Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture

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Abstract: The white-rot fungus *Pycnoporus sanguineus* produces laccase under a range of C/N ratios in submerged liquid culture. Enzyme production was increased 50 fold in the presence of 20 µM xylidine to a maximum of 1368 U L^{-1} in a high carbon low nitrogen medium. Slight repression of enzyme production was observed in high nitrogen culture medium. Other potential inducers were less effective (Tween 80, wood fibres) and reduced the stimulation observed by xylidine alone when included in the same culture medium. Veratryl alcohol failed to stimulate laccase production. Activity of the enzyme activity in crude culture filtrate was stable at temperatures of 35 C and below with a pH optimum of 3.0. The laccase of *P. sanguineus* was identified as a ca 65 kDa protein produced as multiple isoforms.

Key Words: Basidiomycete, laccase, *Pycnoporus sanguineus*, white-rot fungus

INTRODUCTION

Lignin is a highly recalcitrant compound, forming the second most abundant biopolymer in nature after cellulose. Degradation of lignin therefore represents a rate limiting step in carbon flux within environments rich in lignified cell walls. The mineralization of lignin is achieved appreciably only by the white-rot fungi. Most are members of the Basidiomycotina although some higher ascomycetes also cause white-rot (Worral et al 1997).

Enzymes involved in the degradation of lignin oxidize phenolic structures and catalyse the generation of highly reactive radicals that can also degrade non-

phenolic structures in lignins (Boominathan and Reddy 1992, Thurston 1994). The three classes of extracellular lignin modifying enzymes (LME) generally recognized are lignin peroxidase (E.C. 1.11.1.14) (LiP), Mn dependant peroxidase (E.C. 1.11.1.15) (MnP) and laccase (E.C. 1.10.3.2) (Lcc). White-rot fungi variously produce one, two or all three of these phenoloxidase enzymes and this appears to be a ubiquitous feature among this group of fungi (Orth et al 1993). However most research to date has focused on relatively few species, notably *Phanerochaete chrysosporium*. This fungus secretes LiP and MnP as the dominant LME (Reddy and D'Souza 1994), where both enzymes are inducible under specific conditions of low nutrient nitrogen, high oxygen tension, presence of the inducer veratryl alcohol and static cultivation. Laccase is produced in small titres only under nitrogen sufficient conditions (Srinivasan et al 1995). Studies on other fungi have revealed different LME production profiles and physiology (Nerud et al 1991, Orth et al 1993). Most notably that Lcc activity is generally greater than that of either LiP or MnP in most strains studied (Collins and Dobson 1997, Eggert et al 1996b). This makes Lcc producing fungi particularly good candidates for biotechnological applications employing LME, such as biobleaching, biopulping and bioremediation (Reddy 1995).

Most studies on Lcc producing fungi have used temperate strains whereas tropical white-rot fungi have received relatively little attention in comparison. The basidiomycete *Pycnoporus sanguineus* is widely distributed on lignocellulose in tropical forests (Almeida-Filho et al 1993, Gazzano 1990, Ribeiro and Aguiar 1993) and is associated with aggressive white-rot type decay. Here we report on the physiology of LME production by this fungus, in particular attempts to optimize enzyme secretion.

MATERIALS AND METHODS

Organism and cultivation.—The *P. sanguineus* strain CY788 used in this study was isolated from decaying wood in Thailand. Stocks were maintained on malt extract agar slopes (Difco) with periodic transfer. The fungus was identified as potentially ligninolytic by clearance of agar plates [glucose 0.2% (w/v), mycological peptone (Oxoid) 0.01% (w/v), yeast extract (Difco) 0.001% (w/v), agar 1.4% (w/v)] supplemented with 0.02% (w/v) Poly-R (Poly R478, Sigma).

For production of inoculum the fungus was grown on malt extract agar (Difco) plates for 7 d at 25 C in darkness. A whole plate culture was then aseptically transferred to a sterilized Waring blender cup and homogenized with 50 mL distilled water at low speed for 3×10 s. Aliquots of 0.5 mL were then transferred to 250-mL Erlenmyer flasks. Each flask contained 25 mL of a defined growth medium consisting of (g L^{-1} unless indicated): 2,2-dimethylsuccinic acid, 1.46; nitrilotriacetate, 0.15; KH_2PO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; $MnSO_4 \cdot 5H_2O$, 5 mg; NaCl, 10 mg; $FeSO_4 \cdot 7H_2O$, 1 mg; $CoCl_2 \cdot 6H_2O$, 1 mg; $ZnSO_4 \cdot 7H_2O$, 1 mg; $CuSO_4 \cdot 5H_2O$, 0.1 mg; $AlK(SO_4)_2$, 0.1 mg; H3BO₃, 0.1 mg; $NaMoO_4 \cdot 2H_2O$, 0.1 mg; thiamine·HCl, 1 mg; glucose as carbon source (0.1–1.2 g L^{-1} as detailed in the text); ammonium tartrate as nitrogen source (0.24–48 mM as detailed in the text). In some experiments the growth medium was supplemented with yeast extract, 1 mg L^{-1}; Tween 80, 0.05% w/v; veratryl alcohol (3,4, dimethoxybenzoic acid), 1–4 mM; xylidine (2, 5,-dimethylanilline), 5–100 μM; milled and sieved wood (*Fagus sylvatica*) particles (250–500 μm), 1% w/v, as indicated in the text. Final pH of growth medium in all experiments was 4.5. All cultivations were carried out at 25 C on a rotary shaker (125 rpm) with triplicate flasks for each treatment.

Enzyme assays.—Extracellular culture fluids were assayed for enzyme activity after mycelium was removed by centrifugation (30 s at 10 000 g). Lcc activity was determined by measuring the oxidation of 3 mM 2,2′ azino bis(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) in glycine-HCl buffer (pH 3.0) at 420 nm (ϵmax $= 3.6 \times 10^4$ M^{-1} cm^{-1}) and 35 C. In some experiments culture supernatant was preincubated with catalase (1000 units mL^{-1}) (E.C. 1.11.1.6 from *Aspergillus niger*, Sigma C3515) for 30 min at 30 C prior to assay in order to remove any endogenous H_2O_2. In some experiments glycine-HCl buffer was replaced with a sodium acetate or sodium tartrate buffer.

Manganese independent peroxidase activity was measured by adding H_2O_2 (1 mM final concentration) to the laccase assay mixture and subtracting the activity due to laccase alone. Lignin peroxidase activity was determined by measuring the production of veratraldehyde from veratryl alcohol at 310 nm (ϵmax $= 9300$ M^{-1} cm^{-1}) in glycine-HCl buffer (pH 3.0) at 30 C, upon addition of H_2O_2 (1 mM final concentration). Aryl alcohol oxidase was assayed under the same conditions without the addition of H_2O_2. Manganese dependant peroxidase activity was measured by the oxidation of phenol red at 431 nm (ϵmax $= 22\ 000$ M^{-1} cm^{-1}) in the presence of 100 μM $MnSO_4 \cdot 5H_2O$ in glycine-HCl buffer (pH 3.0) at 30 C, upon addition of H_2O_2 (0.5 mM final concentration).

All enzyme assays were carried out using a Hewlett-Packard 8452A UV-visible diode array spectrophotometer. Enzyme activities were expressed as units, with one unit defined as that forming 1 μmole product min^{-1}. All values reported are the mean of three replicates, with standard error of the mean (SE) represented by error bars. Where SE falls within the data point symbols error bars have been omitted.

Protein and biomass determination.—Protein was determined using the method of Lowry et al (1951) using bovine serum albumin as the standard. Fungal biomass was determined by filtering mycelium through three layers of cheesecloth, washing with distilled water, and drying at 105 C overnight.

Determination of molecular weight and isozyme banding pattern.—SDS PAGE was performed as described by Bollag and Edelstein (1992) on crude culture filtrates concentrated 100 fold by centrifugation using a 10 kDa cut-off filter (Ultrafree 15, Millipore). Samples were run with Sigmamarkers wide range protein standards (Sigma M4038), and protein bands visualized using coomassie blue staining. Molecular weight of the laccase band was calculated from relative mobility compared to standards. Nondenaturing PAGE was carried out as described by Bollag and Edelstein (1992). Samples were run and lacase bands detected by staining with 0.05% (w/v) ABTS.

RESULTS

In preliminary experiments strains of *P. sanguineus* were grown on agar plates containing 0.02% (w/v) Poly-R. The strain used in this study was chosen for its ability to rapidly decolorize the dye, which is indicative of LME production (Boominathan and Reddy 1992). Initial experiments in submerged liquid culture revealed superior growth and Lcc production in shaken rather than static or static-oxygen purged conditions (data not shown) and so all subsequent experiments were carried out in shake culture. No LiP, MnP, manganese independant peroxidase or aryl alcohol oxidase activity was detected in any cultivation despite inclusion in the growth medium of the inducer veratryl alcohol (1–4 mM) and static oxygen-purged cultivation, conditions known to favour peroxidase enzyme production in *Phanerochaete chrysosporium* (Reddy and D'Souza 1994).

The Lcc activity of *P. sanguineus* culture filtrates produced linear reaction rates at enzyme assay temperatures up to 40 C. The enzyme was very stable at 4 C, with no significant loss in activity over 8 h. At temperatures of 35 C and below the enzyme was stable over 60 min, with rapid loss in activity occuring after relatively short periods at higher temperatures. A single distinct pH optima of 3.0 was recorded for Lcc activity in culture filtrates. The glycine-HCL buffer used gave higher enzyme activities than sodium tartrate or sodium acetate buffers and so was used in all subsequent experiments (data not shown).

The effect of different carbon (as glucose) and nitrogen (as ammonium tartrate) concentrations in the growth medium on enzyme production were determined (TABLE I). From this data 'low' and 'high' carbon [0.2% (w/v), 0.8% (w/v)] and nitrogen (2.4 mM and 24 mM) concentrations were selected for use in further experiments. Laccase was produced consti-

TABLE I. Influence of growth medium carbon and nitrogen levels on laccase production by *P. sanguineus*

Glucose (% w/v)[a]	Relative laccase production (%)[b]	Ammonium tartrate (mM)[c]	Relative laccase production (%)[b]
0.1	26	0.24	6
0.2	90	2.40	100
0.4	92	12.00	94
0.8	100	24.00	86
1.2	73	48.00	56

[a] With 2.4 mM ammonium tartrate.
[b] Values reported for 7 day old incubations.
[c] With 0.2% w/v glucose.

tutively in liquid culture under all conditions tested. Activity was detectable after 2 d and reached a peak after 7 d cultivation. Optimum Lcc production in non-induced cultures was 22 U L^{-1}. Veratryl alcohol failed to stimulate Lcc production.

Enzyme production was however increased upon addition of the known laccase inducer xylidine (Eggert et al 1996a) (FIG. 1). Stimulation was observed at concentrations from 10–100 μM, with optimum induction about 50 fold (22 U L^{-1} to 1140 U L^{-1}) by the addition of 20 μM xylidine. Lower levels of inducer resulted in less stimulation of activity. Similarly xylidine levels greater than 20 μM resulted in reduced stimulation.

The C/N ratio of culture medium has been reported to affect peroxidase enzyme production (Reddy and D'Souza 1994) and so the effect of different C/N ratios on xylidine induced cultures was assessed (FIG. 2). Optimum enzyme production (1368 U L^{-1}) was achieved in high carbon low nitrogen conditions, although lowest enzyme production (378 U L^{-1}) corresponding to a 72% decrease was recorded in high carbon high nitrogen cultures. In low carbon cultures only slight repression (14%) of Lcc production was observed under high nitrogen conditions. The specific activity of Lcc in cultures varied; low carbon low nitrogen, 4.2 ± 0.0; low carbon high nitrogen, 2.5 ± 0.06; high carbon low nitrogen, 2.0 ± 0.04; high carbon high nitrogen, 0.86 ± 0.27.

Further experiments were carried out using high carbon low nitrogen growth medium to optimize enzyme production by including other potential inducers of Lcc activity (FIG. 3). The addition of 0.05% (w/v) Tween 80 to non induced cultures resulted in a 3 fold stimulation of activity from 22 U L^{-1} to 60 U L^{-1}. However when added to xylidine induced cultures optimum enzyme production was reduced from 1368 U L^{-1} to 914 U L^{-1}. Similarly addition of a 1% (w/v) wood co-substrate enhanced laccase production 12
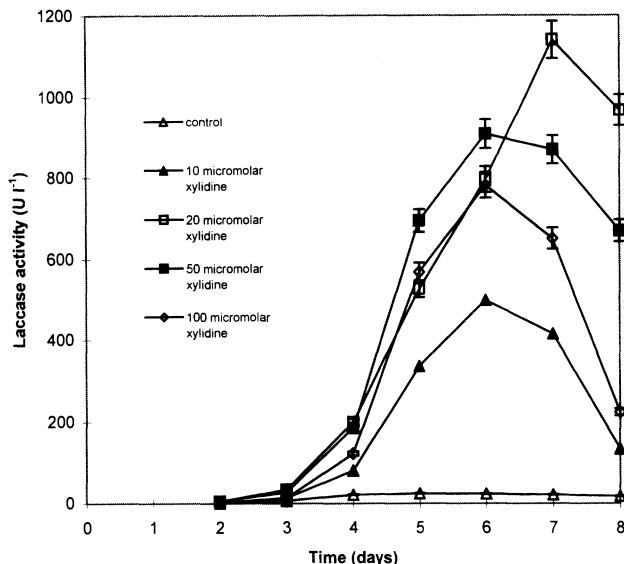
FIG. 1. Stimulation of laccase production by *P. sanguineus* in cultures containing 0.2% w/v glucose, 2.4 mM ammonium tartrate and various amounts of xylidine. Data points represent the mean of three independant replicates. SE falls within data point symbols unless indicated by error bars.
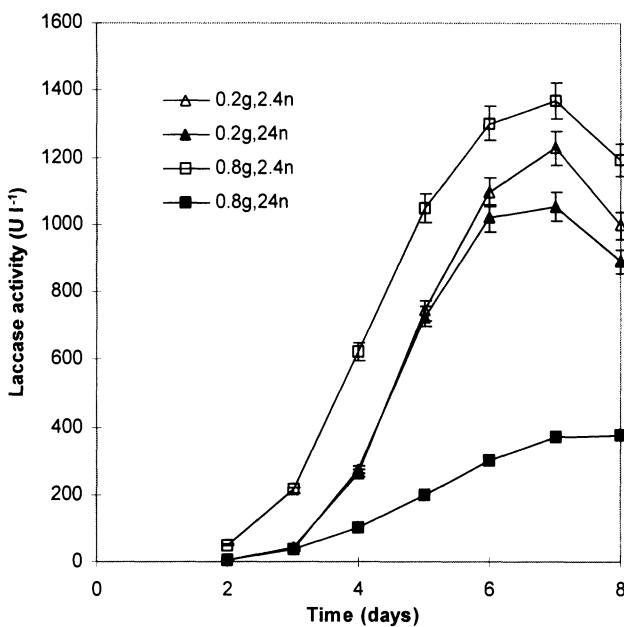
FIG. 2. Effect of C/N ratio on laccase production by *P. sanguineus* in cultures containing 20 μM xylidine. 0.2 g 2.4 n = 0.2% w/v glucose, 2.4 mM ammonium tartrate; 0.2 g 24 n = 0.2% w/v glucose, 24 mM ammonium tartrate; 0.8 g 2.4 n = 0.8% w/v glucose, 2.4 mM ammonium tartrate; 0.8 g 24 n = 0.8% w/v glucose, 24 mM ammonium tartrate. Data points represent the mean of three independant replicates. SE falls within data point symbols unless indicated by error bars.
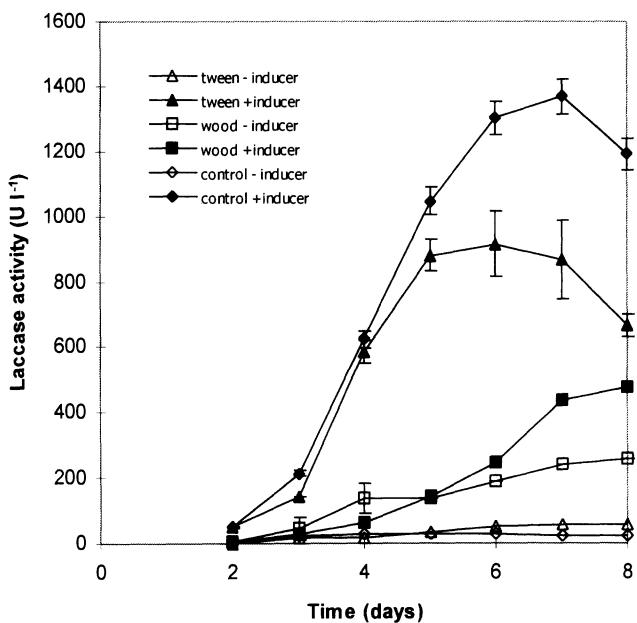
FIG. 3. Effect of the addition of Tween 80 (0.05% w/v) and *Fagus sylvatica* wood particles (1% w/v) on laccase production by *P. sanguineus* in cultures grown in 0.8% w/v glucose, 2.4 mM ammonium tartrate and in the absence (open symbols) and presence (closed symbols) of 20 μM xylidine. Data points represent the mean of three independant replicates. SE falls within data point symbols unless indicated by error bars.

fold to 257 U L^{-1} whereas inclusion of wood in xylidine induced cultures reduced optimum enzyme production almost 3 fold to 477 U L^{-1}.

Electrophoresis of proteins in glucose-grown culture filtrates clearly revealed a single protein band of ca 65 kDa after SDS PAGE (FIG. 4A). Appearance and staining intensity of the band could be correlated with Lcc activity of cultures although, in wood-supplemented cultures multiple banding patterns made visualization of a band corresponding to Lcc production difficult (FIG. 4A). Other (non-Lcc) bands observed in wood-supplemented culture filtrates were probably enzymes associated with utilization of the complex wood substrate. Activity staining of non-denaturing gels suggests glucose-grown cultures of *P. sanguineus* produce two isoforms of the 65 kDa Lcc protein (FIG. 4B). The stronger staining isoform was designated Lcc1 and the weaker staining isoform Lcc2. Cultures supplemented with wood produced only a single Lcc isoform (FIG. 4B). This appeared distinct from either Lcc1 or Lcc2 and so was designated Lcc3.

DISCUSSION

Pycnoporus sanguineus is a fungus commonly associated with aggressive white-rot type wood decay in

tropical climates. In this study Lcc was produced under a variety of culture conditions by this fungus including cultures supplemented with a native wood substrate. Optimum conditions for Lcc production appear to be different from those reported as necessary for other LMEs, notably LiP and MnP in other fungi (Reddy and D'Souza 1994). No LiP, MnP, Mn independent peroxidase or aryl alcohol oxidase was detected in cultures of *P. sanguineus* used in this study under conditions known to favour induction of such enzymes (defined nutrient-limited media, static oxygen-purged cultivation, presence of veratryl alcohol). However it is possible that this fungus may possess peroxidase enzymes requiring as yet unidentified conditions for induction.

Laccase activity was enhanced 50 fold by the addition of 20 μM xylidine to cultures, this is the most significant effect of this inducer in stimulating laccase activity reported for any fungus to date. The maximum level of laccase stimulation previously recorded for this inducer is 10 fold for a strain of *Pycnoporus cinnabarinus* (Eggert et al 1996b). The reduced stimulation observed at higher concentrations of xylidine in this study may be due to toxic effects of xylidine on the fungus. The Lcc of *P. sanguineus* in this study was not stimulated by veratryl alcohol, although in *P. cinnabarinus* this compound is reported to increase Lcc production 2–3 fold (Eggert et al 1996b, Kantelinen et al 1989). The reason for the reduced stimulation of Lcc production in xylidine induced cultures supplemented with Tween 80 is not clear. In studies with *Phlebia radiata* the addition of Tween 80 to veratryl alcohol induced cultures has resulted in increased enzyme production (Kantelinen et al 1989).

The stimulation of Lcc production 12 fold by the addition of wood fibres to non-induced cultures suggests that this fungus could be grown effectively on waste lignocellulose substrates to achieve lignin biodegradation. The enzyme production levels reported by the strain of *P. sanguineus* used in this study are over 3 fold higher than those previously reported for *P. sanguineus* grown on *Eucalyptus grandis* wood chips (Esposito et al 1993). The tropical distribution of this fungus may also make it a particularly good candidate for use in the biotransformation of lignocellulosic wastes such as sago-hampas, cocopeat and bagasse which are produced in large quantities in tropical regions. One study has already shown laccase production by *P. sajor-caju* grown on sago-hampas (Kumaran et al 1997).

Optimal Lcc production by *P. sanguineus* was 1368 U L^{-1}, this is higher than that reported for many other basidiomycetes cultured under similar conditions (Orth et al 1993, Kantelinen et al 1989, Srini-
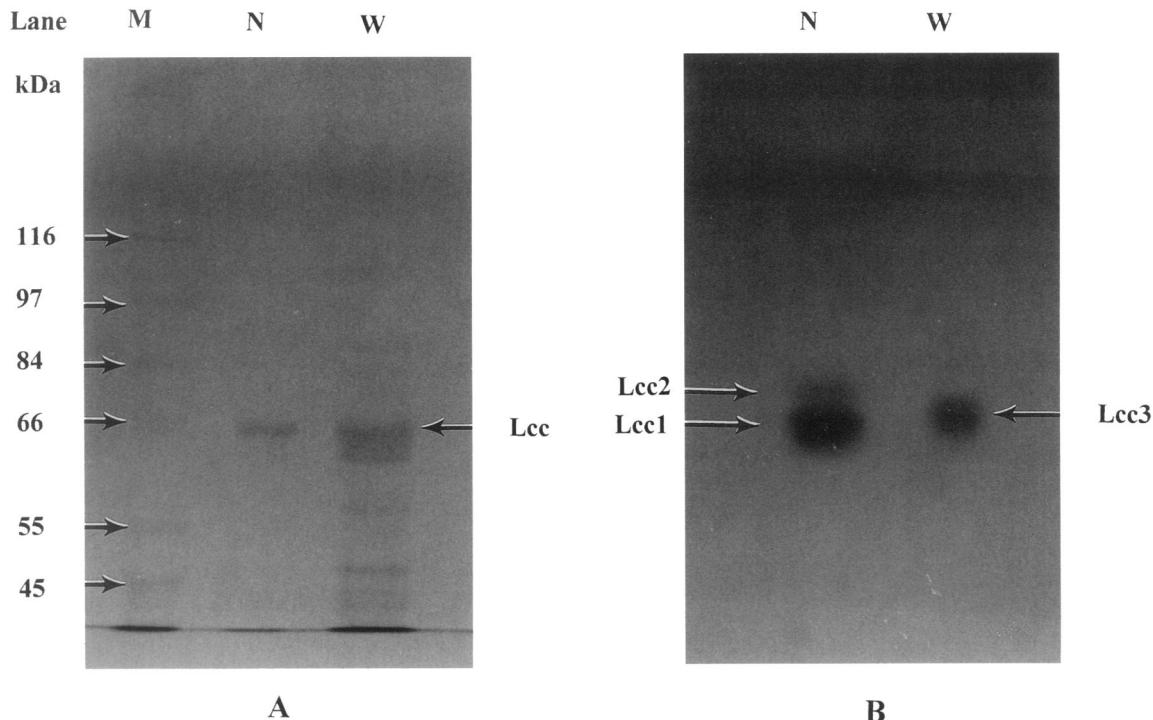
FIG. 4. SDS PAGE (A) and ND PAGE (B) of *P. sanguineus* culture filtrate (×100 fold concentrate). A. Cultures grown in fully defined nutrient medium displayed a single protein band of mol. wt. ca 65 kDa corresponding to laccase production (lane N, arrowed). Cultures supplemented with wood produced multiple bands in the mw region expected for fungal laccases (lane W). Molecular weight markers used (lane M) were Sigmamarkers wide range (Sigma). B. Activity staining of gels revealed two isoforms produced in fully defined growth medium. The more intensely stained isoform was designated Lcc1, whilst the weaker staining isoform was labelled Lcc2. Cultures supplemented with wood produced a single laccase isoform. This appeared to be distinct from Lcc1 and Lcc2 and so was designated as Lcc3.

vasan et al 1995) although it is not clear if such conditions were optimal for each fungus. Conversely few strains are reported to produce Lcc at greater levels than those recorded for *P. sanguineus* in this study under similar growth conditions. Notable exceptions are *Trametes versicolor* (5000 U L^{-1}) (Collins and Dobson 1997) and *Pleurotus sajor-caju* (4000 U L^{-1}) (Buswell et al 1996).

Previous studies among species of *Pycnoporus* are sparse and reveal varied data. The previously reported maximum Lcc activity for *P. sanguineus* is 88.7 U L^{-1} in a malt-extract growth medium (Esposito et al 1993). In contrast to the results reported here this study also identified MnP activity and a Mn independent peroxidase activity in culture filtrates, although LiP was not detected. LME production by *P. cinnabarinus* has been studied in more detail. Optimum Lcc production by this fungus was 10 000 U L^{-1} (Eggert et al 1996b). No LiP or MnP were detected, but very low levels of a Mn independent peroxidase were produced. The purified Lcc from *P. cinnabarinus* has further been shown as essential for lignin degradation by this fungus (Eggert et al 1996a, 1997). Laccase was also reported from *P. coccineus* albeit at rel-

atively lower levels (Oda et al 1991). The nature of the laccase proteins appears to vary greatly between different laccase producing genera however, the Lcc isoforms identified in this study approximate the molecular mass of Lcc from *P. cinnabarinus* and *P. coccineus*, although the number of isoforms reported in each case vary (Eggert et al 1996b, Oda et al 1991).

Laccase production in *P. sanguineus* was slightly repressed by high nitrogen levels in this study. This is a common feature in fungal LiP and MnP production (Reddy and D'Souza 1994) although Lcc responses appear to vary. The Lcc of *P. cinnabarinus* is also repressed slightly by high (24 mM) nitrogen levels (Eggert et al 1996b). Although optimum Lcc production in *T. versicolor* occurs in the presence of 54.3 mM ammonium tartrate (Collins and Dobson 1997) and Lcc activity can only be detected in *P. chrysosporium* under high (24 mM) nitrogen conditions albeit at very low levels (Srinivasan et al 1995). In this study Lcc production was further reduced under conditions of high nitrogen and high carbon. Since cultures grown in high carbon/low nitrogen media were the most active in producing Lcc, this repression was

probably not due to glucose level but rather the overall condition of nutrient sufficiency.

It is the relative nonspecificity of ligninolytic enzymes in catalysing oxidative reactions that has generated interest in their biotechnological applications. Lignin peroxidase and MnP producing white-rot fungi have been demonstrated to decolorize industrial dyes and degrade several polycyclic aromatic hydrocarbons (Reddy 1995). With a greater understanding of Lcc physiology, fungi producing this enzyme are currently the focus of much attention, since Lcc production generally exceeds that of peroxidases. Lignin degradation (Eggert et al 1996b), Dye decolorization (Esposito et al 1993, Schliephake et al 1993), PCB (Ricotta et al 1996) and PAH degradation (Collins et al 1996, Majcherczyk et al 1998) have all been demonstrated by Lcc in the presence of a redox mediator. The white-rot fungus *P. sanguineus* seems a likely candidate for application in these areas.

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