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Production of hydrogen peroxide by aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*

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Summary. Production of extracellular hydrogen peroxide by fungal oxidases is been investigated as a requirement for lignin degradation. Aryl-alcohol oxidase activity is described in extracellular liquid and mycelium of *Pleurotus eryngii* and studied under non-limiting nitrogen conditions. This aryl-alcohol oxidase catalyses conversion of primary aromatic alcohols to the corresponding aldehydes and H_2O_2, showing no activity with aliphatic and secondary aromatic alcohols. The enzyme is stable at pH 4.0–9.0, has maximal activity at 45°–50° C and pH 6.0–6.5, is inhibited by Ag^+, Pb^{2+} and NaN_3, and has a K_m of 1.2 m*M* using veratryl alcohol as substrate. A single protein band with aryl-alcohol oxidase activity was found in zymograms of extracellular and intracellular crude enzyme preparations from *P. eryngii*.

Introduction

Lignin degradation is carried out by a variety of organisms involved during wood rotting and litter decomposition. White-rot basidiomycetous fungi from the order Aphyllophorales are the most effective lignin degraders, and lignocellulose transformation by several species has been investigated (Ander and Eriksson 1978; Crawford 1981; Kirk 1983). However, most of the enzymology of degradation have concentrated on one species, *Phanerochaete chrysosporium* Burd., and several enzymes involved in this process have been studied (Buswell and Odier 1987; Kirk and Farrell 1987).

Following the discovery of lignin peroxidases (Glenn et al. 1983; Tien and Kirk 1983), the search for enzymatic sources for hydrogen peroxide production has been undertaken. Extracellular and intracellular oxidases have been found in ligninolytic cultures of *P. chrysosporium*, producing H_2O_2 from fatty acyl-coenzyme A (Green and Gould 1984), glucose (Kelley and Reddy 1986; Eriksson et al. 1986), methanol (Kuwahara and Asada 1987), glyoxal (Kersten and Kirk 1987) and related compounds. Furthermore, Mn(II)-dependent peroxidases that produce H_2O_2 from reduced nicotine adenine dinucleotide (NADH), reduced nicotine adenine dinucleotide phosphate (NADPH) and certain thiol-containing compounds have been reported (Glenn and Gold 1985; Paszczynski et al. 1986).

The genus *Pleurotus* is well known for the production of edible mushrooms and includes strongly ligninolytic species (Kamra and Zadrazil 1986). The utilization of *Pleurotus* species for the improvement of different lignocellulosic materials for animal fodder has been investigated (Kamra and Zadrazil 1988).

During a study of wheat straw transformation by white-rot fungi a highly selective degradation of lignin by *P. eryngii* (DC) Quel. was found (Valmaseda et al. 1988). Studies on the enzymes involved in lignin degradation by *P. eryngii* have been initiated (Guillén et al. 1988) and the results concerning production and properties of an aryl-alcohol oxidase producing H_2O_2 are presented in this paper.

Materials and methods

Organism and culture conditions. Pleurotus eryngii IJFM A169 was isolated from a fruit-body, and is maintained on 2% malt agar in the Fungal Culture Collection of the CIB (IJFM). Mycelia were cultivated in 100-ml erlenmeyer flasks containing 10 ml culture medium as stationary cultures under 100% oxy-

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gen (Tien and Kirk 1988) (culture conditions A). Samples were taken periodically from three replicate flasks. Modified Czapeck-Dox medium with 1% glucose, 0.2% ammonium tartrate and 0.1% yeast extract was used for experiments involving shake cultures (200 rpm) grown in 1-l erlenmeyer flasks with 200 ml medium (culture conditions B). Samples of 4 ml were taken from five replicate flasks. Inocula were prepared by homogenizing 10-day-old stationary cultures grown in 1-l Roux flasks with 100 ml medium. Homogenates of 0.5 and 5 ml were used as inocula for conditions A and B, respectively. All cultures were grown at 28° C.

Crude enzyme production. Modified Czapeck-Dox medium was used in experiments for enzyme production. Mycelium from 13-day-old cultures was separated by filtration through Whatman No. 1 paper, washed, and a mycelial extract was obtained after homogenization in 280 ml water followed by centrifugation at 13000 *g* for 1 h. Culture filtrate (1.4 l) was concentrated up to 280 ml under reduced pressure at 30° C. Polysaccharide was precipitated from extracellular and intracellular preparations with 30% ethanol (final concentration) and separated by filtration through Whatman No. 1 paper. Filtrates were dialysed overnight against water, concentrated 40 times by ultrafiltration (Amicon PM 10 membrane filter), frozen and stored at −4° C.

Analytical procedures. Reducing sugars were assayed by the method of Somogyi (1945) and Nelson (1944) and proteins by the Coomassie brilliant blue G 250 procedure (Sedmak and Grossberg 1977) using glucose and bovine albumin, respectively, as standards.

Ligninase activity was measured spectrophotometrically as the oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) to veratraldehyde (Tien and Kirk 1984). The H_2O_2 was omitted in the blanks. The concentration of H_2O_2 stock solutions was determined spectrophotometrically from the molar extinction coefficient ($\varepsilon_{230}=81\ M^{-1}\ cm^{-1}$).

Aryl-alcohol oxidase activity was assayed as ligninase activity without H_2O_2 (an oxygen saturated solution of veratryl alcohol was used for kinetic studies). Oxidase activity was also assayed as the production of H_2O_2 by the modified peroxidase coupled procedure described by Kersten and Kirk (1987). In addition to veratryl alcohol, the following alcohols were assayed as substrates for aryl-alcohol oxidase: methanol, ethanol, butanol, benzyl alcohol, 4-methoxybenzyl (=anisyl) alcohol, 3-(4-hydroxy-3-methoxyphenyl)-2-propen-1-ol (=coniferyl alcohol) and 1-(3,4-dimethoxyphenyl) ethanol. International units (U) were used to define the enzymatic activities (μmol min^{-1}).

Electrophoresis was performed in 7.5% polyacrylamide gels (20 μg protein was applied). Proteins were stained with 0.25% Coomassie brilliant blue R-250. Bands with oxidase activity were located by a modification of the staining method for H_2O_2-producing enzymes described by Kersten and Kirk (1987). The nitrocellulose paper was immersed first 30 min in a 100 U ml^{-1} peroxidase solution and then briefly in a solution containing 10 m*M* oxidase substrate and 0.01% phenol red. The treated paper was applied to the gel surface and after 1–3 min clear bands developed against the purple coloured paper. The oxidase activity using coniferyl alcohol as the substrate (10 m*M*) was detected directly over the gel by the production of coniferaldehyde, giving rise to a pale yellow band after 10–15 min.

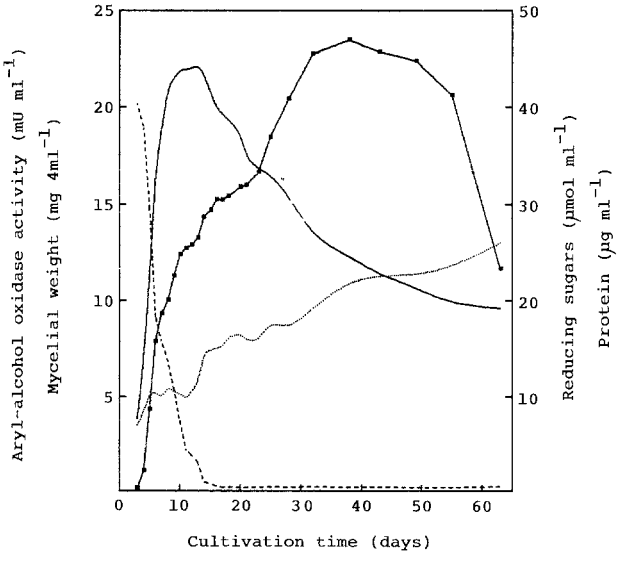

Fig. 1. Evolution of extracellular aryl-alcohol oxidase activity (—■—), mycelial weight (——), proteins (· · ·) and reducing sugars (---) in shake cultures (culture conditions B) of *Pleurotus eryngii*: U=units in μmoles per minute

Results

The production of lignin peroxidase activity, by *P. eryngii* over a 30-day culture period was investigated using the experimental ligninolytic conditions developed for *Phanerochaete chrysosporium* (culture conditions A). A peroxidase that oxidizes veratryl alcohol to veratraldehyde was not found. However, extracellular oxidase activity catalysing this reaction without H_2O_2 was detected. A high variability in the production of this enzyme among replicate flasks was found. Aryl-alcohol oxidase activity was detected from 12 day to the end of the cultivation period. The maximum mycelial weight occurred after 10 days and the highest activity level was 4.5 mU ml^{-1}.

Different culture conditions and sampling procedures (culture conditions B) were used in order to get higher aryl-alcohol oxidase activity levels and reproducible results. The evolution of extracellular aryl-alcohol oxidase, mycelial weight, protein, and reducing sugars is shown in Fig. 1.

Aryl-alcohol oxidase was found in crude extracellular and intracellular enzyme preparations and total activities of 15.6 U and 3.0 U l^{-1} of culture were obtained, respectively (containing 13.4 and 71.7 mg of protein l^{-1}, respectively). Samples from both preparations were electrophoresed to determine whether extracellular and intracellular aryl-alcohol oxidases were the same or not and if respective protein bands demonstrated H_2O_2-generating activity using veratryl alcohol as substrate. A single distinct band with identical electrophor-

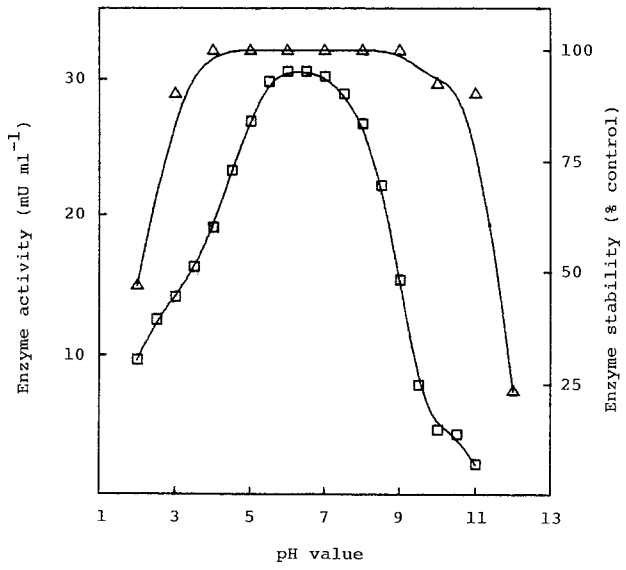

Fig. 2. Effect of pH on the activity (—□—) and stability (—△—) of aryl-alcohol oxidase in 0.1 *M* borate-citrate-phosphate buffer

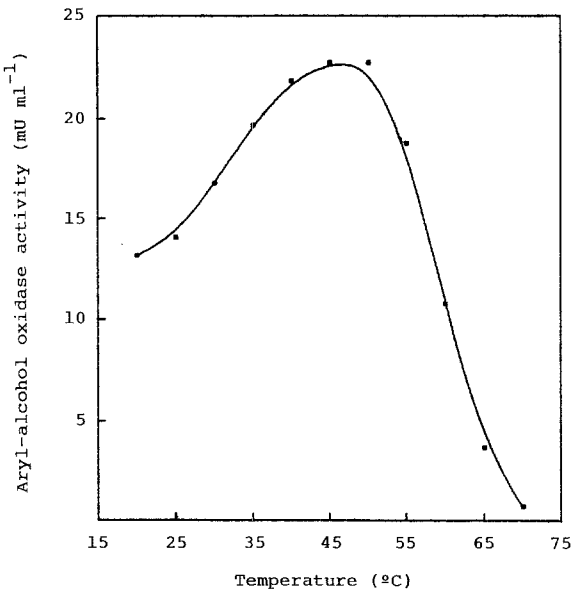

Fig. 3. Effect of temperature on aryl-alcohol oxidase activity

etic mobility was observed with both preparations (Fig. 4B).

Enzyme characteristics in dilutions of the extracellular crude enzyme preparation containing 28 mU ml^{-1} aryl-alcohol oxidase activity were studied. The rate of oxidation of veratryl alcohol was proportional to enzyme concentration and linear for at least 5 min. The optimal pH was 6.0–6.5 and the enzyme was stable from pH 4.0 to 9.0 at room temperature for 24 h (Fig. 2). The highest level of activity was found at 45°–50° C (Fig. 3); activation and deactivation energies of 4.4 and 37.4 Kcal mol^{-1}, respectively, were obtained from Arrhenius plots of data. Thermal stability was determined by incubating the enzyme solution at temperatures ranging from 30° C to 70° C and sampling at fixed intervals over 30 min. The enzyme was stable up to 50° C, had a half-life of 30 min at 60° C and exhibited total inactivation after 10 min at 70° C.

The effect of various cations and enzyme inhibitors on the aryl-alcohol oxidase activity was studied. Oxidation of veratryl alcohol was not affected by the presence of 5.0 m*M* Na^+, K^+, Mn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Sn^{2+} and Hg^{2+}. The reaction was inhibited with 0.5 m*M* Ag^+ and Pb^{2+} by 38% and 63% respectively. Neither 10 m*M* mercaptoethanol, ethylenediaminetetraacetate (EDTA) or CN^- was inhibitory but 10 m*M* NaN_3 inhibited activity by 19%.

A Michaelis-Menten relationship between the veratryl alcohol concentration and the initial velocity of the reaction was found. The Lineweaver-Burk plot yielded a straigt line from which a K_m value of 1.2 m*M* was obtained.

The action of the crude extracellular enzyme was tested against a number of aliphatic and aromatic alcohols. No oxidase activity, assayed as production of H_2O_2, was found with aliphatic alcohols such as methanol, ethanol and butanol. When primary aromatic alcohols were used as substrates, the production of H_2O_2 and the corresponding aldehyde was found (Table 1). However, only coniferaldehyde (and no H_2O_2) was obtained from the unsaturated coniferyl alcohol. Oxidase activity over the secondary alcohol 1-(3,4-dimethoxyphenyl) ethanol was not found. Zymograms from crude extracellular enzymes using benzyl, anisyl or veratryl alcohol as substrates showed the same distinct band corresponding to H_2O_2 production (Fig. 4B). With coniferyl alco-

Table 1. Production of aldehydes and H_2O_2 by aryl-alcohol oxidase from several aromatic alcohols

Alcohol	λ (nm)	ε (1/*M* cm)	Aldehyde (mU/ml)	H_2O_2 (mU/ml)
Benzyl alcohol	240	9600	5.3	3.7
Anisyl alcohol	290	15000	27.7	22.2
Veratryl alcohol	310	9300	14.2	14.2
Coniferyl alcohol	343	56800	0.3	0.0
1-(3,4-Dimethoxyphenyl) ethanol	300	8340	0.0	0.0

λ = wavelength; ε = molar extinction coefficient; U = units in μmoles per minute

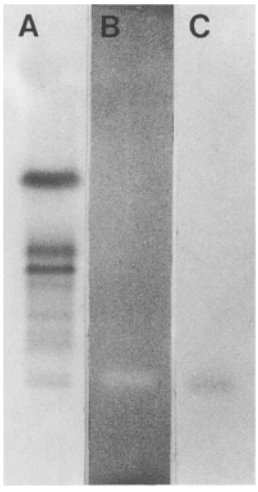

Fig. 4. Gel electrophoresis of crude extracellular enzyme of *P. eryngii* **(A)**, and zymograms of aryl-alcohol oxidase carried out using veratryl alcohol **(B)** and coniferyl alcohol **(C)** as substrates

hol, a pale yellow band due to the coniferaldehide produced was developed with the same electrophoretic mobility found with primary α-alcohols (Fig. 4C). Gels stained for protein (Fig. 4A) showed a single band corresponding to the aryl-alcohol oxidase activity.

Discussion

Lignin peroxidases have been reported in species from the basidiomycetous families Corticiaceae, *P. chrysosporium*, *Phlebia radiata* Fr. (Niku-Paavola et al. 1988) and *Merulius tremellosus* Schrad. (Biswas-Hawkes et al. 1987), and Polyporaceae, *Trametes versicolor* (L.) Lloyd (Dodson et al. 1987). These enzymes have also been found in the ascomycete *Chrysonilia sitophila* (Mont.) von Arx (=*Neurospora sitophila* Shear and B. O. Dodge) (Durán et al. 1987) and in the actinomycete *Streptomyces viridosporus* Parke et al. (Ramachandra et al. 1988). Lignin peroxidases however seem to be absent in several fungi with a high lignin-degrading capacity, including *Pleurotus eryngii*. The distribution and significance of these enzymes in lignin biodegradation is not clear. In this respect, Forrester et al. (1988) recently demonstrated that Mn^{3+} produced by a Mn-dependent peroxidase from *Lentinula edodes* (Berk.) Pegler, is able to oxidize veratryl alcohol as well as lignin model compounds and lignin in the presence of chelating agents.

Aryl-alcohol oxidase (EC 3.1.1.7) from *P. eryngii* oxidizes primary aromatic α-alcohols to the corresponding aldehyde and H_2O_2 (Table 1), catalysing the transfer of two hydrogens from alcohols to molecular oxygen. The same substrate specificity was reported for an enzyme from culture filtrate of *T. versicolor* (Farmer et al. 1960). Enzymes from both sources oxidize anisyl alcohol as a preferred substrate and present a similar relative oxidation rate on coniferyl alcohol (in relation to anisyl alcohol). However, relative oxidation rates of the enzyme from *P. eryngii* over veratryl and benzyl alcohols were respectively 9.1 and 5.3 times higher than those reported for the *T. versicolor* aryl-alcohol oxidase. The optimal pH of both enzymes was the same, although the *P. eryngii* oxidase gave higher activity levels at pH values above the optimum. The results of temperature stability showed that the *P. eryngii* oxidase is more stable than that from *T. versicolor*.

An aryl-alcohol oxidase that oxidizes α,β-unsaturated alcohols (e.g. coniferyl alcohol) was found in culture filtrates of *Fusarium solani* (Mart.) Sacc (Iwahara et al. 1980). Furthermore, this enzyme oxidizes the non-etherified side chain of dehydroconiferyl alcohol and various lignin preparations but has no activity over primary α-alcohols, thus differing from the aryl-alcohol oxidase of *P. eryngii*.

Aryl-alcohol oxidase activity found in *T. versicolor* and *P. eryngii* has been also detected among cell-wall enzymes of *P. sajor-caju* Sing. (Fukuzumi 1987) and in the culture filtrate of *P. ostreatus* (Jacquin) Kummer, *Bjerkandera adusta* (Willd) Karst. and *Fomes lignosus* (Klotzsch) Bres. (Waldner et al. 1988). Studies on enzymatic mechanisms for lignin degradation in these fungi are scarce but, if enzymes such as lignin peroxidases and Mn-dependent peroxidases are involved, aryl-alcohol oxidase would play an important role due to its H_2O_2-producing capacity and affinity for aromatic substrates related to lignin.

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