringinase was additionally stabilized by cross-linking with dextran aldehyde [21].

Immobilization of naringinase from *A. niger* KMS: 7.5 cm<sup>3</sup> of enzyme solution (50 mg·10 mL<sup>-1</sup> 0.1 M phosphate buffer, pH 7.0) was added to 150 mg of carrier. The mixture was incubated at 27 °C for 4 h, shaking at a frequency of 150 rpm.

Cross-linking bound naringinase with dextran aldehyde: After completion of the immobilization stage, 10 cm<sup>3</sup> of 20% dextran aldehyde solution was added to the mixture. Incubation was continued for another 20 h under the same conditions.

After the enzyme stabilization stage using dextran aldehyde, the enzyme-carrier complex was separated from the solution using a neodymium magnet and then rinsed several times with phosphate buffer (0.01 M, pH 7) and distilled water.

#### Immobilization Yield

The activity immobilization yield ( $Y_a$ ) was calculated from the following equation:

$$Y_a = \frac{X_a}{X_{a0}} \times 100\%, \quad (1)$$

where

$X_a$ —the difference between the total activity of the enzyme used for immobilization and the activity of the immobilized enzyme;

$X_{a0}$ —the total activity of the enzyme used for immobilization.

100% yield was defined as the total activity of naringinase ( $X_{a0}$ ) immobilized.

### 3.4. Characteristics of Free, Immobilized and Stabilized Biocatalyst

#### 3.4.1. Effect of pH on the Activity of $\alpha$ -L-rhamnosidase and $\beta$ -D-glucosidase

The effect of the active acidity of the reaction environment on the activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase from free, immobilized, and stabilized naringinase was determined by analyzing the activity of the enzymes at different pH of the mixture. Briefly, 0.1 M McIlvaine buffer (pH 2.5–8.0) was used. The activity of the biocatalysts was tested at 50 °C. The activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase was determined using the methods described in Sections 3.2.1 and 3.2.2. The activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase was determined.

#### 3.4.2. Effect of Incubation in Buffers with Different pH on the Activity of $\alpha$ -L-rhamnosidase, and $\beta$ -D-glucosidase

The stability of free, immobilized, and crosslinked naringinase subunits depending on the pH of the environment was tested by incubating the enzymes for 15 h in buffers with different pH. Briefly, 0.1 M McIlvaine buffer (pH 2.5–8.0) was used. The activity of the biocatalysts was tested at 50 °C in a buffer with an optimal pH of 4.0. The activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase was determined using the methods described in Sections 3.2.1 and 3.2.2.

#### 3.4.3. Effect of Temperature on the Activity of $\alpha$ -L-rhamnosidase and $\beta$ -D-glucosidase

The effect of temperature on changes in the enzymatic activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase of free and immobilized naringinase was studied at temperatures ranging from 30 to 80 °C. The activity of the studied biocatalysts was determined in a 0.1 M acetate buffer with a pH of 4.0. The activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase was determined using the methods described in Sections 3.2.1 and 3.2.2.

#### 3.4.4. Thermal Stability of $\alpha$ -L-rhamnosidase and $\beta$ -D-glucosidase

The thermal stability of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase from free, immobilized, and stabilized naringinase was tested by incubating all enzyme forms at a specified temperature for 60 min. The incubation was carried out at temperatures ranging from 30 to 80 °C. After 60 min, the enzymes were cooled in an ice bath. After that, the remaining activity of the tested enzymes was determined at 50 °C in 0.1 M buffer with a pH of 4.0. The activity of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase was determined using the methods described in Sections 3.2.1 and 3.2.2.

#### 3.4.5. Determination of Kinetic Parameters

The kinetic parameters of naringinase subunits were studied in the decomposition reaction of p-nitrophenyl- $\alpha$ -rhamnopyranoside (pNPR) or p-nitrophenyl- $\beta$ -glucopyranoside (pNPG) by  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, respectively. The assay mixture (1.5 mL) was prepared with either 0.05 mg of free naringinase or 9 mg of immobilized enzyme. The reaction rate was determined at 50 °C in a 0.1 M buffer with a pH of 4.0 using substrate concentrations (pNPR or pNPG) in the reaction mixture equal to 0.4 mM, 0.5 mM, 0.8 mM, 1 mM, 1.5 mM. For each substrate concentration, four samples were prepared. In the first test, the product concentration was determined after 1 min of reaction; in the second test, after 2 min; in the third test, after 3 min; and finally, after 5 min. The initial reaction rate for each substrate concentration was calculated from the derivative of the product formation rate at the start of the reaction.

The maximum reaction rate ( $V_{\max}$ ) and Michaelis constant ( $K_M$ ) were calculated using the Lineweaver–Burk method based on the dependence of the reaction rate on the substrate concentration:

$$\frac{1}{v} = \frac{K_M}{v_{\max}} * \frac{1}{C_s} + \frac{1}{v_{\max}} \quad (2)$$

where

$v$ —enzyme reaction rate [ $\text{mM} \cdot \text{min}^{-1}$ ];

$v_{\max}$ —maximum reaction rate [ $\text{mM} \cdot \text{min}^{-1}$ ];

$K_M$ —Michaelis constant [mM];

$C_s$ —substrate concentration [mM].

#### 3.5. Statistical Analysis

All determinations were performed in triplicate. All values are expressed as mean  $\pm$  standard deviation of the three replicate experiments. STATISTICA v13.3 was used to evaluate the data.

### 4. Conclusions

The naringinase complex was adsorbed onto a magnetic polysaccharide carrier obtained from carob gum activated with polyethyleneimine. In order to strengthen the bond between the enzyme and the carrier, the complex was additionally stabilized by cross-linking with dextran aldehyde. This immobilization procedure was developed for naringinase and described in detail in an earlier article [21]. This approach increased the pH stability of  $\alpha$ -L-rhamnosidase in the wide pH range of 3.0–7.0 and  $\beta$ -D-glucosidase at a pH below 3.0. It expands the possibilities of using the tested enzymes in an acidic environment typical of citrus juices, e.g., in the hydrolysis of naringin in grapefruit juice or hesperidin in orange juice. Immobilization and additional and subsequent stabilization of naringinase increased the activity of both subunits at low temperatures below 40 °C. At these temperatures and with short exposure times, the stability of thermolabile components of citrus fruit juices is higher, which does not cause any noticeable changes in the sensory and nutritional

quality of the products. The stability of  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase increased at temperatures above 50 °C, broadening the potential application of these enzymes in the pharmaceutical industry, e.g., in producing valuable prunin.

The enzymatic characteristics of naringinase subunits, i.e.,  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase, allow their range of activity, optimal pH, and temperature to be determined, which is essential for their practical use. Thanks to the targeted deglycosylation of flavonoids, including hesperidin, naringenin, and rutin, their bioavailability can be increased. For this purpose, it is necessary to preserve the activity of only  $\alpha$ -L-rhamnosidase from the complex naringinase enzyme and to inhibit the second enzyme,  $\beta$ -D-glucosidase, using specific inhibitors or reaction conditions that ensure the selective activity of the enzymatic subunits of naringinase. An analysis of the literature clearly shows that knowledge in this area is limited and needs to be supplemented.

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## Abbreviations

The following abbreviations are used in this manuscript:

|            |                                            |
|------------|--------------------------------------------|
| Hes-7-G    | hesperetin 7-O-glucoside                   |
| pNPR       | 4-nitrophenyl $\alpha$ -L-rhamnopyranoside |
| pNPG       | 4-nitrophenyl $\beta$ -D-glucopyranoside   |
| $v_{\max}$ | maximum reaction rate                      |
| $K_M$      | Michaelis constant                         |

## References

1. Puri, M. Updates on naringinase: Structural and biotechnological aspects. *Appl. Microbiol. Biotechnol.* **2012**, *93*, 49–60. [CrossRef] [PubMed]
2. Golgeri, M.D.B.; Mulla, S.I.; Bagewadi, Z.K.; Faniband, B.; Mishra, P.; Bankole, P.O.; Sharma, S.; Américo-Pinheiro, J.H.P.; Bharagava, R.N.; Romanholo Ferreira, L.F. Microbial naringinase: From microbial source to its current applications in various fields. *Biologia* **2025**, *80*, 977–991. [CrossRef]
3. Cui, Z.; Maruyama, Y.; Mikami, B.; Hashimoto, W.; Murata, K. Crystal Structure of Glycoside Hydrolase Family 78  $\alpha$ -L-Rhamnosidase from *Bacillus* sp. GL1. *J. Mol. Biol.* **2007**, *374*, 384–398. [CrossRef]
4. Zhu, Y.; Jia, H.; Xi, M.; Xu, L.; Wu, S.; Li, X. Purification and characterization of a naringinase from a newly isolated strain of *Bacillus amyloliquefaciens* 11568 suitable for the transformation of flavonoids. *Food Chem.* **2017**, *214*, 39–46. [CrossRef] [PubMed]
5. Slámová, K.; Kapešová, J.; Valentová, K. “Sweet Flavonoids”: Glycosidase-Catalyzed Modifications. *Int. J. Mol. Sci.* **2018**, *19*, 2126. [CrossRef]
6. Ni, H.; Xiao, A.-F.; Wang, Y.Q.; Chen, F.; Cai, H.-N.; Su, W.-J. Development and evaluation of an HPLC method for accurate determinations of enzyme activities of naringinase complex. *J. Agric. Food Chem.* **2013**, *61*, 10026–10032. [CrossRef]
7. Chen, Y.; Ni, H.; Chen, F.; Cai, H.; Li, L.; Su, W. Purification and characterization of a naringinase from *Aspergillus aculeatus* JMUb058. *J. Agric. Food Chem.* **2013**, *61*, 931–938. [CrossRef]
8. Ni, H.; Chen, F.; Cai, H.; Xiao, A.; You, Q.; Lu, Y. Characterization and preparation of *Aspergillus niger* naringinase for debittering citrus juice. *J. Food Sci.* **2012**, *77*, C1–C7. [CrossRef]

9. Soria, F.; Ellenrieder, G.; Grasselli, M.; Navarro Del Cañizo, A.A.; Cascone, O. Fractionation of the naringinase complex from *Aspergillus terreus* by dye affinity chromatography. *Biotechnol. Lett.* **2004**, *26*, 1265–1268. [CrossRef]
10. Bodakowska-Boczniewicz, J.; Garncarek, Z. Use of Naringinase to Modify the Sensory Quality of Foods and Increase the Bioavailability of Flavonoids: A Systematic Review. *Molecules* **2025**, *30*, 2376. [CrossRef]
11. Ribeiro, M.H. Naringinases: Occurrence, characteristics, and applications. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1883–1895. [CrossRef]
12. Li, B.; Wu, B.; Hou, X.; Ding, G. Substrate Selectivities of GH78  $\alpha$ -L-Rhamnosidases from Human Gut Bacteria on Dietary Flavonoid Glycosides. *Molecules* **2025**, *30*, 980. [CrossRef]
13. Roitner, M.; Schalkhammer, T.; Pittner, F. Preparation of prunin with the help of immobilized naringinase pretreated with alkaline buffer. *Appl. Biochem. Biotechnol.* **1984**, *9*, 483–488. [CrossRef]
14. Rajendran, D.S.; Chidambaram, A.; Kumar, P.S.; Venkataraman, S.; Muthusamy, S.; Vo, D.-V.N.; Rangasamy, G.; Vaithyanathan, V.K.; Vaidyanathan, V.K. Three-phase partitioning for the separation of proteins, enzymes, biopolymers, oils and pigments: A review. *Environ. Chem. Lett.* **2023**, *21*, 911–934. [CrossRef]
15. Mazzaferro, L.; Piñuel, L.; Minig, M.; Breccia, J.D. Extracellular monoenzyme deglycosylation system of 7-O-linked X avonoid-rutinosides and its disaccharide transglycosylation activity from *Stilbella fimetaria*. *Arch. Microbiol.* **2010**, *192*, 383–393. [CrossRef] [PubMed]
16. Lee, Y.S.; Huh, J.Y.; Nam, S.H.; Moon, S.K.; Lee, S.B. Enzymatic bioconversion of citrus hesperidin by *Aspergillus sojae* naringinase: Enhanced solubility of hesperetin-7-O-glucoside with in vitro inhibition of human intestinal maltase, HMG-CoA reductase, and growth of *Helicobacter pylori*. *Food Chem.* **2012**, *135*, 2253–2259. [CrossRef] [PubMed]
17. Zou, Y.; Xin, X.; Xu, H.; Yuan, H.; Li, X.; Yu, Y.; Zhao, G. Highly efficient bioconversion of flavonoid glycosides from citrus-processing wastes in solvent-buffer systems. *Green Chem.* **2020**, *22*, 3196–3207. [CrossRef]
18. Vila-Real, H.; Alfaia, A.J.; Bronze, M.R.; Calado, A.R.T.; Ribeiro, M.H.L. Enzymatic Synthesis of the Flavone Glucosides, Prunin and Isoquercetin, and the Aglycones, Naringenin and Quercetin, with Selective  $\alpha$ -L-Rhamnosidase and  $\beta$ -D-Glucosidase Activities of Naringinase. *Enzym. Res.* **2011**, *2011*, 692618. [CrossRef]
19. Li, L.J.; Liu, X.Q.; Du, X.P.; Wu, L.; Jiang, Z.D.; Ni, H.; Li, Q.B.; Chen, F. Preparation of isoquercitrin by biotransformation of rutin using  $\alpha$ -L-rhamnosidase from *Aspergillus niger* JMU-TS528 and HSCCC purification. *Prep. Biochem. Biotechnol.* **2020**, *50*, 1–9. [CrossRef]
20. Urrutia, P.; Arrieta, R.; Torres, C.; Guerrero, C.; Wilson, L. Amination of naringinase to improve citrus juice debittering using a catalyst immobilized on glyoxyl-agarose. *Food Chem.* **2024**, *452*, 139600. [CrossRef]
21. Bodakowska-Boczniewicz, J.; Garncarek, Z. Immobilization of Naringinase from *Aspergillus niger* on a Magnetic Polysaccharide Carrier. *Molecules* **2020**, *25*, 2731. [CrossRef] [PubMed]
22. Bilal, M.; Iqbal, H.M.N. Chemical, physical, and biological coordination: An interplay between materials and enzymes as potential platforms for immobilization. *Coord. Chem. Rev.* **2019**, *388*, 1–23. [CrossRef]
23. Ribeiro, I.A.C.; Ribeiro, M.H.L. Kinetic modelling of naringin hydrolysis using a bitter sweet alfa-rhamnopyranosidase immobilized in k-carrageenan. *J. Mol. Catal. B Enzym.* **2008**, *51*, 10–18. [CrossRef]
24. Bodakowska-Boczniewicz, J.; Garncarek, Z. Immobilizacja naringinazy z *Penicillium decumbens* na magnetycznych polisacharydowych nośnikach. *Pr. Nauk. Univ. Ekon. We Wrocławiu* **2019**, *524*, 9–24. [CrossRef]
25. Ferreira, L.; Afonso, C.; Vila-real, H.; Alfaia, A. Evaluation of the Effect of High Pressure on Naringin Hydrolysis in Grapefruit Juice with Naringinase Immobilised in Calcium Alginate Beads. *Food Technol. Biotechnol.* **2008**, *46*, 146–150.
26. Norouzian, D.; Hosseinzadeh, A.; Inanlou, D.N.; Moazami, N. Various techniques used to immobilize naringinase produced by *Penicillium decumbens* PTCC 5248. *World, J. Microbiol. Biotechnol.* **1999**, *15*, 501–502. [CrossRef]
27. Awad, G.E.A.; Abd El Aty, A.A.; Shehata, A.N.; Hassan, M.E.; Elnashar, M.M. Covalent immobilization of microbial naringinase using novel thermally stable biopolymer for hydrolysis of naringin. *3 Biotech* **2016**, *6*, 14. [CrossRef]
28. Garzón-Alonso, K.E.; Bohorquez-Peña, M.J.; Vargas-Suaza, B.; Rendón-Londoño, J.C.; Mesa, M.; Franco-Tobón, Y.N.; Martínez-Galán, J.P. Hydrolysis of flavanones from orange peel and evaluation of anticancer potential using naringinase immobilized on corn cob powder. *Food Biosci.* **2025**, *69*, 106752. [CrossRef]
29. Serra, I.; Serra, C.D.; Rocchietti, S.; Ubiali, D.; Terreni, M. Stabilization of thymidine phosphorylase from *Escherichia coli* by immobilization and post immobilization techniques. *Enzym. Microb. Technol.* **2011**, *49*, 52–58. [CrossRef] [PubMed]
30. Wang, D.; Zheng, P.; Chen, P.; Wu, D. Immobilization of alpha-L-rhamnosidase on a magnetic metal-organic framework to effectively improve its reusability in the hydrolysis of rutin. *Bioresour. Technol.* **2021**, *323*, 124611. [CrossRef]
31. Wan, W.; Xia, N.; Zhu, S.; Liu, Q.; Gao, Y. A Novel and High-Effective Biosynthesis Pathway of Hesperetin-7-O-Glucoside Based on the Construction of Immobilized Rhamnosidase Reaction Platform. *Front. Bioeng. Biotechnol.* **2020**, *8*, 608. [CrossRef] [PubMed]
32. Wei, B.; Liu, F.; Liu, X.; Cheng, L.; Yuan, Q.; Gao, H.; Liang, H. Enhancing stability and by-product tolerance of  $\beta$ -glucuronidase based on magnetic cross-linked enzyme aggregates. *Colloids Surf. B Biointerfaces* **2022**, *210*, 112241. [CrossRef] [PubMed]

33. Hadadi, M.; Habibi, A. Development of Magnetic Immobilized Cellulase Biocatalysts for Saccharification of Paper Waste. *Catal. Lett.* **2024**, *154*, 5791–5805. [CrossRef]
34. He, G.; Liu, H.; Yang, C.; Hu, K.; Zhai, X.; Fang, B.; Liu, K.; Zulekha; Li, D. A comparison of dual-enzyme immobilization by magnetic nanoparticles and magnetic enzyme aggregates for cascade enzyme reactions. *Biochem. Eng. J.* **2024**, *204*, 109207. [CrossRef]
35. Cavalcante, A.L.G.; Dari, D.N.; Aires, F.I.d.S.; de Castro, E.C.; dos Santos, K.M.; dos Santos, J.C.S. Advancements in enzyme immobilization on magnetic nanomaterials: Toward sustainable industrial applications. *RSC Adv.* **2024**, *14*, 17946–17988. [CrossRef]
36. Yu, C.; Li, Q.; Tian, J.; Zhan, H.; Zheng, X.; Wang, S.; Sun, X.; Sun, X. A facile preparation of immobilized naringinase on polyethyleneimine-modified Fe<sub>3</sub>O<sub>4</sub> magnetic nanomaterials with high activity. *RSC Adv.* **2021**, *11*, 14568–14577. [CrossRef]
37. Ladole, M.R.; Pokale, P.B.; Varude, V.R.; Belokar, P.G.; Pandit, A.B. One pot clarification and debittering of grapefruit juice using co-immobilized enzymes@chitosanMNPs. *Int. J. Biol. Macromol.* **2021**, *167*, 1297–1307. [CrossRef]
38. Kimmins, S.D.; Henríquez, A.; Torres, C.; Wilson, L.; Flores, M.; Pio, E.; Jullian, D.; Urbano, B.; Braun-Galleani, S.; Ottone, C.; et al. Immobilization of Naringinase onto Polydopamine-Coated Magnetic Iron Oxide Nanoparticles for Juice Debittering Applications. *Polymers* **2024**, *16*, 3279. [CrossRef]
39. Luo, J.; Li, Q.; Sun, X.; Tian, J.; Fei, X.; Shi, F.; Zhang, N.; Liu, X. The study of the characteristics and hydrolysis properties of naringinase immobilized by porous silica material. *RSC Adv.* **2019**, *9*, 4514–4520. [CrossRef]
40. Liu, R.; Chen, D.; Fu, H.; Lv, P.; Zhang, D.; He, Y. A facile preparation process of magnetic aldehyde-functionalized Ni<sub>0.5</sub>Zn<sub>0.5</sub>Fe<sub>2</sub>O<sub>4</sub>@SiO<sub>2</sub> nanocomposites for immobilization of Penicillin G Acylase (PGA). *J. Nanosci. Nanotechnol.* **2017**, *17*, 893–899. [CrossRef]
41. Busto, M.D.; Meza, V.; Ortega, N.; Perez-Mateos, M. Immobilization of naringinase from *Aspergillus niger* CECT 2088 in poly(vinyl alcohol) cryogels for the debittering of juices. *Food Chem.* **2007**, *104*, 1177–1182. [CrossRef]
42. Puri, M.; Marwaha, S.S.; Kothari, R.M. Studies on the applicability of alginate-entrapped naringinase for the debittering of kinnow juice. *Enzym. Microb. Technol.* **1996**, *18*, 281–285. [CrossRef]
43. Vila-Real, H.; Alfaia, A.J.; Rosa, M.E.; Calado, A.R.; Ribeiro, M.H.L. An innovative sol-gel naringinase bioencapsulation process for glycosides hydrolysis. *Process Biochem.* **2010**, *45*, 841–850. [CrossRef]
44. Wang, C.; Chen, P.-X.; Xiao, Q.; Chen, J.; Chen, F.-Q.; Yang, Q.-M.; Weng, H.-F.; Fang, B.-S.; Zhang, Y.-H.; Xiao, A.-F. Artificial naringinase system for cooperative enzymatic synthesis of naringenin. *Biochem. Eng. J.* **2022**, *178*, 108277. [CrossRef]
45. Koseki, T.; Mese, Y.; Nishibori, N.; Masaki, K.; Fujii, T.; Handa, T.; Yamane, Y.; Shiono, Y.; Murayama, T.; Iefuji, H. Characterization of an  $\alpha$ -L-rhamnosidase from *Aspergillus kawachii* and its gene. *Biochem. Relev. Enzym. Proteins* **2008**, *80*, 1007. [CrossRef]
46. Thammawat, K.; Pongtanya, P. Isolation, Preliminary Enzyme Characterization and Optimization of Culture Parameters for Production of Naringinase Isolated from *Aspergillus niger* BCC 25166. *Nat. Sci.* **2008**, *42*, 61–72.
47. Gallego, M.V.; Pinaga, F.; Ramon, D.; Valles, S. Purification and characterization of an  $\alpha$ -L-rhamnosidase from *Aspergillus terreus* of interest in winemaking. *J. Food Sci.* **2001**, *66*, 204–209. [CrossRef]
48. Decker, C.H.; Visser, J.; Schreier, P. B-Glucosidases from Five Black *Aspergillus* Species: Study of Their Physico-Chemical and Biocatalytic Properties Keywords: *J. Agric. Food Chem.* **2000**, *48*, 4929–4936. [CrossRef]
49. Muñoz, M.; Holtheuer, J.; Wilson, L.; Urrutia, P. Grapefruit Debittering by Simultaneous Naringin Hydrolysis and Limonin Adsorption Using Naringinase Immobilized in Agarose Supports. *Molecules* **2022**, *27*, 2867. [CrossRef]
50. Zhu, Y.; Jia, H.; Xi, M.; Li, J.; Yang, L.; Li, X. Characterization of a naringinase from *Aspergillus oryzae* 11250 and its application in the debitterization of orange juice. *Process Biochem.* **2017**, *62*, 114–121. [CrossRef]
51. Roitner, M.; Schalkhammer, T.; Pittner, F. Characterisation of naringinase from *Aspergillus niger*. *Monatshefte Für Chem. Chem. Mon.* **1984**, *115*, 1255–1267. [CrossRef]
52. Manzanares, P.; Hetty, C.; Visser, J. Purification and Characterization of Two Different- $\alpha$ -L-Rhamnosidases, RhaA and RhaB, from *Aspergillus aculeatus*. *Appl. Environ. Microbiol.* **2001**, *67*, 2230–2234. [CrossRef]
53. Abbate, E.; Palmeri, R.; Todaro, A.; Blanco, R.M. Production of a  $\alpha$ -L-Rhamnosidase from *Aspergillus terreus* Using Citrus Solid Waste as Inducer for Application in Juice Industry. *Chem. Eng. Trans.* **2012**, *27*, 253–258.
54. Chang, H.-Y.; Lee, Y.-B.; Bae, H.-A.; Huh, J.-Y.; Nam, S.-H.; Sohn, H.-S.; Lee, H.J.; Lee, S.-B. Purification and characterisation of *Aspergillus sojae* naringinase: The production of prunin exhibiting markedly enhanced solubility with in vitro inhibition of HMG-CoA reductase. *Food Chem.* **2011**, *124*, 234–241. [CrossRef]
55. Zdarta, J.; Jędrzak, A.; Kłapiszewski, Ł.; Jesionowski, T. Immobilization of Cellulase on a Functional Inorganic–Organic Hybrid Support: Stability and Kinetic Study. *Catalysts* **2017**, *7*, 374. [CrossRef]
56. Spagna, G.; Barbagallo, R.N.; Casarini, D.; Pifferi, P.G. A novel chitosan derivative to immobilize  $\alpha$ -L-rhamnopyranosidase from *Aspergillus niger* for application in beverage technologies. *Enzym. Microb. Technol.* **2001**, *28*, 427–438. [CrossRef]

57. Keerti; Gupta, A.; Kumar, V.; Dubey, A.; Verma, A.K. Kinetic Characterization and Effect of Immobilized Thermostable beta-Glucosidase in Alginate Gel Beads on Sugarcane Juice. *ISRN Biochem.* **2014**, *2014*, 8. [CrossRef]
58. Spagna, G.; Romagnoli, D.; Angela, M.; Bianchi, G.; Pifferi, P.G. A simple method for purifying glycosidases: Alpha-L-arabinofuranosidase and beta-D-glucopyranosidase from *Aspergillus niger* to increase the aroma of wine. Part, I. *Enzym. Microb. Technol.* **1998**, *22*, 298–304. [CrossRef]

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Article

# Biochemical Characterization of R-Loop Degradation by Chloroplast-Localized RNase H1 from *Arabidopsis thaliana*

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## Abstract

R-loops are three-stranded nucleic acid structures implicated in genome regulation and stability. In *Arabidopsis thaliana*, the chloroplast-localized RNase H1 enzyme (AtRNH1C) is important for chloroplast development and genome integrity; however, its molecular activity has not been experimentally verified. In the present study, we characterized the enzymatic activity of recombinant AtRNH1C toward model R-loops of various structures. Using a set of synthetic R-loop substrates, we demonstrate that AtRNH1C cleaves the RNA within DNA/RNA hybrids with a strong preference for purine-rich sequences, most notably at G↓X dinucleotides. Kinetic assays showed that the enzyme's efficiency is highly dependent on the length of the hybrid duplex but is not affected by a G-quadruplex structure in the single-stranded DNA flap of the R-loop. The most rapid degradation was observed for an R-loop with an 11 nt DNA/RNA hybrid region. This study provides a comparative analysis of chloroplast-localized RNase H1 activity and elucidates its substrate preferences, suggesting that an R-loop with a heteroduplex length closest to the native size found in transcription elongation complexes is the most efficient substrate. These findings suggest that the enzymatic activity of AtRNH1C is sufficient to perform its function in maintaining chloroplast genome stability by the degradation of R-loops in DNA.

**Keywords:** enzyme kinetics; RNase H1; R-loop resolution; *Arabidopsis thaliana*; chloroplast; organelle genome stability

## 1. Introduction

R-loops are three-stranded nucleic acid structures formed by a DNA/RNA hybrid and a displaced single-stranded DNA. Research using the single-stranded DNA ligation-based library preparation method has revealed that R-loops are abundant in the *Arabidopsis* genome, particularly in gene promoters, gene bodies, and regions with GC/AT skews. These structures are also frequently found in repetitive regions, transposable elements, and noncoding RNAs, indicating their involvement in chromatin dynamics, gene expression, and plant-specific silencing pathways like RNA-directed DNA methylation [1,2]. In recent years, R-loops associated with transcription have been identified as a significant contributor to genome instability [3–7]. However, studies have shown that R-loops form independently of replication and that those formed in one cell cycle stage can persist into

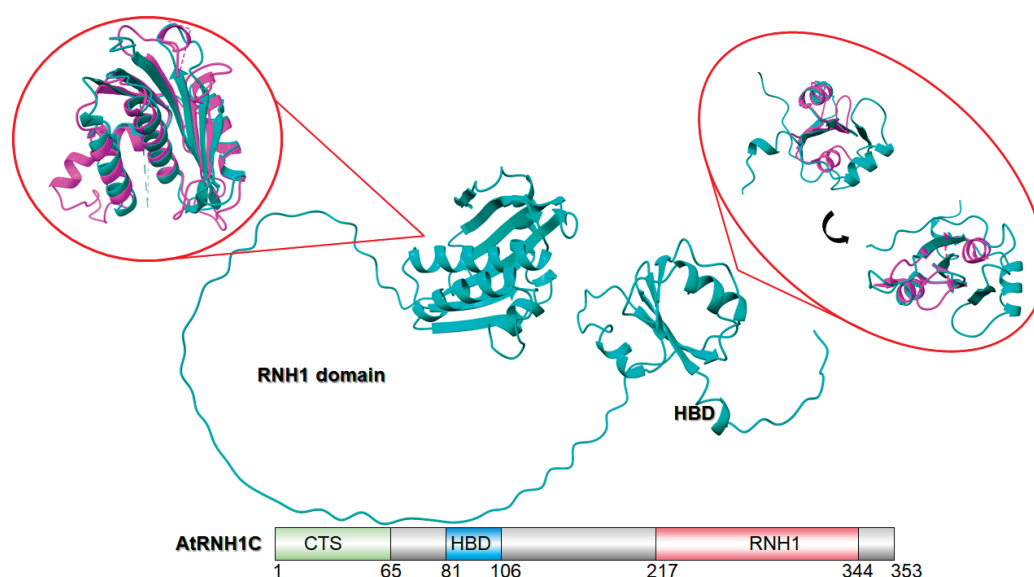
subsequent stages and be transmitted to the next generation [3,8,9]. Thus, R-loops are present throughout the cell cycle and influence a wide range of physiological and pathological processes [3,10,11]. The beneficial functions of R-loops encompass immunoglobulin class switch recombination, the regulation of gene expression, DNA replication, DNA repair, and the control of transcription initiation and termination [12,13]. Nevertheless, the aberrant accumulation of R-loops in the genome, whether in timing or location, exerts detrimental effects. These include transcriptional defects, transcription–replication conflicts, cell cycle arrest, and genomic instability [10,14–16]. This occurs because R-loops can block replication fork progression by provoking transcription–replication conflicts or by recruiting specific endonucleases that cause DNA cleavage. Consequently, cells have evolved multiple mechanisms to maintain R-loop homeostasis, which involve preventing R-loop formation, resolving existing R-loops, or repairing the resulting DNA damage such as fork blocks or DNA breaks [17–20].

Maintenance of R-loop homeostasis depends on a delicate balance between their formation and resolution. The proper regulation of R-loops necessitates a multitude of factors, given their genomic prevalence and broad functional influence. Essential to this control is a dedicated network of enzymes that modulates R-loop levels [18–22]. Helicases, enzymes that unwind nucleic acids, can prevent the formation of secondary structures [23]. Interactome analysis of RNA/DNA hybrids has revealed that many helicases, in addition to other proteins—including topoisomerases, RNA processing factors, and chromatin proteins—function as potential R-loop regulators [24–27]. Rho-n domain-containing protein 1 (RHON1) is the only identified RNA/DNA helicase in plants known to resolve R-loops caused by head-on transcription–replication conflicts in the *Arabidopsis* chloroplast genome [2,28]. By relaxing supercoiled DNA to relieve topological stress, DNA topoisomerases play a key role in replication and transcription. The absence of Topoisomerase I (TOP1) in human cells results in context-dependent alterations to R-loop levels, characterized by an increase at long, highly transcribed genes and a decrease at early replication origin-associated loci [29]. A conserved role is observed in rice, where TOP1 is indispensable for preventing R-loop accumulation on auxin-related genes to ensure proper root development [2]. Chromatin condensation, which influences transcription and replication states, is also associated with genome-wide R-loop formation [30]. This condensation is modulated by various histone modifications and the deposition of histone variants, processes carried out by chromatin remodelers [31]. Although chromatin remodelers are strongly associated with R-loop formation *in vivo* and recent studies have connected them to R-loops [30], direct evidence of their role in R-loop modulation or their mechanistic contribution to R-loop regulation remains scarce. Few plant chromatin remodelers have been directly connected with different stress responses [32,33].

The RNase H enzyme family, which includes RNase H1 and RNase H2, is evolutionarily conserved and specializes in degrading the RNA component of DNA/RNA hybrids [34], making these enzymes key players in R-loop resolution [35,36]. In *A. thaliana*, three genes encode RNase H1-like proteins: RNH1A (nuclear), RNH1B (mitochondrial), and RNH1C (chloroplast) [2,37]. The mitochondrial RNase H1, AtRNH1B, plays a critical role in maintaining R-loop homeostasis to preserve mitochondrial genome integrity and support early embryogenesis [2]. Specifically, AtRNH1B prevents genome instability by suppressing ectopic homologous recombination at intermediate-sized repeats in mitochondrial DNA. When AtRNH1B is absent, the chloroplast-localized RNase H1, AtRNH1C, relocates to mitochondria, compensating for the loss and ensuring genome stability. In chloroplasts, which possess their own genome [38], nucleoids are associated with thylakoid membranes [39]—a location that makes them vulnerable to environmental fluctuations, including DNA-damaging agents produced during photosynthesis [40,41]. Studies indicate

that AtRNH1C is essential for regulating R-loop levels to maintain chloroplast genome stability, a process that requires collaboration with DNA gyrases [2]. Double mutants lacking both AtRNH1B and AtRNH1C demonstrated embryonic lethality due to R-loop accumulation, impaired mitochondrial DNA replication, and mitochondrial dysfunction, highlighting a facultative dual targeting mechanism between organelles [2].

RNase H1s are ubiquitous enzymes found in nearly all living organisms. Their catalytic domain, spanning approximately 150 amino acids (aa), is evolutionarily conserved [42]. In eukaryotes, RNase H1 contains an N-terminal mitochondrial targeting sequence (MTS) [43] and an additional ~50 aa region known as the hybrid binding domain (HBD) [44]. The HBD is linked to the C-terminal catalytic domain by a flexible linker, which is essential for RNase H1 activity [45]. This structural arrangement confers significant flexibility, enabling the N- and C-terminal domains to reposition relative to one another. Figure 1 displays the AlphaFold-predicted structure of AtRNH1C. When superimposed with human RNase H1 (highlighted in red circles in Figure 1, the RNH1 domains show high structural similarity, while the HBD domains exhibit only partial convergence. The HBD is critical for the processive cleavage pattern unique to eukaryotic RNase H1. In yeast, mouse, and human enzymes, deletion of the HBD abolishes processivity, resulting in distributive cleavage of long DNA/RNA substrates—similar to the behavior of *E. coli* RNase H1, which naturally lacks an HBD [46]. A proposed model suggests that processive RNA cleavage involves multiple hydrolysis events triggered by a single HBD-mediated binding event. This mechanism generates short RNA fragments that readily dissociate from the DNA template [47]. Such processive cleavage is essential for efficiently degrading long substrates.



**Figure 1.** AlphaFold-predicted structure of AtRNH1C (AF-F4I7R5-F1-model\_v4) and its domain composition. Red circles include the superposition of AtRNH1C and HBD domains with those of human RNase H1 (human RNH1—PDB ID 2KQ9; human HBD—PDB ID 3BSU). CTS, chloroplast targeting sequence (green on domain composition panel); HBD, hybrid binding domain (blue on domain composition panel); RNH1, RNase H1 domain (red on domain composition panel). The predicted structure represents the mature protein after the CTS has been cleaved.

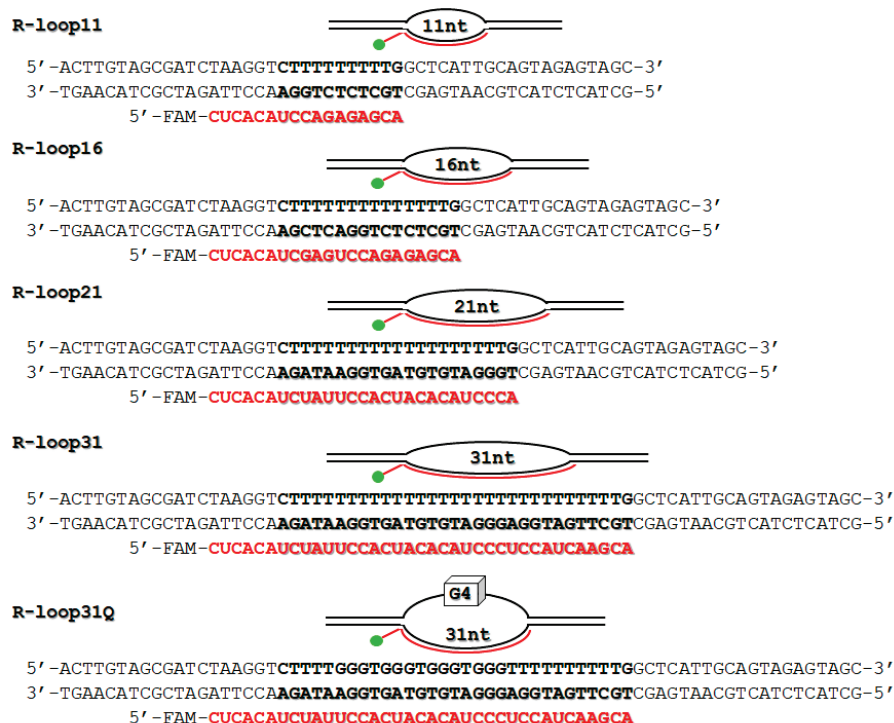
While RNase H1's role in R-loop resolution and genome stability is well-documented in eukaryotic nuclei, the molecular functions of organelle-localized RNase H1 remain poorly understood. In *A. thaliana*, nuclear AtRNH1A and mitochondrial AtRNH1B are dispensable for development under standard laboratory conditions [37], whereas AtRNH1C is essential for chloroplast development [48]. However, its enzymatic activity has yet to be verified experimentally. This work directly addresses this gap by moving beyond genetic evidence

to provide the first direct in vitro biochemical analysis of AtRNH1C. We characterize the enzymatic activity of recombinant AtRNH1C toward model R-loops in vitro. It was shown that AtRNH1C exhibits a strong preference for short R-loop structures (11 bp), which mirrors the natural length of hybrids found in transcription elongation complexes. The kinetic and mechanistic analysis reveals that AtRNH1C operates via a two-step mechanism common to bacterial RNase H1, where the rate-limiting step is the formation of the catalytic complex. The calculated rate constant is remarkably similar to that of *E. coli* RNase H1, underscoring a deeply conserved evolutionary strategy for R-loop resolution across billions of years of evolution.

## 2. Results

### 2.1. Design of R-Loops

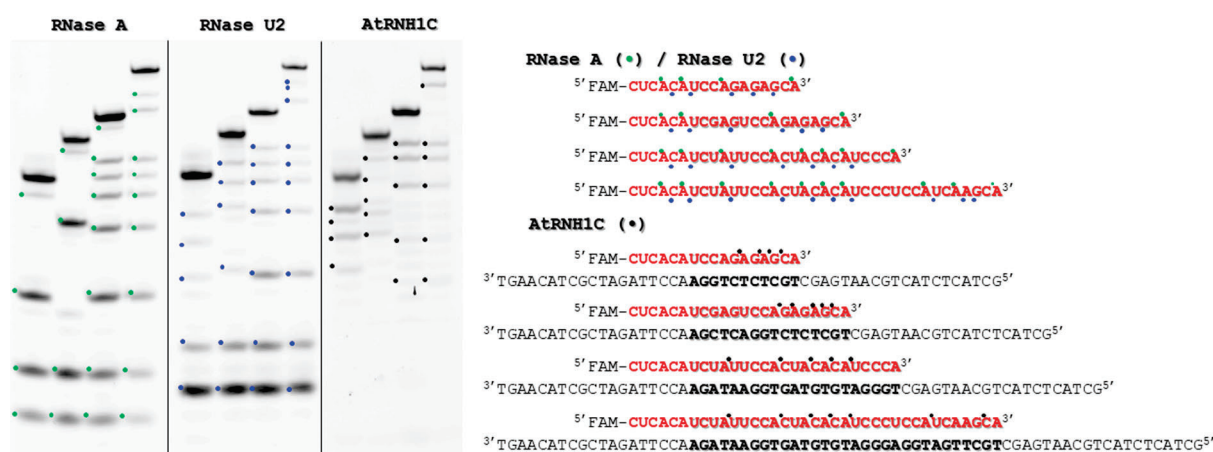
In transcription elongation complexes, a specialized wedge-like structure in RNA polymerase limits the DNA/RNA hybrid to approximately 10–11 base pairs (bp) [49]. Therefore, the R-loop structure with 11 bp heteroduplex length (R-loop11) was used as a “natural” model (Figure 2). To systematically evaluate the influence of hybrid length, extended variants—R-loop16, R-loop21, and R-loop31—were also utilized. All oligonucleotide sequences were deliberately designed to ensure stable R-loop formation and to avoid intramolecular hairpins. Furthermore, the R-loop31Q variant was incorporated, featuring a G-quadruplex (G4) motif in the displaced non-template DNA strand, to probe the effects of DNA secondary structure on AtRNH1C function. This is relevant as G-rich sequences can form stable G4 structures that contribute to R-loop stability and are implicated in regulating genes for stress response and DNA damage [50–52]. The R-loops’ assembly was confirmed using EMSA as previously described [53].



**Figure 2.** Oligonucleotide sequences and model R-loop structures. DNA strands colored black, RNA-primer colored red. Bold sequence denotes the DNA/RNA heteroduplex region. 6-carboxyfluorescein (FAM, green circles) was attached to the 5' end of the RNA-primer.

## 2.2. Sequence Preferences of Purified AtRNH1C

Site and sequence preferences for cleavage by AtRNH1C were identified using R-loops (Figure 2) with different lengths and nucleotide sequences of the hybrid region. The RNases A and U2 were used as control enzymes. The RNA cleavage occurred at the hybrid regions at Pu↓X sites with preferred cleavage at a G↓X dinucleotide (Figure 3).

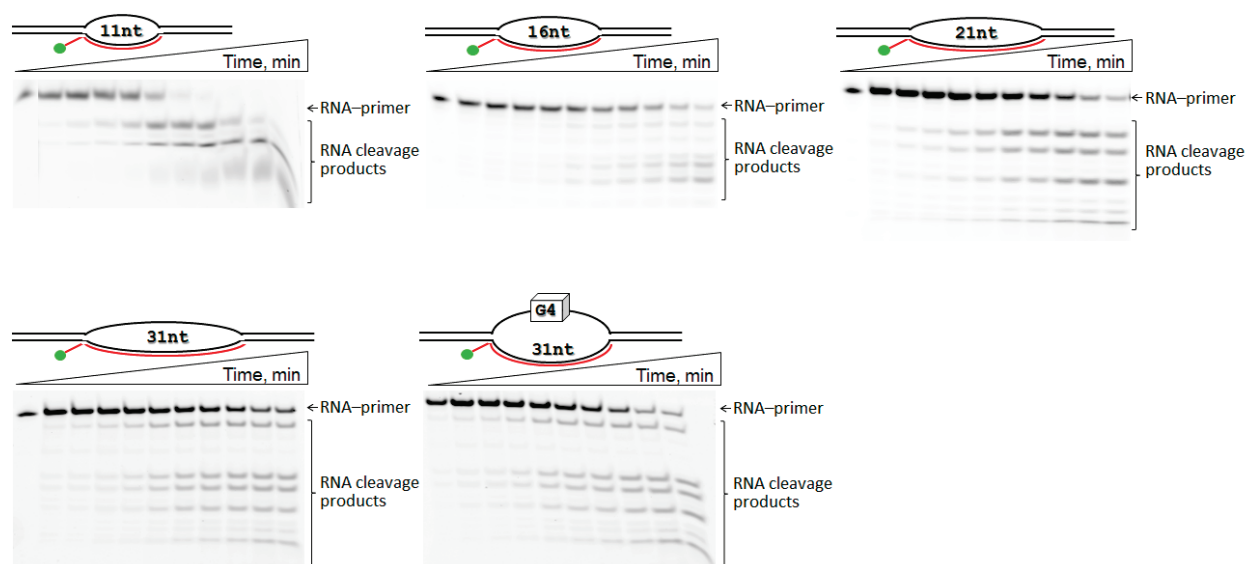


**Figure 3.** Analysis of AtRNH1C cleavage of DNA/RNA heteroduplexes. RNases A and U2 were used as control enzymes. The digestion of RNA (for RNase A and U2) or DNA/RNA heteroduplexes (for AtRNH1C) was performed as described in Section 4. For each substrate, the RNA sequences are shown. RNA chains are colored in red, DNA chains are colored in black and heteroduplex moieties are highlighted in bold. The circles indicate the sites of the enzymes digestion: green for RNase A, blue for RNase U2, and black for AtRNH1C.

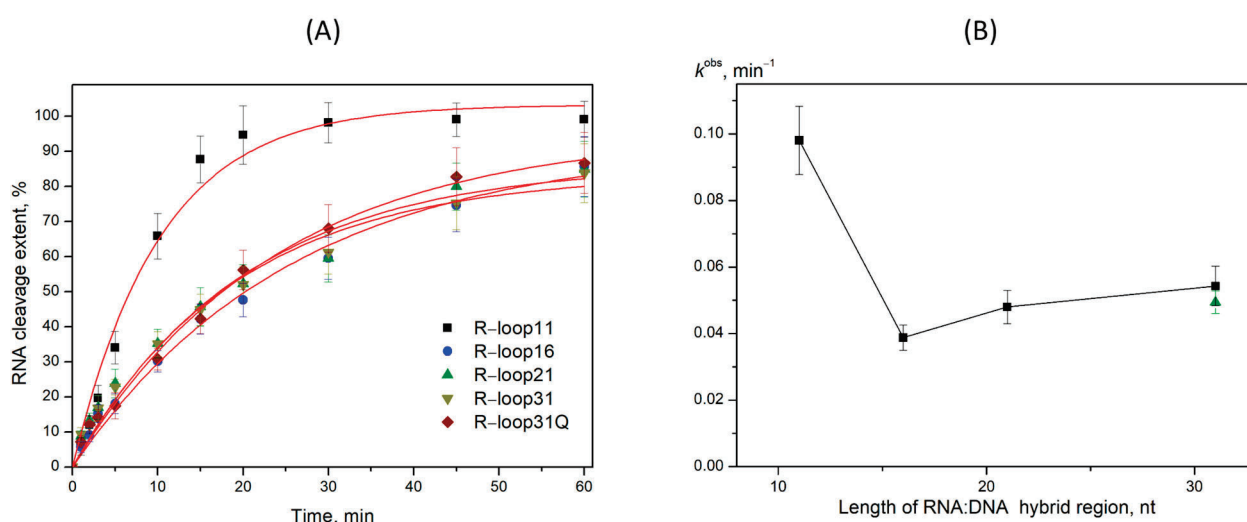
The obtained data demonstrated the AtRNH1C preference for a purine-rich sequence context. It is interesting to note that the efficient cleavage of the RNA strand in the sequence context CA↓AG, with a preference for the G-rich sequence upstream of the cleavage site, has been shown for *E. coli* RNase H1 and the catalytic domain of human RNase H1 [54].

## 2.3. R-Loop Degradation Activity

A kinetic analysis of RNA primer cleavage by AtRNH1C in various R-loops was performed (Figure 4). As shown in the polyacrylamide gels, the full-length RNA primer disappeared over time, with a corresponding accumulation of multiple shorter product bands. For instance, in the R-loop11 substrate, the full-length RNA band was completely depleted within 15–20 min. The accumulation of these shortened RNA products is expected to destabilize the DNA/RNA hybrid, leading to R-loop resolution. The resulting kinetic curves for the cleavage reaction were well fitted by a single-exponential equation, allowing for the determination of the observed rate constant ( $k^{obs}$ ). The values of observed rate constant for R-loop16–31 were decreased with increasing hybrid region length (Figure 5). Moreover, R-loop11 showed the highest  $k^{obs}$  value among the substrates investigated (Figure 5). Recently, we performed the investigation of the kinetic features of the interaction of RNase H1 from *Escherichia coli* (EcRNH1) with similar model R-loops [53]. It was also found that EcRNH1 bound most effectively to R-loops with a 10–11 nt DNA/RNA hybrid region. The rate-limiting step in RNA degradation by EcRNH1 was identified as the formation of the catalytic complex. We therefore propose that the lower cleavage efficiency of AtRNH1C toward the larger R-loop16–31 is caused by both a higher number of potential “landing sites” in the longer heteroduplex and less efficient formation of the catalytic complex.



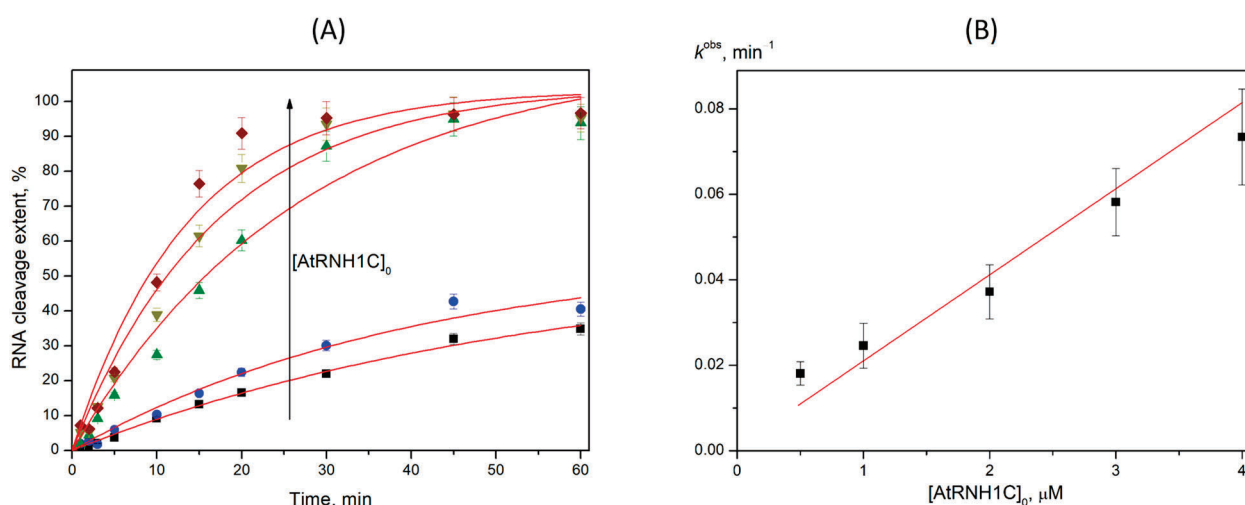
**Figure 4.** PAAG analysis of AtRNH1C-driven cleavage of the RNA in model R-loops. [AtRNH1C] = 2  $\mu$ M, [R-loop] = 1  $\mu$ M. Time: 0, 1, 2, 3, 5, 10, 15, 20, 30, 45, and 60 min. RNA and DNA chains are highlighted in red and black, respectively.



**Figure 5.** Kinetics of RNA cleavage by AtRNH1C in model R-loops. **(A)** Time-dependent cleavage of the RNA strand. The RNA cleavage extent was calculated as the ratio of the summed peak areas for all cleavage fragments to the total peak area (fragments and initial substrate). **(B)** Observed rate constants ( $k^{obs}$ ) derived from the kinetic curves in **(A)**. The data for R-loop31Q is denoted by a triangle ( $\blacktriangle$ ). All data points represent the mean  $\pm$  SD of three independent experiments.

A kinetic analysis of R-loop11 cleavage was conducted using 1  $\mu$ M substrate and AtRNH1C concentrations between 0.5 and 4  $\mu$ M (Figure 6). The cleavage time courses fit well to a single-exponential function, from which the observed rate constant ( $k^{obs}$ ) was derived for each enzyme concentration. A linear relationship was observed between  $k^{obs}$  and the initial AtRNH1C concentration. These data support that the kinetic scheme of AtRNH1C action could be described by a two-step mechanism (Scheme 1) if the rate constant of the catalytic reaction  $k_{cat}$  satisfies the condition  $k_{cat} \gg S \times k_1$ . The same dependence of RNA cleavage was previously reported for R-loop degradation by EcRNH1 [53]. A linear dependence of  $k^{obs}$  on enzyme concentration allows us to calculate an apparent second-order rate constant ( $k_1$ ), which is effectively  $k_{cat}/K_m$  under this regime. The calculated value of  $k_1$  is  $0.020 \pm 0.002 \text{ min}^{-1} \times \mu\text{M}^{-1}$  ( $1.2 \times 10^6 \text{ s}^{-1} \times \text{M}^{-1}$ ) in good agreement

with the rate constant reported for EcRNH1, which ranged from  $0.58 \times 10^6 \text{ s}^{-1} \times \text{M}^{-1}$  to  $6.5 \times 10^6 \text{ s}^{-1} \times \text{M}^{-1}$  depending on the R-loop structure [53]. Therefore, it could be concluded that the efficiency of R-loop degradation by both RNase H1s mostly depends on the rate of the catalytic complex's formation and proceeds by the same mechanism (Scheme 1).



**Figure 6.** Enzyme concentration dependence of R-loop11 cleavage by AtRNH1C. (A) Progress curves of RNA cleavage at varying enzyme concentrations (0.5–4  $\mu\text{M}$ ). The RNA cleavage extent was calculated as the total area of product peaks divided by the sum of the product and initial substrate peak areas. Symbol traces represent experimental data (AtRNH1C,  $\mu\text{M}$ : ■ 0.5; • 1; ▲ 2; ▼ 3; ♦ 4); red curves are the results of fitting to a single-exponential function. Values are the mean of three independent experiments. (B) Linear dependence of the observed rate constant ( $k^{\text{obs}}$ ) on AtRNH1C concentration.



**Scheme 1.** The kinetic scheme of the AtRNH1C-driven cleavage of the R-loop. E: AtRNH1C; S: an R-loop; E•S: the catalytic complex of the R-loop with the enzyme; P: a reaction product.

### 3. Discussion

This study provides the first comparative analysis of the R-loop degradation activity of the chloroplast-localized RNase H1 enzyme from *Arabidopsis thaliana*. The research demonstrates that recombinant AtRNH1C is an active enzyme that cleaves the RNA within DNA/RNA, with a distinct preference for purine-rich sequences—particularly at G↓X dinucleotides. This cleavage specificity aligns with previous observations for both *E. coli* and human RNase H1, suggesting an evolutionarily conserved catalytic mechanism across kingdoms. Kinetic analysis revealed that the enzyme's efficiency is highly dependent on the length of the DNA/RNA hybrid region. The most efficiently processed substrate was R-loop11, which mimics the natural ~11 bp hybrid length maintained by RNA polymerase in transcription elongation complexes. The efficiency of RNA degradation decreased as the hybrid length increased (R-loop16, -21, and -31), indicating that AtRNH1C is exquisitely tuned to resolve the short, transient R-loops that occur co-transcriptionally, rather than processing excessively long, stable structures.

According to the proposed model for DNA/RNA hybrid degradation by human RNase H1 [47], the HBD enhances RNase H1's affinity for DNA/RNA hybrids. In human RNase H1, the long linker (60 aa) appears to moderately enhance substrate binding (approximately 2.5-fold), but it also enables the catalytic domain to interact with the substrate at multiple sites, which may be important for increased processivity [47]. After hydrolysis, when the catalytic RNH1 domain separates from the cleavage product, the HBD may

anchor the enzyme (or at least promote its reassociation) to the substrate, allowing another cleavage event nearby [47]. This processive behavior allows the enzyme to generate smaller nucleotide products compared to the catalytic domain acting alone. It is important to note that processivity was most evident during the degradation of long hybrids and had minimal impact on shorter substrates [46], as only minor differences in degradation product distribution were seen between *E. coli* RNase H1 and mouse RNase H1 [46]. In AtRNH1C, the flexible linker between the HBD and RNH1 domain is 111 aa long. It is reasonable to assume that its effect on substrate binding may also be moderate. We did not investigate the HBD's influence on substrate binding because, despite extensive efforts, we could not produce a soluble recombinant protein of the truncated AtRNH1C without the HBD domain. The model R-loop structures we used contained a DNA/RNA hybrid no longer than 31 bp, so estimating processivity would be highly speculative. What are the possible lengths of R-loops? Studies indicate that R-loops can vary from a few base pairs to several hundred base pairs [12,55]. Although R-loop formation is typically associated with transcription [56], evidence shows that some R-loops can form independently of transcription, especially at DNA damage sites such as double-strand breaks [57], or through mechanisms related to DNA replication or the activity of certain non-B DNA structures and enzymes that can destabilize DNA duplexes. These transcription-independent pathways can still produce the characteristic DNA/RNA hybrid and displaced single-stranded DNA of R-loops, although they may also overlap or interact with transcription-dependent processes [5,58]. Clearly, further experiments are needed to directly test AtRNH1C processivity and the possible effects of the HBD on kinetics, processivity, or substrate preference.

The kinetic data support a two-step mechanism for AtRNH1C action, where the rate-limiting step is the formation of the initial catalytic complex (E•S). Thus, R-loop degradation by AtRNH1C is mostly dependent on the rate of catalytic complex formation and proceeds by the same mechanism as described for EcRNH1. Moreover, the rate constant of the catalytic complex formation for AtRNH1C is in good agreement with the rate constants reported for EcRNH1, suggesting a biologically conserved role of these enzymes in R-loop degradation. This remarkable conservation underscores a fundamental and shared enzymatic strategy for R-loop resolution, despite the phylogenetic distance between bacteria and plants. The presence of a G-quadruplex (G4) structure in the displaced DNA strand (R-loop31Q) did not significantly alter the cleavage rate, suggesting that AtRNH1C's activity is primarily focused on the hybrid duplex itself and is robust against certain DNA secondary structures. The observed rate constant values of RNA cleavage by AtRNH1C in model R-loops show a drop in meaning between 11 and 16 nt followed by a plateau (Figure 5B). According to structural data, the catalytic domain of human RNase H1 interacts with 11 nt of the DNA/RNA hybrid [59]. It can be proposed that in the case of R-loop11, the higher efficacy of RNA cleavage reflected the effective binding of the DNA/RNA hybrid part by the RNH1 domain. The observed substrate preference of AtRNH1C, as well as the most efficient cleavage of the natural-like short R-loop11 generated in a transcription elongation complex, positions RNase H1 as one of important regulators of R-loop homeostasis in chloroplasts.

In a broader biological context, these findings position AtRNH1C as an important guardian of chloroplast genome integrity. Its optimized activity against short, transcription-associated R-loops suggests its role in preventing the accumulation of these structures, which could otherwise lead to transcription–replication conflicts, DNA damage, and impaired organelle function. By efficiently resolving R-loops, AtRNH1C collaborates with other enzymes like DNA gyrases to ensure the smooth progression of replication and transcription, thereby maintaining stability in the genetically vital chloroplast genome.

In summary, this work moves beyond genetic evidence and provides direct in vitro enzymatic validation of AtRNH1C's function. It reveals the conserved kinetic and mechanistic principles that underpin R-loop degradation by RNase H1 enzymes and highlights the specific adaptation of AtRNH1C for maintaining homeostasis in the unique environment of the plant chloroplast. Taken together, this work elucidates the molecular mechanism of a plant organelle-specific RNase H1. Future investigations of the potential protein–protein interactions between AtRNH1C and other known chloroplast R-loop regulators, such as DNA gyrases or the helicase RHON1, as well as the comparison of the enzymatic properties of AtRNH1C with those of other RNase H1 isoforms (mitochondrial and nuclear) can provide the understanding of its functional specialization. For AtRNH1A and AtRNH1B, the canonical RNase H1 activity has been shown [37]; however, kinetic and mechanistic analysis was absent. Taking into account that the presence of AtRNH1B or AtRNH1C is required for viability, a direct kinetic comparison could provide a molecular basis for understanding the facultative dual targeting.

## 4. Materials and Methods

### 4.1. AtRNH1C Expression and Purification

The pGEX-4T-1-AtRNH1C plasmid encoding the wild-type of *A. thaliana* RNH1C was kindly provided by Prof. Qianwen Sun. AtRNH1C was produced in *E. coli* ArcticExpress (DE3) cells (1 L of 2YT medium with 20 µg/mL gentamicin, 10 µg/mL tetracycline, and 50 µg/mL ampicillin, shaking speed of 200 rpm) induced with 0.2 mM 1-thio-β-D-galactopyranoside (added after the optical density at 600 nm reached 0.6–0.7) at 37 °C for 3 h, providing recombinant protein expression in soluble form. The cells were then harvested (20 min at 5000× *g* at 4 °C) and suspended in 20 mM HEPES–KOH (pH 7.8), 140 mM NaCl, 5 mM DTT, and a mixture of protease inhibitors (Inhibitor cocktail, Complete, Mannheim, Germany). Cells were lysed using a French press and all subsequent procedures were performed at 4 °C. The lysate was filtered through a 0.45 µm MCE Syringe Filter (Labfil, ALWSCI Technologies Co., Hangzhou, China). The clarified lysate was incubated with 1 mL of GST-resin (Glutathione Sepharore™ 4B, Uppsala, Sweden) at 4 °C for 1 h with gentle shaking. The resin was washed 3 times by 10 mL of 50 mM Tris–HCl (pH 8.0) with 140 mM NaCl and protein was eluted by 3 mL of 50 mM Tris–HCl (pH 8.0) with 140 mM NaCl and 10 mM reduced glutathione. Protein purity was examined using SDS–polyacrylamide gel electrophoresis. The qualitative assessment of protein purity showed the absence of contaminant proteins (Figure S1). The removal of the GST tag by thrombin resulted in protein precipitation. In order to exclude protein precipitation, the stage of GST tag cleavage was not carried out. The purified AtRNH1C was concentrated with a 30 kDa JetSpin Centrifugal Filter (Jet Bio–Filtration Co., Ltd., Guangzhou, China), and the resulting protein was stored at –20 °C in a buffer consisting of 25 mM Tris–HCl (pH 8.0), 70 mM NaCl, 5mM reduced glutathione, and 50% glycerol. The purified protein yield was 2.1 mg from a 1 L bacterial culture. The enzyme concentration was calculated from the optical density of the protein at 280 nm and a molar extinction coefficient of 68,480 M<sup>–1</sup> × cm<sup>–1</sup>. All experiments were conducted in an autoclaved H<sub>2</sub>O treated with 0.1% DEPC (37 °C, 2 h) unless otherwise stated.

### 4.2. Preparation of R-Loops

The sequences of the model R-loops are shown in Figure 2. DNA oligonucleotides were purchased from Biosset Ltd. (Novosibirsk, Russia). RNA oligonucleotides with a 5'-FAM label were synthesized by the Laboratory of RNA Chemistry at ICBFM SB RAS (Novosibirsk, Russia), as previously described [60]. Oligonucleotide concentrations were determined by measuring absorbance at 260 nm, and the R-loops were assembled according

to an established protocol [53]. Briefly, for R-loops11, -16, -21, and -31, non-template DNA strand, template DNA strand, and RNA primer at a 1:1:1 molar ratio were annealed in  $1\times$  buffer containing 20 mM HEPES-KOH (pH 7.2), 75 mM NaCl, 5 mM  $\text{MgCl}_2$ , and 1 mM DTT. For R-loop31Q, non-template DNA strand, template DNA strand, and RNA primer at a 1:1:1 molar ratio were annealed in  $1\times$  buffer containing 10 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.5), 1 mM EDTA, 1 mM DTT, and 140 mM KCl. The formation of R-loops was analyzed by an electrophoretic mobility shift assay (EMSA) following a previously established method [53]. The EMSA was conducted in a nondenaturing 10% polyacrylamide gel (75:1) using  $0.5\times$  TBE buffer supplemented with 5 mM  $\text{MgCl}_2$ , at a constant current of 10 mA and a temperature of  $4^\circ\text{C}$  (Figure S2). The annealed  $10\times$  R-loop solutions were stored at  $-20^\circ\text{C}$ .

#### 4.3. Cleavage Site Analysis

Site and sequence preferences for cleavage by AtRNH1C were identified using R-loops (Figure 2). For RNases A and U2, used as control enzymes, the cleavage of single-stranded RNA primers was analyzed as these enzymes degrade single-stranded RNA. Partial hydrolysis of single-stranded RNA primers with RNase A was performed as follows: a  $20\ \mu\text{L}$  reaction mixture containing  $1.0\ \mu\text{M}$  substrate and  $13\ \text{pM}$  RNase A in buffer (50 mM Tris-HCl pH 8.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 9% glycerol) was incubated at  $25^\circ\text{C}$  for 1 min [61,62]. For RNase U2, a  $20\ \mu\text{L}$  reaction containing  $1.0\ \mu\text{M}$  substrate and  $2.7\ \mu\text{M}$  enzyme in buffer (50 mM  $\text{AcNH}_4$ , 1 mM EDTA, 1 mM DTT, and 9% glycerol) was incubated at  $25^\circ\text{C}$  for 5 min. For AtRNH1C, a  $20\ \mu\text{L}$  reaction containing  $1.0\ \mu\text{M}$  substrate and  $2.0\ \mu\text{M}$  enzyme in buffer (20 mM HEPES-KOH pH 7.2, 75 mM NaCl, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, and 5% glycerol) was incubated at  $25^\circ\text{C}$  for 30 min. The reactions were stopped by addition of an equal volume of a solution containing 9 M urea, 25 mM EDTA, 0.1% of xylene cyanole, and 0.1% of bromophenol blue followed by incubation at  $96^\circ\text{C}$  for 5 min. The products were analyzed on 15% denaturing TBE-polyacrylamide gels using a vertical Protean II xi unit (Bio-Rad Laboratories, Hercules, CA, USA) at 200–300 V and  $45^\circ\text{C}$ . All data were obtained at three replicates. Gels were visualized with a VersaDoc gel-documenting system (Bio-Rad Laboratories, Hercules, CA, USA).

#### 4.4. Activity Assay

The reaction was conducted at  $25^\circ\text{C}$  in buffer containing 20 mM HEPES-KOH (pH 7.2), 75 mM NaCl, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, and 5% glycerol with various enzyme/substrate ratios (enzyme concentrations, 0.5–4  $\mu\text{M}$ ; R-loop concentration,  $1\ \mu\text{M}$ ). The reaction was stopped at certain time intervals by addition of an equal volume of a solution containing 9 M urea, 25 mM EDTA, 0.1% of xylene cyanole, and 0.1% of bromophenol followed by incubation at  $96^\circ\text{C}$  for 5 min. A no-enzyme control was included in the first lane of all gels. Reaction products were resolved on 15% denaturing TBE-PAGE gels (Bio-Rad Protean II xi unit, Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 200–300 V and  $45^\circ\text{C}$  and imaged using a VersaDoc system (Bio-Rad Laboratories, Hercules, CA, USA). Quantification was performed with the GelAnalyzer 23.1.1 (©Istvan Lazar Jr.), where the transformation degree was calculated as follows: (sum of cleavage fragment peaks)/(sum of product peaks + initial substrate peak). Data from three replicates (mean  $\pm$  SD) were fitted in OriginPro 8.1 (OriginLab Corporation, Northampton, MA, USA) to the equation  $[\text{Product}] = A \times (1 - \exp(-k^{\text{obs}} \times t))$ , where  $A$  is the amplitude,  $k^{\text{obs}}$  is the observed rate constant, and  $t$  is the reaction time. The observed rate constants ( $k^{\text{obs}}$ ) were then plotted against the enzyme concentration and fitted linearly ( $k^{\text{obs}} = k_1 \times [\text{AtRNH1C}]$ ) [63].

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ijms262211125/s1>.

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## References

1. Xu, W.; Xu, H.; Li, K.; Fan, Y.; Liu, Y.; Yang, X.; Sun, Q. The R-Loop Is a Common Chromatin Feature of the Arabidopsis Genome. *Nat. Plants* **2017**, *3*, 704–714. [CrossRef]
2. Zhou, J.; Zhang, W.; Sun, Q. R-loop: The New Genome Regulatory Element in Plants. *J. Integr. Plant Biol.* **2022**, *64*, 2275–2289. [CrossRef]
3. Xu, Y.; Jiao, Y.; Liu, C.; Miao, R.; Liu, C.; Wang, Y.; Ma, C.; Liu, J. R-Loop and Diseases: The Cell Cycle Matters. *Mol. Cancer* **2024**, *23*, 84. [CrossRef]
4. Zhang, S.; Liu, Y.; Sun, Y.; Liu, Q.; Gu, Y.; Huang, Y.; Zeng, Z.; Tang, F.; Ouyang, Y. Aberrant R-Loop-Mediated Immune Evasion, Cellular Communication, and Metabolic Reprogramming Affect Cancer Progression: A Single-Cell Analysis. *Mol. Cancer* **2024**, *23*, 11. [CrossRef]
5. Brickner, J.R.; Garzon, J.L.; Cimprich, K.A. Walking a Tightrope: The Complex Balancing Act of R-Loops in Genome Stability. *Mol. Cell* **2022**, *82*, 2267–2297. [CrossRef]
6. Khan, E.S.; Danckwardt, S. Pathophysiological Role and Diagnostic Potential of R-Loops in Cancer and Beyond. *Genes* **2022**, *13*, 2181. [CrossRef]
7. Marabitti, V.; Valenzisi, P.; Lillo, G.; Malacaria, E.; Palermo, V.; Pichierri, P.; Franchitto, A. R-Loop-Associated Genomic Instability and Implication of WRN and WRNIP1. *Int. J. Mol. Sci.* **2022**, *23*, 1547. [CrossRef]
8. San Martín-Alonso, M.; Soler-Oliva, M.E.; García-Rubio, M.; García-Muse, T.; Aguilera, A. Harmful R-Loops Are Prevented via Different Cell Cycle-Specific Mechanisms. *Nat. Commun.* **2021**, *12*, 4451. [CrossRef] [PubMed]
9. Hirayama, M.; Shinriki, S.; Matsui, H. R-Loops in Normal and Malignant Hematopoiesis. *Front. Hematol.* **2023**, *2*, 1297657. [CrossRef]
10. Skourti-Stathaki, K.; Proudfoot, N.J. A Double-Edged Sword: R Loops as Threats to Genome Integrity and Powerful Regulators of Gene Expression. *Genes Dev.* **2014**, *28*, 1384–1396. [CrossRef] [PubMed]
11. Zhang, F.; Wang, D.; Zhao, G.; Wang, D. Unraveling R-Loops: The Hidden Drivers of Inflammation and Immune Dysregulation. *Medicine* **2025**, *104*, e42833. [CrossRef]
12. Castillo-Guzman, D.; Chédin, F. Defining R-Loop Classes and Their Contributions to Genome Instability. *DNA Repair* **2021**, *106*, 103182. [CrossRef]
13. García-Muse, T.; Aguilera, A. R Loops: From Physiological to Pathological Roles. *Cell* **2019**, *179*, 604–618. [CrossRef] [PubMed]
14. Li, F.; Zafar, A.; Luo, L.; Denning, A.M.; Gu, J.; Bennett, A.; Yuan, F.; Zhang, Y. R-Loops in Genome Instability and Cancer. *Cancers* **2023**, *15*, 4986. [CrossRef] [PubMed]
15. Westover, K.R.; Jin, P.; Yao, B. Bridging the Gap: R-Loop Mediated Genomic Instability and Its Implications in Neurological Diseases. *Epigenomics* **2024**, *16*, 589–608. [CrossRef] [PubMed]
16. Hegazy, Y.A.; Fernando, C.M.; Tran, E.J. The Balancing Act of R-Loop Biology: The Good, the Bad, and the Ugly. *J. Biol. Chem.* **2020**, *295*, 905–913. [CrossRef]

17. Aguilera, P.; Aguilera, A. R-Loop Homeostasis in Genome Dynamics, Gene Expression and Development. *Curr. Opin. Genet. Dev.* **2025**, *92*, 102325. [CrossRef]
18. Zhu, M.; Wang, X.; Zhao, H.; Wang, Z. Update on R-Loops in Genomic Integrity: Formation, Functions, and Implications for Human Diseases. *Genes Dis.* **2025**, *12*, 101401. [CrossRef]
19. Lee, S.Y.; Kwak, M.J.; Kim, J.J. R-Loops: A Key Driver of Inflammatory Responses in Cancer. *Exp. Mol. Med.* **2025**, *57*, 1455–1466. [CrossRef]
20. Niehrs, C.; Luke, B. Regulatory R-Loops as Facilitators of Gene Expression and Genome Stability. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 167–178. [CrossRef]
21. Liu, J.; Li, F.; Cao, Y.; Lv, Y.; Lei, K.; Tu, Z.; Gong, C.; Wang, H.; Liu, F.; Huang, K. Mechanisms Underlining R-Loop Biology and Implications for Human Disease. *Front. Cell Dev. Biol.* **2025**, *13*, 1537731. [CrossRef]
22. Gong, Y.; Liu, Y. R-Loops at Chromosome Ends: From Formation, Regulation, and Cellular Consequence. *Cancers* **2023**, *15*, 2178. [CrossRef]
23. Singleton, M.R.; Dillingham, M.S.; Wigley, D.B. Structure and Mechanism of Helicases and Nucleic Acid Translocases. *Annu. Rev. Biochem.* **2007**, *76*, 23–50. [CrossRef]
24. Cristini, A.; Groh, M.; Kristiansen, M.S.; Gromak, N. RNA/DNA Hybrid Interactome Identifies DXH9 as a Molecular Player in Transcriptional Termination and R-Loop-Associated DNA Damage. *Cell Rep.* **2018**, *23*, 1891–1905. [CrossRef] [PubMed]
25. Song, C.; Hotz-Wagenblatt, A.; Voit, R.; Grummt, I. SIRT7 and the DEAD-Box Helicase DDX21 Cooperate to Resolve Genomic R Loops and Safeguard Genome Stability. *Genes Dev.* **2017**, *31*, 1370–1381. [CrossRef]
26. Weinreb, J.T.; Ghazale, N.; Pradhan, K.; Gupta, V.; Potts, K.S.; Tricomi, B.; Daniels, N.J.; Padgett, R.A.; De Oliveira, S.; Verma, A.; et al. Excessive R-Loops Trigger an Inflammatory Cascade Leading to Increased HSPC Production. *Dev. Cell* **2021**, *56*, 627–640.e5. [CrossRef]
27. Mersaoui, S.Y.; Yu, Z.; Coulombe, Y.; Karam, M.; Busatto, F.F.; Masson, J.; Richard, S. Arginine Methylation of the DDX5 Helicase RGG/RG Motif by PRMT5 Regulates Resolution of RNA:DNA Hybrids. *EMBO J.* **2019**, *38*, e100986. [CrossRef]
28. Yang, Z.; Li, M.; Sun, Q. RHON1 Co-Transcriptionally Resolves R-Loops for Arabidopsis Chloroplast Genome Maintenance. *Cell Rep.* **2020**, *30*, 243–256.e5. [CrossRef] [PubMed]
29. Manzo, S.G.; Hartono, S.R.; Sanz, L.A.; Marinello, J.; De Biasi, S.; Cossarizza, A.; Capranico, G.; Chedin, F. DNA Topoisomerase I Differentially Modulates R-Loops across the Human Genome. *Genome Biol.* **2018**, *19*, 100. [CrossRef]
30. Uruci, S.; Lo, C.S.Y.; Wheeler, D.; Taneja, N. R-Loops and Its Chro-Mates: The Strange Case of Dr. Jekyll and Mr. Hyde. *Int. J. Mol. Sci.* **2021**, *22*, 8850. [CrossRef]
31. Clapier, C.R.; Iwasa, J.; Cairns, B.R.; Peterson, C.L. Mechanisms of Action and Regulation of ATP-Dependent Chromatin-Remodelling Complexes. *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 407–422. [CrossRef] [PubMed]
32. Bhadouriya, S.L.; Mehrotra, S.; Basantani, M.K.; Loake, G.J.; Mehrotra, R. Role of Chromatin Architecture in Plant Stress Responses: An Update. *Front. Plant Sci.* **2021**, *11*, 603380. [CrossRef]
33. Banerjee, S.; Roy, S. An Insight into Understanding the Coupling between Homologous Recombination Mediated DNA Repair and Chromatin Remodeling Mechanisms in Plant Genome: An Update. *Cell Cycle* **2021**, *20*, 1760–1784. [CrossRef] [PubMed]
34. Hyjek, M.; Figiel, M.; Nowotny, M. RNases H: Structure and Mechanism. *DNA Repair* **2019**, *84*, 102672. [CrossRef]
35. Cerritelli, S.M.; Crouch, R.J. Ribonuclease H: The Enzymes in Eukaryotes. *FEBS J.* **2009**, *276*, 1494–1505. [CrossRef]
36. Mach, J. Thrown for a Loop: How RNase H1 and DNA Gyrase Limit R-Loops and Maintain Genome Stability in Chloroplasts. *Plant Cell* **2017**, *29*, 2311–2312. [CrossRef] [PubMed]
37. Kuciński, J.; Chamera, S.; Kmera, A.; Rowley, M.J.; Fujii, S.; Khurana, P.; Nowotny, M.; Wierzbicki, A.T. Evolutionary History and Activity of RNase H1-Like Proteins in Arabidopsis Thaliana. *Plant Cell Physiol.* **2020**, *61*, 1107–1119. [CrossRef]
38. Jarvis, P.; López-Juez, E. Biogenesis and Homeostasis of Chloroplasts and Other Plastids. *Nat. Rev. Mol. Cell Biol.* **2013**, *14*, 787–802, Erratum in *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 147. <https://doi.org/10.1038/nrm3744>. [CrossRef]
39. Powikrowska, M.; Oetke, S.; Jensen, P.E.; Krupinska, K. Dynamic Composition, Shaping and Organization of Plastid Nucleoids. *Front. Plant Sci.* **2014**, *5*, 424. [CrossRef]
40. Oldenburg, D.J.; Bendich, A.J. DNA Maintenance in Plastids and Mitochondria of Plants. *Front. Plant Sci.* **2015**, *6*, 883. [CrossRef]
41. Raven, J.A. Implications of Mutation of Organelle Genomes for Organelle Function and Evolution. *J. Exp. Bot.* **2015**, *66*, 5639–5650. [CrossRef]
42. Katayanagi, K.; Miyagawa, M.; Matsushima, M.; Ishikawa, M.; Kanaya, S.; Ikehara, M.; Matsuzaki, T.; Morikawa, K. Three-Dimensional Structure of Ribonuclease H from E. Coli. *Nature* **1990**, *347*, 306–309. [CrossRef]
43. Cerritelli, S.M.; Frolova, E.G.; Feng, C.; Grinberg, A.; Love, P.E.; Crouch, R.J. Failure to Produce Mitochondrial DNA Results in Embryonic Lethality in Rnaseh1 Null Mice. *Mol. Cell* **2003**, *11*, 807–815. [CrossRef] [PubMed]
44. Cerritelli, S.M.; Crouch, R.J. Cloning, Expression, and Mapping of Ribonucleases H of Human and Mouse Related to Bacterial RNase HI. *Genomics* **1998**, *53*, 300–307. [CrossRef] [PubMed]

45. Wu, H.; Lima, W.F.; Crooke, S.T. Investigating the Structure of Human RNase H1 by Site-Directed Mutagenesis. *J. Biol. Chem.* **2001**, *276*, 23547–23553. [CrossRef]
46. Gaidamakov, S.A. Eukaryotic RNases H1 Act Processively by Interactions through the Duplex RNA-Binding Domain. *Nucleic Acids Res.* **2005**, *33*, 2166–2175. [CrossRef] [PubMed]
47. Nowotny, M.; Cerritelli, S.M.; Ghirlando, R.; Gaidamakov, S.A.; Crouch, R.J.; Yang, W. Specific Recognition of RNA/DNA Hybrid and Enhancement of Human RNase H1 Activity by HBD. *EMBO J.* **2008**, *27*, 1172–1181. [CrossRef]
48. Yang, Z.; Hou, Q.; Cheng, L.; Xu, W.; Hong, Y.; Li, S.; Sun, Q. RNase H1 Cooperates with DNA Gyrase to Restrict R-Loops and Maintain Genome Integrity in Arabidopsis Chloroplasts. *Plant Cell* **2017**, *29*, 2478–2497. [CrossRef]
49. Nudler, E. RNA Polymerase Active Center: The Molecular Engine of Transcription. *Annu. Rev. Biochem.* **2009**, *78*, 335–361. [CrossRef]
50. Yadav, V.; Hemansi, Kim, N.; Tuteja, N.; Yadav, P. G Quadruplex in Plants: A Ubiquitous Regulatory Element and Its Biological Relevance. *Front. Plant Sci.* **2017**, *8*, 1163. [CrossRef]
51. Liu, H.; Chu, Z.; Yang, X. A Key Molecular Regulator, RNA G-Quadruplex and Its Function in Plants. *Front. Plant Sci.* **2022**, *13*, 926953. [CrossRef]
52. Rhodes, D.; Lipps, H.J. G-Quadruplexes and Their Regulatory Roles in Biology. *Nucleic Acids Res.* **2015**, *43*, 8627–8637. [CrossRef]
53. Kuznetsova, A.A.; Kosarev, I.A.; Timofeyeva, N.A.; Novopashina, D.S.; Kuznetsov, N.A. Kinetic Features of Degradation of R-Loops by RNase H1 from Escherichia Coli. *Int. J. Mol. Sci.* **2024**, *25*, 12263. [CrossRef]
54. Kiełpiński, Ł.J.; Hagedorn, P.H.; Lindow, M.; Vinther, J. RNase H Sequence Preferences Influence Antisense Oligonucleotide Efficiency. *Nucleic Acids Res.* **2017**, *45*, 12932–12944. [CrossRef]
55. Chakraborty, P. New Insight into the Biology of R-Loops. *Mutat. Res. Mol. Mech. Mutagen.* **2020**, *821*, 111711. [CrossRef] [PubMed]
56. Palmer, B.; Lee, C.-Y.; Yang, L.; Paul, T.; Myong, S. High-Frequency Transcription Leads to Rapid R-Loop Formation. *J. Biol. Chem.* **2025**, *301*, 108514. [CrossRef] [PubMed]
57. Lim, G.; Hwang, S.; Yu, K.; Kang, J.Y.; Kang, C.; Hohng, S. Translocating RNA Polymerase Generates R-Loops at DNA Double-Strand Breaks without Any Additional Factors. *Nucleic Acids Res.* **2023**, *51*, 9838–9848. [CrossRef]
58. Belotserkovskii, B.P.; Tornaletti, S.; D'Souza, A.D.; Hanawalt, P.C. R-Loop Generation during Transcription: Formation, Processing and Cellular Outcomes. *DNA Repair* **2018**, *71*, 69–81. [CrossRef]
59. Nowotny, M.; Gaidamakov, S.A.; Ghirlando, R.; Cerritelli, S.M.; Crouch, R.J.; Yang, W. Structure of Human RNase H1 Complexed with an RNA/DNA Hybrid: Insight into HIV Reverse Transcription. *Mol. Cell* **2007**, *28*, 264–276. [CrossRef]
60. Kuznetsova, A.A.; Novopashina, D.S.; Fedorova, O.S.; Kuznetsov, N.A. Effect of the Substrate Structure and Metal Ions on the Hydrolysis of Undamaged RNA by Human AP Endonuclease APE1. *Acta Naturae* **2020**, *12*, 74–85. [CrossRef] [PubMed]
61. Kuznetsova, A.A.; Gavrilova, A.A.; Novopashina, D.S.; Fedorova, O.S.; Kuznetsov, N.A. Mutational and Kinetic Analysis of APE1 Endoribonuclease Activity. *Mol. Biol.* **2021**, *55*, 211–224. [CrossRef] [PubMed]
62. Kuznetsova, A.A.; Akhmetgalieva, A.A.; Ulyanova, V.V.; Ilinskaya, O.N.; Fedorova, O.S.; Kuznetsov, N.A. Efficiency of RNA Hydrolysis by Binase from Bacillus Pumilus: The Impact of Substrate Structure, Metal Ions, and Low Molecular Weight Nucleotide Compounds. *Mol. Biol.* **2020**, *54*, 769–776. [CrossRef]
63. Johnson, K.A. *Kinetic Analysis for the New Enzymology*, 1st ed.; KinTek Corporation: Austin, TX, USA, 2019; ISBN 978-1-7339982-0-8.

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