Overexpression of Laccases in *Coprinopsis cinerea*

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Laccases are versatile redox-enzymes that oxidize various phenolic compounds and aromatic amines. Because of the broad substrate specificity, these enzymes are of interest for many different biotechnological applications. White-rot fungi are the major source of laccases. Although basidiomycete species give rise to relatively high enzyme yields, these might not be optimal for applications. Basidiomycetous laccases have been reported to show hyper- or hypo-glycosylation when expressed in ascomycetes. In consequence, enzyme characteristics were found to be altered. Therefore, we use the basidiomycete *Coprinopsis cinerea* as an organism for laccase overproduction. We present a vector system for easy and rapid cloning of promoters and/or genes of interest and show that such constructs can be functional in laccase production.

1. Introduction

1.1. *Laccases: occurrence, structure and biological functions*

The family of multi-copper oxidases (Mco) includes laccases (1.10.3.2), ascorbate oxidases (1.10.3.3), ceruloplasmins (1.16.3.1), bilurubin oxidases, sulochrin oxidases, phenoxazinone synthases, ferroxidases, enzymes contributing to copper-resistance and a few yet not classified proteins (Messerschmidt 1997; Solomon et al. 1996; Hoegger et al. in preparation). Enzymes of this family catalyze the four electron reduction of oxygen to water. Laccas-

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es contain four copper atoms per monomer, which are bound to three distinct copper binding sites (mononuclear Type 1 and Type 2 sites and binuclear Type 3 site; Type 2 and Type 3 together form a trinuclear center). Type 1 Cu is the primary electron acceptor from the substrate. From there, electrons will be transferred to the Type 2/Type 3 cluster, where in two-electron steps the reduction of oxygen to water occurs (Claus 2003). Laccases, the enzymes we are studying, are phenoloxidases that catalyze oxidation of various mono- and di-phenols, ascorbic acid and aromatic amines (Messerschmidt 1997). Other enzymes oxidizing phenols are phenoloxidases are tyrosinases (1.14.18.1), heme peroxidases (1.11.1.7), manganese dependent and independent peroxidases (1.11.1.13) and lignin peroxidases (1.11.1.14). Laccases distinguish in phenoloxidase activity from the two-copper containing tyrosinases by lack of a hydroxylation reaction (cresolase activity). Different from peroxidases, laccase do not need hydrogen peroxide as cosubstrate (Messerschmidt 1997).

Laccases are widely distributed in plants and fungi and few are found in bacteria and insects. Plant laccases are believed to be involved in lignin synthesis (Gavnholt and Larsen 2002), whilst fungal laccases can participate in lignin degradation (Leonowicz et al. 2001). In confrontations with other organisms, laccases are produced as part of defense reactions (Baldrian 2004). Some of the fungal enzymes act in synthesis of melanin and other pigments (Langfelder et al. 2003; Pukkila-Worley et al. 2005). Others seem to have roles in fruiting body formation (Kües and Liu 2000; Wösten and Wessels 2005). Bacterial laccases function in formation of melanin-like pigments, in spore coat formation and in mediating copper tolerance (Endo et al. 2002; Roberts et al. 2002). In insects, laccases likely contribute to cuticle sclerotization (Dittmer et al. 2004).

1.2. *Laccases in industrial applications*

Apart from the broad range of biological functions, laccases are interesting enzymes for many biotechnological applications. By their wide substrate specificity, laccases have a great potential in water and soil bioremediation, for example in degradation of polycyclic aromatic hydrocarbons (PAH) and many other toxic phenolic and non-phenolic compounds (Johannes and Ma-

jcherczyk 2000; Mai et al. 2004). Laccases are very effective in degradation of recalcitrant dyes (Rodríguez et al. 1999, Svobodova et al. 2003). This property is of importance in clearance of effluxes from the textile industry (Kandelbauer et al. 2004) as well as in textile refining, for example in production of "stone-washed jeans" (Pazarlioglu et al. 2005). In addition in the textile industry, laccases might be used as bleaching agent to improve the whiteness of cellulosic fibers (Tzanov et al. 2003). In the pulp and paper industry, laccases are used in bleaching of pulp in order to increase the brightness and the tensile strength of the paper (Addleman and Archibald 1993; Chandra et al. 2004). Laccases have been shown to activate the natural binding forces of lignin, allowing production of medium-density fiber boards (MDF) and particle boards without addition of chemical adhesives (Hüttermann et al. 2001, Mai et al. 2004; Fig. 1). Potential applications in the food industry are in clearance of wine and juices and in enhancing the dough quality in bread baking and the softness of the bread product (Minussi et al. 2002). Laccases are employed in removing phenolic compounds from corks prior to bottling that otherwise would be released and spoil the tastes of wines during storage (Brenna et al. 1994). Laccases can serve as the measuring device in biosensors for detection of phenolic compounds in food, in pharmaceutical formulations and medical research, in waste waters and others (Gomes et al. 2004). Another interesting application of laccases is in the biofuel cell design (Heller et al. 2004).

Figure 1. Medium-density fiber boards (MDF) of 1 cm thickness produced with a raw laccase extract from *Pleurotus ostreatus* (left) and the MDF-pilot plant at the Institute of Forest Botany (right). Details of the laccase-MDF-production process are found in Kharazipour et al. (1997).

1.3. *Production of laccases*

White-rot fungi such as *Pleurotus ostreatus, Trametes versicolor*, and *Coprinopsis cinerea* are natural sources for laccase production (Table 1). Species differ in amount of laccase production (Table 1) and also different strains of a given species (Pelaez et al. 1995). Some species produce laccases in typical fungal growth media, e.g. MYPG containing yeast extract, malt extract, peptone and glucose (Nagai et al. 2002) or soy flour medium (Yaver et al. 1999). More commonly, high laccase production is induced by addition of toxic compounds such as copper or phenols (Palmieri et al. 2000, Terron et al. 2004) or sometimes by non-toxic but expensive vanillin (de la Rubia et al. 2002). Species may produce more than one laccase and individual laccases can be very different in their properties (Table 1). Often, fungal culture supernatants contain mixtures of laccase isoforms and enzymatic activities of different culture batches can vary according to the relative amounts of the different isoforms (Palmieri et al. 2000).

White-rot fungi are most efficient natural laccase producers, but for industrial applications enzyme yields are often not optimal. For example, 2-3 l of concentrated enzyme (8-10 IU/ml) per 10 kg fibre will obtain 1 m^2 of 1 cm-thick MDF boards with a density of 0.8 g cm^{-3} (Schöpper 2002, Schöpper and Rühl, unpublished). In developing the process, commercial available laccase (Novo SP504) or self-produced laccase from *T. versicolor* were used (Kharazipour et al. 1997). Our recent studies with *P. ostreatus* extracts show that enzymes from this species are also effective (Fig. 1A). However, whether enzymes of different sources are more suitable than others remains to be shown. On the market are enzymes from *Agaricus bisporus*, *Rhus vernificera*, *T. versicolor* and *P. ostreatus* (Minussi et al. 2002), indicating that there is an interest in obtaining enzymes from different sources and of different properties for various applications.

To overcome the various problems of laccase production with natural strains (too low enzyme yield, variable compositions of isoenzymes, production of enzymes with suboptimal properties, need for toxic and/or expensive inducers, poorly established fermentation processes, expensive growth media), laccase genes from white-rot fungi were overexpressed in heterologous ascomycetous hosts. Only in some of the cases, recombinant produced enzymes have been further characterized (see Table 2 for examples). Usually,

there are differences in glycosylation compared to the enzymes obtained from the native producer (Schneider et al. 1999, Yaver et al. 1996). Along with this, enzymes have altered properties e.g. in optimal pH and Km values (Sigoillot et al. 2004), making them less suitable for biotechnological applications. In other instances, properties of recombinantly enzymes have been described but data for the original enzymes are not available, e.g. for *Fome lignosus* Lcc, *Pleurotus sajor-caju* Lac4 (Table 2). More than one hundred laccase genes are identified from saprophytic and wood-rotting basidiomycetes (Hoegger et al. in preparation), but only very few of the corresponding enzymes have been identified and characterized. In turn, where specific properties of enzymes are known (e.g. *Trametes trogii*, *T. versicolor*; Garzillo et al. 1998, Claus et al. 2002, see Table 2), the corresponding gene has often still to be identified. From the high number of uncharacterized genes and from the available data on variations between the characterized enzymes, it is clear that the potential of different laccases for industrial applications is yet not exploited in the best possible way.

Table 1
Examples of laccase production in basidiomycetes and enzyme properties[1]

Enzyme	*Laccase activity (IU/ml) in cultutre supernatant[2]*	*Enzyme properties*				
		Mw (kDa)	*pI*	*Optimal pH*	*Optimal temperature (°C)*	*Km (mM)*
Agaricus blazei laccase	5.0	66	4.0	2.3	?	0.063
Ceriporiopsis subvermispora laccase L1	1.1	71	3.4	3.0	?	0.03
Coprinopsis cinerea Lcc1	?	63	3.7-4.0	4.0	?	?
Lentinula edodes Lcc1	1.4	72.2	3.0	4.0	40	0.1
Phellinus ribis laccase	?	76	?	5.0	?	0.207
Pleurotus eryngii laccase I	0.120	65	4.2	4.0	65	?
Pleurotus ostreatus						
POXA1	?	61	6.7	3.0	45-65	9.0
POXA2	?	67	4.0	3.0	25-35	1.2
POXC	?	59	3.3	3.0	50-60	2.8
POXA1b	?	61	6.9	?	?	4.7
POXA3a	?	67	4.1	3.6	35	7.0
POXA3b	?	67	4.3	3.6	35	7.4
Polyporus pinisitus laccase	?	65	3.0	4-5	?	?
Pycnoporus cinnabarinus laccase	18.0	76.5	3.7	?	?	?

1. Data have been taken from the following references: Claus et al. 2002; Eggert et al. 1996; Fukushima and Kirk 1995; Galhaup et al. 2002; Garzillo et al. 1998, 2001; Lomascolo et al. 2002; Min et al. 2001; Munoz et al. 1997; Nagai et al. 2002; Palmieri et al. 1997, 2003; Schneider et al. 1999; Ullrich et al. 2005; Xiao et al. 2003
2. For better comparison, values were converted into IU in cases, where in the original literature laccase activities (determined with ABTS) were given as nkat/ml or as arbitrary units.

Pycnoporus sanguineus GO5 laccase	71.0	70	3.5	?	?	?
Trametes AH28-2 laccase A	9.0	62	4.2	4.5	50	0.025
Trametes pubescens LAP2	15.5	65	2.6	3.0-4.5	50-60	0.014
Trametes trogii laccase	0.8	70	3.3-3.6	3.0-3.5	?	0.03
Trametes versicolor laccase	?	40	3.0	5.0	?	?

White-rot fungi usually have laccase multi-gene families. For example, four laccase genes have been identified in *P. ostreatus* and five non-allelic laccase genes in *Trametes villosa* and in *P. sajor-caju* (Giardiana et al. 1995; Yaver et al. 1996; Soden and Dobson 2001). Recently, we described eight non-allelic laccase genes from *C. cinerea* (Hoegger et al. 2004). Their deduced protein products contain all ten histidine and one cysteine residues spread over four highly conserved amino acid regions known as fungal laccase signature sequences L1-L4 (Fig. 2) that are needed for copper binding. Subsequently, from analysis of the *C. cinerea* genome established by the Broad Institute (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/index.html), nine further non-allelic laccase genes were identified in this species (Kilaru et al., unpublished). On the protein level, only laccase Lcc1 has so far been described (Schneider et al. 1999; Table 1). Lcc1 is the main enzyme produced in liquid culture (Yaver et al. 1999). Designing laccase mutants from this enzyme with improved stability properties has been patented (Schneider et al. 2001).

Gene *lcc1* of *C. cinerea* has been overexpressed in *Aspergillus oryzae* and the recombinant enzyme was found to be hyperglycosylated (Schneider et al. 1999; Table 1, 2). Overexpression of laccases in basidiomycete hosts might not result in such problems. To date, the *Pycnoporus cinnabarinus lac1* and *Coriolus versicolor laccase III* genes were overexpressed in *P. cinnabarinus* and *C. versiocolor*, respectively (Alves et al. 2004, Kajita et al. 2004) but properties of the produced enzymes are not yet known. We now established *C.*

cinerea as a basidiomycete host for overexpression of homologous and heterologous laccases and developed a vector system for quick exchange of promoters and/or genes via *in vivo*-recombination in the yeast *Saccharomyces cerevisiae*. Unlike most other basidiomycetes, *C. cinerea* is very efficient in DNA transformation with up to 1000 transformants per µg DNA (Binninger et al. 1987; Granado et al. 1997). This allows easy screening of bulks of transformants (Granado et al. 1997) and will be of advantage at later stages, for example when screening randomly mutagenised libraries of specific laccase genes. The fungus grows fast in cheap liquid and solid media over a range of temperatures (20-42°C) with the optimum at 37°C, being of benefit for the use as a production organism.

Table 2
Examples of overexpressed basidiomycete laccases[1]

Host-Gene	*Laccase activity (IU/ml) in cultutre supernatant[2]*	*Enzyme properties*				
		Mw (kDa)	*pI*	*Optimal pH*	*Optimal temperature (°C)*	*Km (mM)*
Saccharomyces cerevisiae						
- °*Trametes versicolor* lcc2	0.086	64	?	?	?	?
Pichia pastoris						
– *T. versicolor lcc1*	11.5	67	?	?	?	?
– *T. versicolor lcc4*	1.5	85	?	5.5	?	?
– *Fome lignosus lcc*	9.0	66.5	?	2.4	55	0.177
– *Pleurotus sajor-caju lac4*	?	59	?	3.3	?	2.5
Yarrowia lipolytica						
– *T. versicolor lac111b*	0.23	52	?	3.0	?	0.026
– *Pycnoporus cinnabarinus lac1*	0.44	90	?	?	?	?
Aspergillus niger						
– *P. cinnabarinus lac1*	11.0	70	3.7	4.0	65.0	0.055
Aspergillus oryzae						
– *P. cinnabarinus lac1*	8.0	70	3.5	4.5	65.0	0.055
– *Trametes villlosa lcc1*	3.0	60-70	3.5	2.7	?	0.058
– *Coprinopsis cinerea lcc1*	?	66	3.5	4.0	60-70	0.023

1. Data have been taken from the following references: Brown et al. 2002; Cassland and Jönsson 1999; Hong et al. 2002; Jolivalt et al. 2005; Liu et al. 2003; Madzak et al. 2005; Schneider et al. 1999; Sigoillot et al. 2004; Soden et al. 2002; Yaver et al. 1996.
2. For better comparison values were converted to IU in cases, where in the original literature laccase activities were given as nkat/ml or as arbitrary units.

Protein	L1	L2	L3	L4
Lcc1	HWHGLFQRGTNWADGADGVNQCPI	GTFWYHSHFGTQYCDGLRGPM	HPFHLHGH	GPWFFHCHIEFHLMNGIAIVF
Lcc2	HWHGMFQRGTAWADGPAGVTQCPI	GTFWYHSHHESQYCDGLRGAM	HPFHLHGH	GPWILHCHIDWHLVLGIAVVF
Lcc3	HWHGFLQEGTAWADGPAGVTQCPI	GTFWYHSHHMSQYCDGLRGVM	HPIHLHGH	GPWILHCHIDWHLVLGISVVF
Lcc4	HWHGLLQHGTAWADGSQSVSQCPI	GTFWYHSHFGTQYCDGLRGPF	HPFHLHGH	GPWLFHCHVEFHLQMGIAIVF
Lcc5	HWHGVFQHQSAWADGPDGVTQCPI	GTFWYHSHYGTQYCDGLRGPL	HPFHLHGH	GPWFFHCHIEFHLVTGIAAVF
Lcc6	HWHGLFQKGTNWADGAEGVNQCPI	GTFWYHSHFGTQYCDGLRGPM	HPFHLHGH	GPWFFHCHIEPHLVDGIAIVF
Lcc7	HWHGLFQRGTNWADGAEGVNQCPI	GTFWYHSHFGTQYCDGLRGPM	HPFHIHGH	GPWFFHCHIEPHLVGGIAIVF
Lcc8	HWHGIYQKHTNWADGVAGVNQCPI	GTFWYHSHFRTQYCDGLRGPL	HPFHLHGH	GPWILHCHIDWHLDLGIAIVL
	2 3	3 3	1 2 3	3 1 3 1

Figure 2. Fungal laccase signature sequences of *C. cinerea* laccases. The fungal laccase signature sequences L1-L4 were defined by Kumar et al. (2003) by comparison of 60 different enzymes. The sequences of the *C. cinerea* laccases are from Hoegger et al. (2004) where alignments of the whole proteins can be found. Numbers 1, 2 and 3 beneath the signature sequences refer to residues acting in copper binding at the Type 1, Type 2 and Type 3 sites of laccases, respectively (Piontek et al. 2002)

2. Materials and Methods

C. cinerea monokaryon FA2222 (*A5*, *B6*, *acu-1*, *trp1.1,1.6*; Kertesz-Chaloupková et al. 1998) was transformed by the protocol of Granado et al. (1997). *S. cerevisiae* RH 1385 (Mösch et al. 1990) was used for *in vivo*-recombination (Raymond et al. 1999), *Escherichia coli* XL1-Blue (Stratagene) for plasmid amplification. The yeast shuttle-vector pYSK2 (Kilaru et al., unpublished, Fig. 3) contains the yeast 2µm *ori* and *URA3* selection marker, the ColE1 *ori* (*ori Ec*) and amp^R from *E. coli*, the phage f1(+) *ori*, the *C. cinerea* *pab1* gene (for *para*-aminobenzoic acid synthesis; James et al. 2003) and the *C. cinerea lcc4* gene (Hoegger et al. 2004) under control of the *C. cinerea tub1* promoter (Cummings et al. 1999). pYSK2 can be used alone for transformation of *pab1* strains, but for selection of transformants in monokaryon FA2222, co-transformation with the $trp1^+$ vector pCc1001 (Binninger et al. 1987) is necessary. DNA work was performed by standard protocols (Sambrook et al. 2001). Promoter sequences and/or gene sequences were amplified with suitable chimeric primers from genomic DNA or plasmid subclones (Kilaru et al. unpublished). Recipes of *C. cinerea* media are given in Granado et al. (1997).

3. Results

3.1. *Cloning of promoters and/or genes into pYSK2 by in vivo-recombination in yeast*

The principle of directed cloning promoters and/or genes into vector pYSK2 is documented in Fig. 3. To exchange either a promoter or a gene, a one-step homologous recombination is sufficient. To replace both, promoter and gene, a two-step homologous recombination is required (see Fig. 3). For the latter, at least three chimeric primers have to be designed: i. a chimeric primer having at its 5'end a 30 bp homologous overlap to regions in the vector, e.g. to the *C. cinerea pab1* gene, linked to 20 bp of sequence for a promoter X fragment to be amplified by PCR; ii. a chimeric primer having at its 5'end a 30 bp homologous overlap to regions in the vector, e.g. to the f1(+) *ori*, linked to 20 bp of sequence for a gene Y to be amplified by PCR; iii. a chimeric primer covering the fusion point between a promoter X and a gene Y with a 30 bp extension at the 5' en into either the promoter sequences or the gene sequences, depending on whether the primer is designed for amplifying the gene or the promoter, respectively. A fourth primer required for amplification of the second DNA fragment of interest (either promoter or gene, respectively) does not need to be a chimeric primer but needs to allow amplification of the 30 bp homologous overlap to chimeric primer 3. Upon amplification of promoter X and gene Y by PCR, the DNA fragments are gel-purified and mixed with pYSK2 DNA for transformation into yeast. For suppression of background in yeast transformation by clones without new insert and to enhance recombination frequencies, pYSK2 is previously linearized by suitable restriction enzymes, for example through digestion with *BamH1* and *Kpn1* that cut in pYSK2 only in gene *lcc4* (see Fig. 3). The digested plasmid is purified by gel-electrophoresis. Transformants are plated on yeast selection medium. After two days, grown colonies are analyzed by colony PCR for presence of the expected construct. DNA from positive clones is isolated and transformed into *E. coli* for further plasmid amplification.

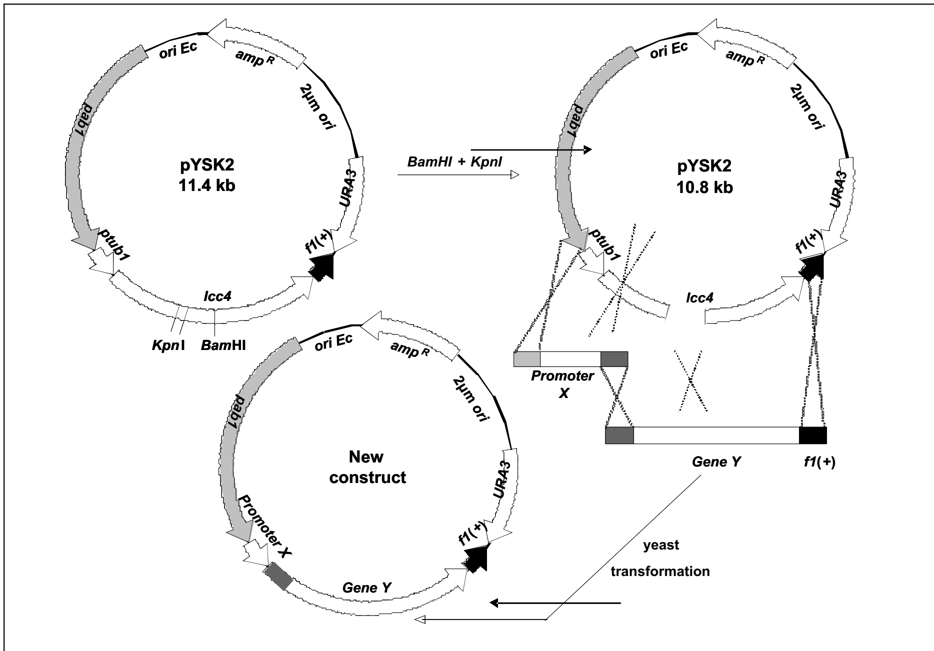

Figure 3. Yeast-shuttle vector pYSK2 and promoter and/or gene replacement strategy via homologous recombination in *Saccharomyces cerevisiae*

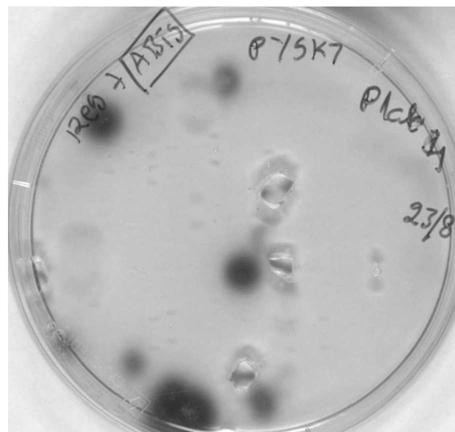

Figure 4. Laccase activity of transformants on regeneration medium plus 0.5 mM ABTS. The pYSK2 derived plasmid with a laccase gene was co-transformed into *C. cinerea* FA2222 protoplasts together with pCc1001 and plated on regeneration medium supplemented with ABTS. The green color around the colonies indicates oxidation of ABTS by secreted laccase.

3.2. Coprinopsis cinerea *transformation*

To test their efficiency in laccase production, pYSK2-derivatives are transformed together with pCc1001 into monokaryon FA2222. Upon transformation, protoplasts are plated onto regeneration medium with the laccase substrate 0.5 mM ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]. Transformants producing functional laccase through activity of a constitutive promoter can be identified after 3 to 5 days of incubation at 37°C by green staining of the regeneration agar due to oxidation of ABTS (Fig. 4). When transferred onto YMG/T medium with 0.5 mM ABTS, constitutive expression of laccase by such transformants is seen throughout growth by staining the medium red-brown (Fig. 5). pCc1001 control transformants of FA2222 neither show staining activity on regeneration agar with ABTS (not shown), nor on YMG/T medium with ABTS (Fig. 5).

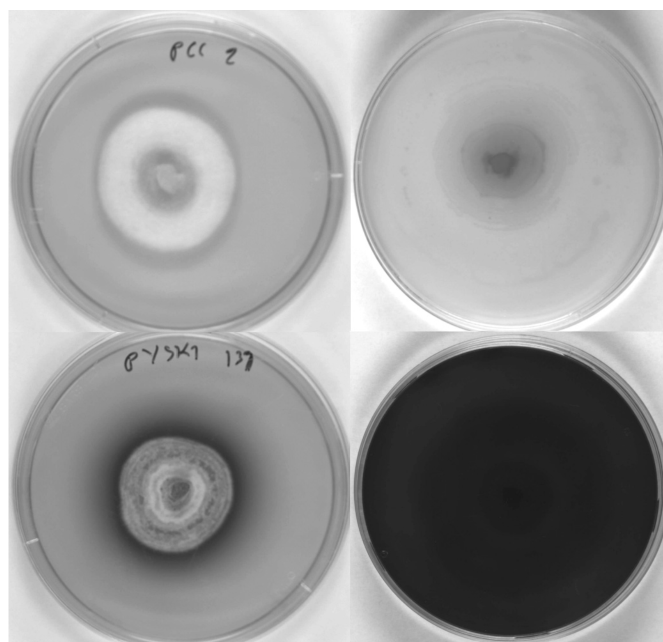

Figure 5. Overexpression of laccase during growth of a *C. cinerea* transformant on YMG/T complete medium plus 0.5 mM ABTS. Upper lane: pCc1001 control transformant at day 4 (left) and day 10 (right) of incubation at room temperature, respectively. Bottom lane: laccase transformant at day 4 (left) and day 10 (right) of incubation at room temperature, respectively.

4. Conclusion

Our study shows that we can express individual laccase genes in *C. cinerea* without other laccase activities in the background. We now can easily isolate individual enzymes for purification and biochemical characterization and, on a larger scale, for biotechnological applications. Enzymes can now be produced within this basidiomycete without the need to add any toxic or expensive laccase inducers. Our strategy for cloning promoters and/or genes via homologous recombination in yeast is simple and rapid. Several different promoters and/or genes from homologous or heterologous origin can be easily combined at the same time in parallel transformations.

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