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MINI-REVIEW

Peculiarities of *Pycnoporus* species for applications in biotechnology

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Abstract The genus *Pycnoporus* forms a cosmopolitan group of four species belonging to the polyporoid white-rot fungi, the most representative group of homobasidiomycetes causing wood decay. *Pycnoporus* fungi are listed as food- and cosmetic-grade microorganisms and emerged in the early 1990s as a genus whose biochemistry, biodegradation and biotechnological properties have since been progressively detailed. First highlighted for their original metabolic pathways involved in the functionalization of plant cell wall aromatic compounds to yield high-value molecules, e.g. aromas and antioxidants, the *Pycnoporus* species were later explored for their potential to produce various enzymes of industrial interest, such as hydrolases and oxidases. However, the most noteworthy feature of the genus *Pycnoporus* is its ability to overproduce high redox potential laccase—a multi-copper extracellular phenoloxidase—as the predominant ligninolytic enzyme. A major potential use of the *Pycnoporus* fungi is thus to harness their laccases for various applications such as the bioconversion of agricultural by-products and raw plant materials into valuable products, the biopulping and biobleaching of paper pulp and the biodegradation of organopollutants, xenobiotics and industrial contaminants. All the studies performed in the last decade show the genus *Pycnoporus* to be a strong contender for white biotechnology. In this review, we describe the properties of *Pycnoporus* fungi in relation to their biotechnological applications and potential.

Keywords *Pycnoporus* · Biotechnology · Enzyme · Laccase · Flavour · Application

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Introduction

The world of fungi offers a fascinating and seemingly endless wealth of biological diversity and forms a valuable resource. Almost 75,000 species of filamentous fungi are known to date, but there may be more than five million (Blackwell 2011). White-rot filamentous fungi form an important ecological group; they cause selective removal of lignin from wood and so have a high potential for biotechnological processes, particularly for lignocellulosic feedstock biorefinery applications. Among these fungi, the Polyporales group, including the genus *Pycnoporus*, is the most representative order of saprotrophic homobasidiomycetes causing wood decay, and its high lignocellulolytic potential is recognized (Alexopoulos et al. 1996). *Pycnoporus* is a genus closely related to *Trametes*, morphologically similar in all its characters, except for the conspicuous bright reddish-orange colour of its basidiocarp (Ryvarden 1991). This colour arises from the synthesis of various pigments of the phenoxazin-3-one type, including cinnabarin, tramesanguin and cinnabarinic acid (Sullivan and Henry 1971). *Pycnoporus* is morphologically characterized by an annual, sessile to effused-reflexed basidiocarp, a dimitic or trimitic hyphal system, generative hyphae with clamps, clavate 4-sterigmate

basidia and cylindrical, slightly curved, hyaline smooth basidiospores (Ryvarden and Gilbertson 1994). Historically, four species were discerned based on their morphological characters (pore size of basidiocarp and basidiospore shape) and their distribution areas (Nobles and Frew 1962; Ryvarden and Johansen 1980): (1) *Pycnoporus cinnabarinus*, a common species distributed especially in the Northern Hemisphere, (2) *Pycnoporus puniceus*, a rare species distributed in Africa, India, Malaysia and New Caledonia (characterized by a basidiocarp with large irregular pores of 1–3/mm), (3) *Pycnoporus sanguineus*, a common species distributed in tropical and subtropical regions and (4) *Pycnoporus coccineus*, distributed in the countries bordering the Indian and Pacific Oceans. The *Pycnoporus* fungi are heterothallic homobasidiomycetes with a tetrapolar mating system (Nobles and Frew 1962). The basidiomycete life cycle (Fig. 1) shows a sexual development from haploid basidiopores, which produce, upon germination, a hypha with one nucleus per cell (monokaryon). Two monokaryotic strains with different mating alleles can fuse and produce a dikaryotic mycelium—characterized by clamp connections—in which the two parental nuclei do not fuse during the vegetative growth. Vegetative growth is maintained until environmental conditions induce fruit-body formation. After karyogamy and meiosis within the basidia, four uninucleate spores are produced and give rise to monokaryotic cell lines (Herpoël et al. 2000; Lomascolo et al. 2002).

In the early 1980s, the species *Phanerochate chrysosporium* was historically the first white-rot model, characterized by a secondary metabolism pattern triggered by nitrogen limitation. Research was then gradually extended to other organisms, among which *Pycnoporus* emerged, in the middle of 1990s, as a genus whose biochemistry, biodegradation and biotechnological properties have since been progressively detailed. The exploration and use of the metabolic capacities of the genus *Pycnoporus* holds great promise for biotechnological applications in view of three main characteristics: (1) its ability to produce various copper and iron metalloenzymes involved in the transformation of plant cell wall aromatic compounds (Moukha et al. 1999; Halaouli et al. 2005), (2) original metabolic pathways involved in the functionalization of these cell wall aromatics to yield high added-value compounds including aromas and antioxidants (Lesage-Meessen et al. 1997; Estrada Alvarado et al. 2003) and (3) its ability to produce fruit-body structures and monokaryotic cell lines in laboratory culture conditions—very rare among basidiomycetes—enabling genetic improvement by both classical genetics and genetically modified-organism (GMO) methods (Alves et al. 2004): the isolation of monokaryotic cell lines offers an alternative to the construction of GMOs providing a simpler genetic and biochemical system than

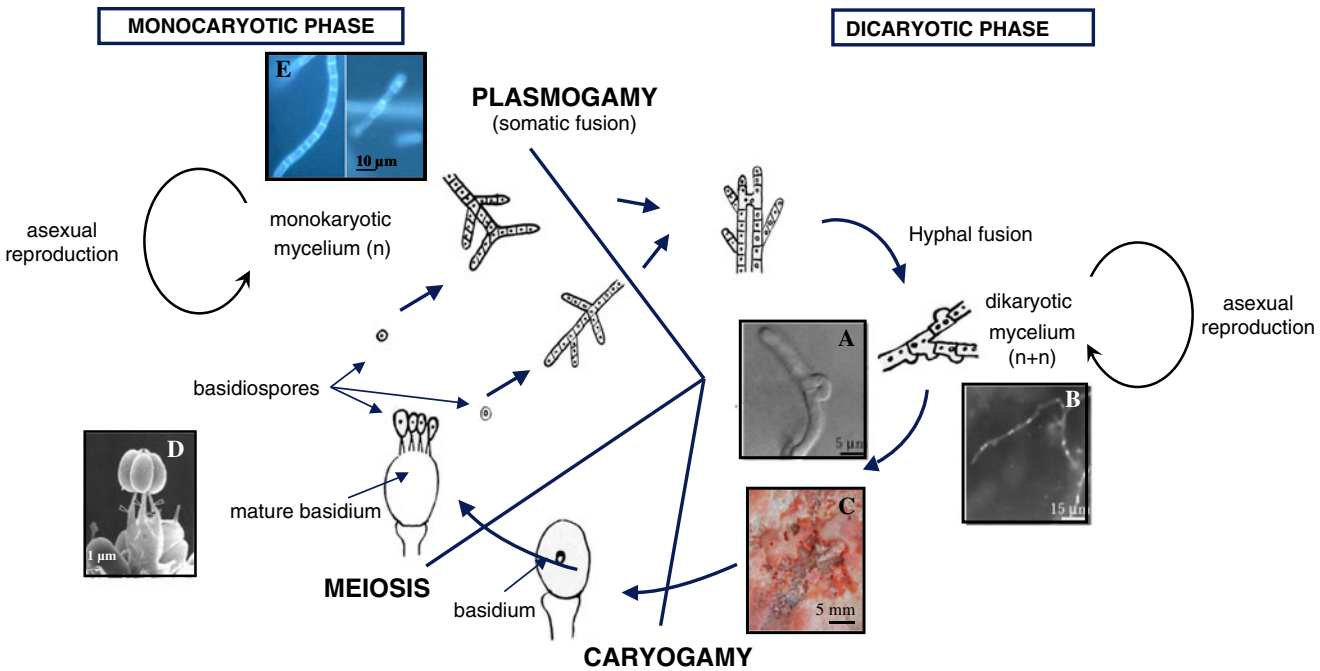

Fig. 1 Scheme of *Pycnoporus* life cycle: clamp connection (**a**), dikaryotic mycelium (**b**), fruit-body-like structure (**c**), basidia with basidiospores (**d**), monokaryotic cell line (**e**); *solid line*, sexual reproduction; *dashed line*, asexual reproduction. Adapted from Herpoël et al. (2000)

the dikaryotic state, the haploid state being easier to handle for mutagenesis and further transformations. Moreover, the enzymes from a non-GMO could also enjoy food-grade status if required for food or cosmetic applications.

The *Pycnoporus* fungi are white-rot basidiomycetes listed as food- and cosmetic-grade microorganisms. *Pycnoporus* fungi are not edible fungi in Europe, but they belong to the traditional pharmacopeia of countries in Africa and South America for the treatment of various illnesses and skin lesions (Smânia et al. 2003). In addition, cinnabarin from *Pycnoporus* fungi has been shown to display antiviral and antibacterial activities against undesirable food bacteria (Smânia et al. 2003) and human pathogenic bacteria such as *Klebsiella pneumoniae* and *Salmonella typhi* (Smânia et al. 1995). An active compound with Leishmanicidal activity was also isolated from *P. sanguineus* (Correa et al. 2006).

Physiologically and biotechnologically, the *Pycnoporus* fungi are easy to cultivate at laboratory and pilot-plant scales. High yields of biomass can be obtained on the following carbohydrate substrates: starch, malt extract, maltose, methyl cellulose, sucrose, dextrose and malt extract broth supplemented with yeast extract and/or phospholipids (Holler and Brooks 1980; Oddou et al. 1999). The glycolytic pathway and pentose-phosphate shunt both seem to be operative in *Pycnoporus*, although the glycolytic pathway is preponderant (Hirono et al. 1978). *Pycnoporus* species have been successfully grown, fully submerged, in 20- to 200-l packed-bed bioreactors (Lonergan et al. 1993, 1995) and in mechanically agitated 2- and 15-l fermentors (Stentelaire et al. 2000; Georis et al. 2003). The fungus was insensitive to fluctuations in both pH (although no buffering was used) and temperature and was capable to produce biomass rapidly. These properties fit *Pycnoporus* to industrial applications. Among basidiomycetes, members of the genus *Pycnoporus* have been shown especially to produce various enzymes of industrial interest, including hydrolases such as xylanase and β-glucosidase (Esposito et al. 1993), invertase (Quiroga et al. 1995), and α-amylase (De Almeida Siqueira et al. 1997). However, the most obviously useful feature of the genus *Pycnoporus* is its ability to overproduce high redox potential laccase—a multi-copper extracellular phenoloxidase— as the predominant ligninolytic enzyme (Eggert et al. 1996a; Lomascolo et al. 2003). A major promise of the *Pycnoporus* fungi lies in the use of their laccases for a variety of applications such as the bioconversion of agricultural by-products and raw plant materials into valuable products, the biopulping and biobleaching of paper pulp, and the biodegradation of organopollutants, xenobiotics and industrial contaminants. All the studies performed in this last decade support the genus *Pycnoporus* as a strong contender for white biotechnology. This review is the first to summarize these findings.

The genus *Pycnoporus* as a cell factory for the production of a wide range of enzymes

Hydrolases

Interest in the enzyme activities of *Pycnoporus* began thirty years ago when the first studies of carboxyl proteinase, chitinase and β-*N*-acetylhexosaminidase were published by Japanese authors (Table 1). The first enzyme, the carboxyl proteinase I_a from *P. coccineus*, was characterized by Ichishima et al. (1980): its major splitting sites, in the B chain of insulin, were Ala-Leu, His-Leu and Phe-Phe. The substrate specificity of this trypsinogen-activating carboxyl proteinase was especially investigated with angiotensin and proangiotensin (Kumagai et al. 1981). A serine carboxypeptidase was then isolated and purified from *P. sanguineus* (Ichishima et al. 1983) and displayed a preference for neutral aliphatic residues and glutamic acid in the penultimate peptide position.

Basidiomycetous fungi have been shown to have significant glycosyl hydrolytic potential and *Pycnoporus* is no exception (Gomez-Alarcon et al. 1989; Esposito et al. 1993). A chitinase and an *N*-acetyl-β-glucosaminidase, both enzymes required for the complete hydrolysis of chitin, were purified from *P. cinnabarinus* extracellular fluid (Ohtakara 1988). The *P. cinnabarinus* chitinase hydrolysed chitin acting on chitooligosaccharides, the mode of action being endo-type, predominantly hydrolysing the second β-*N*-acetylglucosaminide linkage from the non-reducing end (Ohtakara 1988). The β-*N*-acetylhexosaminidase identified in *P. cinnabarinus* hydrolysed chitooligosaccharides at the non-reducing end to *N*-acetylglucosamine, in an exo-type mode (Ohtakara et al. 1981a, b). Biotechnological applicability of *Pycnoporus* hydrolytic enzymes was further demonstrated in the case of glycosidases such as α- and β-galactosidases, α-amylase and β-glucosidase (Table 1). These enzymes are of industrial interest in sugar beet, starch and soymilk processing, in the dairy and fruit product industries and in structural studies of glycoproteins. The α-galactosidase from *P. cinnabarinus* was especially studied by Ohtakara et al. (1984), Mitsutomi et al. (1985), Ohtakara and Mitsutomi (1987), Mitsutomi and Ohtakara (1988) and Mitsutomi et al. (1991). This enzyme hydrolyses α-galactosyl linkages in oligo- and polysaccharides but also displays a galactosyltransferase activity (Mitsutomi and Ohtakara 1988). This highly thermostable α-galactosidase was purified and immobilized on colloidal chitin with glutaraldehyde (Mitsutomi et al. 1985) or on chitosan beads (Ohtakara and Mitsutomi 1987), keeping biochemical properties similar to the native enzyme. The immobilized enzyme was then successfully used for hydrolysis of raffinose in sugar beet molasses in a 30-day continuous process. Later, Mitsutomi et al. (1991)

Table 1 Enzyme activities detected in *Pycnoporus cinnabarinus*, *Pycnoporus coccineus* and *Pycnoporus sanguineus*

Enzyme	Organism	Biochemical and molecular characteristics[a]	Applications	References
Proteinases				
Carboxylproteinase I_a EC 3.4.23.6	*P. coccineus*	p*I* 4.72 Optimal pH 2.5	Proteolytic activity against milk casein	Ichishima et al. 1980 Kumagai et al. 1981
Serine carboxypeptidase EC 3.4.16.1.	*P. sanguineus* ATCC 14622	MW 50 kDa p*I* 4.78 Optimal pH 3.4 Stable up to 60°C K_M, 0.74 mM (2-Glu-Tyr) K_M, 2 mM (bradykinin) K_M, 0.76 mM (angiotensin)		Ichishima et al. 1983
Glycosidases				
β-*N*-Acetylhexosaminidase EC 3.2.1.52	*P. cinnabarinus* IFO 6139	MW, 120 kDa (dimer of 60 kDa) p*I* 5.4 Optimal pH 2.2 (*p*NP-GluNAc) Stable at pH 2–4 Stable up to 50°C K_M, 0.45 mM (*p*NP-GluNAc)		Ohtakara 1988 Ohtakara et al. 1981a Ohtakara et al. 1982
Chitinase EC 3.2.1.14	*P. cinnabarinus* IFO 6139	MW, 38 kDa p*I* 3.6 Optimal pH 4.5 (chitin) Stable at pH 4–8		Ohtakara 1988
Invertase (β-fructofuranosidase) EC 3.2.1.26	*P. sanguineus* IEV 38	MW, 84 kDa (dimer of 42 kDa) Optimal pH 3.85 Stable at pH 3–7.5 K_M, 4.89 mM (sucrose)		Quiroga et al. 1995
α-Amylase EC 3.2.1.1 β-Glucosidase EC 3.2.1.21	*P. sanguineus*	2.5 U/mg (starch) 5 U/mg (*p*NPGlu)		De Almeida Siqueira et al. 1997
Xylanase EC 3.2.1.8		5 U/mg (oat spelt xylan)		
Exo-polygacturonase EC 3.2.1.82	*P. sanguineus* IEV 38	MW, 42 kDa Optimal pH 4.8 Stable at pH 3.8–8 Optimal temperature, 50–60°C Stable up to 60°C K_M, 0.55 mg/ml (PGA)		Quiroga et al. 2009

Table 1 (continued)

Enzyme	Organism	Biochemical and molecular characteristics[a]	Applications	References
α-Mannosidase EC 3.2.1.24	*P. sanguineus* ATCC 14622	MW, 64 kDa p*I* 4.7 Optimal pH 4.5 (α-D-mannan) Stable at pH 3.5–5.5 Stable up to 60°C K_M, 0.9 mM (Manα1–2Man)		Ichishima et al. 1985
β-Galactosidase EC 3.2.1.23	*P. cinnabarinus* IFO 6139	MW, 110 kDa (dimer of 55 kDa) Optimal pH 2.4 Stable at pH 3–6 Stable up to 45°C K_M, 0.95 mM (*p*NP-βgal) K_M, 33 mM (lactose)		Ohtakara et al. 1981b
α-Galactosidase EC 3.2.1.22	*P. cinnabarinus* IFO 6139	MW, 210 kDa (tetramer of 52 kDa) Optimal pH 5 Stable at pH 3–9 Optimal temperature, 75°C Stable up to 75°C K_M, 0.31 mM (*p*NP-αgal)	Immobilization on chitosan beads and hydrolysis of raffinose in sugar beet molasses Enzymatic synthesis of galactooligosaccharides	Ohtakara et al. 1984 Mitsutomi et al. 1985 Ohtakara and Mitsutomi 1987 Mitsutomi and Ohtakara 1988 Mitsutomi et al. 1991
Xylanase EC 3.2.1.8	*P. cinnabarinus* CIRM-BRFM 137	MW, 50 kDa p*I* 4 Optimal pH 5 Optimal temperature, 60°C Stable up to 60°C K_M, 14.2 mg/ml (oat spelt xylan)	Pulp and paper industry	Sigoillot et al. 2002 Herpoël et al. 2002
Oxidative enzymes				
Laccase EC 1.10.3.2	*P. coccineus* IFO4923	MW, 68 kDa p*I* 3.5 Optimal pH 4.5 Stable at pH 4–9 Stable up to 65°C		Oda et al. 1991

Table 1 (continued)

Enzyme	Organism	Biochemical and molecular characteristics[a]	Applications	References
Laccase	*P. coccineus* MUCL 38527	MW, 61.5 kDa p*I* 3.7 Optimal pH 3.5 (ABTS and DMP) Optimal temperature, 60°C Stable up to 60°C K_M, 27 μM (DMP) and 36 μM (ABTS)	Degradation of aromatic compounds in olive oil mill wastewaters	Jaouani et al. 2005 Berrio et al. 2007
Laccase	*P. coccineus* (*Trametes sanguinea*) M85-2	MW, 62 kDa p*I* 3 Optimal pH 5 Stable at pH 5–10 Optimal temperature, 60°C		Nishizawa et al. 1995
Laccase	*P. coccineus* CIRM-BRFM 938	MW, 61.8 kDa Optimal pH 4.5–5 Stable at pH 5–7 Optimal temperature, 65°C Stable up to 78°C K_M, 26 μM (ABTS) Tolerant towards 10–50% organic solvents	Polyphenolic dye decolorization Oxidation on non-phenolic lignin model compounds Oligomerization of rutin (cosmetic application)	Uzan et al. 2010 Uzan et al. 2011
Laccase	*P. sanguineus* CY788	MW, 65 kDa Optimal pH 3 Stable up to 35°C	Growth on waste lignocellulose substrates to achieve lignin biodegradation	Pointing et al. 2000
Laccase	*P. sanguineus* MUCL 41582	MW, 57 kDa Optimal pH 2.5 Low stability at acidic pH Optimal temperature, 50°C Stable up to 75°C Tolerant towards 0.5–1 M NaCl and Na_2SO_4	Polyphenolic dye decolourization	Trovaslet et al. 2007
Laccase	*P. sanguineus* SCC 108	MW, 58 kDa p*I* 6.7 Optimal pH 3–5 Optimal temperature, 55°C Stable up to 75°C K_M,130 μM (ABTS), 52 μM (DMP) and 83 μM (syringaldazine)		Litthauer et al. 2007

Table 1 (continued)

Enzyme	Organism	Biochemical and molecular characteristics[a]	Applications	References
Laccase	*P. sanguineus*	MW, 61.4 kDa Optimal pH 3 Highly stable at pH 2–5 Optimal temperature, 65°C Stable up to 40°C K_M, 77 μM (ABTS), 203 μM (DMP) and 91 μM (syringaldazine)	Polyphenolic dye decolourization	Lu et al. 2007
Laccase	*P. sanguineus* CCT-4518	Lac I MW, 80 kDa Optimal pH 4.8 Optimal temperature, 30°C Stable up to 50°C K_M, 10 μM (syringaldazine) Lac II: MW, 68 kDa optimal pH 4.2 Optimal temperature, 50°C Stable up to 60°C Tolerance towards dimethylsulfoxide K_M, 58 μM (ABTS), 8.3 μM (syringaldazine), 370 μM (guaiacol)		Alves Garcia et al. 2006 Alves Garcia et al. 2007
Laccase	*P. sanguineus* MEXU 25347	MW, 68 kDa p*I* 7 Stable up to 60°C K_M, 239 μM (ABTS), 90 μM (syringaldazine)		Dantan-Gonzalez et al. 2008
Laccase	*P. sanguineus* CIRM-BRFM 66	MW, 59.5 kDa Optimal pH 4–5 Stable at pH 5–7 Optimal temperature, 71°C Stable up to 78°C K_M, 33 μM (ABTS) Tolerant towards 10–50% organic solvents	Polyphenolic dye decolorization Oxidation on non-phenolic lignin model compounds Oligomerization of rutin (cosmetic application)	Uzan et al. 2010 Uzan et al. 2011

Table 1 (continued)

Enzyme	Organism	Biochemical and molecular characteristics[a]	Applications	References
Laccase	*P. sanguineus* CIRM-BRFM 902	MW, 62.9 kDa	Polyphenolic dye decolorization	Uzan et al. 2010
		Optimal pH 4.5–5	Oxidation on non-phenolic lignin model compounds	Uzan et al. 2011
		Stable at pH 5–7	Oligomerization of rutin (cosmetic application)	
		Optimal temperature, 65°C		
		Stable up to 78°C		
		K_M, 32 μM (ABTS)		
		Tolerant towards 10–50% organic solvents		
Laccase	*P. cinnabarinus* CBS 101046	MW, 63 kDa	Degradation of the disazo dye Chicago Sky Blue	Schliephake et al. 2000
		p*I* 3		
		Optimal pH 4.5–5 (syringaldazine)		
		Stable up to 60°C		
		K_M, 30 μM (syringaldazine)		
		K_M, 33 μM (guaiacol)		
Laccase	*P. cinnabarinus* ATCC 200478	MW, 81 kDa	Production of antibacterial compound	Eggert et al. 1995
		p*I* 3.7	Degradation of polyviniyl alcohol	Eggert et al. 1996a
		Optimal pH 4	Lignin degradation	Eggert et al. 1997
		Stable up to 70°C		Larking et al. 1999
Laccase	*P. cinnabarinus* CIRM-BRFM 137	MW, 62 kDa	Paper pulp bleaching	Sigoillot et al. 2001
		p*I* <3.5	Enzymatic delignification	
		Optimal temperature, 50–55°C	Synthesis of biopolymers from agro-residues	Herpoël et al. 2002
		Optimal pH 4	Oxidation of recalcitrant compounds	Sigoillot et al. 2004
		Stable up to 75°C	Oxidative degradation of paper pulp lipophilic extractives	De Wilde et al. 2008
		K_M, 23 μM (ABTS)		Camarero et al. 2008
				Molina et al. 2008
				Ravalason et al. 2009

Table 1 (continued)

Enzyme	Organism	Biochemical and molecular characteristics[a]	Applications	References
Laccase	*Pycnoporus* sp. SYBC-L1	Lac I MW, 55.9 kDa optimal pH 2.5–5.5 Optimal temperature, 65°C Stable up to 60°C Stable for pH 4–10 K_M, 16.6 μM (ABTS) Strong tolerance to cold conditions Lac II MW, 63.1 kDa optimal pH 2.5–5.5 Optimal temperature, 70°C Stable up to 70–80°C Stable for pH 4–10 K_M, 43.5 μM (ABTS) Strong tolerance to cold conditions	Decolourisation of anthraquinone dye	Whang et al. 2010
Tyrosinase EC 1.14.18.1	*P. sanguineus* CBS 614.73	MW, 45 kDa (protyrosinase 68 kDa) p*I* 4.5–5 Optimal pH 6.5–7 Stable at pH 5–8 Under the conditions Optimal temperature, 60–65°C Stable up to 60°C K_M, 1 mM (L-tyrosine) K_M, 0·9 mM (L-DOPA)	Antioxidant synthesis from monophenols Protein cross-linking	Halaouli et al. 2005 Halaouli et al. 2006b
Cellobiodehydrogenase EC 1.1.99.18	*P. cinnabarinus* CIRM-BRFM 137	MW, 92 kDa Optimal pH 4.5 Stable up to 55°C Optimal temperature, 70°C K_M, 111 μM (cellobiose)		Moukha et al. 1999 Sigoillot et al. 2002

MW molecular weight, *pI* isoelectric point

2-Glu-Tyr benzyloxycarbonyl-L-glutamyl-L-tyrosine, *pNP-GluNAc p*-nitrophenyl-β-*N*-acetylglucosaminide, *PGA* polygalacturonic acid, *pNPGlu p*-nitrophenyl-β-D-glucopyranoside, *pNP-βgal p*-nitrophenyl-β-D-galactoside, *pNP-αgal p*-nitrophenyl-α-D-galactoside, *L-DOPA* 3,4-dihydroxyphenylalanine, *DMP* 2,6-dimethoxyphenol, *ABTS* (2,2′-azino-bis-[3-ethylthiazoline-6-sulphonate]

[a] In parenthesis, substrate used for biochemical and kinetic measurements

found that the *P. cinnabarinus* α-galactosidase was able to synthesize galactooligosaccharides, specifically the trisaccharides raffinose, planteose and 3^{G}α-galactosyl sucrose, from the condensation of galactose and sucrose. Such trisaccharides were expected to be utilized as a growth factor for bifidobacteria. In 1995, Quiroga et al. described, for the first time, the isolation and purification of a β-fructofuranosidase (or invertase) from *P. sanguineus*. The enzyme was able to attack sucrose, raffinose, stachyose, inulin and levan, sucrose being the preferred substrate (Table 1). Very recently, a novel exo-polygalacturonase, PGase I, was isolated from *P. sanguineus* grown on citrus fruit pectin (Quiroga et al. 2009), as an exocellular enzyme releasing galacturonic acid as its principal hydrolysis product. Such a pectinase has potential applications in the fruit, paper and textile industries, but also in protoplast fusion technology and plant pathology (Quiroga et al. 2009). Cellulolytic and hemicellulolytic activities were notably evidenced in *P. sanguineus* and *P. cinnabarinus*. An acidic α-D-mannosidase, stable up to 60°C, was isolated from liquid cultures of *P. sanguineus* (Ichishima et al. 1985) and was shown to specifically cleave the 1,2-α-linked side-chain of α-mannan. When cultured on wheat straw solid medium, *P. sanguineus* exhibited exoglucanase activity on microcrystalline cellulose (Avicel), β-glucosidase activity on cellobiose and xylanase activity on oat spelt and birchwood xylans (Quiroz-Castañeda et al. 2009). These enzymatic activities resisted incubation for 1 h at high temperatures (up to 80°C) and were stable in the pH range of 2–8. Thermostable xylanase production (300–3,700 U/l) was obtained from *P. cinnabarinus* grown on natural substrates (cellulose powder, maize and wheat bran and sugar beet pulp) as carbon sources and increased in the presence of Tween 80 as surfactant (Sigoillot et al. 2002).

It is worth noting that the hydrolytic enzymes characterized from *Pycnoporus* generally exhibited high thermal stability, broad pH range activity and remarkable potential in various biotechnological applications. Surprisingly, these results were never taken further by genetic and molecular studies.

Oxidative enzymes

Laccase

Laccases (*p*-diphenol/oxygen oxidoreductases, EC 1.10.3.2) are multi-copper oxidases containing one type-1 copper, the redox potential of which determines the substrates to be oxidized, and three other copper atoms transferring the electrons to O_2, further reduced to water. They catalyse the one-electron oxidation of a wide range of compounds including di-, substituted and polyphenols and di- and aromatic amines to form free radicals, which in turn can produce dimers, oligomers and polymers (Baldrian 2006). In the genus *Pycnoporus*, laccases are extracellular monomeric glycoproteins, produced in both submerged cultures (Eggert et al. 1996a; Lomascolo et al. 2003) and solid-state fermentation of agro-residues (Meza et al. 2006; Vikineswary et al. 2006). Laccase production depends on the cultivation conditions: carbon and nitrogen sources and concentrations (Eugenio et al. 2009), addition of surfactants such as Tween 80 (Gomez-Alarcon et al. 1989) and the presence of inducers and of metal ions such as copper (Hoshida et al. 2005). One of the most effective method for regulating and increasing *Pycnoporus* laccase production is the addition of an appropriate inducer to the medium, which may be aromatic (ferulic acid or 2,5-xylidine) or aliphatic (ethanol, methanol or dimethylsulfoxide), and lignocellulosic agro-residues (Herpoël et al. 2000; Jones et al. 2001; Lomascolo et al. 2002, 2003; Alves Garcia et al. 2006; Valeriano et al. 2009). Likewise, significant *Pycnoporus* intra-genus and intra-species diversity in laccase production has been reported. Wild *Pycnoporus* strains originating from various geographical areas, especially tropical habitats, have been shown to produce up to 17,000 U/l laccase, i.e. 65 mg/l (Lomascolo et al. 2002; Uzan et al. 2010). A monokaryotic strain, *P. cinnabarinus* CIRM-BRFM 137, was further identified as an outstanding overproducer of laccase (266,600 U/l, i.e. 1 g/l) in the presence of ethanol as inducer (Lomascolo et al. 2003). These activities were measured at pH 4 with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS)) as substrate.

Until now, two genes of laccases, named *lac1* (or *lcc3-1*) and *lac 2* (*lcc3-2*), have been isolated from *P. cinnabarinus* and *P. sanguineus* (Eggert et al. 1998; Temp et al. 1999; Alves Garcia et al. 2006, 2007), but Lac 1 seemed to be the isoenzyme mainly expressed in induced culture conditions (Antorini et al. 2002; Lomascolo et al. 2003). Several *Pycnoporus* laccase genes and cDNAs have been cloned and sequenced (NCBI accession numbers AB072703, AB072704, AF025481, AF123571, AF152170, AF 170093, AY147188, AY458017, AY510604, FJ513007, FJ858749, FJ 858750 and FJ585751). These genes are DNA fragments of about 2.1 kbp interrupted by ten introns. The ORFs consist of 11 exons corresponding to sequences of about 1,600–1,900 nucleotides coding for proteins of 518 amino acids (aa) containing a signal peptide of 21 aa. The expression of laccase genes depends on the culture conditions, and a large panel of isoenzymes have been described in the literature (for an overview, see Table 1). The properties of *Pycnoporus* laccases are generally close to those of the high redox potential laccases from *Trametes* spp. More specifically, the main characteristics of *Pycnoporus* laccases are: molecular weight between 57 and 80 kDa, isoelectric point generally acidic but ranging from

3 to 7, Michaelis constant (ABTS as substrate) in the range of 26–239 μM (Table 1). Compared with other white-rot fungal enzymes, *Pycnoporus* laccases display useful biochemical features suitable for biotechnological applications, including high activity optimal temperature (50–65°C), high thermostability and tolerance towards salts and organic solvents (Table 1). Laccases are generally classified into three groups according to the redox potential of the type-1 copper centre: low (*ca.* 0.4–0.5 V), medium (*ca.* 0.5–0.6 V) or high (*ca.* 0.7–0.8 V) (Xu et al. 1996). *Pycnoporus* laccases obviously belong to the group with the highest redox potential, with values of 0.72–0.8 V (Sigoillot et al. 2004; Uzan et al. 2010). Also, the immobilization of *Pycnoporus* laccases on magnetic particles has been successfully achieved (Jiang et al. 2005; Whang et al. 2008). The immobilized enzymes exhibited remarkably improved catalytic capacity and stability properties for various parameters, such as pH, temperature, re-use and storage time, promising economic advantages for large-scale biotechnological applications.

Heterologous expression of the *Pycnoporus* laccase gene has been successfully performed in heterologous eucaryotic hosts including the yeasts *Pichia pastoris*, *Yarrowia lipolytica* and *Saccharomyces cerevisiae* (Otterbein et al. 2000; Hoshida et al. 2005; Mazdak et al. 2005; Romano et al. 2007) and the filamentous fungi *Aspergillus niger* and *Aspergillus oryzae* (Record et al. 2002; Hoshida et al. 2005). In addition, an efficient transformation and expression system was developed for *P. cinnabarinus* (Alves et al. 2005). This was used to transform the monokaryotic strain CIRM-BRFM 44 with the homologous *lac1* gene (Alves et al. 2005). The yields ranged from a few mg per litre in yeasts to about 100 mg/l in *A. niger* and 1 g/l in *P. cinnabarinus*.

Tyrosinase

Tyrosinases (monophenol, *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) are type-3 copper proteins involved in the initial step of melanin synthesis. These enzymes catalyse both the *ortho*-hydroxylation of monophenols and the subsequent oxidation of the resulting *ortho*-diphenols into reactive *o*-quinones, which evolve spontaneously to produce intermediates that associate in dark brown pigments. In fungi, tyrosinases are generally associated with the formation and stability of spores, in defence and virulence mechanisms, and in browning and pigmentation. First characterized from the edible mushroom *Agaricus bisporus* while addressing undesirable enzymatic browning problems during postharvest storage, tyrosinases were found more recently in several other fungi, with useful insights into molecular and genetic characteristics and reaction mechanisms, highlighting their very promising properties for biotechnological applications (Halaouli et al. 2006a). These applications remain limited because native fungal tyrosinases are generally intracellular and produced in low quantities. Halaouli et al. (2005) showed for the first time that several *Pycnoporus* strains were able to produce tyrosinase. The strain *P. sanguineus* CBS 614.73 was identified as the best tyrosinase producer, with tyrosinase production of 45.4 and 163.6 U/g protein per day for monophenolase and diphenolase, respectively. This tyrosinase was monomeric and intracellular; it was purified and characterized (Table 1) and stood apart from other source tyrosinases so far reported in its N-terminal amino acid sequence, kinetic parameters and thermal stability. This tyrosinase proved effective in the synthesis of natural antioxidants and in protein cross-linking (Halaouli et al. 2005). The corresponding tyrosinase-encoding gene (2,204 bp) and cDNA (1,857 nucleotides) were cloned from *P. sanguineus* CBS 614.73. This gene consisted of seven exons and six introns and encoded a predicted protein of 68 kDa, exceeding the mature tyrosinase by 23 kDa (C-terminal proteolytic cleavage). *P. sanguineus* tyrosinase cDNA was over-expressed in *A. niger*, under the control of the strong and constitutive glyceraldehyde-3-phosphate-dehydrogenase promoter. The glucoamylase preprosequence of *A. niger* was used to target the secretion. This construction enabled the production, for the first time, of a fully active recombinant tyrosinase in the extracellular medium of *A. niger* in a yield of *ca*. 20 mg l^{-1} (Halaouli et al. 2006b).

Cellobiose dehydrogenase

Cellobiose dehydrogenase (CDH) is an extracellular oxidoreductase that contains both a protoporphyrin-IX-based heme and a flavin prosthetic group. It is a bifunctional enzyme containing a cellulose-binding domain. It was suggested that CDH could degrade cellulose, hemicellulose and lignin and could participate in wood degradation by white-rot fungi *via* the generation of highly reactive hydroxyl radicals involved in demethylation of lignin. A CDH gene was cloned from *P. cinnabarinus* for the first time by Moukha et al. (1999). mRNA-encoding *P. cinnabarinus* CDH was shown to be induced by cellulose and relatively repressed by cellobiose or glucose in the culture medium. The corresponding protein (92 kDa) was further purified and characterized by Sigoillot et al. (2002) (Table 1).

Others

A partial sequence of a lignin peroxidase-like gene was amplified for the first time in the strain *P. sanguineus* CBS 614.73 (Pointing et al. 2005). Several partial lip- and mnp-

like sequences were obtained from *P. cinnabarinus* in the context of a classification study of peroxidases in the orders Hymenochaetales and Polyporales (Morgenstern et al. 2010). However, members of the genus *Pycnoporus* have never yet been shown to produce any detectable peroxidase during cultivation on a defined growth medium. However, almost all studies on lignin-modifying enzymes produced by *Pycnoporus* spp. have been performed in more or less synthetic liquid media which is not the most favourable condition to detect peroxidase-type activity. Very recently, Liers et al. (2011) have demonstrated the presence of manganese-oxidizing peroxidase activities for *P. cinnabarinus* during beech-wood colonization in solid-state microcosme cultures.

Biotechnological applications

The genus *Pycnoporus* as biological tool for aromatic compound functionalization: utilization and transformation of cell wall aromatic compounds into high added-value products

In the past the only natural sources of flavours were plants. However, active components often occur in tiny amounts and only in exotic plants, making isolation difficult and products expensive. The white-rot basidiomycetes, and especially the genus *Pycnoporus*, represent major biotechnological agents for generating, de novo or by bioconversion, natural aromas for industry when grown in standard media or in the presence of precursors (Asther et al. 1998; Lomascolo et al. 2002). The high demand for natural aroma makes vanillin production by biotransformation a viable alternative to natural and chemical sources. Ferulic acid (4-hydroxy 3-methoxycinnamic acid), a component of plant cell walls with a chemical structure close to that of vanillin, is efficient as a precursor for the production of vanillin. Among 300 food-grade strains of basidiomycetes, the wild dikaryotic strain I-937 of *P. cinnabarinus* was selected for its ability to produce 64 mg/l vanillin after 6 days of culture from 300 mg/l ferulic acid, with a molar yield of 27.5% (Gross et al. 1991). The metabolism of ferulic acid into vanillin by *P. cinnabarinus* I-937 has been established. Vanillic acid, the major degradation product of ferulic acid, is produced by the loss of two carbon atoms from the propenoic chain, and is further metabolized into methoxyhydroquinone through an oxidative decarboxylation or into vanillin and vanillyl alcohol through reductive pathways (Falconnier et al. 1994) (Fig. 2). The major intermediates of [5-^{2}H]-ferulic acid biotransformation by *P. cinnabarinus* have been identified; the phenyl prope-

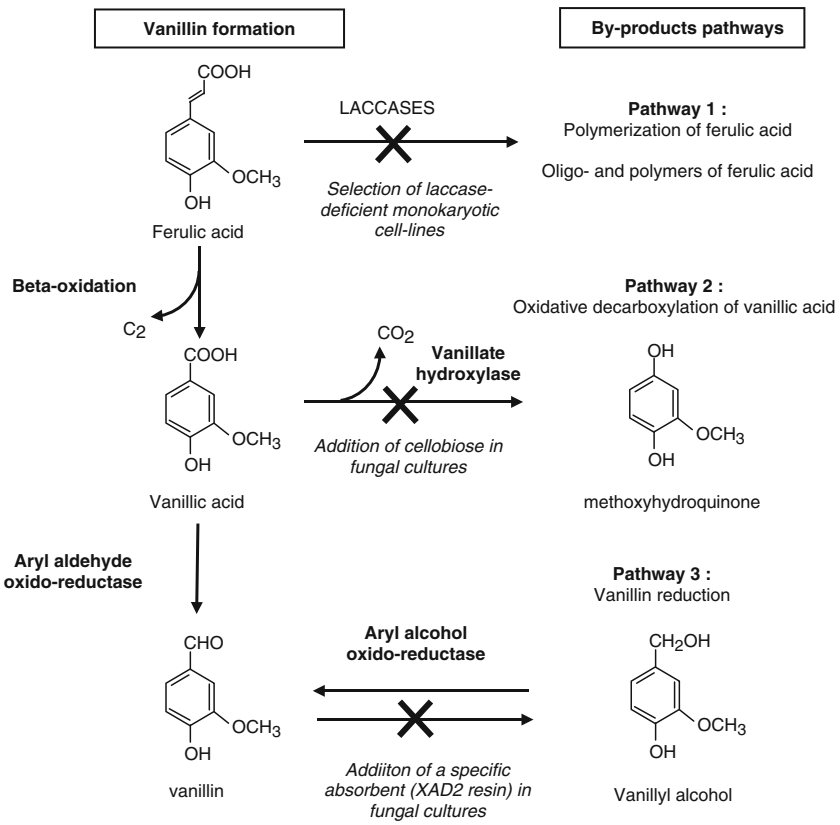

Fig. 2 Metabolic pathways of biotransformation of ferulic acid into vanillin by *P. cinnabarinus*

noic side-chain degradation of ferulic acid appears to be analogous to fatty acid β-oxidation (Krings et al. 2001). The biotransformation yield of vanillin from ferulic acid was lowered by three unwanted by-pass pathways (Fig. 2, pathways 1, 2 and 3) that could be successfully controlled by: (1) the selection of monokaryotic laccase-deficient *P. cinnabarinus* strains, using formal genetics (Lesage-Meessen et al. 1996), (2) the addition of cellobiose to fungal cultures to channel the flow of vanillic acid through the reductive pathway (inhibition of pathway 2) (Lesage-Meessen et al. 1997) and (3) the addition of a selective adsorbent (a hydrophobic cross-linked polystyrene copolymer resin named XAD-2), to trap vanillin before its bioconversion into vanillyl alcohol (Stentelaire et al. 1998). In these conditions, vanillin concentration reached 500 mg/l, with a molar yield of 47%, and vanillin can be easily recovered by desorption from resin using ethyl alcohol (Stentelaire et al. 2000). In addition, a mechanistic model for vanillin production from vanillic acid by *P. cinnabarin*us grown in a 2-l bioreactor was established to predict the evolution of the variables during the growth and biotransformation phases (inoculum, mode and addition time of precursor and cellobiose, aeration and XAD-2 addition). The complete procedure finally led to the setting-up of a simulation model for the process, and the results were supported by the data from five cultures of *P. cinnabarinus* (Bernard et al. 1999). High-density cultures of *P. cinnabarinus* were tested for the optimization of ferulic acid bioconversion into vanillin. A sixfold increase in biomass using glucose-phospholipid mixture as carbon source instead of maltose allowed 760 mg/l vanillin to be produced from ferulic acid in 15 days with a molar yield of 61% (Oddou et al. 1999).

Joint work by various laboratories (EC contract FAIR CT 96–1099) allowed complete processes to be set up to produce natural vanillin from abundant, cheap European agro-industrial by-products (less than 0.2 €/t dry wt.), rich in ferulic acid, such as sugar beet pulp (residues from the sugar industry containing 0.8% dry wt. ferulic acid) and maize brans (residue from the starch industry containing up to 5.5% dry wt. ferulic acid) (Lesage-Meessen et al. 2002). A new two-step process for the production of pure vanillin from autoclaved maize bran has been designed with a limited number of steps involving *Aspergillus niger* and *P. cinnabarinus*. For process economy, two strategies were defined using autoclaved maize bran (Fig. 3). In the first one, the potentialities of *A. niger* grown on sugar beet pulp to produce high levels of polysaccharide-degrading enzymes, including feruloyl esterases, and to transform ferulic acid into vanillic acid were successfully combined for the release of free ferulic acid from autoclaved maize bran (Bonnin et al. 2002). Vanillic acid was then recovered and efficiently transformed into vanillin by the monokaryotic strain of *P. cinnabarinus* MUCL 39533: 767 mg/l of biotechnological vanillin could be produced in the presence of cellobiose and XAD-2 resin after a 14-day bioconversion with a molar yield of 71% (Fig. 3a). In the second strategy, 3-day-old high-density cultures of *P. cinnabarinus* MUCL 39533 were fed with the autoclaved maize bran as a ferulic acid source and *A. niger* culture filtrate as an extracellular enzyme source. Under these conditions, *P. cinnabarinus* was shown to biotransform free ferulic acid released from the autoclaved maize bran by *A. niger* enzymes directly into 584 mg/l vanillin on day 11 with a molar yield of 22% (Fig. 3b). These patented processes (Lesage-Meessen et al. 1995; Bonnin et al. 2000), involving physical, enzymatic and fungal treatments, allowed natural crystalline vanillin, characterized by X-ray diffraction, to be produced from autoclaved maize bran with no purification step (Lesage-Meessen et al. 2002). Such vanillin may be considered

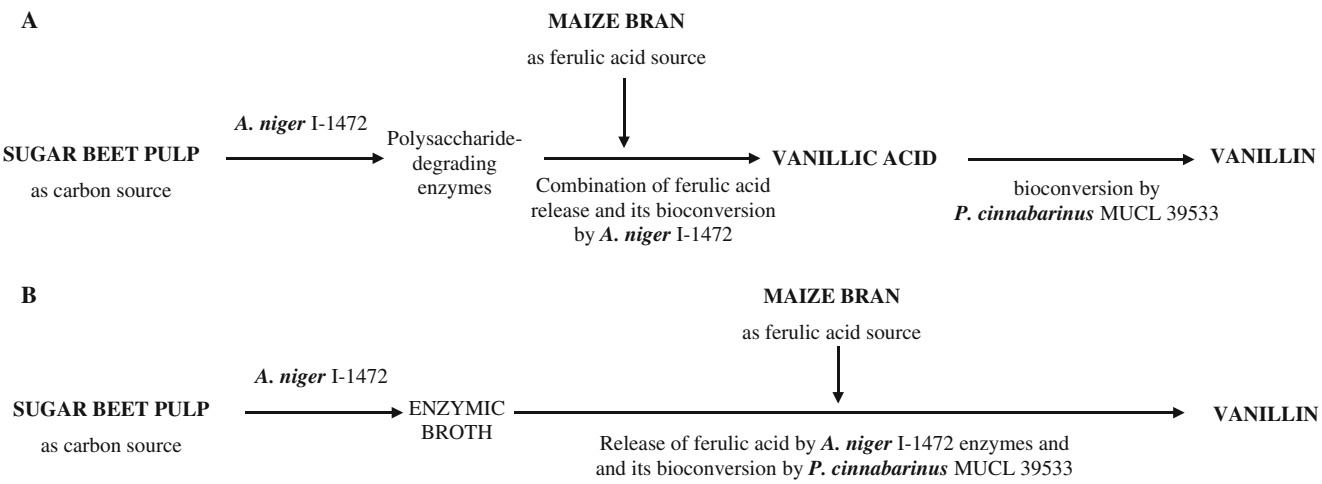

Fig. 3 Different biotechnological two-step processes involving *A. niger* and *P. cinnabarinus* for the production of natural vanillin from maize bran

"natural" according to the European and US legislations, i.e. originating from a natural source and obtained by enzymatic or fungal transformation (EC directive 88/388, OJ N0. L 184, 15 July 1988). Zheng et al. (2007) developed a similar process that would convert ferulic acid prepared from waste residues of rice bran oil into vanillin using the combination of the biotransformation potentials of *A. niger* and *P. cinnabarinus*, previously described. The yield of vanillin reached 2.8 g/l when 5 g/l glucose and 25 g of HZ802 resin were added to the bioconversion medium.

In addition, *P. cinnabarinus* was shown to be able to convert *p*-coumaric acid, a hydroxycinnamic acid found covalently esterified to polysaccharides and lignin in plant cell walls, to *p*-hydroxybenzaldehyde, a component of high organoleptic value present in natural vanilla bean extracts (Estrada Alvarado et al. 2001). The use of a phospholipid-enriched medium induced high-density fungal cultures, which produced 155 mg/l *p*-hydroxybenzaldehyde on culture day 13 with a molar yield of 26%. Based on the different metabolites identified, metabolic pathways of *p*-coumaric acid were suggested. As previously suggested for ferulic acid (Falconnier et al. 1994; Krings et al. 2001), an oxidative side-chain degradation of *p*-coumaric acid led to *p*-hydroxybenzoic and protocatechuic acids, which were then reduced to their corresponding aldehydes and alcohols. Additionally, a reductive pathway of *p*-coumaric acid occurred, leading to 3-(4-hydroxyphenyl)-propanol as the terminal product, as already reported in *Ischnoderma benzoinum* (Krings et al. 1996).

Among aromatic compounds of industrial interest, benzaldehyde (bitter almond aroma) is, like vanillin, widely used in the food and cosmetic industries. The strain *P. cinnabarinus* MUCL 39533 was shown to produce, in a 2-l bioreactor, 100 mg/l benzaldehyde from L-phenylalanine as precursor (Lomascolo et al. 1999). The addition of HP20 resin, a styrene divinylbenzene copolymer highly selective for benzaldehyde, proved an efficient strategy to enhance benzaldehyde production up to 790 mg/l. Described as the organoleptic flavour of orange blossom and wood strawberry, methyl anthranilate (*o*-aminobenzoic acid methyl ester) is widely used in the food and perfumery industries, but there is no readily available source of this compound, owing to its extremely low concentrations in plants. *P. cinnabarinus* has been reported to produce methyl anthranilate de novo in culture conditions combining low nitrogen concentration, maltose as carbon source and uncontrolled culture pH (Gross et al. 1990).

Over the past 25 years, in view of the high demand for natural ingredients, particular attention has been directed to the antioxidant properties of hydroxycinnamic acids, due to their occurrence in nature and their radical scavenging activity. The antioxidant activity of natural phenolic acids depends on the number and relative position of the hydroxyl groups on the ring, which give them reducing properties and hydrogen-donating abilities (Rice-Evans et al. 1996). The cinnamic acids dihydroxylated in the 3,4 position, such as caffeic acid, have a higher radical-scavenging ability than monophenolics like *p*-coumaric acid. Fungal transformation of *p*-coumaric acid into caffeic acid, potentially a strong antioxidant, has been evidenced in *P. cinnabarinus* cultures grown with high *p*-coumaric acid feeding rates. Thus feeding 450 mg/l *p*-coumaric acid to *P. cinnabarinus* cultures grown on glucose medium resulted in the production of 257 mg/l caffeic acid with a molar yield of 21%. Under these conditions, a *p*-coumaric hydroxylating pathway rarely described in fungi was induced at the expense of *p*-hydroxybenzaldehyde (Estrada Alvarado et al. 2003).

Lignin degradation, pretreatment of lignocellulosic biomass, pulp and paper applications

The lignin polymer is highly recalcitrant towards chemical and biological degradation due to its molecular architecture, where different phenolic and non-phenolic units form a complex three-dimensional network linked by a variety of ether and carbon–carbon bonds. The non-phenolic substructures represent about 90% of total lignin and are more recalcitrant to degradation than the phenolic ones. Lignin removal is thus a central aspect in industrial uses of lignocellulosic biomass, such as bioethanol production and manufacture of cellulose-based chemicals and materials, including paper. Geng and Li (2002) showed that *P. cinnabarinus* was capable of oxidatively degrading both phenolic and non-phenolic lignins. The authors evidenced that after incubation of the fungus and lignin preparations for 3 months, over 40% of the non-phenolic lignin and about 70% of the phenolic lignin substructures were degraded. The degradation rate of phenolic lignins was greatly enhanced by the presence of hydroxyl groups and was faster than that of the non-phenolic lignins. According to the studies of Eggert et al. (1997) and Bermek et al. (1998), laccase is essential for lignin degradation and pulp bleaching by *P. cinnabarinus*. However, *Pycnoporus* laccase alone cannot oxidize non-phenolic lignin model compounds in vitro (Eggert et al. 1996b; Uzan et al. 2010). For a while, 3-hydroxyanthranilic acid had been thought to be the natural mediator acting in vivo but it remains a controversial question (Li et al. 2001) until now. Uzan et al. (2010) described the oxidation of monomeric and dimeric non-phenolic lignin model compounds such as veratryl alcohol and adlerol by *P. sanguineus* laccase, testing different natural and synthetic redox mediators at different pH. 1-Hydroxybenzotriazole (HBT), a synthetic mediator, was the most efficient redox mediator and allowed 100% oxidation of veratryl alcohol into veratraldehyde and 86% oxidation of adlerol into adlerone. Given

these findings, *Pycnoporus* fungi are microorganisms of interest for the pretreatment of recalcitrant lignocellulosic biomass, which is the most important step required to remove lignin, and hold potential in utilizing plant residues for the saccharification of cellulosics and the synthesis of biofuels. The biological delignification of tropical lignocellulosic feeedstocks from *Prosopsis juliflora* and *Lantana camara* was carried out with *P. cinnabarinus* under solid-state fermentation conditions with a pre-treatment scalability up to 500 g of substrate (Gupta et al. 2011). The fungal fermentation with 10 g of substrate optimally delignified *P. juliflora* by 11.9% and *L. camara* by 8.4% and enriched their holocellulose content by 3.3% and 4.9%, respectively.

Through their enzyme panel, basidiomycetes, and especially the genus *Pycnoporus*, are also able to degrade the lignin content of wood pulp, and this feature is very promising for the paper pulp industry, as it could allow a substantial reduction in the use of chlorine-containing bleaching agents, thereby reducing their environmental impact. In this context, Herpoël et al. (2002) have developed a biotechnological process using a xylanase and a *P. cinnabarinus* laccase for the delignification of wheat straw pulp. The pulp was delignified by about 47% (compared with untreated pulp). A reduction in the consumption of chlorine chemicals in the bleaching sequence, and a subsequent diminution of the chemical oxygen demand (COD) by about 60%, has also been demonstrated. Enzymatically treated pulp was bleached to 69% ISO brightness by a treatment sequence without addition of chlorinated compounds. To determine the economic feasibility of such an enzymatic process, Sigoillot et al. (2004) produced a recombinant *Pycnoporus* laccase by heterologous expression in *A. niger*. The same promising results (75% delignification of wheat straw kraft pulp) were obtained, showing that a recombinant enzyme-based technology could be considered for reducing process costs. These types of process included redox mediators such as HBT and are called laccase mediator systems (LMS) (Fig. 4). Successful flax alkaline pulp delignification and bleaching could also be achieved by using wild laccases from *P. cinnabarinus* in the presence of HBT as a mediator (Camarero et al. 2004). Up to 20% ISO brightness increase was attained after laccase-HBT treatment, and a decrease in the kappa number from 9 to 3 was simultaneously observed. *P. cinnabarinus* laccase plus HBT gave the best selectivity in lignin removal, determined by the ratio between the decrease in kappa number and pulp viscosity. It is noteworthy that HBT is one of the best laccase redox mediator but (1) it is relatively expensive in terms of bulk applications (such as in the pulp and paper sector), and (2) it can be converted into toxic/mutagenic products during laccase catalysis. The current challenge is now to find cheap and natural mediators. For instance, natural mediators such as syringaldehyde and acetosyringone have been found to perform well for dye decolorization by *Pycnoporus* laccase (Camarero et al. 2005), opening the way to eco-friendly (and potentially cheap) treatments. In another study, an LMS, formed by a *P. sanguineus* fluid enriched in laccase and acetosyringone as mediator proved to be an effective bleaching booster of a *Eucalyptus globulus* kraft pulp totally chlorine-free sequence (Eugenio et al. 2010). This LMS enabled the reduction of hydrogen peroxide load from 94% to 87.4% and the increase in the final brightness from 51% to 59% ISO, compared with conventional bleaching. Ravalason et al. (2009) performed the fusion of a family 1 carbohydrate-binding module (CBM) of *A. niger* cellobiohydrolase B to the laccase of *P. cinnabarinus* CIRM-BRFM 137 for efficient softwood kraft pulp biobleaching. The chimeric enzyme laccase-CBM was able to bind to a cellulosic substrate and, to a greater extent, to softwood kraft pulp. Addition of a CBM was shown to greatly improve the delignification capabilities of the laccase in the presence of HBT as mediator. In addition, chlorine dioxide reduction using 5 U of chimeric enzyme/g of pulp was almost twice than that observed using 20 U of *P. cinnabarinus* laccase/g of pulp. Conferring a carbohydrate-binding capability to the laccase could significantly enhance the biobleaching properties of the enzyme (Ravalason et al. 2009).

In order to develop a new integrated process, Meza et al. (2006) combined (1) the production of laccase, an enzyme usable to bleach paper pulp, (2) the delignification of sugarcane bagasse, a residue of sugar production that can be recycled in the paper industry and (3) the depollution of gaseous effluents such as ethanol, known to be a strong laccase inducer (Lomascolo et al. 2003). Ethanol vapour was blown up through a vapour phase bioreactor packed with bagasse and inoculated with the high redox potential laccase from *P. cinnabarinus* CIRM-BRFM 137. After 28 days, bagasse delignification reached 12%. The fungus-treated bagasse was pulped and refined. An improvement of 35% in the mechanical characteristics (tensile index, breaking strength) of the paper sheets was obtained from biotreated bagasse, with a reduction of 50% in the energy necessary to delignify the pulp. All these results show that enzymatic treatment using *Pycnoporus* has great potential for pulp biobleaching.

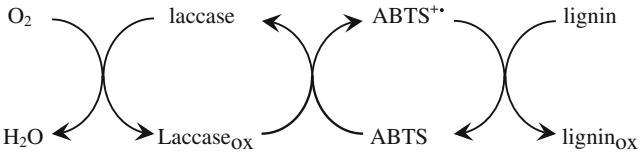

Fig. 4 Schematic representation of laccase/redox mediator concept

Bioremediation, decolourization and detoxification of industrial effluents

Pycnoporus fungi secrete laccases that present a low specificity with regard to substituted aromatic structures and are thus of great interest in wastewater decolourization and detoxification (Thurston 1994). The use of *Pycnoporus* laccase-based techniques for these processes can make bioremediation cost-effective and environmentally friendly. *Pycnoporus* fungi especially demonstrated their ability to decolourize several classes of dyes. Schliephake et al. (1993) first showed the degradation of the diazo dye Chicago Sky Blue by *P. cinnabarinus* in a packed-bed reactor. The degradation was due to the laccase produced by the strain *P. cinnabarinus* tested (Schliephake et al. 2000). Several studies highlighted the efficiency of *Pycnoporus* laccases to degrade azo, triphenylmethane and anthraquinonic dyes (Pointing et al. 2000). Total degradation of reactive blue 38, acid blue 74, reactive blue 19, aniline blue and reactive black 5 was obtained with the *P. cinnabarinus* CIRM-BRFM 137 laccase in the presence of different synthetic or natural mediators (Camarero et al. 2005). Trovaslet et al. (2007) showed high percentages of decolourization obtained by *P. cinnabarinus* and *P. sanguineus* laccases on several dyes such as acid blue 62, acid orange 7 and acid yellow 36 without the addition of a redox mediator (respectively, 80%, 60% and 52% decolourization). Uzan et al. (2010) elected to test the ability of purified laccases from *P. coccineus* and *P. sanguineus* to decolourize a varied range of polycyclic dyes, including, for the first time, Poly-R 478 (a surrogate substrate for lignin biodegradation). The authors showed decolourization even in the absence of a redox mediator. The decolourization of the anthraquinone dye remazol brilliant blue R (RBBR) was consistent with the results of Lu et al. (2007) that showed 94% of RBBR decolourization after incubation at 40°C with a *P. sanguineus* laccase without additional redox mediator. Natural mediators, such as syringaldehyde and acetosyringone, were also found to favour dye decolourization by *Pycnoporus* laccases (Camarero et al. 2005), opening the way to eco-friendly treatments.

Olive oil mill wastewater (OOMW) are pollutant by-products of the olive oil industry. The fungus *P. coccineus* was selected among a wide range of white-rot fungi for its ability to grow on OOMW, without any additional carbon source, strongly decreasing colour and COD in this industrial effluent (Jaouani et al. 2003) as an alternative to physico-chemical methods of detoxification. Oxidation of monomeric phenolic compounds in the different OOMW fractions by the *P. coccineus* laccase could produce radicals leading to polymerization (Jaouani et al. 2005). Similar results have been reported for a *P. coccineus* laccase immobilized on the acrylic epoxy-activated Eupergit C resin (Berrio et al. 2007). Gel filtration profiles of the OOMW treated with this enzyme showed both degradation and polymerization of the phenolic compounds.

Abilities of the white-rot basidiomycete *Pycnoporus* are also very extensive and very attractive in oxidation of several compounds such as polycyclic aromatic hydrocarbons and halogenated derivatives to decrease the toxicity, mutagenicity and carcinogenic properties of these substances. In this context, biotransformation of benzo[a]pyrene in a bench-scale reactor using laccase of *P. cinnabarinus* has been studied (Rama et al. 1998). The authors showed that benzo[a]pyrene concentration was strongly decreased within 3 h in the presence of purified laccase and ABTS as mediator; after 24 h of incubation, most (95%) of this compound was converted into 1,6-, 3,6- and 6,12-quinones in a 2:1:1 ratio. These quinoid-free metabolites proved to be a substrate for microbial populations and were mineralized to carbon dioxide.

Polyvinyl alcohol, a synthetic polymer used in the paper coating and textile industries, can also be degraded by *P. cinnabarinus* (Larking et al. 1999). Another study carried out by Hundt et al. (2000) showed the potential of *Pycnoporus* to biotransform triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether), an antimicrobial compound used in deodorants, soaps and dentifrices. After 48–72 h of incubation in the culture medium of *P. cinnabarinus*, triclosan began to disappear from the supernatant. The metabolites produced were substantially less cytotoxic than triclosan.

The presence of industrial effluents containing heavy metals poses serious environmental problems as they are toxic even at low concentration. White-rot basidiomycetes, and especially the genus *Pycnoporus*, have been used for the removal of heavy metals from aqueous solutions, because of their high tolerance towards these compounds and other adverse conditions (such as low pH), high cell wall binding capacity and high intracellular metal uptake capacity (Yahaya et al. 2009). The following studies suggest that *Pycnoporus* can be used as a low cost, efficient and easily regenerated biosorbent (Zulfadhly et al. 2001) for heavy metals that occur in the environment either naturally (e.g. Cu), or as a result of human activities (e.g. Cd, Hg or Pb). For instance, Mashitah et al. (1999) investigated the capacity of *P. sanguineus* to remove copper ions from aqueous solutions. The authors clearly demonstrated that the sorption capacity for copper increased up to about 10 mg/g of biomass. Yahaya et al. (2009) further showed that copper removal could be improved by the immobilization of *P. sanguineus* cells on alginate beads. Lead Pb(II) biosorption onto immobilized cells of *P. sanguineus* has also been studied (Azila et

al. 2008). At pH 4 and 10 g/l of biosorbent, the immobilized cells of this fungus can remove 97.7% of 200 mg/l of Pb(II) ions. Similarly, Mashitah et al. (2008) have investigated the biosorption of cadmium (II) ions from aqueous solution onto immobilized cells (alginate beads) of *P. sanguineus* in a batch system. It reached 1.36 mg of Cd/g of biomass at pH 6 and 3 g of biomass loading.

Biopolymer synthesis

A recent study was carried out using *Pycnoporus* laccase as a catalyst to develop new potential natural active ingredients from rutin (quercetin-3-rutinoside, one of the best-known naturally occurring flavonoid glycosides) for cosmetic applications (Uzan et al. 2011). Rutin bioconversion reached about 67% for *Pycnoporus* laccases after 24 h incubation. New flavonoid oligomers were synthesized such as dimers and trimers of rutin with one or two *ortho*-quinone moieties or none. These innovative oligorutins, suitable for cosmetic applications, provided some protection against oxidative and inflammatory damage (superoxide radical scavenging activity, inhibitory effects on the cyclooxygenase COX-2 and the human matrix metalloproteinase 3 MMP-3). Laccase-catalysed derivatization may offer a promising route to new medically valuable structures. In addition, laccase from *P. cinnabarinus* can derivatize azoles with hydroxyl benzoic acid methyl or ethyl esters, leading to oligomer products with more and stronger biological activities (antimicrobial activites and stronger cytotoxicity) than the corresponding monomers (Hahn et al. 2010).

Oxidative enzymes from *Pycnoporus* (laccase from *P. cinnabarinus* and tyrosinase from *P. sanguineus*) were also successfully used for the synthesis of biopolymers suitable for the food industry from, for instance, agro-residues such as sugar beet pulp or cereal brans. Ferulic acid has been found ester linked to cell wall polysaccharides in sugar beet pectins or cereal brans such as arabinofuranose residues in maize bran (Saulnier and Thibault 1999). Consequently, these polysaccharide chains are able to gel through ferulic acid covalent cross-linking with oxidizing systems such as laccases. *P. cinnabarinus* laccase was used for the oxidative gelling of sugar beet pectins (Micard and Thibault 1999), soluble wheat arabinoxylans (Figueroa-Espinoza and Rouau 1998) and maize bran arabinoxylans (de Wilde et al. 2008). In the latter case, the gels were heatproof and resistant to freezing down to −20°C as well as to moderate acidic conditions (de Wilde et al. 2008). The *P. sanguineus* CBS 614.73 tyrosinase has been shown to be very effective in the cross-linking of casein, chosen as a model protein substrate for food applications (Halaouli et al. 2005). The use of these enzymes is eco-friendly and food-compatible, since only O_2 (i.e. air and agitation) being required for the cross-linking process.

Conclusions

Pycnoporus fungi have become model lignolytic basidiomycetes whose physiological and biochemical properties have been studied in detail, mainly in liquid media but also in solid-state fermentations. These fungi have demonstrated a significant potential in both white and green biotechnologies. This review is the first attempt to collect the scattered information on *Pycnoporus* species, spread throughout the literature in over 30 years and provides an exhaustive treatment focused on biochemical and biotechnological issues. The *Pycnoporus* genus comes out as a promising microorganism of choice applicable to enzyme biotechnologies, specifically as a producer of various hydrolase and oxidase activities and as a robust biodegradation agent. The lignolytic system of this group is composed mainly of high redox potential blue laccases, easy to produce and purify on a large scale. The remarkable properties of these laccases make the enzymes suitable for a variety of applications such as the bioconversion of agricultural by-products and raw plant materials into valuable products, the biopulping and biobleaching of paper pulp, and the biodegradation of organopollutants, xenobiotics and industrial contaminants. In addition, classical and molecular genetic engineering of the genus *Pycnoporus* is now well established in laboratory conditions, opening the way to applicable industrial processes.

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