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Copper-radical oxidases: A diverse group of biocatalysts with distinct properties and a broad range of biotechnological applications

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ABSTRACT

Copper-radical oxidases (CROs) catalyze the two-electron oxidation of a large number of primary alcohols including carbohydrates, polyols and benzylic alcohols as well as aldehydes and α-hydroxy-carbonyl compounds while reducing molecular oxygen to hydrogen peroxide. Initially, CROs like galactose oxidase and glyoxal oxidase were identified only in fungal secretomes. Since the last decade, their representatives have also been identified in some bacteria. CROs are grouped in the AA5 family of "auxiliary activities" in the database of Carbohydrate-Active enzymes. Despite low overall sequence similarity and different substrate specificities, sequence alignments and the solved crystal structures revealed a conserved architecture of the active sites in all CROs, with a mononuclear copper ion coordinated to an axial tyrosine, two histidines, and a cross-linked cysteine-tyrosyl radical cofactor. This unique post-translationally modified protein cofactor has attracted much attention in the past, which resulted in a large number of reports that shed light on key steps of the catalytic cycle and physico-chemical properties of CROs. Thanks to their broad substrate spectrum accompanied by the only need for molecular oxygen for catalysis, CROs since recently experience a renaissance and have been applied in various biocatalytic processes. This review provides an overview of the structural features, catalytic mechanism and substrates of CROs, presents an update on the engineering of these enzymes to improve their expression in recombinant hosts and to enhance their activity, and describes their potential fields of biotechnological application.

1. Introduction

In nature, the main decomposers of dead plant material are wood-decaying fungi that affect global carbon cycling and nutrient dynamics [12,63], however, some bacteria [13] and other fungi [11] are also involved in lignocellulose breakdown. Meta-omics of these organisms revealed the co-occurrence of polysaccharide-degrading and lignin-degrading enzymes. Since 1998, all enzymes involved in the recognition, breakdown or synthesis of carbohydrate polymers and glycoconjugates have been integrated into the database of Carbohydrate-Active enzymes (CAZy database, http://www.cazy.org) according to their sequence similarities and enzyme mechanisms [14,40]. In 2013, the CAZy database was expanded by integrating a novel class of "Auxiliary activities" comprising enzymes that are involved in the breakdown of lignin [61]. The "Auxiliary activities" (AA) members are divided into ten AA families (AA1-AA10).

Brown-rot fungi cannot directly attack lignin because they do not produce lignin-modifying peroxidases, but degrade lignocellulose using the chelator-mediated Fenton system [62]. White-rot fungi enable lignin degradation by using a broad set of "auxiliary" enzymes including lignin-modifying oxidoreductases like laccases (AA1), lignin peroxidase (LiP, AA2), manganese peroxidase (MnP, AA2) and versatile peroxidase (VP, AA2). The activity of peroxidases is mainly supported by H_2O_2-generating enzymes from the AA3 family, namely aryl-alcohol oxidases (AAOs) and pyranose 2-oxidases, as well as glyoxal oxidases from the AA5 family.

In the catalytic cycle of the above-mentioned H_2O_2-producing enzymes, the two-electron transfer occurs during the oxidation of the substrate and reduction of O_2 to H_2O_2, which is equivalent to dihydrogen transfer between both compounds. Redox cofactors like quinones or flavins usually play a role in hydrogen transfer in redox enzyme catalysis. For instance, AAOs from the AA3 family, which oxidize a broad range of benzylic alcohols to the corresponding aldehydes, contain a

Abbreviations: CRO, copper-radical oxidases; CAZy, carbohydrate-Active enzymes; AA, auxiliary activities; GalOx, galactose oxidases; GLOX, glyoxal oxidase; HRP, horseradish peroxidase; LIP, lignin peroxidase; MnP, manganese peroxidase; VP, versatile peroxidase; 5-HMF, 5-hydroxymethylfurfural; DFF, diformylfuran; HMFCA, 5-hydroxymethyl-2-furancarboxylic acid; FFCA, 5-formyl-2-furancarboxylic acid; FDCA, 2,5-furandicarboxylic acid.

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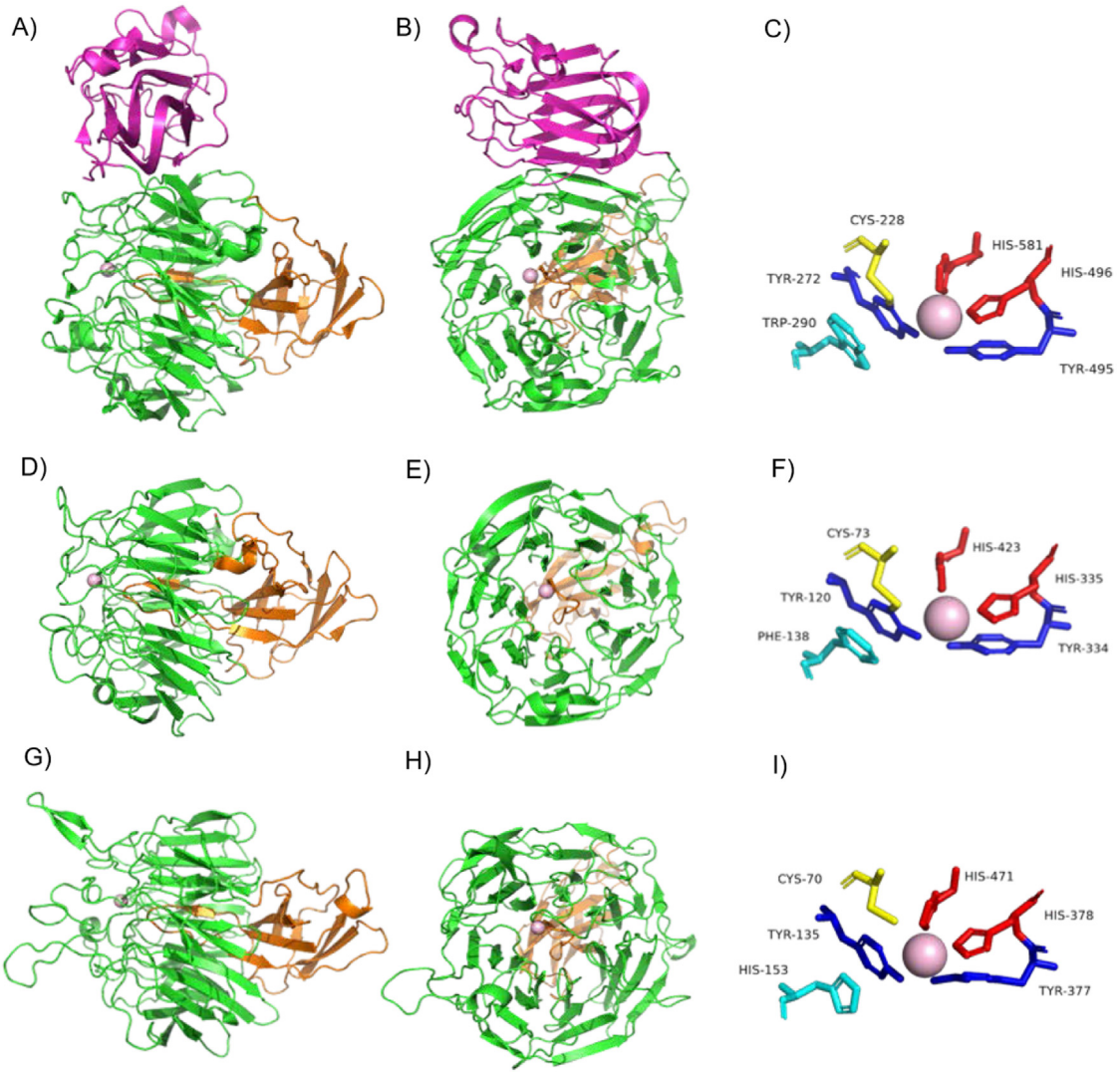

Fig. 1. 3D structure and active site of GalOx (PDB entry 2EIE, A-C) and *Cgr*AlcOx (PDB entry 5C92, D-F) and homology model of GLOX and its catalytic site (G-I). Homology model of GLOX from *Phanerochaete chrysosporium* was built using GalOx as template.

non-covalently bound flavin adenine nucleotide (FAD) cofactor to catalyze this reaction [94,104]. However, none of these organic cofactors are involved in the catalysis by the copper-radical oxidases (CROs). In these oxidases, their exclusive active site with a free radical-coupled copper complex undertakes this task.

This review focuses on the AA5 family that is divided into two subfamilies AA5_1 and AA5_2. Subfamily AA5_1 includes glyoxal oxidases (GLOX) that oxidize a broad range of aldehydes to the corresponding carboxylic acids [115]. The subfamily AA5_2 comprises galactose oxidases (GalOx) that oxidize, besides galactose, a variety of other carbohydrates and primary alcohols to the corresponding aldehydes [111]. In 2015, the subfamily AA5_2 was expanded by two newly discovered CROs that barely oxidize galactose and galactosides, but accept a range of primary aliphatic alcohols instead [127]. Since then, a number of non-GalOx CROs have been assigned to subfamily AA5_2. Despite low sequence similarity and distinct substrate specificities of the AA5 family members, the solved crystal structures of e.g. GalOx and other AA5_2 members are quite similar, with a mononuclear copper ion coordinated to a cross-linked cysteine-tyrosyl radical cofactor, an axial tyrosine and two histidines in their active sites [66,110] (Fig. 1). This review will provide an overview of the properties and natural functions of AA5 enzymes with a focus on commonalities and differences between the members of this family and their potential for application in biotechnology. Since GalOx from the AA5_2 subfamily is considered as a prototypical and the best studied CRO, its structure, mechanism and properties will be described first.

2. AA5_2: Galactose oxidase

2.1. Occurrence

GalOx (D-galactose: oxygen 6-oxidoreductase, EC 1.1.3.9.) are extracellular enzymes produced by some filamentous fungi, predominantly belonging to the genus *Fusarium* [6,7,19,31]. GalOx activity was also detected in *Gibberella fujikuroi* [2], *Alternaria* sp., *Helminthosporium* sp. and *Penicillium album* [36]. The best-studied GalOx originates from *Fusarium graminearum* NRRL 2903, which was initially misidentified as *Polyporus circinatus* [19], later redetermined as *Dactylium dendroides* [69,72] and finally reclassified again as *F. graminearum* NRRL 2903 [73]. The function of GalOx in nature remains elusive and is probably related to the generation of H_2O_2 to support the activity of lignin-degrading peroxidases for weakening the plant cell wall and facilitating the invasion of endophytic fungi in the host [95].

2.2. Structure, properties and maturation

The first crystal structure of GalOx from *F. graminearum* [44,45] was solved in the early nineties of the last century. This enzyme consists of three domains (Fig. 1A and B). The N-terminal domain (designated as domain 1) forms a jelly-roll sandwich composed of eight antiparallel β-strands and contains a carbohydrate-binding site for substrate binding. This domain belongs to carbohydrate-binding module family 32 (CBM32). The central and largest domain (domain 2) consists of seven 'Kelch' motifs forming a classical seven-bladed β-propeller. The copper ion is coordinated by two tyrosines Tyr272 and Tyr495 and two histidines His496 and His581 (Fig. 1C) [110]. Both tyrosines and His496 are provided by domain 2, while His581 is provided by C-terminal domain 3. The C-terminal domain 3 is formed from seven antiparallel β-sheets and has an immunoglobulin-like fold. The active site is located at the surface of domain 2, with the copper ion bound near the surface of the protein in a slight concavity on the wheel axis opposite domain 3. One of the copper-coordinating tyrosines, Tyr272 - the site of the free radical - is covalently bound at C_ε to the sulfur atom of Cys228 via a thioether bond, forming the Cys-Tyr dimer [44]. The thioether bond contributes to the rigidity of the enzyme's active site since it is formed irreversibly and cannot undergo reductive cleavage [112]. The thioether bond is formed post-translationally [89]. The Cys-Tyr dimer is shielded from the solvent by the indole ring of Trp290 which is stacked over the thioether bond. Trp290 has multiple functions in GalOx catalysis. It was reported to stabilize the free radical Cu(I) complex, and mutation of Trp290 to histidine (W290H) decreased stability of the tyrosyl radical [84,92]. Mutagenesis studies revealed that the W290H mutant was significantly less active than the wild-type enzyme [92].

The active site region is hydrophobic and characterized by the presence of several aromatic residues like tyrosine and phenylalanine. Based on a substrate-binding model, Phe194, Arg330, Gln406 and Phe464 restrict the substrate access to the active site. While the side chains of Arg330 and Gln406 form hydrogen bonds with D-galactose, Phe194 and Phe464 build a hydrophobic wall involved in hydrophobic interactions with this substrate [46].

The *galox* gene coding for GalOx from *F. graminearum* has 2043 bp and encodes an inactive precursor polypeptide of 680 amino acid residues. Formation of the catalytically active enzyme requires several sequential post-translational modifications. After proteolytic cleavage of the pre-secretion sequence, copper-dependent cleavage of the N-terminal pro signal peptide occurs, resulting in the premature GalOx of 639 aa that runs at ~68 kDa, which corresponds to the predicted molecular weight of 68.5 kDa. The next maturation step includes the spontaneous formation of the thioether bond between Tyr272 and Cys228 in the presence of O_2 and copper complexes and generation of the Tyr272 radical. Mature GalOx runs at ~65 kDa in the SDS gel due to the cross-link between Tyr272 and Cys228 [89]. Later, it was demonstrated that in the presence of a Cu(I) complex the premature enzyme changed to the mature GalOx within several seconds, while in the presence of Cu(II) this process requires several hours [120]. Furthermore, along with aerobic thioether bond formation, the presence of a catalytically active intermediate was demonstrated also under anaerobic conditions [90].

2.3. Catalytic mechanism

GalOx can exist in three distinct oxidation states: The oxidized state with Cu(II) and tyrosyl radical, the intermediate semi-reduced state with Cu(II) and tyrosine, and the reduced state with Cu(I) and tyrosine. The reaction catalyzed by GalOx starts with substrate binding in front of the copper ion and can be divided into reductive and oxidative half-reaction and follows a ping-pong mechanism [112,116]. According to the initially proposed catalytic mechanism, in the reductive half-reaction, the catalytically active oxidized free-radical Cu(II)-complex (with Cu(II) and tyrosyl radical) is reduced to the non-radical Cu(I)-complex (with Cu(I) and tyrosine) within three steps (Fig. 2). First, a proton is transferred from the alcohol substrate to the axial tyrosinate (Tyr495). It was proposed, that the axial Tyr495 phenolate serves as a general base for abstracting the proton from the coordinated hydroxyl group and was proven to be essential for catalysis [110]. The Y495F mutant is inactive, even though it contains both, copper and the cysteine-tyrosyl radical cofactor [83,91]. In the next step, a hydrogen atom is transferred from the substrate to the tyrosyl radical (Tyr272). This step is considered rate-limiting or at least partially rate-limiting in accordance with spectroscopic studies [116,117]. The remaining alcohol-derived alkoxyl radical is oxidized through electron transfer to the copper ion yielding Cu(I) and the product aldehyde. On the basis of studies with inhibitors, it was suggested that hydrogen atom transfer and electron transfer can occur in a concerted manner [106,107]. More recent theoretical studies revealed that before the initial proton transfer occurred, the radical site can be located at the axial Tyr495. Simultaneously with the proton transfer, the radical is transferred to Tyr272 from the Cys-Tyr-dimer. Thus, it was assumed that the electron transfer from the alkoxyl radical intermediate to Cu(II) cannot be very exothermic, since in such a case the O_2 reduction step would become rate-limiting [42].

In the following oxidative half-reaction, after the product aldehyde is released from the active site, while molecular oxygen takes its place in front of the copper ion, the reduced non-radical Cu(I)-complex is re-oxidized by molecular oxygen yielding the free-radical Cu(II)-complex (with Cu(II) and tyrosyl radical) and hydrogen peroxide (Fig. 2) [112]. It was predicted that one electron is transferred from Cu(I) to the bound O_2, while a hydrogen atom is transferred from the Tyr272 to O_2 (not shown in Fig. 2). After proton transfer from the phenol of Tyr495 H_2O_2 leaves the reaction [109].

The oxidized free-radical Cu(II)-complex is quite stable but can be easily reduced to the catalytically inactive non-radical Cu(II)-complex via one-electron transfer in the presence of a broad range of chemicals [116]. The second one-electron reduction of this complex leads to formation of the non-radical Cu(I)-complex that can react with O_2 and thus re-introduces the enzyme into the reaction cycle. To avoid the accumulation of inactive enzyme during the reaction (which decreases the reaction rate and causes incomplete substrate conversion) mild oxidants like potassium ferricyanide can be added to the reaction for enzyme re-activation [111]. Trp290 stacking to the Cys-Tyr-dimer was found to modulate the redox potential of the oxidized free-radical Cu(II)-complex. While wild-type GalOx was oxidized to the free-radical Cu(II)-complex using potassium ferricyanide ($E^{\circ\prime}$= 424 mV), oxidation of the W290H variant required treatment with cesium octacyanomolybdate ($E^{\circ\prime}$= 892 mV) [92].

Horseradish peroxidase (HRP) was also found to activate GalOx [59,103]. It was suggested that HRP scavenges H_2O_2 from the reaction catalyzed by GalOx thus protecting the latter from deactivation [59]. Later, the role of HRP was re-evaluated and it was suggested, that it acts as an activator of GalOx [103]. This activating effect is still not completely understood but was explained by its function as single-electron oxidant required for the regeneration of the active site radical upon decay [80]. Thus, HRP is typically added to increase GalOx activity during reactions. Since H_2O_2 inhibits and inactivates GalOx [38,58] catalase is usually added to decompose deleterious H_2O_2 to molecular oxygen and water, thereby re-introducing oxygen into the system and reducing the overall need for oxygen [9,33,34,77,80].

Recently, electrochemical activation of GalOx from *F. graminearum* and evolved variants thereof in the presence of several mediators at pH values of 7-9 was studied using cyclic voltammetry [128]. It was demonstrated, that electron transfer rates were dependent on both, the mediator's redox potential and the pH value. It was suggested that oxidation of GalOx by mediators at pH values of 7-9 followed a concerted proton-coupled electron transfer (PCET) mechanism under anaerobic conditions. Additionally, mediated electrochemical (re)activation of GalOx variants was applied during the oxidation of different alcohols. Both electrochemical and HRP-mediated activation of GalOx resulted in similar substrate conversion values and product yields. While the selectivity

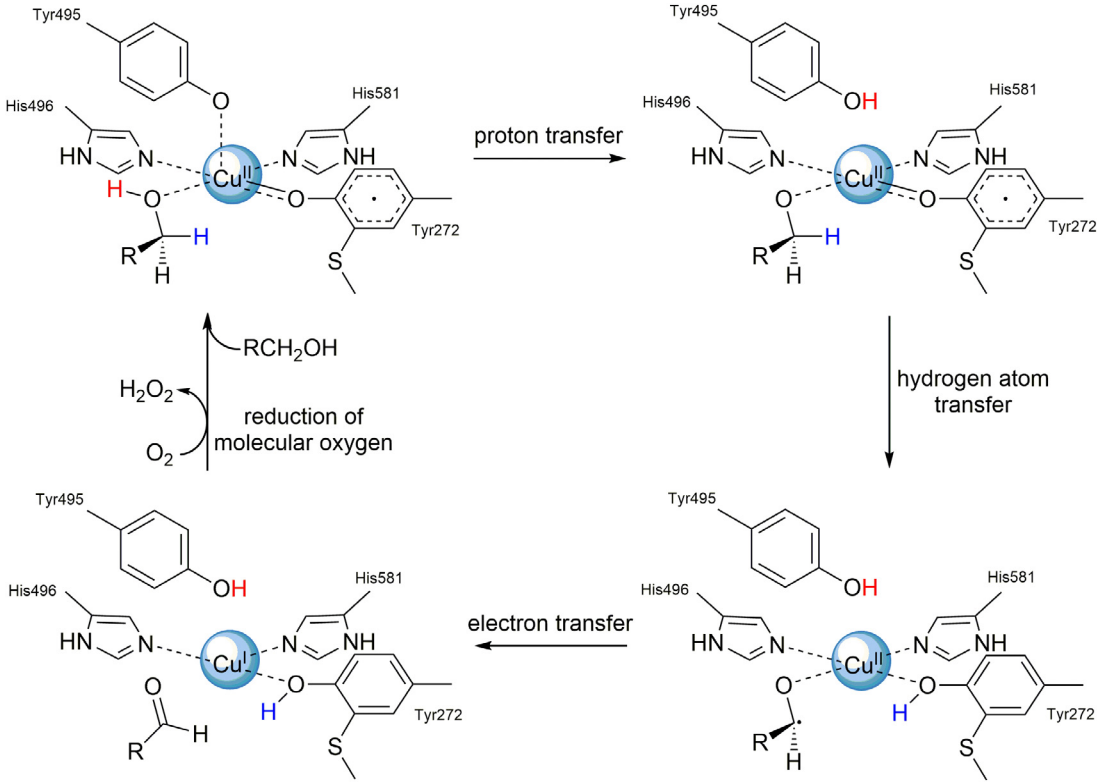

Fig. 2. Proposed catalytic cycle of GalOx from *F. graminearum*.

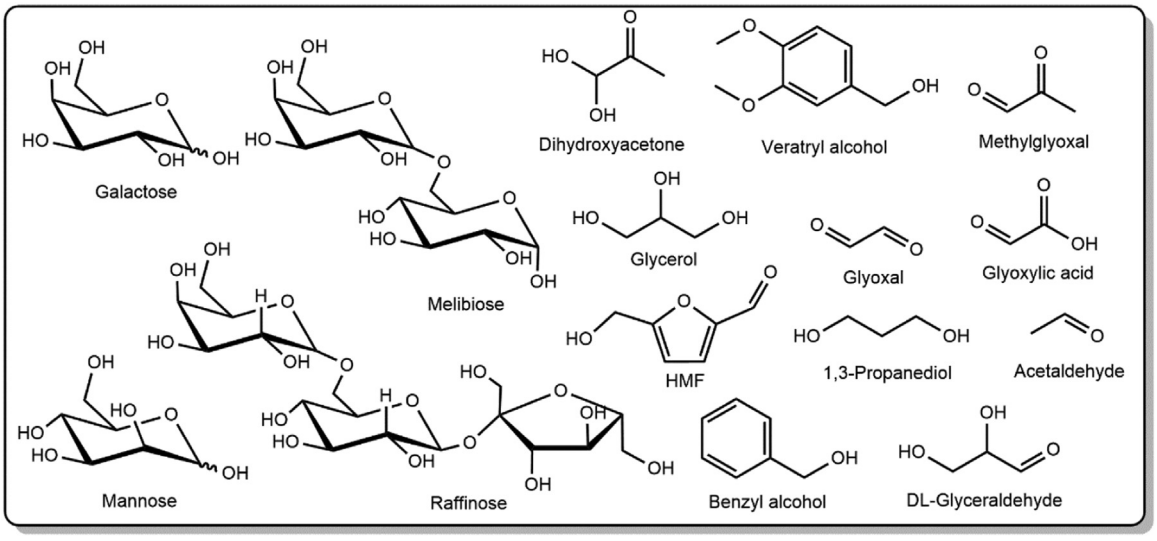

Fig. 3. Some substrates of CROs.

and conversion were sensitive to the operational voltage, no correlation between conversion and the redox potential of the investigated mediators was observed.

2.4. Substrate spectrum

GalOx catalyzes the oxidation of a broad range of primary alcohols to the respective aldehydes (Fig. 3) [119]. While predominantly galactose and galactose-containing carbohydrates are oxidized by GalOx, dihydroxyacetone and benzyl alcohols are accepted as substrates as well [6,96,119]. Notably, GalOx activity with dihydroxyacetone is 4-5 times higher than with D-galactose [96,119]. Non-galactose-containing carbohydrates and aliphatic primary alcohols are not accepted at all or with much lower efficiencies than D-galactose [96]. During the oxidation of D-galactose and glycerol GalOx shows remarkable stereospecificity [55,65]. D-galactose is oxidized by GalOx at the hydroxyl group at position C6 (C6-OH) by abstraction of the pro-*S* C6 hydrogen atom [65]. Among the carbohydrates that are efficiently oxidized by this enzyme at the C6-OH are galactopyranosides, raffinose and melibiose – all carrying an accessible D-galactose moiety [6,96]. GalOx is, however, quite sensitive to the orientation of the hydroxyl group at position C4. Glucose, the C4-epimer of galactose, is not oxidized at all, possibly due to steric interference with the copper ligand Tyr495 [46].

Several studies have dealt with altering the substrate specificity, broadening the substrate spectrum or enhancing the enzymatic activity of GalOx from *F. graminearum*. Sun and colleagues used combinatorial

saturation mutagenesis to introduce glucose 6-oxidase activity in GalOx [99]. Variant A3.E7, which is better expressed and more stable than the wild-type [100], was used as template, and residues Arg330, Phe464 and Gln406 were selected for combinatorial saturation mutagenesis. Variant M-RQ, with mutations R330K and Q406T compared to A3.E7, showed 20-times higher activity towards D-glucose than the parental enzyme. Introduction of W290F into M-RQ generated variant M-RQW with 100-fold improved oxidation of D-glucose compared to A3.E7. Oxidation of D-glucose at the C6-OH has not been observed in nature before [99]. Moreover, M-RQW accepted other primary alcohols much better than the wild-type, which was explained by the significantly augmented accessibility to the active site by the introduced mutations. For instance, the specific activity of M-RQW towards 2-pyridine methanol, hardly accepted by the wild-type, was even higher compared to the specific activity of the wild-type towards D-galactose. Furthermore, dihydroxyacetone, the best substrate of GalOx, was better oxidized by M-RQW.

The R330K mutant of GalOx exhibited an 8-fold higher catalytic efficiency (k_{cat}/K_M) during D-fructose oxidation than the wild-type enzyme [26]. GalOx variants generated via directed evolution demonstrated a 19-fold improved activity with methyl-α-D-galactopyranoside or a 16-fold increased activity towards the natural polysaccharide guar isolated from the guar plant [28]. Among others, these variants carried mutation C383S, which was suggested to provide more space and allow for tighter substrate binding as reflected by decreased K_M values. Wilkinson et al. constructed several single, double and triple mutants at positions Cys383, Tyr436 and Val494, which were identified in the aforementioned evolved GalOx variants [122]. Among the generated mutants, the variant C383S/Y436H displayed a 5.3-fold increase in catalytic efficiency during D-galactose oxidation, and the variant C383S/V494A - a 4.9-fold increase in catalytic efficiency during methyl-α-D-galactopyranoside oxidation. Besides kinetic studies on the constructed variants, the crystal structure of the C383S variant has been solved. The structure revealed that the substitution C383S, which is located in a pocket behind the active site, leads to strong H-bonding to a neighboring water molecule. However, a specific reason for the beneficial effect of this mutation was not found.

Directed evolution was also applied to enable oxidation of secondary alcohols which are not accepted by wild-type GalOx. The GalOx variant M_3 (S10P, M70V, P136, G195Q, W290F, R330K, Q406T, V494A, N535D) identified by Sun and colleagues [99], was used as a template for random mutagenesis in a study aiming at the construction of GalOx variants for enantioselective oxidation of 1-phenylethanol [30]. Variant $M_{3\text{-}5}$ differing in only one amino acid (K330M) to the parental M_3 variant showed a more than 100-fold increase in catalytic efficiency for 1-phenylethanol compared to M_3. Likewise, activity of $M_{3\text{-}5}$ towards other secondary alcohols was higher compared to M_3. Moreover, $M_{3\text{-}5}$ showed high enantioselectivity in the reaction with1-phenylethanol (99 % *ee* for the *R*-enantiomer).

2.5. Heterologous expression

Isolation of fungal GalOx from natural sources has been reported by several researchers [2,3,19,37,116]. However, low level of secretory expression and purification issues along with the need for stable and high production levels for simple mutagenesis and screening of GalOx variants with new properties stimulated the search for and development of heterologous expression systems.

As shown in Table 1, *galox* genes from several representatives of the genus *Fusarium* were heterologously expressed in different hosts like *Aspergillus nidulans* [8,70], *A. oryzae* and *F. venetatum* [125], *Pichia pastoris* [98,111,118] and *Escherichia coli* [16,27,79,99].

As aforementioned, the *galox* gene includes a pre-pro signal sequence for secretion. While in *A. nidulans* and *A. oryzae* the pre-pro signal sequence was efficiently removed from the heterologously expressed and secreted *F. graminearum* GalOx, processing of this construct in *P. pastoris* was inefficient and resulted in a mixture of mature and partly processed GalOx as confirmed by N-terminal sequencing and mass spectrometry [118]. In contrast, the pre-pro signal peptide of *F. oxysporum* GalOx was efficiently processed in *P. pastoris:* Only a single protein band of purified GalOx appeared on an SDS-gel [79]. Expression of GalOx from *F. graminearum* in *P. pastoris* with either the glucoamylase signal peptide (gla) or the α-factor secretion signal peptide from *S. cerevisiae* was also very efficient. One protein band at ~65 kDa appeared on an SDS-gel after protein purification from the culture supernatant, and mass spectrometry revealed a single N-terminus of secreted GalOx [26,118].

Recombinant expression of GalOx from *F. graminearum* with the leader peptide in *E. coli* gave only low amounts of active enzyme [70], but was used as a starting point for construction of improved GalOx variants by directed evolution [28,100]. As a result, *F. graminearum* GalOx variant A3.E7 carrying mutations S10P, M70V, P136 (silent), G195E, V494A and N535D was obtained, which displayed a 1.7-fold increase in catalytic efficiency and 18-fold improved expression (10.8 mg/L of purified enzyme) compared to the wild type [100]. Remarkably, although GalOx originates from fungus and is secreted by its natural source, it was successfully expressed in *E. coli* and did not need tedious refolding procedures [31,78,79,98]. This might be explained by the lack of glycosylation sites in GalOx from *F. graminearum* present in many other extracellular ligninolytic fungal enzymes like AAO, laccase, MnP, GLOX and other AA5_2 family members, which could be expressed as active proteins only in eukaryotic hosts. Expression of N-terminally His-tagged wild-type GalOx from *F. graminearum* and the variant A3.E7, both lacking the pre-pro signal peptide, in *E. coli* and *P. pastoris* were compared [98]. In *E. coli*, volumetric productivity of the A3.E7 variants reached 180 U/L/h, while the expression of the wild-type was approximately 10-times lower. In *P. pastoris*, expression of the wild-type was more efficient than of the A3.E7 variant and reached 610 U/L/h when the α-factor secretion signal peptide from *S. cerevisiae* was used.

A set of GalOx variants was constructed to improve expression of active GalOx in *E. coli* [27]. At first, silent mutations were introduced within the codons 2-7 of the mature GalOx sequence. The GO-N6 variant with silent mutations within Ala2, Ser3 and Ile6 displayed the highest expression. By introducing mutations present in the variant A3.E7 (denoted as M1), generated by Sun et al., into the GO-N6 variant GO-N6M1 was created, which was produced in *E. coli* BL21 Star (DE3) under optimized expression conditions at a concentration of 240 mg/L. Catalytic constants of GO-N6M1 and A3.E7 variants of GalOx were similar [100].

3. AA5_2: Non-GalOx CROs

3.1. Occurrence

In 2015 subfamily AA5_2 that encompassed until then only GalOx was expanded by two newly discovered CROs - *Cgr*AlcOx and *Cgl*AlcOx from *Colletotrichum graminicola* and *C. gloeosporioides Nara gc5*, respectively [127]. Both enzymes barely oxidized galactose and galactosides but accepted a range of primary aliphatic alcohols. Since then, other CROs with low or negligible activity towards galactose, all of them from ascomycetous fungi, have been described and assigned to AA5 subfamily 2. Many of these enzymes originate from the genus *Colletotrichum* including *Cg*RaOx and *Cgr*AAO from C. *graminicola* [5,66], *Cor*AlcOx from *C. orbiculare* [10] and *Ch*AlcOx from *C. higginsianum* [74]. Other CROs have been identified in *F. graminearum* (*Fgr*AAO) and *F. oxysporum* (*Fox*AAO) [17], *Penicillium rubens* (*Pru*AA5_2=*Pru*AlcOx) [71] and *Magnaporte oryzae* (anamorph *Pyricularia oryzae*) (*Mor*AlcOx=*Po*AlcOx=*Por*AlcOx) [10,18,74]. Only recently, the scope of non-GalOx AA5_2 members was further broadened by the discovery of new CROs, two of them from basidiomycetous fungi [18,87]. Among AA5_2 members, *Cg*RaOx, *Uma*RafOx and *Phu*RafOx were designated as raffinose oxidases due to their distinct specificity to raffinose and low activity towards galactose while other AA5_2 members like *Pru*AlcOx, *Cgl*AlcOx and *Por*AlcOx accepted a broad range of linear and benzylic alcohols.

Table 1
Heterologously expressed GalOx.

Source	Host; vector	N-terminal signal peptide	GalOx	Molecular mass (kDa)	Protein concentration (mg/L)	Volumetric activity (U/L)	Specific activity (U/mg)	Reference
F. graminearum	*A. nidulans*; G191	pre-pro	Galactose oxidase	65	35	80,000	1093	[8]
Fusarium spp.	*A.oryzae;* pEJG23	pre-pro	rGOase	66	100	-	-	[125]
Fusarium spp.	*P. pastoris;* pPICZB	gla	GAOX	68	500		1400	[118]
F. graminearum	*P. pastoris* GS115; pPIC3.5 (intracellular)	none	GalOx	65 and 68	-	14,000	10	[98]
F. graminearum	*P. pastoris* SMD1168H; pPICZαC	α-factor	GalOx	65	280	73,000	260	[98]
F. graminearum	*E. coli* BL21 (DE3); pET16b+	none	GalOx	65	-	-	10	[98]
F. graminearum	*E. coli* BL21 (DE3); pET16b+	none	A3.E7	65	-	3,600[a]	180[a]	[98]
F. graminearum PH-1	*E. coli* BL21 DE3); pET21a	none	GAO	66	0.25	16.2	63.9	[16]
F. graminearum	*E. coli* BL21 Star (DE3); pET101D	none	GO-N6M1	66	240	215,815	-	[27]
F. oxysporum G12	*E. coli* BL21(DE3); pET21a	pre-pro	GalOx	72	5.7	380	65.4	[79]
F. oxysporum G12	*P. pastoris;* pPIZB	pre-pro	GalOx	70	10.6	700	63.2	[79]
F. oxysporum G12	*P. pastoris;* pPICZαA	α-factor	GalOx	70	3	200	61	[79]
F. sambucinum	*E. coli* BL21 (DE3); pET21a	none	GalOx	68.5	4.4	704	159	[78]
F. subglutinans	*E. coli* Rosetta (DE3); pTrcHis2B	none	GaoA	74 and 77	1.38	1,732	1255	[31]

[a] after incubation with 0.5 mM $CuSO_4$.

The natural function of the non-GalOx AA5_2 members has not been clarified yet. As supposed for other H_2O_2-producing extracellular fungal oxidases, they might be involved in lignin degradation by fueling ligninolytic peroxidases with H_2O_2. Besides this, a potential role of these enzymes in pathogenesis is also possible [5,71]. *Cg*RaOx from the phytopathogenic fungus *C. graminicola* and *Pru*AlcOx oxidize raffinose, the latter one being used by a stachyose synthase of the plant to produce stachyose [105]. Both, raffinose and stachyose function as reactive oxygen species (ROS) scavengers in plants [105]. Thus, inhibition of their synthesis might assist fungal attack by weakening oxidative stress response in plants. *Cor*AlcOx and *Por*AlcOx from phytopathogenic fungi were found to oxidize long-chain alcohols of the plant cuticle to the corresponding aldehydes. The latter may function as key signal molecules to prime the fungus for plant infection [10]. During plant infection by fungi, both *Cor*AlcOx and *Por*AlcOx were found to be co-secreted with a heme peroxidase by specialized cells appressoria that puncture the plant cuticles. The peroxidase-CRO pair was found in a head-to-head arrangement in genomes of several *Colletotrichum* and *Magnaporthe* species [10]. Notably, the heterologously expressed *Cor*AlcOx and *Por*AlcOx were activated *in vitro* for oxidation of primary aliphatic alcohols by adding their respective tandem-peroxidase. Activation effect was even stronger than with HRP, which might indicate that the AlcOx/peroxidase pairs in these phytopathogenic fungi function as redox partners in nature [10].

3.2. Structure

To date, two crystal structures of non-GalOx representatives are available. Both enzymes, *Cgr*AlcOx (Fig. 1D and E) and *Cgr*AAO, originate from *C. graminicola* [66,127]. The crystal structures of both enzymes have a high similarity to the structure of GalOx from *F. graminearum*, apart from the missing CBM32 domain (N-terminal domain 1 in GalOx) [127]. In *Cgr*AlcOx and *Cgr*AAO two domains were identified – an N-terminal domain – that displays seven 'Kelch' motifs forming a seven-bladed β-propeller and contains the copper-radical active site, and a C-terminal domain displaying a nine-stranded β-barrel. Interestingly, a sequence analysis revealed that some of the non-GalOx AA5_2 CROs contain a CBM32 domain on their N-terminus, while other members contain a PAN_1 or cell wall integrity and stress response component (WSC) domain or none of those. Since CBM32 enables binding of carbohydrates like galactose in GalOx [1], the absence of CBM32 in *Cgr*AlcOx and *e.g. Cgl*AlcOx might explain their low activity with galactose and galactosides [127]. The biological functions of PAN_1 and WSC domains are diverse and include mediation of protein-protein and protein-carbohydrate interaction. *Cg*RaOx contains an N-terminal PAN_1 domain while both other raffinose oxidases, *Uma*RafOx and *Phu*RafOx, have neither CBM32, PAN_1 nor WSC domain. This indicates that additional carbohydrate- or protein-binding modules are not essential for the oxidation of carbohydrates by the mentioned CROs. *Por*AlcOx contains a WSC domain, that was shown to mediate the binding of the enzyme to xylans and fungal chitin/β-1,3-glucan to attach this enzyme to plant or fungal cell walls [74]. Deletion of the WSC domain did not affect the enzyme's catalytic activity.

Common to all CROs, non-GalOx AA5_2 members possess a conserved mononuclear copper-radical active site. The copper ion is coordinated by a crosslinked Cys-Tyr dimer (Cys228-Try272 in GalOx from *F. graminearum*), an axial tyrosine (Tyr495 in GalOx) and two histidines (His496 and His581 in GalOx) (Fig. 1C). EPR analysis confirmed that isolated *Cgr*AlcOx contains a Cu(II) site and a one-electron reduced cysteine-tyrosine cofactor. However, in contrast to GalOx *Cgr*AlcOx cannot be oxidized by ferricyanide, which indicates that the cysteine-tyrosine cofactor possesses a lower redox potential than in GalOx.

As above mentioned, in GalOx from *F. graminearum,* a residue stacking to the Cys-Tyr dimer is Trp290 which is considered a key residue for substrate (galactose) binding and catalysis and modulates the redox potential of the oxidized free-radical Cu(II)-complex [8,92]. Alcohol oxidases from *Fusarium* as well as *Afl*AlcOx and *Pru*AlcOx that demonstrate a noticeable activity with D-galactose possess tryptophan at this position, like GalOx. In *Cgr*AlcOx the homologous position 138 is occupied by phenylalanine (Figs. 1F and 4). *Cgr*AlcOx accepts a broad range of substrates but essentially failed to oxidize galactose or galactosides. The F138W mutant lost activity towards alkanols, while oxidation of 2,4-hexadienol and cinnamyl alcohol was improved [127]. However, activity of this variant towards D-galactose did not increase. Thus, it can be assumed that the galactose oxidizing activity of AA5_2 enzymes is not affected solely by the presence of tryptophan at this position. Other AA5_2 members like *Cgl*AlcOx, *Por*AlcOx and *Asy*AlcOx possess phenylalanine at the corresponding position as in *Cgr*AlcOx, while e.g. in raffinose oxidases *Cg*RaOx, *Uma*RafOx and *Phu*RafOx this position is occupied by tyrosine (Fig. 4). The effect of Tyr334 in *Cgr*AAO that corresponds to Trp290 in GalOx, on catalytic properties and substrate specificity was studied in a mutagenesis study [66]. The variant Y334W showed a strong increase in specific activity towards carbohydrates but decreased activity towards diols, aryl alcohols and furans. On the other hand, for the Y334F variant the specific activities for all tested substrates

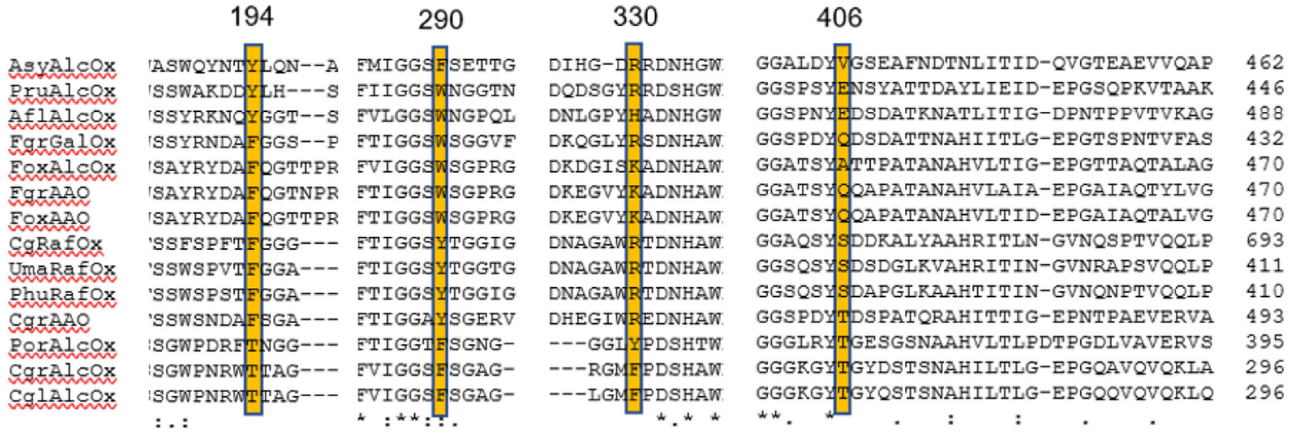

Fig. 4. Excerpt of a multiple sequence alignment of AA5_2 enzymes. Amino acid residues involved in substrate binding in GalOx from *F. graminearum* are highlighted in orange.

were comparable to wild-type or even higher. Specific activity on raffinose increased 4-fold.

In GalOx from *F. graminearum*, two phenylalanines, Phe194 and Phe464 form a hydrophobic wall for the interaction with substrates [99]. While Phe464 is highly conserved among AA5_2 family members, the amino acid residue at position 194 varies. The characterized alcohol oxidases *Fox*AlcOx, *Fgr*AAO, *Fox*AAO and *Cgr*AAO with activity towards raffinose possesses phenylalanine, while *Afl*AlcOx and *Pru*AlcOx, both oxidizing D-galactose, have tyrosine at the corresponding position (Fig. 4).

In GalOx, Arg330 and Gln406 form hydrogen bonds with D-galactose [99]. The amino acid residue at position 406 varied in non-GalOx AA5_2 CROs: Glutamine was found only in two of the galactose-accepting non-GalOx AA5_2 members (*Fox*AAO and *Fgr*AAO), while serine occupied this position in raffinose oxidases *Cg*RaOx, *Uma*RafOx and *Phu*RafOx. At positions homologous to Arg330 in GalOx from *F. graminearum*, arginine is present in raffinose oxidases, raffinose-oxidizing *Cgr*AAO and D-galactose-accepting *Pru*AlcOx. Notably, *Asy*AlcOx that barely accepts carbohydrates possesses an arginine as well while e.g. *Afl*AlcOx with moderate activity with D-galactose has a histidine at this position.

3.3. Properties and substrate scope

The length of non-GalOx members of AA5_2 subfamily varies between ~500 and 900 amino acids. So far, these enzymes were exclusively expressed in *Pichia pastoris* under the control of the methanol-inducible *AOX1* promoter with the α-factor signal peptide of *S. cerevisiae* for secretion. Protein yields reached up to 250 mg per liter of culture and molecular masses of purified enzymes ranged between ~65 kDa and 130 kDa [18,85]. While some of these CROs are glycosylated up to 30 % as reported for e.g. *Cg*RaOx [5], others are barely glycosylated like *Por*AlcOx or *Phu*RafOx [18]. Interestingly, deglycosylation of *Cg*RaOx did not affect its activity upon raffinose oxidation [5].

The substrate scope of non-GalOx AA5_2 members is quite broad and includes carbohydrates, polyols, diols, primary alcohols, aryl alcohols and furans (Figs. 3 and 5). These enzymes often demonstrate their highest activity towards raffinose, glycerol, benzyl or veratryl alcohol accompanied by a moderate to high activity with aryl alcohols. In comparison to these enzymes, GalOx usually showed its highest activity towards D-galactose or D-galactosides and oxidized aryl alcohols at much lower rates.

The AA5_2 members with high activity towards raffinose, so-called, raffinose oxidases (*Cg*RaOx, *Uma*RafOx and *Phu*RafOx) can be distinguished from those preferring benzylic alcohols as substrates (e.g. *Fgr*AAO, *Fox*AAO, *Fox*AlcOx and *Afl*AlcOx) or exhibiting high activity towards a broader range of substrates (*Cgr*AlcOx, *Cgl*AlcOx, *Por*AlcOx and *Pru*AlcOx) (Fig. 5).

Among the three raffinose oxidases, *Cg*RaOx showed the highest activity with raffinose followed by glycerol and D-melibiose, while *Phu*RafOx and *Uma*RafOx preferred glycerol over raffinose. In general, the substrate profiles of *Phu*RafOx and *Uma*RafOx, with a sequence identity of 80 %, seem quite similar. Their low specific activity towards D-galactose and different specificity towards carbohydrates distinguish the three raffinose oxidases from GalOx. For instance, *Cg*RaOx accepted the di- and trisaccharides melibiose and raffinose but failed to oxidize stachyose or polysaccharides, which are accepted by GalOx from *F. graminearum* [5].

Some of the AA5_2 enzymes oxidize raffinose as well but have not been designated as “raffinose oxidases” because their activity towards other substrates like diols, benzylic alcohols or furan derivatives is much higher. For instance, although raffinose was found a poor substrate for *Cgr*AAO, its catalytic efficiency for raffinose (71.5 M^{-1} s^{-1}) was threefold higher as compared to raffinose oxidase *Phu*RafOx. For comparison, catalytic efficiencies of *Cgr*AAO during oxidation of aryl alcohols were up to 90-fold higher and the highest catalytic efficiency of this enzyme of 19,400 M^{-1} s^{-1} was reported for 5-hydroxymethylfurfural (5-HMF).

*Fgr*AAO, *Fox*AAO, *Fox*AlcOx, *Afl*AlcOx, *Asy*AlcOx and *Cgr*AAO showed high activity towards benzylic alcohols while linear alcohols and carbohydrates were less “good” substrates. For instance, *Fgr*AAO and *Fox*AAO with a sequence identity of 90 % to each other and of 62 % to GalOx from *F. graminearum* for each, demonstrate their highest activity with veratryl alcohol and *m*-anisyl alcohol. *Fox*AlcOx with 77 % sequence identity to both enzymes showed the highest specific activity with benzyl alcohol. Among AA5 enzymes, *Fox*AlcOx possesses the highest K_M value and the lowest k_{cat} value during galactose oxidation [18]. Intriguingly, *Fgr*AAO and *Fox*AAO showed a clear preference for *m*-anisyl alcohol with a methoxy-substitution at the *meta*-position over *p*-anisyl alcohol with a methoxy-substitution at the *para*-position while other CROs accepted both equally well (Fig. 5). Specific activity of *Fgr*AAO and *Fox*AAO towards *m*-anisyl alcohol was more than 12-times higher than towards *p*-anisyl alcohol [17]. A strong preference for *meta*-substituted benzylic alcohols over *para*-substituted ones has also been described for GalOx from *F. graminearum* [119], which demonstrates the overlap in substrate profiles within AA5_2 family members. *Afl*AlcOx and *Asy*AlcOx also showed the highest specific activities with benzyl alcohol, while the best substrate for *Cgr*AAO was 5-HMF. Nonetheless, benzylic alcohols like veratryl alcohol can also be considered good substrates for *Cgr*AAO because they were oxidized with catalytic efficiencies exceeding those measured with carbohydrates or linear alcohols by two orders of magnitude [66].

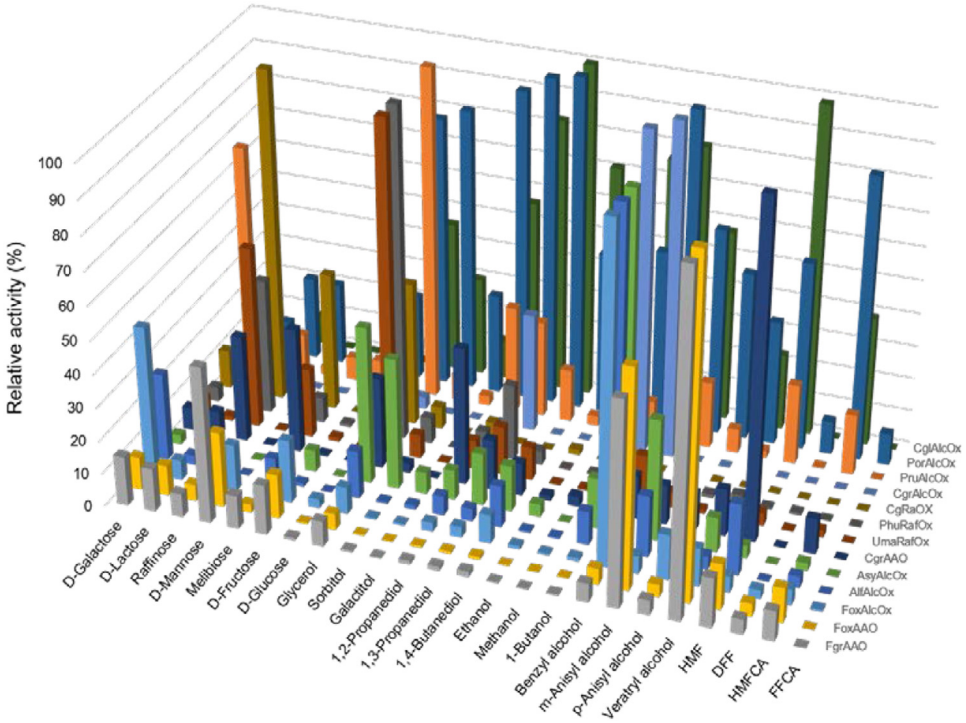

Fig. 5. Relative activity of several AA5_2 members towards a broad range of substrates. Activity towards the "best" substrate was set to 100 %.

*Cgl*AlcOx, *Cgr*AlcOx, *Por*AlcOx and *Pru*AlcOx displayed high activity towards a broad range of substrates rather than preferring a small number of compounds. They catalyze the oxidation of polyols like glycerol, diols like 1,2-propanediol, benzylic alcohols like benzyl alcohol and furans like 5-HMF (no data for *Cgr*AlcOx) with high activities [18,127]. *Pru*AlcOx demonstrated a noticeable activity towards D-galactose with catalytic efficiency being the highest among the non-GalOx AA5_2 members but much lower compared to GalOx [18]. *Cgr*AlcOx exhibited significant activity towards long-chain aliphatic alcohols like hexan-1-ol and octan-1-ol [127]. Recently, *Cde*AlcOx from *C. destructivum* and *Cta*AlcOx from *C. tabacum* with 90 % sequence identity to *Cgr*AlcOx were also found to oxidize aliphatic alcohols like octan-1-ol and decan-1-ol [85]. With catalase and HRP added to the reaction mixture, *Cgr*AlcOx and *Cde*AlcOx enabled the almost complete oxidation of hexan-1-ol and octan-1-ol to the corresponding aldehydes within 15 min [85,85]. Without HRP and catalase, conversion of both substrates reached only around 50 % after 16 h. Catalase removes the produced hydrogen peroxide, while HRP has an activating effect on CROs, similar as described above for GalOx. The addition of catalase and HRP to the reactions catalyzed by AA5_2 family members to improve substrate oxidation has been reported in several studies [9,77,85].

*Cgl*AlcOx can be considered the most active AA5_2 member described so far with a catalytic efficiency of 2,700,000 M^{-1} s^{-1} during 5-HMF oxidation [18]. By this, *Cgl*AlcOx is even more active towards 5-HMF than a bacterial HMF-oxidase which has a 380-times lower catalytic efficiency [29].

4. AA5_1: Glyoxal oxidases

4.1. Occurrence and function in nature

After GalOx, GLOX belong to the most intensively studied copper radical oxidases. Unsimilar to AA5_2 family members, GLOX catalyze the oxidation of aldehydes to carboxylic acids. Genes encoding for GLOX were identified in many white-rot fungi [22]. The first GLOX was reported in 1987 by Kersten and Kirk as a new H_2O_2-producing enzyme found in the extracellular fluid of the wood-rot degrader *Phanerochaete chrysosporium* [54]. Peroxide-generating activities were not observed with externally added primary wood-derived carbohydrates like glucose, cellobiose or xylose but with their intermediary metabolites - aldehydes and α-hydroxy-carbonyl substances. Two of these intermediates, methylglyoxal and glyoxal were detected together with GLOX in the extracellular fluid of *P. chrysosporium*, and since then considered physiological substrates for this oxidase. Whereas the highest activity was measured with methylglyoxal, enzyme activity with glyoxal was much lower. However, glyoxal concentration in extracellular fluid was substantially higher than methylglyoxal concentration [54]. There are several sources of glyoxal in fungal extracellular fluids: Starting from glycolaldehyde derived from lignin [51]; via the peroxidation of linoleic acid catalyzed by MnP [108]; or via sugar fragmentation by hydroxyl radical [64]. The product of glyoxal oxidation is glyoxylic acid, which is a better substrate for GLOX than glyoxal [52]. Further metabolism leads to oxalic acid, which might serve as chelator for Mn^{3+} produced by MnP for lignin degradation [56]. Analysis of the secretome of *P. chrysosporium* degrading aspen mechanical pulp revealed the co-occurrence of LiP, MnP and GLOX [25], indicating that GLOX might serve as hydrogen peroxide producer for ligninolytic peroxidases. Furthermore, experimental data suggested a mutual regulation between LiP and GLOX during lignin degradation, which will be described below.

Along with the physiological role of GLOX as an enzyme producing H_2O_2 for oxidative ligninolysis by peroxidases, other functions were reported as well. For example, the morphological phenotype of *glo*1 mutants of the phytopathogenic fungus *Ustilago maydis* indicated that H_2O_2 produced by the glyoxal oxidase Glo1 does not only support activities of other enzymes but also plays a role in filamentous growth and pathogenic development of *U. maydis* [60]. Among others, H_2O_2 might be required for the maturation of cell walls or can be used as a source of more active superoxide radicals. Another suggested function of Glo1 is protection from methylglyoxal that at high concentrations can lead to severe cell damage [130]. A glyoxal oxidase-like GLOXrg identified and cloned from wild Chinese grape *Vitis pseudoreticulata* was suggested to be involved in the resistance of this plant towards *Uncinula necator*

that causes powdery mildew of grape [129]. Recently, a putative GLOX, GLX1, was identified in *Trichoderma virens* [20]. Silencing of the *glx1* gene in *T. virens* resulted in mutants that lost glyoxal oxidase activity, were not able to grow directionally and showed delayed hyphal growth. This suggests a role of GLX1 in controlling hyphal growth and morphology rather than in ligninolysis.

Similar to the AA5_2 family members, GLOX was reversibly inactivated during purification or dialysis and remained inactive unless activation involving peroxidase activity [54,57]. However, while as mentioned above for the activation of GalOx, peroxidase alone can fulfill this task, peroxidase-mediated GLOX activation requires the presence of a peroxidase substrate. Under conditions mimicking those of ligninolytic cultures, GLOX was activated in the presence of LiP with its substrate veratryl alcohol [52]. Peroxidase alone did not demonstrate this reactivation effect. In nature, the reversible inactivation of GLOX in the absence of a peroxidase substrate may prevent the accumulation of high H_2O_2 concentrations *in vivo* and thus protect LiP from inactivation [52,57]. The same (re)activating effect on GLOX was achieved with HRP but again in combination with its substrates methoxybenzenes [52]. Further investigation of this enzyme expressed in recombinant *A. nidulans* (rGLOX) revealed a basal activity also in the absence of an activating coupled peroxidase system [53,57]. After an initial phase of fast oxygen consumption, the enzyme was inactivated by increasing concentrations of hydrogen peroxide [57]. However, the same concentration of externally added H_2O_2 caused only a partial enzyme inactivation, which suggested a complex mechanism of activity control. rGLOX was activated in the presence of LiP and lignin or its oxidized intermediates veratryl alcohol or methoxybenzene, whereas the phenolic products of lignin degradation with lower redox potential - guaiacol or catechol - added as substrates for LiP inactivated rGLOX [52]. On the basis of these experimental data, it was suggested that mutual regulation of LiP and GLOX is required for the controlled radical formation and minimized radical coupling, forcing lignin depolymerization [57].

For GLOX, it has been reported that the addition of catalytic amounts of hydrogen peroxide to the peroxidase system is required to reduce the lag phase [52]. However, in a coupled system with HRP and its artificial substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), HRP would immediately use the initially added hydrogen peroxide for ABTS oxidation. In order to elucidate if ABTS cation radicals generated by HRP can serve as the activating species for GLOX, their concentration was continuously measured in a spectroelectrochemical cell, where the catalytic activity of rGLOX from *P. chrysosporium* was monitored based on O_2 consumption [124]. These measurements indicated that ABTS cation radicals act as activating species for GLOX. After activation, the enzyme catalyzed multiple turnovers of methylglyoxal, and enzyme activity increased at increasing concentrations of the activating species. Additionally, GLOX activity was found to be restrained by low O_2 concentrations. This study provided additional evidence that *in vivo*, GLOX activity is regulated by O_2 concentration and by peroxidase activity during radical formation.

Purified GLOX from *P. chrysosporium* has been found to be activated also by Cu^{2+} [52] and high-redox potential inorganic oxidants [115]. The same reactivation prior to substrate turnover was reported for GLOX from *P. cinnabarinus* [23] and *U. maydis* [60], whereas GLOX from *Myceliophthora thermophile* did not require any activation [48].

4.2. Properties and structure

The purified GLOX from the extracellular liquid of *P. chrysosporium* is a glycoprotein of approximately 68 kDa with a degree of glycosylation of around 15 %. Sequence analysis revealed five potential N-glycosylation sites [53]. Spectroscopic studies on GLOX from *P. chrysosporium* including resonance Raman spectroscopy has revealed the presence of a free radical coupled copper catalytic motif in the active site, which has previously been identified in GalOx from *F. graminearum* [115].

Similar to GalOx, in GLOX the mononuclear copper ion is coordinated to a cross-linked cysteine-tyrosine radical cofactor. Despite the low sequence identity between GalOx from *F. graminearum* and GLOX from *P. chrysosporium* of around 20 %, certain similarity in positioning of the critical residues and structural motifs allowed to identify four Cu coordinating residues, His378, His471, Tyr135 and Tyr377 in the active site of GLOX (Fig. 1I). Tyr135 is the radical-forming Cu ligand cross-linked to the adjacent Cys70. Cys70, Tyr135, Tyr377 and His378 building the active site of GLOX are located in a catalytic domain with a 'Kelch' motif forming a seven-fold β-barrel structure (Fig. 1G and H). A second domain was also modeled that builds a cap to the seven-fold β-barrel and possesses a loop that contains the His471 [51,114]. Sequence comparison also revealed that GLOX lacks an N-terminal domain with carbohydrate-binding site present in GalOx [114]. The essential role of the residues Cys70, Tyr135 and Tyr377 was confirmed by mutagenesis. Based on these data, the axial Tyr377 serves as a general base and is involved in proton abstraction from the coordinated hydroxyl group, thus activating the substrate for oxidation [114]. Compared to GalOx, the stacking tryptophan (Trp290) seems to be replaced with histidine (His153) in GLOX, which explains a higher redox potential (0.64 V vs 0.43 V) [47] and a 43-fold lower stability of the GLOX radical than the GalOx radical [113]. In non-GalOx AA5_2 enzymes tryptophan, phenylalanine or tyrosine can be found at the corresponding position (Fig. 4). These differences between AA5_2 enzymes and GLOX emphasize the importance of the residue shielding the Cys-Tyr dimer for the Cu-protein radical stability [92] and probably contributes to the catalytic differences between AA5_1 and AA5_2 family members [51].

4.3. Substrate spectrum

Typically, GLOX catalyze the two-electron oxidation of simple aldehydes and α-hydroxycarbonyls (Fig. 3) [115]. The best characterized native GLOX from *P. chrysosporium* after reactivation with a peroxidase system, accepted methylglyoxal, glyoxal, glycolaldehyde, acetaldehyde, formaldehyde, glyoxylic acid, dihydroxyacetone, DL-glyceraldehyde and acetol as substrates [52]. Whereas the k_{cat} values were in the same range for all substrates, K_M values differed significantly and ranged from 0.64 mM for methylglyoxal to 42 mM for DL-glyceraldehyde. Considering the catalytic efficiency k_{cat}/K_M methylglyoxal seems to be the best substrate followed by glyoxylic acid. The highest k_{cat} value was measured with formaldehyde, and the lowest one with glyoxylic acid [52]. Later, the same GLOX was heterologously expressed in *P. pastoris* and *T. reesei* at high level [123]. For expression in *T. reesei* the enzyme sequence including the native signal peptide was cloned under the on glucose highly active cDNA1 promoter and for expression in *P. pastoris* – under the control of the AOX1 promoter for methanol induced expression. Both recombinant GLOX forms were glycosylated: While GLOX produced in *T. reesei* carried Man5 glycosylation, the enzyme expressed in *P. pastoris* had the high-mannose type N-glycosylation. Generally, the enzyme expressed in *T. reesei* displayed slightly lower specific activities, which were explained by the lower copper loading of 51.9 % compared to 65.5 % for the enzyme expressed in *P. pastoris*. Both variants were active towards the substrates accepted by the enzyme isolated from the original source, however, both the K_M and k_{cat} values were slightly lower. Additionally, glycerol was also identified as substrate. Affinity to this substrate was low with K_M of > 600 mM but with the highest k_{cat} value among all substrates. Glycerol was also oxidized by some non-GalOx AA5_2 members with *Pru*AlcOx showing the lowest K_M of < 40 mM and a 17-fold higher catalytic efficiency than GLOX from *P. chrysosporium* [18]. As glycerol is lacking a carbonyl/formyl group typical for GLOX substrates, activity of GLOX towards this substrate was explained by its structural similarity to dihydroxyacetone. Another explanation is based on structural similarity of the primary alcohols to *gem*-diols, derived from aldehydes [23].

Two GLOX from *Pycnoporus cinnabarinus Pci*GLOX1 and *Pci*GLOX2 were produced in *A. niger* [24]. The natural signal peptides of both

GLOX were replaced by the 24-amino-acid glucoamylase pre-pro sequence from *A. niger*. Both recombinant GLOX accepted the substrates described for GLOX from *P. chrysosporium*. Nevertheless, the two enzymes demonstrated significant differences in their catalytic properties between each other and compared to GLOX from *P. chrysosporium*. *Pci*GLOX2 accepted more substrates and converted them with higher activity than *Pci*GLOX1. The best substrate for both enzymes was glyoxylic acid. *Pci*GLOX1 exhibited the highest catalytic efficiency with this substrate of more than 2000 mM^{-1} s^{-1} (as compared to 17 mM^{-1} s^{-1} for *Pci*GLOX2), followed by methylglyoxal, glyoxal and glycerol. Glycerol was accepted as substrate by both GLOX, but k_{cat} of the more active *Pci*GLOX1 was 7-times lower than that of GLOX from *P. chrysosporium* [23]. Products were analyzed by HPLC. As expected, glyoxylic acid was converted to oxalic acid and methylglyoxal to pyruvate. In the case of glyoxal conversion catalyzed by the more active *Pci*GLOX1 one peak corresponding to oxalic acid appeared, while a second peak corresponding to the primary product glyoxylic acid appeared only in the *Pci*GLOX2 catalyzed reaction. Furthermore, GLOX from *P. cinnabarinus* oxidized 5-HMF mainly to 5-hydroxymethyl-2-furancarboxylic acid (HMFCA) [24]. MtGLOX from *M. thermophile* generally demonstrated lower activity than other reported GLOX [48]. Among different substrates, this enzyme preferred 5-HMF, methylglyoxal and glycerol.

5. Other AA5 enzymes

In 2021, Daou et al. identified a new CRO, *Tr*LOx, in the genome of *T. reesei* [21]. Although phylogenetic analysis showed that this enzyme belongs to the AA5_1 subfamily, biochemical characterization revealed overlapping properties of *Tr*LOx across the AA5 family. The enzyme oxidized both, alcohols and aldehydes with a preference for alcohols. The highest activity was measured with dihydroxyacetone, the best substrate of GalOx, and the second highest activity with methylglyoxal, a typical substrate of GLOX. Furfuryl alcohol, veratryl alcohol and 2-phenylethanol were accepted by *Tr*LOx as well while carbohydrates like galactose were poor substrates and oxidation of glyoxylic acid and glycerol, known substrates of GLOX, was quite slow. Thus, *Tr*LOx showed activities of AA5_1 and AA5_2 members. The gene-encoding sequence of *Tr*LOx contains five predicted WSC domains, which was shown to bind to chitin, but not cellulose [20]. A WSC domain was also found in *Por*AlcOx where it was shown to mediate binding of the enzyme to xylans and fungal chitin/β-1,3-glucan [74]. The function of the WSC domain in *Tr*LOx remains, however, unknown.

A membrane-associated mononuclear radical copper oxidase, GlxA, was identified in the bacterium *Streptomyces lividans* [15]. GlxA has a different tertiary structure, different spectroscopic properties and a distinct substrate profile compared to GalOx and other AA5 family members and was therefore not assigned to either AA5 subfamily. The enzyme contains three domains as GalOx from *F. graminearum*, and first sphere copper coordinating residues in GlxA and GalOx are identical. However, domain 2 is located on top of domain 1, shielding the copper site from the solvent. Moreover, while in GalOx Trp290 is stacked over the thioether bond, the benzene ring of Trp288 in GlxA is π-π stacked over the copper ligand Tyr289 which probably leads to the distinct absorbance spectrum of GlxA. Among several substrates tested, activity was observed only with glycolaldehyde. D-galactose, D-glucose and glycerol were hardly accepted, and glyoxal, a substrate of GLOX, was not oxidized at all. Construction of a *S. lividans glxA* null-mutant indicated that GlxA is required for the synthesis or localization of glycan at hyphal tips.

Genes with homology to *galox* were also identified in *Stigmatella aurantiaca* and *S. coelicolor* A3(2) [97,121]. However, oxidation of galactose by these enzymes was not shown. For instance, the GalOx-like SCO2837 protein from *S. coelicolor* A3(2) converted some primary alcohols to aldehydes and showed the highest activity with glycolaldehyde, similar to GlxA [121]. However, glyoxal was hardly accepted as substrate.

6. Application

Due to the broad range of substrates accepted by AA5 family members their potential fields of application are quite diverse. The ability to catalyze the regioselective oxidation of primary alcohols renders these enzymes attractive for the synthesis of flavor and fragrance compounds or pharmaceuticals as well as for the production of the bio-plastics precursor 2,5-furandicarboxylic acid (FDCA) from 5-HMF (Fig. 6). These and other potential applications in biotechnology will be described in the following sub-chapter.

6.1. Production of FDCA

AA5 enzymes from both subfamilies have been intensively investigated for the synthesis of the bio-based plastic precursor FDCA from 5-HMF. On the one hand, 5-HMF can be oxidized by e.g. GalOx, GLOX and *Cgr*AAO to diformylfuran (DFF) (Fig. 6). DFF can be further oxidized to 5-formyl-2-furancarboxylic acid (FFCA) by some AA5_2 enzymes like *Cgl*AlcOx, *Por*AlcOc and *Pru*AlcOx [18] while others, like *Cgr*AAO, mt-GLOX and GalOx, failed to oxidize DFF [66]. Oxidation of FFCA to yield FDCA has been reported with *Pci*GLOX and some AA5_2 enzymes like *Cgl*AlcOx and *Por*AlcOx. On the other hand, 5-HMF can be converted by *Pci*GLOX to HMFCA which can be further oxidized to FFCA by e.g. AA5_2 enzymes *Afl*AlcOx, *Pru*AlcOx, *Cgl*AlcOx and *Por*AlcOx. Starting from 5-HMF, *Cgl*AlcOx with the highest activity towards this substrate catalyzed its conversion to a mixture of FFCA and FDCA in a ratio of 91:9 [18], while e.g. *Fgr*AAO and *Fox*AAO produced a mixture of DFF and FFCA [17].

A complete conversion of 5-HMF to FDCA with one of the mentioned AA5 enzymes alone could not be achieved so far. To improve the conversion of 5-HMF to FDCA, Daou et al. combined *Pci*GLOX3 with an AAO from *U. maydis* (*Uma*AAO), that oxidized 5-HMF within 2 h to DFF. This bienzymatic approach yielded FFCA and FDCA in a ratio of 86:14 [24]. In several systems, GalOx or variants thereof were exploited in combination with other enzymes for the synthesis of FDCA from 5-HMF via DFF [9,67,81]. To this end, GalOx variant $M_{3\text{-}5}$ [30] and a periplasmic aldehyde oxidase (PaoABC) were used in a tandem sequential process [67]. In the first step, $M_{3\text{-}5}$ completely converted 50 mM 5-HMF to DFF. In the second step, PaoABC was added which completely oxidized DFF to FFCA and further to FDCA within 3 h. A combination of both enzyme activities in one step yielded only 56 % FDCA after 17 h. This was due to the fast production of HMFCA from 5-HMF by PaoABC, which was not accepted by GalOx variant $M_{3\text{-}5}$ and only slowly converted by PaoABC to FFCA, followed by rapid oxidation to FDCA. Later, GalOx variant $M_{3\text{-}5}$ was found to oxidize HMFCA very efficiently when added as the sole substrate [68]. However, in a bienzymatic cascade with PaoABC, the produced FDCA might inhibit HMFCA oxidation by GalOx $M_{3\text{-}5}$. After HRP and catalase were added and lower amounts of PaoABC were applied in this system, complete conversion of 100 mM 5-HMF to FDCA was achieved within 6 h [68]. Lower concentrations of PaoABC resulted in reduced formation of the undesired HMFCA from 5-HMF, which facilitated oxidation of 5-HMF to DFF by GalOx, followed by efficient oxidation of DFF and FFCA by PaoABC.

Selective oxidation of 5-HMF to DFF by GalOx was improved by optimizing reaction conditions and enzyme engineering [9]. GalOx variant $M_{3\text{-}5}$ was used as starting point for construction of several libraries with pairwise mutated active site residues [9]. The best performing variant M_4 ($M_{3\text{-}5}$ + Y329L/M330F) was used for the construction of further active site libraries that were screened at low oxygen levels for improved 5-HMF oxidation. The best variant, $M_{6\text{-}A}$ (M_4 + F290W/S291S), showed a 50 % increase in apparent k_{cat} value for 5-HMF and 2-times lower K_M value for oxygen compared to M_4. By merging the hits from different mutant libraries, variant $M_{7\text{-}2A}$ was constructed and applied for conversion of 5-HMF in 0.2 L volume. Within this setup, a conversion of 56 % with a productivity of 2.6 g/L/h was achieved [9].

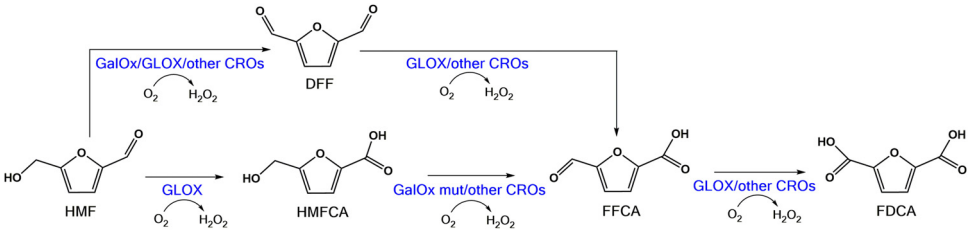

Fig. 6. Application of CROs in the synthesis of the bio-plastic precursor FDCA. Other CROs" include non-GalOx members of AA5_2.

Another report described a two-enzyme system with a recombinant GalOx variant and an unspecific peroxygenase (UPO) for 5-HMF oxidation to furnish FDCA [49]. Recombinant GalOx was also applied in a three-enzyme approach consisting of GalOx, UPO and AAO to produce FDCA from 5-HMF [50]. Within this setup, GalOx was applied for the oxidation of HMFCA to FFCA.

6.2. Synthesis of pharmaceuticals, flavor and fragrance compounds

CROs are potential biocatalysts for the synthesis of flavor and fragrance ingredients. For example, *Cgr*AlcOx converted the long-chain alcohol octan-1-ol within minutes to octanal, a valuable fragrance compound used in perfumes [85]. Recently, a bienzymatic cascade for the synthesis of the (-)-menthol precursor (*R*)-citronellal from geraniol by exploiting a non-GalOx AA5_2 CRO (*Cgr*AlcOx) and an old yellow enzyme (OYE) has been established [86]. First, *Cgr*AlcOx catalyzed the oxidation of geraniol to geranial which was then converted by OYE2 from *S. cerevisiae* with high enantioselectivity to (*R*)-citronellal with an *ee* of 95.9 %. By replacing OYE2 by OYE GluER from *Gluconobacter oxydans* with opposite enantioselectivity (*S*)-citronellal was obtained (99.2 % *ee*).

A one-pot two-enzyme system comprising of GalOx and ω-transaminase was developed for the amination of benzylic alcohols and cinnamic alcohols and applied for the biocatalytic synthesis of the antifungal agent naftifine [35]. Furthermore, by combining GalOx and ω-transaminase from *Chromobacterium violaceum* a biocatalytic cascade for the production of galactose derivatives with an amino group at the C6-position was established [4]. GalOx variants were also applied in one-pot reaction cascades either with aldolase for the production of amino sugars or with xanthine dehydrogenase or aldehyde oxidase for the synthesis of lactams [41]. Thereby, GalOx variants catalyzed the oxidation of amine alcohols to aldehydes. Recently, a one-pot enzymatic cascade has been developed employing GalOx and imine reductase for the synthesis of enantiopure L-3-aminopiperidine and L-3-aminoazepane from amino alcohols [32].

An evolved GalOx variant was applied for desymmetrization of prochiral 2-ethynylglycerol via oxidation to (*R*)-2-ethynylglyceralde hyde, a key intermediate in the synthesis of the HIV reverse transcriptase translocation inhibitor islatravir [43]. Besides GalOx and HRP, four other enzymes were used to establish a biocatalytic cascade starting from 2-ethynylglycerol to produce islatravir at high yield and with high stereoselectivity.

6.3. Other applications

Another interesting substrate for AA5 enzymes is glycerol, a prochiral compound and the main unavoidable by-product in biodiesel production. Due to the growing interest in biofuels and increasing production of biodiesel, a large surplus of glycerol is generated. Different valorization methods on the basis of the AA5 enzymes have been reported. On the one hand, glycerol can be valorized through desymmetrization to yield D- or L-glyceraldehyde. Most AA5_2 enzymes showed a preference for producing L-glyceraldehyde [18]. Only *Cgr*AlcOx, *Cgl*AlcOx and *Por*AlcOx showed a preference for D-glyceraldehyde formation. On the other hand, GLOX was shown to convert glycerol to the final product glyceric acid [93].

Since the 1980s, GalOx has been applied for selective oxidation of galactose-containing mono-, oligo- and polysaccharides [75,76,126]. GalOx and its engineered variants were successfully used for labeling of galactose-containing membrane oligosaccharides and glycoproteins [39,82,88]. Furthermore, due to its high specificity towards D-galactose compared to D-glucose, GalOx was recognized as an attractive biosensor candidate for detecting lactose or galactose in blood or urine [101,102].

7. Concluding remarks

Copper radical oxidases build a versatile group of enzymes that harbor in their active sites a mononuclear copper and a cross-link between one of the tyrosines involved in the copper ion binding and an adjacent cysteine. In the catalytically active state, the modified tyrosine is oxidized to a tyrosyl radical. Due to this unique cofactor CROs have attracted considerable attention in basic research. A prototypical CRO GalOx has been extensively studied for almost thirty years. Its physicochemical and catalytic properties, structure and catalytic mechanism have been elucidated in detail. GLOX also belongs to the one of most intensively studied CROs, however, no crystal structure has been solved yet. Recent reports reflect the increasing interest in this group of oxidases in applied research. Newly identified and characterized CROs possess distinct catalytic properties and different substrate spectra compared to GalOx and GLOX and thus significantly expand the toolbox of these useful biocatalysts. For instance, CROs were identified that oxidize various carbohydrates not accepted by GalOx, or with activity towards 5-HMF exceeding that of a bacterial HMF-oxidase. By protein engineering activity of CROs could be increased and substrate preference altered, which paves the way for new biotechnological processes. However, predominantly methods of directed evolution and semi-rational design were applied because three-dimensional structures were solved only for four CROs. Moreover, although these structures revealed highly conserved copper coordinating residues, amino acids that interact with substrates differ. Obviously, more crystal structures are required to help in understanding the substrate preferences of different CROs and in engineering more efficient and more selective biocatalysts.

The range of CROs application can be extended towards deactivation of harmful aldehydes and engineering of biosensors for medicine and food production and preservation. CROs can also be exploited as H_2O_2 suppliers for peroxidases and peroxygenases to support the latter in biocatalytic delignification of plant biomass and in biocatalysis. In this regard, the potential of CROs at a higher industrially relevant scale has not been evaluated yet and will also move into focus in the near future.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Given her role as Editorial Board Member, Vlada B. Urlacher had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Dr. Shengying Li.

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References

[1] D.W. Abbott, J.M. Eirin-Lopez, A.B. Boraston, Insight into ligand diversity and novel biological roles for family 32 carbohydrate-binding modules, Mol. Biol. Evol. 25 (2008) 155–167, doi:10.1093/molbev/msm243.
[2] K. Aisaka, O. Terada, Production of galactose-oxidase by *Gibberella fujikuroi*, Agric. Biol. Chem. 45 (1981) 2311–2316, doi:10.1080/00021369.1981.10864879.
[3] D. Alberton, L.S. de Oliveira, R.M. Peralta, I.P. Barbosa-Tessmann, Production, purification, and characterization of a novel galactose oxidase from *Fusarium acuminatum*, J. Basic Microbiol. 47 (2007) 203–212, doi:10.1002/jobm.200610290.
[4] V. Amalga, F. Mollerup, E. Jerk, F. Blame, J. Carpi, A.E. Kristine, E. Schulte, M. Voss, U. Bornscheuer, J. Deska, E.R. Master, Biocatalytic production of amino carbohydrates through oxidoreductase and transaminase cascades, ChemSusChem 12 (2019) 848–857, doi:10.1002/cssc.201802580.
[5] M. Andberg, F. Mollerup, K. Parikka, S. Koutaniemi, H. Boer, M. Joanne, E. Master, M. Tankage, K. Kraus, A novel *Colletotrichum graminicola* raffinose oxidase in the AA5 family, Appl. Environ. Microbiol. 83 (2017) e01383–17, doi:10.1128/AEM.01383-17.
[6] G. Avigad, C. Asensio, B.L. Horecker, D. Amaral, The D-galactose oxidase of *Polyporus circinatus*, J. Biol. Chem. 237 (1962) 2736–2743.
[7] I.P. Barbosa-Tessmann, D.A. Da Silva, R.M. Peralta, C. Kemmelmeier, A new species of *Fusarium* producer of galactose oxidase, J. Basic Microbiol. 41 (2001) 143–148, doi:10.1002/1521-4028(200107)41:3/4<143::Aid-Jobm143>3.0.Co;2-G.
[8] A.J. Baron, C. Stevens, C. Wilmot, K.D. Seneviratne, V. Blakeley, D.M. Dooley, S.E.V. Phillips, P.F. Knowles, M.J. McPherson, Structure and mechanism of galactose oxidase. The free radical site, J. Biol. Chem. 269 (1994) 25095–25105.
[9] W.R. Birmingham, A.T. Pedersen, M.D. Gomes, M.B. Madsen, M. Breuer, J.M. Woodley, N.J. Turner, Toward scalable biocatalytic conversion of 5-hydroxymethylfurfural by galactose oxidase using coordinated reaction and enzyme engineering, Nat. Commun. 12 (2021) 4946, doi:10.1038/s41467-021-25034-3.
[10] B. Bissaro, S. Kodama, H. Hage, D. Ribeaucourt, M. Haon, S. Grisel, A. Simaan, F. Beisson, S. Forget, H. Brumer, M.N. Rosso, R. O'Connell, M. Lafond, Y. Kubo, J.G. Berrin, Unravelling the role of alcohol copper radical oxidases in fungal plant pathogens (2021), doi:10.21203/rs.3.rs-493001/v1.
[11] C.G. Bjordal, Evaluation of microbial degradation of shipwrecks in the Baltic Sea, Int. Biodeterior. Biodegrad. 70 (2012) 126–140, doi:10.1016/j.ibiod.2012.01.012.
[12] M.A. Bradford, R.J. Warren, P. Baldrian, T.W. Crowther, D.S. Maynard, E.E. Oldfield, W.R. Wieder, S.A. Wood, J.R. King, Climate fails to predict wood decomposition at regional scales, Nat. Clim. Change 4 (2014) 625–630, doi:10.1038/Nclimate2251.
[13] T.D. Bugg, M. Ahmad, E.M. Hardiman, R. Rahmanpour, Pathways for degradation of lignin in bacteria and fungi, Nat. Prod. Rep. 28 (2011) 1883–1896, doi:10.1039/c1np00042j.
[14] B.L. Cantarel, P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics, Nucleic Acids Res. 37 (2009) D228–D233, doi:10.1093/nar/gkn663.
[15] A.K. Chaplin, M.L.C. Petrus, G. Mangiameli, M.A. Hough, D.A. Svistunenko, P. Nicholls, D. Claessen, E. Vijgenboom, J.A.R. Worrall, GlxA is a new structural member of the radical copper oxidase family and is required for glycan deposition at hyphal tips and morphogenesis of *Streptomyces lividans*, Biochem. J. 469 (2015) 433–444, doi:10.1042/Bj20150190.
[16] W. Choosri, R. Paukner, P. Wuhrer, D. Haltrich, C. Leitner, Enhanced production of recombinant galactose oxidase from *Fusarium graminearum* in *E. coli*, World J. Microbiol. Biotechnol. 27 (2011) 1349–1353, doi:10.1007/s11274-010-0585-2.
[17] M. Cleveland, M. Lafond, F.R. Xia, R. Chung, P. Mulyk, J.E. Hein, H. Brumer, Two *Fusarium* copper radical oxidases with high activity on aryl alcohols, Biotechnol. Biofuels 14 (2021) 138, doi:10.1186/s13068-021-01984-0.
[18] M.E. Cleveland, Y. Mathieu, D. Ribeaucourt, M. Haon, P. Mulyk, J.E. Hein, M. Lafond, J.G. Berrin, H. Brumer, A survey of substrate specificity among Auxiliary Activity Family 5 copper radical oxidases, Cell. Mol. Life Sci. 78 (2021) 8187–8208, doi:10.1007/s00018-021-03981-w.
[19] J.A.D. Cooper, W. Smith, M. Bacila, H. Medina, Galactose oxidase from *Polyporus circinatus*, Fr. J. Biol. Chem. 234 (1959) 445–448.
[20] F.K. Crutcher, M.E. Moran-Diez, I.V. Krieger, C.M. Kenerley, Effects on hyphal morphology and development by the putative copper radical oxidase glx1 in *Trichoderma virens* suggest a novel role as a cell wall associated enzyme, Fungal Genet. Biol. 131 (2019) 103245, doi:10.1016/j.fgb.2019.103245.
[21] M. Daou, A. Bisotto, M. Haon, L.O. Correia, B. Cottyn, E. Drula, S. Garajova, E. Bertrand, E. Record, D. Navarro, S. Raouche, S. Baumberger, C.B. Faulds, A putative lignin copper oxidase from *Trichoderma reesei*, J. Fungi 7 (2021) 643, doi:10.3390/jof7080643.
[22] M. Daou, C.B. Faulds, Glyoxal oxidases: their nature and properties, World J. Microbiol. Biotechnol. 33 (2017) 87, doi:10.1007/s11274-017-2254-1.
[23] M. Daou, F. Piumi, D. Cullen, E. Record, C.B. Faulds, Heterologous production and characterization of two glyoxal oxidases from *Pycnoporus cinnabarinus*, Appl. Environ. Microbiol. 82 (2016) 4867–4875, doi:10.1128/AEM.00304-16.
[24] M. Daou, B. Yassine, S. Wikee, E. Record, F. Duprat, E. Bertrand, C.B. Faulds, *Pycnoporus cinnabarinus* glyoxal oxidases display differential catalytic efficiencies on 5-hydroxymethylfurfural and its oxidized derivatives, Fungal Biol. Biotechnol. 6 (2019) 4, doi:10.1186/s40694-019-0067-8.
[25] A. Datta, A. Bettermann, T.K. Kirk, Identification of a specific manganese peroxidase among ligninolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay, Appl. Environ. Microbiol. 57 (1991) 1453–1460, doi:10.1128/aem.57.5.1453-1460.1991.
[26] S.E. Deacon, K. Mahmoud, R.K. Spooner, S.J. Firbank, P.F. Knowles, S.E.V. Phillips, M.J. McPherson, Enhanced fructose oxidase activity in a galactose oxidase variant, ChemBioChem 5 (2004) 972–979, doi:10.1002/cbic.200300810.
[27] S.E. Deacon, M.J. McPherson, Enhanced expression and purification of fungal galactose oxidase in *Escherichia coli* and use for analysis of a saturation mutagenesis library, ChemBioChem 12 (2011) 593–601, doi:10.1002/cbic.201000634.
[28] S. Delagrave, D.J. Murphy, J.L.R. Pruss, A.M. Maffia, B.L. Marrs, E.J. Bylina, W.J. Coleman, C.L. Grek, M.R. Dilworth, M.M. Yang, D.C. Youvan, Application of a very high-throughput digital imaging screen to evolve the enzyme galactose oxidase, Protein Eng. Des. Sel. 14 (2001) 261–267, doi:10.1093/protein/14.4.261.
[29] W.P. Dijkman, M.W. Fraaije, Discovery and characterization of a 5-hydroxymethylfurfural oxidase from *Methylovorus* sp. strain MP688, Appl. Environ. Microbiol. 80 (2014) 1082–1090, doi:10.1128/aem.03740-13.
[30] F. Escalettes, N.J. Turner, Directed evolution of galactose oxidase: generation of enantioselective secondary alcohol oxidases, ChemBioChem 9 (2008) 857–860, doi:10.1002/cbic.200700689.
[31] C.B. Faria, F.F. de Castro, D.B. Martim, C.A.L. Abe, K.V. Prates, M.A.S. de Oliveira, I.P. Barbosa-Tessmann, Production of galactose oxidase inside the *Fusarium fujikuroi* species complex and recombinant expression and characterization of the galactose oxidase GaoA protein from *Fusarium subglutinans*, Mol. Biotechnol. 61 (2019) 633–649, doi:10.1007/s12033-019-00190-6.
[32] G.J. Ford, N. Kress, A.P. Mattey, L.J. Hepworth, C.R. Baldwin, J.R. Marshall, L.S. Seibt, M. Huang, W.R. Birmingham, N.J. Turner, S.L. Flitsch, Synthesis of protected 3-aminopiperidine and 3-aminoazepane derivatives using enzyme cascades, Chem. Commun. 56 (2020) 7949–7952, doi:10.1039/d0cc02976a.
[33] S.M. Forget, F.R. Xia, J.E. Hein, H. Brumer, Determination of biocatalytic parameters of a copper radical oxidase using real-time reaction progress monitoring, Org. Biomol. Chem. 18 (2020a) 2076–2084, doi:10.1039/c9ob02757b.
[34] S.M. Forget, F.R. Xia, J.E. Hein, H. Brumer, Determination of biocatalytic parameters of a copper radical oxidase using real-time reaction progress monitoring, Org. Biomol. Chem. 18 (2020b) 2076–2084, doi:10.1039/c9ob02757b.
[35] M. Fuchs, K. Tauber, J. Sattler, H. Lechner, J. Pfeffer, W. Kroutil, K. Faber, Amination of benzylic and cinnamic alcohols via a biocatalytic, aerobic, oxidation-transamination cascade, RSC Adv. 2 (2012) 6262–6265, doi:10.1039/c2ra20800h.
[36] J.M. Gancedo, C. Gancedo, C. Asensio, Widespread occurrence of galactose oxidase and glucose oxidase in fungi, Arch. Biochem. Biophys. 119 (1967) 588–590, doi:10.1016/0003-9861(67)90498-5.
[37] E.P.L. Gasparotto, S.C.C. Abrao, S.Y. Inagaki, D.J. Tessmann, C. Kemmelmeier, I.P.B. Tessmann, Production and characterization of galactose oxidase produced by four isolates of *Fusarium graminearum*, Braz. Arch. Biol. Technol. 49 (2006) 557–564, doi:10.1590/S1516-89132006000500004.
[38] G.A. Hamilton, P.K. Adolf, J. Dejersey, G.C. Dubois, G.R. Dyrkacz, R.D. Libby, Trivalent copper, superoxide, and galactose oxidase, J. Am. Chem. Soc. 100 (1978) 1899–1912, doi:10.1021/ja00474a042.
[39] H. Heitzmann, F.M. Richards, Use of the avidin-biotin complex for specific staining of biological membranes in electron microscopy, Proc. Natl. Acad. Sci. USA 71 (1974) 3537–3541, doi:10.1073/pnas.71.9.3537.
[40] B. Henrissat, A classification of glycosyl hydrolases based on amino acid sequence similarities, Biochem. J. 280 (Pt 2) (1991) 309–316, doi:10.1042/bj2800309.
[41] S. Herter, S.M. McKenna, A.R. Frazer, S. Leimkühler, A.J. Carnell, N.J. Turner, Galactose oxidase variants for the oxidation of amino alcohols in enzyme cascade synthesis, ChemCatChem 7 (2015) 2313–2317, doi:10.1002/cctc.201500218.
[42] F. Himo, L.A. Eriksson, F. Maseras, P.E.M. Siegbahn, Catalytic mechanism of galactose oxidase: a theoretical study, J. Am. Chem. Soc. 122 (2000) 8031–8036, doi:10.1021/ja994527r.
[43] M.A. Huffman, A. Fryszkowska, O. Alvizo, M. Borra-Garske, K.R. Campos, K.A. Canada, P.N. Devine, D. Duan, J.H. Forstater, S.T. Grosser, H.M. Halsey, G.J. Hughes, J. Jo, L.A. Joyce, J.N. Kolev, J. Liang, K.M. Maloney, B.F. Mann, N.M. Marshall, M. McLaughlin, J.C. Moore, G.S. Murphy, C.C. Nawrat, J. Nazor, S. Novick, N.R. Patel, A. Rodriguez-Granillo, S.A. Robaire, E.C. Sherer, M.D. Truppo, A.M. Whittaker, D. Verma, L. Xiao, Y. Xu, H. Yang, Design of an in vitro biocatalytic cascade for the manufacture of islatravir, Science 366 (2019) 1255–1259, doi:10.1126/science.aay8484.
[44] N. Ito, S.E.V. Phillips, C. Stevens, Z.B. Ogel, M.J. McPherson, J.N. Keen, K.D.S. Yadav, P.F. Knowles, Novel thioether bond revealed by a 1.7 Å crystal structure of galactose oxidase, Nature 350 (1991) 87–90, doi:10.1038/350087a0.

[45] N. Ito, S.E.V. Phillips, C. Stevens, Z.B. Ogel, M.J. McPherson, J.N. Keen, K.D.S. Yadav, P.F. Knowles, 3-Dimensional structure of galactose-oxidase - an enzyme with a built-in secondary cofactor, Faraday Discuss. 93 (1992) 75–84, doi:10.1039/fd9929300075.
[46] N. Ito, S.E.V. Phillips, K.D.S. Yadav, P.F. Knowles, Crystal structure of a free radical enzyme, galactose oxidase, J. Mol. Biol. 238 (1994) 794–814, doi:10.1006/jmbi.1994.1335.
[47] J.M. Johnson, H.B. Halsall, W.R. Heineman, Redox activation of galactose oxidase: thin-layer electrochemical study, Biochemistry 24 (1985) 1579–1585, doi:10.1021/bi00328a001.
[48] M.A.S. Kadowaki, M.O. de Godoy, P.S. Kumagai, A.J. da Costa, A. Mort, R.A. Prade, I. Polikarpov, Characterization of a new glyoxal oxidase from the thermophilic fungus *Myceliophthora thermophila* M77: Hydrogen peroxide production retained in 5-hydroxymethylfurfural oxidation, Catalysts 8 (2018) 476, doi:10.3390/catal8100476.
[49] L. Kalum, M.D. Morant, H. Lund, J. Jensen, J. Lapainaite, N.H. Soerensen, S. Pedersen, L.H. Ostergaard, F. Xu, Enzymatic oxidation of 5-hydroxymethylfurfural and derivatives thereof, Patent WO2014015256A3 (2014).
[50] A. Karich, S.B. Kleeberg, R. Ullrich, M. Hofrichter, Enzymatic preparation of 2,5-furandicarboxylic acid (FDCA)-a substitute of terephthalic acid-by the joined action of three fungal enzymes, Microorganisms 6 (2018) 12, doi:10.3390/microorganisms6010005.
[51] P. Kersten, D. Cullen, Copper radical oxidases and related extracellular oxidoreductases of wood-decay Agaricomycetes, Fungal Genet. Biol. 72 (2014) 124–130, doi:10.1016/j.fgb.2014.05.011.
[52] P.J. Kersten, Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase, Proc. Natl. Acad. Sci. USA 87 (1990) 2936–2940, doi:10.1073/pnas.87.8.2936.
[53] P.J. Kersten, D. Cullen, Cloning and characterization of cDNA encoding glyoxal oxidase, a H_2O_2-producing enzyme from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*, Proc. Natl. Acad. Sci. USA 90 (1993) 7411–7413, doi:10.1073/pnas.90.15.7411.
[54] P.J. Kersten, T.K. Kirk, Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*, J. Bacteriol. 169 (1987) 2195–2201, doi:10.1128/jb.169.5.2195-2201.1987.
[55] A.M. Klibanov, B.N. Alberti, M.A. Marletta, Stereospecific oxidation of aliphatic alcohols catalyzed by galactose oxidase, Biochem. Biophys. Res. Commun. 108 (1982) 804–808, doi:10.1016/0006-291x(82)90900-7.
[56] I.C. Kuan, M. Tien, Stimulation of Mn peroxidase activity: a possible role for oxalate in lignin biodegradation, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 1242–1246, doi:10.1073/pnas.90.4.1242.
[57] B. Kurek, P.J. Kersten, Physiological regulation of glyoxal oxidase from *Phanerochaete chrysosporium* by peroxidase systems, Enzyme Microbiol. Technol. 17 (1995) 751–756, doi:10.1016/0141-0229(95)00003-N.
[58] L.D. Kwiatkowski, M. Adelman, R. Pennelly, D.J. Kosman, Kinetic mechanism of the Cu(II) enzyme galactose oxidase, J. Inorg. Biochem. 14 (1981) 209–222, doi:10.1016/S0162-0134(00)80001-X.
[59] L.D. Kwiatkowski, D.J. Kosman, On the role of superoxide radical in the mechanism of action of galactose oxidase, Biochem. Biophys. Res. Commun. 53 (1973) 715–721, doi:10.1016/0006-291x(73)90152-6.
[60] B. Leuthner, C. Aichinger, E. Oehmen, E. Koopmann, O. Muller, P. Muller, R. Kahmann, M. Bolker, P.H. Schreier, A H_2O_2-producing glyoxal oxidase is required for filamentous growth and pathogenicity in *Ustilago maydis*, Mol. Genet. Genom. 272 (2005) 639–650, doi:10.1007/s00438-004-1085-6.
[61] A. Levasseur, E. Drula, V. Lombard, P.M. Coutinho, B. Henrissat, Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes, Biotechnol. Biofuels 6 (2013) 41, doi:10.1186/1754-6834-6-41.
[62] A. Levasseur, A. Lomascolo, O. Chabrol, F.J. Ruiz-Duenas, E. Boukhris-Uzan, F. Piumi, U. Kues, A.F. Ram, C. Murat, M. Haon, I. Benoit, Y. Arfi, D. Chevret, E. Drula, M.J. Kwon, P. Gouret, L. Lesage-Meessen, V. Lombard, J. Mariette, C. Noirot, J. Park, A. Patyshakuliyeva, J.C. Sigoillot, A. Wiebenga, H.A. Wosten, F. Martin, P.M. Coutinho, R.P. de Vries, A.T. Martinez, C. Klopp, P. Pontarotti, B. Henrissat, E. Record, The genome of the white-rot fungus *Pycnoporus cinnabarinus*: a basidiomycete model with a versatile arsenal for lignocellulosic biomass breakdown, BMC Genom. 15 (2014) 486, doi:10.1186/1471-2164-15-486.
[63] N. Lustenhouwer, D.S. Maynard, M.A. Bradford, D.L. Lindner, B. Oberle, A.E. Zanne, T.W. Crowther, A trait-based understanding of wood decomposition by fungi, Proc. Natl. Acad. Sci. USA 117 (2020) 11551–11558, doi:10.1073/pnas.1909166117.
[64] P. Manini, P. La Pietra, L. Panzella, A. Napolitano, M. d'Ischia, Glyoxal formation by Fenton-induced degradation of carbohydrates and related compounds, Carbohydr. Res. 341 (2006) 1828–1833, doi:10.1016/j.carres.2006.03.027.
[65] A. Maradufu, G.M. Cree, A.S. Perlin, Stereochemistry of dehydrogenation by D-galactose oxidase, Can. J. Chem. 49 (1971) 3429–3437, doi:10.1139/v71-575.
[66] Y. Mathieu, W.A. Offen, S.M. Forget, L. Ciano, A.H. Viborg, E. Blagova, B. Henrissat, P.H. Walton, G.J. Davies, H. Brumer, Discovery of a fungal copper radical oxidase with high catalytic efficiency toward 5-hydroxymethylfurfural and benzyl alcohols for bioprocessing, ACS Catal. 10 (2020) 3042–3058, doi:10.1021/acscatal.9b04727.
[67] S.M. McKenna, S. Leimkühler, S. Herter, N.J. Turner, A.J. Carnell, Enzyme cascade reactions: synthesis of furandicarboxylic acid (FDCA) and carboxylic acids using oxidases in tandem, Green Chem. 17 (2015) 3271–3275, doi:10.1039/c5gc00707k.
[68] S.M. McKenna, P. Mines, P. Law, K. Kovacs-Schreiner, W.R. Birmingham, N.J. Turner, S. Leimkühler, A.J. Carnell, The continuous oxidation of HMF to FDCA and the immobilisation and stabilisation of periplasmic aldehyde oxidase (PaoABC), Green Chem. 19 (2017) 4660–4665, doi:10.1039/c7gc01696d.
[69] M.J. McPherson, Z.B. Ögel, C. Stevens, K.D.S. Yadav, J.N. Keen, P.F. Knowles, Galactose oxidase of *Dactylium dendroides* - gene cloning and sequence-analysis, J. Biol. Chem. 267 (1992) 8146–8152.
[70] M.J. McPherson, C. Stevens, A.J. Baron, Z.B. Ogel, K. Seneviratne, C. Wilmot, N. Ito, I. Brocklebank, S.E.V. Phillips, P.F. Knowles, Galactose oxidase: Molecular analysis and mutagenesis studies, Biochem. Soc. Trans. 21 (1993) 752–756, doi:10.1042/bst0210752.
[71] F. Mollerup, V. Amalga, K. Parikka, Y. Mathieu, H. Brumer, M. Tankage, E. Master, A family AA5_2 carbohydrate oxidase from *Penicillium rubens* displays functional overlap across the AA5 family, PLoS One 14 (2019) e0216546, doi:10.1371/journal.pone.0216546.
[72] M.K. Nobles, C. Madhosingh, *Dactylium dendroides* (Bull.) Fr. misnamed as *Polyporus circinatus* Fr, Biochem. Biophys. Res. Commun. 12 (1963) 146–147, doi:10.1016/0006-291x(63)90251-1.
[73] Z.B. Ögel, D. Brayford, M.J. McPherson, Cellulose-triggered sporulation in the galactose oxidase-producing fungus *Cladobotryum (Dactylium) dendroides* NRRL 2903 and its reidentification as a species of *Fusarium*, Mycol. Res. 98 (1994) 474–480.
[74] S. Oide, Y. Tanaka, A. Watanabe, M. Inui, Carbohydrate-binding property of a cell wall integrity and stress response component (WSC) domain of an alcohol oxidase from the rice blast pathogen *Pyricularia oryzae*, Enzyme Microbiol. Technol. 125 (2019) 13–20, doi:10.1016/j.enzmictec.2019.02.009.
[75] K. Parikka, A.S. Leppanen, L. Pitkanen, M. Reunanen, S. Willfor, M. Tankage, Oxidation of polysaccharides by galactose oxidase, J. Agric. Food Chem. 58 (2010) 262–271, doi:10.1021/jf902930t.
[76] K. Parikka, E. Master, M. Tankage, Oxidation with galactose oxidase: multifunctional enzymatic catalysis, J. Mol. Catal. B 120 (2015) 47–59, doi:10.1016/j.molcatb.2015.06.006.
[77] K. Parikka, M. Tankage, Oxidation of methyl α-D-galactopyranoside by galactose oxidase: products formed and optimization of reaction conditions for production of aldehyde, Carbohydr. Res. 344 (2009) 14–20, doi:10.1016/j.carres.2008.08.020.
[78] R. Paukner, P. Staudigl, W. Choosri, D. Haltrich, C. Leitner, Expression, purification, and characterization of galactose oxidase of *Fusarium sambucinum* in *E. coli*, Protein Expr. Purif. 108 (2015) 73–79, doi:10.1016/j.pep.2014.12.010.
[79] R. Paukner, P. Staudigl, W. Choosri, C. Sygmund, P. Halada, D. Haltrich, C. Leitner, Galactose oxidase from *Fusarium oxysporum* - expression in *E. coli* and *P. pastoris* and biochemical characterization, PLoS One 9 (2014) e100116, doi:10.1371/journal.pone.0100116.
[80] A.T. Pedersen, W.R. Birmingham, G. Rehn, S.J. Charnock, N.J. Turner, J.M. Woodley, Process requirements of galactose oxidase catalyzed oxidation of alcohols, Org. Process Res. Dev. 19 (2015) 1580–1589, doi:10.1021/acs.oprd.5b00278.
[81] Y.Z. Qin, Y.M. Li, M.H. Zong, H. Wu, N. Li, Enzyme-catalyzed selective oxidation of 5-hydroxymethylfurfural (HMF) and separation of HMF and 2,5-diformylfuran using deep eutectic solvents, Green Chem. 17 (2015) 3718–3722, doi:10.1039/c5gc00788g.
[82] J.B. Rannes, A. Ioannou, S.C. Willies, G. Grogan, C. Behrens, S.L. Flitsch, N.J. Turner, Glycoprotein labeling using engineered variants of galactose oxidase obtained by directed evolution, J. Am. Chem. Soc. 133 (2011) 8436–8439, doi:10.1021/ja2018477.
[83] M.P. Reynolds, A.J. Baron, C.M. Wilmot, S.E. Phillips, P.F. Knowles, M.J. McPherson, Tyrosine 495 is a key residue in the active site of galactose oxidase, Biochem. Soc. Trans. 23 (1995) 510S, doi:10.1042/bst023510s.
[84] M.P. Reynolds, A.J. Baron, C.M. Wilmot, E. Vinecombe, C. Stevens, S.E.V. Phillips, P.F. Knowles, M.J. McPherson, Structure and mechanism of galactose oxidase: Catalytic role of tyrosine 495, J. Biol. Inorg. Chem. 2 (1997) 327–335, doi:10.1007/s007750050139.
[85] D. Ribeaucourt, B. Bissaro, V. Guallar, M. Yemloul, M. Haon, S. Grisel, V. Alphand, H. Brumer, F. Lambert, J.G. Berrin, M. Lafond, Comprehensive insights into the production of long chain aliphatic aldehydes using a copper-radical alcohol oxidase as biocatalyst, ACS Sustain. Chem. Eng. 9 (2021) 4411–4421, doi:10.1021/acssuschemeng.0c07406.
[86] D. Ribeaucourt, G.T. Hofler, M. Yemloul, B. Bissaro, F. Lambert, J.G. Berrin, M. Lafond, C.E. Paul, Tunable production of (*R*)- or (*S*)-citronellal from geraniol via a bienzymatic cascade using a copper radical alcohol oxidase and old yellow enzyme, ACS Catal. 12 (2022) 1111–1116, doi:10.1021/acscatal.1c05334.
[87] D. Ribeaucourt, S. Saker, D. Navarro, B. Bissaro, E. Drula, L.O. Correia, M. Haon, S. Grisel, N. Lapalu, B. Henrissat, R.J. O'Connell, F. Lambert, M. Lafond, J.G. Berrin, Identification of copper-containing oxidoreductases in the secretomes of three *Colletotrichum* species with a focus on copper radical oxidases for the biocatalytic production of fatty aldehydes, Appl. Environ. Microbiol. 87 (2021) e0152621, doi:10.1128/AEM.01526-21.
[88] G.P. Roberts, S.K. Gupta, Use of galactose oxidase in histochemical examination of mucus-secreting cells, Nature 207 (1965) 425–426, doi:10.1038/207425a0.
[89] M.S. Rogers, A.J. Baron, M.J. McPherson, P.F. Knowles, D.M. Dooley, Galactose oxidase pro-sequence cleavage and cofactor assembly are self-processing reactions, J. Am. Chem. Soc. 122 (2000) 990–991, doi:10.1021/ja993385y.
[90] M.S. Rogers, R. Hurtado-Guerrero, S.J. Firbank, M.A. Halcrow, D.M. Dooley, S.E. Phillips, P.F. Knowles, M.J. McPherson, Cross-link formation of the cysteine 228-tyrosine 272 catalytic cofactor of galactose oxidase does not require dioxygen, Biochemistry 47 (2008) 10428–10439, doi:10.1021/bi8010835.
[91] M.S. Rogers, P.F. Knowles, A.J. Baron, M.J. McPherson, D.M. Dooley, Characterization of the active site of galactose oxidase and its active site mutational variants Y495F/H/K and W290H by circular dichroism spectroscopy, Inorg. Chim. Acta 276 (1998) 175–181, doi:10.1016/S0020-1693(97)06142-2.

[92] M.S. Rogers, E.M. Tyler, N. Akyumani, C.R. Kurtis, R.K. Spooner, S.E. Deacon, S. Tamber, S.J. Firbank, K. Mahmoud, P.F. Knowles, S.E.V. Phillips, M.J. McPherson, D.M. Dooley, The stacking tryptophan of galactose oxidase: A second-coordination sphere residue that has profound effects on tyrosyl radical behavior and enzyme catalysis, Biochemistry 46 (2007) 4606–4618, doi:10.1021/bi062139d.
[93] T. Roncal, C. Munoz, L. Lorenzo, B. Maestro, M.D.D. de Guerenu, Two-step oxidation of glycerol to glyceric acid catalyzed by the *Phanerochaete chrysosporium* glyoxal oxidase, Enzyme Microbiol. Technol. 50 (2012) 143–150, doi:10.1016/j.enzmictec.2011.11.007.
[94] A. Serrano, J. Carro, A.T. Martinez, Reaction mechanisms and applications of aryl-alcohol oxidase, Enzymes 47 (2020) 167–192, doi:10.1016/bs.enz.2020.05.005.
[95] N.P. Shetty, H.J.L. Jorgensen, J.D. Jensen, D.B. Collinge, H.S. Shetty, Roles of reactive oxygen species in interactions between plants and pathogens, Eur. J. Plant Pathol. 121 (2008) 267–280, doi:10.1007/s10658-008-9302-5.
[96] A. Siebum, A. van Wijk, R. Schoevaart, T. Kieboom, Galactose oxidase and alcohol oxidase: scope and limitations for the enzymatic synthesis of aldehydes, J. Mol. Catal. B-Enz. 41 (2006) 141–145, doi:10.1016/j.molcatb.2006.04.003.
[97] B. Silakowski, H. Ehret, H.U. Schairer, *fbfB*, a gene encoding a putative galactose oxidase, is involved in *Stigmatella aurantiaca* fruiting body formation, J. Bacteriol. 180 (1998) 1241–1247, doi:10.1128/Jb.180.5.1241-1247.1998.
[98] O. Spadiut, L. Olsson, H. Brumer, A comparative summary of expression systems for the recombinant production of galactose oxidase, Microb. Cell Fact. 9 (2010) 68, doi:10.1186/1475-2859-9-68.
[99] L.H. Sun, T. Bulter, M. Alcalde, I.P. Petrounia, F.H. Arnold, Modification of galactose oxidase to introduce glucose 6-oxidase activity, ChemBioChem 3 (2002) 781–783, doi:10.1002/1439-7633(20020802)3:8<781::Aid-Cbic781>3.0.Co;2-8.
[100] L.H. Sun, I.P. Petrounia, M. Yagasaki, G. Bandara, F.H. Arnold, Expression and stabilization of galactose oxidase in *Escherichia coli* by directed evolution, Protein Eng. 14 (2001) 699–704, doi:10.1093/protein/14.9.699.
[101] J. Tkac, P. Gemeiner, E. Sturdik, Rapid and sensitive galactose oxidase-peroxidase biosensor for galactose detection with prolonged stability, Biotechnol. Tech. 13 (1999) 931–936, doi:10.1023/A:1008966413722.
[102] J. Tkac, E. Sturdik, P. Gemeiner, Novel glucose non-interference biosensor for lactose detection based on galactose oxidase-peroxidase with and without co-immobilised beta-galactosidase, Analyst 125 (2000) 1285–1289, doi:10.1039/b001432j.
[103] P. Tressel, D.J. Kosman, *O,O*-dityrosine in native and horseradish peroxidase-activated galactose oxidase, Biochem. Biophys. Res. Commun. 92 (1980) 781–786, doi:10.1016/0006-291x(80)90771-8.
[104] V.B. Urlacher, K. Koschorreck, Pecularities and applications of aryl-alcohol oxidases from fungi, Appl. Microbiol. Biotechnol. 105 (2021) 4111–4126, doi:10.1007/s00253-021-11337-4.
[105] W. Van den Ende, Multifunctional fructans and raffinose family oligosaccharides, Front. Plant Sci. 4 (2013) 247, doi:10.3389/fpls.2013.00247.
[106] R.M. Wachter, B.P. Branchaud, Molecular modeling studies on oxidation of hexopyranoses by galactose oxidase. An active site topology apparently designed to catalyze radical reactions, either concerted or stepwise, J. Am. Chem. Soc. 118 (1996) 2782–2789, doi:10.1021/ja9519896.
[107] R.M. Wachter, B.P. Branchaud, Thiols as mechanistic probes for catalysis by the free radical enzyme galactose oxidase, Biochemistry 35 (1996) 14425–14435, doi:10.1021/bi961369x.
[108] T. Watanabe, N. Shirai, H. Okada, Y. Honda, M. Kuwahara, Production and chemiluminescent free radical reactions of glyoxal in lipid peroxidation of linoleic acid by the ligninolytic enzyme, manganese peroxidase, Eur. J. Biochem. 268 (2001) 6114–6122, doi:10.1046/j.0014-2956.2001.02557.x.
[109] J.W. Whittaker, Oxygen reactions of the copper oxidases, Essays Biochem 34 (1999) 155–172, doi:10.1042/bse0340155.
[110] J.W. Whittaker, Galactose oxidase, Adv. Protein Chem. 60 (2002) 1–49, doi:10.1016/s0065-3233(02)60050-6.
[111] J.W. Whittaker, Free radical catalysis by galactose oxidase, Chem. Rev. 103 (2003) 2347–2363, doi:10.1021/cr020425z.
[112] J.W. Whittaker, The radical chemistry of galactose oxidase, Arch. Biochem. Biophys. 433 (2005) 227–239, doi:10.1016/j.abb.2004.08.034.
[113] M.M. Whittaker, D.P. Ballou, J.W. Whittaker, Kinetic isotope effects as probes of the mechanism of galactose oxidase, Biochemistry 37 (1998) 8426–8436, doi:10.1021/bi980328t.
[114] M.M. Whittaker, P.J. Kersten, D. Cullen, J.W. Whittaker, Identification of catalytic residues in glyoxal oxidase by targeted mutagenesis, J. Biol. Chem. 274 (1999) 36226–36232, doi:10.1074/jbc.274.51.36226.
[115] M.M. Whittaker, P.J. Kersten, N. Nakamura, J. SandersLoehr, E.S. Schweizer, J.W. Whittaker, Glyoxal oxidase from *Phanerochaete chrysosporium* is a new radical-copper oxidase, J. Biol. Chem. 271 (1996) 681–687, doi:10.1074/jbc.271.2.681.
[116] M.M. Whittaker, J.W. Whittaker, The active site of galactose oxidase, J. Biol. Chem. 263 (1988) 6074–6080, doi:10.1016/S0021-9258(18)68751-4.
[117] M.M. Whittaker, J.W. Whittaker, Ligand interactions with galactose oxidase: mechanistic insights, Biophys. J. 64 (1993) 762–772, doi:10.1016/S0006-3495(93)81437-1.
[118] M.M. Whittaker, J.W. Whittaker, Expression of recombinant galactose oxidase by *Pichia pastoris*, Protein Expr. Purif. 20 (2000) 105–111, doi:10.1006/prep.2000.1287.
[119] M.M. Whittaker, J.W. Whittaker, Catalytic reaction profile for alcohol oxidation by galactose oxidase, Biochemistry 40 (2001) 7140–7148, doi:10.1021/bi010303l.
[120] M.M. Whittaker, J.W. Whittaker, Cu(I)-dependent biogenesis of the galactose oxidase redox cofactor, J. Biol. Chem. 278 (2003) 22090–22101, doi:10.1074/jbc.M300112200.
[121] M.M. Whittaker, J.W. Whittaker, *Streptomyces coelicolor* oxidase (SC02837p): A new free radical metalloenzyme secreted by *Streptomyces coelicolor* A3(2), Arch. Biochem. Biophys. 452 (2006) 108–118, doi:10.1016/j.abb.2006.06.020.
[122] D. Wilkinson, N. Akumanyi, R. Hurtado-Guerrero, H. Dawkes, P.F. Knowles, S.E.V. Phillips, M.J. McPherson, Structural and kinetic studies of a series of mutants of galactose oxidase identified by directed evolution, Protein Eng. Des. Sel. 17 (2004) 141–148, doi:10.1093/protein/gzh018.
[123] L. Wohlschlager, F. Csarman, M. Zrilic, B. Seiboth, R. Ludwig, Comparative characterization of glyoxal oxidase from *Phanerochaete chrysosporium* expressed at high levels in *Pichia pastoris* and *Trichoderma reesei*, Enzyme Microbiol. Technol. 145 (2021) 109748, doi:10.1016/j.enzmictec.2021.109748.
[124] L. Wohlschlager, D. Kracher, S. Scheiblbrandner, F. Csarman, R. Ludwig, Spectroelectrochemical investigation of the glyoxal oxidase activation mechanism, Bioelectrochemistry 141 (2021) 107845, doi:10.1016/j.bioelechem.2021.107845.
[125] F. Xu, E.J. Golightly, P. Schneider, R.M. Berka, K.M. Brown, J.A. Johnstone, D.H. Baker, C.C. Fuglsang, S.H. Brown, A. Svendsen, A.V. Klotz, Expression and characterization of a recombinant *Fusarium* spp. galactose oxidase, Appl. Biochem. Biotechnol. 88 (2000) 23–32, doi:10.1385/Abab:88:1-3:023.
[126] M. Yalpani, L.D. Hall, Some chemical and analytical aspects of polysaccharide modifications. II. A High-yielding, specific method for the chemical derivatization of galactose-containing polysaccharides: oxidation with galactose oxidase followed by reductive amination, J. Polym. Sci. Pol. Chem. 20 (1982) 3399–3420, doi:10.1002/pol.1982.170201213.
[127] D.L. Yin, S. Urresti, M. Lafond, E.M. Johnston, F. Derikvand, L. Ciano, J.G. Berrin, B. Henrissat, P.H. Walton, G.J. Davies, H. Brumer, Structure-function characterization reveals new catalytic diversity in the galactose oxidase and glyoxal oxidase family, Nat. Commun. 6 (2015) 10197, doi:10.1038/ncomms10197.
[128] S.G. Zhang, S. Ruccolo, A. Fryszkowska, A. Klapars, N. Marshall, N.A. Strotman, Electrochemical activation of galactose oxidase: mechanistic studies and synthetic applications, ACS Catal. 11 (2021) 7270–7280, doi:10.1021/acscatal.1c01037.
[129] B.J. Zhou, X.P. Wang, Y.J. Wang, cDNA cloning, expression, protein purification, and characterization of a novel glyoxal oxidase related gene from *Vitis pseudoreticulata*, Biol. Plant. 51 (2007) 458–466, doi:10.1007/s10535-007-0096-7.
[130] M.P. Kalapos, Methylglyoxal in living organisms. Chemistry, biochemistry, toxicology and biological implications, Toxicol Lett 110 (1999) 145–175, doi:10.1016/s0378-4274(99)00160-5.