Molecular Biotechnology (2019) 61:633–649
https://doi.org/10.1007/s12033-019-00190-6

ORIGINAL PAPER

Production of Galactose Oxidase Inside the *Fusarium fujikuroi* Species Complex and Recombinant Expression and Characterization of the Galactose Oxidase GaoA Protein from *Fusarium subglutinans*

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Published online: 8 June 2019

Abstract
Galactose oxidase catalyzes a two-electron oxidation, mainly from the C6 hydroxyl group of D-galactose, with the concomitant reduction of water to hydrogen peroxide. This enzyme is secreted by *Fusarium* species and has several biotechnological applications. In this study, a screening of galactose oxidase production among species of the *Fusarium fujikuroi* species complex demonstrated *Fusarium subglutinans* to be the main producer. The truncated *F. subglutinans gaoA* gene coding for the mature galactose oxidase was expressed from the prokaryotic vector pTrcHis2B in the *E. coli* Rosetta™ (DE3) strain. The purified recombinant enzyme presented temperature and pH optima of 30 °C and 7.0, respectively, K_M of 132.6 ± 18.18 mM, V_{max} of 3.2 ± 0.18 µmol of H_2O_2/min, k_{cat} of 12,243 s^{-1}, and a catalytic efficiency (k_{cat}/K_M) of 9.2×10^4 M^{-1} s^{-1}. In the presence of 50% glycerol, the enzyme showed a T_{50} of 59.77 °C and was stable for several hours at pH 8.0 and 4 °C. Besides D-(+)-galactose, the purified enzyme also acted against D-(+)-raffinose, α-D-(+)-melibiose, and methyl-α-D-galactopyranoside, and was strongly inhibited by SDS. Although the *F. subglutinans gaoA* gene was successfully expressed in *E. coli*, its endogenous transcription was not confirmed by RT-PCR.

Keywords Galactose oxidase · Heterologous expression · *Fusarium subglutinans* · *Fusarium fujikuroi*

Introduction

Galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) is a monomeric metalloenzyme with a copper ion as a cofactor. It is an extracellular protein naturally secreted by different species of the *Fusarium* genus, among which the following stand out: *Fusarium austroamericanum* (NRRL 2903) (formerly *Polyporus circinatus* and *Dactylium dendroides*) [1–3], which belongs to the species complex *Fusarium graminearum* [4]; *Fusarium subglutinans* (formerly *Fusarium moniliformis* f. sp. *subglutinans*), *Fusarium moniliforme*, and *Gibberella fujikuroi*, which belong to the *Fusarium fujikuroi* species complex [5–8]; and *Fusarium acuminatum* [8, 9]. Secretion of galactose oxidase is also reported for species of other genera, such as *Alternaria* sp. *Helminthosporium* sp., and *Penicillium album* [5]. As regards bacteria, galactose oxidase-like genes have been described: e.g., the *fbfB* gene from *Stigmatella aurantiaca*, which encodes a protein involved in fruiting body formation, and the *Streptomyces coelicolor* laterally acquired *SCO2837* gene, which encodes the SCO2837 protein that processes primary alcohols to aldehydes and is required for the aerial bacterial development during osmotic stress [10–12]. However, no galactose activity has been ascribed to these bacterial genes.

Galactose oxidase is one of the most studied oxidases, due to its mechanism and practical applications. Its catalytic action results in the stereo-specific oxidation of two electrons of many primary alcohols to their corresponding aldehydes, with the concomitant reduction of oxygen to hydrogen peroxide [13–15]. This enzyme is highly selective for D-galactose and oxidizes galactose residues either

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as monosaccharides or polysaccharides and glycoconjugates that contain galactose at the non-reducing end [14]. The best substrate reported so far is dihydroxyacetone, which is more than three times better than D-galactose [13]. The role that galactose oxidase plays in filamentous fungi physiology is unclear, although it seems to be related to hydrogen peroxide production, which can cause lesions in the plant cell wall and facilitate fungal invasion [16]. Other alcohol oxidases, such as the glyoxal oxidase from *Phanerochaete chrysosporium*, produce hydrogen peroxide, which may play some part in lignin degradation together with lignin peroxidase [17]. However, the enzyme galactose oxidase is widely applied in different biotechnological processes. Among its practical applications, it is worth mentioning: (1) biosensors for D-galactose, lactose, and other substrates [18]; (2) enzymatic biocatalyst in aldehydes, carbohydrates, and other molecular synthesis [19]; (3) histochemical staining studies, including cancer detection using a cytochemical staining method to detect the Gal-β(1→3)-GalNAc disaccharide in body fluid glycoproteins [20]; (4) synthesis of paper strength additives in the paper industry [21]; (5) glycosylation of recombinant proteins produced in bacteria [22]; and (6) glycoproteomic studies [23].

According to Cordeiro et al. [24], there are three intronless orthologous gene lineages within the galactose oxidase family in *Fusarium* spp., namely *gaoA*, *gaoB*, and *gaoC*. The first galactose oxidase cloned and sequenced gene was the *F. austroamericanum gaoA* gene [25]. It has 2043 bp and, therefore, codes a 680-amino-acid residue protein. This gene has already been heterologously expressed in *Aspergillus nidulans*, *Aspergillus oryzae*, *Fusarium venenatum*, *Pichia pastoris*, *Escherichia coli*, and in an in vitro system [26–33].

The mature and active *F. austroamericanum* galactose oxidase GaoA protein has 639 amino acid residues. It is the product of the *gaoA* gene, a 680-amino-acid residue inactive precursor protein. The maturation process involves two cleavages, the first removes the *N*-terminal secretory signal peptide (also known as pre-sequence), which contains 24 amino acid residues, and the second removes the *N*-terminal maturation peptide (also known as pro-sequence), which contains 17 amino acid residues. The removal of the pro-sequence is an autocatalytic event, which requires the presence of copper [13]. Further steps in the maturation process lead to a thioether bond formation between the active site residues Tyr272 and Cys228, which creates a tyrosyl radical that is essential for enzyme catalysis [13, 14].

Other *Fusarium* galactose oxidase genes have also been studied. The galactose oxidase *gaoB* gene from *Fusarium venenatum* was cloned and heterologously expressed in *F. venenatum* [34]. The *gaoB* genes from *Fusarium verticillioides* and *F. subglutinans* were cloned, sequenced, and used as biomarkers for the detection of these species [24, 35]. While the *gaoA* gene from *Fusarium sambucinum* was cloned and expressed in *E. coli* [36], the *gaoA* gene from *Fusarium oxysporum* was cloned and expressed in *E. coli* and *Pichia pastoris* [37].

This study reports a screening of galactose oxidase production among species of the *F. fujikuroi* species complex. In this screening, *F. subglutinans* was found to be the major producer. The *F. subglutinans gaoA* gene was cloned, sequenced, and successfully expressed in *E. coli* and the recombinant enzyme was characterized. In addition, the endogenous transcription of the *gaoA* gene was evaluated in *F. subglutinans*.

Materials and Methods

Microorganisms

The fungi used in this study are described in Table 1. These isolates were maintained in potato dextrose agar (PDA) (for 1 L: 15 g dextrose, 20 g agar, filtrate of 200 g of peeled potato cooked in 400 mL of distilled water) slants with trimestral transfers. *E. coli* TOP10® and DH5α™ strains were used for plasmid propagation and as prokaryotic expression systems. *E. coli* BL21 Star™ (DE3) and Rosetta™ (DE3) strains were used as prokaryotic expression systems. These *E. coli* strains were stored in Luria–Bertani (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0), with 50% glycerol, at − 20 °C.

Screening of Galactose Oxidase Production Among Species of the *F. fujikuroi* Species Complex

The isolates (Table 1) were cultured in the liquid medium formulated by Markus et al. [38], which was prepared as described by Cordeiro et al. [24]. A 125-mL Erlenmeyer flask containing 25 mL of the liquid culture medium was inoculated with a smashed fragment (1 cm^3) from a fresh PDA slant culture and was incubated for 3 days at 25 °C, in the dark, under agitation (100 rpm). Afterward, a 500-µL aliquot (2%, v/v) of the obtained culture was used to inoculate a new 125-mL flask containing 25 mL of the same liquid medium. These flasks were incubated for 3 days in the same conditions. The cultures were subsequently filtered using filter paper and/or centrifuged (1500*g*, 5 min). The enzyme was assayed in the filtrate or supernatant of three independent culture flasks.

In an attempt to increase *F. subglutinans* UnB 379 enzyme production, the fungus was cultured in the same conditions, but the medium was supplemented with 0.4% (w/v) corn flour, yellow corn grits, oat flakes, rice, or Bulgur wheat, before autoclaving. The solid substrates were used as additional carbon sources.

Table 1 Fungi isolates used in this work and galactose oxidase production

Specie	Strain	Galactose oxidase (U/L of filtrate)[§]
Fusarium verticillioides	MGA 5-1*	0
F. verticillioides	MGA 45-1*	0
F. verticillioides	MGI 1-1*	0
F. verticillioides	PG 5-2*	0
F. verticillioides	PG 6-1*	0
F. verticillioides	CO 2-2*	0
F. verticillioides	BAN 4-2*	0
F. verticillioides	BAN 5-2*	0
F. verticillioides	RV 21-2*	0
F. verticillioides	RV 25-1*	0
F. verticillioides	CPÃ 1-1*	0
F. verticillioides	RV 29-2*	0
F. verticillioides	CMA 2-1*	0
F. verticillioides	CMA 7-2*	0
F. verticillioides	CMA 9-1*	0
F. verticillioides	CML 767[#]	0
Fusarium sacchari	CML 769[#]	0
Fusarium fujikuroi	CML 793[#]	0
Fusarium proliferatum	CML 770[#]	9.7 ± 2.8^{b}
Fusarium thapsinum	CML 775[#]	0
Fusarium nygamai	CML 797[#]	0
Fusarium circinatum	CML 791[#]	0
Fusarium konzum	CML 776[#]	15.2 ± 14.9^{b}
F. subglutinans	UnB 379[&]	258 ± 80^{a}

* Previously obtained from maize grains [35]

[#]Generously donated by Dr. Pfenning—Mycological Collection of Lavras (CML) from the Federal University of Lavras and originally obtained from the Kansas State University Collection, USA

[&]Generously donated by Dr. Dianese—University of Brasília (UnB) and previously reported as a galactose oxidase producer [8]

[§]Values are the averages and the standard deviations from data individually obtained in the filtrate of three culture flasks

[a]Enzyme activity at 10 min of reaction

[b]Enzyme activity at 2 h of reaction

DNA Extraction

The fungus *F. subglutinans* was cultured as described above and the mycelia mass was collected by filtration in sterile gauze. After being ground to a powder in a mortar with liquid nitrogen, a 300-µL aliquot of the ground mycelia was transferred to a microcentrifuge tube and the genomic DNA was extracted according to the protocol designed by Koenig et al. [39]. The DNA concentration was determined by absorbance at 260 nm and by fluorometry, using the Qubit Quantitation Fluorometer and the Quant-it™ dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). The DNA final concentration was adjusted to 100 ng/µL in TE buffer and the DNA was kept frozen at − 20 °C.

Construction of the pET101/D-TOPO®-*F. subglutinans gaoA* Gene and Expression Analysis

Initially, to see if the *F. subglutinans gaoA* gene could be expressed in *E. coli*, the following studies were performed. Most of the work regarding the *F. austroamericanum* GaoA recombinant expression in *E. coli* used the truncated gene, without the 5′ region coding the pre-pro sequence of 41 amino acids. Thus, the *F. austroamericanum* GaoA (GenBank M86819) and the *F. verticillioides* GaoA (GenBank DAA34000.1) proteins were aligned to verify the corresponding pre-pro sequences in the latter protein. As there was no sequenced genome for *F. subglutinans* and *F. verticillioides* and *F. subglutinans* belong to the *F. fujikuroi* species complex and are closely related, a primer pair (FW 5′-CACCATGGCCTCAGCTCCTCTCGGCACTGCTATC, and RV 5′-TTACTGAGTAACGAGAAGAGTACTGG) was designed to amplify the truncated *F. verticillioides gaoA* gene (BK007072.1), without the pre-pro sequence. The FW primer had an extra 5′ sequence (CACC) to allow directional cloning in the pET101/D-TOPO® (Thermo Fisher Scientific, USA) and a translation start ATG codon (underlined). The RV primer had a stop codon (underlined). These primers were used in a PCR with the *F. subglutinans* UnB 379 strain genomic DNA as follows: 1× enzyme buffer; 1.0 mM $MgSO_4$; 0.3 mM of each dNTP, 25 pmol of each primer; 2.5 U of Platinum® *Pfx* DNA Polymerase, and 400 ng of genomic DNA in a total volume of 25 µL. The reaction consisted of 25 cycles of 1 min at 94 °C; 1 min at 58 °C, and 2 min at 68 °C. The reactions were heated for 5 min at 94 °C before cycling and for 10 min at 68 °C after cycling. The PCR was then loaded in a 1% agarose gel and the amplified fragment of approximately 2000 bp was gel purified using the S.N.A.P™ Gel Purification Kit (Thermo Fisher Scientific, USA), following the manufacturer's instructions. After fluorometric quantification with the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, USA), the DNA fragment was cloned into the pET101® vector from the Champion™ pET Directional TOPO® Expression Kit, according to the vendor's protocol. The recombinant plasmid was used to transform the *E. coli* TOP10® strain [40]. Next, it was recovered by alkaline lysis [41] and analyzed by restriction digestion.

As regards expression analysis, a 60-ng aliquot of the pET101®-*F. subglutinans gaoA* was used to transform the *E. coli* BL21 Star™ (DE3) strain [40]. The transformed bacteria were inoculated in 10 mL of LB medium containing ampicillin (50 µg/mL) and cultured overnight at 37 °C, under agitation of 100 rpm. Aliquots of 500 µL of this culture were used to inoculate flasks of 250-mL containing

50 mL of the LB-2X-ampicillin medium [2% (w/v) tryptone, 1% (w/v) yeast extract, 2% (w/v) NaCl, ampicillin 50 µg/mL] or 50 mL of ZYM-5052-ampicillin auto-induction medium described by Studier [42], with modifications [1% tryptone, 0.5% yeast extract, 0.5% glycerol, 0.05% glucose, 0.2% monohydrated lactose, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4{\cdot}7H_20$, 10 µM $MnSO_4{\cdot}H_20$, 2 µM $CuSO_4{\cdot}5H_20$, 10 µM, $ZnSO_4{\cdot}7H_2O$, 25 mM sodium succinate dibasic hexahydrated, 20 µM $CaCl_2{\cdot}2H_2O$, 50 µM $FeCl_3$, 50 µg/mL ampicillin]. After incubation for 4 h at 37 °C under agitation (100 rpm), when an optical density of 0.6–0.8 at 620 nm was achieved, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added (final concentrations of 1 mM) to the flask containing LB-2X and to one of the two flasks containing ZYM-5052, so as to induce protein expression (flasks incubated at 25 °C for 16–18 h). The cells were subsequently collected by centrifugation (1500*g*, 5 min) and resuspended in 5.0 mL of the lysis buffer described by Deacon and McPherson [33], with modifications (50 mM phosphate buffer, pH 8.0; 0.5 M NaCl; 5 mM $MgCl_2{\cdot}6H_2O$; 1% Triton X-100). After vortex agitation and 40 min incubation on ice, 1 mM PMSF, 150 µg/mL lysozyme, and 2 U/mL DNase I were added. The mixture was incubated for 2 h at 4 °C. The samples were then dialyzed twice against 50 mM phosphate buffer, pH 7.0, containing 0.4 mM $CuSO_4{\cdot}5H_2O$. After centrifugation (9000*g*, 5 min), galactose oxidase activity was tested in the supernatant.

Galactose Oxidase Activity Assay

Galactose oxidase activity was evaluated using colorimetric analysis, which uses horseradish peroxidase and *o*-dianisidine, based on the estimation of the colored oxidized *o*-dianisidine [43]. The assay mixture consisted of: 0.5 mL of the sample, 0.1 mL of 0.5 M D-galactose (25 mM final) as the substrate and 1.4 mL of the following reactive mixture: 0.04 mg/mL peroxidase (Sigma Cat. P-8125) (6 U/mL), 0.2 mg/mL *o*-dianisidine (Sigma cat. D-3252) previously dissolved in a small volume of methanol (2 mg/mL) and 50 mM phosphate buffer, pH 7.0. A blank without substrate was used to establish the zero point of absorbance. The reactive mixture was always tested with hydrogen peroxide to make sure the peroxidase was active. Unless otherwise stated, the reaction was incubated for 10 min at 30 °C before the absorbance was read at 430 nm. An enzyme unit is defined as the amount of enzyme necessary for the oxidation of 2 µmol of *o*-dianisidine (corresponding to the oxidation of 1 µmol of galactose) per min, under the conditions described above. A *o*-dianisidine oxidation calibration curve was performed with 8.2–82 µmol of *o*-dianisidine in 1.4 mL of 50 mM phosphate buffer, pH 7.0, containing 0.04 mg/mL peroxidase, and addition of 0.6 mL of H_2O_2 at a final concentration of 4.35 mM.

The Entire *F. subglutinans gaoA* Gene Cloning and Sequencing

Next, the entire *F. subglutinans gaoA* gene was cloned, sequenced, and analyzed. Primers (FW 5′-ATGAAGTCC TTTTGGACACTTGC and RV 5′-TTACTGAGTAACGAG AAGAGTACTGG) were designed targeting the entire coding region of the *F. verticillioides gaoA* gene (GenBank BK007072.1). The designed primers were used in a PCR, as described above. An additional adenosine was then added at the 3′ extremities by incubating the PCR with 1 U of Platinum *Taq*® DNA polymerase at 72 °C for 10 min. The amplified DNA was phenol:chloroform (pH 8.0) (1:1) purified and ethanol precipitated. The DNA fragment was subsequently cloned into the TA Cloning Kit vector pCR®2.1 (Thermo Fisher Scientific, USA), following the manufacturer's instructions, and the recombinant plasmid was used to transform the *E. coli* TOPO10® strain [40]. The recombinant plasmid was subsequently recovered by alkalyne lysis [41].

The cloned gene was sequenced at the Center for Human Genome Studies (University of São Paulo, São Paulo, Brazil) with the forward and reverse M13 universal primers and internal primers to the gene, which were designed based on the *F. verticillioides gaoA* gene sequence (GenBank BK007072.1). Each DNA strand was sequenced at least twice and a contig was generated using the BioEdit program [44], through which the GC content was also calculated. Presence of introns and ORF was analyzed using the Augustus gene prediction program (http://augustus.gobics.de/).

The *F. subglutinans* GaoA Protein In Silico Analysis

The *F. subglutinans* GaoA amino acids sequence was obtained translating the sequenced gene in the ExPASy Translate program (https://www.expasy.org/). A structural model for the *F. subglutinans* mature GaoA protein, without the amino terminal pre-pro sequence, was generated using the Modeller program [45], employing the *F. austroamericanum* GaoA protein structure (PDB 1gofA) as a template [46]. The modeled PDB structure was visualized and colored using the CCP4MG program [47]. The protein was also analyzed for structural domains using the Conserved Protein Domain Database (CDD) from NCBI (https://www.ncbi.nlm.nih.gov) and Pfam from EMBL-EBI (https://pfam.xfam.org/). The putative *N*-glycosylation sites, and molecular weight and pI, were calculated using the NetCGlyc 1.0 server and Compute pI/Mw program, respectively, from ExPASy.

Blast searches were carried out with the *F. subglutinans* and *F. austroamericanum* GaoA protein sequences in order

to retrieve other GaoA proteins from *Fusarium* genera species from GenBank. The sequences thus obtained were aligned and the alignment was used to build a phylogenetic tree by employing the neighbor-joining method [48] of the MEGA 7.0 program [49]. Bootstrap analyses were conducted (1000 heuristic replicates) to assess the confidence limits of the branching [50]. Values higher than 70% in the bootstrap test of phylogenetic accuracy indicated reliable grouping among proteins. Pairwise deletion was used to remove gaps, because a complete removal could eliminate a large part of phylogenetically meaningful sites. The phylogenetic distances were computed using the maximum composite likelihood method [51] and the number of amino acid substitutions per site. The grouping was also performed using other methods, such as maximum parsimony, minimum evolution, and UPGMA with similar results.

Construction of the pET21a(+)- and pTrcHis2B-*F. subglutinans gaoA* Gene and Expression Analysis

The truncated *F. subglutinans gaoA* gene, which codes the mature *F. subglutinans* GaoA protein, without the pre-pro sequence, was amplified and cloned into the pET21a(+) (Novagen, USA) expression vector. A pair of primers (FW 5′-TTCATATGGCCTCAGCTCCCATCGGCAC and 5′-TTGCGGCCGCCTGAGTAACGAGAAGAG) was designed based on the cloned and sequenced gene. Restriction sites for the enzymes *Nde*I and *Not*I were included in the forward and reverse primers, respectively (underlined). The stop codon was removed in the reverse primer so as to insert a histidine tag provided by the vector. A PCR was performed with 1× enzyme buffer, 2.0 mM $MgSO_4$, 0.2 mM of each dNTP, 25 pmol of each primer, 1.0 U of Platinum® *Taq* DNA Polymerase High Fidelity (Thermo Fisher Scientific, USA), and 200 ng of the pCR2.1-*gaoA* recombinant constructed plasmid as DNA template. The PCR cycling consisted of an initial incubation of 5 min at 94 °C, followed by 25 cycles of 1 min at 94 °C, 1 min at 60 °C, 2 min at 68 °C, and a final incubation at 68 °C for 10 min. To remove the plasmid used as template, the amplified DNA fragment was electrophoresed in an agarose gel and purified from the gel by means of the Wizard® SV Gel PCR Clean-Up System Kit (Promega, USA). A deoxyadenosine was then added to the 3′ extremities of the 2 Kb amplified DNA fragment, as described above, and the PCR product was cloned into the pGEM®-T Vector (Promega, USA), following the manufacturer's instructions. The truncated gene was subsequently transferred from the pGEM®-T vector (Promega, USA) to the pET21a(+) expression vector using digestion and ligation technology [41]. The recombinant plasmid was transformed into the *E. coli* DH5α strain [40], recovered by alkaline lysis, and analyzed using restriction digestion [41].

The truncated *F. subglutinans gaoA* gene was also cloned into the pTrcHis2B (Thermo Fisher Scientific, USA) expression vector. To do so, the primers FW 5′-TTGGTACCGCC TCAGCTCCCATCGGCAC and RV-TTTCTAGATTCTG AGTAACGAGAAGAGTAC were designed. The forward primer contained a restriction site for the *Kpn*I enzyme (underlined) and no start codon, which was provided by the vector. The reverse primer had a restriction site for the *Xba*I enzyme (underlined), no stop codon to allow a His tag to be inserted by the vector, and two extra nucleotides to keep the gene in-frame (double underlined). The DNA was amplified in a 50-µL PCR containing 1.0 U of Platinum *Pfx*® DNA polymerase (Thermo Fisher Scientific, USA), 1× enzyme buffer, 1.0 mM $MgSO_4$, 0.3 mM of each dNTP, 25 pmol of each primer, and 200 ng of the recombinant pCR2.1-*gaoA* plasmid. The gene was then cloned into the pCR2.1 vector and transferred to the pTrcHis2B (Thermo Fisher Scientific, USA) vector as described above.

The pET21a(+)-*gaoA* construction expression analysis was performed in the *E. coli* Bl21 Star™ (DE3) strain. The recombinant pTrcHis2B-*gaoA* expression analysis was carried out in the *E. coli* DH5α, TOP10®, and Rosetta™ (DE3) strains. For this, 40–60 ng of each vector containing the truncated *gaoA* gene were used to transform the *E. coli* strains, according to Chung et al. [40]. The transformed Rosetta™ (DE3), DH5α, and TOP10® strains were inoculated onto LB agar plates containing ampicillin (50 µg/mL). Various colonies of each plate were used to inoculate 10 mL of LB-ampicillin (50 µg/mL). As regards the *E. coli* Bl21 Star™ (DE3) strain, the transformed bacteria were used to directly inoculate 10 mL of LB with ampicillin (50 µg/mL), instead of spreading them on the LB plates. The bacteria were grown overnight at 37 °C with stirring (100 rpm). A 500-µL aliquot of each culture was used to inoculate 250-mL flasks containing 50 mL of the ZYM-5052 auto-induction medium described by Studier [42], with modifications. After 4 h at 37 °C under agitation of 100 rpm, 1 mM IPTG was added to the media and the temperature was reduced to 25 °C. After 18 h, the cells were collected by centrifugation (1500*g*, 5 min), resuspended, and lysed as described above. The samples were then dialyzed twice against 100 mM ammonium acetate buffer, pH 7.0, containing 0.4 mM $CuSO_4 \cdot 5H_2O$. After centrifugation (9000*g*, 5 min), the galactose oxidase activity was tested in the supernatant.

RNA Extraction and Analysis

For RNA extraction, the *F. subglutinans* UnB 379 strain was cultivated as described above for enzyme production, without substrate supplementation. The mycelium was collected by filtration on sterile gauze, and the RNA was extracted using the PureLink™ Micro-to-Midi Total RNA Purification System Kit (Thermo Fisher Scientific, USA), as follows.

Cells were macerated to a powder in a mortar with liquid nitrogen and a 100-µL aliquot of the powder was transferred to a sterile microtube. A volume of 500 µL of lysis solution containing 5 µL of β-mercaptoethanol was then added. The mixture was kept on ice and homogenized by passing it through a sterile needle (18G).

After centrifugation (12,000*g*, 2 min), the supernatant was transferred to another microtube, to which 300 µL of absolute ethanol was added. After homogenization, the RNA was purified using the kit affinity column, according to the manufacturer's protocol. The RNA concentration and purity were estimated by reading at 260 nm and $A_{260/280}$, respectively.

One microgram of total RNA was treated for 15 min at 25 °C with 1 U amplification-grade DNase I and 1× DNase I buffer (Thermo Fisher Scientific, USA), in a total volume of 10 µL. An aliquot of 8 µL of this mixture (0.8 µg of RNA) was used in the reverse transcriptase reaction (RT) using the SuperScritp ™ First-Strand Synthesis System for RT-PCR Kit (Thermo Fisher Scientific, USA), with oligo $(dT)_{12–18}$ primer included, following the manufacturer's instructions.

The primers used in the PCR were designed to amplify both the entire *F. subglutinans gaoA* gene (described above), as well as the entire *F. subglutinans gaoB* gene (FW 5′-ATG AAGTCTTTCTACTCCTTGGCCTTGTGC and RV 5′-TCA GACAGTGACCTTAATGGTGCTAGCAACG) [24]. The PCR mixture contained: 1× enzyme buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer, 1.5 U of Platinum® *Taq* DNA polymerase (Thermo Fisher Scientific, USA), and 4 µL of the RT reaction containing the cDNA as template, in a total volume of 25 µL. The PCR was performed with an initial incubation of 5 min at 94 °C and 25 cycles of 1 min and 30 s at 94 °C, 1 min and 30 s at 58 °C, and 2 min at 72 °C. After 25 cycles, the reactions were subjected to 72 °C for 10 min. The control contained 4 µL of RNA solution treated with DNAse I instead of cDNA. To verify the DNA amplification, an aliquot of 10 µL of PCR was analyzed in 1% agarose gel, as described above.

The RNA folder WebServer program (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was used to predict the truncated *F. subglutinans gaoA* gene mRNA 5′ structure with its minimal free energy and the Rare Codon Analyser program (https://www.biologicscorp.com/tools/RareCodonAnalyzer) was used to predict the mRNA Codon Adaptation Index (CAI) value.

Expression and Purification of the *F. subglutinans* GaoA Galactose Oxidase

The pTrcHis2B-*gaoA* recombinant vector system and the *E. coli* Rosetta™ (DE3) strain were used for GaoA enzyme production and purification by immobilized metal affinity chromatography (IMAC). For this, a volume of 400 mL of ZYM-5052 auto-induction medium, in a 2-L flask, was inoculated with 4 mL of an overnight LB-ampicillin medium (50 µg/mL) culture, obtained as described above. The culture was carried out at 37 °C, 100 rpm, for 4 h. IPTG was then added at a final concentration of 1 mM and the flask was incubated at 25 °C, 100 rpm, for 18 h. Cells were collected by centrifugation (4300*g*, 5 min). The obtained pellet was resuspended in 10 mL of lysis buffer (50 mM phosphate buffer, pH 7.0, 150 µg/mL of lysozyme, 1 mM PMSF, 50 mM NaCl, 5 mM $MgCl_2{\cdot}6H_2O$, 2 U/mL DNase I) and incubated at 4 °C for 1 h. The suspension was then sonicated (48 cycles, 5 s on, 5 s off, 40% of amplitude) in an ice bath. The obtained homogenate was submitted to centrifugation (9000*g*, 5 min), and the supernatant was applied (1 mL/min) into a 5-mL HisTrap™ HP column (GE Healthcare Life Sciences, USA), which was equilibrated with washing buffer (100 mM phosphate buffer, pH 7.0, 100 mM NaCl, 20 mM imidazole). Afterward, the column was washed with 20 mL of washing buffer and the recombinant protein was eluted with an imidazole gradient (150 and 300 mM) in 100 mM phosphate buffer, pH 7.0, containing 100 mM NaCl. Fractions of 1.5 mL were collected and those with the highest galactose oxidase activity were pooled and dialyzed twice against 100 mM ammonium acetate buffer, pH 7.0, containing 0.4 mM $CuSO_4{\cdot}5H_2O$, and once omitting copper. The protein was kept frozen at − 20 °C without glycerol addition, until use.

SDS-PAGE Analysis

Protein concentration was determined according to Bradford [52], using bovine albumin as the standard. Enzyme purity was analyzed in a denaturating polyacrylamide gel (SDS-PAGE). An aliquot of 10 µg of supernatant protein was suspended in 20 µL of sample dilution buffer [20% (v/v) glycerol, 1% (w/v) SDS, 0.03 mg/mL bromophenol blue, 125 mM tris-base, pH 6.8, 0.72 M β-mercaptoethanol]. A 100-µL aliquot of the eluate containing 9.2 µg of the purified recombinant protein was precipitated with 11 µL of 100% Trichloroacetic acid (TCA), washed with absolute ethanol, air-dried, and resuspended in 20 µL of sample dilution buffer. All samples were boiled for 10 min before being applied in a 7.5% polyacrylamide resolving gel, pH 8.9, with a 4.5% stacking gel, pH 6.8, in the Tris–glycine buffer system [53]. The relative molecular mass of the recombinant galactose oxidase was measured using molecular weight markers (Thermo Fisher Scientific, USA). The gel was stained with Coomassie Blue.

Mass Spectrometry

To confirm the identity of the purified protein, peptide mass fingerprint (PMF) MALDI-TOF mass spectrometry was

carried out using gel bands obtained from the SDS-PAGE gel. The protein bands cut from the gel were discolored with 25 mM ammonium bicarbonate solution containing 50% (v/v) acetonitrile. The gel was then dehydrated with 100% acetonitrile, dried in a vacuum chamber, and rehydrated with a 10 µg/mL trypsin solution in 40 mM ammonium bicarbonate and 10% acetonitrile. This mixture was incubated overnight at 37 °C to generate the protein band peptides. A 2-µL aliquot of the resulting peptide solution was mixed with an equal volume of saturated HCCA (α-cyano-4-hydroxycinnamic acid) matrix solution. Next, the mixture was placed on a metal plate for analysis in a MALDI-TOF/MS AutoFlex II mass spectrometer (Bruker Daltonics, USA). Peak acquisition was carried out in the positive polarity ion reflector mode. The spectrometer was calibrated following the protocol provided by the manufacturer (acceleration voltage of 20 kV). The generated mass spectra were analyzed using the FlexAnalysis 3.0 software (Bruker Daltonics, USA). The Swiss-Prot database employing the MASCOT 2.0 program [54] was used to identify the proteins, considering carbamidomethylation of cysteine and possible methionine oxidation. The expressed protein from the pTrc2B plasmid was analyzed in the PeptideMass program of ExPASy to predict the trypsin fragment sizes and sequences and compare them to the obtained results.

Enzyme Characterization

Activity against different substrates was verified by replacing D-(+)-galactose with D-(+)-glucose, sucrose, lactose monohydrate, D-(+)-raffinose, α-D-(+)-melibiose, maltose, glycerol, methyl-α-D-galactopyranoside, or guar gum. Except for guar gum, which was used at 1%, all substrates were used at a final concentration of 25 mM.

The optimal temperature was found by ranging the enzyme reaction temperature from 20 to 60 °C. Thermal stability was evaluated by incubating the purified enzyme in 100 mM ammonium acetate buffer, pH 7.0, for 10 min at 20, 30, 40, 50, 55, and 60 °C, before the enzyme assay, in the absence or presence of 25% or 50% (v/v) glycerol (pH 7.0). In addition, enzyme stability over time was determined by incubating the purified enzyme, in the presence of 50% (v/v) glycerol, for a period of 4 h at 25 and 50 °C, before the enzyme assay.

The optimal pH was determined by varying the enzyme reaction pH from 5 to 9, using the Britton and Robinson buffer (0.1 M boric acid, 0.1 M acetic acid, 0.1 M phosphoric acid). The pH stability was evaluated by diluting the enzyme five times in Britton and Robinson buffer, pHs 6.0, 7.0, and 8.0, and incubating it for several h at 4 °C, before the enzyme assay was performed with 0.1 M phosphate buffer, pH 7.0.

The Michaelis–Menten constant, K_M, and the maximum reaction velocity, V_{max}, were determined by non-linear regression (GraphPad Prism 8.0 program), using the results obtained by varying the galactose concentration from 30 mM to 300 mM, in the enzymatic assay, and in the presence of 50% (v/v) glycerol. The purified enzyme (0.317 µg) was added to the enzyme reaction containing 50 mM phosphate buffer, pH 7.0. The mixture was incubated at 30 °C for 5 min, as described above.

Effect of Potential Inhibitors and Activators

Different compounds, namely NaCl, $MgCl_2 \cdot 6H_2O$, KCl, EDTA, CTAB, SDS, glycerol, and Tween 80, were added to the reaction mixture to verify their effects on the galactose oxidase activity. All compounds were added to a final concentration of 5 mM, apart from glycerol (25%) and Tween 80 (2.5%). Relative activity was measured as the percentage of remaining activity in relation to a reaction without any compound addition. The same procedure was carried out with the reactive mixture alone, without addition of galactose oxidase, to verify the extent to which the peroxidase in this system was inhibited or activated by the same compounds. For this, reactions were carried out with 1.4 mL of the reactive mixture of the galactose oxidase enzyme assay described above and 0.6 mL of H_2O_2 (23 µM final), without any addition of the purified enzyme or galactose.

Results and Discussion

Production of Native Galactose Oxidase by *F. fujikuroi* Species Complex Isolates

The screening results for galactose oxidase production are listed in Table 1. The *F. subglutinans* UnB 379 strain was the best producer among *F. fujikuroi* isolates. On the other hand, *F. proliferarum* and *F. konzum* demonstrated potential to produce the enzyme, as was verified by enzyme assays with 2 h incubation. The addition of solid substrates to the medium did not improve galactose oxidase activity production by *F. subglutinans*.

The *F. fujikuroi* complex includes at least 50 distinct species or phylogenetic lineages that group into three clades: the American, African, and Asian [55]. Regarding the distribution of galactose oxidase production, the first reports were published when representative isolates were all called *Fusarium moniliformis* (sexual stage: *Gibberella fujikuroi*), i.e., before the establishment of this species complex [5–7]. In a previous study by our group [8] and in this study, only the *F. subglutinans* UnB 379 strain was found to produce significant amounts of galactose oxidase among other *F. subglutinans* isolates or species of the *F. fujikuroi* species

complex. Therefore, it is reasonable to suppose that *F. subglutinans* is the main galactose oxidase producer in the above-mentioned complex.

The results obtained from the RT-PCR analysis (Fig. 1) did not show the presence of the *F. subglutinans gaoA* gene mRNA, which indicates that it is transcribed in insufficient amounts to be detected under the employed culture conditions. However, the *F. subglutinans gaoB* mRNA expression was confirmed by RT-PCR, what was previously reported [24]. In agreement with our results, Brown et al. [56] demonstrated in an EST analysis (GenBank DR659031.1) that the *F. verticillioides gaoB* gene, unlike the *gaoA* gene, is expressed when this fungus is grown in a solid state fermentation using corn meal or shoot and root biomass of maize seedlings as substrates. Each galactose oxidase gene is present in a single copy in the *F. fujikuroi* species complex genomes sequences. The expression of multiple genes could explain the high level of galactose oxidase production in *F. subglutinans*. On the other hand, why other *Fusarium* species, even having the *gao* genes, do not express any active galactose oxidase is unknown. Regarding gene expression regulation of *gao* genes, it is only known that in *F. graminearum*, the best galactose oxidase species producer, the production of extracellular galactose oxidase and the occurrence of *gaoA* gene mRNA are dependent on the carbon source used in the liquid culture medium [57].

Since the most representative species of galactose oxidase production in the *F. fujikuroi* species complex is *F. subglutinans*, it would be interesting to characterize its protein. However, its expression level does not allow protein purification. As *gaoB* and *gaoC* genes are not well known, together with the fact that the *gaoA* gene from other *Fusarium* species was previously expressed in bacteria, we decided to clone and express the latter to characterize the expressed protein.

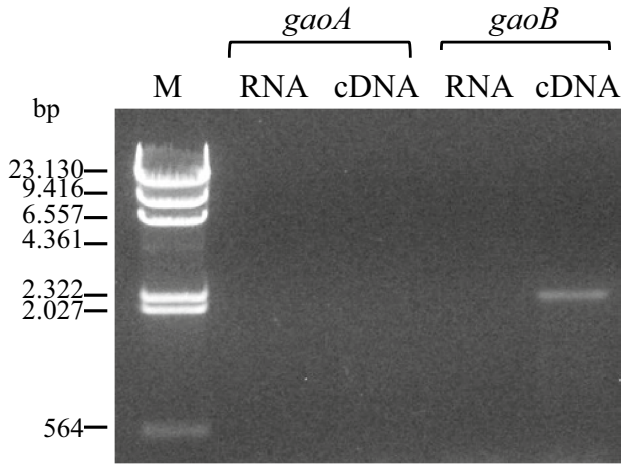

Fig. 1 RT-PCR. Agarose gel (1%) stained with ethidium bromide containing the RT-PCR using primers for the amplification of the entire coding region of the *F. subglutinans* UnB 379 *gaoA* or *gaoB* genes. RNA and cDNA indicate the lanes in which the reactions were performed with DNase I-treated RNA and cDNA, respectively. The molecular marker (M) used was λDNA/*Hin*dIII

The *F. subglutinans gaoA* Gene and GaoA Protein

The *gaoA* gene from the *F. subglutinans* UnB 379 strain was amplified, cloned, and sequenced. The obtained gene sequence was deposited in GenBank with the accession number KM279622.1. The cloned gene has a GC content of 50.24%, an open reading frame (ORF) of 2046 bp, which encodes a 681-amino-acid residue protein, and no introns. The phylogenetic analysis of the *F. subglutinans* GaoA protein in relation to other *Fusarium* species GaoA proteins is shown in Fig. 2. The *F. subglutinans* GaoA protein has an identity of approximately 80% with the GaoA protein of *F. austroamericanum* (AN AAA16228.1) [25] and 95% with the GaoA proteins of *F. verticillioides* (AN DAA34000.1) [24] and *F. oxysporum* (AN AHA90705.1) [36]. This similarity made the *F. fujikuroi* and *F. oxysporum* complexes group together by means of their GaoA proteins. This indicates that *F. subglutinans* and *F. oxysporum* are more phylogenetically related, which agrees with data derived from total genome analysis pertaining to the phylogenetic relationships between *F. graminearum*, *F. oxysporum* f. sp. *lycopersic*i, and *F. verticillioides* [58], and with phylogenetic analysis with molecular markers such as the nucleotide sequences of the largest (RPB1) and second largest (RPB2) RNA polymerase II subunits genes [59].

Similar to other GaoA proteins, the *F. subglutinans* GaoA protein has no putative *N*-glycosylation sites either, but presents *N*-terminal secretory and maturation sequences and indicates conservation of important active site amino acids (Fig. 3). In silico analysis revealed that the protein derived from the cloned gene has an *N*-terminal F5/8 type C domain (PF00754) with a galactose-binding domain-like (CL0202), with an *E* value of $7.5 \times e^{-21}$, which consists of a globular unit with a β-sandwich structure. The central and largest part of the protein contains a Kelch motif domain (PF01344; CL0058), with an *E* value of $6.9 \times e^{-08}$, which has pseudo symmetry of seven repeated structures of four β-conformation segments forming a β-pleated antiparallel sheet. The third domain is a *C*-terminal domain of unknown function (DUF1929; PF09118), with an *E* value of $4.9 \times e^{-24}$ (Figs. 3, 4).

The in silico tridimensional model obtained for the mature *F. subglutinans* GaoA protein, built by comparison with the *F. austroamericanum* GaoA structure (PDB 1GOF) [46], is shown in Fig. 4a, b. This model confirms the three-domain existence with the cupric ion present near the central axis of the Kelch domain and β-conformation predominance. Details of the modeled *F. subglutinans* GaoA active site are shown in Fig. 4c. The amino acids directly involved in the

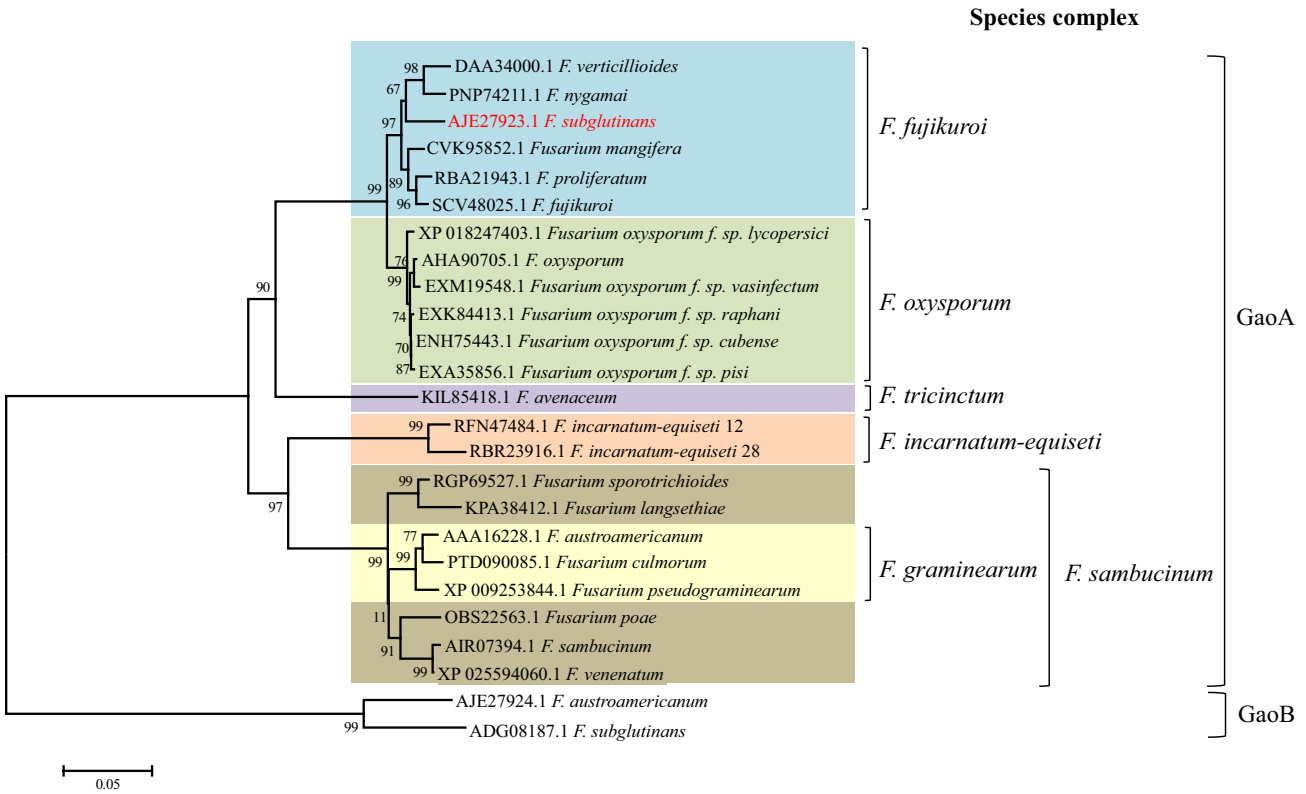

Fig. 2 The phylogenetic relationships among *Fusarium* spp. GaoA protein sequences. The phylogenetic history was inferred using the neighbor-joining method [48]. The optimal tree with the sum of branch length = 1.07431682 is shown. The percentages of replica trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [50]. The tree is drawn to scale, with branch lengths in number of amino acid substitutions per site. The analysis involved 25 amino acid sequences. All ambiguous positions were removed. There were a total of 691 positions in the final dataset. The phylogenetic tree was built in MEGA7 [49]. The *F. austroamericanum* and *F. subglutinans* GaoB proteins were used as an outgroup. The *Fusarium* species complexes reported by O'Donnell et al. [4, 55] are indicated (Color figure online)

galactose oxidase catalysis are conserved in the active site of the *F. subglutinans* GaoA protein (Cys230, Tyr274, Trp292, Tyr497, His498, and His583). The thioether bond presence between Cys230 and Tyr274 was also predicted (Fig. 4c). This bond decreases the redox potential of the tyrosyl/tyrosine pair creating an internal cofactor, which works together with the copper ion to catalyze the two-electron oxidation reaction [60]. The Trp292 indole ring is stacked with Tyr274 and, similar to the Trp290 of the *F. austroamericanum* GaoA protein, appears to protect the tyrosyl radical [61].

F. subglutinans gaoA Gene Expression Analysis

Most studies involving the heterologous expression of *gaoA* genes from *Fusarium* species have used pET plasmids in *E. coli* BL21 strains [31–33, 36, 37]. The truncated *F. subglutinans gaoA* gene, which was amplified with primers targeting the *F. verticillioides gaoA* gene and cloned into the pET101 plasmid, was expressed in the *E. coli* BL21 Star™ (DE3) strain cultured in LB-2X induced with 1 mM IPTG (0.27 U/mL of culture medium) and in ZYM-5052 induced with 1 mM IPTG (0.34 U/mL of culture medium). The LB-2X and ZY media were used before to express the *F. austroamericanum* GaoA protein [31, 33]. Enzyme production in ZYM-5052 was a little bit higher than in LB-2X. The auto-induction ZYM-5052 medium can vigorously enhance *E. coli* growth as it has several ions which improve bacterial metabolism and increase enzyme expression. Another difference between LB and ZYM-5052 is that the latter is buffered, which better maintains the culture pH around 7.0. However, although ZYM-5052 is an auto-induction medium, no enzyme was expressed without IPTG induction. One explanation for this is that although ZYM-5052 has lactose, a precursor for allolactose, which can induce expression, the presence of IPTG allows a stronger expression.

Considering the low level of expression of the *F. subglutinans* GaoA protein from pET101® and that it could not be Ni-affinity-chromatography purified because it did not have a His-tag, we decided to clone and sequence the entire *F. subglutinans gaoA* gene to design a better primer for its 5′

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F. subglutinans         MKSFWTLAFYLGNANAVAISQP--ASKAETPEGSLQFLSLRASAPIGTAINRDKWKVTCDSQHQGDECSK
F. oxysporum            MKPLWTLALYLGSASAVAISQP--AAKAETPEGSLQFLSLRASAPIGTAINRDKWRVTCDSQHEGDECSK
F. austroamericanum     MKHLLTLALCFSSINAVAVTVPHKAVGTGIPEGSLQFLSLRASAPIGSAISRNNWAVTCDSAQSGNECNK
F. sambucinum           -MYLLSLALCFSSINAVAINNPHKASGAEKPEGSLQFLSLRASAPIGSAISRNNWAVTCDSAQPGNECNK
                           : :**: :.. .***:. *  *  :  ******************:**.*::* *****  : *:**.*

F. subglutinans         AIDGDRNTFWHTNWAAGATNDPKPPHTITIDMGSSQNVNGLSVLPRQDGSDHGWIGRHNVFLSTDGKNWG
F. oxysporum            AIDGDRDTFWHTAWAAGGTNDPKPPHTITIDMGSSQNVNGLSVLPRQDGSDHGWIGRHNVFLSTDGKNWG
F. austroamericanum     AIDGNKDTFWHTFY--GANGDPKPPHTYTIDMKTTQNVNGLSMLPRQDGNQNGWIGRHEVYLSSDGTNWG
F. sambucinum           AIDGNRDTFWHTFY--GANGDPKPPHTYNIDMKTTQNVNGLSMLPRQDGSQNGWIGRHEVYLSSDGKTWG
                        ****:::***** :  *...******* .*** ::*******:******.::******:*:**:**..**

F. subglutinans         NAVATGTWFADITEKYSNFETRPARYVRLVAVTEANDQPWTSIAEINVFNAASYTSPQPGLGLWGPTLDF
F. oxysporum            DAVATGTWFADNTEKYSNFETRPARYVRLVTVTEANDQPWTSIAEINVFKAASYTSPQPGLGRWGPTLDF
F. austroamericanum     SPVASGSWFADSTTKYSNFETRPARYVRLVAITEANGQPWTSIAEINVFQAS SYTAPQPGLGRWGPTIDL
F. sambucinum           SPVATGSWFADSTTKYSNFETRPARYVRLVALAEANGQPWTSIAEINVYQASSYTAPQPGLGRWGPTIDL
                        . **:*:**** * ****************:::***.***********::*:***:****** ****:*:

F. subglutinans         PIVPVAAAVEPTSGKVLVWSSYKNDAFGGSPGGVTLTSTWDPSTGVISQRTVTVTKHDMFCPGISMDGNG
F. oxysporum            PIVPVAAAVEPTSGKVLVWSSYRNDAFGGSPGGVTLTSTWDPSTGVISQRTVTVTKHDMFCPGISMDGNG
F. austroamericanum     PIVPAAAAIEPTSGRVLMWSSYRNDAFGGSPGGITLTSSWDPSTGIVSDRTVTVTKHDMFCPGISMDGNG
F. sambucinum           PIVPAAAAIEPTSGRVLMWSSYRNDAFGGSPGGVTLTSSWDPSSGIVSDRTVTVTKHDMFCPGISMDGNG
                        ****.***:*****:**:****:**********:****:****:*::*:*********************

F. subglutinans         QVVVTGGNNAEKTSLYDSSSDSWIPGPDMKVARGYQFSATLSNGRVFTIGGSWSGGTFEKNGEVYDPSSK
F. oxysporum            QVVVTGGNDAQKTSLYDSSSDSWIPGPDMKVARGYQSSATLSNGRVFTIGGSWSGGIFEKNGEVYDPSSK
F. austroamericanum     QIVVTGGNDAKKTSLYDSSSDSWIPGPDMQVARGYQSSATMSDGRVFTIGGSWSGGVFEKNGEVYSPSSK
F. sambucinum           QIVVTGGNDAKKTSLYDSPSDSWVPGPDMQVARGYQSSATMSDGRVFTIGGSWSGGIFEKNGEVYSPSSK
                        *:******:*:******* ****:*****:****** ***:*:************* ********.****

F. subglutinans         TWTSLPGALVKPMLTADQQGIYRSDNHGWLFGWKKGSVFQAGPSTAMNWYYTSGKGNTKSAGKRQSSRGT
F. oxysporum            TWTSLPGALVKPMLTADQQGLYRSDNHGWLFGWKKGSVFQAGPSTAMNWYYTTGNGDVKSAGKRQSSRGT
F. austroamericanum     TWTSLPNAKVNPMLTADKQGLYRSDNHAWLFGWKKGSVFQAGPSTAMNWYYTSGSGDVKSAGKRQSNRGV
F. sambucinum           TWTSLPNAKVNPMLTADKQGLYRSDNHAWLFGWKKGSVFQAGPSTAMNWYYTSGSGDVKSAGKRQSNRGV
                        ******.* *:******:**:******.************************:*.*:.********.**.

F. subglutinans         DPDAMCGNAVMYDAVKGKILTFGGSPSYQDSDATTNAHIITIGEPGSTPKTVFASNGLYYPRTFHTSVIL
F. oxysporum            DPDAMCGNAVMYDAVKGKILTFGGSPSYQDSDATTNAHIITISEPGSTPKTVFASNGLYYPRTFHTSVIL
F. austroamericanum     APDAMCGNAVMYDAVKGKILTFGGSPDYQDSDATTNAHIITLGEPGTSPNTVFASNGLYFARTFHTSVVL
F. sambucinum           APDAMCGNAVMYDAVQGKILTFGGSPDYQDSDATTNAHIITLGEPGSTPKTVFASNGLYFARTFHTSVVL
                         **************:**********.***************:.***::*:*********:  *******:*

F. subglutinans         PDGNVFITGGQQRGIPFADSTPQLTPELYVPNDDTFYKQQPNSIVRVYHSISLLLPDGRVFNGGGGLCGD
F. oxysporum            PDGNVFITGGQQRGIPFADSTPQLTPELYVPNDDTFYKQQPNSIVRVYHSISLLLPDGRVFNGGGGLCGD
F. austroamericanum     PDGSTFITGGQRRGIPFEDSTPVFTPEIYVPEQDTFYKQNPNSIVRVYHSISLLLPDGRVFNGGGGLCGD
F. sambucinum           PDGSTFITGGQRRGIPFEDSTPVLTPEIYVPEQDTFYKQN PNSIVRVYHSISLLLPDGRVFNGGGGLCGD
                        ***..******:***** **** :***:***::******:*****************************

F. subglutinans         CSTNHFDAQIYTPNNLYDSNGKLATRPKITNVSAKSAKVGGKITISTDTS IKQASLIRYGTSTHTVNTDQ
F. oxysporum            CDTNHFDAQIYTPNNLYDSNGKLATRPKITKVSAKSVKVGGKITITADTSIKQASLIRYGTST HTVNTDQ
F. austroamericanum     CTTNHFDAQIFTPNYLYNSNGNLATRPKITRTSTQSVKVGGRITISTDSSISKASLIRYGTAT HTVNTDQ
F. sambucinum           CTTNHFDAQIFTPNYLYDSNGNLATRPKITRTSTQSVKVGGRVTISTDY SIRKASLIRYGTATHTVNTDQ
                        * ********:*** **:***:********..*:::*.****::**::* ** :********:********

F. subglutinans         RRIPLSLRSTGSGNSYSFQVPSDSGIALPGYWMLFVMNSAGVPSI ASTLLVTQ
F. oxysporum            RRIPLSLRRTGTGNSYSFQVPSDSGIALPGYWMLFVMNSAGVPSVASTLLVTQ
F. austroamericanum     RRIPLTLTNN-GGNSYSFQVPSDSGVALPGYWMLFVMNSAGVPSVASTIRVTQ
F. sambucinum           RRIPLTLMNN-GGNSYSFQVPSDTGIALPGYWMLFVINSAGVPSVASTIRVTQ
                        *****:*  .  ***********:*:**********:*******:***: ***
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Fig. 3 Alignment of *Fusarium* GaoA proteins. *F. subglutinans* (AJE27923.1), *F. oxysporum* (AHA90705.1), *F. austroamericanum* (AAA16228.1), and *F. sambucinum* (AIR07394.1). GaoA proteins were aligned using Clustal Omega. Conserved active site amino acids are in red. The *F. austroamericanum* GaoA protein secretion signal peptide is in green and the maturation peptide is in blue. The *F. subglutinans* GaoA protein was predicted as a three-domain protein (colored in yellow, blue, and green). (*) identical residues; (:) non-conserved substitutions; (.) semi-conserved substitutions (Color figure online)

internal sequence, targeting the coding region for the mature protein. In addition, it was hypothesized that the resulting mRNA could be better translated with the correct codons. Nevertheless, no galactose activity was detected from the construct pET21a(+)-truncated *F. subglutinans gaoA* gene transformed in the *E. coli* Bl21 Star™ (DE3) strain, which was grown in ZYM-5052 medium induced with 1 mM IPTG. The vectors pET101® and pET21a(+) have the same T7 RNA polymerase promoter. However, the pET101 vector provides the transcribed mRNA two RBS sequences, while the pET21a(+)vector provides only one. This fact may explain, at least in part, why no expression could be

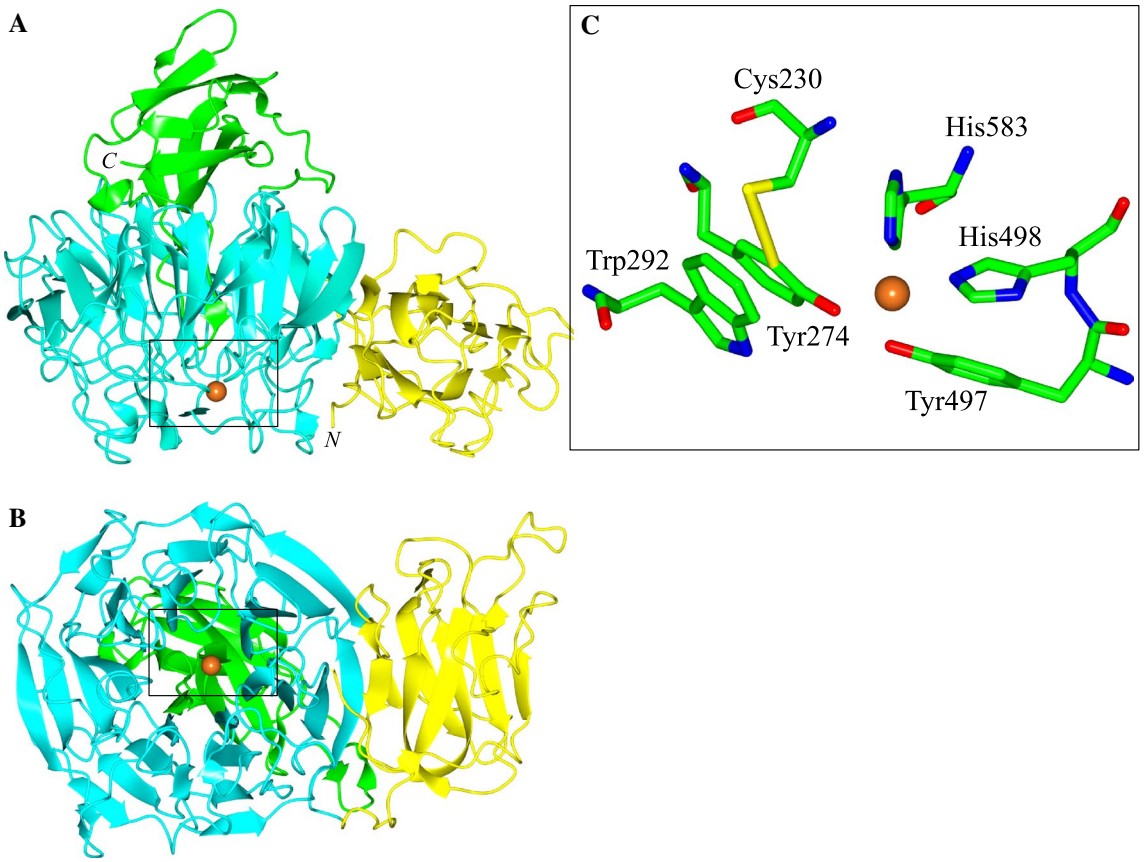

Fig. 4 Theoretical three-dimensional structural model of the mature *F. subglutinans* GaoA protein. **a** The structural model was built using the *F. austroamericanum* GaoA structure as the template (PDB-1gofA) [46]. Protein domain I is shown in yellow, domain II in blue, and domain III in green, as shown in Fig. 3. The active site is shown in the inset and the copper ion is shown in orange. *N* and *C* indicate the protein amino and carboxy-terminus, respectively. **b** Horizontal 90° rotation of picture A to the front. In evidence is the Kelch domain, a pseudo-symmetric structure of seven structures. **c** The conserved amino acid residues of the active site (Color figure online)

achieved with pET21a(+). In fact, the predicted structure of the *F. subglutinans gaoA* mRNA 5′ first 228 nucleotides (without the pre-pro sequence) has a minimum free energy of −70.10 kcal/mol. The same region of the *F. austroamericanum ga*oA mRNA sequence has a predicted structure with a minimum free energy of −56.30 kcal/mol. In agreement with this, Deacon and McPherson [34] obtained *F. austroamericanum gaoA* gene mutants with mRNA structures having lower minimum free energy, which increased expression in *E. coli*.

Efficient expression was obtained when the truncated *F. subglutinans gaoA* gene was expressed from pTrcHis2B only in the *E. coli* Rosetta™ (DE3) strain (1.37 U/mL culture medium) cultured in ZYM5052 medium induced with 1 mM IPTG. The vector pTrcHis2B has a mini cistron, which provides effective translational restart and, therefore, is useful for expression of eukaryotic proteins in *E. coli*. The truncated *F. subglutinans gaoA* gene mRNA has a CAI value of 0.6, which is lower than the value of 0.62 from the truncated *F. subglutinans gaoA* gene mRNA, indicating that it has a higher quantity of rare codons. The better translation initiation provided by the pTrcHis2B plasmid, in combination with the rare codon tRNAs in the *E. coli* Rosetta™ (DE3) strain, may have been responsible for the improved galactose oxidase expression.

F. subglutinans GaoA Protein Expression and Purification

The truncated *F. subglutinans gaoA* gene cloned in pTrcHis2B was successfully expressed in the *E. coli* Rosetta™ (DE3) strain. However, this construction expression did not occur in *E. coli* TOP10 or DH5α strains. The expressed GaoA protein was purified with a production of 1.38 mg/L (1732 U/L) of culture liquid medium with a specific activity of 1255 U/mg. This production is superior to the 258 U/L (Table 1) native production by *F. subglutinans* obtained in this study and also superior to the production of 0.6 mg/L obtained by Deacon and McPherson [33] and Sun et al. [29] of wild-type *F. austroamericanum*

GaoA protein expressed in *E. coli*. However, Paukner et al. [36] obtained a production of 5.7 mg/L of active and soluble *F. oxysporum* GaoA protein expressed in *E. coli*, with a specific activity of 65.4 U/mg; and Paukner et al. [37] obtained 4.4 mg/L of active and soluble *F. sambucinum* GaoA protein expressed in *E. coli*, with a specific activity of 159 U/mg. Nevertheless, the higher specific activity obtained for the *F. subglutinans* GaoA protein in this study indicates that this protein was efficiently produced, purified, and activated.

SDS-PAGE analysis (Fig. 5a) shows that the galactose oxidase was purified to homogeneity and that two bands (77 kDa for one and 74 kDa for the other) were observed, in agreement with the predicted molecular weight of 72.5 kDa for the enzyme, with the additional *N*- and *C*-terminal tails coded by the plasmid. Although most recombinant and native GaoA proteins have a molecular size around 70 kDa [13, 15, 36, 37, 62], Aisaka and Terada [7] found a molecular weight of 90 kDa for the native *G. fujikuroi* galactose oxidase protein using gel filtration. No other GaoA protein of the *F. fujikuroi* species complex has been purified and studied.

The predicted pI of the purified recombinant *F. subglutinans* GaoA protein is 6.65 with the additional *N*- and *C*-sequences and 7.63 without. Aisaka and Terada [7] found a lower pI of 3.7 for the native *G. fujikuroi* galactose oxidase protein using isoelectric focusing. The native *F. austroamericanum* GaoA protein is reported to have a pI of 12 [63]. A more neutral protein is more adequate for histochemical studies because it is not inhibited by contaminants and is less reactive with cell membrane glycoconjugates [64].

Both purified protein bands found in the SDS-PAGE analysis (Fig. 5a) were confirmed to be galactose oxidase by mass spectrometry with the same trypsin produced 1457.77 mass *N*-terminal peptide (SAAGTASAPIGTAINR) (Fig. 5b–d). However, the presence of two protein bands implies that the purified enzyme was not fully processed, as reported for the *F. austroamericanum* GaoA protein [26, 62]. One possible cause is the incomplete thioether bond formation between Cys230 and Tyr274, because it

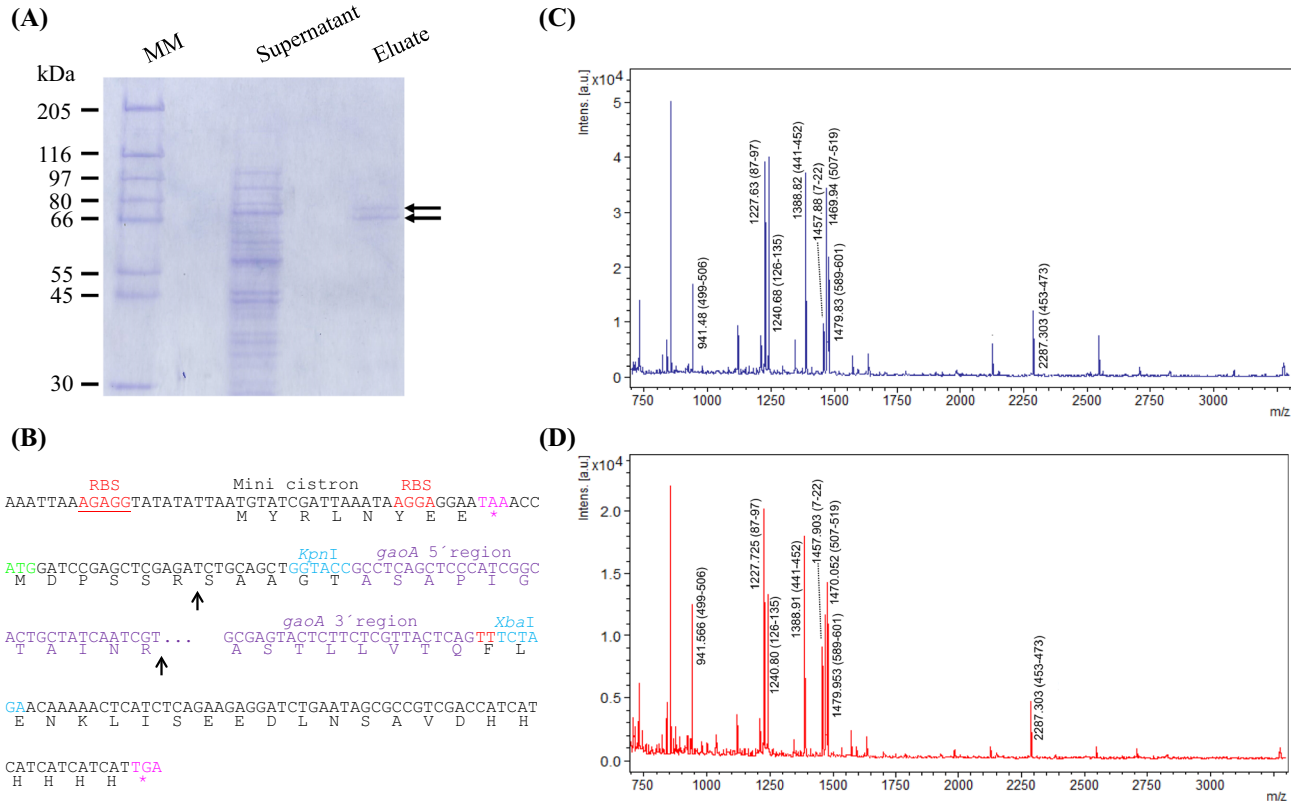

Fig. 5 SDS-PAGE of the *F. subglutinans* GaoA protein expressed from pTrcHis2B in the *E. coli* Rosetta™ (DE3) strain and MALDI-TOF peptide mass map of the expressed protein. **a** Proteins were developed in a 7.5% discontinuous gel. The supernatant was obtained after cell lysis and centrifugation, as described above. An aliquot of approximately 10 µg of the supernatant proteins and the recombinant purified protein was applied in the gel. The positions of the molecular weight markers are indicated. The gel was stained with Coomassie Blue. **b** The pTrcHis2B-*F. subglutinans* GaoA construction showing the minicistron and the trypsin cut position into the *N*-terminal fragment (arrows). **c**, **d** Superior and inferior protein band, respectively, mass spectrometry analysis. The peptides were generated by sequential digestion using trypsin. The peak labels (in the right or left of the peaks, or indicated with a dotted line) correspond to the ion mass of the obtained peptide fragments and their positions in the *F. subglutinans* GaoA expressed from pTrcHis2B (Color figure online)

prevents the complete unfolding by SDS treatment and the fully processed enzyme migrates faster in SDS-PAGE [26, 62]. However, in the mass spectrometry analysis, the peptide GYQFSATLSNGR (mass of 1300.62), which contains Tyr274, was not identified in either protein band, indicating that it could be joined to the peptide HDMFCPGISMDGNGQVVVTGGNNAEK (mass of 2677.18), which contains Cys230 and was also unidentified due to the parameters of the used equipment (Fig. 5c, d). Mass spectrometry was used before to search for the GaoA thioether bond [36]. Therefore, it is reasonable to conclude that other maturation processes may have occurred and given rise to the protein bands. In fact, Firbank et al. [65] determined the structure of the *F. austroamericanum* GaoA precursor protein at 1.4 Å and identified structural differences between the precursor and the mature protein in five regions of the main chain, in addition to the significant differences among key residues of the active site between the two forms.

Table 2 *F. subglutinans* GaoA activity against different substrates

Substrate	Final concentration	Enzymatic activity (relative activity %)
D-(+)-Galactose	25 mM	100
D-(+)-Glucose	25 mM	0
Sucrose	25 mM	0
Lactose	25 mM	3
D-(+)-Raffinose	25 mM	223
α-D-(+)-Melibiose	25 mM	99
Glycerol	25 mM	0
Methyl-α-D-galactopyranoside	25 mM	123
Guar gum	1%	0

Biochemical Properties of the *F. subglutinans* Recombinant GaoA Protein

The *F. subglutinans* GaoA protein displayed activity against different sugars such as D-(+)-raffinose, α-D-(+)-melibiose, and methyl-α-D-galactopyranoside, besides D-(+)-galactose, presenting preference for D-(+)-raffinose (Table 2), which is similar to other *Fusarium* GaoA proteins [9, 13, 37]. The fact that the *F. subglutinans* GaoA protein oxidizes galactose derivatives with substitutes at the carbon-1 and has no activity against D-(+)-glucose confirms its stereo specificity.

Considering the high sequence and structural identity between the *F. subglutinans* GaoA protein and other GaoA proteins, the former would, predictably, have similar optima pH and temperature. In fact, the optima pH and temperature of the purified enzyme were 7.0 and 30 °C, respectively (Fig. 6a, d), which is very close to the parameters of the native *F. austroamericanum* galactose oxidase [13, 29, 63,

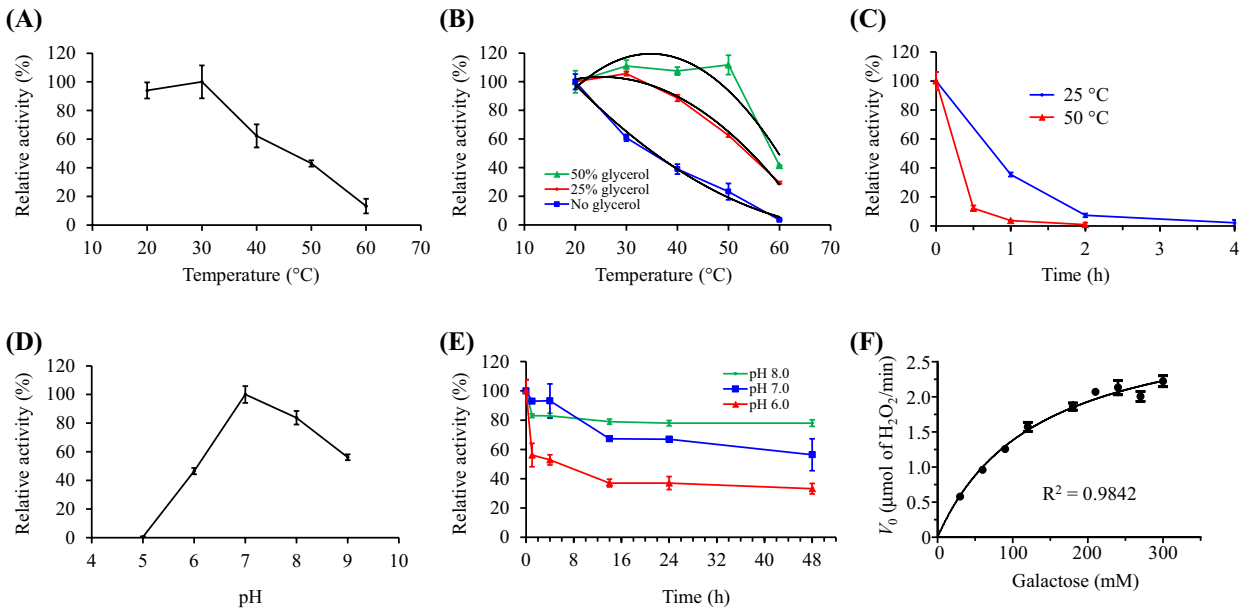

Fig. 6 *F. subglutinans* GaoA characterization. **a** Optimum temperature at pH 7.0. **b** Thermal stability. The enzyme was assayed after a 10-min incubation at temperatures ranging from 20 to 60 °C, at pH 7.0, in the absence or presence of 25 or 50% (v/v) glycerol. **c** Thermal stability over time at pH 7.0 and in 50% (v/v) glycerol. **d** Optimum pH. **e** pH stability over time at 4 °C. **f** The *F. subglutinans* GaoA protein saturation curve showing the relation between the substrate concentration and reaction rate. Enzyme activity was measured at 30 °C, in 50 mM phosphate buffer, pH 7.0, for 5 min, in 50% (v/v) glycerol. All the results are the means and standard deviations of the analyses carried out in triplicate

64], the native *F. acuminatum* galactose oxidase [9], and the recombinant *F. sambucinum* GaoA protein [37].

In contrast, the *F. subglutinans* GaoA protein T_{50} (temperature at which the enzyme loses 50% of its activity after a 10-min incubation), calculated from the second order polynomial regression curve equation, was 35.47 °C (Fig. 6b), which is much lower than the T_{50} found for the *F. austroamericanum* and *F. acuminatum* enzymes (64.6 °C and 64.6 °C, respectively) [9, 29]. However, thermal stability increased to 54.45 and 59.77 °C when 25% or 50% (v/v) glycerol was added, respectively (Fig. 6b). Although the *F. sambucinum* GaoA protein remained stable after 24 h at 30 °C [37], the *F. subglutinans* GaoA protein showed little stability at 25 and 50 °C over time, losing almost all its activity after 4 h of incubation in the presence of 50% glycerol (Fig. 6c). On the other hand, the *F. subglutinans* GaoA protein presented greater stability at pH 8.0 and 4 °C, remaining 80% active after 48 h of incubation (Fig. 6e). These results highlight that small changes in amino acid sequence and structure have modified the enzyme characteristics regarding pH and temperature stability.

At 30 °C, pH 7.0, in 50% (v/v) glycerol presence, the *F. subglutinans* GaoA protein presented a Michaelian dependency on galactose concentration (Fig. 6e), with a K_M of 132.6 ± 18.18 mM for galactose, V_{max} of 3.2 ± 0.18 µmol of H_2O_2/min, k_{cat} of 12,243 s^{-1}, and k_{cat}/K_M of 9.2×10^4 M^{-1} s^{-1} (Fig. 6f). Similar results were obtained when the purified recombinant enzyme was examined in the absence of 50% (v/v) glycerol. The enzyme K_M is higher than those found for galactose of other *Fusarium* GaoA proteins, such as 71 mM and 57 mM for the recombinant *F. austroamericanum* GaoA protein [26, 29], 16.2 mM for the native *F. acuminatum* GaoA protein [9], and 47 mM and 61 mM for the recombinant *F. oxysporum* and *F. sambucinum* GaoA proteins, respectively [36, 37]. This indicates that the enzyme characterized in this study has lower affinity for galactose compared to other characterized GaoA proteins. However, the specificity constant (k_{cat}/K_M) of the recombinant *F. subglutinans* GaoA protein is higher than the value of the recombinant *F. sambucinum* GaoA protein of 0.89×10^3 M^{-1} s^{-1} [37], the recombinant *F. oxysporum* GaoA protein of 2 mM^{-1} s^{-1} [36], the recombinant *F. graminerarum* GaoA protein of 1.88×10^4 M^{-1} s^{-1} [29], and the native *F. graminerarum* GaoA protein of 1.46×10^4 M^{-1} s^{-1} [29]. The higher value of k_{cat}/K_M of the *F. subglutinans* GaoA protein indicates that it has higher efficiency compared to the other characterized GaoA proteins.

The effect of various compounds on the recombinant *F. subglutinans* GaoA protein activity is shown in Table 3. No tested compounds affected the peroxidase activity of the enzyme assay, which confirmed their action on galactose oxidase (Table 3). Monovalent and divalent cations (K^+, Na^+, Mg^{2+}) cations had almost no observed effect on the enzyme activity, confirming earlier results for other galactose oxidases [9, 37]. This reaffirms that the purification method used in this study, which employs Na^+ and Mg^{2+}, did not inhibit the enzyme. The anionic detergent SDS significantly inhibited the enzyme; whereas the cationic detergent CTAB was a weak inhibitor. This indicates that enzyme activity depends more on positive charges in its structure. In addition, the enzyme presented higher activity in the presence of Tween 80, a non-ionic detergent. In former studies, Tween 80 was shown to improve the absorbance reading of the galactose oxidase reaction [9], and other detergents, such as Triton X-100, were already used in the galactose oxidase reaction assay [5]. In fact, Triton X-100 was used in this study in the expression analysis and the information that neutral detergent does not affect enzyme activity is important to reaffirm the obtained results. EDTA did not significantly inhibit the *F. subglutinans* GaoA protein. Similar results were obtained for other *Fusarium* GaoA proteins [1, 7, 9]. Glycerol substantially improved the recombinant *F. subglutinans* GaoA protein activity (Table 3), which was also observed for the native *F. acuminatum* GaoA protein [9]. The role of glycerol in stabilizing enzymes is well known [66, 67] and its effect on the recombinant *F. subglutinans* GaoA protein may be related, at least in part, to the increased enzyme thermal stability shown above.

Table 3 Impact of potential inhibitors and activators on the galactose oxidase activity of *F. subglutinans*

Compound	Concentration	Relative galactose oxidase activity (%)	Relative peroxidase activity (%)
NaCl	5 mM	90	101
$MgCl_2 \cdot 6H_2O$	5 mM	107	101
KCl	5 mM	90	98
EDTA	5 mM	81	100
CTAB	5 mM	77	121
SDS	5 mM	4	105
Glycerol	25%	774	104
Tween 80	2.5%	316	99

Conclusions

Among several species of the *F. fujikuroi* species complex, *F. subglutinans* was found to be the largest galactose oxidase producer. The *F. subglutinans gaoA* gene was cloned, sequenced, and expressed from the pTrcHis2B plasmid in the *E. coli* Rosetta™ (DE3) strain. For the first time, the heterologous expression of a GaoA protein from the *F. fujikuroi* species complex was achieved and a new expression system for galactose oxidase was reported. Moreover, the recombinant *F. subglutinans* GaoA protein has been purified

and characterized. The purified enzyme presented a high catalytic efficiency. In addition, it is predicted to be a neutral protein, what is good for its possible application in histochemical studies.

Acknowledgments The authors thank CNPq (Conselho Nacional de Pesquisa e Desenvolvimento—Ministry of Science and Technology—Brazil) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Grant 001—Ministry of Education, Brazil) for the students' scholarships. The authors are also thankful to the Department of Biochemistry from the Federal University of Paraná, Brazil, for the use of its MS facility.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

References

1. Cooper, J. A. D., Smith, W., Bacila, M., & Medina, H. (1958). Galactose oxidase from *Polyporus circinatus* Fr. *Journal of Biological Chemistry, 234,* 445–448.
2. Nobles, M. K., & Madhosingh, C. (1963). *Dactylium dendroides* (Bull.) Fr. misnamed as *Polyporus circinatus* Fr. *Biochemistry and Biophysics Research Communications, 12,* 146–147. https://doi.org/10.1016/0006-291X(63)90251-1.
3. Ögel, Z. B., Brayford, D., & McPherson, M. J. (1994). Cellulose triggered sporulation in the galactose oxidase-producing fungus *Cladobotryum* (*Dactylium*) *dendroides* NRRL 2903 and its re-identification as a species of *Fusarium*. *Mycological Research, 98,* 474–480. https://doi.org/10.1016/S0953-7562(09)81207-0.
4. O'Donnell, K., Ward, T. J., Geiser, D. M., Kistler, H. C., & Aoki, T. (2004). Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology, 41,* 600–623. https://doi.org/10.1016/j.fgb.2004.03.003.
5. Gancedo, J. M., Gancedo, C., & Asensio, C. (1967). Widespread occurrence of galactose oxidase and glucose oxidase in fungi. *Archives of Biochemisry and Biophysics, 119*(1), 588–590. https://doi.org/10.1016/0003-9861(67)90498-5.
6. Aisaka, K., & Terada, O. (1981). Production of galactose oxidase by *Gibberella fujikuroi*. *Agricultural and Biological Chemistry, 45*(10), 2311–2316. https://doi.org/10.1080/00021369.1981.10864879.
7. Aisaka, K., & Terada, O. (1982). Purification and properties of galactose oxidase from *Giberella fujikuroi*. *Agricultural and Biological Chemistry, 46,* 1191–1197. https://doi.org/10.1080/00021369.1982.10865226.
8. Barbosa-Tessmann, I. P., Silva, D., Peralta, R. M., & Kemmelmeier, C. (2001). A new species of *Fusarium* producer of galactose oxidase. *Journal of Basic Microbiology, 41,* 143–148.
9. Alberton, D., Oliveira, L. S., Peralta, R. M., & Barbosa-Tessmann, I. P. (2007). Production, purification and characterization of a novel galactose oxidase from *Fusarium acuminatum*. *Journal of Basic Microbiology, 47,* 203–212. https://doi.org/10.1002/jobm.200610290.
10. Silakowski, B., Ehret, H., & Schairer, H. U. (1998). *fbfB*, a gene encoding a putative galactose oxidase, is involved in *Stigmatella aurantiaca* fruiting body formation. *Journal of Bacteriology, 180*(5), 1241–1247.
11. Whittaker, M. M., & Whittaker, J. W. (2006). *Streptomyces coelicolor* oxidase (SCO2837p): A new free radical metalloenzyme secreted by *Streptomyces coelicolor* A3(2). *Archives of Biochemistry and Biophysics, 452*(2), 108–118. https://doi.org/10.1016/j.abb.2006.06.020.
12. Liman, R., Facey, P. D., van Keulen, G., Dyson, P. J., & Del Sol, R. (2013). A laterally acquired galactose oxidase-like gene is required for aerial development during osmotic stress in *Streptomyces coelicolor*. *PLoS ONE, 8*(1), e54112. https://doi.org/10.1371/journal.pone.0054112.
13. Whittaker, J. W. (2005). The radical chemistry of galactose oxidase. *Archives of Biochemistry and Biophysics, 443,* 227–239. https://doi.org/10.1016/j.abb.2004.08.034.
14. Turner, N. J. (2011). Enantioselective oxidation of C–O and C–N bonds using oxidases. *Chemical Reviews, 111,* 4073–4087. https://doi.org/10.1021/cr200111v.
15. Solomon, E. I., Heppner, D. E., Johnston, E. M., Ginsbach, J. W., Cirera, J., Qayyum, M., et al. (2014). Copper active sites in biology. *Chemical Reviews, 114,* 3659–3853. https://doi.org/10.1021/cr400327t.
16. Shetty, N. P., Jørgensen, H. J. L., Jensen, J. D., Collinge, D. B., & Shetty, H. S. (2008). Roles of reactive oxygen species in interactions between plants and pathogens. *European Journal of Plant Pathology, 121,* 267–280. https://doi.org/10.1007/s10658-008-9302-5.
17. Kersten, P. J., & Kirk, T. K. (1987). Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. *Journal of Bacteriology, 169*(5), 2195–2201.
18. Kanyong, F., Krampa, D., Aniweh, Y., & Awandare, G. A. (2017). Enzyme-based amperometric galactose biosensors: A review. *Mikrochimica Acta, 184,* 3663–3671. https://doi.org/10.1007/s00604-017-2465-z.
19. Monti, D., Ottolina, G., Carrea, G., & Riva, S. (2011). Redox reactions catalyzed by isolated enzymes. *Chemical Reviews, 111,* 4111–4140. https://doi.org/10.1021/cr100334x.
20. Shamsuddin, A. M. (1996). A simple mucus test for cancer screening. *Anticancer Research, 16,* 2193–2199.
21. Parikka, K., Leppänen, A. S., Pitkänen, L., Reunanen, M., Willförd, S., & Tenkanen, M. (2010). Oxidation of polysaccharides by galactose oxidase. *Journal of Agricultural and Food Chemistry, 58,* 262–271. https://doi.org/10.1021/jf902930t.
22. Henderson, G. E., Isett, K. D., & Gerngross, T. U. (2011). Site-specific modification of recombinant proteins: A novel platform for modifying glycoproteins expressed in *E. coli*. *Bioconjugate Chemistry, 22,* 903–912. https://doi.org/10.1021/bc100510g.
23. Ramya, T. N. C., Weerapana, E., Cravatt, B. F., & Paulson, J. C. (2013). Glycoproteomics enable by tagging sialic acid or galactose-terminated glycans. *Glycobiology, 23*(2), 211–221. https://doi.org/10.1093/glycob/cws144.
24. Cordeiro, F. A., Faria, C. B., & Barbosa-Tessmann, I. P. (2010). Identification of new galactose oxidase genes in *Fusarium* spp. *Journal of Basic Microbiology, 50,* 527–537. https://doi.org/10.1002/jobm.201000078.
25. McPherson, M. J., Ögel, Z. B., Stevens, C., Yadav, K. D. S., Keen, J. N., & Knowles, P. F. (1992). Galactose oxidase of *Dactylium dendroides*. Gene cloning and sequence analysis. *Journal of Biolological Chemistry, 267,* 8146–8152.
26. Baron, A. J., Stevens, C., Wilmot, C., Seneviratne, K. D., Blakeley, V., Dooley, D. M., et al. (1994). Structure and mechanism of galactose oxidase. The free radical site. *Journal of Biolological Chemistry, 269,* 25095–25105.
27. Whittaker, M. M., & Whittaker, J. W. (2000). Expression of recombinant galactose oxidase by *Pichia pastoris*. *Protein Expression and Purification, 20,* 105–111. https://doi.org/10.1006/prep.2000.1287.

28. Xu, F., Golightly, E. J., Schneider, P., Berka, R. M., Brown, K. M., Johnstone, J. A., et al. (2000). Expression and characterization of a recombinant *Fusarium* spp. galactose oxidase. *Applied Biochemistry and Biotechnology, 88,* 23–32. https://doi.org/10.1385/ABAB:88:1-3:023.
29. Sun, L., Petrounia, I. P., Yagasaki, M., Bandara, G., & Arnold, F. H. (2001). Expression and stabilization of galactose oxidase in *Escherichia coli* by directed evolution. *Protein Engineering, 14,* 699–704. https://doi.org/10.1093/protein/14.9.699.
30. Wilkinson, D., Akumanyi, N., Hurtado-Guerrero, R., Dawkes, H., Knowles, P. F., Phillips, S. E. V., et al. (2004). Structural and kinectic studies of a series of mutants of galactose oxidase identified by directed evolution. *Protein Engineering, 17,* 141–147. https://doi.org/10.1093/protein/gzh018.
31. Choosri, W., Paukner, R., Wührer, P., Haltrich, D., & Leitner, C. (2010). Enhanced production of recombinant galactose oxidase from *Fusarium graminearum* in *E. coli*. *World Journal of Microbiology and Biotechnology, 27*(6), 1349–1353. https://doi.org/10.1007/s11274-010-0585-2.
32. Spadiut, O., Olsson, L., & Brumer, H., III. (2010). A comparative summary of expression systems for the recombinant production of galactose oxidase. *Microbial Cell Factories, 9*(68), 1–13. https://doi.org/10.1186/1475-2859-9-68.
33. Deacon, S. E., & McPherson, M. J. (2011). Enhanced expression and purification of fungal galactose oxidase in *Escherichia coli* and use for analysis of a saturation mutagenesis library. *ChemBioChem, 12,* 593–601. https://doi.org/10.1002/cbic.201000634.
34. Golightly, E., Berka, R. M., & Rey, M. W. (2001). Polypeptides having galactose oxidase activity and nucleic acids encoding same. *US Patent, 6*(277), 612.
35. Faria, C. B., Abe, C. A. L., Silva, C. N., Tessmann, D. J., & Barbosa-Tessmann, I. P. (2012). New PCR assays for the identification of *Fusarium verticillioides*, *Fusarium subglutinans*, and other species of the *Gibberella fujikuroi* complex. *International Journal of Molecular Sciences, 13,* 115–132. https://doi.org/10.3390/ijms13010115.
36. Paukner, R., Staudigl, P., Choosri, W., Haltrich, D., & Leitner, C. (2015). Expression, purification, and characterization of galactose oxidase of *Fusarium sambucinum* in *E. coli*. *Protein Expression and Purification, 108,* 73–79. https://doi.org/10.1016/j.pep.2014.12.010.
37. Paukner, R., Staudigl, P., Choosri, W., Sygmund, C., Halada, P., Haltrich, D., et al. (2014). Galactose oxidase from *Fusarium oxysporum*—Expression in *E. coli* and *P. pastoris* and biochemical characterization. *PLOS ONE, 9*(6), e100116. https://doi.org/10.1371/journal.pone.0100116.
38. Markus, Z., Miller, G., & Avigad, G. (1965). Effect of culture conditions on the production of D-galactose oxidase by *Dactylium dendroides*. *Applied Microbiology, 13,* 686–693.
39. Koenig, R. L., Ploetz, R. C., & Kistler, H. C. (1997). *Fusarium oxysporum* f. sp. *cubense* consists of a small number of divergent and globally distributed clonal lineages. *Phytopathology, 87,* 915–923. https://doi.org/10.1094/PHYTO.1997.87.9.915.
40. Chung, C. T., Niemela, S. L., & Miller, R. H. (1989). One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. *Proceedings of the National Academy of Sciences USA, 86,* 2172–2175. https://doi.org/10.1073/pnas.86.7.2172.
41. Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). New York: Cold Spring Harbor Laboratory Press.
42. Studier, F. W. (2005). Protein production by auto-induction in high-density shaking cultures. *Protein Expression and Purification, 41,* 207–234. https://doi.org/10.1016/j.pep.2005.01.016.
43. Tressel, P. S., & Kosman, D. J. (1982). Galactose oxidase from *Dactylium dendroides*. *Methods in Enzymology, 89,* 163–171.
44. Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41,* 95–98.
45. Webb, B., & Sali, A. (2016). Comparative protein structure modeling using modeller. *Current Protocols in Bioinformatics, 54,* 561–5637. https://doi.org/10.1002/cpbi.3.
46. Ito, N., Phillips, S. E. V., Stevens, C., Ogel, Z. B., Mcpherson, M. J., Keen, J. N., et al. (1991). Novel thioether bond revealed by 1,7 Å crystal structure of galactose oxidase. *Nature, 350,* 87–90. https://doi.org/10.1038/350087a0.
47. McNicholas, S., Potterton, E., Wilson, K. S., & Noble, M. E. M. (2011). Presenting your structures: The CCP4MG molecular-graphics software. *Acta Crystallographica*, *D67*, 386–394.
48. Saitou, N. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4,* 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.
49. Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution, 33,* 1870–1874. https://doi.org/10.1093/molbev/msw054.
50. Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution, 39,* 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x.
51. Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences USA, 101,* 11030–11035. https://doi.org/10.1073/pnas.0404206101.
52. Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of dye-binding. *Analytical Biochemistry, 72,* 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.
53. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bactheriophage T4. *Nature, 227,* 680–685. https://doi.org/10.1038/227680a0.
54. Perkins, D. N., Pappin, D. J., Creasy, D. M., & Cottrell, J. S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis, 20*(18), 3551–3567. https://doi.org/10.1002/(SICI)1522-2683(19991201)20:18%3c3551:AID-ELPS3551%3e3.0.CO;2-2.
55. O'Donnell, K., Cigelnik, E., & Nirenberg, H. I. (1998). Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia, 90*(3), 465–493. https://doi.org/10.2307/3761407.
56. Brown, D. W., Cheung, F., Proctor, R. H., Butchko, R. A. E., Zheng, L., Lee, Y., et al. (2005). Comparative analysis of 87,000 expressed sequence tags from the fumonisin-producing fungus *Fusarium verticillioides*. *Fungal Genetics and Biology, 42,* 848–861. https://doi.org/10.1016/j.fgb.2005.06.001.
57. Ögel, Z. B., & Ozilgen, M. (1995). Regulation and kinetic modeling of galactose oxidase secretion. *Enzyme and Microbial Technology, 17*(10), 870–876. https://doi.org/10.1016/0141-0229(94)00145-H.
58. Ma, L. J., van der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M. J., Di Pietro, A., et al. (2010). Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature, 464,* 367–373. https://doi.org/10.1038/nature08850.
59. O'Donnell, K., Rooney, A. P., Proctor, R. H., Brown, D. W., McCormick, S. P., Ward, T. J., et al. (2013). Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria. *Fungal Genetics and Biology, 52,* 20–31. https://doi.org/10.1016/j.fgb.2012.12.004.
60. Lee, Y. K., Whittaker, M. M., & Whittaker, J. W. (2008). The electronic structure of the Cys-Tyr˙ free radical in galactose oxidase

determined by EPR spectroscopy. *Biochemisry, 47,* 6637–6649. https://doi.org/10.1021/bi800305d.
61. Rogers, M. S., Tyler, E. M., Akyumani, N., Kurtis, C. R., Spooner, R. K., Deacon, S. E., et al. (2007). The stacking tryptophan of galactose oxidase, a second-coordination sphere residue that has profound effects on tyrosyl radical behavior and enzyme catalysis. *Biochemistry, 46,* 4606–4618. https://doi.org/10.1021/bi062139d.
62. Rogers, M. S., Baron, A. J., McPherson, M. J., Knowles, P. F., & Dooley, D. M. (2000). Galactose oxidase pro-sequence cleavage and cofactor assembly are self-processing reactions. *Journal of the American Chemical Society, 122*(5), 990–991. https://doi.org/10.1021/ja993385y.
63. Ettinger, M. J., & Kosman, D. J. (1981). Chemical and catalytic properties of galactose oxidase. In T. G. Spiro (Ed.), *Copper proteins* (pp. 219–261). New York: Wiley.
64. Mazur, A. W. (1991). Galactose oxidase. Selected properties and synthetic applications. *ACS Symposium Series, 466,* 99–110. https://doi.org/10.1021/bk-1991-0466.ch008.
65. Firbank, S. J., Rogers, M. S., Wilmot, C. M., Dooley, D. M., Halcrow, M. A., Knowles, P. F., et al. (2001). Crystal structure of the precursor of galactose oxidase: An unusual self-processing enzyme. *Proceedings of the National Academy of Sciences USA, 98,* 12932–12937. https://doi.org/10.1073/pnas.231463798.
66. Gianfreda, L., & Scarfi, M. R. (1991). Enzyme stabilization: state of the art. *Molecular and Cellular Biochemisrtry, 100*(2), 97–128. https://doi.org/10.1007/BF00234161.
67. Iyer, P. V., & Ananthanarayan, L. (2008). Enzyme stability and stabilization—Aqueous and non-aqueous environment. *Process Biochemistry, 43*(10), 1019–1032. https://doi.org/10.1016/j.procbio.2008.06.004.