Review

Thermostable α-Amylases and Laccases: Paving the Way for Sustainable Industrial Applications.

Nivedita Jaiswal * and Pundrik Jaiswal

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Review

Thermostable α-Amylases and Laccases: Paving the Way for Sustainable Industrial Applications

Nivedita Jaiswal [1,*] and Pundrik Jaiswal [2]

[1] Department of Biochemistry, University of Lucknow, Lucknow-226007, UP, India
[2] LCDB /National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland-20892, USA
* Correspondence: nive_june29@yahoo.co.in

Abstract: The growing demand in industrial and biotechnological settings for more efficient enzymes with enhanced biochemical features, particularly thermostability and thermotolerance, necessitates a timely response. Renowned for their versatility, thermostable enzymes, offer significant promise across a range of applications, including agricultural, medicinal, and biotechnological domains. This comprehensive review summarizes the structural attributes, catalytic mechanisms, and the relationships between structure and function of two major classes of thermostable enzymes: α-amylases and laccases. These enzymes serve as valuable models for understanding the structural basis of protein thermostability. Commercial significance of these enzymes and researchers' interest in further optimization and innovation, this article can greatly contribute to ongoing research on thermostable enzymes and aiding industries in optimizing production processes. It also give insights to the exploration of suitable strategies and factors for enhancing thermostability, resulting in heightened multipronged stability and notable enhancements in the enzymes' industrial applicability.

Keywords: thermostable enzymes; α-amylases, laccases; industrial applications

Introduction

The enduring significance of thermostable enzymes stems from their diverse applications across industries, including food, pharmaceuticals, and biotechnology. In the realm of industrial biocatalysis, enzymes face stringent demands, necessitating robust and thermostable biocatalysts to meet high industrial standards. Enzymes characterized as thermostable possess intrinsic stability, enabling them to endure elevated temperatures well beyond 50 °C, reaching as high as 80 °C, 90 °C or even more [1,2]. These enzymes maintain their structural integrity and distinctive features under such extreme conditions. This inherent stability provides significant biotechnological advantages compared to mesophilic enzymes (active optimally at 25 to 50 °C) or psychrophilic enzymes (active optimally at 5 to 25 °C). Thermostable enzymes are more easily purified through heat treatment (as one of the initial step), exhibit increased resilience and resistance to chemical denaturants, allow for higher substrate concentrations, contribute to lower viscosity, pose fewer risks of microbial contamination, and often lead to higher reaction rates [1]. This heightened stability allows them to thrive in harsh environments, including applications like enzymatic bioremediation of xenobiotics and innovative green processes [3]. The demand for industrially relevant thermostable enzymes has created a pressing need to identify easily accessible, cost-effective, and process-friendly sources. This review is motivated by the rapid strides in discovering novel thermostable enzymes from unconventional sources. Among these enzymes, α-amylases and laccases stand out as crucial players in industries such as pulp and paper, starch processing, textiles, detergents, fuels, alcohols, and pharmaceuticals, constituting the major consumers of these thermostable enzymes. Alpha-amylase patents distributed across different categories: in biofuels, beverages, pharmaceuticals, detergents, food, animal feed, and textiles, were found deposited in intellectual property databases [4]. Besides being widespread in plants, animals, fungi, unicellular eukaryotes like eubacteria and archaea,

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'Dictyo-type' α-amylase is also found to be widespread and may be ancestral in the Unikonts, a clade comprising animals, fungi (Opisthokonts) and amoebozoa [5]. The activity of α-amylase has been observed to be associated with the neurodegenerative diseases such as Alzheimer's disease. Studies also show that α-amylase synthesis is linked with mTOR (Mechanical Target of Rapamycin) signaling pathways [6]. mTOR serves as a key regulator of celluar growth by controlling the both anabolic and catabolic processes [7,8].

Another dimension of the profound interest in thermostable enzymes lies in the exploration of the thermodynamic stability of proteins. Investigating the interplay between stability, flexibility, or plasticity and catalytic efficiency adds a layer of understanding to these enzymes' properties. The heightened interest in thermostable enzymes has catalyzed a focus on developing enzymes with enhanced thermostability or thermotolerance through genetic engineering or site-directed mutagenesis, revolutionizing the attainment of desired enzyme properties. The preference for enzymatic processes, particularly thermostable ones, over conventional methods in various industries is attributed to their rapid and specific action, along with advantages in energy, time, raw material, and chemical savings. Crucially, their environmentally friendly nature further underscores their appeal. Moreover, conducting processes at elevated temperatures using thermostable enzymes not only diminishes the threat of microbial contamination, a notable advantage, but also serves to lower substrate viscosity, enhance transfer rates, and augment solubility in the course of reaction procedures [2].

Thermostable enzymes, beyond their inherent thermostability, exhibit favorable characteristics such as a wide pH tolerance and resistance to organic solvents, positioning them as superior to other enzyme groups. This underscores the need for continued efforts in screening and isolating novel sources, developing innovative purification approaches to enhance yield and purity, and ultimately harnessing thermostable enzymes for diverse industrial applications. The present review article is an effort to address thermostable *α*-amylases and laccases indicating their varied sources of origin, structural characteristics and catalytic mechanism, structure-function relationships as well as factors attributing and various strategies resulting thermostability. Besides, the prevailing challenges that exist in order to cope up with aiming for further research and advances in the field of thermostable enzymes providing insights into future direction in the field of thermostable enzymes, have been focused on.

Thermostable α-Amylases

Thermostable *α*-amylases, constituting the largest share of industrial enzyme sales at approximately 25%, play a pivotal role across diverse sectors such as fermentation, textile, food, detergent, brewing, biorefinery, paper, and pharmaceutical industries [9,10], as illustrated in Figure 1 and also listed in Table 1. Particularly crucial in starch liquefaction processes, these enzymes serve as valuable models for studying thermal adaptation in proteins [11,12]. Widely distributed in nature, *α*-amylases originate from various sources, including microbes, animals, and plants, with a notable presence in germinating seeds where they contribute significantly to carbohydrate metabolism [13,14].

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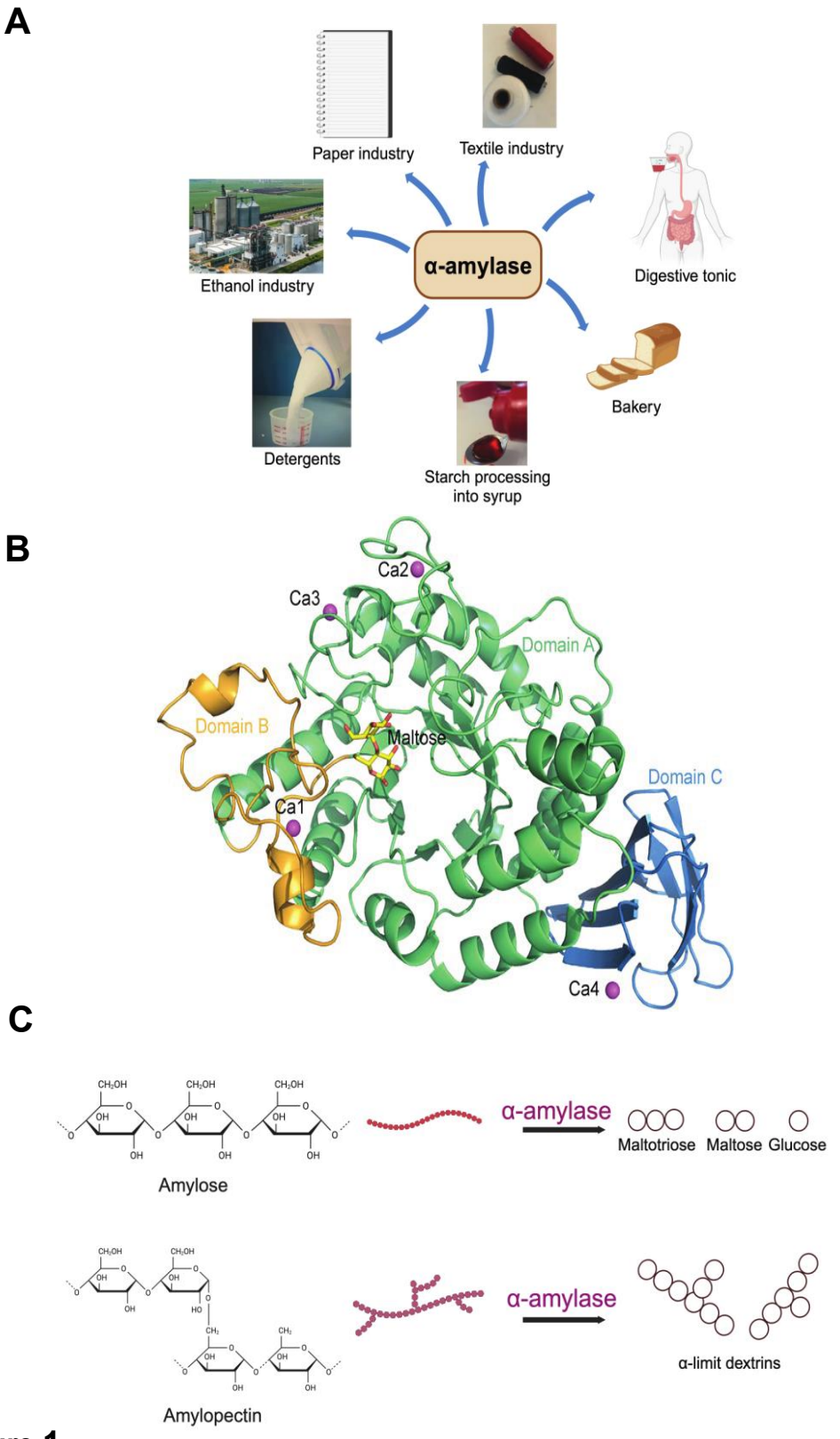

Figure 1

Figure 1. Schematic representations of -amylases industrial applications, structural relation with thermostablilty and mechanism of catalytic functions. A. An illustration model of the -amylases uses in the diverse commercial applications. B. Structural depiction of the crystal structure of -amylase from Anoxybacillus species (TASKA, PDB 5A2A [134]) showing a single polypeptide chain folding with the relative positions of the three structural domains: a) Domain A, the catalytic domain, b)

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Domain B, that constitutes a significant portion of the substrate binding cleft, responsible for notable variations in size, structure, and substrate specificity among different α-amylases, and c) Domain C, forming the C-terminal segment of the sequence. Domains A, B and C are shown in green, orange, and blue, respectively and the calcium ions in magenta. C. Illustration depicting the chemical structure of starch (comprising amylose and amylopectin) and the enzymatic conversion into sugar units [135]. The catalytic mechanism of α-amylases involves the cleavage of the internal α-glycosidic bonds in polysaccharides like starch, glycogen, and others, leading to the hydrolysis of these bonds and the production of α-anomeric mono- or oligosaccharides.

Table 1. Commercially available α-amylases and their industrial applications.

Microbial source of α amylases	Commercial Name of α- amylase	Manufacturer	Industrial applications
Aspergillus oryzae	Fructamyl® FHT	Erbslöh Geisenheim AG	Beverage industry
Bacillus licheniformis	Liquozyme® SC DC	Novozymes	Liquefaction for ethanol production
Bacillus amyloliquefaciens	BAN®	Novozymes	Oat starch liquefaction
Bacillus licheniformis	Termamyl®	Novozymes	Adjunct liquefaction
Aspergillus oryzae	Fungamyl	Novozymes	Baking
Bacillus subtilis	Validase BAA	IMCD Germany	Food and Feed
Bacillus subtilis	Zylozyme™ AA	Kemin Industries	Biofuel
Bacillus licheniformis	Bioconvert ALKA	Noor Enzymes	Biofuel
Genetically modified microorganism	Stainzyme® Plus Evity® 48 T	Novozymes	Detergent
Genetically modified microorganism	Aquazym®	Novozymes	Textile

Listed are commercial α-amylases, each derived from specific microbial sources, offering various industrial purposes. Sources: https://erbsloeh.com/; https://www.novozymes.com/en/products/ethanol/liquefaction/liquozyme; https://www.novozymes.com/en/products/baking/freshness/ban?utm_source; https://www.novozymes.com/en/products/plant-based-foods/plant-based-dairy/termamyl; https://www.novozymes.com/en/products/baking/flour-correction/fungamyl?utm_source; https://www.imcd.de/en/trade-names/food-and-nutrition-BG3/validase-a5u690000000fr0AAA/; https://www.kemin.com/na/en-us/markets/biofuels/products/zylozyme-aa; http://www.enzyme-india.com/amylase-enzymes-biofuel.html; https://www.novozymes.com/en/products/laundry/starch-stains/stainzyme-plus-evity-48-t; https://www.novozymes.com/en/products/pulp-paper/aquazym-480-l.

Established in 1998, the CAZy Carbohydrate-Active Enzymes (CAZymes) offers users online access [15] (http://www.cazy.org) that is regularly updated to a sequence-based family classification linking sequences with the specificities and three-dimensional structures of enzymes that are involved in the assembly alteration, and degradation of oligo-and polysaccharides [16]. The enzymes that are currently covered in the CAZy database that catalyze the breakdown, biosynthesis or modification of carbohydrates and glycoconjugates are:

a) Glycosyl Hydrolases (GHs): hydrolysis and/or rearrangement of glycosidic bonds.
b) Glycosyl Transferases (GTs): formation of glycosidic bonds.
c) Polysaccharide Lyases (PLs): non-hydrolytic cleavage of glycosidic bonds.
d) Carbohydrate Esterases (CEs): hydrolysis of carbohydrate esters.
e) Auxiliary Activities (AAs): redox enzymes that act in conjunction with CAZymes.

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In recent years, there has been significant growth in the CAZy classification system, including the introduction of new families and the establishment of subfamilies within existing ones [17]. Currently, of the 189 class glycoside hydrolases (GH) families in total, four are considered α-amylase families: i) GH13— proven to be the most abundant and largest α-amylase family composed of a $(\beta/\alpha)_8$ barrel structure; (ii) GH57— the second and smaller α-amylase family composed of a $(\beta/\alpha)_7$ barrel structure; (iii) GH119— a very small family related to GH57; (IV) GH126— composed of $(\alpha/\alpha)_6$ barrel structure [18]. Families GH13, GH57 and GH119 employ the α-retaining mechanism of α-amylase involving two catalytic residues in the active site; a glutamic acid/base catalyst and an aspartate as the nucleophile and the anomeric carbon remains in the same position as it is mediated by the double-displacement mechanism. In contrast, GH126 employ the inverting reaction mechanism in which an anomeric carbon position is shifted from β to α through a single-displacement mechanism [19]. The α-amylase family GH13 has already been divided into 47 subfamilies, with additional subfamilies still emerging. Nature doesn't always provide enzymes with the desired properties; hence protein engineering has been proposed as an alternative method to enhance the physicochemical traits of enzymes [20]. Leveraging solved crystal structures, a structure-guided consensus approach is recognized as an effective and dependable method for refining enzyme properties [21]. Employing this approach to enhance the thermostability of α-amylase without additional Ca^{2+} would benefit process efficiency and reduce the cost of the starch liquefaction process [22,23] This on other hand, requires both a starting enzyme with which to work and adequate structural information to guide the modifications. In the related efforts, Li and coworkers [21] showed from their experimental observations that malto-hexaose-forming α-amylsae from *Bacillus stearothermophilus* (AmyMH) is an adequate starting point from which to design a more thermostable α-amylase without added Ca^{2+}. Previously, attention was directed towards a loop situated in domain B across various bacterial α-amylases as a means to enhance their thermostability. Suzuki and coworkers [24] suggested that eliminating the equivalent loop formed by R176-G177 (according to BAA numbering) could significantly boost the thermostability of *Bacillus amyloliquefaciens* α-amylase (BAA). This depiction has been replicated in several other bacterial α-amylases from different species, yielding similar improvements in thermostability [25,26]. Furthermore, the enhanced thermostability of *Bacillus licheniformis α-amylase* (BLA) was achieved by removing amide-containing side chains through the mutation of N190F (according to BLA numbering [27].

Despite the escalating demand for thermostable enzymes in multiple industries, the production and properties of *α*-amylases have been restricted by their susceptibility to extremes of pH, temperature, external conditions, and catalytic efficiency [28,29]. In this context, thermostable enzymes play a critical role in withstanding the high temperatures inherent in industrial processes [10]. Microbial sources, especially fungi and bacteria, particularly those belonging to the Bacillus genus (e.g., *B. subtilis, B. stearothermophilus, B. licheniformis, B. amyloliquefaciens*), are often favored for industrial production due to their cost-effectiveness, consistency, efficient use of time and space, and ease of process modification and optimization [11].

The ongoing quest for novel thermostable *α*-amylases has extended to plant sources, seeking alternatives that meet the standards set by microbial sources. Plant-derived sources such as barley [30], mung bean [31], potato [32,33], soybean [12], radish [34], red pitaya peel [35], wheat [36], broad bean [14], and sword bean [37] have been explored. Reports of thermostable *α*-amylases have emerged from plant sources cultivated in local areas, including *Vicia faba* (65 °C), red pitaya (*Hylocereus polyrhizus*) peel (70 °C), wheat (*Triticum aestivum*) seeds (68 °C), soybean (*Glycine max*) seeds (70 °C), mung bean (*Vigna radiata*) seeds (65 °C), and sword bean (*Canavalia gladiate*) seeds (70 °C). Some sources of thermostable *α*-amylases and their respective industrial applications is listed in Table 2.

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Table 2. Microbial Sources of thermostable α-amylases.

Source of α-amylases	Optimum Temperature	Industrial applications	References
Actinomadura keratinilytica sp. Cpt29	70 °C	Laundry detergent additive	[138]
Aeribacillus pallidus BTPS-2	70 °C	Starch liquefcation	[139]
Anoxybacillus vranjensis ST4	60–80 °C	Starch hydrolysis	[140]
Bacillus amyloliquefaciens BH072	60 °C	Food processing	[141]
Bacillus cereus SP-CH11	65 °C	Food processing	[142]
Bacillus licheniformis AT70	60 °C	Starch degradation	[143]
Bacillus licheniformis NH1 strain	70 °C	Laundry detergent additive	[144]
Bacillus licheniformis So-B3	70 °C	Hydrolyzing raw starch	[145]
Bacillus sp. isolate A3-15	100 °C	Textile industry	[146]
Bacillus tequilensis TB5	60 °C	Textile de-sizer	[147]
Chromohalobacter sp. TVSP 101	65 °C	Starch hydrolysis	[148]
Geobacillus thermoleovorans	80 °C	Improvement of washing efficiency of detergents	[149]
Germinated wheat seeds (*Triticum aestivum*)	68 °C	Starch processing	[36]
Haloterrigena turkmenica	55 °C	Agricultural residues treatment	[150]
Paecilomyces variotii	60 °C	Starch degradation	[151]
Rhizomucor miehei	75 °C	Food processing	[152]
Rhizopus oligosporus	60 °C	Laundry detergent additive	[153]
Soybean (*Glycine max*) seeds	75 °C	Starch liquefaction	[12]
Tepidimonas fonticaldi strain HB23	80 °C	Laundry detergent additive	[154]
Thermomyces dupontii	60 °C	Maltose syrup production	[155]

Listed are sources of α-amylases from different microbes with their optimum temperature which can be useful in the industrial purposes.

Structural Characteristics and Catalytic Mechanism of α-Amylases

The architecture of α-amylases consists of a single polypeptide chain that folds into three distinctive domains (Figure 1): a) Domain A, the catalytic domain characterized by an N-terminal $(\beta/\alpha)_8$ barrel (also known as TIM barrel) structure, featuring eight parallel β-strands forming a barrel shape surrounded by eight α-helices; b) Domain B, an irregular β-rich structure that constitutes a significant portion of the substrate binding cleft, responsible for notable variations in size, structure, and substrate specificity among different α-amylases; and c) Domain C, forming the C-terminal segment of the sequence [38–40]. Some maltogenic amylases exhibit an additional D-domain after the C-domain, the function of which remains currently unknown [41].

The catalytic mechanism of α-amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) classifies them as endozymes within the glycosyl hydrolase (GH13 family), targeting internal α-glycosidic bonds in polysaccharides like starch, glycogen, and others, leading to the hydrolysis of these bonds and the production of α-anomeric mono- or oligosaccharides [42], as depicted in Figure 1. In plants, α-amylases play a crucial role in the degradation of stored starch in germinating seeds, releasing sugars essential for proper plant growth [43]. Unlike many other amylases exclusive to hydrolyzing α-D-(1,4)-glycosidic bonds, maltogenic amylases exhibit remarkable catalytic versatility, hydrolyzing both α-D-(1,4)- and α-D-(1,6)-glycosidic bonds and participating in transglycosylation reactions, transferring glycosyl units to the C3, C4, or C6 hydroxyl groups of diverse acceptor mono- or disaccharides [44].

With only a few exceptions, nearly all identified α-amylases exhibit structural stability attributed to the presence of a calcium ion located at the interface between domains A and B. This stability is disrupted upon the removal of the calcium ion, leading to a noticeable decline in catalytic activity. A

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prominent illustration of this phenomenon is the α-amylase from *Bacillus licheniformis* (BLA), a hyperthermostable enzyme extensively utilized in biotechnology for starch and complex carbohydrate breakdown at temperatures reaching 110 °C, and also serving as a crucial component in detergents and baking additives [45]. The elucidation of BLA's structure in the metal-containing state, coupled with cmalliomparisons to the apo-enzyme (calcium-depleted form), has provided insights into how metal ions regulate enzyme activity. Studies have revealed that the addition of calcium chelators significantly reduces the stability of BLA [46,47], resulting in a heightened susceptibility to proteolysis [48]. This serves as compelling evidence of the indispensable stabilizing role played by the calcium ion. Consequently, *α*-amylases are proposed as a novel category of metallo-enzymes distinguished by a prosthetic group—an alkaline-earth metal instead of a transition element. This group primarily serves a structural role, akin to disulfide bridges [49]. Calcium ions are believed to contribute structurally, positioned too distantly from the active site to directly participate in catalysis [50–52]. The heightened thermostability of the enzyme, attributed to calcium ions, is explained by their salting-out effect on hydrophobic residues within the protein, inducing a more compact structural conformation [53]. In certain *α*-amylases, the presence of a chloride ion in the active site enhances catalytic efficiency by inducing conformational changes around the active site, potentially by elevating the pKa (acid dissociation constant) of a hydrogen-donating residue in the active site.

Factors Contributing to Stability in Thermostable α-Amylases

Thermostable enzymes serve as valuable models for comprehending the physico-chemical factors governing protein thermostability. Identifying the structural characteristics implicated in thermal stability facilitates the engineering and design of more robust enzymes for diverse industrial applications. Over the past two decades, *α*-amylase has emerged as a crucial model system for investigating the thermal adaptation of enzymes [40].

Studies based on amino acid sequence prediction, including the distribution of amino acids and dipeptide composition, help discern factors contributing to thermostability, distinguishing thermophilic and mesophilic proteins. Despite sequence identity and structural similarity, thermophilic proteins exhibit higher frequencies of charged, hydrophobic, and aromatic amino acids compared to mesophilic counterparts [54]. The presence of fewer cysteines in thermophilic proteins is associated with their oxidation at higher temperatures. Sequence analysis proves valuable in predicting protein thermostability when structural information is lacking. Comparing dynamic features of mesophilic proteins and their thermophilic homologs with increased sequence identity and structural similarity but distinct thermostability offers insights into the physical basis of protein stability [55]. Calcium ions play crucial roles in determining the structure, function, and stability of thermophilic *α*-amylases, providing resistance or tolerance to thermal inactivation by maintaining correct protein conformation [56–59]. The removal of calcium ions irreversibly inactivates barley *α*-amylase, while calcium ion addition restores activity in certain bacterial *α*-amylases [60]. Mostly, all α-amylases are Ca^{2+}-dependent with some exceptions of Ca^{2+}-independent α-amylases [61–64]; and also, some α-amylases that are inhibited by Ca^{2+} [65,66]. The role of α-amylases is mainly structural because their catalytic sites are far away from the calcium-binding sites [52,53]. Several studies have been made on the effect of calcium ions on the activity and stability of α-amylases from thermophiles which may help in determining the mechanism of Ca^{2+}-binding proteins in the presence of extreme thermal environment as was investigated by Liao and coworkers in which they studied the influence of calcium ions on the structure and thermal characterization of *α*-amylase (AGXA) from thermophilic *Anoxybacillus* sp.GXS-BL [67].

Multiple factors contribute to thermostability, such as increased hydrogen bonds, ionic and electrostatic interactions, hydrophobic interactions, disulfide bonds, metal binding, salt bridges, ion pairs, aromatic clusters, sidechain-sidechain interactions, shorter surface loops, GC-rich codons, charged amino acid ratios, amino acid preferences, post-translational modifications, and solute accumulation [11]. Thermophilic proteins are characterized by more rigid and compact packing density, lower thermal motion, decreased flexibility, shorter surface loops, stabilization by heat-stable

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chaperones, reduced water-accessible hydrophobic surface, decreased entropy difference between folded and unfolded states, increased proline frequency, and decreased thermolabile residue occurrence compared to mesophilic counterparts [11,12].

In terms of industrial applications, enzyme stability is crucial, with a focus on thermodynamic and long-term stability. Various strategies for stability enhancement, including immobilization, addition of stabilizing agents, chemical modification, protein engineering, and genetic engineering through cloning and expression of thermostable α-amylase genes, have been explored. Site-directed mutagenesis and the revolutionary approach of directed evolution have emerged as promising strategies for thermostabilization [68,69]. Economic considerations in starch processing industries drive the need for α-amylases active at higher temperatures, and ongoing research has shifted the focus from stability engineering to pH activity profile and substrate specificity engineering, resulting in the development of α-amylases with novel and improved properties.

Thermostable Laccases

Laccases are recognized as environmentally friendly proteins and green biocatalysts, setting them apart from other oxidases. Unlike certain oxidases, laccases do not depend on toxic H_2O_2 or any mediator for the reduction reaction, and they exclusively generate water as the end product by reducing molecular oxygen. These glycoproteins exist in monomeric, dimeric, and tetrameric forms, displaying the ability to oxidize a wide range of inorganic substrates, aromatic compounds, and organic compounds. Primarily employed for the breakdown of chemical contaminants, laccase's low substrate specificity and monoelectronic oxidation of substrates in various complexes make it versatile [70]. Although laccase effectively degrades emerging contaminants, its application on a large scale necessitates features like reusability, thermostability, and operational stability. Achieving these characteristics often involves techniques such as immobilization and the production or isolation of robust laccase variants with desired attributes.

Laccase, also identified as benzenediol:oxygen oxidoreductase (EC 1.10.3.2), stands as a crucial enzyme with a central role in diverse biological processes. This copper-containing enzyme serves as a catalyst for oxidizing a wide array of organic and inorganic compounds, facilitating the reduction of oxygen molecules to produce water as a byproduct. Its versatility in mediating oxidative reactions has sparked considerable interest in scientific research and industrial applications across various fields, including biotechnology, environmental science, and agriculture (Figure 2). In this context, there is a pressing need to delve deeper into the properties, functions, and applications of laccase, exploring its significance in different domains.

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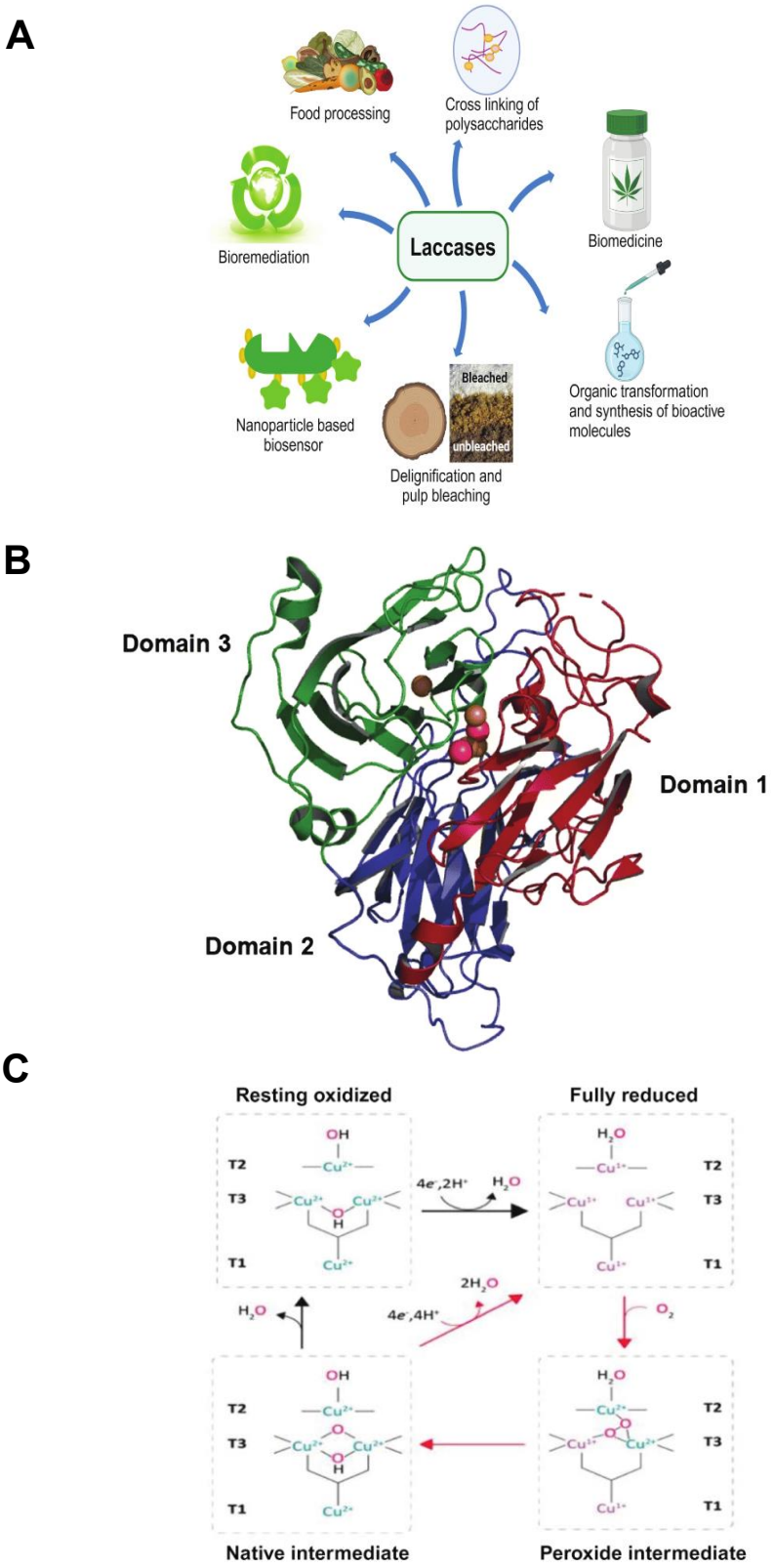

Figure 2

Figure 2. Schematic representations of laccases industrial applications, structural relation with thermostablilty and mechanism of catalytic functions. A. A comprehensive model of laccases uses in the diverse industrial applications. B. Structural representation of the crystal structure of the laccase from Bacillus subtilis (PDB 1GSK, [136]) showing the polypeptide chain with relative postioning of the three homologous cupredoxin domains, domain 1, 2, and 3 arranged, sequentially. Domain 2 joins

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and positions domains 1 and 3. Domain 1, 2, and 3 are depicted in red, blue, and green. C. Illustration representing the catalytic mechanism of laccases: the substrate-induced reduction of the T1 copper, followed by the transfer of electrons to the TNC and subsequent reduction of O2 (adapted from [94,137]. The first step is the oxidation of the substrate by mononuclear copper T1, which serves as an electron acceptor, converting Cu2+ to Cu+ oxidation state. After removing an electron from the substrate, an unstable cationic radical is formed which is oxidized by a second enzymatic reaction or undergo non-enzymatic reactions, such as hydration or polymerization. The electrons removed from the substrate at the T1 site are transferred to the T2/T3 center for conversion of O2 to H2O. Complete reduction of molecular oxygen to water requires four molecules of reducing substrate. Thus, the stoichiometry of the enzymatic reaction of the catalytic mechanism of laccases is represented by the equation: $4RH + O2 \rightarrow 4R + 2H2O$, where RH signifies the substrate.

Laccase exhibits a broad distribution in nature, being present in bacteria, fungi, plants, and insects. This widespread occurrence underscores the fundamental importance of laccase in nature and its relevance in various biological and ecological contexts. The diverse functions of laccases are contingent upon their source organism, contributing to processes such as lignin degradation, pigmentation, fruiting body formation, fungal morphogenesis, detoxification, sporulation, and pathogenesis in fungi; melanin formation, and endospore coat protein synthesis in bacteria; and lignification, wound healing, and iron oxidation in plants [71–76]. Thermostable laccases are advantageous for the eco-friendly remediation of hazardous synthetic dyes, particularly in the treatment of high-temperature dyeing wastewater. Table 3 lists some commercially available laccases with their industrial applications.

Table 3. Commercially available Laccases and their industrial applications.

Source of Laccases	Commercial Name of Laccase	Manufacturer	Industrial applications
Myceliophthora thermophila laccase expressed in *Aspergillus oryzae*	Denilite™ I	Novozymes [156]	Textile
	Denilite™ II	Novozymes	
	Zylite	Zytex Biotech Private Limited [156]	
	Ecostone LC10	AB Enzymes GmbH	
	IndiStar	Genencor International Inc.	
	Novoprime Base 268	Novozymes [157]	
	Primagreen Ecofade LT100	Genencor International Inc. [158]	
	Novozym® 51,003	Novozymes [159]	
White-rot fungi (*Phanerochaete chrysosporium*, *Trametes versicolor*)	Lignozym® Process	IBB Netzwerk GmbH [160]	Paper Food processing
	Laccase Y120	Amano Enzyme [161]	
	Novozym® 51,003	Novozymes [159]	
Filamentous fungi and yeasts	Suberase®	Novozymes [162]	Brewing
Genetically engineered bacterial laccase	MetZyme® LIGNO™	MetZen [163]	Bio-refinery

Listed are commercial α-Laccases with their specific microbial sources, serving different industrial purposes. Sources: https://www.novozymes.com/en; https://www.zytex.com/; https://www.abenzymes.com/en/your-industry/textiles/; https://www.textileweb.com/doc/genencor-launches-the-indistar-coloradjust-sy-0001.

Examples of laccase sources reported from bacteria include *Azospirrullum lipoferum* [77], *Anabaena azollae* [78], *Bacillus subtilis* [79], *Streptomyces cyaneus* [80], *S. lavendulae* [81], and *Marinomonas mediterranea* [82]. In fungi, laccases are widely distributed in ascomycetes, basidiomycetes, and deuteromycetes, encompassing various species such as *Trametes* (*Coriolus*) *versicolor*, *T. hirsute*, *T.*

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ochracea, *T. villosa*, *T. gallica*, *Cerrena maxima*, *Phlebia radiata*, *Coriolopsis polyzona*, *Lentinus tigrinus*, *Pleurotus eryngii*, *Myceliophthora thermophila*, *Aspergillus*, *Curvularia*, *Penicillium*, *Chaetomium thermophile*, *Mycelia sterlia*, *Cantharellus cibarius*, *Lactarius piperatus*, *Russula delica*, *Thelephora terestris*, *Agaricus*, *Marasmius*, *Tricholoma*, and *Volvariella* [83].

In contrast to fungal laccases, bacterial laccases exhibit higher activity and greater stability at elevated temperatures, high pH, and high concentrations of chloride and copper ions [84–86]. Laccases have also been reported in various plants, including Japanese lacquer tree (*Rhus vernicifera*), lacquer, mango, mung bean, peach, pine, prune, sycamore, *Pinus taeda*, *Populus trichocarpa*, *Acer pseudoplatanus*, *Liriodendron tulipifera*, *Zinnia elegans*, tobacco (*Nicotiana tabacum*), *Zea mays*, *Lolium perenne*, *Leucaena leucocephala*, and *Carica papaya*. Plant laccases generally exhibit a higher molecular mass compared to fungal laccases, attributed to the increased glycosylation in plant laccases (22-45%) compared to fungal counterparts (10-25%) [87]. Glycosylation significantly influences various aspects of laccase functionality, including copper retention, thermal stability, and enzymatic activity. Some sources of thermostable laccases with industrial applications is listed in Table 4.

Table 4. Microbial Sources of thermostable Laccases.

Source of Laccases	Optimum Temperature	Industrial applications	References
Agaricus bisporus CU13	55 °C	Decolorization of synthetic dyes	[164]
Alcaligenes faecalis XF1	80 °C	Decolorization of synthetic dyes	[165]
Azospirillum lipoferum	70 °C	Ecological role in the process of root colonization	[166]
Bacillus altitudinis SL7	55 °C	Bioremediation of lignin contaminated wastewater from pulp and paper industries	[167]
Bacillus sp. MSK-01	75 °C	Proposed as an anti-proliferative agent to cancer cells	[168]
Bacillus sp. PC-3	60 °C	Functionalization of chitosan film for antimicrobial activity	[169,170]
Bacillus subtilis	60 °C	Biodegradation of the fungicide	[171]
Bacillus subtilis strain R5	55 °C	Degradation of synthetic dyes	[172]
Caldalkalibacillus thermarum TA2.A1	70 °C	Lignin degradation	[123]
Coprinopsis cinerea	70 °C	Wastewater treatment	[173]
Enterobacter sp. AI1	60 °C	Degradation and detoxification of synthetic dyes	[153]
Galerina sp. HC1	60 °C	Demethylation of lignin	[174]
Ganoderma lucidum KMK2	60 °C	Decolorization of reactive dyes	[175]
Ganoderma multipileum	70 °C	Biodegradation of chromium	[176]
Geobacillus stearothermophilus MB600	90 ℃	Biodegradation of pollutants	[70]
Geobacillus yumthangensis	60 °C	Degradation of organic pollutants	[169]
Klebsiella pneumoniae	70 ℃	Decolorization of synthetic dyes	[177]
Lactobacillus plantarum J16 CECT 8944	60 °C	Eliminating toxic compounds present in fermented food and beverages	[178]
Litopenaeus vannamei	>90 °C	Marine bioremediation	[179]
Lysinibacillus fusiformis	80 °C	Removal of sulfonamides and tetracyclines residues	[180]
Setosphearia turcica	60 °C	Decolorization of malachite green	[181]
Staphylococcus haemolyticus	60 °C	Textile finishing	[182]

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Streptomyces ipomoeae CECT 3341	60 ± 6 °C	Decolorization and detoxification of textile dyes	[183]
Thermobaculum terrenum	80 °C	Protein engineering studies	[184]
Thermus sp. 2.9	70 °C	Delignification of Eucalyptus biomass	[185]
Trametes maxima IIPLC-32	50–70 °C	Detoxification of phenolic inhibitors in lignocellulosic biomass	[186]
Trametes orientalis	80 °C	Decolorization and bioremediation of synthetic dyes	[187]
Trametes trogii	70 °C	Modification of kraft lignin	[188]
Leucaena leucocephala	80 °C	Decolorization of synthetic dyes	[98,189]
Carica papaya	70 °C	Dye decolorization	[98,189]

Listed are sources of laccases from diverse microbes with specific optimal temperatures which are proven to be useful in industrial applications.

Laccase has gained attention for its versatile applications in biotechnological fields, including dye decolorization, biopulping, biobleaching, xenobiotic degradation, food processing, biopolymer modification, ethanol production, biosensor development, drug and organic synthesis, among others [83]. However, a common challenge with many isolated laccases is their relatively low enzyme activity yield and sensitivity to extreme conditions like temperature, pH, and metal ions. These limitations can impede their widespread use in large-scale commercial and industrial applications. Addressing these challenges, thermostability emerges as a crucial attribute. Thermostable laccases not only facilitate enzyme reactions at elevated temperatures with enhanced rates but also mitigate the risk of microbial contamination. They are particularly valuable in applications such as biobleaching of pulp and the treatment of colored industrial effluents. Thermostable laccases have been predominantly reported from thermophilic bacteria and fungi. Numerous approaches are currently under investigation to enhance laccase activity and mitigate thermal enzyme inactivation. While common strategies involve chemical modifications and enzyme immobilization on solid supports, these methods often pose challenges in terms of synthetic complexity and sustainability, leading to high costs. Recent studies explore the use of co-solvents like polyethylene glycol and ionic liquids, showing promise in improving reaction kinetics for enzymes like alcohol dehydrogenase and α-chymotrypsin. Additionally, deep eutectic solvents (DESs) have emerged as environmentally friendly alternatives for biotechnological applications, owing to their compatibility with enzymes [88]. DESs typically consist of a hydrogen bond acceptor (HBA), such as a salt, and a hydrogen bond donor (HBD), like polyols and sugars. The interaction between HBA and HBD forms hydrogen bonds, resulting in DESs with a significantly lower melting temperature compared to their individual components. Noteworthy properties associated with DESs include low flammability, low vapor pressure, high solvability, low volatility, thermal and chemical stability, and broad polarity. A specific category within DESs, referred to as natural deep eutectic solvents (NADESs), is exclusively prepared using raw materials derived from natural sources, such as sugars and amino acids [89]. This natural origin makes NADESs suitable for various food-related applications, including applications in food packaging. Multiple patents have been filed predominately from fungi between 2008 and 2019, which are related to various applications and obtained from multiple sources.

Structural Characteristics and Catalytic Mechanism of Laccases

The three-dimensional structure of most of the laccases from bacteria, fungi, and plants reveals a polypeptide chain organized into three cupredoxin-like domains, domain 1, 2 and 3 arranged sequentially, as depicted in Figure 2 [83]. The function of domain 2 is to join and position domains 1 and 3, and a trinuclear cluster (TNC) is formed at the interface between domains 1 and 3. Laccases, being copper-containing glycoproteins, exist in either dimeric or tetrameric forms, with each monomer housing four copper atoms. These copper sites in laccases fall into three distinct groups: Type-1 (blue copper center), Type-2 (normal copper), and Type-3 (coupled binuclear copper centers) [90,91], each exhibiting characteristic electronic paramagnetic resonance (EPR) signals.

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Type-1 copper centers coordinate with two histidines, one cysteine, and one methionine as ligands, imparting the deep blue color to laccases with an intense electronic absorption band near 600 nm (ε = 5000 M^{-1} cm^{-1}). However, certain laccases, like the "white laccase" in *Pleurotus ostreatus* or 'yellow laccases,' exhibit variations in absorption due to altered oxidation states or the presence of additional elements [92,93]. Type-2 copper, coordinated by two histidines and a water molecule, lacks visible spectrum absorption and is typically positioned near Type-3 copper. Type-3 copper, coordinated by three histidines and a hydroxyl bridge, displays electron absorption around 330 nm. The structure and properties of these copper centers classify laccases into low-redox potential (bacteria and plants) and high-redox potential (basidiomycetes, especially white-rot fungi) categories, influencing their suitability for diverse applications.

The catalytic mechanism of laccase relies on the distributed copper atoms across three distinct centers (Figure 2). Three key steps characterize laccase-mediated catalysis:

1. Type-1 Copper Reduction by Reducing Substrate: Laccase initiates the reaction by accepting electrons from the substrate, reducing the Type-1 copper center.
2. Internal Electron Transfer: Electron transfer occurs from Type-1 to Type-2 and Type-3 copper centers, forming a trinuclear cluster.
3. Reduction of Oxygen to Water: The trinuclear copper cluster reduces molecular oxygen to water, concluding the catalytic cycle.

The overall reaction for laccase catalysis can be summarized as: $4RH + O_2 \rightarrow 4R\bullet + 2H_2O$ (Equation 1) Here, RH represents the substrate molecules, and laccase oxidizes these substrates (4RH), generating free radicals (4R•) and reducing molecular oxygen (O_2) to water ($2H_2O$). This underscores laccases' pivotal role in generating free radicals for diverse reactions, including polymerization. The oxidative capabilities of laccases can be enhanced in the presence of specific low-molecular-weight compounds functioning as redox mediators, for example ABTS, 1-hydroxybenzotriazole (HBT), violuric acid, 2,2,6,6,-tetramethyl-1-piperidinyloxy free radical (TEMPO), etc. The combination of laccases with these compounds in 'laccase-mediator systems' has proven to significantly broaden the substrate range of the enzymes and enhance their efficiency in oxidizing resistant molecules or complex polymers [94].

Structure-Function Relationship among Laccases

Laccases, renowned for their capacity to catalyze the oxidation of various compounds, demonstrate versatility in function and industrial applications. Among their diverse functions, lignification/delignification stands out as particularly significant due to its involvement in various industrial processes such as pulp and paper manufacturing, biobleaching, bioenergy production, biomass conversion, biofuel production, and environmental pollutant removal. Laccases also play roles in polymerization/depolymerization of lignin, fungal pathogenesis, wound healing, sclerotization, morphogenesis, sporulation, pigmentation, fruiting body formation, melanin formation, and endospore coat protein synthesis [83,95–99].

In the plant lignification process, monolignols undergo polymerization through dehydrogenation, facilitated by enzymes including laccases found in the cell wall. Experimental studies reveal that laccases from various plant species efficiently oxidize monolignols, contributing to dehydrogenative polymer formation [100–102]. Laccase expression, mainly in the secondary xylem, has been reported in trees like *Populus trichocarpa* and *Pinus taeda*, suggesting involvement in plant lignin biosynthesis. Plant transformation studies using laccase gene constructs further support this involvement [103–105]. In fungi, laccases mediate lignin biodegradation, breaking down lignin polymer through oxidative processes, releasing phenolic compounds [106]. As discussed before, directed evolution stands as a potent protein engineering strategy, employing iterative cycles of random mutagenesis and selection under specific selective pressures. This approach aims to fine-tune the inherent characteristics of native enzymes, adapting them to the rigorous conditions of industrial operations or instilling them with new properties. Thus, by employing a combination of

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enzyme-directed evolution and rational design, successfully engineered fungal laccases, produced in yeast, has been investigated to function effectively under alkaline pH and high temperatures. These optimized conditions align with the requirements commonly found in the kraft process and the manufacture of fiberboard.

Despite sharing similar molecular architecture, plant and fungal laccases exhibit wide phylogenetic, physicochemical, and functional diversity [83,107,108]. Plant laccases participate in lignin biosynthesis, while fungal laccases are involved in lignin degradation or depolymerization. The redox potential of laccase is crucial, with fungal laccases (higher redox potential) capable of acting on both phenolic and non-phenolic subunits, contributing to lignin degradation. Plant laccases, with a lower redox potential, polymerize lignin by facilitating the coupling of phenoxy radicals [109].

pH dependence of fungal and plant laccases is proposed as a factor influencing their dual role in lignin degradation or synthesis [110,111]. Fungal laccases typically have low pH optima, adapted to acidic growth conditions, while plant laccases, being intracellular, have pH optima closer to the physiological range. Differences in pH optima may be linked to the dual function of laccases. Hakulinen and coworkers [112] have reported that the architectural differences at the C-terminal end of *M. albomyces* and *T. versicolor* laccases might be responsible for their role in lignification and delignification, respectively.

The three-dimensional structure of laccases, leading to an altered microenvironment at the enzyme's active site, is suggested as the basis for their dual action in lignin biosynthesis and degradation. Structural distinctions in the C-terminal region have been reported, contributing to the role of laccases in lignification and delignification. Computational studies using bioinformatic tools have provided insights into the molecular basis of lignin synthesis and degradation, offering valuable information for future strategies aiming to modify laccase structure in plants and fungi to improve lignin biosynthesis and biodegradability [113].

Major Strategies to Enhance Thermostability

α-Amylase serves as a crucial industrial biocatalyst in the process of starch liquefaction and also stands as a significant model enzyme for exploring thermal adaptation in proteins. Presently, there's a surge in demand for enzymes, particularly those adaptable to industrial applications, prompting researchers to delve into diverse sources like metagenomes [114]. Despite this, bacterial sources continue to dominate the industrial landscape due to their diversity and requisite properties, notably stability and functionality at high temperatures commonly encountered in industrial processes [115]. Enhancing the thermostability of an enzyme primarily involves three strategies. The *first* entails sourcing extremophiles in hopes of enzyme behavior mirroring that of its host. The *second* strategy involves shielding the enzyme structure through immobilization on suitable matrices like cloisites or via the addition of certain cations, crowding agents, and deep eutectic solvents [116–118]. However, both strategies have inherent limitations, such as the rarity of finding natural sources with desired industrial-grade properties and the inability of many enzymes to be stabilized using additives. Thus, consideration of a *third* strategy involving protein structure design or protein engineering to meet thermal stability demands arises [119]. This involves modifying key features of protein structure critical for thermal adaptation, including enhancing rigidity, reducing loop length, optimizing core packing, intensifying surface hydration, and designing stabilizing interactions like salt bridges and hydrophobic interactions.

Numerous studies have explored the enhancement of enzyme thermostability through protein engineering. Protein engineering on hotspot residues is recognized as a highly effective strategy for enhancing both the stability and activity of enzymes. Computer modeling was employed to delve deeper into the structural underpinnings of the variance in thermostability between the wild-type enzyme and its variants. Thus, suggesting that augmenting the number of salt bridges and hydrophobic interactions surrounding Lys209 serves as the primary mechanism driving the enhanced compactness of the enzyme's protein structure [120]. Yuan's group [121] studies on improving thermostability of *Bacillus amyloliquefaciens* alpha-amylase by multipoint mutations. Rational protein design, a top-down approach, aids in identifying necessary modifications for

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achieving thermal stability but is hindered by the extensive information required for reliable predictions for each protein. An alternative approach to circumvent these limitations involves random alteration of protein structure, such as random mutagenesis, recombination, and targeted mutagenesis combined with computational biology, termed Computer-Aided Directed Evolution of Enzymes (CADEE). For instance, Suzuki's group [24] utilized site-directed mutagenesis of the BAA gene, deleting Arg176 and Gly177 while substituting Ala for Lys269, to engineer a thermostable mutation.

Wang's group [122] endeavored to enhance the thermostability of α-amylase through combinatorial coevolving-site saturation mutagenesis, a pivotal strategy in directed protein evolution. Similarly, a directed evolution approach using a combination of random and site-directed mutagenesis was adopted to enhance the laccase activity of *Caldalkalibacillus thermarum* strain TA2.A1 for its application in lignin degradation [123]. Li's group [21] employed structure-based rational design to enhance the thermostability of AmyMH, the maltohexaose-forming α-amylase from Bacillus stearothermophilus, in the absence of added Ca^{2+}. Thermostability of α-amylase is enhanced upon mutatiting S187D/N188T, A269K/S187D, and A269K/S187D/N188T via site-directed mutagenesis in *B. licheniformis* [124]. Through a systematic approach to enzyme engineering, which combined enzyme-directed evolution and rational design, Rodríguez-Escribano and coworkers [125] successfully altered the optimal pH of the laccase for lignin phenol oxidation from acidic to basic with an objective to cultivate laccases capable of operating under extreme conditions of high temperature and pH, characteristic of industrial wood conversion processes into kraft pulp and fiberboard. From a structural perspective, integrative strategies like enhancing substrate affinity, introducing electrostatic interactions, alleviating steric hindrance, increasing flexibility of the active site, N- and C-terminal engineering, and augmenting intramolecular and intermolecular hydrophobic interactions are well-established for improving both activity and thermostability [126].

Current Challenges, Research Aims and Recent Advances in the Field of Thermostable α-Amylases and Laccases

Among the various commercially available enzymes, α-amylases seem to be the most versatile enzymes in the industrial enzyme sector no doubt due to the abundance of starch, with the applications ranging from the conversion of starch to sugar syrups, and the production of cyclodextrins for the pharmaceutical industry. With increase in their application spectrum, the research is focused on developing new α-amylases with more thermophilic, thermotolerant and pH tolerant characteristics to improve starch gelatinization, decrease media viscosity, accelerate catalytic reactions and decrease the risks of bacterial contamination. The most thermostable α-amylase currently used in industrial processes is from *Bacillus licheniformis* [127]. It remains active for several hours at 90 °C. One extracellular enzyme from *Pyrococcus woesei* was isolated that is active between 40 °C and 130 °C with an optimum at 100 °C and pH 5.5 [128]. In spite of this, retaining high α-amylase activity at pH around 4.0 is still desired for industrial starch processing. But there seemed to be no great progress in essence, and it needs some huge technological advances. Nevertheless, the structural and dynamic features of amylase may give some inspiration to understand or improve other enzymes' thermostability, as the heat resistance is always a subject of unfailing interest [27]. In spite of the great significance of α-amylase in biotechnology, the greatest challenge of ensuring its stability for economic viability has to be taken into account. To address this challenge, recent attention has been directed towards enhancing both the functionality and stability of α-amylase. Various emerging technologies, including irradiation, pulsed electric field, high pressure, and sonication, have been employed to improve its secondary structure, thermal stability, and overall performance thereby resulting in economic benefits. These latest technologies stated in a recent review article, offer potential avenues for enhancing the stability and efficiency of α-amylase, thereby contributing to its utility in various industrial processes [129]. By optimizing these techniques, researchers aim to overcome the limitations associated with α-amylase stability and unlock its full potential for applications in biotechnology and related fields. Similarly, the current utilization of laccases in industry appears to be restricted in comparison to their potential. Efforts in research should prioritize

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reducing production costs and enhancing the tools for precise control of reactions on specific polyphenols and other substrates targeted by these enzymes. Primary challenges related to the industrial deployment of laccases involve production expenses and the broad range of substrates they can act upon. While the extensive substrate diversity of laccases offers advantages for biodegradation purposes, it also presents hurdles in their commercial utilization within biocatalysis due to by-product formation resulting from free radical chemistry. Recent developments have introduced new areas of application such as plastic degradation, diagnostic tool development, among others. Therefore, it is reasonable to anticipate a rise in the number of patented innovations in the foreseeable future. Consequently, laccases are poised to penetrate a broader range of industrial sectors, potentially supplanting conventional methods with more environmentally sustainable production routes.

Certainly, addressing the significant expenses associated with laccase manufacturing and purification stands as a paramount challenge that needs resolution to facilitate the enzyme's widespread utilization [130]. Various strategies have been investigated to mitigate enzyme production costs, such as on-site manufacturing, utilizing economical raw materials for enzyme synthesis, exploring novel enzymes with improved activity rates and versatile characteristics, employing cost-effective purification methods, and immobilizing enzymes. In order to fully envision the utilization of laccase on an industrial scale, its activity must be accurately quantifiable on complex substrates and within intricate matrices. Therefore, it becomes imperative to reliably quantify laccase activity on such substrates. Industrial applications often involve substrates much more intricate than those typically assessed using spectrophotometry (by monitoring the appearance of a coloured oxidation product), such as lignin, effluents, and textile dyes. Moreover, the complexity of matrices and mixtures necessitates the development of alternative analytical methods. A recent study [131] outlined several methods, including FTIR, fluorimetry, calorimetry, electrochemistry, and electron paramagnetic resonance, which could address this challenge. However, substantial optimization efforts are essential in the upcoming years before these methods can be considered routine analyses.

Future Directions in the Field of Thermostable Enzymes

In order to utilize thermostable enzymes for industrial purposes, it’s crucial to produce the enzyme on a large scale at a low cost. However, the traditional approach to enzyme production and purification is time-consuming and inefficient. Therefore, employing cloning, purification, and over-expression techniques for such enzymes using a suitable expression system can effectively address this issue. It’s evident that further research is necessary in upcoming studies on thermostable enzymes to fully harness its industrial potential. There is significant potential for enhancing the thermal stability of enzymes. Studies that delve into assessing the thermostability of the mutant enzymes by examining disparities in model structures between the wild-type and mutant ones. Such analysis offers theoretical benchmarks for refining and developing thermostable enzymes. Additionally, introducing tailor-made approaches through systemic enzyme engineering, combining enzyme-directed evolution and rational design is an adapted endeavor nowadays aiming to furnish extremophilic biocatalysts capable of industrial applications. The generic techniques of protein purification and recovery that includes filtration followed by membrane ultrafiltration, precipitation followed by dialysis, freeze and thaw followed by centrifugation and chromatographic techniques demands several steps which are expensive and demand considerable time and energy [132]. Thus, there is an ample opportunity for enhancement in this connection. Nonetheless, encountering challenges persists in discovering a new enzyme with verified activity, largely due to the fact that many proteins are forecasted solely on sequence similarity, leaving their functions hypothetical. It is imperative to experimentally characterize predicted proteins to ascertain sequence-to-function correlations.

Given the biotechnological significance of α-amylase, its substantial stability represents a paramount challenge for ensuring its economic feasibility. Hence, there has been a surge in interest in enhancing both its functionality and stability. In pursuit of this objective, a recent review has

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outlined the utilization of a blend of emerging technologies alongside traditional approaches on α-amylases from diverse sources.

Environmental and health concerns in chemical hair dyeing, such as, laccases have attracted considerable interest due to their capability for cross-coupling polymerization of phenolic monomers and their high oxidation potential. For instance, a thermostable bacterial laccase derived from *Brevibacillus agri* (LacT) has demonstrated significant potential for widespread utilization in the hair dyeing industry as a substitute for conventional chemical hair dyes [133].

With the emerging advances in thermostable laccase and its current application in lignin-first, in future research should focus more on the interaction between thermostable laccases and lignin substrates. At present, the utilization of thermostable laccases exhibiting exceptional characteristics in various environments, their applications have mainly been confined to textile industry, paper industry and the oxidation of small molecular substrates. To date, only a handful of thermostable laccases have been employed in reactions involving macromolecular lignin, with thorough investigations into their mechanisms still lacking, thus demanding more extensive research on this. Moreover, for the specialized discovery of laccases, there is a necessity for further exploration and enhancement of methods in involving metagenomic DNA extraction or enrichment from thermal environments.

Concluding Remarks

Thermostable enzymes not only provide valuable insights into the thermodynamic stability of proteins from a fundamental standpoint but also contribute to understanding the intricate relationship between stability, flexibility, and catalytic efficiency. α-Amylases and laccases, considered as exemplary representatives of thermostable enzymes, hold immense significance in biotechnological and industrial applications. The heightened interest in these enzymes has sparked intensified research endeavors aiming to discover new and improved variants of α-amylases and laccases. Consequently, the current scenario emphasizes the need to explore additional sources of thermostable enzymes or enhance the thermotolerance of existing enzymes through genetic modifications or site-directed mutagenesis, with the goal of achieving specific and desired properties in these enzymes.

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Compliance with ethical standards

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