Heterologous Production and Characterization of Two Glyoxal Oxidases from Pycnoporus cinnabarinus
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ABSTRACT
The genome of the white rot fungus Pycnoporus cinnabarinus includes a large number of genes encoding enzymes implicated in lignin degradation. Among these, three genes are predicted to encode glyoxal oxidase, an enzyme previously isolated from Phanerochaete chrysosporium. The glyoxal oxidase of P. chrysosporium is physiologically coupled to lignin-oxidizing peroxidases via generation of extracellular H₂O₂, and utilizes an array of aldehydes and α-hydroxycarbonyls as the substrates. Two of the predicted glyoxal oxidases of P. cinnabarinus, GLOX1 (PciGLOX1) and GLOX2 (PciGLOX2), were heterologously produced in Aspergillus niger strain D15#26 (pyrG negative) and purified using immobilized metal ion affinity chromatography, yielding 39 and 5 mg of protein for PciGLOX1 and PciGLOX2, respectively. Both proteins were approximately 60 kDa in size and N-glycosylated. The optimum temperature for the activity of these enzymes was 50°C, and the optimum pH was 6. The enzymes retained most of their activity after incubation at 50°C for 4 h. The highest relative activity and the highest catalytic efficiency of both enzymes occurred with glyoxylic acid as the substrate. The two P. cinnabarinus enzymes generally exhibited similar substrate preferences, but PciGLOX2 showed a broader substrate specificity and was significantly more active on 3-phenylpropionaldehyde.

IMPORTANCE
This study addresses the poorly understood role of how fungal peroxidases obtain an in situ supply of hydrogen peroxide to enable them to oxidize a variety of organic and inorganic compounds. This cooperative activity is intrinsic in the living organism to control the amount of toxic H₂O₂ in its environment, thus providing a feed-on-demand scenario, and can be used biotechnologically to supply a cheap source of peroxide for the peroxidase reaction. The secretion of multiple glyoxal oxidases by filamentous fungi as part of a lignocellulolytic mechanism suggests a controlled system, especially as these enzymes utilize fungal metabolites as the substrates. Two glyoxal oxidases have been isolated and characterized to date, and the differentiation of the substrate specificity of the two enzymes produced by Pycnoporus cinnabarinus illustrates the alternative mechanisms existing in a single fungus, together with the utilization of these enzymes to prepare platform chemicals for industry.

Lignocellulose degradation is a key step in carbon recycling. Due to the importance of lignocellulosic material as a renewable source of platform chemicals and a potential substitute for fossil-derived ones, there is increasing interest in improving the efficiency of the use of lignocellulose through industrial biotechnology approaches. Various naturally occurring microorganisms are well adapted to degrade the cellulose, hemicellulose, and lignin components of the lignocellulosic biomass and use them as carbon and energy sources (1). White rot fungi are particularly adapted to degrade lignin, the most recalcitrant polymer in biomass, through the production of a group of extracellular lignin-degrading enzymes, such as class II peroxidases, laccases, and peroxide-generating enzymes, such as aryl alcohol oxidases and glyoxal oxidases (2).

Phanerochaete chrysosporium was for years the most studied white rot lignin-degrading fungus, and these studies contributed to most of the current understanding of the enzymology behind lignin degradation. However, the recent expansion in genome analyses has shown that the repertoire of genes varies substantially among white rot fungi. For example, P. chrysosporium lacks genes encoding laccases but features 15 class II peroxidases and a single glyoxal oxidase gene (3), whereas a recent analysis has identified 5 laccase, 9 class II peroxidase, and 3 glyoxal oxidase genes in Pycnoporus cinnabarinus (4). Extracellular H₂O₂-generating enzymes, such as glyoxal oxidase (GLOX), are thought to be involved in lignin degradation by providing hydrogen peroxide, which is necessary for peroxidase activity (5). Other fungal enzymes demonstrated to generate H₂O₂ include sugar oxidases, methanol oxidase, and aryl-alcohol oxidase (6). These auxiliary activity (AA) enzymes (7) have been classified within the Carbohydrate-Active Enzymes database (CAZY; http://www.cazy.org), where they are currently divided into 13 families on the basis of their catalytic modules, reaction mechanisms, and substrate specificities.

In this classification, copper radical oxidases (CROs) form the family AA5, all of which are characterized by the presence of a free radical-coupled copper ion acting as a two-electron redox catalytic site (8). Subfamily AA5_1 includes GLOX and structurally

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related copper CROs, whereas subfamily AA5_2 contains galactose oxidases (7). Although GLOX and galactose oxidase share a conserved active site, the enzymes differ in their number of domains, redox potential, free radical stability, and substrate specificity (5). Beyond GLOX, other members of the AA5_1 subfamily (CRO) differ from GLOX by their structure, regulation, and, at least in one case, substrate specificity (9).

GLOX is responsible for the oxidation of aldehydes, leading to the formation of carboxylic acids and the simultaneous reduction of dioxygen to hydrogen peroxide (8):

\[
\text{RCHO} + O_2 + H_2O \rightarrow \text{RCOOH} + H_2O_2
\]

They catalyze the oxidation of aldehydes and alpha-hydroxy-carbonyls derived from carbohydrates (10) and lignin degradation (5). Some of these substrates have been found in cultures of lignin-degrading fungi; therefore, they are likely to be the in vivo substrates for GLOX (9). GLOX also can act as a detoxifying agent for carbonyls derived from carbohydrates (10) and lignin degradation (5). Some of these substrates have been found in cultures of lignin-oxidized to date (12,13), and more studies are needed to understand the role of these enzymes.

Seven genes encoding enzymes belonging to the AA5_1 family, including 3 GLOX enzymes and 4 CROs, were previously identified in the genome of P. cinnabarinus (4). However, only two of the GLOX enzymes (P. cinnabarinus GLOX1 [PciGLOX1] and P. cinnabarinus GLOX2 [PciGLOX2]) were apparently secreted into the culture medium under the defined growth conditions, as identified through proteomic analysis of the secretome (4). This study describes the production and characterization of these enzymes.

**MATERIALS AND METHODS**

**Strains and culture conditions.** P. cinnabarinus strain BRFM137 was obtained from the Centre International de Ressources Microbiennes-Champignons filamenteurs (CIRM-CF; https://www6.inra.fr/cirm_eng/Filamentous-Fungi). Aspergillus niger strain D15#26 (pyrG negative) (14) was used for the heterologous expression of the synthesized coding genes of the two PciGLOX enzymes. The pyrG gene encodes orotidine-5′-phosphate decarboxylase, which is an enzyme essential for the biosynthesis of pyrimidine nucleotides, and thus, mutants lacking this enzyme require uracil or uridine for growth (15). Escherichia coli strain JM109 (Promega, Charbonnieres, France) was used for vector storage and propagation and was cultured on YT medium (85 mM NaCl, 1% yeast extract, 1.6% Bacto tryptone) containing ampicillin (final concentration, 100 µg/ml).

A. niger transformants were isolated on the basis of selective growth on uridine-free solid minimal medium, which contained MgSO\(_4\)\_7\(H_2O\) (2 mM), sorbitol (0.8 M), glucose (35.5 mM), Na\(_2\)NO\(_3\) (70 mM), KCl (7 mM), KH\(_2\)PO\(_4\) (11 mM), and 1× trace elements solution (the 1,000× stock solution contained ZnSO\(_4\)\_7\(H_2O\) [76 mM], H\(_3\)BO\(_3\) [178 mM], MnCl\(_2\)\_4\(H_2O\) [25 mM], Fe\(_3\)O\(_2\)\_7\(H_2O\) [18 mM], CoCl\(_2\)\_6\(H_2O\) [7.1 mM], CuSO\(_4\)\_5\(H_2O\) [6.4 mM], Na\(_2\)MoO\(_4\)\_2\(H_2O\) [6.2 mM], and EDTA\_Na\(_2\)\_7\(H_2O\) [174 mM]). Positive transformants were cultured in liquid production medium containing Na\(_2\)SO\(_4\) (70 mM), KCl (7 mM), glucose (277.5 mM), MgSO\(_4\)\_7\(H_2O\) (2 mM), 1× trace elements solution, Na\(_2\)HPO\(_4\) (200 mM), and vitamin solution. The pH of the production medium was adjusted to 5.5 with 1 M citric acid prior to inoculation and maintained at this pH during growth.

**Cloning and transformation.** The genes coding for PciGLOX1 and PciGLOX2 (scf184747.g48 and scf184747.g42, respectively) were previously identified in the sequenced genome of P. cinnabarinus (4). The open reading frames (ORFs) of the two genes were predicted from their genomic sequences by translating the six frames and identifying splicing sites according to the general GTRMTG...YAG pattern. This prediction was further supported using the Augustus gene prediction website (http://bioinf.uni-greifswald.de/augustus/) and by aligning the sequence of the ORF with the sequences of similar genes from other organisms using blastx (NCBI). The signal peptides of PciGLOX1 (MKWTSLLSLPPLAP LALG) and PciGLOX2 (MFQTLHLLFLVTVGRLA) were replaced by the 24-amino-acid glucoamylase (GLA) prepro sequence from A. niger (MGFRSLALSLVGCLANVSKR). A sequence encoding 6×His tag was also added downstream of the coding region of the genes to facilitate protein purification. The cDNA sequences of the two PciGLOX enzymes containing the above-mentioned modifications were codon optimized for A. niger expression and synthesized (GeneArt; Thermo Fisher Scientific, Saint Aubin, France). The synthetic genes were then cloned in the HindIII/MluI site of the pAN52.4 vector. The vectors were propagated in E. coli strain JM109 (Promega). A. niger was cotransformed following the protocol described by Punt and van den Hondel (16) with pAB4.1 containing the pyrG gene and pAN52.4 vectors containing the recombinant genes. Positive transformants were isolated on minimal medium on the basis of their ability to grow in the absence of uridine.

**Protein production.** Thirty different clones of each of PciGLOX1 and PciGLOX2 along with two negative controls were cultured to check protein production by enzymatic activity tests. Nine clones of PciGLOX1 and 13 clones of PciGLOX2 tested positive for GLOX activity. No activity was detected in the negative controls. For each of the two proteins, two positive clones showing maximum activity were selected, and the corresponding proteins were further purified using His SpinTrap columns (GE Healthcare Life Sciences, Buc, France). The protein produced was analyzed by SDS-PAGE and Western blotting in order to verify the presence of the 6×His tag. From the positive clones, one clone each of PciGLOX1 and PciGLOX2 was used for large-scale production. About 4×10^6 spores from the positive clones, stored in physiological water, were used to inoculate 100 ml of production medium in 250-ml baffled flasks. Eight flasks were prepared for each protein. The cultures were then incubated at 30°C with shaking at 130 rpm for 11 days. The pH of the cultures was monitored every day and adjusted when needed to pH 5.5 using sterilized citric acid (1 M).

**Enzyme purification.** The culture media were harvested on day 11 of growth, pooled, and filtered through Miracloth (Merck Millipore, Darmstadt, Germany). The filtrate (600 ml) was collected and filtered through glass fiber filter (GF/D, GF/A, and GF/F; Whatman; GE Healthcare Life Sciences) and a 0.22-µm pore-size polyethersulfone membrane (Express Plus; Merck Millipore). Filtrates were concentrated to 100 ml using a Vivacell 250 concentration unit with a 10-kDa molecular-mass-cutoff polyethersulfone membrane (Sartorius, Aubagne, France). The pH of the concentrated filtrate was adjusted to 7.8 with NaOH (1 M) and sterile filtered (pore size; 0.22 µm; Express Plus; Membrane Millipore) again. His-tagged proteins were purified by immobilized metal affinity chromatography (IMAC) using an Äkta purifier (GE Healthcare Life Sciences). The samples were loaded onto a 5-ml HisTrap column prepacked with Ni Sepharose and equilibrated with binding buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM imidazole). The His-tagged proteins were gradually eluted with 30% and 100% elution buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 500 mM imidazole). Recovered recombinant proteins were then dialyzed against 50 mM sodium phosphate, pH 7. The total protein concentration was determined by the Bradford assay using a Bio-Rad protein assay kit (Bio-Rad, Marnes-la-Coquette, France) and bovine serum albumin as a standard, and the concentration of the purified proteins was determined spectrophotometrically at 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

**Protein characterization.** Purified proteins were fractionated on a 12% SDS-polyacrylamide gel, which was then stained with Coomassie blue. The molecular mass of the proteins was estimated according to the standard markers PageRuler prestained protein ladder (10 to 180 kDa; Thermo Fisher Scientific). For the Western blot analysis, proteins were blotted onto a nitrocellulose membrane (Invitrogen, Cergy-Pontoise, France). The membrane was
incubation. In 2,2-dimethylsuccinate buffer at pH 4, 4.5, 5, 5.5, and 6. For both experi-
s amended. Similarly, the pH stability was analyzed by incubating the enzyme at 70, 80°C. The enzyme was cooled in ice before the activity was mea-
sed. Substrate specificity was determined by the same assay with 1 mM CuSO4 for 30 min at 800 rpm and 25°C (17).

**Phylogenetic study and sequence alignments.** Copper radical oxidase protein models were downloaded from the Joint Genome Institute (JGI) web portals for the Polyporales *Trametes versicolor, Dichotomius squelans, Ganoderma sp., Phanerochaete carnosa, Bjerkandera adusta,* and *Phlebia brevispora.* CRO sequences for the Polyporales *Phanerochaete chrysosporium* were derived from GenBank (9), and the *Pycnoporus cin-
nabarius* sequences are described in this work. Also retrieved from the JGI were models for a representative Agaricales, *Pleurotus ostreatus,* Agra-
ricus bisporus, a member of the Auriculariales; *Auricularia delicata*; the Corticales species *Punctularia strigosozonata*; and a Russulales, *Stereum hir-
sutum.* All sequences were manually trimmed of secretion signals and, where present, N-terminal repeats of the WSC domain (a cell wall stress-
response component). Alignments and phylogenetic trees were con-
structed using the ClustalW program within the MegaAlign module (ver-
sion 11.0.0) of DNAStar software (Madison, WI). Bootstrap values were gen-
erated with 1,000 trials, and the Kimura distance formula was used to calculate amino acid substitutions.

**Accession number(s).** The sequences of *PciGLOX1* and *PciGLOX2* are available in GenBank under accession numbers KU215437 and KU215438, respectively.

**RESULTS**

**Phylogenetic analysis and protein production.** *PciGLOX* se-
quences (*PciGLOX1, PciGLOX2,* and *PciGLOX3,* referred to as *Pycc_2052* in Fig. 1) were confidently embedded within the GLOX clade among other Polyporales (Fig. 1). The genomic se-
quences of both *PciGLOX1* and *PciGLOX2* contained 3 introns with strong position homology and signal peptide sequences of 18 and 20 amino acids, respectively. The cDNA sequences of *PciGLOX1* and *PciGLOX2* consisted of open reading frames of 1,671 and 1,680 bp encoding 556- and 559-amino-acid proteins, respectively. The identity between the two *PciGLOX* proteins was 85%, and the amino acids at the active site were found to be highly conserved (Fig. 2).

**Protein production and purification.** The codon-optimized gene sequences containing an upstream GLA prepro sequence from *A. niger* and a downstream N-terminal 6×His tag were cloned in the pAN52.4 vector. The plasmids were used for the transformation of *A. niger* protoplasts, and positive clones were selected and used for the large-scale production of *PciGLOX1* and *PciGLOX2.* Protein production was followed at days 7, 9, and 11 by measuring activity against methyglyoxal in the culture supernatant. An increase in the total activity measured from 9,093 nkat to 43,293 nkat was observed during *PciGLOX1* production; however, *PciGLOX2* activity was not detectable in the culture medium before ultrafiltration. The recombinant *PciGLOX1* and *PciGLOX2* proteins were purified 20.9- and 607.8-fold using IMAC, yielding 59 mg and 5 mg, respectively, from 600 ml of culture (Table 1). The purified protein was green and turned into light purple after dialysis against sodium phosphate (pH 7) buffer. The purified proteins were shown to be homogeneous using SDS-
PAGE (see Fig. S1a in the supplemental material). The molecular mass was estimated to be 70 kDa for both enzymes (see Fig. S1a in the supplemental material). The N-terminal amino acid sequences obtained were APSA and APST for *PciGLOX1* and
PciGLOX2, respectively, which correspond to the predicted sequences. These results show that the recombinant proteins were properly processed from the N terminus.

The obtained molecular mass of 70 kDa was larger than the predicted molecular mass for both proteins (58 kDa calculated using ProtParam-ExPaSy), suggesting the presence of glycosylation. The NetNGlyc (version 1.0) server (accessible via http://www.cbs.dtu.dk/services/NetNGlyc) predicted 5 and 6 N-glycosylation sites for PciGLOX1 and PciGLOX2, respectively, supporting the results observed with SDS-PAGE. The presence of N-glycosylation sites was confirmed by treating both enzymes with PNGase F, and the resulting deglycosylated proteins were about 60 kDa in mass, corresponding to the theoretical mass of the PciGLOX proteins (see Fig. S1b in the supplemental material). On the basis of the findings on SDS-polyacrylamide gels, the glycosylation percentage was about 14% for both enzymes. The two PciGLOX proteins had a pI of 6 (data not shown). This result was in agreement with the pI predicted from the cDNA sequences (5.8 for PciGLOX1 and 5.5 PciGLOX2).

**Effect of pH and temperature.** The two PciGLOX enzymes showed narrow optimum activity at pH 6 (Fig. 3a), with only 20% relative activity at pH 5. The pH stability was determined by incubating the enzymes in buffer at a pH of between 4 and 6 for 15, 30, 60, 120, and 240 min. The two PciGLOX enzymes were found to lose activity gradually with time, with only 40% and 60% activity remaining after 4 h of incubation at 30°C (Fig. 3b and c). The extent of activity loss over time was similar for all tested pH conditions.

The optimum temperature was found to be 50°C for both enzymes, which had similar temperature profiles (Fig. 4a). Both enzymes retained 60% activity at 75°C. Thermal stability (at 30 to 80°C) was also determined by incubating the enzymes for 15, 30, 60, 120, and 240 min at these temperatures. The two enzymes behaved in a similar way at the tested temperatures (Fig. 4b and c). Only about 20% of the activity was lost after 4 h of incubation at 50°C. The proteins retained 50% of their activity after 2 h at 60°C and completely lost activity after a 15-min incubation at 70°C and 80°C.

**Substrate specificity and kinetics.** The two PciGLOX enzymes showed broad substrate specificity (Table 2). Specifically, PciGLOX2 was active on a wider number of substrates than PciGLOX1. PciGLOX2 was more active than PciGLOX1 on di-glyceraldehyde, dihydroxycetone, glycerol, and formaldehyde and was the only one active on 3-phenylpropionaldehyde. The highest activity was found on glyoxylic acid (192% for PciGLOX1 and 132% for PciGLOX2 relative to their activities on methylglyoxal). Neither of the enzymes was able to oxidize sugars or methanol. The kinetic parameters of both enzymes on selected substrates are presented in Table 3. The highest catalytic efficiency was found for glyoxylic acid (2,136.363 s⁻¹ mM⁻¹ for PciGLOX1 and 17 s⁻¹ mM⁻¹ for PciGLOX2), followed by methylglyoxal, glyoxal, and glycerol. The catalytic efficiency of PciGLOX1 for methylglyoxal, glyoxal, and glyoxylic acid was significantly higher than that of PciGLOX2.

The conversion of methylglyoxal, glyoxal, and glyoxylic acid by both PciGLOX enzymes to their corresponding carboxylic acids was analyzed by HPLC. When glyoxylic acid was used as the substrate, a decrease in the peak representing glyoxylic acid was observed, and a new peak corresponding to oxalic acid appeared at 8.97 min (see Fig. S2a in the supplemental material). In the case of glyoxal as the substrate, a decrease in the peak representing glyoxal was observed, and a new peak corresponding to oxalic acid appeared only in the PciGLOX2 reaction (see Fig. S2b in the supplemental material). In the reactions with methylglyoxal, a peak corresponding to pyruvic acid was present (see Fig. S2c in the supplemental material).
DISCUSSION

Two glyoxal oxidases from *P. cinnabarinus* were heterologously produced in *A. niger* and biochemically characterized. The genes encoding these proteins, along with the glox3 gene, were found on the same scaffold and positioned in the same transcriptional direction, thus forming a cluster (4). In *P. chrysosporium*, three CRO-encoding genes related to but structurally distinct from the GLOX gene were grouped on the same scaffold along with eight class II peroxidases (3). However, in *P. cinnabarinus*, the glox and peroxidase clusters were located on different scaffolds (4).

By comparing the sequences of the two *Pci*GLOX proteins described in this paper and the previously characterized GLOX from *P. chrysosporium* (GenBank accession no. AAA87595, referred to as PhGLOX in this paper) (20), a 74% similarity was found (Fig. 1). The alignment further showed that the amino acid residues responsible for ligating the copper ion active site and characteristic of CROs (Cys73, Tyr138, His380, Tyr379, and His473) (21) were highly conserved in the *Pci*GLOX and *Ph*GLOX proteins. These residues are also conserved in galactose oxidases (22), suggesting that GLOX and galactose oxidases should follow a similar mechanism of action for the oxidation of their substrates. This might be explained by the distinctive function of the radical copper complex of these enzymes acting as a two-electron redox-active site, in contrast to other free radical enzymes that show single-electron reactivity, such as ribonucleotide reductase (8).

The production of GLOX proteins in *Aspergillus* species was previously performed by Kersten and Cullen (23), and the yield of proteins was between 10 and 20 mg per liter of culture, similar to the yield obtained for *PciGLOX2* in this study. However, a five times larger amount of *PciGLOX1* was obtained. Before buffer exchange the purified proteins were green. This color was previously assigned to the oxidized active form of the protein (24). The purple form of the enzyme obtained after dialysis against 50 mM sodium phosphate (pH 7) was also reported previously when the enzyme was dialyzed into potassium phosphate at the same pH and down to pH 4.53 (24). The two purified glycosylated proteins were similar in size to *Ph*GLOX (68 kDa), which was also found to be N-glycosylated, with glycosylation accounting for about 16% of the total protein mass (12, 23). Contrary to the optimum temperature for *Ph*GLOX, which is 30°C (25), the optimum temperature for both *Pci*GLOX described here was 50°C, and activity was still high at 55°C.

![FIG 2 ClustalW alignment of highly expressed *Pycnoporus cinnabarinus* (Pci) GLOX-like proteins with *P. chrysosporium* (Phach) GLOX. Identical residues are highlighted in red. Arrows, residues essential for catalysis.](image-url)

<table>
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<th>Total activity (nkat)</th>
<th>Amt of protein (mg)</th>
<th>Act. act (nkat · mg⁻¹)</th>
<th>Activity yield (%)</th>
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¹ND, not detected.
detected at 75 to 80°C. The two PciGLOX enzymes were found to be similarly affected by temperature and were relatively stable at 50°C after 4 h of incubation. The activities of both enzymes were found to be highly dependent on the buffer used, since the use of different buffers with the same pH was found to alter the activities of the enzymes. For this reason, the pH profile test was limited to the pH range of sodium 2.2-dimethylsuccinate (pH 4 to 6), the buffer in which both enzymes were found to be the most active. In addition, over this pH range, there was no effect on HRP activity (18). The optimum pH obtained under the tested conditions for both PciGLOX enzymes was 6, which is in agreement with that previously reported for PhGLOX (26, 27). However, our results differ from those obtained by Son et al. (25), who showed that the optimum pH for the recombinant PhGLOX was 5. In our study, it was noticed that the enzymes were very sensitive to minor pH changes and they were less active at pH 5, which is a change of only 1 pH unit from the optimal pH. However, the results showed that the enzymes retained about 50% of their activity after incubation for 4 h under all tested pH conditions.

Similar to PhGLOX (12), a 1.4-fold increase in activity was observed when PciGLOX1 was incubated with 1 mM CuSO4 for 30 min at 25°C. However, no similar effect on the activity was detected for PciGLOX2. This might be explained by the fact that the large amount of PciGLOX1 produced in the culture medium requires more copper ions to ensure complete ion activation. The presence of copper(II) ions (e.g., in the form of CuSO4) has previously been found to be required for the activation of galactose oxidase (28).

The two purified GLOX proteins were active only in the presence of both HRP and ABTS in the reaction mixture, as HPLC analysis demonstrated (data not shown). PciGLOX appears to be activated when the enzyme is oxidized by one electron or the copper is reduced by one electron, as has been previously proposed for PhGLOX (8). This activation mechanism is thought to be directed by lignin peroxidase and veratryl alcohol (12). Furthermore, the activation by peroxidase (lignin peroxidase or HRP) was reported to be possible only when a high-redox-potential peroxidase substrate was used in the reaction (29). The activation of GLOX by peroxidase might be a mechanism used by fungi to regulate peroxide generation and thus control peroxidase action in vivo (24, 29). GLOX has also been found to be activated by high-redox-potential inorganic oxidants, including manganese chelates (Mn3+/H2O2 EDTA) (24), possibly relating this enzyme to manganese peroxidase, which is responsible for the generation of Mn3+/H2O2.

HPLC analysis also showed that the substrates were not completely converted even when the reaction time was increased, suggesting that the enzyme is inactivated at some point during the
reaction. This inactivation has been attributed in part to the inhibitory effect of peroxide accumulation on GLOX (29). Further investigations by Roncal et al. (27) showed that the inactivation was triggered by components that are not initially present in the reaction medium or that are present in a different state at first. These components could be products of the reaction or other generated compounds directly inhibiting the enzyme or interfering with its mechanism of action under the coupled reaction conditions (27).

The two PciGLOX enzymes had broad substrate specificity. The enzymes were unable to oxidize sugars and methanol, showing that they are not sugar or methanol oxidases. Substrates included methylglyoxal, glyoxal, glyoxylic acid, glyceraldehyde, dihydroxyacetone, and glycerol, which are compounds derived from carbohydrate metabolism. The nature of the substrates could explain the production of H2O2 for peroxidase activity. Recently, it was shown that, when the primary products of their reactions (see Fig. S2 and S3 in the supplemental material). As shown by HPLC, while the only product of PciGLOX1 on glyoxal was oxalic acid, which is the final product of the reaction, in the case of PciGLOX2, another peak corresponding to glyoxylic acid, the intermediate compound of this reaction, was also detected (see Fig. S2b in the supplemental material). The absence of the first product in the reaction of PciGLOX1 can be explained by the higher catalytic efficiency (more than 100 times) of this enzyme compared to that of PciGLOX2 in oxidizing this compound (Table 3). These findings suggest that the PciGLOX enzymes are implicated in sequential reactions or their substrate preference evolves during fungal growth. This characteristic could be biotechnologically important, since it is possible to perform multiple oxidation steps using one enzyme. Recently, Yin and coworkers (31) showed that two fungal alcohol oxidases from Colletotrichum graminicola and Colletotrichum gloeosporioides belonging to the AA5 family were incapable of oxidizing sugars but were efficient in the oxidation of a diverse number of aliphatic alcohols, including glycerol. Considering the broad specificity of PciGLOX2, oxidases belonging to the AA5 family could be rather promiscuous regarding their substrate specificity.

As a hydrogen peroxide-generating enzyme, GLOX is thought to be part of the lignin-degrading enzyme machinery by providing H2O2 for peroxidase activity. Recently, it was shown that, when P. chrysosporium was grown on solid spruce wood, a significant up-regulation of manganese and lignin peroxidases and auxiliary enzymes, including GLOX, was observed during extracellular oxidation, which was detected by the presence of oxidant-sensing beads with the ligninolytic peroxidases in fungi. A major difference between the two PciGLOX enzymes and PhGLOX is that the best substrate for the former was glyoxylic acid instead of methylglyoxal. Both GLOX enzymes were active in the majority of the reported substrates of PhGLOX (26, 27); however, PciGLOX2 was also found to act on 3-phenylpropionaldehyde. Activity on glycerol is interesting, since it is a sugar alcohol lacking the formyl group present in the other substrates. Glycerol is structurally similar to dihydroxyacetone, a molecule found to be a substrate for PhGLOX (26) and PciGLOX. It has also been proposed that the gem diol form obtained after hydration of aldehydes to acetals is the actual substrate recognized by GLOX (24). Primary alcohols are structural analogues of gem diols, which derive from aldehydes, and this might explain the activity of GLOX on glycerol (27). In a similar way, carbonyl-containing substrates are suggested to be hydrated by solvent coordinated to the copper in the active site, and this form of the substrates is recognized by the enzyme (24).

In some cases, the PciGLOX enzymes were able to also act on the primary products of their reactions (see Fig. S2 and S3 in the supplemental material). As shown by HPLC, while the only product of PciGLOX1 on glyoxal was oxalic acid, which is the final product of the reaction, in the case of PciGLOX2, another peak corresponding to glyoxylic acid, the intermediate compound of this reaction, was also detected (see Fig. S2b in the supplemental material). The absence of the first product in the reaction of PciGLOX1 can be explained by the higher catalytic efficiency (more than 100 times) of this enzyme compared to that of PciGLOX2 in oxidizing this compound (Table 3). These findings suggest that the PciGLOX enzymes are implicated in sequential reactions or their substrate preference evolves during fungal growth. This characteristic could be biotechnologically important, since it is possible to perform multiple oxidation steps using one enzyme. Recently, Yin and coworkers (31) showed that two fungal alcohol oxidases from Colletotrichum graminicola and Colletotrichum gloeosporioides belonging to the AA5 family were incapable of oxidizing sugars but were efficient in the oxidation of a diverse number of aliphatic alcohols, including glycerol. Considering the broad specificity of PciGLOX2, oxidases belonging to the AA5 family could be rather promiscuous regarding their substrate specificity.

### Table 2: Substrate specificity of PciGLOX1 and PciGLOX2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PciGLOX1</td>
</tr>
<tr>
<td>Methylglyoxal</td>
<td>100</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>40</td>
</tr>
<tr>
<td>Glyoxylic acid</td>
<td>192</td>
</tr>
<tr>
<td>3-Phenylpropionaldehyde</td>
<td>NDb</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>6</td>
</tr>
<tr>
<td>Veratraldehyde</td>
<td>ND</td>
</tr>
<tr>
<td>4-Hydroxybenzaldehyde</td>
<td>ND</td>
</tr>
<tr>
<td>2,4-Dimethoxybenzaldehyde</td>
<td>ND</td>
</tr>
<tr>
<td>α-Butyrolactone</td>
<td>ND</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>ND</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>ND</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>ND</td>
</tr>
<tr>
<td>D-Methanol</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The standard activity test was performed with 10 mM substrate, and the values were calculated relative to those for methylglyoxal.*

*ND, not detected under these conditions.*

### Table 3: Kinetic parameters of PciGLOX1 and PciGLOX2 with different substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PciGLOX1</th>
<th>PciGLOX2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V&lt;sub&gt;max&lt;/sub&gt; (nmol mg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>K&lt;sub&gt;m&lt;/sub&gt; (mM)</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>Methylglyoxal</td>
<td>1,289</td>
<td>1.3</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>1,422</td>
<td>13.1</td>
</tr>
<tr>
<td>Glyoxylic acid</td>
<td>3,193</td>
<td>0.088</td>
</tr>
</tbody>
</table>

*V<sub>max</sub>, maximum velocity; K<sub>m</sub>, Michaelis-Menten constant; k<sub>cat</sub>, turnover number.*

*The standard test was used; however, the concentration of substrate was varied.*
bearing a fluorometric dye (32). However, secretomic analysis of P. cinnabarinus cultures on different substrates does not clearly establish the relation between these enzymes since peroxidases were detected only in solid-state fermentation, whereas the PciGLOX enzymes were present in liquid and solid cultures (4). On the other hand, the activation by peroxidases reported previously and also in this work could support the synergistic activity between GLOX and peroxidases. The amino acids involved in copper binding were highly conserved in the PciGLOX enzymes, as well as in PhGLOX, which might explain the similar characteristics between these enzymes. However, the PciGLOX enzymes were more active on glyoxylic acid, contrary to the findings for PhGLOX, whose best substrate was methylglyoxal. In addition, a difference in catalytic efficiency and substrate preference was observed between the PciGLOX1 enzymes and PciGLOX2, which either acted alone or more efficiently on some of the substrates. In light of these differences, the multiplicity of GLOX genes in P. cinnabarinus may reflect an adaptation to shifting substrate compositions and availability during decay of complex woody cell walls. A better understanding of the 3-dimensional structure and the mechanism of action of these enzymes is needed to explain the results obtained in this study.

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