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Review

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

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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 connection between structural configuration and functional activity of two major classes of thermostable enzymes: α-amylases and laccases. These enzymes serve as valuable models for understanding the structural foundation behind the thermostability of proteins. By highlighting the commercial importance of thermostable enzymes and the interest these generate among researchers in further optimization and innovation, this article can greatly contribute to ongoing research on thermostable enzymes and aiding industries in optimizing production processes via immobilization, use of stabilizing additives, chemical modification, protein engineering (directed evolution and mutagenesis), and genetic engineering (through cloning and expression of thermostable genes). It also gives insights to the exploration of suitable strategies and factors for enhancing thermostability like increasing substrate affinity; introducing electrostatic, intramolecular, and intermolecular hydrophobic interactions; mitigating steric hindrance; increasing flexibility of an active site; and N- and C-terminal engineering, thus resulting in heightened multipronged stability and notable enhancements in the enzymes' industrial applicability.

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

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1. Introduction

The enduring significance of thermostable enzymes stems from their diverse applications across various industries, including food, pharmaceuticals, and biotechnology [1,2]. 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 in bacteria, fungi, and plants [3,4], contrary to human enzymes having an optimal temperature of ~37 °C. The thermostable enzymes maintain their structural integrity and distinctive features under such extreme conditions. This inherent stability provides significant biotechnological advantages compared to mesophilic enzymes (functioning at their best between 25 and 50 °C) or psychrophilic enzymes (ideally active between 5 and 25 °C). Besides being widespread in plants, animals, fungi [5–7], and unicellular eukaryotes like eubacteria and archaea, 'Dictyo-type' α-amylase is also reported to be ubiquitous and may be an ancestor of the Unikonts, a clade that includes amoebozoa, fungi (Opisthokonts), and animals. The activity of α-amylase has been observed to be associated with neurological conditions like Alzheimer's. Studies also show that α-amylase synthesis is linked with mTOR (Mechanical

Target of Rapamycin) signaling pathways [8]. mTOR serves as a key regulator of cellular growth by controlling both anabolic and catabolic processes [9,10]. The widespread occurrence of laccases in fungi, bacteria, animals, plants, and insects highlight varying biological importance of laccases like lignification in plants and delignification in fungi and bacteria.

Thermostable enzymes are more easily purified through heat treatment (as one of the initial steps) [11], exhibit increased resilience and resistance to chemical denaturants, permit elevated levels of substrate concentrations, aid in reducing viscosity, provide less chances of microbial contamination, and frequently cause greater rates of reaction [3]. This heightened stability allows them to thrive in harsh environments, including applications like the enzymatic bioremediation of xenobiotics and innovative green processes [12]. The demand for industrially relevant thermostable enzymes has created a critical requirement to identify easily accessible, economical, and feasible 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 [13]. The use of laccases and their potential expansion in industrial sectors are evident in several process patents under various trade names like Denilite™, Zylite, Novozyme® 51,003, Suberase®, MatZyme® LIGNO™, etc. [14–17]. Thus, laccases show great promise as a replacement for traditional chemical processes in various industries including pulp and paper, textiles, bioremediation, pharmaceuticals, and nanobiotechnology [18–23]. α-Amylase patents distributed across different categories, in biofuels, beverages, pharmaceuticals, detergents, food, animal feed, and textiles, were found to be deposited in intellectual property databases [24].

Another dimension of the profound interest in thermostable enzymes lies in the exploration of the thermodynamic stability of proteins [25–27]. Investigating how catalytic efficiency, stability, and flexibility interact with each other 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 [28,29]. 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 [30]. Crucially, their environmentally friendly nature further underscores their appeal, especially being used as detergent additives [31–33], textile de-sizers [34], starch and food processing [35–37] treatment of agricultural residues [36], detoxification of phenolic inhibitors in lignocellulosic biomass [38,39], and decolorization and detoxification of synthetic dyes [40–46]. 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 [4].

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 [2,11,19]. 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 [4,46]. 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, and structure–function relationships as well as factors and strategies attributing to thermostability. Additionally, this review focuses on the prevailing challenges that exist in the field of thermostable enzymes, offering suggestions for further study and developments for the future of thermostable enzymes.

2. Thermostable α-Amylases

2.1. An Overview

Thermostable α-amylases, constituting the largest share of industrial enzyme sales (approximately 25%), play a pivotal role in a variety of sectors such as food, detergent, textile, fermentation, brewing, biorefinery, paper, and therapeutic industries [13,47], also being illustrated in Figure 1 and listed in Table 1. Particularly crucial in starch liquefaction processes, these enzymes serve as valuable models for studying thermal adaptation in proteins [30]. 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 [48,49].

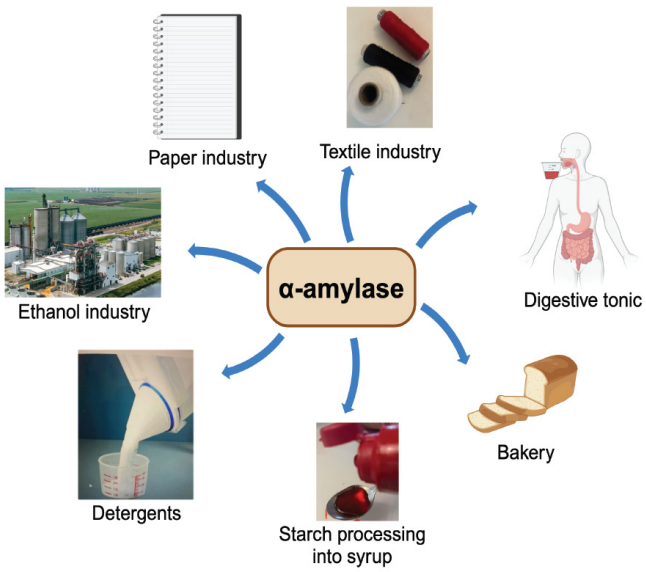

Figure 1. An illustration of the α-amylases used in various industrial applications.

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

Sources 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.

Established in 1998, the CAZy Carbohydrate-Active Enzymes (CAZymes) offer users online access (http://www.cazy.org, accessed on 17 June 2024) that is updated on a regular basis to a sequence-oriented family categorization [50]. This database connects sequences to the three-dimensional structures and specificities of enzymes involved in oligo- and polysaccharide construction, modification, and destruction [51]. The enzymes that are

currently covered in the CAZy database that facilitate the biosynthesis, degradation, or alteration of glycoconjugates and carbohydrates are

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

The CAZy classification system has grown significantly in recent years, including the introduction of new families and the establishment of subfamilies within existing ones [52]. Currently, out of the 189 class glycoside hydrolase (GH) families in total, 4 are considered α-amylase families [53], described here as follows:

(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 member of the α-amylase family composed of a $(\beta/\alpha)_7$ barrel structure;
(iii) GH119—a little family associated with GH57;
(iv) GH126—composed of a $(\alpha/\alpha)_6$ barrel structure.

The α-retaining mechanism of α-amylase is utilized by the families GH13, GH57, and GH119. It involves two catalytic residues in the active site: an aspartate and a glutamic acid/base catalyst. The aspartate retains the nucleophile and the anomeric carbon in the same position, because it is mediated by the double-displacement mechanism. In contrast, GH126 employs the inverting reaction mechanism in which an anomeric carbon position is shifted from β to α through a single-displacement mechanism [54]. There are currently 47 subfamilies within the α-amylase family GH13, and more subfamilies are continuously arising. Since nature sometimes fails to give enzymes the appropriate and ideal characteristics, the engineering of proteins has been suggested as a suitable technique to improve the enzymes' physical and chemical characteristics [55]. The structure-driven consensus method is acknowledged as a reliable and efficient way to refine the characteristics of enzymes by using solved crystal structures [56]. Employing this approach to enhance the thermostability of α-amylase without additional Ca^{2+} would increase effectiveness of the process and lower the cost of starch liquefaction processes [57,58]. On the other hand, this needs sufficient structural information to direct the alterations in addition to a starting enzyme. In the related efforts, through their experimental observations, Li and co-workers showed that malto-hexaose-forming forming α-amylase from *Bacillus stearothermophilus* (AmyMH) is a suitable beginning point for designing a more thermostable α-amylase without the need for additional Ca^{2+} [56]. In the past, efforts to improve the thermostability of different bacterial α-amylases have focused on a loop located in domain B. Suzuki and co-workers suggested to remove the analogous loop formed by R176-G177 (according to *Bacillus amyloliquefaciens* α-amylase (BAA) numbering), stating that it could substantially improve BAA thermostability [59]. A number of additional bacterial α-amylases from various species have reproduced this picture, with a comparable increase in thermostability [60,61]. 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) [62].

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 [63,64]. In this context, thermostable enzymes play a critical role in withstanding the high temperatures inherent in industrial processes [47]. Microbial sources—fungi and bacteria, in particular, especially those in the *Bacillus* genus, such as *B. licheniformis* [31,65,66], *B. amyloliquefaciens* [67], *B. subtilis* [68], B. cereus [69], B. tequilensis [70], *and B. stearothermophilus* [56,71]—are frequently chosen for industrial applications because of their affordability, consistency, and ease of optimizing and modifying the process. Other bacterial and fungal sources reported for varied industrial appli-

cations are *Actinomadura keratinilytica* [72], *Aeribacillus pallidus* [73], *Anoxybacillus vranjensis* [2], *Chromohalobacter* sp. [74], *Geobacillus thermoleovorans* [75], *Paecilomyces variotii* [6], etc.

The ongoing quest for novel thermostable α-amylases has extended to plant sources, seeking alternatives that meet the standards set by microbial sources. Reports of thermostable α-amylases have emerged from plant sources cultivated in local areas, including sword bean (70 °C, [76]), broad bean (65 °C, [49]), potato tuber (60 °C, [77]), red pitaya peel (70 °C, [78]), radish (60 °C, [79]), wheat (68 °C, [56]), soyabean seeds (70 °C, [30]), and mung bean (65 °C, [80]). Some sources of thermostable α-amylases and their respective industrial applications are listed in Table 2.

Table 2. Sources of thermostable α-amylases.

Source of α-Amylases	Optimum Temperature	Industrial Applications	References
Actinomadura keratinilytica sp. Cpt29	70 °C	Laundry detergent additive	[72]
Aeribacillus pallidus BTPS-2	70 °C	Starch liquefaction	[73]
Anoxybacillus vranjensis ST4	60–80 °C	Starch hydrolysis	[2]
Bacillus amyloliquefaciens BH072	60 °C	Food processing	[67]
Bacillus cereus SP-CH11	65 °C	Food processing	[69]
Bacillus licheniformis AT70	60 °C	Starch degradation	[65]
Bacillus licheniformis NH1 strain	70 °C	Laundry detergent additive	[81]
Bacillus licheniformis So-B3	70 °C	Hydrolyzing raw starch	[66]
Bacillus sp. *isolate A3-15*	100 °C	Textile industry	[34]
Bacillus tequilensis TB5	60 °C	Textile de-sizer	[70]
Chromohalobacter sp. TVSP 101	65 °C	Starch hydrolysis	[74]
Geobacillus thermoleovorans	80 °C	Improvement in washing efficiency of detergents	[75]
Germinated wheat seeds (*Triticum aestivum*)	68 °C	Starch processing	[35]
Haloterrigena turkmenica	55 °C	Agricultural residue treatment	[36]
Paecilomyces variotii	60 °C	Starch degradation	[6]
Rhizomucor miehei	75 °C	Food processing	[37]
Soybean (*Glycine max*) seeds	75 °C	Starch liquefaction	[30]
Tepidimonas fonticaldi strain HB23	80 °C	Laundry detergent additive	[33]
Thermomyces dupontii	60 °C	Maltose syrup production	[7]

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

2.2. Structural Features and Mechanism of Action of α-Amylases

The structure of α-amylase is composed of a single polypeptide chain folded into three independent domains (Figure 2, [27]) described as follows:

(a) Domain A is the catalytic domain that is identified by an N-terminal $(\beta/\alpha)_8$ barrel, which is also referred to as a TIM barrel. This structure consists of eight parallel β-strands forming a barrel shape surrounded by eight α-helices.
(b) Domain B, which makes up a large part of the substrate binding cleft, has an irregular β-rich structure, responsible for notable variations in size, structure, and substrate specificity among different α-amylases.
(c) Domain C, which makes up the C-terminal portion of the sequence [82–84].

After the C-domain, some maltogenic amylases exhibit an additional D-domain, the purpose of which remains unknown yet [85].

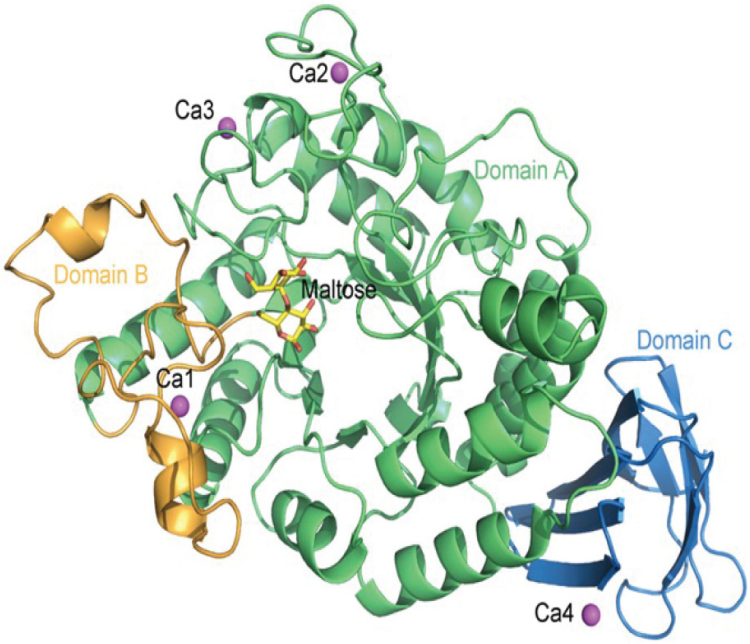

Figure 2. A structural depiction of the crystal structure of α-amylase from *Anoxybacillus* species (TASKA, PDB 5A2A [27]) showing a single polypeptide chain folding with the relative positions of the three structural domains: (a) domain A, the catalytic domain; (b) domain B that constitutes a significant 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.

The α-amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are classified as endozymes in the Glycosyl Hydrolase (GH13) family based on their catalytic mechanism. They form bonds between internal α-glycosidic linkages in polysaccharides including glycogen, starch, and others, causing these bonds to hydrolyze and produce α-anomeric mono- or oligosaccharides [86], as depicted in Figure 3 [87]. α-Amylases in plants are vital for the breakdown of starch contained in sprouting seeds, releasing sugars required for healthy growth of the plant [88]. Maltogenic amylases demonstrate exceptional diversity in catalysis, as they can hydrolyze both α-D-(1,4)- and α-D-(1,6)-glycosidic linkages and take part in transglycosylation processes. This involves the transfer of the glycosyl units to the C3, C4, or C6 hydroxyl groups of various acceptor mono- or disaccharides, in contrast to many other amylases that are limited to hydrolyzing α-D-(1,4)-glycosidic bonds [89].

Nearly every known α-amylase, with very few exceptions, has structural stability, which is linked to the existence of a calcium ion at the interface between domains A and B. This stability is disrupted when the calcium ion is removed, which causes a noticeable decline in catalytic activity. A 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 [90]. Elucidating the structure of BLA, when it contains metals, with comparisons to the calcium-depleted form (apo-enzyme), has provided insights into how metal ions regulate enzyme activity. Research has shown that the stability of BLA is dramatically decreased when calcium chelators are added [91,92], resulting in a heightened susceptibility to proteolysis [93]. This serves as compelling evidence of the indispensable stabilizing role played by the calcium ion. As a result, it is suggested that α-amylases represent a new class of metallo-enzymes that are identified by an alkaline-earth metal prosthetic group—rather than a transition element. The main function of the group is structural, similar to disulfide bridges [94]. Because they are positioned too far away from the active site to be directly

involved in catalysis, calcium ions are thought to contribute structurally [95–97]. The increased 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 [98]. A chloride ion at the active site of some α-amylases increases catalytic performance by bringing about modifications in conformation surrounding the active site and potentially by raising the pKa (acid dissociation constant) of a residue in the active site that donates hydrogen.

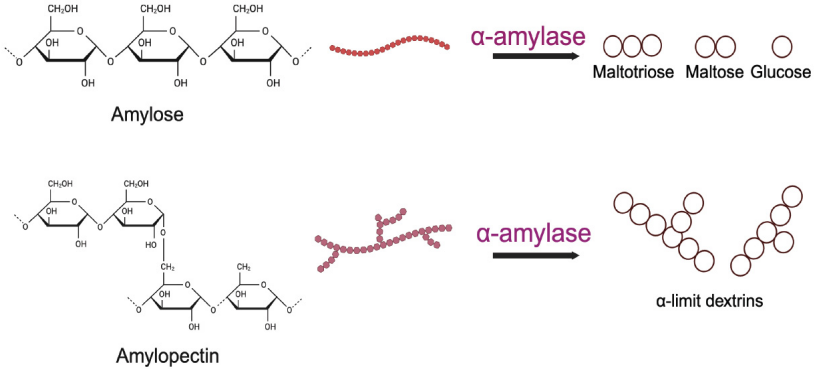

Figure 3. An illustration depicting the chemical structure of starch (comprising amylose and amylopectin) and the enzymatic conversion into sugar units [87]. The catalytic mechanism of α-amylases involves the internal α-glycosidic bond cleavage in polysaccharides like starch, glycogen, and others, leading to the hydrolysis of these bonds and the production of α-anomeric mono- or oligosaccharides.

2.3. Factors Contributing to Stability in Thermostable α-Amylases

Multiple factors contribute to thermostability, like increased hydrogen bonding, hydrophobic interactions, ionic and electrostatic interactions, disulfide bonds, metal binding, salt bridges, ion pairs, aromatic clusters, sidechain–sidechain interactions, shorter surface loops, GC-rich codons, charged amino acid ratios, preferences for amino acids, post-translational changes, and accumulation of solutes [99]. Thermophilic proteins are characterized by more rigid and compact packing density, lower thermal motion, decreased flexibility, shorter surface loops, stabilization by heat-stable 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 [30,99].

In terms of industrial applications, enzyme stability is crucial, with a focus on thermodynamic and long-term stability. Numerous techniques to improve stability such as immobilization, modifying chemicals, stabilizing additives, and expressing thermostable α-amylase genes through cloning, protein, and genetic engineering have been explored. Site-directed mutagenesis and the revolutionary approach of directed evolution have emerged as promising strategies for thermostabilization [100,101]. Economic considerations in starch processing industries drive the need for α-amylases active at higher temperatures, and with continued study, the emphasis has changed from engineering for stability to engineering for substrate specificity and pH activity, leading to the creation of novel and enhanced features in α-amylases.

Calcium ions are essential for determining the stability, functionality, and structure of thermophilic α-amylases, providing resistance or tolerance to thermal inactivation by maintaining correct protein conformation [102–105]. The elimination of calcium ions irreversibly inactivates barley α-amylase, while calcium ion addition restores activity in certain bacterial α-amylases [106]. With a few Ca^{2+}-independent exceptions, the majority of α-amylases are Ca^{2+}-dependent [31,107–109] and also, some α-amylases are inhibited by Ca^{2+} [110,111]. Because of the distance between their catalytic centers and calcium-binding locations, α-amylases primarily have a structural role [97,98]. Numerous investigations have been conducted regarding the impact of calcium ions on the stability and activity of

α-amylases derived from thermophiles, which may help in determining the mechanism of Ca^{2+}-binding proteins in the presence of an extreme thermal environment as was investigated by Liao and co-workers, in which they studied the effect of calcium ions on the thermal characteristics and structure of thermophilic *Anoxybacillus* sp. GXS-BL α-amylase (AGXA) [112].

3. Thermostable Laccases

3.1. An Overview

Laccases are recognized as environmentally friendly proteins and green biocatalysts, setting them apart from other oxidases [46]. Unlike certain oxidases, laccases do not depend on toxic H_2O_2 or any mediator for the reduction reaction, and by reducing molecular oxygen, they only produce water as the final product. These glycoproteins may oxidize a broad variety of inorganic substrates, and exist in monomeric, dimeric, and tetrameric forms, displaying the ability to oxidize a variety of organic, inorganic, and aromatic compounds. Laccase is a versatile enzyme that is mostly used to breakdown chemical pollutants due to its low selectivity for substrates and ability to monoelectronically oxidize substrates in a variety of complexes [71]. Although laccase effectively degrades emerging contaminants [113–116], 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 known as benzenediol/oxygen oxidoreductase (EC 1.10.3.2), is an essential enzyme involved in diverse biological processes. This copper-containing enzyme serves as a catalyst to oxidize a variety of inorganic and organic compounds, allowing oxygen molecules to be reduced and water to be produced 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 4). 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.

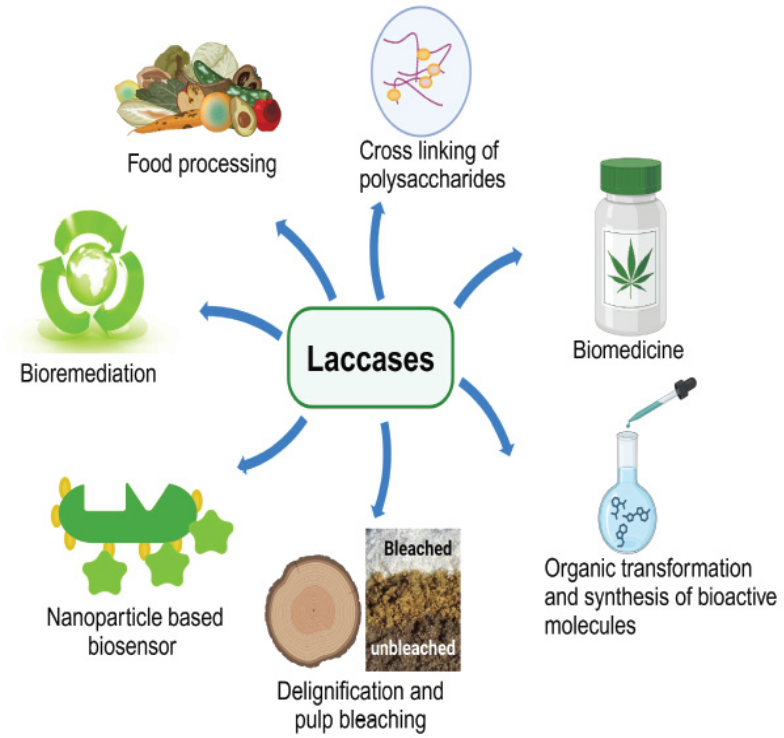

Figure 4. A comprehensive depiction of laccases used in diverse industrial applications.

In nature, laccase is widely distributed and can be found in fungi, bacteria, plants, animals, and insects. This widespread occurrence underscores the fundamental importance of laccase in nature and its relevance in various biological and ecological contexts [117]. The diverse functions of laccases are contingent upon their source organism; they support a variety of biological processes, like synthesis of endospore coat proteins, production of melanin, degradation of lignin, pigmentation, formation of fruiting bodies, fungal morphogenesis, fungal pathogenesis, fungal morphogenesis, lignification, detoxification, wound healing, sporulation, and iron oxidation in plants [118–123]. Thermostable laccases are advantageous for the eco-friendly remediation of hazardous synthetic dyes [124], 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 [17]	Textile
	Denilite™ II	Novozymes	
	Zylite	Zytex Biotech Private Limited [17]	
	Ecostone LC10	AB Enzymes GmbH	
	IndiStar	Genencor International Inc.	
	Novoprime Base 268	Novozymes [125]	
	Primagreen Ecofade LT100	Genencor International Inc. [126]	
	Novozym® 51,003	Novozymes [15]	
White-rot fungi (*Phanerochaete chrysosporium*, *Trametes versicolor*)	Lignozym® Process Laccase Y120	IBB Netzwerk GmbH [127]	Paper
	Novozym® 51,003	Amano Enzyme [128]	Food processing
		Novozymes [15]	
Filamentous fungi and yeasts	Suberase®	Novozymes [14]	Brewing
Genetically engineered bacterial laccase	MetZyme® LIGNO™	MetZen [16]	Bio-refinery

Examples of laccase sources reported from bacteria include *Azospirillum lipoferum* [129], *Anabaena azollae* [130], *Bacillus subtilis* [131], *Streptomyces cyaneus* [132], *S. lavendulae* [133], and *Marinomonas mediterranea* [134]. Ascomycetes, basidiomycetes, and deuteromycetes are the fungal taxa that contain laccases. These include species like *Trametes hirsute*, *T. ochracea*, *T. villosa*, *T. gallica*, *Cerrena maxima*, *Lentinus tigrinus*, *Phlebia radiata*, *Coriolopsis polyzona*, *Pleurotus eryngii*, *Thelephora terrestris*, *Myceliophthora thermophila*, *Russula delica*, *Aspergillus*, *Marasmius*, *Agaricus*, *Tricholoma*, *Penicillium*, *Volvariella*, *Curvularia*, *Chaetomium thermophile*, *Lactarius piperatus*, *Mycelia sterlia*, *and Cantharellus cibarius* [83].

Bacterial laccases are more active and stable at high pH levels, rising temperatures, and high concentrations of copper and chloride ions than fungal laccases [135–137]. There also have been reports of laccases in a variety of plants, such as *Pinus taeda*, *Rhus vernicifera*, *Liriodendron tulipifera*, *Zinnia elegans*, *Populus trichocarpa*, *Acer pseudoplatanus*, *Nicotiana tabacum*, *Zinnia elegans*, *Leucaena leucocephala*, *Carica papaya*, *Lolium perenne*, *Zea mays*, etc. 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%) [138]. Glycosylation significantly influences various aspects of laccase functionality, including copper retention, thermal stability, and enzymatic activity. Some sources of thermostable laccases with industrial applications are listed in Table 4.

Table 4. Sources of thermostable laccases.

Source of Laccases	Optimum Temperature	Industrial Applications	References
Agaricus bisporus CU13	55 °C	Decolorization of synthetic dyes	[42]
Alcaligenes faecalis XF1	80 °C	Decolorization of synthetic dyes	[43]
Azospirillum lipoferum	70 °C	Ecological role in the process of root colonization	[117]
Bacillus altitudinis SL7	55 °C	Bioremediation of lignin contaminated wastewater from pulp and paper industries	[23]
Bacillus sp. MSK-01	75 °C	Proposed as an anti-proliferative agent to cancer cells	[139]
Bacillus sp. PC-3	60 °C	Functionalization of chitosan film for antimicrobial activity	[21,22]
Bacillus subtilis	60 °C	Biodegradation of the fungicide	[113]
Bacillus subtilis strain R5	55 °C	Degradation of synthetic dyes	[68]
Caldalkalibacillus thermarum TA2.A1	70 °C	Lignin degradation	[140]
Coprinopsis cinerea	70 °C	Wastewater treatment	[114]
Enterobacter sp. AI1	60 °C	Degradation and detoxification of synthetic dyes	[32]
Galerina sp. HC1	60 °C	Demethylation of lignin	[141]
Ganoderma lucidum KMK2	60 °C	Decolorization of reactive dyes	[44]
Ganoderma multipileum	70 °C	Biodegradation of chromium	[115]
Geobacillus stearothermophilus MB600	90 °C	Biodegradation of pollutants	[71]
Geobacillus yumthangensis	60 °C	Degradation of organic pollutants	[22]
Klebsiella pneumoniae	70 °C	Decolorization of synthetic dyes	[40]
Lactobacillus plantarum J16 CECT 8944	60 °C	Eliminating toxic compounds present in fermented food and beverages	[116]
Litopenaeus vannamei	>90 °C	Marine bioremediation	[1]
Lysinibacillus fusiformis	80 °C	Removal of sulfonamides and tetracycline residues	[20]
Setosphearia turcica	60 °C	Decolorization of malachite green	[41]
Staphylococcus haemolyticus	60 °C	Textile finishing	[19]
Streptomyces ipomoeae CECT 3341	60 ± 6 °C	Decolorization and detoxification of textile dyes	[39]
Thermobaculum terrenum	80 °C	Protein engineering studies	[26]
Thermus sp. 2.9	70 °C	Delignification of Eucalyptus biomass	[142]
Trametes maxima IIPLC-32	50–70 °C	Detoxification of phenolic inhibitors in lignocellulosic biomass	[38]
Trametes orientalis	80 °C	Decolorization and bioremediation of synthetic dyes	[45]
Trametes trogii	70 °C	Modification of kraft lignin	[18]
Leucaena leucocephala	80 °C	Decolorization of synthetic dyes	[143]
Carica papaya	70 °C	Dye decolorization	[11]

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 biobleaching, ethanol production, modification of biopolymers, decolorization of dyes, food processing, biopulping, development of biosensors, degradation of xenobiotics, and synthesis of drugs and organic compounds, among others [144]. However, a common challenge with many isolated laccases is their relatively low enzyme activity yield and susceptibility to harsh environmental conditions such as pH, temperature, and metal ions. These limitations can impede their extensive use in significant industrial and commercial applications. Addressing these challenges, thermal tolerance emerges as a crucial attribute. Thermostable laccases not only facilitate enzyme reactions at elevated temperatures with increased prevalence but also help in reducing the chance of contamination from microbes. They are particularly valuable in applications such as pulp biobleaching and colored industrial wastewater treatment. Most thermophilic fungi and bacteria have been observed to produce thermostable laccases. Numerous approaches are currently under investigation to enhance laccase activity and mitigate thermal enzyme inactivation. While common strategies involve chemical alterations and the immobilization of enzymes on solid surfaces, the methods frequently pose challenges in terms of synthetic complexity and sustainability, leading to high costs.

3.2. Structural Characteristics and Catalytic Mechanism of Laccases

The majority of laccases found in fungi, bacteria, and plants have three sequentially placed domains similar to cupredoxin (domain 1, 2, and 3) [144], as depicted in Figure 5 [25]. Domain 2 is responsible for connecting and arranging domains 1 and 3, and at the interface where domains 1 and 3 meet, a trinuclear cluster (TNC) is created. Laccases are copper-containing glycoproteins, and can be found in either dimeric or tetrameric forms, having four copper atoms in each monomer. Type-1 (blue copper center), type-2 (normal copper), and type-3 (coupled binuclear copper centers) are the three different groups of copper sites found in laccases [145,146], each exhibiting distinctive signals of electronic paramagnetic resonance (EPR).

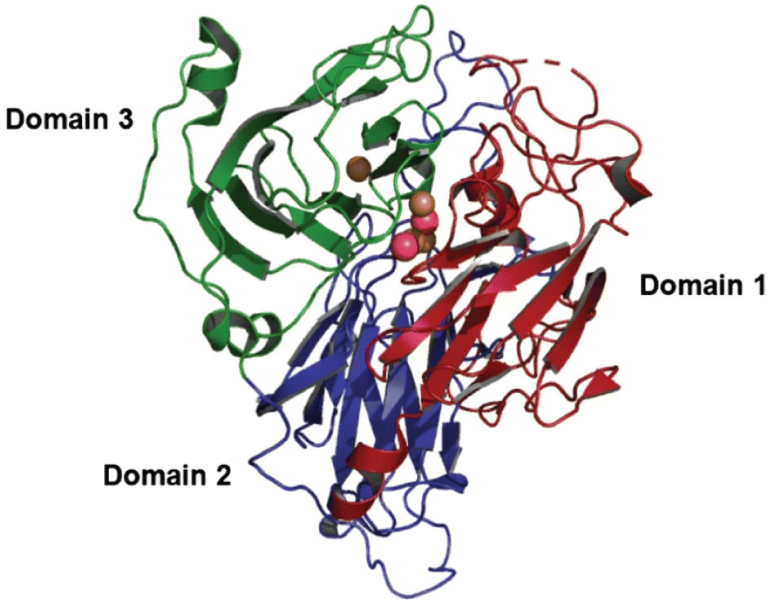

Figure 5. A structural depiction of the crystal structure of the laccase from *Bacillus subtilis* (PDB 1GSK, [25]) showing the polypeptide chain with relative positioning of the three homologous cupredoxin domains, domain 1, 2, and 3, arranged sequentially. Domain 2 joins and positions domains 1 and 3. Domain 1, 2, and 3 are depicted in red, blue, and green.

When two histidines, one cysteine, and one methionine combine together as ligands, type-1 copper centers impart a deep blue color with a strong 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 [147,148]. Coordinated by two histidines and one water molecule, type-2 copper is usually found close to type-3 copper and does not exhibit a visible light spectrum. Three histidines and a hydroxyl bridge coordinate together with type-3 copper, which exhibits electron absorption at 330 nm. Laccases are categorized into low redox potential (bacteria and plants) and high redox potential (basidiomycetes, particularly white-rot fungi) groups based on the structure and characteristics of these copper centers, influencing their suitability for diverse applications.

The dispersed copper atoms over three different sites are essential to catalytic activity of laccase (Figure 6). Three key steps characterize catalysis by laccase [149].

1. Type-1 Reduction of Copper by Reducing Substrate: Laccase initiates the reaction by accepting electrons from the substrate, reducing the type-1 copper center.
2. Internal Electron Transfer: A trinuclear cluster is formed when electrons are transferred from type-1 to type-2 and type-3 copper centers, forming a trinuclear cluster.
3. Oxygen Reduction to Water: The catalytic cycle is completed when the trinuclear copper cluster reduces molecular oxygen to produce water.

The overall reaction for laccase catalysis can be summarized as

$$4RH + O_2 \rightarrow 4R\bullet + 2H_2O \quad (1)$$

Here, RH represents the substrate molecules, and laccase oxidizes these substrates (4RH), generating free radicals (4R•) and reducing molecular oxygen (O_2) to water ($2H_2O$).

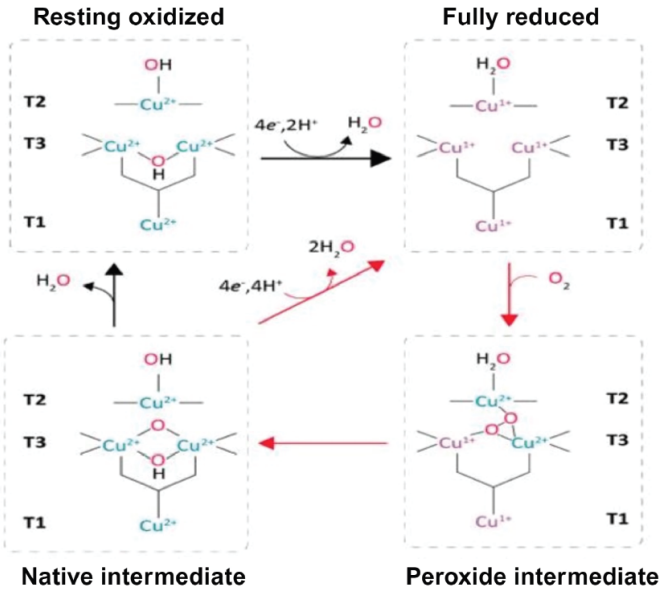

Figure 6. An illustration representing the catalytic mechanism of action of laccases, the substrate-induced decrease in T1 copper, after which the electrons are transferred to the TNC and subsequent reduction of O_2 occurs (adapted from [149,150]). As an electron acceptor, mononuclear copper T1 oxidizes the substrate in the initial phase, converting Cu2+ to the Cu+ oxidation state. After the removal of an electron from the substrate, an unstable cationic radical is produced, which is oxidized by a second enzymatic reaction or undergoes 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 the conversion of O_2 to H_2O. Four molecules of the reducing substrate are needed for the complete reduction of molecular oxygen to water. Thus, the stoichiometry of the enzymatic reaction of the catalytic mechanism of laccases is represented by the equation 4RH + O2 → 4R + 2H2O, where RH signifies the substrate.

This underscores laccases' pivotal role in generating free radicals for diverse reactions, including polymerization. Certain low-molecular-weight substances that act as redox mediators, for example, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), abbreviated as ABTS; violuric acid, 1-hydroxybenzotriazole (HBT); 2,2,6,6,-tetramethyl-1-piperidinyloxy free radical (TEMPO), etc., can increase the oxidative potential of laccases. It has been demonstrated that combining laccases with the above-stated compounds to create 'laccase-mediator systems' greatly expands the substrate range of the enzymes and increases their effectiveness in oxidizing resistant molecules or complex polymers [150].

3.3. Structure–Function Relationship among Laccases

Laccases are widely used in industries and exhibit diversity in their function, as seen by their ability to catalyze the oxidation of diverse compounds. Among their diverse functions, lignification and delignification stand out as particularly significant because they are involved in a number of industrial processes such as the production of pulp and paper, production of biofuels, biobleaching, bioenergy production, conversion of biomass, and removal of pollutants from the environment. The polymerization and depolymerization of lignin, wound healing, pigmentation, fruiting body creation, fungal pathogenesis, sclerotization, morphogenesis, sporulation, melanin generation, and endospore coat protein synthesis are additional applications that laccases are involved in [144,151,152].

Plant lignification involves the polymerization of monolignols through dehydrogenation, which is aided by enzymes including laccases found in the cell wall. Experiments show that laccases from different plant species effectively oxidize monolignols, helping in the synthesis of a dehydrogenative polymer [153–155]. 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 [156–158]. In fungi, laccases mediate lignin biodegradation, breaking down the lignin polymer through oxidative processes, releasing phenolic compounds [159]. Some reports of bacteria involved in lignin degradation have also been made [140,142].

Plant and fungal laccases have a wide range of physicochemical, functional, and phylogenetic diversity, despite having a comparable molecular architecture [144,160,161]. Fungal laccases are engaged in the breakdown or depolymerization of lignin, whereas plant laccases are involved in lignin biosynthesis. The redox potential of laccase is crucial, with fungal laccases (higher redox potential) being capable of influencing both phenolic and non-phenolic subunits, contributing to the degradation of lignin. Plant laccases, with a lower redox potential, are capable of lignin polymerization by facilitating the phenoxy radical coupling [162].

pH dependence of fungal and plant laccases is proposed as a factor influencing their dual role in lignin degradation or synthesis [163,164]. Fungal laccases typically have low pH optima and hence they are suited to acidic growing conditions, while plant laccases are intracellular and hence have pH optima closer to the physiological range. The dual role of laccases may be linked to these variations in pH optima. According to Hakulinen and co-workers, roles of *Melanocarpus albomyces* and *T. versicolor* laccases in lignification and delignification, respectively, may be due to structural variations at the C-terminal ends [165].

The dual activity of laccases in lignification and delignification is proposed to be based on their three-dimensional structure, which modifies the microenvironment at the active site of an enzyme. 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 underpinnings of lignin biosynthesis and breakdown, providing important insights for future plans seeking to alter the structure of laccase in fungi and plants to enhance the biodegradability and biosynthesis of lignin, respectively [166].

4. Major Strategies to Enhance Thermostability

Thermostable enzymes serve as valuable models for comprehending the physicochemical factors governing protein thermostability. Identifying the structural characteristics implicated in thermal stability facilitates the engineering and production of more resilient enzymes for industrial applications [28,29]. Research utilizing the prediction of amino acid sequences, encompassing amino acid distribution and dipeptide composition, helps 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 [167]. The presence of fewer cysteines in thermophilic proteins is associated with their oxidation at higher temperatures. A sequence analysis proves to be valuable in predicting protein thermostability when structural information is lacking. Understanding the molecular underpinnings of protein stability can be gained by contrasting the dynamic properties of mesophilic proteins with those of their thermophilic homologs that have different thermostability but higher and similarity in structure and identity in sequences.

α-Amylase has been an essential model system for examining enzymes' ability to withstand thermal tolerance [84]. 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 is a surge in demand for enzymes, particularly those adaptable to industrial applications, prompting researchers to delve into diverse sources like metagenomes [168]. 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 [169]. 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 [170–172]. 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, the consideration of a third strategy involving protein structure design or protein engineering to meet thermal stability demands arises [173]. This involves modifying key features of protein structure critical for thermal adaptation, including enhancing rigidity, reducing loop length, maximizing surface hydration and core packing, and designing stabilizing interactions like hydrophobic interactions and salt bridges. The various strategies usually employed for enhancing thermostability of enzymes are also represented in Figure 7.

As discussed before, directed evolution stands as a potent protein engineering strategy, employing iterative cycles of random mutagenesis and selection under specific selective pressures [99]. 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 enzyme-directed evolution and rational design, successfully engineered fungal laccases, produced in yeast, have 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 [18,141].

Numerous studies showed the enhancement in enzyme thermostability through protein engineering [26]. Protein engineering on hotspot residues is recognized as a highly effective approach for enhancing both enzyme stability and activity. 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, it was suggested that augmenting the number of salt bridges and hydrophobic interactions surrounding K209 serves as the primary mechanism operating the enhanced compactness of the enzyme's protein structure [174]. Yuan and co-workers [175] studied multipoint mutations, improving thermostability of *Bacillus amyloliquefaciens* α-amylase. Rational protein design,

a top-down approach, aids in identifying necessary modifications for 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 the 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 and co-workers [59] utilized site-directed mutagenesis of the BAA gene, deleting R176 and G177 while substituting A for K269, to engineer a thermostable mutation.

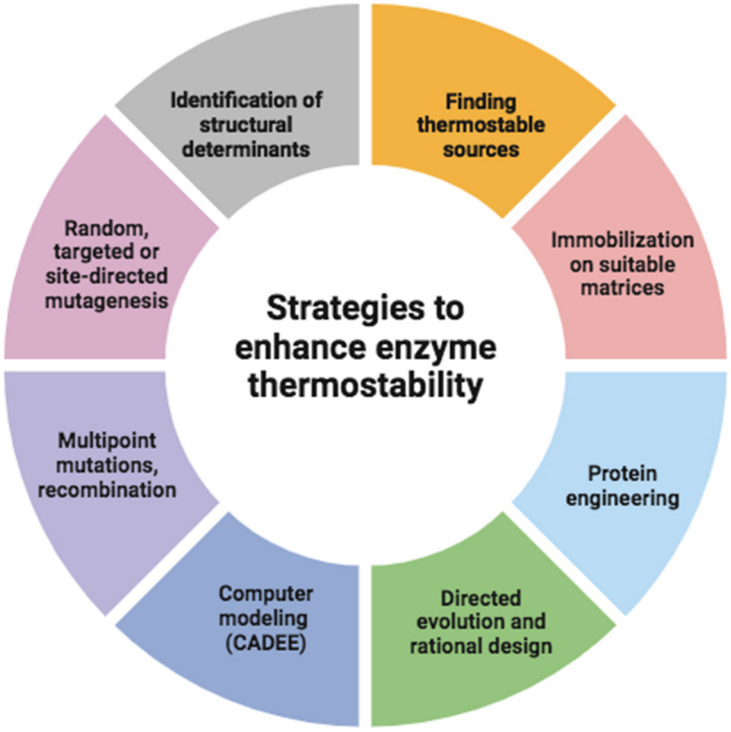

Figure 7. A comprehensive representation of various strategies employed to enhance thermostability in enzymes: finding natural thermostable sources with desired industrial-grade properties; immobilization on suitable matrices like cloisites or addition of stabilizing additives, crowding agents, deep eutectic solvents, and certain cations; protein engineering involving modifications like enhancing rigidity, reduction in loop length, increasing surface hydration and core packing, and inclusion of hydrophobic interactions and salt bridges; directed evolution and rational protein design using random mutagenesis and selection under specific selective pressures; computational biology, also termed as Computer-Aided Directed Evolution of Enzymes (CADEE); multipoint mutations including random mutagenesis, recombination, site-directed and targeted mutagenesis, and mutagenesis via combinatorial coevolving-site maturation; and identifying and integrating strategies like increasing active site flexibility, enhancing substrate affinity, N- and C-terminal engineering, and alleviating steric hindrance.

Wang and co-workers [176] endeavored to increase thermostability of α-amylase through mutagenesis via combinatorial coevolving-site saturation, a pivotal strategy in directed protein evolution. Similarly, a directed evolution approach that combines random and site-directed mutagenesis was adopted to enhance the laccase activity of *Caldalkalibacillus thermarum* strain TA2.A1 for its application in lignin degradation [140]. Li and co-workers [56] utilized a structure-based rational design approach to enhance the thermostability of AmyMH, α-amylase from *Bacillus stearothermophilus* that forms maltohexaose, without the addition of Ca^{2+}. Thermostability of α-amylase is enhanced upon mutating S187D/N188T, A269K/S187D, and A269K/S187D/N188T via site-directed mutagenesis in *B. licheniformis* [177]. Through a systematic approach to enzyme engineering, which

combined enzyme-directed evolution and rational design, Rodríguez-Escribano and coworkers [178] successfully altered the ideal pH of the laccase to oxidize lignin phenol from an acidic to basic state, with an objective to produce laccases that can function in harsh environments with high pH and temperature, a feature of industrial procedures used to convert wood to fiberboard and kraft pulp. Integrating strategies like introducing electrostatic interactions, increasing flexibility of the active site, enhancing substrate affinity, N- and C-terminal engineering, alleviating steric hindrance, and augmenting intramolecular and intermolecular hydrophobic interactions are well established for improving both activity and thermostability [179].

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

Among the various commercially available enzymes, α-amylases appear to be the most adaptable enzymes in the industrial enzyme field due to the abundance of starch, such as converting starch to sugar syrups, and producing cyclodextrins for the pharmaceutical industry. The development of new α-amylases with more thermophilic, thermotolerant, and pH-tolerant properties is the focus of research due to their expanding application spectrum. These enzymes can speed up catalytic reactions, improve starch gelatinization, reduce media viscosity, and lower the risks of bacterial contamination. *Bacillus licheniformis* produces the most thermostable α-amylase that is currently utilized in commercial application processes. It continues to be active for several hours at 90 °C. An extracellular enzyme that is active between 40 °C and 130 °C with an optimum at 100 °C and pH 5.5 was isolated from *Pyrococcus woesei* [180]. Nevertheless, for commercial starch processing, maintaining high α-amylase activity at a pH of approximately 4.0 is still preferred. However, there did not seem to be much of an advancement, and significant technological advancements. However, as heat resistance is a constant area of interest, the structural and dynamic characteristics of α-amylase may provide some inspiration to comprehend or enhance the thermostability of other enzymes [62]. Despite α-amylase's great significance 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 sonication, high pressure, pulsed electric field, and irradiation, have all been used to enhance its secondary structure, thermal stability, and overall efficiency, 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 [181]. 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 best utilization. Lowering manufacturing expenses should be the top priority for research efforts and enhancing the tools for precise control of reactions on particular polyphenols and other substrates targeted by such enzymes. Primary difficulties 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 the production of byproducts resulting from chemistry of free radicals. Recent developments have introduced new areas of application such as plastic degradation and 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 [182].

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 [183]. Various strategies have been investigated to mit-

igate 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 [182]. For laccases to be completely realized as an industrial tool, their activity needs to be precisely measured on complicated substrates and complex matrices. It is therefore crucial to accurately measure laccase activity on these substrates. Industrial applications frequently deal with substrates like lignin [18,38,140,141], effluents [113,114], and textile dyes [39] that are far more complex than those that are normally evaluated using spectrophotometry (by checking for the development of a colored oxidized product). Moreover, the complexity of matrices and mixtures necessitates the development of alternative analytical methods. A recent study [184] outlined several methods, including fluorimetry, Fourier transform infrared spectroscopy (FTIR), calorimetry, electron paramagnetic resonance, and electrochemistry, which could address this challenge. However, substantial optimization efforts are essential in future years prior to these techniques being considered for regular evaluations.

6. Concluding Remarks and Future Directions in the Field of Thermostable Enzymes

To apply thermostable enzymes in industrial settings, it is imperative to develop the enzyme on a large scale inexpensively. But the conventional approach of purifying and producing enzymes 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 [7,31,40,41,59,113,157]. It is 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 delve into assessing the thermostability of mutant enzymes by examining disparities in model systems contrasting the mutant and wild types. Such an analysis offers theoretical benchmarks for refining and developing thermostable enzymes. Additionally, introducing tailor-made approaches through systemic enzyme engineering, which combines rational design with enzyme-directed evolution, is an adapted endeavor nowadays, aiming to furnish extremophilic biocatalysts capable of industrial applications [140,173]. The generic techniques of protein purification and recovery that include filtration followed by membrane ultrafiltration, precipitation followed by dialysis, and freezing and thawing followed by centrifugation and chromatographic techniques demand several steps, which are expensive and demand considerable time and energy [181,182]. Thus, there is 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, this review has outlined the utilization of a blend of emerging technologies alongside traditional approaches on α-amylases from diverse sources.

Regarding environmental and health concerns in chemical hair dyeing, laccases have attracted considerable interest due to their capability of phenolic monomers' cross-coupling polymerization and their high oxidation potential. For instance, a thermostable laccase derived from the bacteria *Brevibacillus agri* (LacT) has demonstrated significant potential for widespread utilization within the hair coloring industry as a substitute for conventional chemical hair dyes [124].

With the emerging developments with thermostable laccase and its use in lignin, first, future research should focus more on the interaction between thermostable laccases and lignin substrates. At present, there is the utilization of thermostable laccases exhibiting exceptional characteristics in various environments; their applications have mainly been

confined to the textile industry and paper industry and the oxidation of small substrates. To date, just a handful of uses of thermostable laccases have occurred 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 the further exploration and enhancement in methods involving extraction or enrichment of metagenomic DNA from thermal settings. 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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