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Fungal Extremozymes: A Potential Bioresource for Green Chemistry

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Abstract

Green chemistry is a rapidly evolving area that aims to reduce the hazards released to the environment during various chemical processes and improve the efficiency of bioconversion. Application of green chemistry could therefore lead to less environmental pollution and better economic outcomes. Enzymes, as biocatalysts, are expected to play a central role in green chemistry owing to their reusability, catalytic efficiency and specificity. Besides, biocatalytic reactions result in no toxic waste in contrast to chemical processes that require careful disposal. However, the use of enzymes in chemical reactions presents various challenges, including stability and unwanted side-reactions. Fungi have drawn significant attention in recent years as a new source of enzymes that could be used in harsh conditions to improve various industrial processes, such as biofuel production and biomass conversion. Combined with modern bioengineering techniques, fungal extremozymes have emerged as promising tools in future applications. Also, structural information has provided new insights into the function and stability of various fungal extremozymes. This review is focused on latest progress in fungal extremozymes, in particular their structural features as well as the current research efforts to improve their properties for better use in green chemistry applications.

Keywords: fungus; biocatalysis; crystal structure; biomass degradation; enzyme improvement; enzyme stability

1. Introduction

Green chemistry has become a worldwide approach toward sustainable growth. New processes using materials which do not emit pollutants and toxic waste to the environment are being pursued. The development, however, of new safer methods that produce less harmful products is not an easy task and presents many challenges. Enzymes have become a great tool for scientists in the field of green chemistry (Table 1) (Anna Calarco 2015). As enzymes act as biocatalysts, they require mild conditions to function, thus they can save key resources such as energy or water (Sheldon and Woodley 2018). Also, enzymes are attractive alternatives owing to their minimal impact on the environment and low costs. Taking into account the benefits of green chemistry, enzyme biocatalysis is now found in various traditional chemical processes in several fields (Cipolatti et al. 2019). This change is going to expand to even more areas owing to new emerging technologies in enzyme engineering.

Table 1. Enzymes with potential applications in green chemistry.

EC number	Enzyme class	Catalyzed reactions	Representative enzymes	Polymer synthesis	Polymer modification
1	Oxidoreductases	Redox reactions by electron transfer	peroxidase, laccase, tyrosinase, glucose oxidase	Yes	Yes
2	Transferases	Transfer of a functional group from one compound (donor) to another	phosphorylase, glycosyltransferase, acyltransferase	Yes	Yes

		compound (acceptor)			
3	Hydrolases	Hydrolysis of various bonds	glycosidase (cellulase, amylase, xylanase, chitinase, hyaluronidase), lipase, protease, peptidase, feruloyl esterase, nitrilase	Yes	Yes
4	Lyases	Cleavage of C-C, C-O, C-N and other bonds by using other than hydrolysis or oxidation	decarboxylase, aldolase, dehydratase	No	No
5	Isomerases	Either racemization or epimerization of chiral centres; isomerases are subdivided according to their substrate specificity	racemase, epimerase, isomerase	No	Yes
6	Ligases	Linking of two molecules with concurrent hydrolysis of the diphosphate-bond in ATP or a similar triphosphate	ligase, synthase, acyl CoA synthase	No	No

Fungi have received significant attention in recent years (de Cassia Pereira et al. 2015; Hyde et al. 2019), especially as a reservoir of extremozymes for use in many biotechnological applications (Table 2). Thermophilic fungi, in particular, have drawn considerable interest owing to their ability to grow at a temperature of 50 °C or above, and at a minimum of 20 °C or above (Maheshwari et al. 2000). Various thermophilic fungi have been isolated in recent years and the enzymes they produce have been investigated at functional and structural level. In this review, an up-to-date information on structure-function aspects of fungal extremozymes is presented and the current research efforts to improve their properties for better use in green chemistry applications are discussed.

Table 2. Fungal enzymes with industrial importance.

Enzyme	Preferred Properties	Representative fungi	Applications
Cellulases	Thermostable	*Melanocarpus albomyces, Chaetomium thermophilum, Humicola insolens*	Biofuel, biorefinery, paper and pulp industry
Xylanases	Alkaliphilic, thermophilic	*Humicola insolens, Melanocarpus albomyces, Sporotrichum thermophile*	Fuels, chemicals, paper, brewing industry
Feruloyl esterases	Thermophilic	*Talaromyces cellulolyticus,*	Food, cosmetics, chemical synthesis

		Sporotrichum thermophile	
Laccases	Thermophilic, Alkaliphilic	*Sporotrichum thermophile, M. albomyces, C. thermophilum, T. versicolor*	Bioenergy, pulp and denim treatment, food and beverages, wastewater, bioenergy
Lipases	Thermotolerant	*Candida sp*., *Penicillum sp*., *Aspergilllus sp.*	Detergents, therapeutics, food supplements
Nitrilases	Thermostable	*Ascomycota*	Organic synthesis, bioremediation
Transaminases	Thermostable	*Thermomyces stellatus*	Organic synthesis
Tyrosinases	Thermostable	*Agaricus bisporus*, *Neurospora crassa*, *Aspergillus oryzae, Aspergillus niger*	Organic synthesis, bioremediation
Keratinases	Thermotolerant, Alkaliphilic	*A. fumigatus, M. thermophila*	Animal feed, medicine, detergents

2. Cellulases

The enzymatic hydrolysis of cellulose to its constituent monosaccharides for the low-cost production of food and biofuels has attracted considerable attention in recent years. Cellulose is the most abundant and renewable non-fossil carbon source on Earth and accounts for 20-50% in dry weight of the plant cell wall material. Compared to current industrial procedures such as heat, mechanical, and acid treatment of cellulose, cellulose degradation by enzymes is considered a more environment-friendly process (Wilson 2009). However, cellulose is the most refractory carbohydrate polymer to enzymatic degradation amongst all polysaccharides of the plant cell wall. Thus, efficient enzymatic degradation of cellulose to glucose requires the synergistic action of endocellulases (E.C.3.1.1.4), exocellulases (cellobiohydrolases, CBH, E.C.3.2.1.91) and β-glucosidases (E.C.3.2.1.21). Endoglucanases initiate hydrolysis by cutting internal glycosidic linkages in a random fashion, resulting in a swift decrease of polymer length and a gradual increase in the reducing sugar concentration. Exocellulases act upon either the reducing or the non-reducing ends to release cello-oligosaccharides and cellobiose units. In the last step, β-glucosidases cleave cellobiose to release glucose molecules (Vlasenko et al. 2010).

Owing to the difficulties for its breakdown, cellulose is subjected to higher temperatures to swell and become more susceptible to breaking. Besides, the use of higher temperatures in industrial processes offers additional advantages such as substrate and product solubility, reduced hydrolysis time, and minimum risk of microbial contamination. Enzymatic hydrolysis by thermophilic cellulases has therefore become a key step for efficient biomass degradation (Atalah et al. 2019).

Cellulases are classified into 13 glycoside hydrolase (GH) families (http://www.cazy.org): 1, 3, 5, 6, 7, 8, 9, 12, 26, 44, 45, 48 and 61. Thermophilic fungal cellulases are found in families 1, 3, 5, 6, 7, 12, 45 and 61 (Li and Papageorgiou 2019). GH61 family members are now identified as Cu(II) ion-dependent lytic polysaccharide monooxygenases (LPMOs) and have been included in the auxiliary activity families of the CAZy database (Busk and Lange 2015).

2.1. Cellulase production and characterization

Heterologous host organisms, such as *E. coli*, yeast, and filamentous fungi have been used for the expression of cloned cellulase genes of thermophilic fungi (Li and Papageorgiou 2019). The majority of the recombinant cellulases expressed in yeast and filamentous fungi are glycosylated (Li et al. 2009; Takashima et al. 1999). Glycosylation could be a contributing factor to the thermostability improvement of cellulases according to previous reports (Meldgaard and Svendsen 1994). The mechanism, however, is still unknown. It has been reported that N-glycosylation could increase solubility and reduce aggregation (Ioannou et al. 1998; Kayser et al. 2011). Also, analysis of protein structures deposited in the Protein Data Bank has also indicated a decrease in protein dynamics upon N-glycosylation without significant global or local structural changes (Lee et al. 2015).

Thermophilic fungal cellulases are usually single polypeptides although some β-glucosidases exist as dimers (Gudmundsson et al. 2016; Mamma et al. 2004). The molecular weight of thermophilic fungal cellulases has a wide range (30-250 kDa) with different carbohydrate contents (2%-50%). The majority of the purified cellulases from thermophilic fungi exhibit similar optimal pH and temperature. Indeed, thermophilic fungal cellulases are found active in the pH range 4.0-7.0 and display a high-temperature maximum at 50-80 °C for activity (Li and Papageorgiou 2019). Also, they exhibit remarkable thermal stability and are stable at 60 ºC with longer half-life at 70, 80 and 90 °C than those from mesophilic fungi.

2.2. Primary and three-dimensional (3-D) structure

2.2.1 Primary structure

Most cellulases are characterized by a modular structure. Typically, endocellulases and cellobiohydrolases consist of four modules: a signal peptide that facilitates secretion, a cellulose-binding domain (CBD) for the enzyme's attachment to the substrate, a catalytic domain (CD) used for the hydrolysis of the substrate, and a hinge region (linker) which is usually post-translationally glycosylated and rich in Ser, Thr, and Pro residues.

CBDs consist of less than 40 amino acids and interact with cellulose through a flat platform-like hydrophobic binding site that is thought to be complementary to the flat surfaces presented by cellulose crystals (Shoseyov et al. 2006). Studies have shown that deletion of the CBDs present in *T. reesei* Cel7A and Cel6A and *Humicola grisea* CBH1 reduces significantly enzymatic activity toward crystalline cellulose (Takashima et al. 1998), suggesting that the efficient hydrolysis of crystalline cellulose requires tight interactions to cellulose through the CBDs. Aromatic residues in CBDs have been suggested to affect the cellulose-binding ability and enzymatic activity (Takashima et al. 2007).

Variations in the primary structure have been identified. *Talaromyces emersonii* CBHII, for instance, has a modular structure (Murray et al. 2003) whereas *T. emersonii* CBH1 consists solely of a catalytic domain (Grassick et al. 2004). Similar variations are found in *Chaetomium thermophilum* CBHs (CBH1, CBH2 and CBH3) where CBH1 and CBH2 consist of a typical CBD, a linker, and a CD whereas CBH3 only comprises a catalytic domain and lacks a CBD and a hinge region (Li et al. 2009). However, cellulases without CBDs can still be efficiently used (Le Costaouëc et al. 2013; Pakarinen et al. 2014).

2.2.2. Three-dimensional (3-D) structural details

3-D structures of thermophilic fungal cellulases from families 1, 5, 6, 7, 12 and 45 have been reported. Details have been recently reviewed (Li and Papageorgiou 2019).

2.3. Improvement of thermophilic fungal cellulases

Two main research approaches are presently in use for improvement and modification of enzyme function: structure-based rational site-directed mutagenesis and random mutagenesis through directed evolution. Detailed knowledge of the 3-D structure of a protein is required for site-directed mutagenesis. In contrast, the directed evolution approach is not limited by the absence of structural details but requires an efficient method for high-throughput screening (Labrou 2010).

2.3.1. Thermostability improvement

Generally, the mechanism of protein thermostability has been studied more extensively in thermophilic bacteria and hyperthermophilic archaea (Pack and Yoo 2004; Trivedi et al. 2006). However, a common thermostability mechanism has not yet been established. Several contributing factors to protein thermostability have been proposed. An increase in ion pairs on the protein surface and a more hydrophobic interior have been put forward as the major factors of improved protein thermostability (Taylor and Vaisman 2010). Nevertheless, compared with thermophilic proteins from thermophilic bacteria and hyperthermophilic archaea, the understanding of the nature and mechanism of thermostability in proteins from thermophilic fungi is relatively poor and additional studies are needed.

Despite that cellulases from thermophilic fungi are already thermostable, additional increase of their thermostability is desirable for industrial applications. error-Use of error-prone PCR, for example, in *Melanocarpus albomyces* Cel7B resulted in the improvement of the enzyme and the identification of two positive thermostable mutants (Voutilainen et al. 2007). Also, introduction of extra disulfide bridges to the catalytic module of *Talaromyces emersonii* Cel7A gave rise to three mutants with improved thermostability as revealed by Avicel hydrolysis efficiency at 75 °C (Voutilainen et al. 2010).

Mutants of three Cys residues of the thermostable *Humicola grisea* Cel12A were found to affect the stability of the enzyme (Sandgren et al. 2005). A report of fold-specific thermostability through variations in amino acid compositions of endoglucanases have provided new strategies for thermostability improvement (Yennamalli et al. 2011).

Random mutagenesis and recombination of beneficial mutations were employed for the construction of a chimeric Cel6A cellobiohydrolase (Wu and Arnold 2013). Consequently, increased hydrophobic interactions and reduced loop flexibility by introduction of Pro residues were found to improve thermostability.

A computational approach, namely SCHEMA, which uses protein structure data to generate new sequences with minimal structure disruption when they are introduced in chimeric proteins has been employed to create thermostable fungal cellulases (Heinzelman et al. 2009). Application of SCHEMA using the high-resolution structure of *Humicola insolens* CBHII as a template resulted in a collection of CBHII chimeras with high thermostablility (Varrot et al. 2003).

Improvement of cellulase stability in detergent solutions has also been pursued (Otzen et al. 1999). The anionic surfactant C12-LAS can inactivate *H. insolens* Cel45 endoglucanase because of the positive charges at the surface of the enzyme (Otzen et al. 1999). Mutation of surface residues R158E/R196E was found to improve stability, most likely by preventing C12-LAS from binding to the protein.

2.3.2. Activity improvement

Improvement of cellulase activity has been pursued in recent years by using site-directed mutagenesis and directed evolution. However, the lack of general rules for site-directed mutagenesis and the limitations of screening methods have resulted in only a few successful examples of cellulase mutants with considerably higher activity than the wild-type enzymes (Percival Zhang et al. 2006). Directed evolution of *Chaetomium thermophilum* CBHII produced mutants that were able to retain more than 50% of their activity at 80 °C for 1 h while the wild type abolished all of its activity under the same conditions (Wang et al. 2012).

Addition or replacement of a CBD to alter the enzyme characteristics and to improve hydrolytic activity has also been tried (Limon et al. 2001; Shoseyov et al. 2006; Szijarto et al. 2008; Takashima et al. 1999).

2.4. Glycosidic bond synthesis

Glycosynthases are engineered enzymes able to catalyze the synthesis of glycosidic bonds (Hayes and Pietruszka 2017). They are best described as retaining GH mutants in which the catalytic nucleophile (Asp, Glu) has been replaced by a non-nucleophilic residue, usually a smaller uncharged amino acid. Cellulase engineering to produce glycosynthases by site-directed mutagenesis has been actively pursued (Shaikh and Withers 2008). The first glycosynthase from thermophilic fungus was derived from *Humicola insolens* Cel7B following mutation of catalytic residue Glu197 to Ala. The resultant Cel7B E197A glycosynthase was able to catalyze regio- and stereo-selective glycosylation in high yield (Fort et al. 2000). Three mutants of the *H. insolens* Cel7B

E197A glycosynthase, namely E197A/H209A and E197A/H209G double mutants, and Cel7B E197A/H209A/A211T triple mutant, were subsequently produced and characterized (Blanchard et al. 2007). The results suggested that appropriate active site mutations could modulate the regioselectivity of the glycosylation reaction. Apart from glycosynthases, use of β-glucosidases for the synthesis of various glycoconjugates, such as alkyl glucosides and aminoglycosides, has also been pursued in recent years. A GH3 β-glucosidase from the thermophilic fungus *Myceliophthora thermophila* was found to act as an efficient biocatalyst in alkyl glycoside synthesis (Karnaouri et al. 2013).

3. Xylanases

Xylanases (EC 3.2.1.8; endo-β-1,4-xylanases) are the main enzymes that hydrolyze internal bonds in xylan, a particularly resistant to degradation component of plant cell walls and the second most abundant polysaccharide in nature after cellulose (Chadha et al. 2019). Apart from their use in the conversion of lignocellulose biomass into fermentable sugars, xylanases have also been successfully employed in the saccharification of agrowaste, such as wheat straw, corn cobs, birch and spruce biomass.

Xylanases are found in GH families 3, 5, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 62, 98 and 141. Their majority, however, belongs to the GH10 and GH11 families based on sequence considerations. GH11 xylanases are more specific than GH10 xylanases as the former do not act on substituted forms of xylans.

Sporotrichum thermophile (syn *Myceliophthora thermophila*), a major thermophilic fungus isolated from soil in eastern Russia, is a powerful cellulolytic organism that produces a variety of enzymes with immense industrial potential (Karnaouri et al. 2014). S *thermophile* synthesizes a complete set of enzymes, including GH10 and GH11 xylanases (Bala and Singh 2016). Characterization of *S. thermophile* GH10 and GH11 xylanases has been reported (Basit et al. 2018).

A xylanase from the thermophilic and thermotolerant fungus *Myceliophthora heterothallica* F.2.1.4. has been purified and characterized (de Oliveira Simões et al. 2019). The enzyme has 27 kDa MW and displays maximum activity at pH 4.5 and 65-70 °C. It maintains more than 80% of its residual activity when exposed to (i) temperatures between 30 and 60 °C for 1 h and (ii) pH 5-10 for 24 h at 4 and 25 °C.

A GH10 xylanase PspXyn10 produced by the mesophilic fungus *Penicillium sp.* has been characterized and found to exhibit thermostability (Shibata et al. 2017). The molecular weight of PspXyn10 was estimated to be 55 kDa and found to contain a CBM. Its optimal temperature and pH for xylanase activity were 75 °C and pH 4.5, respectively. PspXyn10 retained more than 80% of its xylanase activity after incubation at 65 °C for 10 min.

A purified xylanase produced by *T. aurantiacus* M-2 was found to be acidophilic and thermostable (Ping et al. 2018). Its relative molecular mass was approximately 31.0 kD. The purified xylanase exhibited maximum activity at 75 °C and pH 5.0, and it was stable after treatment at a pH range from 2.0 to 10.0 or a temperature range from 30 °C to 80 °C for 2h. A GH10 xylanase from *Aspergillus fumigatus* var. *niveus* (AFUMN-GH10) contains no carbohydrate-binding module. The enzyme was able to retain its activity in a pH range from 4.5 to 7.0, with an optimal temperature at 60 °C (Velasco et al. 2019).

3.1. Structural details

Homology modelling studies of GH10 and GH11 xylanases from S. *thermophile* suggested structural similarities and only minor differences with other fungal xylanases (Basit et al. 2018). The crystal structure of a GH10 xylanase from the fungus *Fusarium oxysporum* (PDB id 3u7b) has been reported (Dimarogona et al. 2012). The structure is similar to that of other GH10 xylanases and is characterized by a $(\beta/\alpha)_8$-barrel fold. Differences have been identified in the loop regions. Sequence alignment and homology modelling suggested the presence of a long loop between strand β6b and helix α6 that may play a role in the catalytic efficiency of the enzyme.

The GH11 xylanases exhibit a β-jelly-roll fold. The structure of xylanase XynCDBFV, a GH11 xylanase from ruminal fungus *Neocallimastix patriciarum* has been determined and the catalytic residues Glu109 and Glu202 were identified (Cheng et al. 2014). The structure of a GH11 xylanases from *Fusarium oxysporum* has been elucidated to 1.56-Å resolution and deposited to the PDB (pdb ID 5jrm; Gomez S, Payne AM, Savko M, Fox GC, Shepard WE, Fernandez FJ, Vega MC, unpublished). The structure of an acidophilic GH11 xylanace (XynC) from *Aspergillus kawachii*, a filamentous fungus used for brewing the Japanese distilled spirit shochu, has been determined to 2.0 Å resolution (Fig. 1). The structure is characterized by a negatively-charged surface, which was postulated to be responsible for the stability of the enzyme in acidic environments (Fushinobu et al. 1998).

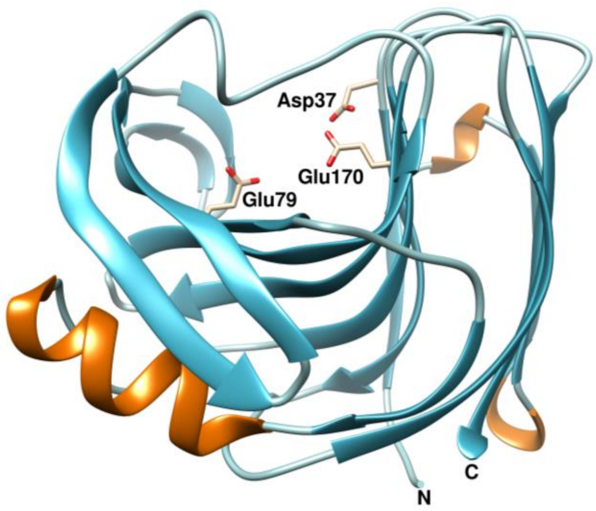

Fig. 1. Crystal structure of *Aspergillus kawachii* XynC (PDB id 1bk1). The catalytic residues Glu79 (nucleophile), Glu170 (acid-base catalyst), and Asp37 are shown as sticks and labelled. N- and C-termini are depicted. Figures of crystal structures were created with Chimera (Pettersen et al. 2004).

3.2. Xylanase improvement

The GH10 xylanase Xyn10A_ASPNG from *Aspergillus niger* (Song et al. 2015) was subjected to iterative saturation mutagenesis (ISM). After four rounds of ISM, a quintuple mutant 4S1 (R25W/V29A/I31L/L43F/T58I) was generated with improved thermostability compared to the wild type. The 4S1 mutant retained 30% of its initial activity after 15 min heating at 65°C and its melting temperature T_m increased by

17.4°C compared to the wild type. For comparison, the wild-type enzyme retained 0.2% of its initial activity after heat treatment for 10 min at 60°C and was completely inactivated after 2 min at 65°C. Although each of the five mutations in 4S1 was found to contribute to thermoresistance, their synergistic action was suggested to be responsible for the dramatic improvement of the 4S1 enzyme thermotolerance.

An N-terminal region (NTR) has been identified in XynCDBFV from the ruminal fungus *Neocallimastix patriciarum* (Cheng et al. 2014). In the truncated mutant, it was clearly shown that the NTR plays a role in the catalytic activity of XynCDBFV and is required for the thermophilic functions of the enzyme. Removal of NTR resulted in a truncated mutant that retained 61.5% and 19.5% enzymatic activity at 55 °C and 75 °C, respectively, compared with the wild-type enzyme. Elimination of a disulfide bond in the C4A/C172A mutant resulted in 23.3% activity. These results suggested that NTR plays a role in XynCDBFV thermostability, and the Cys-4/Cys-172 disulfide bond is critical to the NTR-mediated interactions. Further modifications included four single mutants by substituting residues from 87 to 90 by site-directed mutagenesis (Han et al. 2019). Temperature stability measurements showed promising enhancement of thermostability for all four single mutants. The mutants retained 50% of their activities after incubation at the optimal temperature 60℃ for 1 h, while the retained activity for wild-type XynCDBFV was only 20.94% at the same condition. The increase in thermostability was attributed to a novel hydrogen bonding interaction. However, the enzyme activity of the single mutants was compromised with their thermostability.

Combined mutations displayed an antagonistic effect due to the closed contact of the mutated residues

A novel GH11 endoxylanase (Liu et al. 2019) was constructed via DNA shuffling by using the catalytic domains of two xylanases as parent sequences: *Bacillus amyloliquefaciens* xylanase A (BaxA), which is mesophilic and xylanase A (TfxA) from *Thermomonospora fusca*, a thermophilic soil bacterium. Notably, TfxA, one of the most thermostable xylanases, was able to retain 96% of its catalytic activity after incubation at 75°C for 18 h.

A thermostable GH11 xylanase TlXynA from *Thermomyces lanuginosus* was mutated to improve its pH-tolerance using a rational structure-based approach (Wu et al. 2020).

A thermostable Xyn10A of *A. fumigatus* Z5, which belongs to GH10 family and has a CBM1 domain linked to its C-terminus by a Ser/Thr-rich linker was studied (Miao et al. 2018). Removal of the CBM1 domain had little effect on the thermostability but further truncation of the linker region significantly decreased its stability at high temperatures.

Protein engineering of a GH11 xylanase from *Aspergillus fumigatus* RT-1 was performed near the active site and at the N-terminal region to improve the catalytic efficiency of the enzyme towards pretreated kenaf (Damis et al. 2019). A 13.9-fold increase in catalytic efficiency for a double mutant showed the most effective hydrolysis reaction. The enhanced catalytic efficiency resulted in an increase in sugar yield of up to 28% from pretreated kenaf. In addition, another mutant showed improved thermostability and acid stability. Notably, these mutations were located at distances less than 15 Å from the active site and at putative secondary binding sites away from the active site.

Lytic polysaccharide monooxygenases (LPMOs) are capable of breaking down xylans. A xylan-active LPMO from *Pycnoporus coccineus Pc*AA14B LPMO and a GH30_7 family xylanase (*Tt*Xyn30A) from *Thermothelomyces thermophila* were found to act synergistically with a family GH11 endoxylanase (*An*Xyn11) in the degradation of xylan-containing substrates, resulting in an increase of the released total oligosaccharides (Zerva et al. 2020).

The replacement of an N-terminal segment in *Ao*Xyn11, a mesophilic family 11 xylanase from *Aspergillus oryzae,* by the corresponding N-terminal of *Ev*Xyn11^{TS}, a hyperthermotolerant family 11 xylanase, led to a hybrid xylanase with improved thermostability (Yin et al. 2013). The new xylanase, NhXyn11^{57}, was overexpressed in *Pichia pastoris* and its temperature optimum was 75 °C, much higher than that of AoXyn11. AoXyn11 and NhXyn11^{57} were thermostable at 40 and 65 °C, respectively. A poly-threonine helix from the thermostable GH10 family xylanase XynAF0 from the thermophilic composting fungus *Aspergillus fumigatus* Z5 was introduced to the C-terminal of another GH10 xylanase, improving its thermostability (Li et al. 2019). Thus, the creation of hybrid xylanases can be another strategy for thermostability modifications in this family of enzymes.

Aspergillus kawachii produces, apart from XynC, a second GH11 xylanase, namely XynB, which is neutrophilic. Mutants to adjust the pH optimum of xylanases have been studied. In acidophilic GH11 xylanases, the residue adjacent to the acid/base catalyst is Asp whereas in neutrophilic and alkaliphilic GH11 xylanases is Asn. A D37N mutation in *Aspergillus kawachii* XynC GH11 xylanase (Fig. 1) raised the pH optimum of XynC from 2.8 to 5.5, whereas an N43D mutation in A. *kawachii* XynB GH11 lowered the pH optimum of XynB from 4.2 to 3.6 (Fushinobu et al. 2011).

4. Feruloyl esterases

Feruloyl esterases (FAEs; EC 3.1.2.72) are enzymes that catalyze the hydrolysis of ester bonds between ferulic (hydroxycinnamic) acid (FA) and plant cell wall polysaccharides (Dilokpimol et al. 2016). They act as accessory enzymes of plant biomass degradation to facilitate the access of other enzymes, such as xylanases, xylosidases, and arabinofuranosidases, to sites of action during biomass conversion. FAEs act synergistically with xylanases to release FA from cell-wall material. Based on their catalytic properties, FAEs have also attracted a great deal of attention in recent years for use in the food, pharmaceutical, and cosmetics industries as synthetic tools of novel hydroxycinnamates with enhanced antioxidant activity and custom-made lipophilicity (Faulds 2010; Koseki et al. 2009). Recent amino-acid based sequence analysis has classified fungal FAEs in 13 subfamilies (SFs) (Dilokpimol et al. 2016) as a replacement of the old classification system of ABCD, which was based on substrate specificity (Crepin et al. 2004). Based on the analysis of 1000 fungal FAEs, SF5 and

SF7 FAEs were found suitable for biorefinery applications, such as the production of biofuels, where a complete degradation of the plant cell wall is desired (Underlin et al. 2020). In contrast, SF6 FAEs are promising enzymes for industrial applications that require a high release of only FA and *p*-coumaric acid, which are needed as precursors for the production of biochemicals. Finally, FAEs of SF1, 9 and 13 display an overall low release of hydroxycinammates from plant cell wall-derived and natural substrates. A feruloyl esterase from *Aspergillus terreus* (Mäkelä et al. 2018) was used to expand the Carbohydrate Esterase 1 (CE1) family of the CAZy database. Phylogenetic analysis showed that the CE1 family can be subdivided into 5 groups to include more members of fungal FAEs.

An FAE from the thermophilic fungus *Sporotrichum thermophile* has shown stability at the pH range 5.0-7.0 and retained 70% of its activity after 7 h at 50 °C and lost 50% of its activity after 45 min at 55 °C and after 12 min at 60 °C (Topakas et al. 2004). Characterization of an FAE (*Tc*FaeB) from *Talaromyces cellulolyticus*, a high cellulolytic-enzyme producing fungus, has been reported (Watanabe et al. 2015). Thermal stability measurement using differential scanning calorimetry showed that *Tc*FaeB has a *Tm* value of 70 °C and optimum optimum temperature of the enzyme was estimated to be 65 °C at pH 4.5–6.5, suggesting that this enzyme may be applicable for biomass saccharification processes.

4.1. Structural features of FAEs

FAEs exhibit an α/β hydrolase fold with a Ser-His-Asp catalytic triad (Hermoso et al. 2004). The FA has been found to bind to a shallow surface pocket able to accommodate the methoxy and hydroxyl moieties of the substrate owing to the presence of hydrophobic and hydrogen bonding specificity determinants (Prates et al. 2001). Although fungal FAEs show structural similarity to lipases, their catalytic sites are different with more hydrophobic residues present in the active site of lipases (McAuley et al. 2004). Fungal and bacterial FAEs show different topology of secondary structure elements, leading to suggestion that bacterial FAEs diverged earlier than the fungal FAEs and the lipases from a common ancestor.

The crystal structure of an FAE from *Fusarium oxysporum* has been reported (Dimarogona et al. 2020). Similar to other FAEs, the structure revealed a large lid domain covering the active site with a potential regulatory role (Fig. 2). This lid domain, however, is absent in *A. niger* FAE (McAuley et al. 2004) and more fungal structures should be determined to understand its exact role. A disulphide bond brings together the serine and histidine residues of the catalytic triad. A number of differences were identified, mainly in the metal coordination site and the substrate binding pocket.

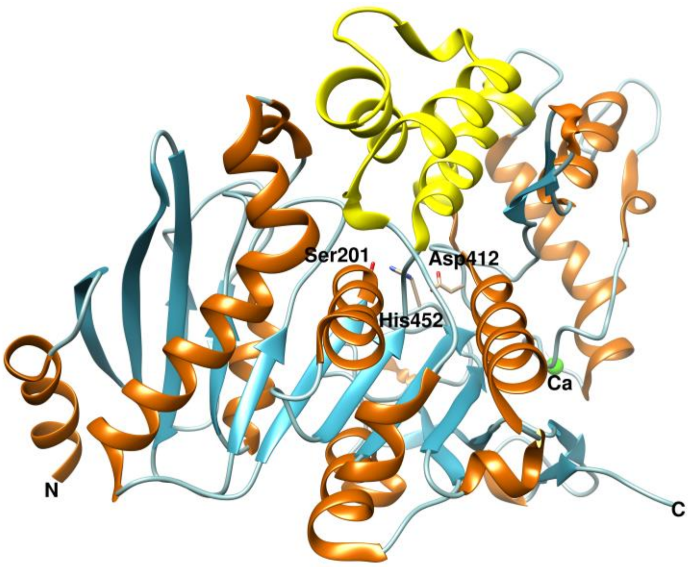

Fig. 2. Crystal structure of an *F. oxysporum* FAE (PDB id 6fat). The catalytic triad is shown. The characteristic lid is colored in yellow. A bound Ca ion is depicted as a green sphere.

4.2. Improvement efforts of FAEs

Directed evolution studies have been reported in the filamentous fungus *Aspergilus niger* FAE (*An*Fae) (Zhang et al. 2012). The resultant mutant exhibited 80% residual activity after heat treatment at 90 °C for 15 min and an increase in half-life from 15 min to >4000 min at 55 °C. The thermostable mutant displayed significantly enhanced performance compared to the parental *An*FaeA, suggesting it could be useful in biotechnological applications. Directed evolution has also been applied to EstF27 identified from a soil metagenomic library (Cao et al. 2015). Structural analysis showed that a new disulfide bond and hydrophobic interactions formed by the mutations may play an important role in stabilizing the protein.

The effect of glycosylation has been studied in feruloyl esterase 1a from *Myceliophthora thermophila* (Bonzom et al. 2019). Heterologous expression of the enzyme in three different hosts (*M. thermophila*, *P. pastoris*, *E. coli*) revealed that the enzyme produced in *E. coli* had the lowest catalytic efficiency compared to its glycosylated form. Moreover, differences were found depending on the degree of glycosylation, suggesting that careful choice of the expression host should be considered for enzyme optimization depending on the specific biotechnological application.

Examples of chimeric enzymes have been reported. *An*FAEA and a dockerin from *Clostridium thermocellum* were connected and the resultant protein was expressed in *A. niger* and characterized (Levasseur et al. 2004), showing higher enzymatic activity than the parental *An*FAEA. A type A FAE from *A. awamori* (*Aw*FaeA) and family 42 carbohydrate-binding module (AkCBM42) from glycoside hydrolase family 54 α-L-arabinofuranosidase of *A. kawachii* have been used to create a chimeric enzyme (Koseki et al. 2010). The new enzyme was found to be more thermostable than *Aw*FaeA.

5. Laccases

Laccases (EC 1.10.3.2; p-diphenol:dioxygen oxidoreductases) belong to the family of multi-copper oxidases and can oxidize a wide range of aromatic and non-aromatic compounds, such as substituted phenols, non-phenolic compounds and some inorganic ions (Agrawal et al. 2018). Owing to their ability to degrade and detoxify a wide range of phenolic and non-phenolic compounds, including lignin, laccases have received broad attention for use in several industrial and biotechnological applications, including eco-friendly synthesis of fine chemicals and the gentle derivatization of biologically active compounds (Mikolasch and Schauer 2009). Fungal laccases are characterized by higher redox potential compared to the bacterial ones, thus they are more suitable for use in a wide range of substrates and act as ‘green biocatalysts’ in various areas of biotechnology, including bioremediation (Mäkelä et al. 2020) and bio-bleaching (Baldrian 2006). Fungal laccases have been purified from wood-rotting white-rot basidiomycetes and to a lesser extent from other groups of fungi (other groups of basidiomycetes, ascomycetes and imperfect fungi) (Baldrian 2006). Glycosylation (typically between 10%-25%) has been detected in fungal laccases and it was suggested that it protects the enzymes from protease degradation in addition to the structural role it plays (Maestre-Reyna et al. 2015; Yoshitake et al. 1993).

Fungal laccases require only molecular oxygen as co-substrate, resulting in significant cost savings and reduced protein inactivation compared to the use of H_2O_2 as co-substrate in peroxidases. Moreover, the biocatalytic process products are water and a corresponding radical derived from the reducing substrate which afterwards evolves to the formation of insoluble polymers that are easily removable by filtration or other conventional technologies (Songulashvili et al. 2016)

A laccase from *Sporotrichum thermophile* exhibits temperature and pH optima of 60 °C and 3.0, respectively. The enzyme was found stable in organic solvents, such as DMSO and ethanol, and has been used in decolourisation of six synthetic dyes (Kunamneni et al. 2008). In the field of bioelectrocatalysis, fungal laccases, for example, *Trametes versicolor* laccase (*Tv*L), have been intensively studied because of their high redox potential compared to the bacterial ones that makes them suitable for use in enzymatic biofuel cells (EBFCs) (Arregui et al. 2019).

Municipal wastewater is characterized by high alkalinity and high concentration of metal ions. Thus, laccases from alkaliphilic fungi have been studied for use in such environments (Prakash et al. 2019). Metagenomic studies of Soda Lake have led to the identification of various fungi with laccase-like oxidase activity suitable for degradation of phenolic compounds.

Thermophilic fungi have been also a source of laccases with thermostability properties (Hildén et al. 2009). Thermostable laccases have found use in various industrial applications, such as pulp and denim bleaching, and in the food and beverage industry as stabilizers (Osma et al. 2010). Among ascomycetes, significant thermostability has been detected in a laccase from the thermophilic fungus *Melanocarpus albomyces* which can retain its activity for 24 h at 50°C and for 2 h at 60°C (Kiiskinen et al. 2002). A laccase produced *by C. thermophilum* have been characterized and sequenced (Chefetz et al. 1998). The enzyme retains activity for 1 h at 70°C and has half-lives of

24 and 12 h at 40 and 50 °C, respectively. It is also stable at a wide pH range, from pH 5 to pH 10.

5.1. Structural details of laccases

Fungal laccases are enzymes with 520-550 amino acids and a molecular weight of 60-70 kDa in their glycosylated form. They exist mainly as monomers although there are cases of homodimeric, heterodimeric, and multimeric laccases. The three-dimensional structure of several fungal laccases has been reported. A characteristic of fungal laccases is the presence of 10 histidines with 8 of them belonging to four HXH motifs (Sitarz et al. 2016).

Laccases are characterized by an active centre that contains four copper ions, each identified based on its spectroscopic properties. The T1 copper ('blue' copper) exhibits a strong absorption around 600 nm and is paramagnetic, T2 ('non-blue' copper) is also paramagnetic with an absorbance at 610 nm, and T3 contains a diamagnetic spin-coupled copper–copper pair with an absorbance at 330 nm. The T1 copper site and the T2/T3 trinuclear copper cluster are connected to each other through a strongly conserved internal electron transfer pathway. The substrates are oxidized by the T1 copper and the extracted electrons are transferred, probably through a highly conserved His-Cys-His tripeptide motif, to the T2/T3 site, where molecular oxygen is reduced to water (Mehra et al. 2018).

The crystal structure of a laccase produced by *M. albomyces* has been determined (Hakulinen et al. 2002). The molecule is divided into three domains (Fig. 3). Domain 1 includes residues involved in the binding of coppers at the trinuclear site. Domain 2 includes residues that are involved in the binding of reducing substrates. Domain 3 contains residues that participate in the binding of coppers at the mononuclear and trinuclear sites as well as in substrate binding. Electron-donating organic substrate molecules are bound in a hydrophobic pocket of domain 2.

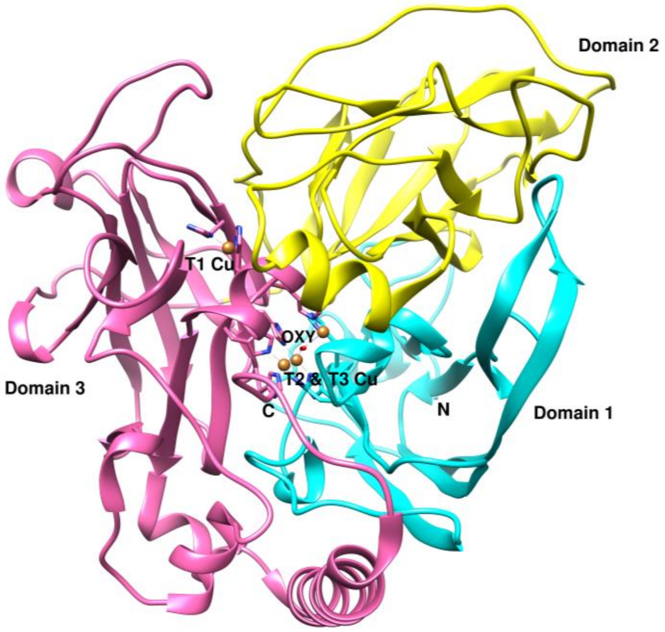

Fig. 3. Crystal structure of *M. albomyces* laccase (PDB id 1gw0). The three domains are shown in different colors. Copper ions are depicted as brown spheres. The oxygen atom is colored in red.

5.2. Improvement of fungal laccases

Thermostable fungal laccase chimeras have been generated by the SCHEMA-RASPP computational approach (Mateljak et al. 2019). The most thermostable variant showed a 5-fold increase (up to 108 min) of its thermal inactivation half-life at 70 °C. Interestingly, ancient laccases have recently emerged as a promising source of novel laccases (Gomez-Fernandez et al. 2020). A fungal Mesozoic laccase (dated back 250-500 million years) was resurrected and showed strikingly high heterologous expression and pH stability. Directed evolution, rational, and semi-rational approaches to improve laccase function have been reported (Mate and Alcalde 2015). N-glycosylation sites are found mostly conserved in fungal laccases and they have been suggested as potential modulators of the laccase properties (Ernst et al. 2018).

6. Lipases

Lipases (EC 3.1.1.3) catalyze the hydrolysis and synthesis of acylglycerol and other water-insoluble esters. They are important industrial enzymes that have found use in food, chemical, drug, biodiesel and detergent industries. As flavor and fragrance compounds are esters formed by short-chain carboxylic acids and alcohols, the use of lipases for the enzymatic synthesis of flavour esters offers a more efficient, economically benign, and promising alternative approach compared to the traditional methods of chemical synthesis or extraction from natural sources (Dhake et al. 2013; Verma 2019). Biodiesel consists of fatty acid alkyl esters (FAAE) derived from triglycerides (TGs) by transesterification with alcohols. Thus, fungal lipases have been used in biodiesel production either free and immobilized or as whole cells and fermented solids (Aguieiras et al. 2015).

Thermophilic fungi have been a promising source of new thermostable lipases. Lipases from thermophiles are mostly used in waste-water treatments. Using solid-state fermentation, a thermostable lipase from *Thermomyces lanuginosus* was obtained (Avila-Cisneros et al. 2014). The enzyme exhibited thermostability and it has been used in various applications, from chemical synthesis and biodiesel production to transesterification reactions.

Thermophilic lipases are also tools in the pulp industry as the processing of lignocellulosic material leads to the formation of pitch, a substance rich in esters able to clog machines. A lipase from *Aspergillus oryzae* has been used to control pitch formation (Gutiérrez et al. 2009).

Psychrophilic and alkaliphilic lipases are usually added to the detergent formulation as polymer-degrading agents (Joseph et al. 2008). *Talaromyces thermophilus* produces a thermoactive and alkaline lipase which retains activity at pH 9.5 (Romdhane et al. 2010). The enzyme is stable at 60 °C and retains 65% of its enzyme activity after 30 min incubation at 70 °C. Its half-activity is retained after incubation for 40 min at 80 °C. The optimum pH for the enzyme activity was 9.0 and the lipase was stable from pH 8.0 to 12.0. Higher frequency of hydrophobic amino acids, such as Ala, Val, Leu and Gly in thermostable lipases from *T. lanuginosus* has been suggested for increased thermal stability (Zheng et al. 2011).

A lipase from the biotrophic fungus *Sporisorium reilianum* SRZ2 (SRL) with 73% amino acid sequence identity to *Candida antarctica* lipase B (CALB) was cloned and overexpressed in *Pichia pastoris* retained 75% of its activity at pH 3 to 11 for 72 h and it has been suggested to act as a thermophilic fungal lipase (Shen et al. 2020). Moreover, LipG7 from the Antarctic filamentous fungus *Geomyces sp.* P7 retained 100% of its initial activity after 1 h of incubation at 100°C (Florczak et al. 2013). An acidic and thermostable lipase with a preference for the medium-chain length *p*-nitrophenyl esters (C12) rather than short and long-chain length substrates has been characterized from the thermophilic fungus *Neosartorya fischeri* P1 (Sun et al. 2016).

6.1. Structural details of lipases

Lipases have a common α/β- hydrolase fold, a catalytic triad (Ser-His-Asp/Glu) similar to that found in serine proteases and a lid covering the active site (Holmquist 2000). The lid is displaced upon activation and opens up the binding pocket, thus making the active site accessible to the substrate (Stauch et al. 2015) (Fig. 4). The ester hydrolysis mechanism of lipases is similar to that of carboxyl esterases and serine proteases. It involves a nucleophilic attack on the carbonyl carbon of the ester bond by the catalytic triad, leading to the formation of an acyl-enzyme intermediate and alcohol. This acyl-enzyme intermediate in second nucleophilic attack is hydrolysed by water and yields carboxylic acid.

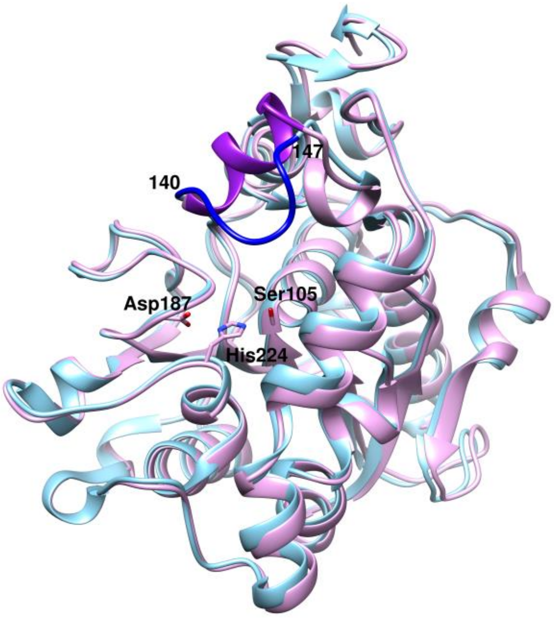

Fig. 4. Lid displacement in CALB. The conformational change of residues 140-147 is shown after superposition of the closed conformation structure (PDB id 5a71; cyan) onto the open conformation structure (PDB id 1tca; pink). The residues of the catalytic triad are shown as sticks.

6.2. Improvement of catalytic efficiency in lipases

There have been enormous efforts to increase the catalytic efficiency and thermal properties of the lipases. The Lipase Engineering Database (LED; http://www.led.uni-stuttgart.de) provides up-to-date information about the updated and engineered lipases. Lipases are synthetically designed, genetically engineered, cloned and expressed using suitable expression systems in recombinant organisms. The methods employed (Bornscheuer et al. 2002) for this purpose are:

- Physicochemical methods (e.g., immobilization and solubilization)
- Reaction engineering methods (e.g., use of acyl donors and ionic liquids)
- Molecular biology methods (e.g., rational protein design and directed evolution)

Notably, the conversion through directed evolution of the catalytic Ser to Cys in CALB along with four mutations resulted in 40-fold higher activity lipases (Cen et al. 2019), suggesting that similar approaches could be useful for the fungal lipases.

7. Nitrilases

Nitrilases (NLases; EC 3.5.5.-) catalyse the hydrolysis of different nitriles to corresponding amides and acids. They can be used in organic synthesis to improve industrial biocatalysis (Gong et al. 2017). Bacterial NLases have been characterized in detail. According to their substrate specificity, they are classified as aromatic NLases, aliphatic NLases, and arylacetoNLases. In contrast, studies of fungal nitrilases have been slow (Martínková 2019). Fungal nitrilases belong to aromatic nitrilases and arylacetonitrilases. A recent bioinformatics search identified various nitrilases in fungi (Rucká et al. 2020). In general, fungal nitrilases are considered advantageous over the bacterial ones regarding activity, thermostability, and selectivity (Wu et al. 2013).

7.1 Structural details of nitrilases

No structure of a fungal nitrilase is currently available. Insights into the catalytic mechanism have been provided by an archaeal nitrilase (Raczynska et al. 2011). A 2-fold dimer symmetry has been identified with each subunit of the dimer characterized by an αββα sandwich fold, resulting in a super-sandwich αββααββα structure formed by the association of the two subunits. Dimerization is achieved through multiple approaches, including interactions of the extended C-terminal of each subunit and interactions between arginine and glutamate residues that form salt bridges. The binding pocket lies close to the inter-subunit interface while a binding loop assists the binding of the substrate. A Lys residue was suggested as the acid in the catalytic reaction.

7.2 Improvement

Site-directed mutagenesis of a fungal nitrilase from *Gibberella intermedia* resulted in mutants with higher catalytic activity and increased stability (Gong et al. 2016). Notably, point mutations near the active site of an *A. niger* nitrilase were able to change the enantioselectivity of the enzyme (Petříčková et al. 2012). Mutagenesis studies on bacterial nitrilases to improve their thermostability have been carried out and may apply to their fungal counterparts as well (Xu et al. 2018).

8. Transaminases

Transaminases (TAs), or aminotransferases, are enzymes that catalyze the transfer of an amino group from an amino donor to an acceptor for chiral amino acid or amine synthesis (Guo and Berglund 2017). Owing to their excellent enantioselectivity, environmental friendliness, and compatibility with other enzymatic or chemical systems, TAs have drawn attention in the area of biocatalysis. The most known example of use of a TA is in the synthesis of the antidiabetic drug sitagliptin (Savile et al. 2010). An *in silico* strategy for sequence-based prediction of substrate specificity and enantiopreference revealed 17 novel (R)-selective TAs, many of them from fungi (Höhne et al. 2010).

8.1 Structural details of TAs

The structure of a puruvate TA from the fungus *Nectria haematococca* (Sayer et al. 2014) has been determined and provided initial insights into the R-enantioselectivity of the TAs. Also, the structure of an (R)-selective amine TA from *A. fumigatus* has been elucidated (Thomsen et al. 2014). The enzyme has the typical fold found in class IV of PLP-dependent enzymes and its overall structure is similar to that of a branched-chain amino acid aminotransferase from *T. thermophilus* and D-amino-acid amino-transferase from *Bacillus sp*. YM-1 (Fig. 5). An N-terminal α-helical extension has been found that was suggested to play a role in the enzyme's stability. The structure of an ω-transaminase from *Aspergillus terreus* has been solved and found to exhibit also the class IV fold (Łyskowski et al. 2014).

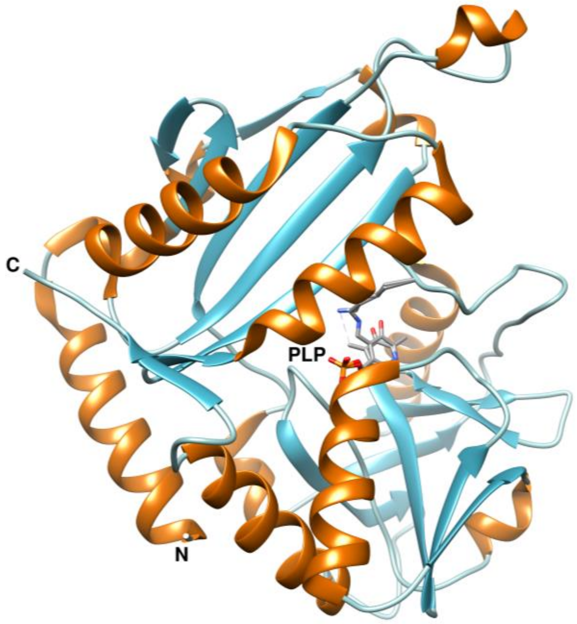

Fig. 5. Crystal structure of *A. fumigatus* (R)-selective amine TA (PDB id 4chi). The PLP cofactor is depicted in sticks.

8.2 TA improvement

A thermostable (R)-TA (Huang et al. 2017) from *A. terreus* (*At*RTA) has been characterized. A homolog of *At*RTA from the thermotolerant fungus *Thermomyces stellatus* (*Ts*RTA) has been reported (Heckmann et al. 2020). The thermostability of *Ts*RTA (40% retained activity after 7 days at 40 °C) was initially attributed to its tetrameric form in solution. However, subsequent studies of *At*RTA revealed that the enzyme also exists predominantly as a tetramer but, in contrast to *Ts*RTA, it is inactivated within 48 h at 40 °C. The engineering of a cysteine residue to promote disulfide bond formation across the dimer-dimer interface stabilized both enzymes, with *Ts*RTA_G205C mutant retaining almost full activity after incubation at 50 °C for 7 days.

9. Tyrosinases

Tyrosinases (EC 1.14.18.1, monophenol, o-diphenol:oxygen oxidoreductases) together with laccases (EC 1.10.3.2) form two subgroups of phenoloxidases. As in laccases, tyrosinases are also copper-containing enzymes. In tyrosinases, the copper ions are known as CuA and CuB (Ba and Vinoth Kumar 2017).

Tyrosinases are found in bacteria, fungi and plants. Fungal tyrosinases from *Agaricus bisporus*, *Neurospora crassa*, *Aspergillus oryzae* and *Aspergillus niger* have been largely explored (Agarwal et al. 2017). Detailed characterization of a tyrosinase from *T. reesei* has also been reported (Selinheimo et al. 2006). Tyrosinases have been used either in a free or immobilized form for the removal of micro-pollutants in the environment (Ba and Vinoth Kumar 2017). Besides, tyrosinases are employed in L-DOPA synthesis and biosensor development (Min et al. 2019).

9.1 Structural details of tyrosinases

Crystal structures of an *Aspergillus oryzae* fungal tyrosinase are available (Fujieda et al. 2020) and have provided information into the copper movements during the catalytic reaction (Fig. 6). No structures are currently available for tyrosinases from extremophilic fungi.

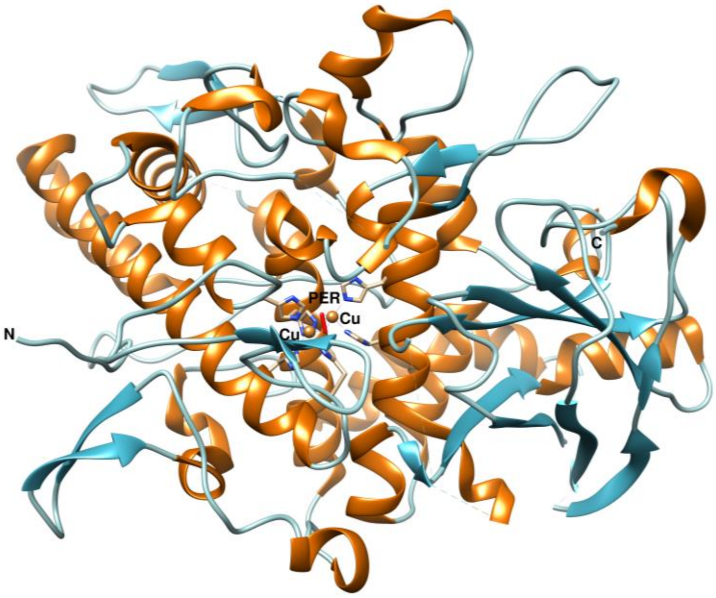

Fig. 6. Crystal structure of *A. oryzae* tyrosinase mutant (C92A) in the presence of peroxide (PDB id 6jub). Cu atoms are shown as brown spheres and peroxide is colored in red.

9.2 Tyrosinase improvement

The use of D-DOPA in Parkinson’s disease has been proposed as more effective than the use of L-DOPA. As fungal tyrosinases have lower affinity for D-tyrosine, improvement of their catalytic activity against D-tyrosine has been pursued (Ali et al. 2020). Thermostable tyrosinases have been proposed for the removal and bioconversion of phenol from wastewater (Lee et al. 1996). Immobilization of the enzyme appears to have beneficial effects. Indeed, an immobilized mushroom tyrosinase has been found to work at high temperatures (100 °C) and in organic solvents (Wu et al. 2018). Also, immobilized *Aspergillus niger* tyrosinase has shown an increased thermal and pH stability (Agarwal et al. 2016). A computational approach which has been carried out to improve the thermostability of a bacterial tyrosinase (Guo et al. 2015) may be applicable for fungal enzymes as well.

10. Keratinases

Keratinases are key enzymes used for the degradation of agricultural and industrial keratin waste. Keratin is the most abundant insoluble structural fibrous protein and a major constituent of hair, nails, horn, wool, skin and feather. Chemical and physical treatment of keratin is currently unfriendly to the environment, leading to a product with poor digestibility, variable nutrient quality and destruction of several amino acids (Mabrouk 2008). Keratinases have been identified in a wide variety of microorganisms (Brandelli et al. 2010). A crude enzyme preparation from *Aspergillus fumigatus* (Santos et al. 1996) showed remarkable thermostability by retaining ~90% of its original activity at 70°C for 1.5 h. Besides, a novel thermophilic *M. thermophila* strain GZUIFR-H49-1 with potential applications for production of thermostable keratinase has been identified (Liang et al. 2011). Also, screening of almost 300 fungi species revealed that *Aspergillus flavus* was the most productive fungus in keratinolytic enzymes (Friedrich et al. 1999).

10.1 Structural details of keratinases

Structural details of keratinases have been obtained from bacterial sources and the crystal structures of five keratinolytic enzymes are known (Qiu et al. 2020).

Phylogenetic and structure-based analysis has shown the presence of other keratinases in various extremophiles, including fungi. The structure of the keratinase fervidolysin has suggested a distant relationship with pro-subtilisin E using structural alignment (Kim et al. 2004; Kluskens et al. 2002). The structure also suggested a functional relationship of fervidolysin to the fibronectin-like domains of the human promatrix metalloprotease-2 that degrades the fibrous polymeric substrate gelatin. The structure of a novel heat-stable keratinase (Wu et al. 2017) from the feather-degrading thermophilic bacterium *Meiothermus taiwanensis* WR-220 has shown similarities with the overall fold of the catalytic domain of fervidolysin.

10.2 Improvement

Computational techniques to improve the thermostability of bacterial keratinases have been applied (liu et al. 2013) and they may be extended to other keratinases of industrial interest. A synergistic action of keratinases with LPMOs has been proposed (Lange et al. 2016), thus offering additional strategies to improve keratinase performance.

11. Conclusions

Fungal extremozymes are promising alternatives for use in green chemistry. However, a systematic functional and structural characterization is necessary to understand better their stability, behavior in extreme environments, catalytic mechanism, synergism, and evolutionary relationships. Currently, the two most preferable approaches for producing novel enzyme variants are site-directed mutagenesis and directed evolution. Further improvement of fungal extremozymes will help in developing better and more versatile enzymes for their use either alone or in mixtures with other enzymes for green chemistry applications.

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