Polish Journal of Microbiology
2006, Vol. 55, No 4, 309–319

Effects of Culture Conditions on Production of Extracellular Laccase by *Rhizoctonia praticola*

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Received 10 July 2006, revised 22 September 2006, accepted 9 October 2006

Abstract

It was found that the soil-dwelling fungus *Rhizoctonia praticola* 93A was capable to produce laccase in submerged cultures. Effects of culture conditions on the enzyme biosynthesis in shaken flask and aerated bioreactor cultures were evaluated to improve the yields of the process. Production of extracellular laccase was considerably intensified by the addition of Cu^{2+} to a carbon-limited and nitrogen-sufficient culture medium (C/N = 0.98). When an optimized medium containing glucose (2 g/l) and L-asparagine (1.5 g/l) was used and enzyme synthesis was stimulated by addition of 5 μM Cu^{2+} before inoculation, maximal laccase activities obtained in a batch cultivation were, approximately, 1000 nkat/l. Under these conditions, addition to the medium of the aromatic inducer 2,5-xylidine (1 mM) led to a 10-fold increase in laccase activity. Laccase productivity in shaken flask cultures was also enhanced (to more than 4000 nkat/l on day 3) by using a medium with the initial pH of 7.5. Such a high value of the optimal medium pH for laccase production by *R. praticola* is exceptional among the ligninolytic fungi. In fermenter fungal cultures supplemented with cupric ions, the highest laccase activity (about 4000 nkat/l after 3 days' cultivation) was reached after 24-h incubation using a bioreactor with the aeration rate of 2 l/min, the agitation speed of 200 rpm, and a constant medium pH of 8.0.

Key words: *Rhizoctonia praticola*, laccase, inducers, shaken and fermenter cultures

Introduction

Laccases (EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductases) are copper-containing enzymes which use molecular oxygen to oxidise various aromatic and non-aromatic compounds by the radical-catalysed reaction mechanism (Leonowicz *et al.*, 2001). Because of their broad substrate specificity, soluble or immobilised laccases can potentially be used in textile dye bleaching, pulp delignification, effluent detoxification, production of washing powder, removal of phenolics from effluents, treatment of must, wine and fruit juices, transformation of steroids and antibiotics and in biosensors (Mayer and Staples, 2002).

Laccases are commonly found in plants, fungi, insects, and bacteria. The best known laccases producers and a major source of these enzymes are ligninolytic organisms, such as white-rot fungi (Claus, 2004). Unfortunately, production of laccases by fungi is associated with secondary metabolism, the main drawback of which is limited yield of the enzyme under the growth-limiting conditions (Moreira *et al.*, 2000). At present, research and application are somewhat stymied by rather low yields of the enzyme (Gianfreda *et al.*, 1999), as well as by difficulties in efficient heterologous overexpression of laccases in an active form (Jönsson *et al.*, 1997). The problem of increasing the yield of ligninolytic enzymes in fungal cultures is, therefore, of constant interest to researches (Tien and Kirk, 1984).

Fungal laccase production is influenced by many typical culturing parameters, such as medium composition, carbon and nitrogen ratio, pH, temperature, and aeration ratio (Arora and Gill, 2001). In white-rot fungi, extracellular laccases are constitutively produced in small amounts (Bollag and Leonowicz, 1984), but their production can be considerably enhanced by a wide variety of inducing substances, mainly aromatic or phenolic compounds related to lignin or lignin derivatives (Farnet *et al.*, 1999). In addition, laccase

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production can be modulated by nitrogen and carbon concentrations in the culture medium, as well as by heavy metal ions, especially Cu^{2+} (Galhaup and Haltrich, 2001; Galhaup *et al.*, 2002).

Rhizoctonia praticola is a soil-dwelling plant-pathogenic fungus which causes root rot. It produces an inducible laccase belonging to the class of blue oxidases (Xu *et al.*, 1998; Janusz, 2005). The laccase from this source has a unique property of detoxifying chlorinated phenolic pollutants at a broad range of pH (pH 3.0–10) and temperatures (5–55°C) (Shuttleworth *et al.*, 1986; Dec and Bollag, 1990; Cho *et al.*, 1999). The extracellular enzyme from *R. praticola* had been well characterised (Bollag *et al.*, 1979), but operational conditions favorable to the production of increased amounts of laccase in submerged fungal cultures had not been investigated in detail. Keeping in mind the potential applications of laccase in various bioprocesses, the present study was undertaken to find the best culture conditions for the overproduction of laccase by *R. praticola* in shaken flasks and aerated fermenter cultures, and to search for the most effective inducers of the enzyme synthesis. The results of this study may contribute to commercialisation of the production of this valuable enzyme.

Experimental

Materials and Methods

Chemicals. Syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine), ferulic acid (4-hydroxy-3-methoxycinnamic acid), veratric acid (3,4-dimethoxybenzoic acid), *o*-anisidine (2-methoxyaniline), *p*-anisidine (4-methoxyaniline), and antifoam B emulsion were supplied by Sigma-Aldrich (St. Louis, MO, USA). 2,5-Xylidine (2,5-dimethylaniline) was purchased from Fluka (Buchs, Switzerland), while L-asparagine came from Merck (Darmstadt, Germany). All other products were of a reagent or an analytical grade and were purchased locally.

Fungal strain, media, and culture conditions. *Rhizoctonia praticola* was obtained from the culture collection of the Laboratory of Soil Microbiology (Pennsylvania State University, USA), and is deposited in the fungal strains collection of the Department of Biochemistry (Maria Curie-Skłodowska University, Poland) under the strain number 93A. Stock cultures of fungus were stored at 4°C on GPY agar slants (per litre: 1.0 g of glucose, 0.5 g of peptone, 0.1 g of yeast extract, 20 g of agar). For inoculations, pieces of mycelium taken from agar slants were grown for 7 days at 28°C in stationary conical flasks with the Lindeberg-Holm (pH 5.5) (Lindeberg and Holm, 1952) or the Czapek-Dox medium (pH 6.8) (Leonowicz *et al.*, 1984). Mycelial mats were subsequently collected and broken in a Waring blender (three times for 15 s at 10 000 rpm), and the homogenates were used as inocula for shaken flask and aerated bioreactor cultures. After inoculation with 2.5% (v/v) mycelial suspension, the shaken flask cultures were run for up to 14 days at 28°C in 100-ml wide mouth Erlenmeyer flasks (each with 40 ml of the Lindeberg-Holm or the Czapek-Dox medium) placed on an orbital rotary shaker at 180 rpm.

Out of the media tested for laccase production, the medium developed by Lindeberg and Holm (1952) was chosen for the experiments and optimised with respect to the initial pH, and carbon (0.5, 1.0, 2.0, 5.0, 10, 15 g/l glucose), nitrogen (0.25, 0.5, 1.0, 1.5, 2.0, 5.0, 10 g/l L-asparagine) and copper concentrations ($CuSO_4 \times 5\ H_2O$ over the range of 0–300 μM). Copper concentration in the unsupplemented medium was 0.2 μM.

Putative aromatic laccase inducers (ferulic and veratric acids, *o*- and *p*-anisidines, and 2,5-xylidine) were dissolved in ethanol as stock solutions and sterilised by filtration (Sterivex-GS filter unit, 0.22 μm; Millipore Corp.). These were added to the growing fungal cultures on the third day of incubation, so that their final concentration in the optimised Lindeberg-Holm medium was 0.02, 1.0, or 5.0 mM. The concentration of ethanol in the growth medium was always less than 0.5% and an equivalent amount of ethanol was added to control flasks without aromatic inducers.

Bioreactor-scale cultivations were performed at 28°C in a 2.5 l glass fermenter (BioFlo III, New Brunswick Scientific, Edison, NY, USA) containing 2 l of the optimised Lindeberg-Holm medium. The fermenter was sterilised (121°C, 30 min) and seeded with mycelial suspension (10% of the total volume). The culture was run for 5 days at an aeration rate of 1 or 2 l air/min and stirrer speeds of 100, 200, or 300 rpm. Antifoam B emulsion was added to break the foam. The pH was either not regulated, or was automatically maintained at 7.5, 8.0, and 8.5 value.

Samples of the culture media were harvested from shaken flasks or the fermenter at specified time intervals and analysed for laccase activity and pH. Submerged cultures were performed in three independent experiments, and analyses were carried out at least in duplicate. The values reported here are mean values with standard deviations being less than 10% in all cases.

Assays. Laccase activity in the culture supernatant was measured spectrophotometrically at 525 nm in a Shimadzu UV-Vis 160A spectrophotometer (Tokyo, Japan) using syringaldazine as a substrate (Leonowicz and Grzywnowicz, 1981). Enzyme and substrate blanks were included. One unit (nano katal, nkat) of laccase activity was defined as the amount of enzyme catalysing the production of one nanomole of coloured product [quinone, $\varepsilon^M = 65\ 000/(M \times cm)$] per second at 25°C and pH 7.4. The activity was expressed as nano katals per litre of culture medium (nkat/l).

Results and Discussion

It had been reported by other authors that secretion of laccase by different white-rot fungi was strictly dependent upon growth conditions (Niku-Paavola *et al.*, 1990; Arora and Gill, 2001; Hess *et al.*, 2002). Our experiments were undertaken for determining the effect of different media, initial pH, nitrogen, carbon and

inducer concentrations, as well as agitation and aeration rates of the culture on the increase of laccase production by *R. praticola* in shaken flask and aerated bioreactor cultures.

Enzyme production in shaken cultures. The first step of the studies was determination of the optimum medium composition for extracellular laccase synthesis by *R. praticola*. Fungal shaken flask cultures were run for 14 days using two culture media (Czapek-Dox or Lindeberg-Holm) commonly used for the production of ligninolytic enzymes by fungi. Typical culture profiles are depicted in Fig. 1. The results clearly showed that in the Lindeberg-Holm medium laccase productivity reached the maximum level (on 8th day) faster than in the Czapek-Dox medium (on 11th day). Enzyme activity in the Lindeberg-Holm medium declined very slowly towards the end of the incubation, in contrast to the Czapek-Dox medium, where a sharp decrease was observed. Taking these results into consideration, the Lindeberg-Holm medium was chosen for further experiments.

To determine the optimum C/N ratio in the Lindeberg-Holm medium, different variants of this medium were prepared. The concentration of carbon (added as glucose) varied from 0.5 to 15 g/l (2.78–83.34 mM) while that of nitrogen (added as L-asparagine) from 0.25 to 10 g/l (1.89–75.69 mM). The C/N ratio ranged from 0.037 to 44.10. Titres of laccase were measured for 14 days in shaken flask cultures. Fig. 2 illustrates a topographic dependence of laccase activity upon glucose and L-asparagine concentrations. The results indicated that enzyme activity reached its maximum (420 nkat/l on day 6) in cultures with a C/N ratio of 0.98, *i.e.*, containing 2 g/l glucose (11.12 mM) and 1.5 g/l L-asparagine (11.34 mM), respectively. These optimum C and N concentrations were used in further studies. Under these nutritional conditions (a strict carbon limitation and a sufficient amount of nitrogen), the enzyme productivity was six times higher than that obtained in the original Lindeberg-Holm medium used as a control (glucose 10 g/l, L-asparagine 1 g/l; C/N ratio of 7.35). It was found that an increase in glucose concentration from 5 to 15 g/l significantly suppressed laccase activity. In contrast, a rise in nitrogen concentration (from 0.25 to 2.0 g/l) enhanced enzyme synthesis yields.

On the basis of the above data, we postulate that synthesis of laccase by *R. praticola* is the result of a carbon-based regulatory mechanism rather than an outcome of nitrogen limitation. Also, studies of Kwon and Anderson (2001) revealed that expression of laccases in a wheat-pathogenic *Fusarium proliferatum* isolate was regulated by the nutritional status of the fungus and was greater on a carbon-limited than on a nitrogen-limited medium. Opposite results were obtained in batch and fed-batch cultivations of *Trametes*

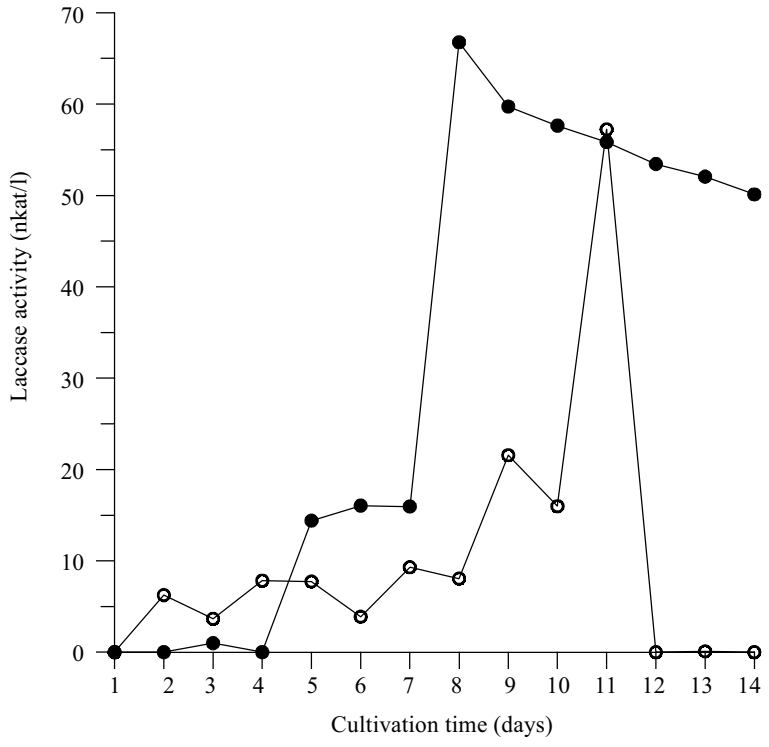

Fig. 1. Time course of laccase production in Lindeberg-Holm (●) and Czapek-Dox (o) media during shaken flask cultures of *Rhizoctonia praticola*.

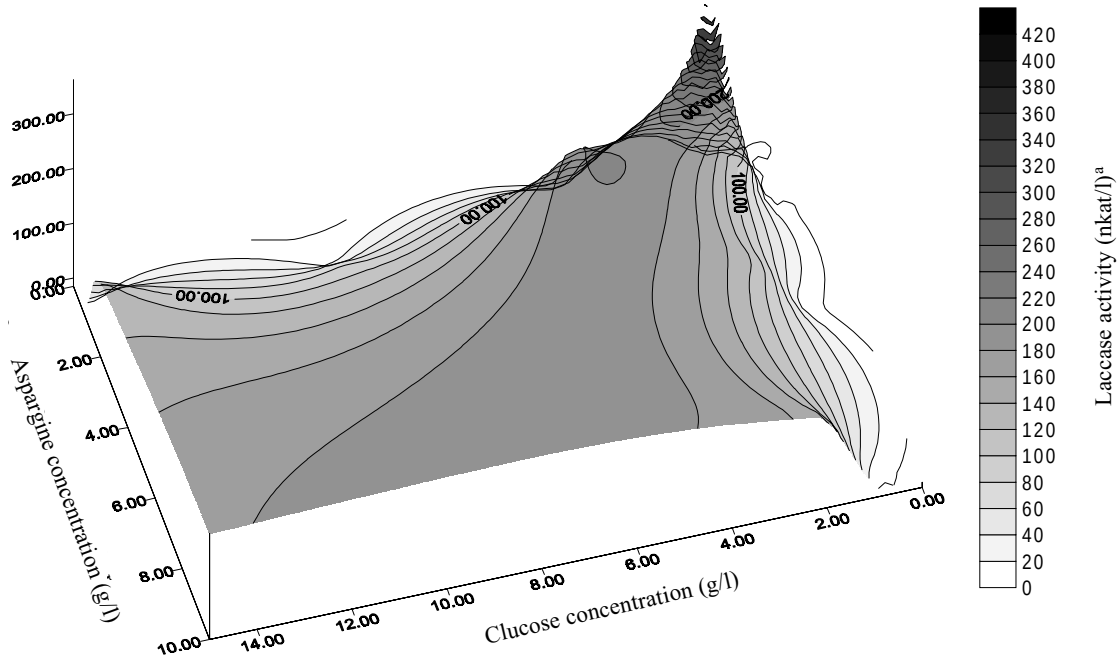

Fig. 2. Effect of nitrogen (L-asparagine) and carbon (glucose) concentrations on laccase activity of *R. praticola* grown in shaken flask cultures in Lindeberg-Holm medium.

[a] The values given are maximum activities reached during cultivation.

pubescens, in case of which high glucose and peptone levels in the medium (40 g/l and 10 g/l, respectively) gave rise to maximum activity of extracellular laccase (Galhaup *et al.*, 2002). Higher nitrogen levels are often required in order to enhance laccase production (Gianfreda *et al.,* 1999; Pointing *et al.*, 2000; Galhaup and Haltrich, 2001; Chen *et al*., 2003), but with certain fungi nitrogen-limited culture conditions stimulate the formation of this enzyme (Niku-Paavola *et al*., 1990; Pointing *et al.*, 2000; Baldrian and Gabriel, 2002).

The influence of Cu^{2+} concentration on the production of extracellular laccase in 10-day shaken flask cultures of *R. praticola* is shown in Fig. 3. Total Cu^{2+} concentrations varied within the range of 0 to 300 μM, and these ions were added to the culture medium before inoculation with fungal suspension. As the data obtained demonstrate, all Cu^{2+}-containing cultures of *R. praticola* showed higher laccase activity and produced the enzyme within a shorter incubation period (3 days) when compared with Cu^{2+}-free cultures where maximum laccase production was delayed until day 6. Maximum laccase activity (about 1 000 nkat/l) for this fungus was observed in the presence of 5 μM Cu^{2+}. Increased concentrations of copper (from 10 to 300 μM Cu^{2+}) repressed laccase production (to 54% at 300 μM Cu^{2+}).

Cupric ions had been found to be strong stimulants of laccase activity also in experiments carried out by Giardina *et al.* (1999) as well as Galhaup *et al.* (2002), in which up to 50 times higher levels of the enzyme were obtained in induced, compared to non-induced, cultures. In the present study, both the time of Cu^{2+} ions supplementation and Cu^{2+} ions concentration were important for obtaining an increased laccase activity. Their supplementation before the inoculation of culture resulted in markedly increased laccase titres. The optimal copper concentration for the enzyme production by *R. praticola* 93A in shaken flask cultures was 5 μM. Thus, approximately 2.5 times higher yields of the enzyme were obtained in cultures containing Cu^{2+} as compared to Cu^{2+}-free cultures. The optimal Cu^{2+} concentration was significantly lower than that (2.0 mM, added after 4 days of incubation) reported by Galhaup and Haltrich (2001) for submerged cultures of *T. pubescens*, but was still within the range of 2 to 600 μM used in typical cultivation media for the production of laccase both in wild-type and recombinant strains of different basidiomycete fungi (Farnet *et al.*, 1999; Palmieri *et al.*, 2000; Chen *et al.*, 2003). In another study, Baldrian and Gabriel (2002) showed that laccase production by *Pleurotus ostreatus* increased 8 times during stationary cultivation in nitrogen-limited medium supplemented with 1.0 mM $CuSO_4$ after 12 day incubation. It had also been reported (Palmieri *et al.*, 2000) that the induction of laccase in *P. ostreatus* occurred when the fungus was cultivated in a nutrient-rich medium supplemented with 150 μM $CuSO_4$ at the time of inoculation. A Cu^{2+} dose of 1.0 mM was also required for enhancement of laccase synthesis by *T. multicolor* in bioreactor cultures (Hess *et al.,* 2002).

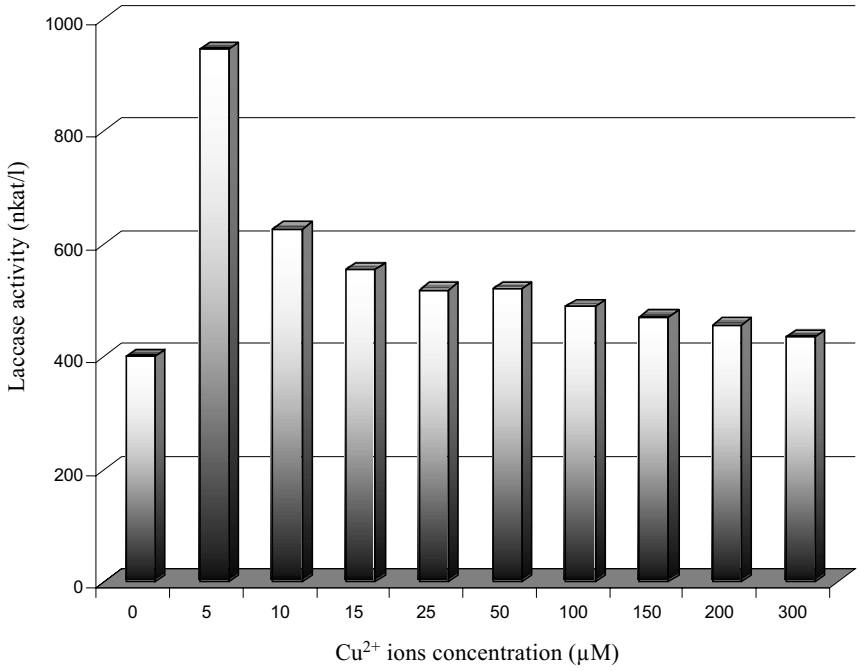

Fig. 3. Effect of Cu^{2+} concentration on the production of laccase by *R. praticola* in shaken flask cultures. Cu^{2+} ions were added to the medium before inoculation with the fungus (on day 0). Values represent maximum enzyme activity measured on day 6 (control without Cu^{2+}) and 3 (samples with Cu^{2+}).

Production of laccase is very often enhanced by phenolic and aromatic compounds related to lignin or lignin derivatives such as guaiacol or ferulic acid (Gianfreda *et al*., 1999). The question arises whether the same substances affect the formation of extracellular laccase by *R. praticola* in a similar way. To check this, several putative inducers that have a proven effect on laccase synthesis in other fungi were added at varying concentrations (0.02, 1.0 and 5.0 mM) to an actively growing culture of *R. praticola* on the third day of incubation. Results of these shaken-flask experiments are summarised in Table I. Three out of five different compounds tested, namely, xylidine, and *o*- and *p*-anisidines, showed an inductive effect on laccase production at almost all concentrations used. They increased enzyme activity to the maximum level (approximately 10, 2, and 4 times, respectively) at the concentration of 1.0 mM compared to the control culture medium containing no aromatic compound. However, the particular maxima of laccase activity were shifted to day 7 (for xylidine and *o*-anisidine) or even day 9 (in the case of *p*-anisidine) compared to the control, which reached its maximum after 3 days of cultivation. Addition of the other substances, *i.e.*, ferulic and veratric acids, to the culture medium reduced laccase levels significantly, and veratric acid, used at the dose of 5.0 mM, caused a slight increase in enzyme titres.

Xylidine, the most effective inducer, stimulated laccase production in *R. praticola* by a factor of 10 at the concentration of 1.0 mM. This contrasts with the results of earlier investigations on this fungus (Bollag and Leonowicz, 1984), in which the authors showed that *R. praticola* laccase was not affected by xylidine when grown at 24 ºC in sugar-rich shaken and stationary cultures. The difference in the findings might be attributed to the higher concentration of xylidine used in the cited experiments. On the other hand, our results were consistent with those of other studies carried out for several fungal strains by different research groups (Galhaup and Haltrich, 2001; Jang *et al.*, 2002; Nyanhongo *et al.*, 2002; Chen *et al.,* 2003; Rancaño *et al.*, 2003), which reported that xylidine, one of the most common and most often used stimulants, elevated laccase production in various *Trametes* species and in *Volvariella volvacea*. Moreover, our results are also in accordance with investigations performed by Rogalski *et al*. (1991), who revealed that out of several tested laccase elicitors, xylidine led to the highest laccase activities in *Phlebia radiata.*

Anisidines are known to induce laccase in *Rhizoctonia* species (Shuttleworth *et al.*, 1986; Crowe and Olsson, 2001), and such specific induction may operate *via* receptor-mediated transcriptional activation (Fernandez-Larrea and Stahl, 1996). As expected, we also found *p*-anisidine to be a powerful inducer of laccase in *R. praticola*. On the other hand, ferulic and veratric acids repressed laccase production significantly in this fungus although these substances are effective inducers for other fungi, such as *V. volvacea*,

Table I
Laccase activity in shaken flask cultures of *R. praticola* exposed to various aromatic compounds[a]

Inducer	Concentration (mM)	Enzyme activity (nkat/l) Cultivation time (day)							
		2	3	4	5	6	7	8	9
Control	0.00	841.5 ± 61.1	960.1 ± 63.3	764.0 ± 62.6	658.0 ± 45.9	653.0 ± 35.1	572.0 ± 39.0	532.0 ± 32.3	494.6 ± 31.3
Ferulic acid	0.02	–	445.6 ± 34.0	384.2 ± 26.5	365.7 ± 27.2	329.0 ± 22.8	280.6 ± 19.4	245.6 ± 20.5	234.3 ± 20.3
	1.00	–	373.1 ± 27.4	328.9 ± 29.1	351.7 ± 32.8	272.7 ± 22.4	226.7 ± 15.8	220.6 ± 20.9	116.4 ± 11.2
	5.00	–	146.7 ± 9.2	114.2 ± 9.6	85.0 ± 6.1	50.6 ± 2.5	39.0 ± 3.4	41.8 ± 2.8	0.0 ± 0.0
Veratric acid	0.02	–	605.9 ± 43.1	528.1 ± 35.1	495.0 ± 41.2	442.7 ± 31.4	472.7 ± 32.2	242.4 ± 23.9	377.9 ± 25.8
	1.00	–	648.2 ± 44.7	582.6 ± 47.4	528.8 ± 40.7	511.1 ± 31.8	475.8 ± 40.7	409.5 ± 35.0	383.9 ± 27.5
	5.00	–	727.4 ± 50.1	857.7 ± 59.9	722.8 ± 59.4	659.5 ± 43.7	578.3 ± 50.0	550.4 ± 35.1	504.4 ± 37.5
Xylidine	0.02	–	1342.8 ± 71.9	1592.6 ± 106.1	1441.8 ± 95.2	1240.7 ± 82.6	1225.3 ± 86.5	904.9 ± 56.9	828.5 ± 49.6
	1.00	–	360.5 ± 16.5	468.0 ± 29.9	1015.8 ± 71.2	2942.1 ± 193.4	9254.1 ± 498.7	7283.5 ± 449.9	4029.4 ± 302.7
	5.00	–	900.7 ± 51.3	857.8 ± 54.7	811.9 ± 58.9	740.0 ± 47.9	609.1 ± 44.1	603.1 ± 31.5	599.8 ± 30.1
o-Anisidine	0.02	–	857.2 ± 52.9	1205.3 ± 60.1	938.3 ± 58.4	901.7 ± 56.1	857.3 ± 39.9	685.8 ± 40.4	583.0 ± 34.6
	1.00	–	49.2 ± 4.6	481.7 ± 39.9	535.7 ± 37.7	536.5 ± 22.9	1875.4 ± 114.9	1028.3 ± 89.8	624.2 ± 48.6
	5.00	–	37.1 ± 1.9	42.1 ± 2.5	42.8 ± 2.2	55.7 ± 3.5	78.8 ± 5.5	29.6 ± 1.6	22.5 ± 1.42
p-Anisidine	0.02	–	1242.7 ± 61.8	1611.5 ± 91.6	1563.1 ± 99.4	1502.2 ± 92.2	1269.9 ± 88.6	1191.0 ± 71.2	864.5 ± 65.0
	1.00	–	489.1 ± 35.1	782.9 ± 50.1	791.0 ± 44.9	1392.5 ± 65.3	2027.9 ± 122.3	3246.4 ± 246.4	3478.9 ± 216.7
	5.00	–	873.7 ± 54.8	1106.3 ± 64.8	923.0 ± 64.9	729.1 ± 45.5	717.4 ± 59.2	509.2 ± 32.3	62.0 ± 5.4

[a] Lindeberg-Holm medium with optimised carbon and nitrogen concentrations and 5 μM Cu^{2+} (added before inoculation, *i.e.*, on day 0) was used. Each inducer was introduced into the medium on the third day of cultivation.

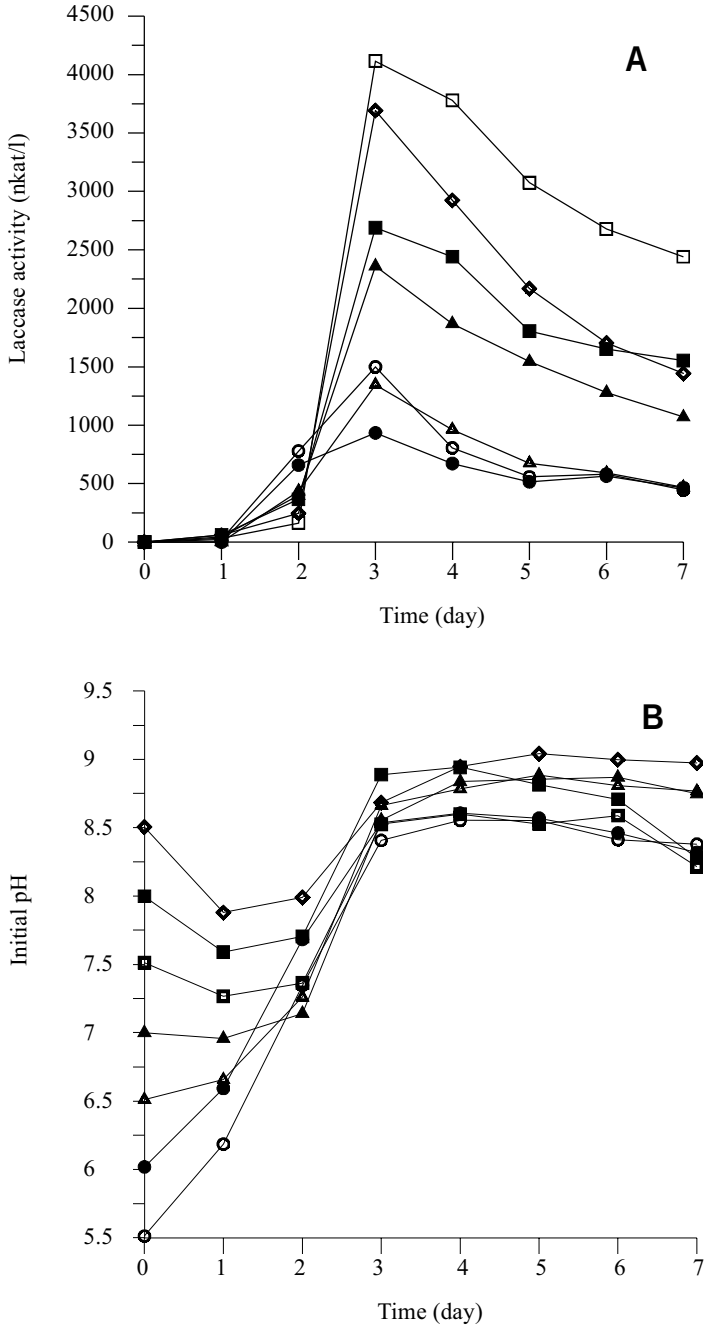

Fig. 4. The effect of initial medium pH (**A**) and changes in the pH of culture medium as a function of time (**B**) on laccase biosynthesis during shaken flask cultures of *R. praticola* in optimised Lindeberg-Holm medium containing Cu^{2+} (5 μM, added before inoculation).
Initial pH of the original medium was 5.5. Symbols: pH 5.5 (○), pH 6.0 (●), pH 6.5 (△), pH 7.0 (▲), pH 7.5 (□), pH 8.0 (■), pH 8.5 (◇).

Marasmius quercophilus, *Pleurotus eryngii*, and *P. radiata* (Rogalski and Leonowicz, 1992; Muñoz *et al.*, 1997; Farnet *et al.,*1999; Chen *et al.,* 2003).

In preliminary experiments, the pH of the original Lindeberg-Holm medium was 5.5. To find the most suitable conditions for laccase production in shaken flask cultures, we determined enzyme activity at pHs between 5.5 to 8.5. It follows clearly from the data in Fig. 4 that the initial medium pH of 7.5 was the most conducive to enzyme production, yielding, in shaken flask cultures, over 4000 nano katals of laccase per litre of culture broth after 3 days of cultivation. This activity was approximately three times higher than that obtained at the initial pH of 5.5 (about 1400 nkat/l).

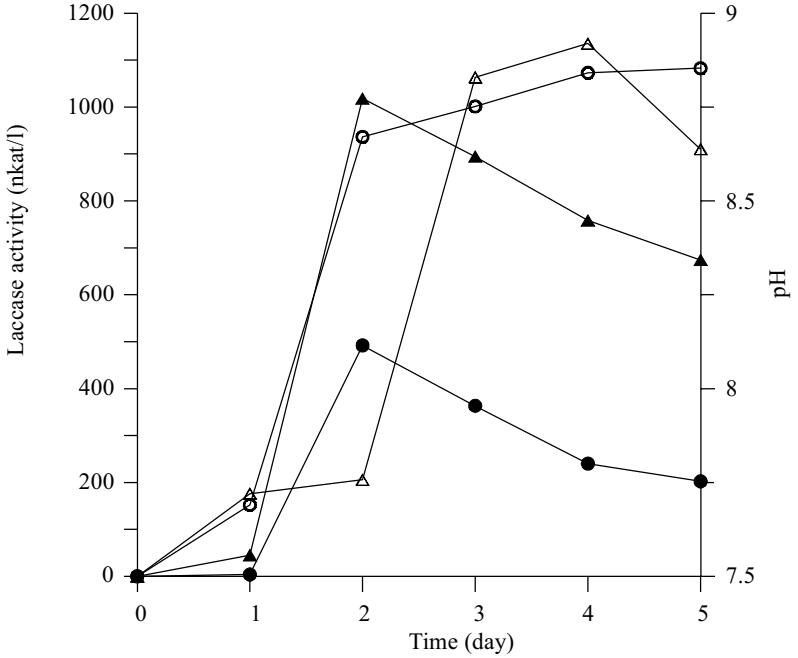

Fig. 5. Laccase production (▲, ●) and changes in pH (Δ, o) during fermenter cultures of *R. praticola* in optimised Lindeberg-Holm medium containing Cu^{2+} (▲, Δ) and in a Cu^{2+}-free medium (●, o). Cu^{2+} ions (5 μM) were added to the medium before inoculation. Culture conditions: pH-value not controlled (the initial medium pH was 7.5); stirrer speed 300 rpm; aeration rate 1 l/min.

The results also demonstrated the high correlation between laccase production and changes in the medium pH during cultivation. In all cases, the enzyme was detected in the medium when the fungus alkalised the medium to a pH above 7.5. Such a high value of the initial medium pH (7.5), which is indispensable for the production of laccase by *R. praticola*, is exceptional among ligninolytic fungi. Most fungal laccases reach their maximum activity when the initial pH of the nutrient medium ranges from 4 to 6 (Galhaup *et al.*, 2002; Jang *et al.*, 2002; Chen *et al.*, 2003). A newly isolated strain of *T. modesta* resembles *R. praticola* in requiring a relatively high initial pH (*i.e.* 6.95) to produce the highest titres of laccase (Nyanhongo *et al.*, 2002); however, the laccase from *R. praticola* is of particular interest because it also has an unusually high pH optimum for its catalytic activity. Most fungal laccases have optima in the acidic region (below pH 5), whereas laccase isolated from *R. praticola* as well as enzymes obtained lately from *Melanocarpus albomyces* and *Pleurotus ostreatus* have their optimum activity at a neutral pH (Bollag and Leonowicz, 1984; Shuttleworth *et al.*, 1986; Kiiskinen *et al.*, 2002; Pozdnyakova *et al.*, 2004).

Enzyme production in fermenter cultures. Laccase production by *R. praticola* was also carried out in 2-litre batches in a 2.5 l fermenter. Biosynthesis conditions were fixed, taking into account the previous results obtained in agitated flask cultures (the optimised Lindeberg-Holm production medium with the determined Cu^{2+} dose and C/N ratio, initial pH of the medium, and incubation temperature). During the first stage, we compared laccase activities achieved by *R. praticola* in bioreactor cultures performed on the optimised Lindeberg-Holm medium with and without cupric ions. As Fig. 5 shows, the fungus reached the highest titres of laccase activity (over 1000 nkat/l) when it was cultivated in the presence of the fixed dose of Cu^{2+}. This activity was two times higher than in the Cu^{2+}-free culture, but still much lower (even by 75%) than that obtained in optimised shake flask cultures. In the fermenter culture, however, the enzyme productivity reached maximum levels within a shorter incubation period (2 days).

In order to increase laccase yields in aerated fermenter cultures, some operating parameters affecting enzyme production by *R. praticola* were optimised. The effects of increasing the stirrer speed (from 100 to 300 rpm) and the aeration rate (from 1 to 2 l per min) on laccase production are shown in Fig. 6. The results indicate that laccase activity reached maximum levels (2400 nkat/l on day 2) in cultures with the aeration rate of 2 l/min and the agitation speed of 200 rpm. The effects of stirrer speed and aeration rate agree with data obtained for fungal laccases in other studies (Moreira *et al.*, 2000; Galhaup *et al.*, 2002; Rancaño *et al.*, 2003).

It is significant that in all fermenter cultures carried out without pH regulation, the fungus alkalised the medium during cultivation, and pH rose from 7.5 to above 8.8. Therefore, the influence of stabilisation of

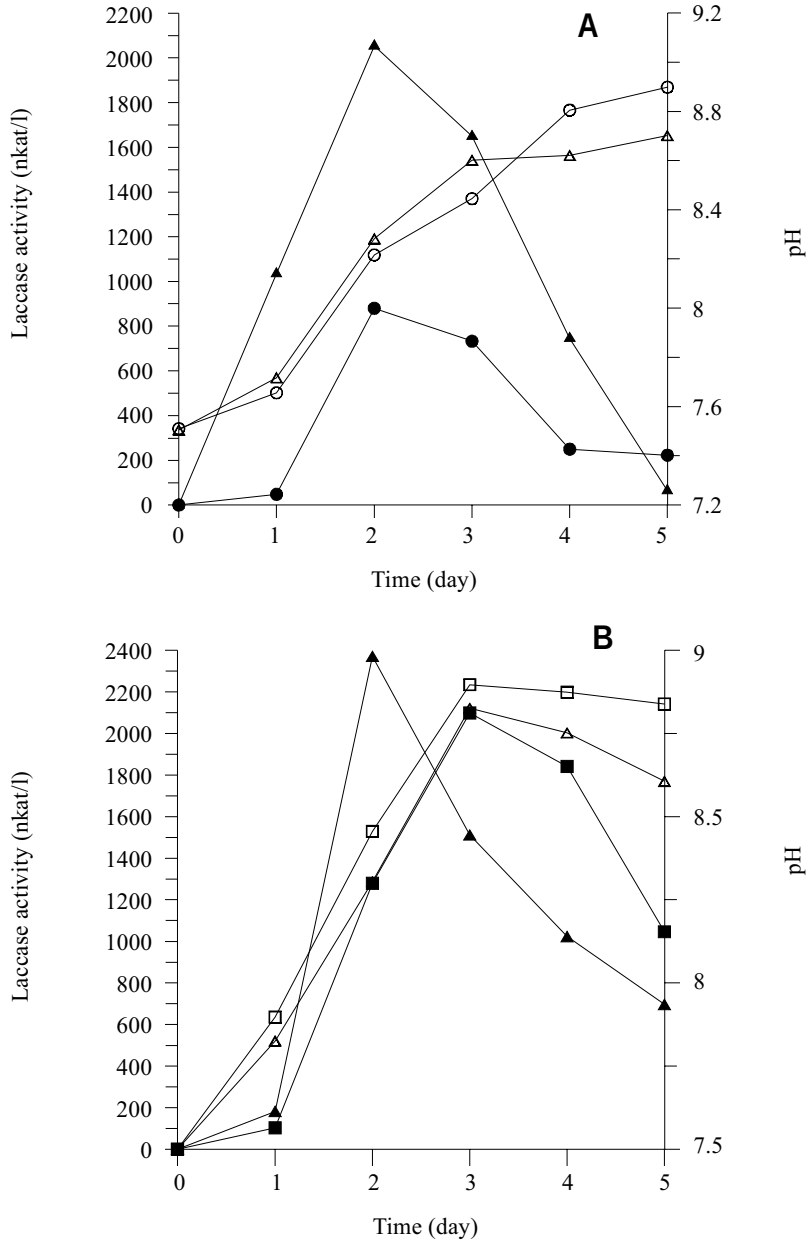

Fig. 6. Time course of laccase production (▲, ■, ●) and changes in pH (Δ, □, o) during fermenter cultures of *R. praticola* in optimized Lindeberg-Holm medium.

The fungal cultures were run at the aeration rates of 1 (**A**) and 2 l (**B**) air per min and with the stirrer speeds of 100 (■, □), 200 (▲, Δ), and 300 rpm (●, o). Cu^{2+} ions (5 μM) were added to the medium before inoculation. Culture conditions: pH value not controlled (the initial medium pH was 7.5).

medium pH after 24 h incubation on laccase activity had to be estimated (Fig. 7). The obtained data show that the use of an automatic pH control set at pH 8 significantly induced laccase productivity. Under these conditions, the highest enzyme activity of 4000 nkat/l was reached after 3-day incubation. It was almost two times higher than that obtained in a fermenter culture with a non-stabilised pH value. Consequently, the application of optimised medium and culture conditions as well as the use of a bioreactor maintaining the pH value on the same level enabled us to obtain an increased laccase yield (in average 4000 nkat/l) within a short time (2–3 days), both in shake flask and aerated fermenter cultures of *R. praticola*.

In conclusion, the present study reveals that the soil-dwelling plant-pathogenic fungus *R. praticola*, strain 93A, represents a new source of extracellular laccase which requires high values of the initial medium pH (7.5 to 8.0) to produce the highest titres of the enzyme. The study also shows that an appropriate combination of culture conditions, *i.e.*, the use of a C-limited medium supplemented with an adequate dose of Cu^{2+} ions before inoculation, stabilisation of medium pH, and maintenance of stirring speed and aeration rate at

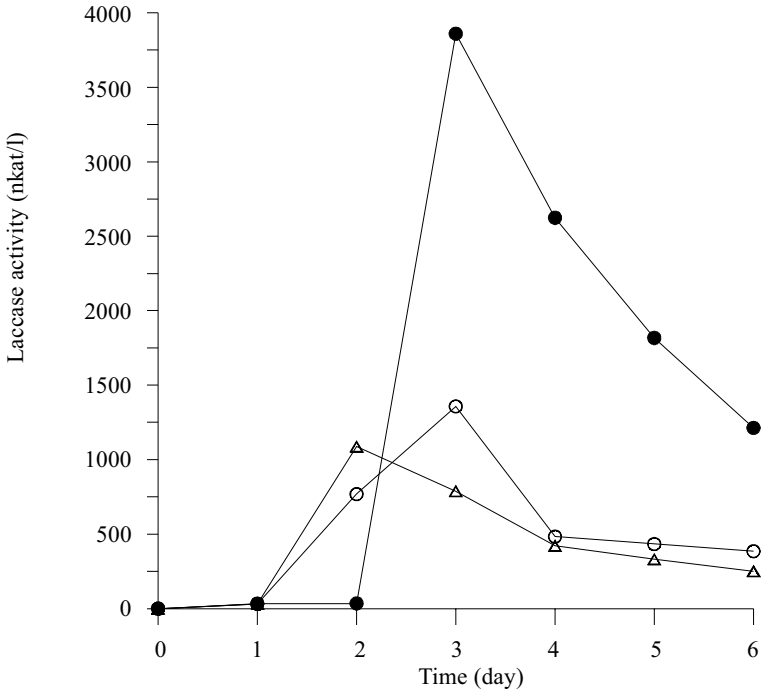

Fig. 7. Effect of stabilisation of medium pH on laccase production by *R. praticola* in fermenter cultures in optimised Lindeberg-Holm medium.

The pH was automatically maintained at a value of 7.5 (o), 8.0 (●) and 8.5 (Δ) after 24-h incubation. Cu^{2+} ions (5 μM) were added to the medium before inoculation. Culture conditions: stirrer speed 200 rpm; aeration rate 2 l/min.

the optimised level increases laccase production by *R. praticola* 93A within a short period of incubation. These results applied in a large scale fermenter culture could prove to be of an economic advantage. Future experiments with the use of immobilised mycelium could lead to determining the optimal conditions for repeated batch or continuous fungal cultures, resulting in another potential cost reduction in laccase production.

Acknowledgments. This work was financially supported by the Polish State Committee for Scientific Research (Grant PBZ-KBN-098/T09/2003).

Literature

Arora D.S. and P.K. Gill. 2001. Effects of various media and supplements on laccase production by some white rot fungi. *Biores. Technol.* **77**: 89–91.

Baldrian P. and J. Gabriel. 2002. Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. *FEMS Microbiol. Lett*. **206**: 69–74.

Bollag J.-M. and A. Leonowicz. 1984. Comparative studies of extracellular fungal laccases. *Appl. Environ. Microbiol.* **48**: 849–854.

Bollag J.-M., R.D. Sjoblad and S.-Y. Liu. 1979. Characterization of an enzyme from *Rhizoctonia praticola* which polymerizes phenolic compounds. *Can. J. Microbiol.* **25**: 229–233.

Chen S., D. Ma, W. Ge and J.A. Buswell. 2003. Induction of laccase activity in the edible straw mushroom, *Volvariella volvacea*. *FEMS Microbiol. Lett.* **218**: 143–148.

Cho N.-S., J. Rogalski, M. Jaszek, J. Luterek, M. Wojtas-Wasilewska, E. Malarczyk, M. Fink-Boots and A. Leonowicz. 1999. Effect of coniferyl alcohol addition on removal of chlorophenols from water effluent by fungal laccase. *J. Wood Sci*. **45**: 174–178.

Claus H. 2004. Laccases: structure, reactions, distribution. *Micron* **35**: 93–96.

Crowe J.D. and S. Olsson. 2001. Induction of laccase activity in *Rhizoctonia solani* by antagonistic *Pseudomonas fluorescens* strains and a range of chemical treatments. *Appl. Environ. Microbiol.* **67**: 2088–2094.

Dec J. and J.-M. Bollag. 1990. Detoxification of substituted phenols by oxidoreductive enzymes through polymerization reactions. *Arch. Environ. Contamin. Toxicol.* **19**: 543–550.

Farnet A.-M., S. Tagger and J. LePetit. 1999. Effects of copper and aromatic inducers on the laccases of the white-rot fungus *Marasmius quercophilus*. *C.R. Acad. Sci. Paris, Sciences de la vie/Life Sciences* **322**: 499–503.

Fernandez-Larrea J. and U. Stahl. 1996. Isolation and characterization of a laccase gene from *Podospora anserina*. *Mol. Gen. Genet.* **252**: 539–551.

Galhaup C. and D. Haltrich. 2001. Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Appl. Microbiol. Biotechnol.* **56**: 225–232.

Galhaup C., H. Wagner, B. Hinterstoisser and D. Haltrich. 2002. Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. *Enzyme Microb. Technol.* **30**: 529–536.

Gianfreda L., F. Xu and J.-M. Bollag. 1999. Laccases: a useful group of oxidoreductive enzymes. *Biorem. J.* **3**: 1–25.

Giardina P., G. Palmieri, A. Scaloni, B. Fontanella, V. Faraco, G. Cennamo and G. Sannia. 1999. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. *Biochem. J.* **34**: 655–663.

Hess J., C. Leitner, C. Galhaup, K.D. Kulbe, B. Hinterstoisser, M. Steinwender and D. Haltrich. 2002. Enhanced formation of extracellular laccase activity by the white-rot fungus *Trametes multicolor*. *Appl. Biochem. Biotechnol*. **98–100**: 229–241.

Jang M.Y., W.R. Ryu and M.H. Cho. 2002. Laccase production from repeated batch cultures using free mycelia of *Trametes* sp. *Enzyme Microb. Technol.* **30**: 741–746.

Janusz G. 2005. Comparative studies of fungal laccases. Ph.D. Thesis, Maria Curie-Sklodowska University, Lublin, Poland.

Jönsson L.J., M. Saloheimo and M. Penttilä. 1997. Laccase from the white-rot fungus *Trametes versicolor*: cDNA cloning of *lcc1* and expression in *Pichia pastoris*. *Curr. Genet.* **32**: 425–430.

Kiiskinen L.L., L. Viikari and K. Kruus. 2002. Purification and characterization of a novel laccase from the ascomycete *Melanocarpus albomyces*. *Appl. Microbiol. Biotechnol*. **59**: 198–204.

Kwon S.-I. and A.J. Anderson. 2001. Laccase isozymes: production by an opportunistic pathogen, a *Fusarium proliferatum* isolate from wheat. *Physiol. Mol. Plant Pathol.* **59**: 235–242.

Leonowicz A., N.-S. Cho, J. Luterek, A. Wilkolazka, M. Wojtas-Wasilewska, A. Matuszewska, M. Hofrichter, D. Wesenberg and J. Rogalski. 2001. Fungal laccase: properties and activity on lignin. *J. Basic Microbiol.* **41**: 185–227.

Leonowicz A., R.U. Edgehill and J.-M. Bollag. 1984. The effect of pH on the transformation of syringic and vanillic acids by the laccases of *Rhizoctonia praticola* and *Trametes versicolor*. *Arch. Microbiol*. **137**: 89–96.

Leonowicz A. and K. Grzywnowicz. 1981. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. *Enzyme Microb. Technol.* **3**: 55–58.

Lindeberg G. and G. Holm. 1952. Occurrence of tyrosinase and laccase in fruit bodies and mycelia of some *Hymenomycetes*. *Physiol. Plant.* **5**: 100–114.

Mayer A.M. and R.C. Staples. 2002. Laccase: new functions for an old enzyme – a review. *Phytochemistry* **60**: 551–565.

Moreira M.T., A. Torrado, G. Feijoo and J.M. Lema. 2000. Manganese peroxidase production by *Bjerkandera* sp. BOS55. 2. Operation in stirrer tank reactors. *Bioprocess Eng.* **23**: 657–661.

Muñoz C., F. Guillén, A.T. Martinez and M.J. Martinez. 1997. Induction and characterization of laccase in the ligninolytic fungus *Pleurotus eryngii*. *Curr. Microbiol.* **34**: 1–5.

Niku-Paavola M.-L., E. Karhunen, A. Kantelinen, L. Viikari, T. Lundell and A. Hatakka. 1990. The effect of culture conditions on the production of lignin modifying enzymes by the white-rot fungus *Phlebia radiata*. *J. Biotechnol.* **13**: 211–221.

Nyanhongo G.S., J. Gomes, G. Gübitz, R. Zvauya, J.S. Read and W. Steiner. 2002. Production of laccase by a newly isolated strain of *Trametes modesta*. *Biores. Technol.* **84**: 259–263.

Palmieri G., P. Giardina, C. Bianco, B. Fontanella and G. Sannia. 2000. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **66**: 920–924.

Pointing S.B., E.B.G. Jones and L.L.P. Vrijmoed. 2000. Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. *Mycologia* **92**: 139–144.

Pozdnyakova N.N., J. Rodakiewicz-Nowak and O.V. Turkovskaya. 2004. Catalytic properties of yellow laccase from *Pleurotus ostreatus* D1. *J. Mol. Cat. B- Enzymatic* **30**: 19–24.

Rancaño G., M. Lorenzo, N. Molares, S. Rodriguez Couto and Á. Sanromán. 2003. Production of laccase by *Trametes versicolor* in an airlift fermentor. *Proc. Biochem.* **39**: 467–473.

Rogalski J. and A. Leonowicz. 1992. *Phlebia radiata* laccase forms induced by veratric acid and xylidine in relation to lignin peroxidase and manganese-dependent peroxidase. *Acta Biotechnol.* **12**: 213–221.

Rogalski J., T. Lundell, A. Leonowicz and A. Hatakka. 1991. Influence of aromatic compounds and lignin on production of ligninolytic enzymes by *Phlebia radiata*. *Phytochemistry* **30**: 2869–2872.

Shuttleworth K.L., L. Postie and J.-M. Bollag. 1986. Production of induced laccase by the fungus *Rhizoctonia praticola*. *Can. J. Microbiol.* **32**: 867–870.

Tien M. and T.K. Kirk. 1984. Lignin-degrading enzyme from *Phanerochaete* chrysosporium: Purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. *Proc. Natl. Acad. Sci. USA* **81**: 2280–2284.

Xu F., R.M. Berka, J.A. Wahleithner, B.A. Nelson, J.R. Shuster, S.H. Brown, A.E. Palmer and E.I. Solomon. 1998. Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. *Biochem. J.* **334**: 63–70.