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Full Length Research Paper

Biochemical characterisation of two novel laccases from *Magnaporthe grisea*

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Laccases are widely distributed oxido-reductases that catalyse the biological oxidation-reduction of polyphenols with a concomitant reduction of molecular oxygen to water. Genome analysis of *Magnaporthe grisea* using bioinformatic approach showed the presence of multiple laccases, which encode proteins with three domains of multicopper oxidase. The transcript levels of all *M. grisea* laccases were analysed by quantitative RT-PCR, in order to study their expression patterns in normal and nitrogen starved conditions. The highest relative expression was observed for MGG_08127 (*Mg*Lac1) in normal conditions. The highest induction was observed for MGG_02876 (*Mg*Lac2) in nitrogen starvation. Since total fungal protein extracts would contain multiple laccases, heterologous gene expression, purification and further enzyme characterisation was carried out to analyse the function of these two laccases from *M. grisea.* Thus, we identified a novel multifunctional laccase, *Mg*Lac2, in *M. grisea* which showed lignin-like dye decolourising activity, 1, 8-dihydroxynapthalene (DHN) polymerisation ability and also ferroxidase activity. Its optimum pH and maximum thermostability were at 4 to 4.5 and 30°C, respectively. *Mg*Lac1 also showed dye decolourization activity, its optimum pH and maximum thermostability were at 4 to 5 and 30°C, respectively. We found that the laccases expressed in normal conditions and in conditions which mimic pathogenicity are different biochemically.

Key words: *Magnaporthe* **grisea**, laccase, dye decolourization, 1, 8-dihydroxynapthalene (DHN) polymerisation, ferroxidase activity, glutathione-S-transferase (GST).

INTRODUCTION

Laccases are copper-containing enzymes catalyzing the monoelectronic oxidation of a number of phenolic compounds and aromatic amines at the expense of molecular oxygen. These enzymes are widely distributed among plants and fungi (Hatakka, 1994). The proposed physiological roles of these enzymes, biosynthetic in plants or biodegradative in fungi are not yet clear. Plant laccases participate in the radical-based mechanisms of lignin polymer formation (Sterjiades et al., 1992; Liu et al., 1994; Boudet, 2000; Ranocha et al., 2002; Hoopes and Dean, 2004), whereas in fungi, laccases probably have roles in morphogenesis, fungal plant pathogen-host interaction, stress responses and lignin degradation (Thurston, 1994). Laccase occurrence has also been reported in prokaryotes (Claus, 2003). Among prokaryotes, enzymes with laccase-like activity have been described participating in pigmentation, electron transport, sporulation, metal oxidation, and UV and H_2O_2 resistance among others (Claus, 2003; Givaudan et al., 1993; Faure et al., 1994; Faure et al., 1996; Alexandre et al., 1999; Francis and Tebo, 2001; Hullo et al., 2001; Kim et al., 2001; Enguita et al., 2003).

Laccases belong to the multi-copper oxidase family

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Abbreviations: ***Mg*Lac1,** *Magnaporthe* **grisea** laccase 1; ***Mg*Lac2,** *Magnaporthe* ***grisea*** laccase 2; **GST,** glutathione-S-transferase; **DHN,** 1, 8-dihydroxynapthalene; **ABTS,** 2, 2'-azinobis-bis-3-ethylbenzthiazolinesulphonate.

containing at least four copper ions essential for enzyme activity, coordinated in three different redox sites: Type 1, site where substrate oxidation takes place; and Types 2 and 3, trinuclear cluster where reduction of molecular oxygen and release of water take place (Claus, 2004). Laccases are suitable for use in biotechnology; they may be used as a pulp-bleaching agent in the paper industry, in dye decolourization in textile industries, for bioremediation and drug analysis, among other uses (Mayer and Staples, 2002; Jordaan and Leukes, 2003; Ryan et al., 2003). Laccases have the advantage that they do not need H_2O_2 for substrate oxidation like peroxidases and have a broader substrate spectrum compared to tyrosinases (Saito et al., 2003).

Genome analysis of *Magnaporthe grisea* using bioinformatic approach revealed the presence of twelve putative laccases having three multicopper oxidase domains. *M. grisea* is the causal agent of rice blast disease, which is one of the most serious diseases on cultivated rice (Ou, 1985). It is capable of destroying enough rice to feed 60 million people every year (Zeigler et al., 1994). Due to the experimental tractability and socioeconomic impact of rice blast, the fungus has served as an important model organism in the studies aimed at understanding the biology of fungal plant pathogens (Talbot, 2003; Valent, 1990). Several studies have suggested that lack of nutrients is one of the signals that control expression of pathogenicity genes in various fungal pathogens of plants (Snoeijers et al., 2000) and humans (Lengeler et al., 2000). Starvation stress has also been implicated as a key influence on fungal gene expression during growth of *M. grisea* within the host plants, that is, starvation stress mimics pathogenicity in *M. grisea* (Talbot et al., 1997).

In this report, we describe heterologous expression, purification and characterisation of two laccases, MGG_08127 and MGG_02876 from *M. grisea*. MGG_08127 and MGG_02876 were named as *Mg*Lac1 and *Mg*Lac2, respectively, for our own convenience. In the present study, for the first time, we demonstrate that *Mg*Lac2 of *M. grisea* oxidises a lignin-like dye, polymerises dihydroxynapthalene and also has ferroxidase activity.

MATERIALS AND METHODS

Fungal strain, media and culture conditions

M. grisea isolate B157, belonging to the international race IC9 was previously isolated in our laboratory from infected rice leaves (Kachroo et al., 1994). The fungus was grown and maintained on YEG medium (glucose, 1 g; yeast extract, 0.2 g; H_2O to 100 ml) or oatmeal agar (Hi-Media, Mumbai, India) at 28°C. The composition of complete media (CM), minimal media (MM) and MM-N (used for nitrogen starvation) were as reported earlier (Talbot et al., 1997). Mycelia used for RNA extraction and total protein extraction were obtained by growing the mycelia in liquid medium for 3 days at 28°C.

Total RNA extraction and cDNA synthesis

Fungal biomass grown in liquid media was frozen in liquid nitrogen. Total RNA was isolated using TRIZOL reagent (Invitrogen Life Technologies, California, USA). The quality of isolated RNA was checked by electrophoresis on formaldehyde gels and quantified by UV spectrophotometry. 5 µg of total RNA was used to synthesise the first strand cDNA using MuMLV reverse transcriptase (Fermentas GmBH, St. Leon-Rot, Germany) and random hexamer in 20 µl reaction system.

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed by monitoring the increase in fluorescence of the SYBR Green dye on LightCycler system (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. Each qRT-PCR quantification was carried out in triplicate using primers for each individual gene (Table 1). Thermal cycling conditions consisted of 2 min at 95°C followed by 40 cycles of 10 s at 95°C, 10 s at 54°C, and 10 s at 72°C. The data were normalized against *tubulin* gene.

GenBank ID, national center for biotechnical information (NCBI)

GenBank ID of *Mg*Lac1 is MGG_08127 and that of *Mg*Lac2 is MGG_02876.

Heterologous expression of *Mg*Lac1 and *Mg*Lac2 in *Saccharomyces cerevisiae*

MgLac1 was amplified by PCR using the following forward primer: 5'ATGAATCTTCGGGACACCATCT 3' and reverse primer 5' TTATCTCCTCAAACCAGACTCCA 3'. *MgLac2* was amplified by PCR using the following forward primer: 5' GGGGATCCCGATGGGTATCATGCAGGGGATG 3' and reverse primer 5' GCAAGCTTGGTTAAACACCGCTGTCGATCTG 3'. The amplified PCR products were gel purified and sequenced. The vectors pEG(KT)*MgLac1* and *pEG*(KT)*MgLac2* were constructed by cloning the coding region of *Mg*Lac1 and *Mg*Lac2 translationally in frame with glutathione-S-Transferase (GST) gene in pEG(KT) (Mitchell et al., 1993). *S. cerevisiae* strain, s288C, was transformed using the recombinant plasmids by 'one step transformation' method (Chen et al., 1992).

Enzyme purification and characterisation

Enzyme purification

The GST*Mg*Lac1 and GST*Mg*Lac2 fusion proteins were purified from total protein extract of the transformed *S. cerevisiae*. Two days old cultures of *S. cerevisiae* cells (OD_{600} ~1) harbouring the expression constructs was inoculated into 100 ml 1X YNB (yeast nitrogen base) containing 2% ethanol. The cultures were incubated at 28°C by shaking for 30 h, until the OD_{600} was about 0.6 and expression was induced by the addition of 4% galactose, followed by further incubation at 28°C for 5 h. Cells were harvested by centrifugation (4°C, 10000 g, for 15 min) and pellets were resuspended in 1 ml of ice-cold PBS. Cell lysis was achieved by

Table 1. List of primers of 12 multicopper oxidases and tubulin used for the qRT-PCR.

Gene id	Primer sequence	
MGG_08127.5	Forward	5' CCTGCCAGCGCGAATTACG 3'
	Reverse	5' CGACCTCCACTGCCTTTGGG 3'
MGG_02876.5	Forward	5' AAGACGGTGTGCCTGGTGTGA 3'
	Reverse	5' AGAAGACCATTGGGCCAACG 3'
MGG_13464.5	Forward	5' ACCACTCTCACTTCTCCGGG 3'
	Reverse	5' GGATCAGCTCGTACTGGATGCG 3'
MGG_0579.5	Forward	5' CGGGCTCGACCGTGACTTA 3'
	Reverse	5' TTCCCAGGTCCTCGTCGTAGTT 3'
MGG_09139.5	Forward	5' ATGTATGGCGGCATCGTCATCA 3'
	Reverse	5' GGCCGTTGGACAGAATCGGAG 3'
MGG_11608.5	Forward	5' ACGTGACCAACAACATGCAGAC 3'
	Reverse	5' GGGTGGCGCGGAACTTGTA 3'
MGG_00551.5	Forward	5' CAGATGGTGTTGTCCGCGAT 3'
	Reverse	5' TTCTGCTGAATACCGTGCCAGTG 3'
MGG_07771.5	Forward	5' AGCGGGACAGCGTTCAAAT 3'
	Reverse	5' ATAGGGCCGTACAACCCATCG 3'
MGG_07220.5	Forward	5' CGGCTCCAACGAGATGGAT 3'
	Reverse	5' CGATGATAAGAGGACCACGCAG 3'
MGG_02156.5	Forward	5' GTCCCATGAAGGAGCTGATCGC 3'
	Reverse	5' ACGTTGACCATCCTGACCAGGTA 3'
MGG_14307.5	Forward	5' ACTATGAGATCCGACCCGACATT 3'
	Reverse	5' CCAGGCCAACAACAACGTCC 3'
MGG_09102.5	Forward	5' GCATCGACGAGCACGAGTTC 3'
	Reverse	5' GTCACCCTCACGGCGTAGT 3'
β Tubulin	TubL1	5' GAGTCCAACATGAACGATCT 3'
	TubR1	5' GTACTCCTCTTCCTCCTCGT 3'

sonication. Lysed cells were centrifuged (13000 g, for 10 min) at 4°C to remove insoluble material and cleared supernatants were used to purify fusion protein. The GST fusion proteins were purified by affinity chromatography using a glutathione-sepharose matrix under mild conditions, using the GST purification module as per manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Protein concentrations were estimated by Bradford method (Bradford 1976). Around 200 to 300 ng of purified proteins were loaded on 12% SDS–PAGE.

Thrombin protease cleavage

Removal of the GST moiety from the proteins of interest was accomplished through a thrombin protease cleavage site located between the GST moiety and the recombinant proteins. GST from the fusion protein was removed using Thrombin Cleavage Capture Kit (Novagen, Merck KGaA, Darmstadt, Germany) as per the manufacturer's instructions. Cleaved GST was easily removed by a second round of chromatography on the glutathione column. The purified proteins were checked on 12% SDS–PAGE.

Raising polyclonal antibodies

The purified protein fraction was used to raise polyclonal antibodies in rabbit. 5 $g_l$of purified *Mg*Lac1 and *Mg*Lac2 in 0.5 ml of 1X PBS were emulsified with an equal volume of Freund's complete adjuvant and used immediately for subcutaneous injection in rabbit. Two booster doses of antigen- adjuvant mixture were given after every 4 weeks. 15 ml of blood were drawn 7 days after the second booster dose. The antibody titre was estimated by indirect-enzyme linked immuno sorbent analysis (ELISA) using anti-rabbit IgG (Bangalore Genie, India) as the secondary antibody.

Enzyme activity

Kinetic measurements were carried out at 30°C, with initial velocity measurements performed in 3 ml glass cuvettes with 1 cm path lengths. Reactions were initiated by the addition of purified proteins. The velocities of protein catalyzed reactions were measured at 420 nm for 2,2'-azinobis-bis-(3-ethylbenzthiazolinesulphonate) (ABTS), 530 nm for syringaldazine 477 nm for 2,6-dimethoxyphenol (DMP), 390 nm for catechol, 390 nm for pyrogallol, 320 nm for phloroglucinol, 390 nm for hydroquinone, 468 nm for guaiacol, and 275 nm for L-tyrosine. Km and Vmax for the afore-mentioned were determined using the Michaelis-Menten's equation. Graph Pad Prism Software (La Jolla, CA, USA) was used for the calculations.

Enzyme inhibition

Enzyme inhibition studies were performed by pre-incubation of the

enzyme with the inhibitor for a certain period of time (2 to 10 min) to ensure that the inhibition is complete. The subsequent kinetic measurements were carried out by the addition of proper substrate and then monitoring the substrate's decline or the formation of the product. Four potential inhibitors (sodium azide, cysteine, EDTA (ethylenediaminetetraacetic acid) and Cl^- (chloride)) were evaluated to test the inhibition of both laccases.

pH optimum

Three substrates (ABTS, catechol, and pyrogallol) were used to determine the effect of pH on enzyme activity of the purified protein. The pH optima were determined over the range of pH 3 to 9 and a 0.1 M Britton- Robinson buffer (Xu, 1996) was used to check the enzyme activity.

Temperature effect and thermostability

The effect of temperature on laccase activity was determined spectrophotometrically following the laccase-catalysed oxidation of ABTS for 2 min at temperatures ranging from 25 to 90°C, at 5°C intervals. Britton-Robinson buffer (Xu, 1996) was used for all the reactions. Thermostability of both the laccases was determined by following the oxidation of ABTS after pre-incubation of laccase for 1 h at different temperatures (20 to 60°C). The reactions were initiated by the addition of substrate.

Protein extraction and western blotting

Total intracellular protein was extracted from wild type *M. grisea* mycelium grown in CM for 24 h. *M. grisea* biomass was crushed into fine powder in liquid nitrogen and resuspended in 1X PBS containing 1 mM PMSF. The extract was clarified by centrifuging at 13000 g for 15 min at 4°C. For secretory proteins, culture filtrate was concentrated with an Amicon Stirred Cell protein concentrator with a 3 kDa cutoff. For western blot analysis 15 µg of intracellular and secretory proteins were electrophoresed on a 12% SDS–PAGE and transferred to a nitrocellulose membrane (Hybond ECL, GE Healtcare, Buckinghamshire, England). The membrane was probed with polyclonal antibodies raised in rabbit followed by a 1:1000 dilution of goat anti-rabbit alkaline phosphatase labelled secondary antibody (Bangalore Genei, Bangalore) and detected with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Dye decolourisation assay

Dye decolourising activity was estimated by diluting 20 ml of a 0.2% (w/v) Remazol brilliant blue dye solution (in H_2O) to 4 ml citrate-phosphate buffer, pH 4.5. Laccases (400 U) were added and decolourisation was assayed after 0, 10, 20, 30, 45, 60, 75 and 90 min at 23°C. Decolourisation was monitored by diluting 0.5 ml of the dye solutions to 2 ml with a 10 mM sodium azide solution in 10 mM citrate-phosphate buffer, pH 4.5. At the selected time intervals, the ratio of absorbance (A_{591}/A_{500}) was measured for the azide-dye solution (Lozovaya et al., 2006). Decolourisation assays were performed in triplicate, and absorbances were compared with that of a control, that is, identical solution without the enzyme (Edens et al., 1999).

Polymerisation of dihroxynapthalene (DHN)

DHN polymerisation was estimated using chromotropic acid disodium salt dehydrate (1,8- dihroxynapthalene-3,6-disulfonic acid disodium salt; 4,5- dihroxynapthalene-2,7-disulfonic acid disodium salt) (Sigma Chemical, St. Louis, MO, USA), by the addition of 15 U of laccase to a 2 ml solution of 1 mM DHN in 5 mM citrate-phosphate buffer, pH 4.5, in 50% ethanol. The polymerisation solution was incubated at 23°C and monitored on nanodrop spectrophotometer (Nanodrop Technologies, USA) by scanning the solution from 320 to 520 nm at 0 to 120 min. An identical control solution without the enzyme that auto-oxidized was also scanned (Edens et al., 1999).

Ferroxidase activity

Ferroxidase activity was determined using ferrous sulfate as the electron donor and 3-(2-pyridyl)- 5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) as a specific chelator to bind ferrous iron remaining at the end of the reaction. Reactions were carried out in disposable cuvettes containing 100 µM ferrous sulfate in 100 mM sodium acetate buffer, and pH 5.0. The reactions were quenched by the addition of ferrozine to a final concentration of 1.5 mM, and the rate of Fe^{2+} oxidation was calculated from the decreased absorbance at 560 nm using a molar absorptivity of e_{560}= 25,400/M /cm for the Fe^{2+}-ferrozine complex (Hoopes and Dean, 2004).

RESULTS

Expression profiling of laccases of *M. grisea*

High laccase activity was reported in liquid cultures of *M. grisea* as early as 24 h (Iyer and Chattoo, 2003). Therefore, quantitative real time PCR (qRT-PCR) was carried out to monitor the mRNA expression of all the twelve multicopper oxidase genes. Nitrogen starvation-stress induces expression of a large number of genes expressed during growth of the fungus in plant tissue, particularly during disease symptom outbreak (Talbot et al., 1997). We, therefore, checked the relative expression of 12 multicopper oxidases *of M. grisea* in normal condition versus nitrogen starved condition. qRT-PCR was carried out using 12 laccase specific primers (Table 1). The calibrator used was *TUB* (*β-tubulin*), where the primers were designed to amplify 78 bp fragments.

Relative expression of MGG_08127 was highest in the normal hyphal condition (Figure 1A). Most of the multicopper oxidases showed 2 to 4 fold induction under nitrogen starvation condition, except *MgLac2* (MGG_02876) which showed 15-fold induction (Figure 1B). Therefore these two genes were selected for further analysis and were named as *MgLac*1 (MGG_08127) and *MgLac*2 (MGG_02876), respectively.

Enzyme purification

Bioinformatics analysis of *Mg*Lac1 and *Mg*Lac2 protein revealed them to be laccases. To characterise their enzymatic properties, we cloned the full-length coding

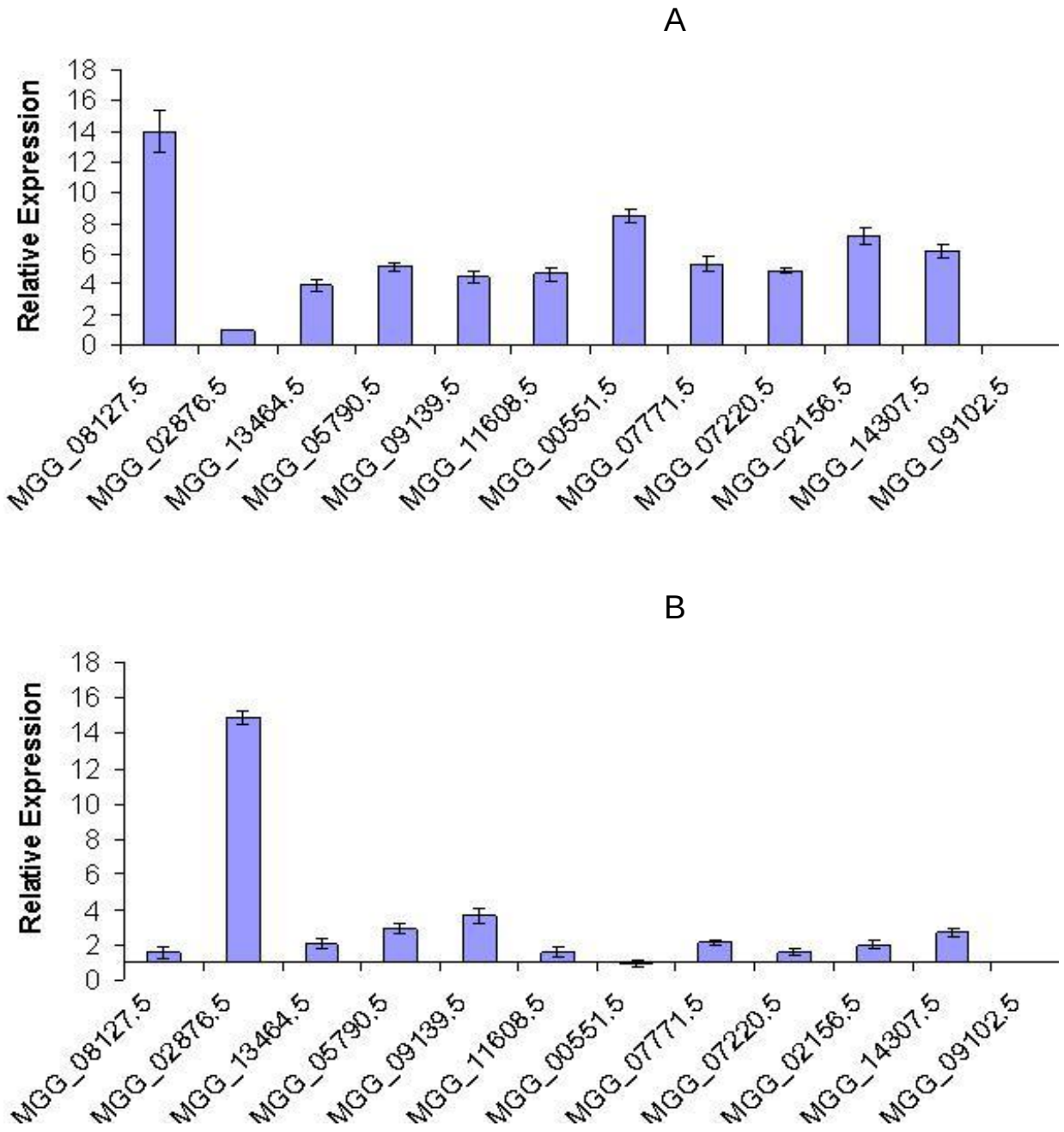


Figure 1. (A) Relative expression of 12 multicopper oxidases in *M. grisea*. The relative expression level was compared with the lowest expressing multicopper oxidase. MGG_08127 (*MgLac1*) was the highest expressing multicopper oxidase. The values given are the average of triplicates. Bars indicate mean ± SD. (B) Response of the 12 multicopper oxidases to nitrogen starvation. *M. grisea* was grown in CM for 48 h and then in nitrogen starvation media for 24 h. The transcript levels were analysed by qRT-PCR. The relative induction levels were compared with the basal levels observed in the normal condition. 15-fold induction was observed for MGG_02876 (*MgLac2*). The values given are the average of triplicates. Bars indicate mean ± SD.

sequence of *Mg*Lac1 and *Mg*Lac2. The expression plasmid constructs were generated containing *Mg*Lac1 and *Mg*Lac2 translationally in frame with glutathione-S-Transferase (GST) gene in pEG(KT) vector. The constructs pEG(KT) *MgLac*1 and pEG(KT) *MgLac*2 were expressed in *S. cerevisiae* (s288C) and induced by 4%

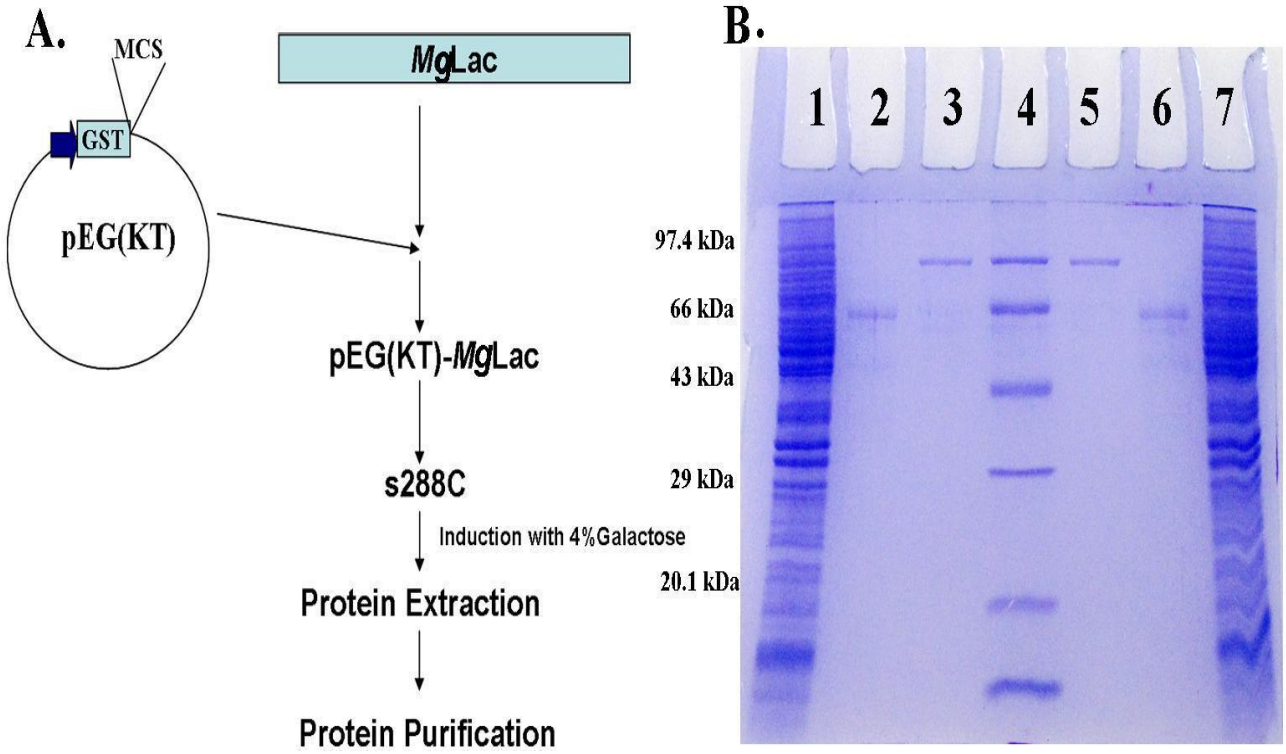


Figure 2. Heterologous expression and purification of *Mg*Lac1 and *Mg*Lac2. (A) Steps involved in the construction of expression vectors pEG(KT)*MgLac1* and pEG(KT)*MgLac2* which were cloned in pEG(KT) vector and expressed in *S. cerevisiae* strain, s288C. (B) The fusion proteins were purified by affinity chromatography using glutathione-sepharose as affinity matrix and checked on 12% SDS–PAGE. Lane 1. Crude protein extract of s288C transformed with pEG(KT)*MgLac1* plasmid; Lane 2. Purified *Mg*Lac1; Lane 3. Purified GST-*Mg*Lac1; Lane 4. Molecular weight marker; Lane 5. Purified GST-*Mg*Lac2; Lane 6. Purified *Mg*Lac2; Lane 7. Crude protein extract of s288C transformed with pEG(KT)*MgLac2* plasmid.

galactose (Figure 2A). *Mg*Lac1 and *Mg*Lac2 were expressed from the pEG(KT) vector as a fusion protein with GST at its N-terminus; we will refer to these fusion proteins as GST-*Mg*Lac1 and GST-*Mg*Lac2. The fusion proteins were purified using glutathione sepharose affinity matrix. The GST tag was removed from fusion proteins as described in materials and methods. Both *Mg*Lac1 and *Mg*Lac2 resolved as a single fragment of 66 kDa on SDS-PAGE when stained with Coomassie Blue (Figure 2B). The purified *Mg*Lac1 and *Mg*Lac2 were subsequently used for *in-vitro* analysis of enzyme properties.

Localisation of *Mg*Lac1 and *Mg*Lac2

*Mg*Lac1 and *Mg*Lac2 were predicted to be extracellular using program Wolf PSORT (Horton et al., 2007). To validate extracellular localisation of *Mg*Lac1 and *Mg*Lac2 western blot analysis was carried out using antibodies raised in rabbit. The polyclonal antibodies titre was found to be 1600.

Our analysis revealed a clear band of 66 kDa in culture filtrate of *M. grisea* which corresponds to the relative molecular weight (Mr) of both *Mg*Lac1 and *Mg*Lac2. Such band was not observed in case of intracellular proteins of *M. grisea* confirming that the *Mg*Lac1 and *Mg*Lac2; both are secretory proteins (Figure 3).

_Mg_Lac1 and _Mg_Lac2 are laccases

Enzyme activity

The purified *Mg*Lac1 and *Mg*Lac2 were further tested for their laccase activity using common laccase substrates such as ABTS, syringaldazine and DMP. *MgLac*1 K_m values were observed to be 0.1035 ± 0.02 mM (ABTS) and 1.113 ± 0.28 mM (Syringaldazine). The V_{max} values were observed to be 0.0205 ± 0.0007 s^{-1}(ABTS) and 0.0158±0.0042 s^{-1}(Syringaldazine). *Mg*Lac2 K_m values were observed to be 0.1301 ± 0.02 mM (ABTS) and 1.243 ± 0.32 mM (Syringaldazine). The V_{max} values were observed to be 0.0168 ± 0.0004 s^{-1}(ABTS) and 0.0143 ±0.0048 s^{-1}(Syringaldazine). However, both *Mg*Lac1 and

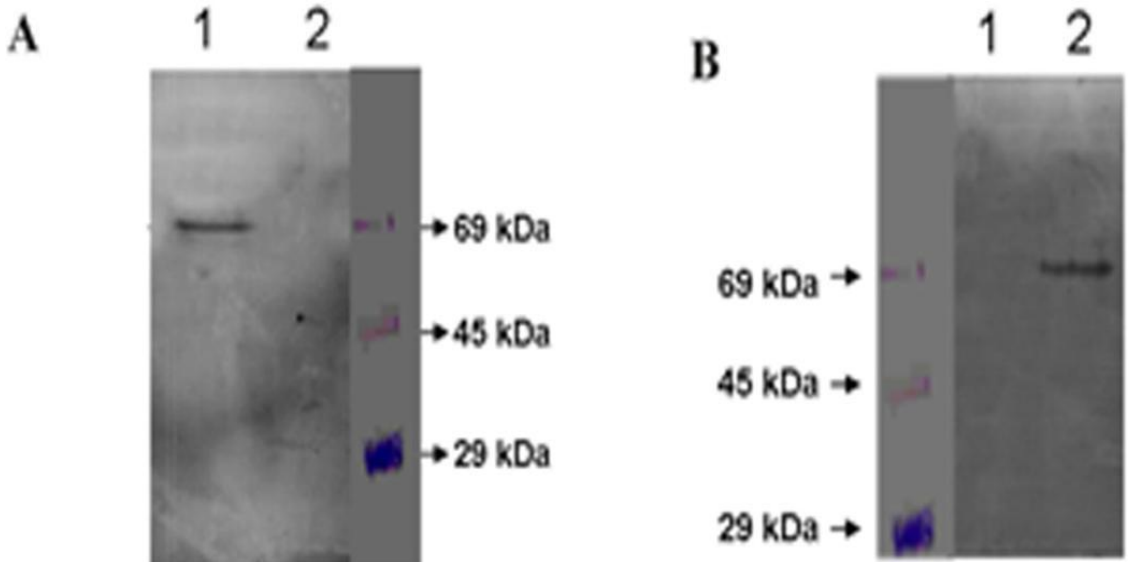


Figure 3. Western blot analysis. (A) Western blot analysis of *Mg*Lac1 of *M. grisea.* Lane 1. culture filtrate; Lane 2. intracellular proteins. 15 µg of protein was loaded in each well. (B) Western blot analysis of *Mg*Lac2 of *M. grisea.* Lane 1. intracellular proteins; Lane 2. culture filtrate. 15 µg of protein was loaded in each well.

*Mg*Lac2 were unable to oxidise a common laccase substrate, DMP. Significant differences were found in the affinity constants (K_M) and substrate preferences of both laccases towards other phenolic substrates. *Mg*Lac1 oxidised catechol, pyrogallol and phloroglucinol with K_m values 1.047 ± 0.16, 1.301 ± 0.16 and 0.389 ± 0.05 mM, respectively. The Vmax values for *Mg*Lac1 were observed to be 0.0267 ± 0.0008 s^{-1} (Catechol), 0.0168 ± 0.0004 s^{-1} (Pyrogallol) and 0.0115 ± 0.0006 s^{-1}(Phloroglucinol). *Mg*Lac2 oxidised catechol, pyrogallol and hydroquinone with K_m values 7.432 ± 1.06, 2.509 ± 0.29 and 2.088±0.39 mM, respectively. The V_{max} values for *Mg*Lac2 were observed to be 0.0110 ± 0.0016 s^{-1} (Catechol), 0.0187 ± 0.0007 s^{-1} (Pyrogallol) and 0.0097 ± 0.0005 sec^{-1}(Hydroquinone). Both enzymes did not oxidise tyrosine, thus, proving that they are not tyrosinases.

Enzyme inhibition

To characterise the laccases further, general inhibitors which form stable copper complexes, were tested using ABTS as substrate. It was observed that *Mg*Lac1 was more susceptible to common laccase inhibitor sodium azide with IC_{50} of 0.63 ± 0.03 mM whereas other inhibitors, cysteine (IC_{50}, 0.73 ± 0.01 mM) and EDTA (IC_{50}, 2.41 ± 0.21 mM) showed inhibition to a lesser extent. In comparision with *Mg*Lac1, *Mg*Lac2 was more susceptible to inhibition by cysteine with IC_{50} of 0.28 ± 0.07 mM than sodium azide (IC_{50}, 0.37 ± 0.02 mM) and EDTA (IC_{50}, 3.55 ± 0.27 mM).

pH optimum

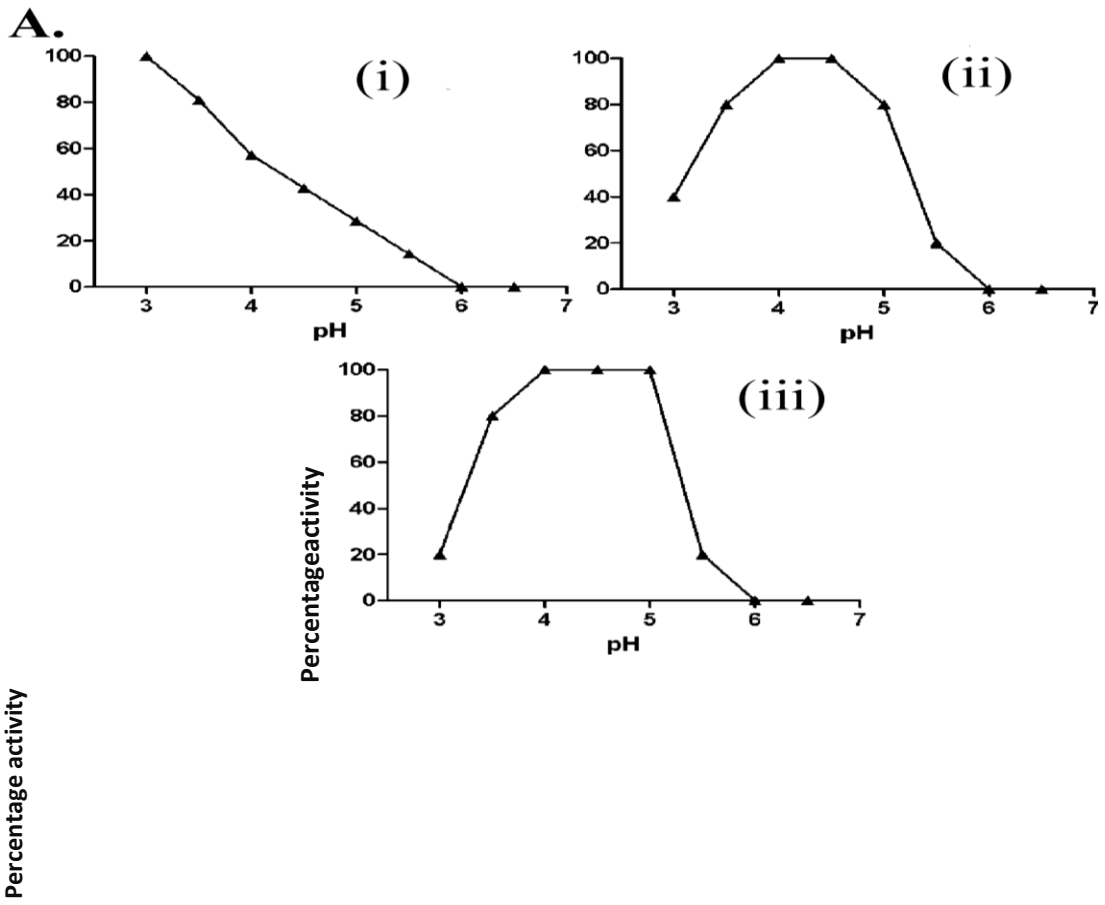
The enzyme activity of *Mg*Lac1 and *Mg*Lac2 were measured in pH range of 3 to 8. The optimum pH for *Mg*Lac1 activity was at pH 4 to 5 (Figure 4A). The optimum pH for *Mg*Lac2 activity was at pH 4 to 4.5 (Figure 4B). The enzyme activity of both the laccases decreased abruptly at pH higher than 5, with no activity after pH 6. The pH optimums determined for both laccases were acidic, which is representative of typical laccases.

Temperature effects and thermostability

Both proteins showed enzymatic activity over a remarkably wide range of temperatures. Significant enzyme activity was observed over the temperature range of 25 to 80°C. Maximum enzyme activity of *Mg*Lac1 was observed at 80°C and maximum thermostability at 30°C, when stored for 60 min (Figure 5A). However, maximum enzyme activity of *Mg*Lac2 was observed at 70°C with maximum thermostability at 30°C, when stored for 60 min (Figure 5B).

***MgLac*1 and *MgLac*2 showing dye decolourisation activity**

The purified *Mg*Lac1 and *Mg*Lac2 were also used for determining decolourisation activity for Remazol brilliant blue dye. To control for the presence of possible


A.
(i)
(ii)
(iii)
100
80
60
40
20
0
3
4
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pH
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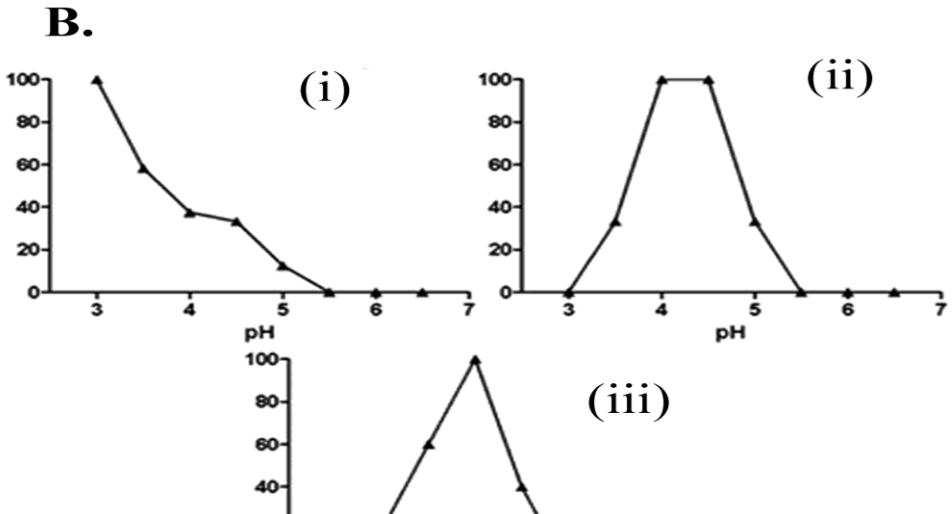
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Figure 4. Effect of pH on activity of *Mg*Lac1 and MgLac2. (A) Effect of pH on activity of *MgLac*1 with different substrates. (i) ABTS; (ii) Catechol; (iii) Pyrogallol. The pH optimum was determined over a range of pH 3 to 9. (B) Effect of pH on activity of *MgLac*2 with different substrates. (i) ABTS;(ii) Catechol; (iii) Pyrogallol. The pH optimum was determined over a range of pH 3 to 9.

contaminating enzyme activity not detectable by SDS-PAGE, a fraction of empty vector transformed *S. cerevisiae* strain, s288C purified in an identical fashion was used as a negative control. Under our experimental conditions *Mg*Lac1 and *Mg*Lac2 were able to decolourise Remazol Brilliant Blue dye (Figure 6A). We utilised this lignin-like dye decolourisation assay to assess a possible role of these laccases in lignin degradation. Laccases are

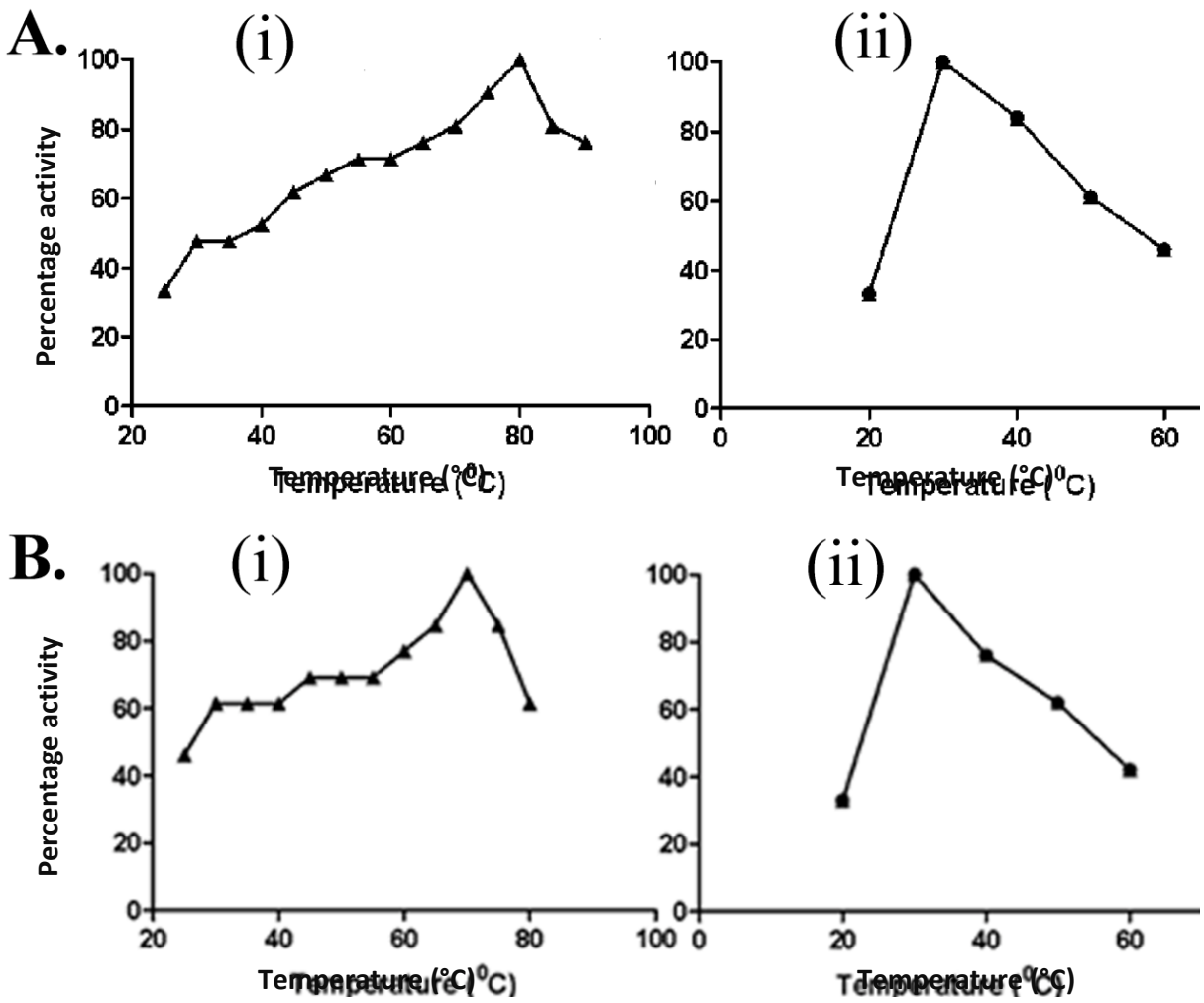

Figure 5. Effect of temperature on activities of *Mg*Lac1 and *Mg*Lac2. (A) (i) Temperature of *Mg*Lac1 maximum activity was determined at temperatures ranging from 25 to 90^{0}C. (ii) Thermostability of *Mg*Lac1 was determined at temperatures ranging from 20 to 70^{0}C. (B) (i) Temperature of *Mg*Lac2 maximum activity was determined at temperatures ranging from 25 to 90^{0}C. (ii) Thermostability of *Mg*Lac2 was determined at temperatures ranging from 20 to 60^{0}C.

known to be involved in lignin degradation in fungi (Crestini and Argyropoulos, 1998; Goodell et al, 1998; Ardon et al, 1998).

*Mg*Lac2 shows DHN polymerised potential

*Mg*Lac2 polymerised DHN to a higher molecular mass melanin. An increase of 0.1 optical density units was observed with maximum absorbance at 348 nm (Figure 6B). In contrast, *Mg*Lac1 did not show any DHN polymerisation. As a negative control, identical fraction from empty vector transformed *S. cerevisiae* strain, s288C was used. However, no enzyme activity was observed in the empty vector transformed *S. cerevisiae* fraction. Such DHN polymerisation ability has also been observed in a secretory laccase of *Gaeumannomycesgraminis* var. *tritici* (Edens et al., 1999).

*Mg*Lac2 also shows ferroxidase activity

In addition to the earlier properties of *Mg*Lac2, we found that this laccase possessed iron oxidase activity as well. Laccase-catalyzed Fe(II) oxidation was measured by the addition of purified laccase to a reaction mixture containing ferrous sulfate in metal-free buffer. Similar fraction from empty vector transformed *S. cerevisiae*

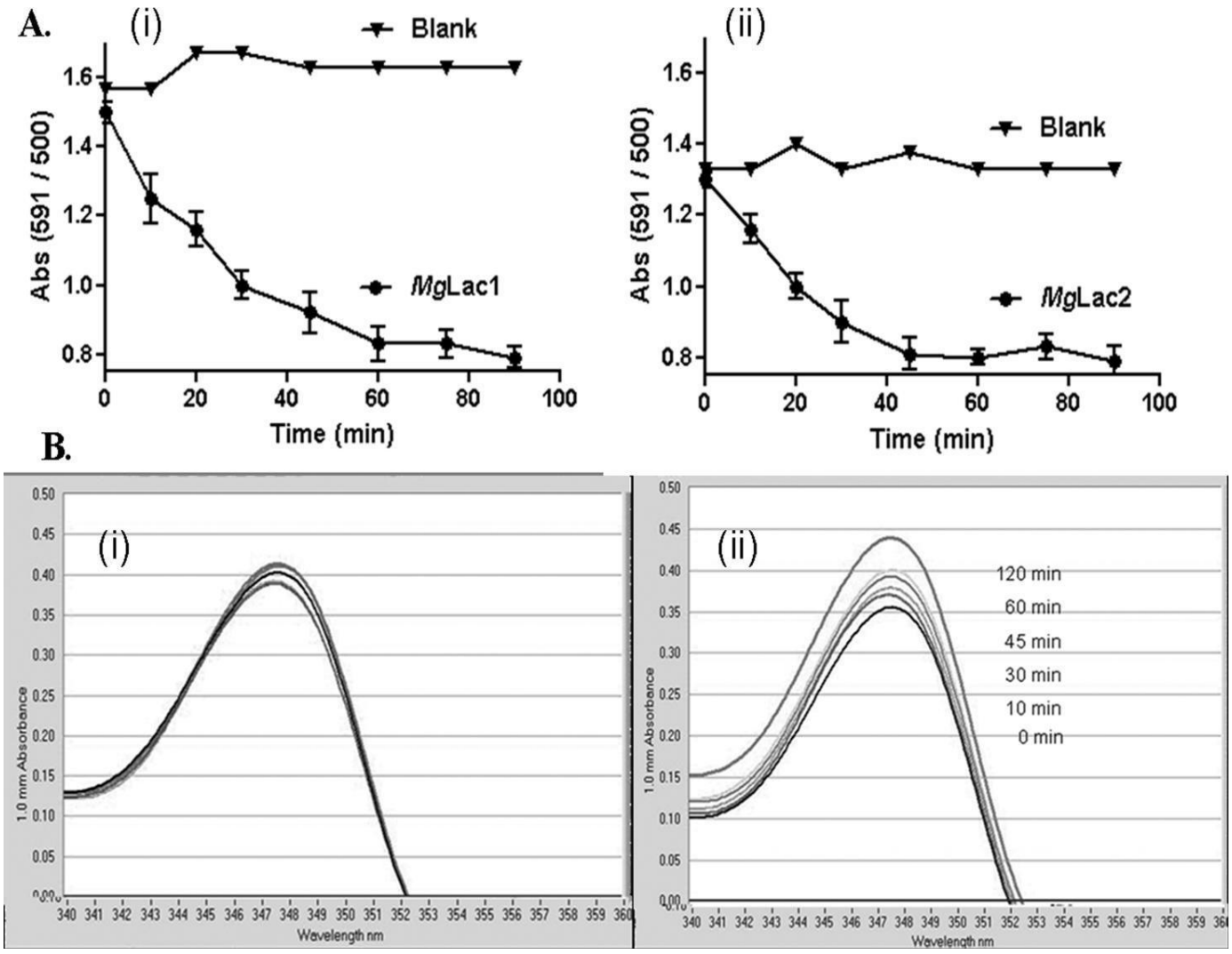

Figure 6. Dye decolourisation and DHN polymerisation. (A) Dye decolourisation activity of *Mg*Lac1 (i) and *Mg*Lac2 (ii) was estimated using Remazol brilliant blue R. Purified laccases were added, and decolourisation was monitored spectrophotometrically after indicated time points at 23^0C. (B) DHN polymerisation of *Mg*Lac2 was estimated using Chromotropic acid disodium salts dehydrate. Purified laccase was added, and polymerisation was monitored spectrophotometrically by scanning the solution from 320 to 520 nm at 0 to 1 20 min. (i) Control (ii) *Mg*Lac2.

served as a negative control and did not show any enzyme activity. The specific ferroxidase activity was $1.574 \times 10^{-6} \pm 0.26$ U/mg protein. Such ferroxidase activity was also observed in laccase of *Cryptococcus neoformans* (Liu et al., 1999).

DISCUSSION

Laccases are ubiquitous enzymes in fungi and higher plants and have been shown to play important roles in developmental cycle of various fungi (Thurston, 1994). High laccase activity was detected in liquid cultures of *M. grisea* as early as 24 h (Iyer and Chattoo, 2003). Therefore, we hypothesised that the high expression of laccase during very early stages may be important for infection. *MgLac2* was induced by 15-fold in nitrogen starvation in *M. grisea*.

The molecular masses of *Mg*Lac1 and *Mg*Lac2 were found to be in the range of 60 and 80 kDa, which are typical for laccases (Thurston, 1994). *Mg*Lac1 and *Mg*Lac2 exhibited very low K_m values for ABTS indicating very high binding affinity towards this substrate and oxidised syringaldazine efficiently, but there was no activity towards L-tyrosine which proved that both of them were not tyrosinases. Thus, *Mg*Lac1 and *Mg*Lac2 were

classified as a true laccases based on their substrate specificity. Laccases are known to act on *p*-diphenol as well as *o*-diphenol, but they mostly have a higher affinity and activity toward the *p*-diphenols (Xu, 1996). Under current investigation *Mg*Lac2 showed affinity towards para-substituted phenol, that is, hydroquinone, but this was not the case with the *Mg*Lac1, since no laccase activity was observed towards hydroquinone. *Mg*Lac2 had higher affinity (according to K_m values) toward pyrogallol than the compounds having two adjacent hydroxyl groups such as catechol. To characterise the enzyme further, several general inhibitors, especially compounds able to form stable copper complexes were tested using ABTS as substrate. These inhibitors are not laccase specific and their application for phenoloxidase originates from results obtained with other metalloenzymes (Johannes and Majcherczyk, 2000). Sodium azide (NaN_3), that complex with the coppers in the active site, was observed to inhibit *Mg*Lac1 efficiently. Sulfhydryl organic compound, cysteine, which has a reducing effect on the copper-containing active site of laccases, was found to be the most efficient inhibitor of *Mg*Lac2.

The pH optimums obtained for *Mg*Lac1 and *Mg*Lac2 were acidic range and thus represented typical laccases. Laccases tend to react differently to varying pH with different substrates. The reasons for dependency of laccases to act on different substrates have been previously elucidated (Xu, 1996). The dependence of laccase on pH usually renders a bell-shaped profile as with catechol and pyrogallol. With ABTS there is rather a monotonic decline than a bell-shaped profile as reported (Xu, 1996).

Laccases are reported to be involved in lignin degradation in white-rot basidiomycetes *Trametes versicolors* (Crestini and Argyropoulos, 1998), *Lentinus edode* (Goodell et al, 1998) and *Pleurotus ostreatus* (Ardon et al, 1998). In phytopathogen, *Gaeumannomyces graminis* var. *tritici,* laccase is known to be involved in melanisation (Edens et al., 1999). Ferroxidase activity was also observed in *Cryptococcus neoformans* laccase (Liu et al., 1999). In our experimental conditions *Mg*Lac1 and *Mg*Lac2 both were able to decolourise Remazol Brilliant Blue suggesting a possible role in lignin degradation.

In addition to the decolourisation activity, *Mg*Lac2 also showed DHN polymerisation potential and ferroxidase activity. This is the first report of a single fungal laccase showing three above mentioned activities. Role of laccase in iron metabolism was reported in *C. neoformans*. It was speculated that laccase oxidation of ferrous iron may be important during infection in addition to its role in melanin production. The fact that *C. neoformans* laccase is expressed only during very early stages of infection would be consistent with its role in iron acquisition and in protection of fungal cells from hydroxyl radical attack from host cells during the initial adaptation to the host environment (Jung et al., 2006).

In this study we present biochemical evidence that laccases expressed in normal conditions and in conditions which mimic pathogenicity are different, that is, their substrate preferences and inhibition. Laccase which is highly induced in nitrogen starvation that is, *Mg*Lac2, produced by *M. grisea,* may be involved in lignin degradation and/or melanin synthesis and/or iron oxidation. May be multicopper oxidase-dependent iron uptake systems are present in this fungus and this laccase-iron oxidase activity might protect the fungus in concert with other laccase products (melanin) by maintaining Fe in an oxidized form, thereby decreasing production of antifungal hydroxyl radicals. Future studies will focus on the role(s) of other laccases of *M. grisea* in melanization, delignification, and/or protection from antimicrobial compounds.

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