Physiological roles and metabolism of fungal aryl alcohols

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Ed de Jong

Physiological roles and metabolism of fungal aryl alcohols

Proefschrift
ter verkrijging van de graad van doctor
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Cover: Photograph of a 3X magnified under-surface of the maze fungus, *Daedalea quercina*. This saprophytic, wood-degrading fungus grows on deciduous trees and is especially common on oak trees and stumps. The Dutch name for this fungus is "Doolhofzwam".

Stellingen

1. Sommige gechloreerde aromaten zijn niet zeldzaam in het milieu, en omdat ze door schimmels produceerd worden, zijn het zeker geen xenobiotica.

   Dit proefschrift

2. De door schimmels geproduceerde gechloreerde aromaten zijn potentiële bouwstenen voor natuurlijke dioxines.

   Dit proefschrift

3. Het definiëren van groei van schimmels op veratryl alcohol enkel en alleen op grond van de radiale groeisnelheid is twijfelachtig.

   Collett O (1992) *Material und Organismen* 27:67-77

4. In tegenstelling tot wat Khan en Overend beweren, kunnen laccases ook geel van kleur zijn.

   Khan AW, Overend RP (1990) *FEMS Microbiol Lett* 66:215-220
   Mohoroshi N, Wariishi H, Muraiso C, Nagai T, Haraguchi T (1987) *Mokuzai Gakkaishi* 33:218-225
   Sariaslani FS (1989) *Crit Rev Biotechnol* 9:171-257

5. De conclusie van Shimada en collega's, dat lignine peroxidase een rol speelt bij de biosynthese van veratryl alcohol, wordt onvoldoende door experimentele gegevens onderbouwd.

   Shimada M, Nakatsubo F, Kirk TK, Higuchi T (1981) *Arch Microbiol* 129:321-324
   Shimada M, Ohta A, Kurosaka H, Hattori T, Higuchi T, Takahashi M (1989) *Am Chem Soc Symp Ser* 399:412-425

6. Wanneer maar twee parameters gemeten zijn, moet een tijdschrift niet accepteren dat deze gegevens in een driedimensionaal staafdiagram weergegeven worden.

   Roy B, Ackermann H-W, Pandian S, Picard G, Goulet J (1993) *Appl Environ Microbiol* 59:2914-2917
   Thomas AD, Booth IR (1992) *J Gen Microbiol* 138:1829-1835

7. Er zit een boel rommel in de Dommel.

Labuitje Industriële Microbiologie 1989

8. Blijkens de verkeerssituatie in Wageningen is het afregelen van stoplichten een moeilijk vak.

9. De huidige inspraakprocedures bij een stadsvernieuwingsproject doen de kwaliteit van het project in de regel geen goed en frustreren de interesse bij de rest van de bevolking om in de toekomst mee te denken.

Stadsverniewingsproject Heerenstraat, Wageningen

10. Promoveren doe je niet vanwege een betere baan en hogere salariëring in de toekomst, maar vooral omdat het doen van wetenschappelijk onderzoek je interesseert.

Intermediair 22 oktober 1993

11. Ondanks de toenemende vergrijzing is volledige werkgelegenheid alleen nog te bereiken door vergaande arbeidstijdverkorting.

Stellingen behorende bij het proefschrift "Physiological roles and metabolism of fungal aryl alcohols" van Ed de Jong.

Wageningen, 1 december 1993

Voor Judith
Aan mijn ouders

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

LIGNIN BIODEGRADATION BY WHITE-ROT FUNGI

The major structural elements of woody tissue are cellulose, hemicellulose and lignin. Wood and other vascular tissues contain generally 20-30% lignin. Lignin gives the plant strength, it serves as a barrier against microbial attack and it acts as a water impermeable seal across cell walls of the xylem tissue. The compound originates from a random polymerization reaction of *p*-coumaryl alcohol radicals and their methoxy substituted counterparts (Dean and Eriksson 1992). These aspecific reactions create a high molecular weight, heterogenous, three-dimensional, optical inactive molecule containing both ether and carbon-carbon linkages. This structure imposes unusual restrictions on biodegradative systems responsible for the initial attack. The systems must be extracellular and non-specific. Lignin, the most abundant aromatic polymer on earth, is not degraded by hydrolytic enzymes in contrast to cellulose and hemicellulose. The most rapid and extensive degradation of lignin described to date is caused by the white-rot fungi (Kirk and Farrell 1987, Buswell 1992). Under ligninolytic conditions these fungi produce several extracellular enzymes (peroxidases and oxidases) and secondary metabolites. Despite the fact that native lignin is potentially capable of providing enough energy to sustain fungal growth, white-rot fungi can not use lignin as sole source of carbon and energy. Thus, an easily metabolizable cosubstrate such as cellulose or hemicellulose, must be available. Most white-rot fungi degrade lignin in a secondary metabolic (idiophasic) process, which starts only after nitrogen, carbon or sulphur are limiting. For a few fungi, e.g. *Lentinula edodes* (shiitake) and *Pleurotus ostreatus* (oyster mushroom) , nitrogen limitation is not a

prerequisite for lignin degradation. Presently, it is unclear whether such fungi exhibit a clear separation between primary and secondary metabolism or if lignin biodegradation is regulated differently in these organisms (Kirk and Farrell 1987). Other physiological parameters that in general stimulate lignin biodegradation are a high oxygen tension, static culture conditions and a pH between 3.5 and 5.5 (Kirk and Farrell 1987, Buswell 1992). The fact that the initial degradation of lignin, catalyzed by the extracellular enzymes, is non-specific and results in a potpourri of random reactions made Kirk and Farrell (1987) describe this process as "enzymatic combustion". This unique enzyme system, capable of degrading lignin, gives white-rot fungi several distinct advantages for biotechnological purposes above other microbes. Several potential applications have been suggested for white-rot fungi, the most important ones will be discussed.

POTENTIAL APPLICATIONS OF WHITE-ROT FUNGI

Biopulping of wood. Production of high quality pulps in high yields with a selective removal of the lignin moiety, and without substantial destruction of the cellulose and hemicellulose, is a major goal. At present two main processes are used to manufacture such paper pulps. In the kraft process the wood chips are cooked at high temperature in an alkaline environment while in the sulfite process the addition of sodium sulfite is used. Both processes use immense amounts of energy, huge quantities of chemicals which are not always regenerable, and create vast amounts of toxic effluents. Also in the production of thermomechanical pulps substantial amounts of energy are required. The use of white-rot fungi or their enzymes offer many potential advantages in reducing the above mentioned drawbacks of the current processes (Boominathan and Reddy 1992). The main disadvantages of the fungal process are the facts that the biological delignification step is too slow and not selective enough to compete with conventional pulping methods.

Recently, promising biomechanical pulping results have been obtained with the white-rot fungus *Ceriporiopsis subvermispora*. Fungal pretreatment of both soft- and hardwood chips resulted in energy savings of approximately 40% and in a considerable increase in both tear-strength and burst index compared to untreated controls. The fungal treatment causes only slight weight losses (5%) and a limited decrease of optical properties. The process has been patented and current research focuses on the physico-chemical basis of this biopulping efficiency, the decrease of the fungal incubation time and how the process can be scaled up economically (Blanchette et al. 1992, Aktar et al. 1992, Aktar et al. 1993).

Biobleaching of paper pulp. The kraft pulping process leaves approximately 10% of the lignin in the pulp. Chlorination followed by alkaline extraction is a very efficient way to degrade the remaining lignin, but an important drawback is the production of a waste water containing toxic chloroaromatics. Several laboratories have focused on replacing the chlorination step by biological bleaching with white-rot fungi (Boominathan and Reddy 1992, Kantelinen et al. 1993, Onysko 1993). *Trametes versicolor* can biobleach kraft pulp in the absence of physical hyphal-fibre contact and with no loss of paper strength properties (Kirkpatrick et al. 1990, Archibald 1992). This indicates that time needed for biobleaching can be substantially reduced by using 'concentrated' extracellular fluid instead of the whole fungus (Archibald 1992). However, this process still awaits commercialization. Rather unexpected was the use of xylanases to improve the biobleachability of kraft pulps. These enzymes have now successfully been used both in the laboratory and on an industrial scale (Viikari et al. 1992). Recently, xylanases have been tested that are active at pH 9 at 65 °C for two hours (Högman et al. 1992). Future research should be directed to preparations of cellulase-free xylanases with better specificities that are active in the high alkalinity and temperature of kraft cooking.

Waste water treatments. The dark coloured waste waters of the pulp and paper industries contain high molecular weight, modified and often chlorinated lignins. Conventional aerated waste water treatments reduce the biological and chemical oxygen demands but are not very effective in removing colour and high molecular weight chlorolignins (Boominathan and Reddy 1992). Both colour and chloroaromatics concentration are readily removed by several white-rot fungi, including *Phlebia radiata* (Lankinen et al. 1991), *Phanerochaete chrysosporium* (Fukui et al. 1992) and *Trametes versicolor* (Roy-Arcand and Archibald 1991, Bourbonnais and Paice 1992). The MYCOPOR (mycelial colour removal) process, using *Phanerochaete chrysosporium* and a continuous trickling filter system, eliminates both colour and chlorolignins efficiently, and is now to run at a pilot plant scale at an Australian pulp mill (Jaklin-Farcher et al. 1992). Surprisingly, Bergbauer and Eggert (1992) recently reported that chlorine-free bleaching effluent had a higher toxicity and was more difficult to degrade by *T. versicolor* and *P. chrysosporium* than two chlorinated effluents. These results demonstrate that a simple replacement of chlorine bleaching with other methods does not really solve the problem of the toxic and recalcitrant waste waters of the pulp and paper industries.

Improved digestibility of lignocellulosic materials. White-rot fungi can potentially be used to improve the digestibility of lignocellulosic materials (Hatakka et al. 1989). Lignification limits the rumen digestibility of polysaccharides and even a partial delignification can give major increases in animal productivity (Reid 1989). In the rain forests of southern Chile this process occurs spontaneously resulting in white decomposed wood. This ecosystem is called "Palo podrido" and it is characterized by a selective degradation of lignin by fungi, such as *Ganoderma* and *Armillaria* spp., and bacteria. The cellulose is conserved and can be used as feed for cattle (Ríos and Eyzaguirre 1992, Bechtold et al. 1993). Lignocellulosic materials can also be used to produce fungal food protein (Leonowicz et al. 1991). The benefits of both processes can be combined. Several white-rot fungi (e.g. *Lentinula edodes*, shiitake and *Pleurotus ostreatus*, oyster mushroom) produce highly appreciated, edible fruiting bodies, while in the same time the digestibility of the lignocellulosic growth substrate is increased (Rajarathnam et al. 1987, Moyson and Verachtert 1991). The feasibility of the process can be increased by combining the improved digestibility or production of edible fruiting bodies with the extraction of ligninolytic enzymes (Hatakka et al. 1990, Mishra et al. 1990). The potential uses of white-rot fungi to improve nutritional quality of crops residues are hampered because the results are both very fungal species- and crop-specific (Zadražil 1985, Jung et al. 1992). The major challenge is now to adapt and design simple, inexpensive equipment for the upgrading of lignocellulosic materials and to test the process in actual operation on the farm.

Fungal production of renewable raw materials from lignocellulose. The uncertain future petroleum supplies have focused the attention on alternative raw materials as petroleum substituents, particulary for carbon-based chemicals. Wood is composed of 70-80% polymeric carbohydrates and 20-30% of lignin. Carbohydrates occur in close association with lignin in the plant cell walls and are only partly available for hydrolysis with microbial enzymes. Lignocellulosic materials treated by white-rot fungi are better substrates for this enzymic saccharification. White-rot fungi can also potentially be used for the production of monomeric aromatic compounds and for the solubilization of coal and related substances to chemicals and fuels (Boominatan and Reddy 1992). All this research is still in a very preliminary stadium and much needs to be done before white-rot fungi are employed in biotechnological processes to produce sugars and chemicals.

Degradation of xenobiotic compounds. The random and non-specific nature of the lignin-degrading system also attacks other compounds containing an aromatic structure, such as many xenobiotic compounds. It has been shown that the lignin-

degrading system of white-rot fungi is involved in the initial oxidation of a wide range of pollutants including azo dyes, chlorophenols, polycyclic aromatic hydrocarbons (PAH) and dioxins (Aust 1990, Field et al. 1993). The fact that the ligninolytic system is active extracellularly makes white-rot fungi far better candidates for the bioremediation of soil containing highly apolar pollutants compared with non-ligninolytic microorganisms. Non-ligninolytic organisms use intracellular processes to degrade the aromatic pollutants, so the limited bioavailability (dissolution and diffusion) is a key factor in their slow biodegradation rate (Volkering et al. 1992). Xenobiotics bound to humic compounds are mineralized by *Phanerochaete chrysosporium* at about the same rate as the organic carbon from the humic compounds, again demonstrating the aselective nature of the ligninolytic system (Haider and Martin 1988). The first promising results of using white-rot fungi for *in situ* bioremediation of contaminated soils and wood have appeared (Lamar and Dietrich 1990, Lamar and Dietrich 1992, Morgan et al. 1993).

Azo dyes are the largest class of commercially produced dyes and are used for dyeing and printing of natural and synthetic fibres, leather, furs and paper. The compounds often have mutagenic, carcinogenic and toxic potential, also to men. About 10-15% of the dye is lost in the effluent of textile mills and of dye-stuff factories and the commonly used waste water treatments do not remove these dyes sufficiently. *Phanerochaete chrysosporium* was able to oxidize and mineralize many sulfonated and otherwise substituted azo dyes at a high rate (Pasti-Grigsby et al. 1992, Spadaro et al. 1992, Paszczynski et al. 1992). These results show that in addition to waste waters of the pulp and paper industry white-rot fungi can also be used for the clean-up of dye industry effluents.

Biosynthesis of flavours. At the moment a great interest exists in aromas of biological origin, because consumers prefer such compounds over synthetic aromas. The production of flavours, e.g. terpenes, lactones and aromatics, is ubiquitous among micro-organisms, especially fungi (Janssens et al. 1992). Also several white-rot fungi produce odorous components (Gallois et al. 1990, Janssens et al. 1992). Especially the production of aromatic compounds is noteworthy. In chapter 2 the important roles of the *de novo* biosynthesized aryl alcohols in the physiology of white-rot fungi will be discussed. Several fungal strains with a high flavour production have been described. *Bjerkandera adusta* biosynthesizes veratryl alcohol, a compound with a milky, vanilla pungent odour assessment (Berger et al. 1986). Benzyl alcohol is produced by *Phlebia radiata* and has initially a roasted but later a harsh smell (Gross et al. 1989). The main components of the smell of *Ischnoderma benzoinum* are benzaldehyde (almond odour) and anisaldehyde (anise-like odour) (Berger et al. 1987). Veratraldehyde has

a milky, chocolate aroma and is produced by *Dichomitus squalens* (Gallois et al. 1990). The production of flavours by white-rot fungi has considerable potential (Gallois et al. 1990). The yields are very much dependent on the growth conditions, and are likely to be improved in future.

OUTLINE OF THIS THESIS

The lignin biodegradation research at the Wageningen Agricultural University has been initiated in 1987 at the Division of Forest Techniques and Wood Science, Department of Forestry to investigate the possibilities of white-rot fungi and/or their extracellular ligninolytic enzymes for the biopulping of wood. The research was continued in a joint program with the ATO, Agrotechnological Research Institute in Wageningen and financed by the Dutch government to investigate the potential of hemp (*Cannabis sativa*) as source for paper pulp production. The fundamental aspects of lignin biodegradation and the interactions between fungi and aryl alcohols presented in this thesis have been studied at the Division of Industrial Microbiology in cooperation with forementioned groups and with others.

Only a few fungal species that cause white-rot have been thoroughly characterized. Thus it was decided to start the research by isolating and characterizing new strains having better properties than the then known strains. The isolation and screening for fungi with a high peroxidative activity is described in chapter 3. One of the newly isolated and most active strains, *Bjerkandera* sp. BOS55, isolated by F.P. de Vries at the ATO, was studied in more detail. In addition to lignin peroxidase and manganese peroxidase, this organism produced a third extracellular enzyme, manganese independent peroxidase (chapter 4). The strain also produced a wide range of secondary metabolites. In addition to veratryl alcohol, (chlorinated) anisyl alcohols and their corresponding aldehydes were produced (chapter 5). The chlorinated anisyl alcohols have an important physiological role as substrates for aryl alcohol oxidase, generating extracellular H_2O_2 for the peroxidases (chapter 6). Chapter 7 describes the production of chlorinated aromatics by *Bjerkandera* spp. in the natural environment. Astonishing high concentrations of the chlorinated aromatics were produced in the environment by many other common wood and forest-litter degrading fungi.

The production of physiological important secondary metabolites by white-rot fungi and their subsequent metabolization have been dealt with more extensively. Especially, the important functions of aryl alcohols in the physiology of white-rot fungi have been addressed (chapter 2). Secondary metabolites are not only produced but

are also readily metabolized by fungi. *Penicillium simplicissimum*, not a white-rot fungus, was studied since it used veratryl alcohol as sole source of carbon and energy (chapter 8). This organism also produces a very interesting intracellular vanillyl alcohol oxidase. This enzyme has characteristics completely different from the extracellular aryl alcohol oxidases of white-rot fungi, although both enzymes are FAD-dependent flavoproteins (chapter 9). Finally, in chapter 10 concluding remarks are made on the ligninolytic system of white-rot fungi, the potential of these fungi for biotechnological applications and the implications of chlorinated aromatic compounds produced in natural environments.

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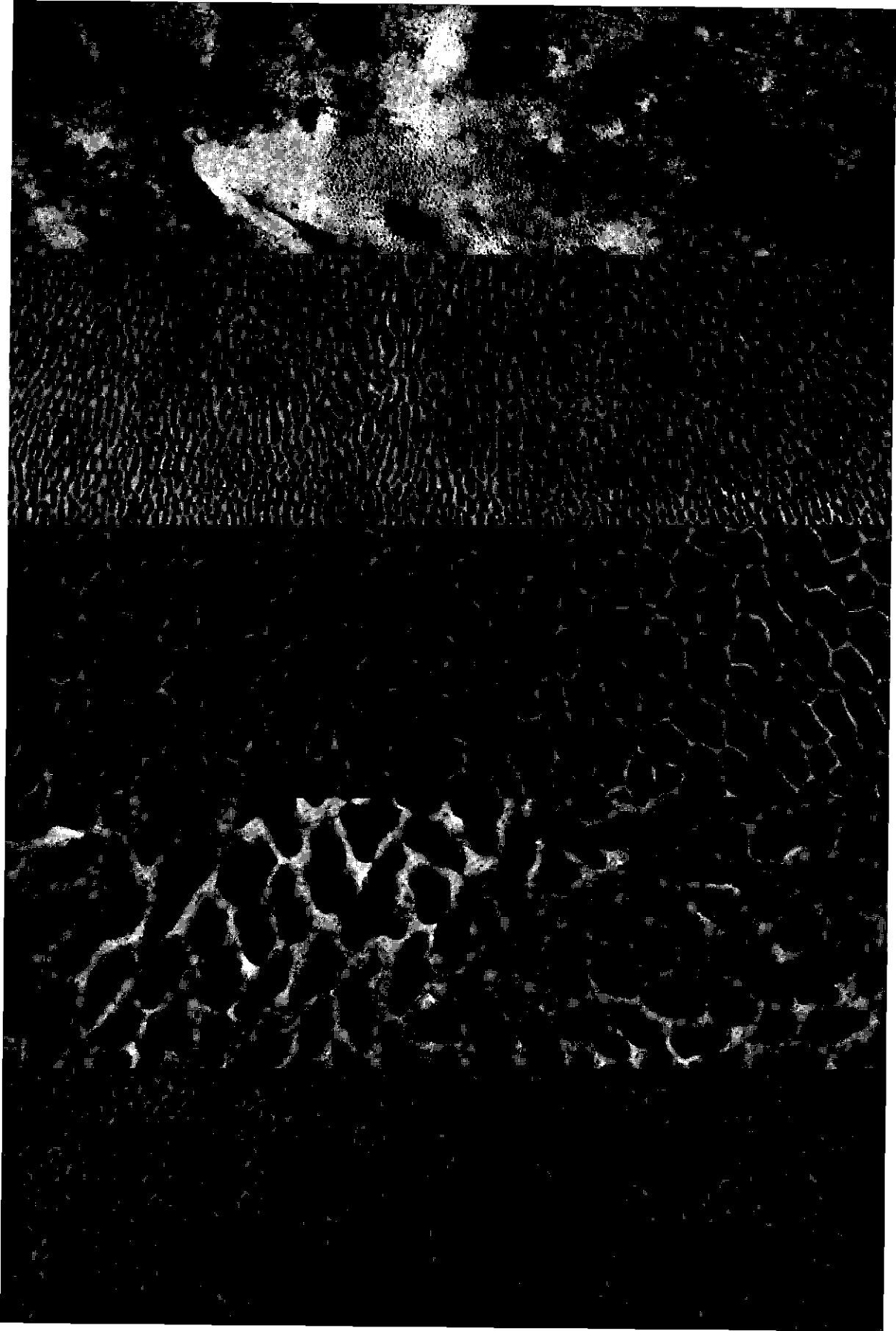
Many wood-degrading fungi
have pores
beneath their caps

They are named polypores and they often
grow in brackets

From top to bottom these photographs show
3X magnified
under-surfaces of hymenium,
which is a fertile layer
containing or carrying
the spores,
of

Bjerkandera adusta,
Trametes gibbosa,
Hexagonia nitida - an Mediterranean sp,
an undetermined polypore from
Ivory Coast
and
Daedaleopsis confragosa



CHAPTER 2

ARYL ALCOHOLS IN THE PHYSIOLOGY OF LIGNINOLYTIC FUNGI

Ed de Jong, Jim A. Field and Jan A.M. de Bont

SUMMARY

White-rot fungi have a versatile machinery of enzymes which work in harmony with secondary aryl alcohol metabolites to degrade the recalcitrant, aromatic biopolymer lignin. This review will focus on the important physiological roles of aryl (veratryl, anisyl and chlorinated anisyl) alcohols in the ligninolytic enzyme system. Their functions include stabilization of lignin peroxidase, charge-transfer reactions and as substrate for oxidases generating extracellular H_2O_2. The aryl alcohol / aldehyde couple is well protected against degradation by the fungi's extracellular ligninolytic enzymes and their concentration in the extracellular fluid is highly regulated by intracellular enzymes.

FEMS Microbiology Reviews (1994) In press

CONTENTS

INTRODUCTION

Lignin, the most abundant aromatic polymer on earth, is found in all higher plants. The compound gives the plant strength, it serves as a barrier against microbial attack and it acts as a water impermeable seal across the cell walls in the xylem tissue [1]. The biosynthesis of lignin arises from a phenol oxidase catalyzed coupling reaction of *p*-coumaryl alcohol and it's methoxy substituted counterparts (Figure 1). The structural features of this heterogenous polymer impose unusual restrictions on its biodegradability. The initial attack must be extracellular, nonspecific and nonhydrolytic.

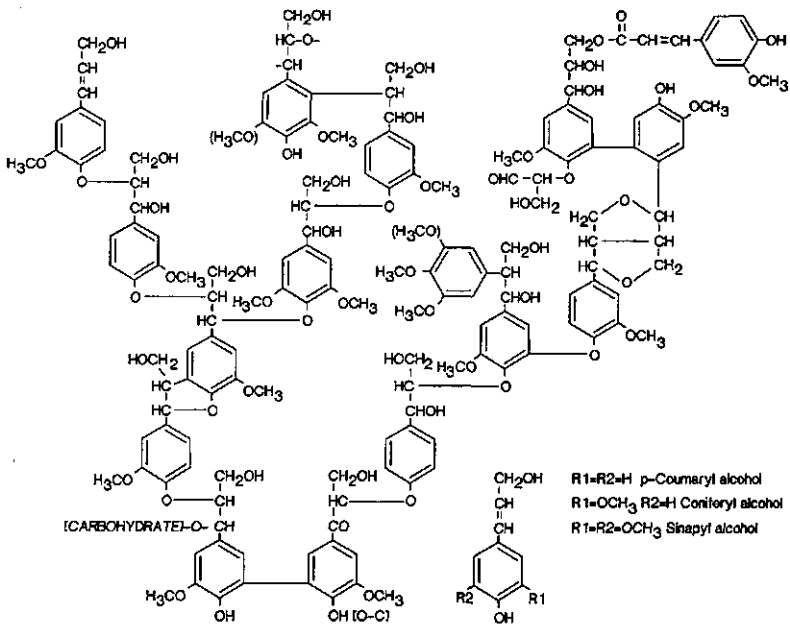


Figure 1. Schematic structural formula for lignin, adapted from Adler [2]. The three precursor alcohols of lignin are shown at the lower right. One electron oxidation of the precursors and subsequent polymerization reactions produces lignin.

White-rot fungi, belonging to the basidiomycetes, are responsible for the fastest and most extensive degradation of lignin [3]. The white-rot fungus *Phanerochaete chrysosporium* (anamorph: *Sporotrichum pulverulentum*) has been used extensively as a model organism to study the physiological requirements and enzymes required for lignin biodegradation. Lignin cannot be degraded as a sole source of carbon and energy, consequently lignin degradation only occurs when other readily available cosubstrates (e.g. hemicellulose, cellulose) are available. Many environmental factors

are known to influence the degradation rate of lignin by this fungus including oxygen tension, culture agitation, choice of buffer, mineral concentrations (especially Ca and Mn). Lignin degradation by *P. chrysosporium* occurs during secondary (idiophasic) metabolism, triggered by carbon, nitrogen or sulfur limitation [3-5]. The extracellular ligninolytic system of *P. chrysosporium* consists of lignin peroxidase (LiP) [6,7], manganese peroxidase (MnP) [8], H_2O_2-producing enzymes including glyoxal oxidase (GLYOX) [9]) and the secondary metabolite veratryl alcohol synthesized *de novo* [10] (Figure 2).

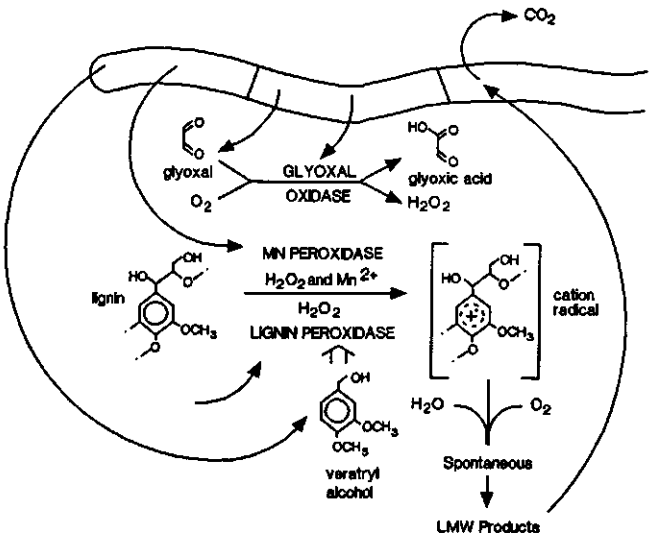


Figure 2. The ligninolytic system of the white-rot fungus *Phanerochaete chrysosporium.* (adapted from Kersten et al. [11]).

Research on lignin biodegradation has accelerated tremendously during the last 15 years in response to potential applications in several areas. The use of white-rot fungi for biopulping and bleaching has been investigated [12,13]. Also the potential of white-rot fungi and their enzymes to improve the digestibility of lignocellulosic feed has been studied [14,15]. Lignin degradation functions in a non-specific manner, consequently other compounds that have an aromatic structure are also highly susceptible for attack by white-rot fungi and their ligninolytic enzymes. Therefore white-rot fungi can potentially be applied to clean-up toxic waste sites [16-18].

In recent years several reviews have appeared dealing with lignin biodegradation in general [3,4,19] and selected aspects, including the mechanism of lignin model compound degradation [20,21], production of lignin peroxidase [6], genetic aspects of ligninolytic enzymes [22], ultrastructural localization of lignin

degradation and enzymes [23,24] and biotechnological applications [25].

An intriguing aspect of the ligninolytic complex of white-rot fungi is the *de novo* biosynthesis of aromatic metabolites, including veratryl, anisyl and chlorinated anisyl alcohols. The most enigmatic attribute of these secondary metabolites is their purpose. Fungi frequently expend a considerable amount of their available energy to biosynthesize these compounds, yet the selective advantage(s) for doing so are not obvious [26]. Moreover, it seems contradictory for white-rot fungi to biosynthesize aromatic compounds like veratryl alcohol when simultaneously huge amounts of analogous aromatic metabolites are formed from lignin biodegradation [27-29]. The objectives of this paper are to give an overview of the aryl alcohol metabolite biosynthesis and their physiological functions in the ligninolytic complex of white-rot fungi. In addition, we will evaluate which enzymes ligninolytic fungi possess to regulate the amount of monomeric aromatic metabolites in their extracellular environment.

BIOSYNTHETIC ROUTES FOR SUBSTITUTED ARYL ALCOHOLS

Both brown- and white-rot fungi synthesize a wide range of aromatic compounds with a methoxygroup at the *para*-position (Table 1). It is anticipated that these kind of compounds are produced via the shikimate pathway [52]. However, in most cases neither intermediates nor enzymes have been characterized nor have labelling studies been performed to confirm that the shikimate pathway is involved. Several white-rot fungi produce *de novo* veratryl alcohol (Table 1). *P. chrysosporium* biosynthesizes this compound via *L*-phenylalanine, 3,4-dimethoxycinnamyl alcohol and veratrylglycerol (Figure 3) [53]. The enzyme phenylalanine ammonia-lyase is common among basidiomycetous fungi [54,55]. Its action results in the recycling of nitrogen, often the growth limiting nutrient [3,56]. What enzymes are involved in the hydroxylation reactions is not known. Also the final steps in the biosynthesis, the oxidation of 3,4-dimethoxycinnamyl alcohol to veratrylglycerol and the subsequent Cα-Cβ bond cleavage yielding veratraldehyde and glycolaldehyde, are not well characterized. Another white-rot fungus, *T. versicolor* also metabolized added 3,4-dimethoxycinnamyl alcohol via veratrylglycerol and veratraldehyde [57]. *In vitro* these conversions are catalyzed by lignin peroxidase [56]. However, the addition of catalase to idiophasic cultures of *P. chrysosporium* did not halt veratryl alcohol biosynthesis [58], and a lignin peroxidase negative mutant was still capable of converting exogenous 3,4-dimethoxycinnamate to veratryl alcohol [56]. This suggests that lignin peroxidase is not needed in the biosynthesis route.

Table 1. The production of aryl alcohols/aldehydes by basidiomycetes.

Fungus *	Type †	VA ‡	AA	BA	CA	DA	References
Armillaria ostoyae	WR	○		○,●			[30]
Armillaria mellea	WR	○,●	●	○,●			[30]
Bjerkandera adusta	WR	○,●	○,●	○,●	○,●	○,●	[31-33]
Bjerkandera sp. BOS55	WR	○,●	○,●		○,●	○,●	[31,32]
Camarophyllus virgineus			●				[34]
Coriolopsis occidentalis	WR	○					[34]
Daedalea juniperina			●				[36]
Dichomitus squalens	WR	●	●	○			[30]
Gloeophyllum odoratum (syn. *Osmoporus odoratus*, *Trametes odorata*)	BR		●				[37,38]
Hypholoma capnoides						○,●	[32]
Hypholoma fasciculare	WR					○,●	[32]
Hypholoma sublateritium						○,●	[32]
Ischnoderma benzoinum	WR	●	○,●	●			[39]
Lentinula edodes	WR	○					[29,30]
Lepista diemii					●		[40]
Oudemansiella mucida	WR					○,●	[32]
Phanerochaete chrysosporium	WR	○,●					[10,31,41]
Phlebia radiata	WR	○		○,●			[41,42,43]
Pholiota squarrosa						●	[32]
Pleurotus ostreatus	WR		●				[30, this work]
Pycnoporus cinnabarinus	WR	○					[3,44]
Ramaria sp. 158.	WR				●	●	[32,45]
Trametes gibbosa	WR	○,●					[46]
Trametes hirsuta	WR	●					[46]
Trametes suaveolens			●				[47]
Trametes versicolor (syn. *Coriolus versicolor*)	WR	○			●		[32,45,48]

Fungus *	Type †	VA ‡	AA	BA	CA	DA	References
Trichaptum pergamenum (syn. *Hirschioporus pergamenus*)	WR	O,●		●			[30]

* The production of only unsubstituted phenyl alcohols has been reported for several other basidiomycetes, including *Heterobasidion annosum* (syn. *Fomes annosus*), *Lenzitus betulina*, *Phellinus* spp., *Polyporus* spp., *Poria* spp., *Nigroporus durus* (syn. *Polyporus durus*) and *Tyromyces sambuceus* [30,33,49,50,51]. The structure of the secondary metabolites is shown in Fig. 7.

† Fungi who give white-rot (WR) or brown-rot (BR) decay of wood.

‡ The production of veratryl (VA), anisyl (AA), benzyl (BA), 3-chloro-anisyl (CA) and 3,5-dichloro-anisyl alcohol (O) and aldehyde (●) by fungi.

Many white-rot fungi also produce anisyl alcohol and aldehyde as well as their chlorinated derivatives (Table 1). However, the route by which they are biosynthesized by basidiomycetes is not known and should be elucidated by further research.

The production of secondary metabolites is very much dependent on the strain, the growth substrate and the growth conditions [30]. Consequently, the absence of a certain secondary metabolite under any given culture condition does not exclude its production by the fungus under other conditions.

Methylation of phenolic metabolites. Many secondary metabolites contain methoxy groups (Table 1), indicating the presence of a methylating system in white-rot fungi. Methylation is also a common reaction in the catabolism of aromatic compounds by white-rot fungi. When grown on lignocellulose as substrate, lignin degradation metabolites, including vanillate [27,29] and syringate [59], are methylated. Subsequent reduction of veratrate contributes significantly to the veratryl alcohol titer (Figure 3), parallel to *de novo* biosynthesis [29]. It has been suggested that methylation of phenolic compounds prevents polymerization reactions by phenol oxidases and detoxifies the culture broth. Recently, a *S*-adenosyl methionine-dependent *O*-methyltransferase was purified from the white-rot fungus *P. chrysosporium* and characterized. This enzyme catalyzed the *para*-specific methylation of vanillate and syringate [60]. The enzyme has no activity with ferulate, isoferulate or caffeate, suggesting that it is not involved in the proposed biosynthesis route of veratryl alcohol (Figure 3) [53].

The brown-rot fungi *Lentinus lepideus* [56,61,62] and *Piptoporus betulinus* (syn.

Polyporus betulinus) [30] only produce methylated aromatic acids e.g. methyl anisate. Methyl *p*-methoxycinnamate was produced when the brown-rot fungus *L. lepideus* was incubated with methyl *p*-coumarate and [$^{14}CH_3$]-methionine. The [$^{14}CH_3$]-methionine lable was found both in the methyl and in the methoxy group [61]. From this organism two distinct *S*-adenosyl methionine-dependent *O*-methyltransferases were partly purified, one catalyzing the formation of methyl esters from free aromatic acids while the other catalyzes the *para*-specific methylation of these methyl esters [63,64].

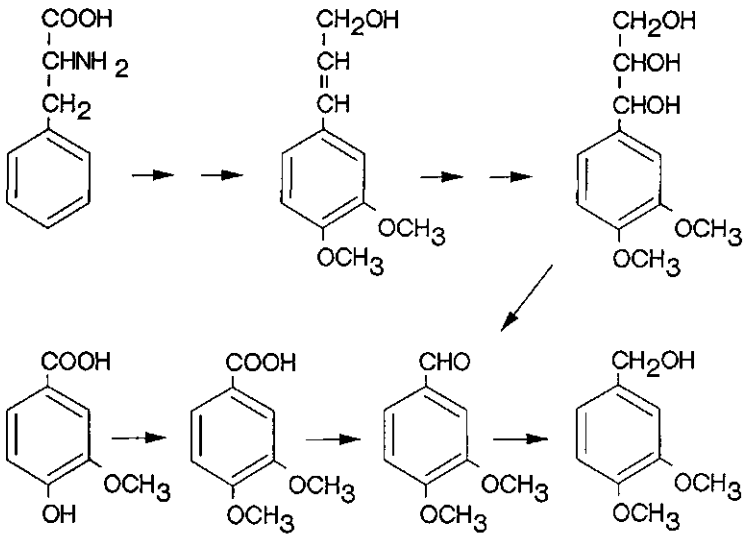


Figure 3. The secondary metabolite veratryl alcohol originates from *de novo* biosynthesis via phenylalanine, veratrylglycerol and veratraldehyde or from the lignin biodegradation intermediate vanillate, via methylation and subsequent reduction. (adapted from [29,53]).

Another methyl donor used by basidiomycetes in the biosynthesis of methoxy groups and esters is chloromethane (CH_3Cl) which has a primary role in the methylation of aromatic compounds, such as acids or phenols [65]. Hymenochaetaceae, a widely distributed family of wood-rotting bracket fungi (including *Phellinus* spp.) release significant amounts of chloromethane (CH_3Cl) in the environment [66,67]. Also several edible mushrooms from the orders of Boletales and Agaricales (*Boletus edulis* and *Agaricus bisporus*) produce CH_3Cl [68]. The methyl group of chloromethane is also derived through a *S*-adenosyl methionine transferase [69]. At least two distinct chloromethane-utilizing systems occur in *Phellinus pomaceus*. One system is involved in methylating the carboxylic acids and the other system methylates the phenolic groups [65]. Harper and colleagues [43] showed that CH_3Cl could serve as a methyl donor in the biosynthesis of veratryl alcohol. Fungi belonging to families of Coriolaceae

and Polyporaceae (*P. chrysosporium*, *T. versicolor* and *P. radiata*) do not emit detectable amounts of CH_3Cl, but when they are grown in the presence of C^2H_3Cl high levels of C^2H_3 were incorporated both in the 3- and 4-*O*-methyl groups of veratryl alcohol. However, the addition of CH_3Cl to cultures of *P. chrysosporium* did not result in increased levels of veratryl alcohol [70]. In *P. chrysosporium* two distinct systems are simultaneously involved in methylating reactions, one using *S*-adenosyl methionine as methyl donor and the other using CH_3Cl as methyl donor [71]. The results show that CH_3Cl is a common intermediate in methylating systems of basidiomycetous fungi, although it is not always produced in detectable amounts. Future research should focus on the characterization of this unknown enzyme system responsible for the methyl transfering reactions from chloromethane to aromatic compounds. Besides methylation also xylosylation reactions are possible with the hydroxy groups.

Xylosylation of veratryl and vanillyl alcohol. The white-rot fungus *T. versicolor* xylosylates both secondary metabolites and lignin biodegradation intermediates. Kondo et al. [72] showed that veratryl and vanillyl alcohol are glycosylated (Figure 4). The phenolic hydroxyl group is much more extensively glycosylated than the alcoholic hydroxyl group. Several roles have been suggested for these glycosylation reactions in lignin biodegradation. The prevention of polymerization reactions by phenol oxidases, the conversion of oligomeric lignin molecules to more hydrophilic compounds and the detoxification of phenolic compounds are possible explanations. A possible example of the latter role is the xylosylation of 2-chlorobenzyl alcohol by *T. versicolor* [73], but all possible roles need further confirmation.

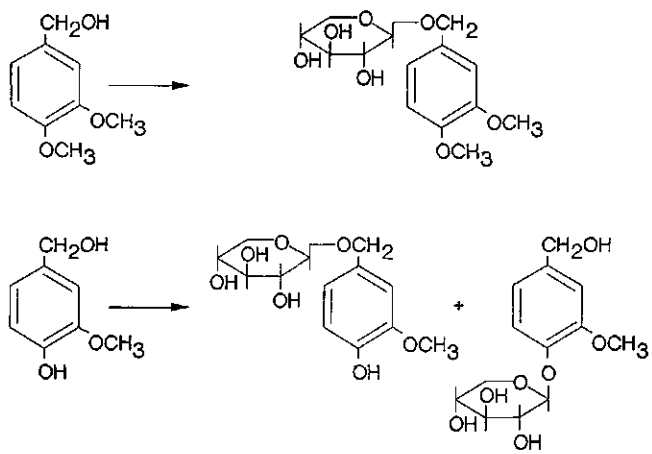


Figure 4. Xylosylation reaction of veratryl and vanillyl alcohol by *Trametes versicolor*. (adapted from [72]).

Chlorination of aromatic compounds. The fact that basidiomycetes can produce chlorinated compounds has been known for some time [74-76]. It is anticipated that haloperoxidases are involved in most biohalogenation reactions [75]. From terrestrial fungi two different classes of extracellular chloroperoxidases have been described. *Caldariomyces fumago* produces a heme-containing chloroperoxidase [77], while a dematiaceous hyphomycete, *Curvularia inaequalis*, produces an vanadium-dependent chloroperoxidase [78]. The sources and properties of both heme- and vanadium-containing chloroperoxidases have been reviewed recently [77]. The occurrence of chloroperoxidases in basidiomycetes has not yet been described. Although lignin peroxidase can brominate veratryl alcohol, it is unreactive with chloride [79].

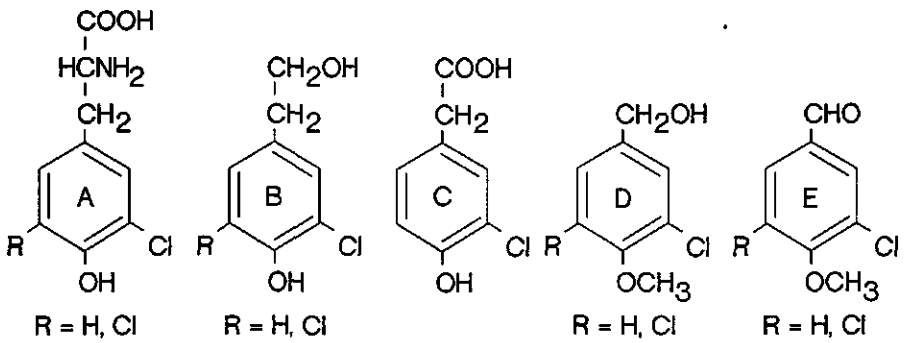


Figure 5. Naturally produced chlorinated amino acids derivatives, including chlorinated tyrosines (A), tyrosols (B), *p*-hydroxypheylacetate (C) and chlorinated anisyl metabolites (CAM) (D and E).

Table 1 shows that several basidiomycetes also produce chlorinated anisyl alcohols and aldehydes. The chlorinated anisyl metabolites (CAM) are produced both under laboratory conditions [31,40,80] and in the environment [32]. It is anticipated that phenylalanine or tyrosine is also a precursor in the biosynthesis of CAM compounds. The production of chlorinated amino acids has been reported for several marine organisms. 3-Chlorotyrosine has been isolated (Figure 5) from the welk *Buccinum undatum* [81] and the cuticle of the horseshoe crab *Limulus polyphemus* [82], the latter also produces 3,5-dichlorotyrosine. From cuticular proteins of a terrestrial insect (locust, *Schistocerca gregaria*) 3-chlorotyrosine has also been purified [83]. Also in the culture fluids of fungi, chlorinated amino acid derivatives have been found. *Caldariomyces fumago* produced chlorinated tyrosols (Figure 5) [84]. The chlorination of tyrosol is catalyzed *in vitro* by chloroperoxidase. The basidiomycete *Marasmius palmivorus* produced 3-chloro-4-hydroxyphenylacetate (Figure 5) [52], which is perhaps derived from 3-chlorotyrosine via deamination and decarboxylation.

LIGNINOLYTIC ENZYMES OF WHITE-ROT FUNGI

The preceding paragraph has shown that the biosynthesis of secondary metabolites is common among white-rot fungi, thus it is interesting to know if these metabolites have a role in the ligninolytic system of those fungi. To answer this question first the extracellular ligninolytic enzyme system will be explained.

Peroxidases, laccases and H_2O_2-producing oxidases are important enzymes of the extracellular ligninolytic machinery secreted by white-rot fungi. Table 2 summarizes the extracellular peroxidases and oxidases described in white-rot fungi. The table clearly demonstrate that the ligninolytic enzyme complex is not so homogeneous among white-rot fungi as once thought [3]. However, it should be kept in mind that the detection of ligninolytic enzymes in the culture broth is often difficult because of the association of the enzymes to the fungal mycelium [85] and/or the occurrence of inhibitors in the culture broth [86-89].

The reactions catalyzed by phenol oxidizing enzymes (laccases and peroxidases) are very similar. Laccase and the different peroxidases (including lignin peroxidase (LiP), manganese peroxidase (MnP) and horseradish peroxidase (HRP)) can oxidize phenolic compounds creating phenoxy radicals [20,21,90], while non-phenolic compounds are oxidized to the corresponding cation radicals. All phenolic compounds are oxidized by phenol oxidases, whereas the different enzymes have complete dissimilar substrate ranges for non-phenolic compounds. Laccase can only oxidize compounds with a relatively low ionization potential, including 1,2,4,5-tetramethoxybenzene ($E_{1/2}$ < = 0.81 V versus a saturated calomel electrode), to the corresponding cation radical [91-93]. Non-phenolic compounds with higher ionization potentials ($E_{1/2}$ < = 1.06-1.12 V) are still readily oxidized by both LiP, MnP, HRP. LiP is an extraordinary peroxidase since it can oxidize non-phenolic aromatic compounds with very high ionization potentials such as 1,2-dimethoxybenzene ($E_{1/2}$ = 1.5 V) and veratryl alcohol [92,94]. The exact oxidizing activity of MnP is at the moment still unclear [93,95-97].

First it is important to discuss the characteristics of each ligninolytic enzyme. With this information, it will be possible to explain the physiological roles and the metabolism of the *de novo* biosynthesized aryl alcohols.

Table 2. The production of extracellular oxidative enzymes by basidiomycetes.

Fungus	Type *	Ligninolytic enzymes †						References
		LiP	MnP	Per	Lac	Glyox	AAO	
Agaricus bisporus					+			[98]
Armillaria mellea	WR	-	+		+			[99]
Armillaria ostoyae	WR	-	+		+			[99]
Bjerkandera adusta	WR	+	+	+	+	+	+	[100-103]
Bjerkandera sp. BOS55	WR	+	+	+	+	+	+	[101,104,105]
Ceriporiopsis subvermispora	WR	-	+		+	-		[106]
Coprinus cinereus				+	-			[98,107,108]
Coriolopsis occidentalis	WR	+	+					[35]
Daedaleopsis confragosa	WR	-	+		+	-	-	[101]
Dichomitus squalens	WR	-	+	+	+	+	-	[46,109]
Ganoderma australis	WR		+					[110]
Ganoderma valesiacum		-	+		+			[46]
Gleophyllum trabeum	BR	-	-	+	-			[111,112]
Inonotus (Phellinus) weirii	WR	-	-	+				[113]
Junghuhnia separabilima	WR	+		+	+			[87]
Lentinula edodes	WR	+/-	+		+			[114-116]
Merulius (Phlebia) tremellosus	WR	+/-	+		+			[88,114,117]
Panus tigrinus	WR	-	+		+			[118]
Phanerochaete chrysosporium	WR	+	+	-	-	+	-	[6-9,101]
Phanerochaete flavido alba	WR	+	+					[114]
Phanerochaete magnolia	WR	+	+					[114]
Phlebia brevispora	WR	+	+	-	+	+		[106,119]
Phlebia ochraceofulva	WR	+	-		+			[120]
Phlebia radiata	WR	+	+		+	+		[114,121,122]
Phlebia subserialis	WR	-	+					[114]
Phellinus igniarius	WR	-	-		+			[111]
Phellinus pini	WR	+	+					[114]

Fungus	Type *	Ligninolytic enzymes †						References
		LiP	MnP	Per	Lac	Glyox	AAO	
Piptoporus betulinus	BR	-	+		-			[111]
Pleurotus ostreatus	WR	-	+		+		+	[100,102,123-125]
Pleurotus sajor-caju	WR	-	+		+		+	[126,127]
Polyporus ostreiformis	BR	+						[128]
Pycnoporus cinnabarinus	WR	-	-	+	+			[46]
Stereum hirsutum	WR	-	+	-	+			[46]
Trametes gibbosa	WR	+	+	-	+			[46]
Trametes hirsuta	WR	+	+	-	+			[46]
Trametes versicolor	WR	+	+		+	+	+	[101,129,130]
Trametes villosa	WR	-	+		+	+	-	[101]

* Fungi which give a white-rot (WR) or brown-rot (BR) type of wood decay.

† Extracellular ligninolytic enzymes detected, including lignin peroxidase (LiP), manganese peroxidase (MnP), peroxidase (Per), Laccase (Lac), aryl alcohol oxidase (AAO) and glyoxal oxidase (GLYOX).

Lignin peroxidase. Lignin peroxidase (LiP, EC 1.11.1.-) is an extracellular lignin degrading enzyme discovered in ligninolytic cultures of *P. chrysosporium* [6,7]. The enzyme is produced by many (Table 2) but not by all white-rot fungi. Together with other enzymes, LiP is thought to constitute the major component of the lignin degrading system of *P. chrysosporium*. However, with *in vitro* experiments only polymerization reactions of lignin were noticed [131] leading to doubts about the essential role of LiP *in vivo* [132]. Several facts indicate that LiP is important in lignin and xenobiotic degradation by LiP-producing white-rot fungi. The enzyme can depolymerize dilute solutions of lignin *in vitro* [133], can oxidize and depolymerize a variety of dimers and oligomers structurally related to lignin *in vitro* [20,21] and LiP catalyzes the production of activated oxygen species [134]. Furthermore, the observation was made that cultures supplemented with LiP, degrade lignin much faster than reference cultures which received no enzyme [135]. It has also been suggested that LiP plays a much more important role in the degradation of synthetic ^{14}C-lignin to $^{14}CO_2$ than MnP [114,119,136]. The reaction mechanisms of lignin model compound oxidation have been extensively reviewed [20,21].

The *P. chrysosporium* enzyme is a monomeric *N*- and probably *O*-glycosylated protein with four disulfide bonds [137,138]. Its pH-optimum near 3 is unusually low [139]. LiP contains one iron protoporphyrin IX as prosthetic group and has the same catalytic cylce as horseradish peroxidase (HRP).

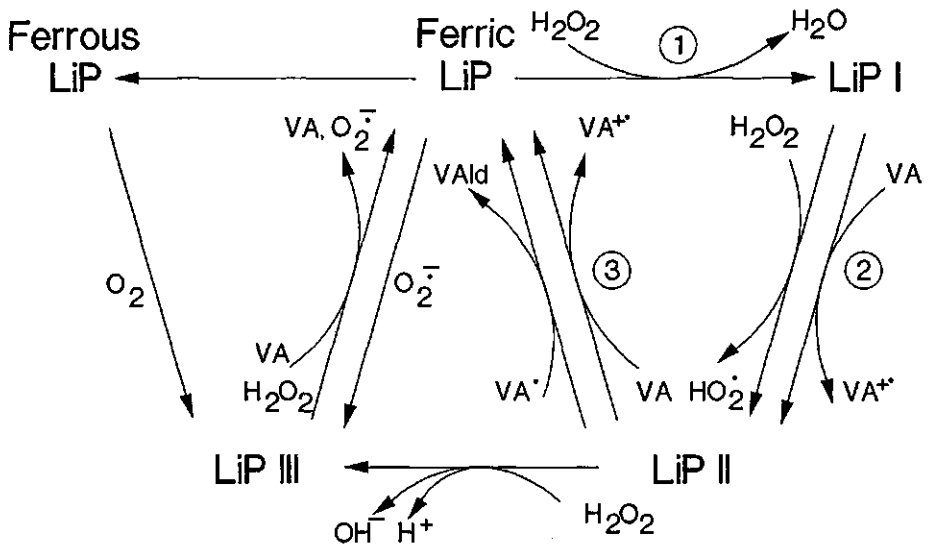


Figure 6. Interrelationships between the oxidized intermediates of lignin peroxidase. Catalytic cycle denoted by reactions 1, 2 and 3. VA = veratryl alcohol; $VA^{\cdot+}$ = veratryl alcohol cation radical; $VA^{\cdot}$ = veratryl alcohol radical (adapted from [121,149]).

In Figure 6, the five oxidation states are shown. Reaction 1 of ferric enzyme with H_2O_2 yields the compound I, ferryl iron (Fe^{IV}) porphyrin cation radical intermediate, which is 2 oxidizing equivalents above the resting state. One-electron reduction of compound I with a reducing substrate (e.g. veratryl alcohol [121,140]) or H_2O_2 yields the iron oxo intermediate, compound II (reaction 2). This intermediate still contains ferryl iron (Fe^{IV}) but no longer has the porphyrin cation radical. Finally a single one electron reduction step returns the enzyme to its native state (reaction 3), completing the catalytic cycle [141-143]. In the absence of reducing substrate, compound II is further oxidized by H_2O_2 to compound III, a species with limited catalytic ability [144-147]. Compound III is stable, but it is inactivated rapidly in the presence of excess H_2O_2 [148]. Compound III can return to the resting ferric state either spontaneously or in the presence of H_2O_2 and a reducing substrate like veratryl alcohol [149].

The enzyme is produced as a set of closely related isozymes with molecular weights ranging from 38 to 43 kilodaltons, and is encoded by different genes in *P.*

chrysosporium [139,150], *P. radiata* [121,151], *B. adusta* [152] and *T. versicolor* [129,153]. The expression of LiP genes is regulated by an inverse function of the Mn^{II} concentration [114,154,155]. The Mn^{II} concentration has no influence on the amount of veratryl alcohol biosynthesized [156]. Intracellularly, the concentrations of cAMP regulate the LiP gene expression at the level of transcription [157]. The influence of several culture parameters on the production of LiP by *P. chrysosporium* has been reviewed [5]. Both the strain and the growth conditions alter the balance and the nature of the different isozymes [158-160].

Manganese Peroxidase. In addition to LiP, white-rot fungi produce extracellular manganese peroxidase (MnP, E.C. 1.11.1.-) under ligninolytic conditions (Table 2). The enzyme was first discovered in the culture fluid of *P. chrysosporium* [8]. The MnP/Mn^{II}/malonate system oxidizes several phenolic substrates including lignin and xenobiotic model compounds [17,161,162]. Furthermore, the Mn^{III}-chelator complex is a freely diffusible oxidant (mediator), and consequently it can oxidize lignin within the woody matrix. Purified enzyme can give depolymerization of a synthetic lignin (DHP) [163], and also degrades high molecular weight chlorolignins [164]. MnP is the predominant enzyme involved in kraft pulp bleaching [89,165] and the decolorization of bleach plant effluents [155].

MnP is a glycoprotein (M_r 46000) and contains one iron protoporphyrin IX prosthetic group [166,167]. The enzyme exists as several closely related isozymes and are encoded by several different genes in *P. chrysosporium* [159,168] and *T. versicolor* [129,153]. The expression of MnP genes is regulated by a direct function of the Mn^{II} concentration [109,114,154-156,169]. Intracellularly, the concentrations of cAMP regulate the MnP gene expression at the level of transcription [157]. Both spectral characteristics and the catalytic cycle of MnP are very similar to LiP and horseradish peroxidase (HRP, EC 1.11.1.7) [141,170-173]. However, Mn^{II} is required for the reduction of compound II back to the native ferric enzyme in order to complete the catalytic cycle [170,172].

After oxidation by MnP, Mn^{III} must form a complex with a chelator before it can oxidize phenolic substrates [173]. Organic acids are good chelators [166,172,174] and basidiomycetes are producers of oxalic acid [134,173,175-177], malonic acid [173], pyruvic acid [178] and malic acid [179]. Mn^{III}/oxalate and Mn^{III}/malonate form very stable complexes, which probably function *in vivo*. Malonate facilitates Mn^{III} dissociation from the enzyme and has a relatively low Mn^{II} binding constant [173]. The Mn^{III}/malate complex is not stable, decomposition by H_2O_2 leads to the formation of molecular oxygen and Mn^{II} [180].

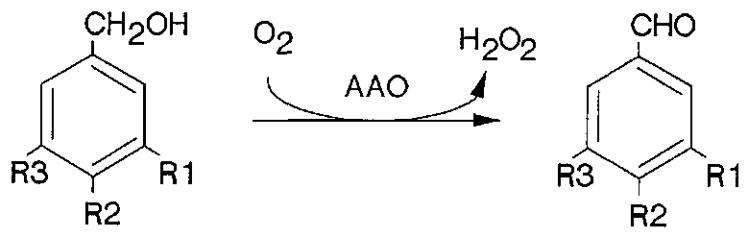
Other phenol oxidases can behave like MnP under special conditions. LiP is

capable of Mn^{III} generation in the presence of a suitable charge-transfer mediator, e.g. veratryl alcohol [181,182]. Laccase and peroxidases from horseradish (HRP), *Arthromyces ramosus* and *Coprinus macrorhizus* can generate Mn^{III} ions in the presence of a phenol (i.e. *m*-cresol or 3-hydroxybenzoate), Mn^{II}, H_2O_2 and pyrophosphate [183]. The major difference between MnP and other phenol oxidases is the ability of MnP to directly oxidize Mn^{II} to Mn^{III} in the absence of a (non)-phenolic co-substrate. Consequently, all phenol oxidases can potentially perform the same mediator reaction as MnP by producing the diffusible Mn^{III} oxidant. Further research is necessary to investigate the role, if any, of this MnP-independent formation of Mn^{III} ions *in vivo*.

Other peroxidases. Several reports have appeared where extracellular peroxidative activies other than LiP or MnP were detected. *Bjerkandera adusta* [100,104], *Junghuhnia separabilima* [87], *Coprinus cinereus* [107,108], *Inonotus weirrii* [113], *Pycnoporus cinnabarinus* and *Dichomitus squalens* [46]) all produce unique extracellular peroxidases. These not so well characterized enzymes show no reactivity with veratryl alcohol in the standard ligninase assay [184] and are, in terms of the reactions catalyzed, comparable with HRP.

Laccase. Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper containing enzymes catalyzing the four one-electron oxidations of a variety of mostly polyhydric phenolic compounds and simultaneous four-electron reduction of dioxygen to water [185]. The enzyme is produced by almost all white-rot fungi (Table 2) [111,186], with *P. chrysosporium* being a notable exception [3]. Its production is dependent on the culture conditions [98,123,187], and both constitutive and inducible forms of laccase are known. The inducible forms in general have a higher activity [188]. Laccase has been linked many times with lignin degradation [21,189]. This is in part because of the widespread production of laccase by white-rot fungi, but also because the reactions of laccase with phenolic lignin substructure models are similar to the mechanisms reported for lignin peroxidase [21]. However, in *in vitro* experiments using purified enzyme, lignin polymerization reactions predominate. The addition of purified laccase also gives a limited demethylation of kraft pulp [165]. It has also been speculated that laccase has a role in toxicity reduction of phenolic compounds by the polymerization reactions [190]. All laccases are glycoproteins [185]. *A. bisporus* laccase contains equal amounts of *O*- and *N*-linked carbohydrates [191]. It has been proposed that the *N*-linked carbohydrate chains protect *T. versicolor* laccase III from proteolysis [192]. In general laccase contains four copper ions, allthough also enzymes with two, three and six copper atoms are known [193].

H_2O_2 producing enzymes. The extracellular peroxidases need H_2O_2 for activity. Interestingly, one of the enzymes that generates H_2O_2 is extracellular aryl alcohol oxidase (AAO). This enzyme oxidize aromatic alcohols to aldehydes and reduces O_2 to H_2O_2 (Figure 7). AAO activity has been found in cultures of *T. versicolor* [130], *Pleurotus sajor-caju* [126], *P. ostreatus* [124], *P. eryngii* [194-196] and *B. adusta* [103,197]. Also extracellular α,β-unsaturated alcohol oxidase of *Fusarium* spp. [198] and intracellular vanillyl alcohol oxidase of *Penicillium simplicissimum* [199] have been described. These enzymes of the hyphomycetes do not resemble the extracellular AAO's of basidiomycetes. It is interesting to note that the intracellular vanillyl alcohol oxidase needs a hydroxy group in the para-position [199] while extracellular AAO shows reduced or no activity with 4-hydroxy-substituted aryl alcohols [124,195]. AAO of *P. eryngii* is a glycoprotein of 72.6 kDa and contains FAD as a prosthetic group. The enzyme has the highest activity with non-phenolic aryl alcohols but also shows activity with aliphatic alcohols with a conjugated double bond, its activity is strongly increased by an additional conjugated double bond [195].



Substates:
Benzyl alcohol; R1=R2=R3=H
Anisyl alcohol; R1=R3=H, R2=OCH_3
Veratryl alcohol; R1=R2=OCH_3, R3=H
3-Chloro-anisyl alcohol; R1=Cl, R2=OCH_3, R3=H
3,5-Dichloro-anisyl alcohol; R1=R3=Cl, R2=OCH_3

Figure 7. Reaction mechanism of aryl alcohol oxidase (AAO) and the chemical structure of secondary metabolites, which are all substrates of the enzyme (adapted from [105,126,195,197]).

AAO is not the only system available to white-rot fungi to produce extracellular H_2O_2. Many other extracellular and intracellular oxidases are known which provide hydrogen peroxide. Extracellular glyoxal oxidase [9,200] is thought to be very important for H_2O_2 generation (Table 2). MnP can generate H_2O_2 by catalyzing the oxidation of NAD(P)H [201] and also extracellular pyranose 2-oxidase has been detected [105,202].

Several intracellular enzymes producing H_2O_2 have been reported from white-rot fungi, including glucose-1-oxidase (EC 1.1.3.4) [203], pyranose-2-oxidase (EC 1.1.3.10) [204,205], methanol oxidase (EC 1.1.3.13) [206] and fatty acyl-CoA oxidase

[207].

SIMULTANEOUS OCCURRENCE OF LIGNINOLYTIC ACTIVITY AND ARYL ALCOHOL PRODUCTION

It is important to know if the secondary metabolites and the ligninolytic enzymes are produced concurrently. The production of veratryl alcohol by *P. chrysosporium* and the onset of ligninolysis are closely linked. Both appear at the onset of idiophase [208]. Moreover, culture conditions which stimulate lignin degradation, such as an elevated oxygen tension [53] and high cAMP levels [209], increase the veratryl alcohol titer, whereas factors that decrease lignin degradation, such as culture agitation [53] and high levels of glutamate or ammonium nitrogen, also decrease the veratryl alcohol titer [209,210]. However, the results obtained with both mutant and revertant strains of *P. chrysosporium* have not unequivocally revealed a linkage between lignin decomposition and veratryl alcohol biosynthesis [211-213]. Also *Bjerkandera* spp. produce several secondary metabolites [31,33] simultaneously with their ligninolytic enzymes [105]. In this fungus, both the LiP-production and the veratryl alcohol biosynthesis are strongly stimulated by N-rich glucose/peptone medium [102,105]. Because the ligninolytic enzymes and secondary metabolites are produced simultaneously, it is interesting to see what physiological roles aryl alcohols have in the ligninolytic system.

PHYSIOLOGICAL ROLES OF ARYL ALCOHOLS

Numerous fungi produce *de novo* aryl alcohols and aldehydes (Table 1) and several roles have been suggested for these compounds, particularly veratryl alcohol, in the physiology of white-rot fungi:

1) induction of ligninolytic enzymes by aryl alcohols
2) stabilization of lignin peroxidase by veratryl alcohol
3) role of veratryl alcohol as a charge-transfer mediator
4) veratryl alcohol and the formation of activated oxygen species
5) aryl alcohols as substrates for H_2O_2-producing enzymes

The different aspects of those physiological roles will be discussed in the following chapter.

Induction of ligninolytic enzymes by aryl alcohols. The effect of aryl alcohols on the induction of the ligninolytic system has been studied by adding these compounds to growing cultures. Cultures of *P. chrysosporium* supplemented with exogenous veratryl alcohol demonstrated an earlier appearance of lignin degradation [208] and a higher lignin degradation rate [135]. This stimulation of lignin degradation was found with many LiP-producing white-rot fungi, including *P. radiata* [214] and *T.versicolor* [100]. The effect of other aryl alcohols on lignin degradation rates is not known.

The addition of veratryl alcohol induced LiP production by *P. chrysosporium* [58,215,216] and *P. radiata* [217]). In *T. versicolor* on the other hand, veratryl alcohol did not increase LiP production [218]. However, a direct correlation between veratryl alcohol addition and the increase of LiP mRNA or the translation of LiP proteins has yet to be proven [219]. Veratryl alcohol not only induces LiP but may also enhance the formation of MnP and laccase in *P. radiata* [217,220]. It is interesting to note that aromatic compounds including veratryl alcohol and veratrate, which themselves are not laccase substrates, are able to induce laccase production [122,220-222].

Other aryl alcohols, including anisyl and vanillyl alcohol, are in general less effective in inducing LiP and MnP production [58,222]. However, addition of anisyl alcohol to cultures of the white-rot fungus *P. tigrinus* stimulated MnP production by 2.5 fold while veratryl alcohol had no inducing effect [223]. In several fungi laccases are induced upon addition of various aromatic compounds, including 2,5-xylidine and ferulic acid [122,188,221,222,224,225].

Aryl alcohol oxidase appears to be a constitutive enzyme in both *Pleurotus* spp. [195] and *B. adusta* [102] and is not induced by veratryl alcohol [124]. However, light stimulates AAO activity in *P. eryngii* [196].

During lignin biodegradation significant amounts of monomeric aromatic compounds are found in the culture fluids [27,29]. However, it should be kept in mind that these concentrations (μM range) are considerably lower than used in the induction experiments. Consequently, the results of the induction experiments should be interpreted with caution. Physiological roles of the aryl alcohols other than induction are probably more important in the *in vivo* situation.

Stabilization of lignin peroxidase by veratryl alcohol. Purified LiP's are irreversibly inactivated in the presence of H_2O_2 [145,226,227] and it is suspected that these enzymes may also be inactivated by H_2O_2 under physiological conditions [228]. Consequently, some mechanism must exist to stabilize LiP or they would be of little value for the fungus. Haemmerli et al. [229] were the first to propose that veratryl alcohol has a stabilizing effect on LiP. Veratryl alcohol can conserve LiP activity by closing the catalytic cycle at the level of the reactions 1,2 and 3 in Figure 6. The

formation of the rather unstable and catalytically less active compound III would thus be avoided. Furthermore, in the presence of H_2O_2, veratryl alcohol can convert compound III back to the resting ferric state [149] (Figure 6). Inactivation of LiP is prevented if the veratryl alcohol/H_2O_2 ratio is greater than 200 at a H_2O_2 concentration of 10 μM [227]. The oxidation of anisyl alcohol, a poor LiP substrate, is increased by the addition of veratryl alcohol [230], and this stimulation can be explained by prevention of enzyme inactivation [231]. Phenolic compounds, including guaiacol and vanillyl alcohol, are as good substrates for LiP as is veratryl alcohol. However, these compounds do not have a role in preventing enzyme inactivation [232].

Role of veratryl alcohol as charge-transfer mediator. Harvey and others [230,233] proposed that veratryl alcohol can act as a charge-transfer mediator in lignin degradation. They suggested that veratryl alcohol is oxidized via cation radical intermediates, which are known to be powerful charge-transfer reagents [234] that can oxidize large hydrophobic molecules like lignin. Simultaneously, the catalytic cycle of LiP is closed, thereby preventing the formation of LiP compound III (Figure 6). The veratryl alcohol cation radical is reduced to veratryl alcohol and ready for another LiP catalyzed charge-transfer reaction.

The formation of veratryl alcohol radical-cations and their ability to participate in charge-transfer reactions has been questioned. Free radical cations derived from veratryl alcohol were not detected with ESR techniques [142]. Nevertheless there are several indications that the veratryl alcohol cation radical is formed in the LiP-catalyzed oxidation of veratryl alcohol. The oxidation of several methoxybenzenes generates ESR detectable cation radicals [92,235]. One of the tested compounds, 1,4-dimethoxybenzene, has a comparable effect as veratryl alcohol in enhancing the oxidation of anisyl alcohol and 4-methoxymandelate [230]. Furthermore, the production of several quinones and lactones in veratryl alcohol oxidation can only be explained when radical cations are produced [236-238]. It has been proposed that veratryl alcohol can function as charge-transfer mediator between the redox cycles of cellobiose:quinone oxidoreductase and LiP [239,240]. Also the veratryl alcohol mediated formation of active oxygen species point to charge-transfer reactions (Figure 6, 9 and 10) [134,149,181]. Indeed, with NMR spectroscopy it was shown that radical intermediates are produced during LiP-catalyzed veratryl alcohol oxidation [241].

The fact that cation radicals are formed does not prove the role of veratryl alcohol as charge-transfer mediator in the oxidation of a third compound. Several groups have intensively researched the oxidation of anisyl alcohol, 4-methoxymandelate, lignin model compounds and dyes with LiP or with simple LiP mimics (hemin) in the presence and absence of veratryl alcohol [133,231,233,242-245].

Since anisyl alcohol and 4-methoxymandelate have higher redox potentials than veratryl alcohol itself, it is unlikely they would be more easily oxidized to cation radicals than veratryl alcohol. However, follow-up reactions, like the irreversible decarboxylation of 4-methoxymandelate into anisaldehyde, suggests that the occurrence of the 4-methoxymandelate radical is still a possibility. This hypothesis is supported by the fact that laccase decarboxylates 4-hydroxymandelate into stoichiometric amounts of 4-hydroxybenzaldehyde. The initially formed 4-hydroxymandelate radical is probably not stable enough to give polymerization reactions (Figure 8) [246].

The results obtained with anisyl alcohol do not fit into the charge-transfer hypothesis. At H_2O_2-concentrations which are low enough to avoid LiP inactivation, LiP is able to directly catalyse the oxidation of anisyl alcohol in the absence of veratryl alcohol [231] and with simple hemin mimics veratryl alcohol even repressed anisyl alcohol oxidation [245]. This result suggest that veratryl alcohol functions as a LiP stabilisator.

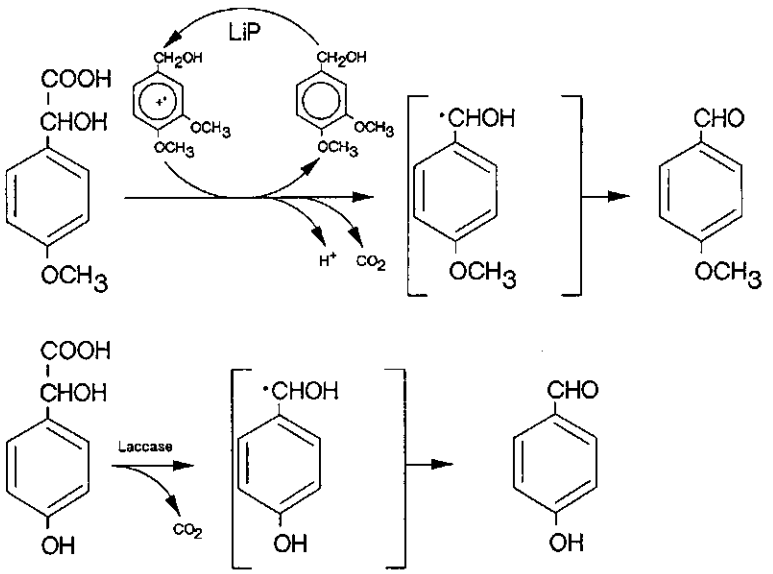


Figure 8. Proposed mechanism for veratryl alcohol radical cation as a charge-transfer mediator in the oxidation of 4-methoxymandelic acid. The bottom reaction shows the stoichiometric conversion of 4-hydroxymandelic acid in 4-hydroxybenzaldehyde (adapted from [230,233,246]).

However, during the LiP catalyzed oxidation of 4-methoxymandelate, veratryl alcohol can act as a charge-transfer mediator (Figure 8). The oxidation of veratryl alcohol to veratraldehyde by LiP was completely inhibited by 4-methoxymandelate

[233]. Veratraldehyde formation commenced only after depletion of 4-methoxymandelate, while in the absence of veratryl alcohol no anisaldehyde was formed. A similar inhibition of veratryl alcohol oxidation was also seen during the oxidation of several azo dyes [244]. Fawer et al. [227] demonstrated with flow injection analysis a mediator role for veratryl alcohol. This mediated oxidation of a third substrate, e.g. lignin, only functioned when all components were present simultaneously, and was not observed when lignin was separated from the site of veratryl alcohol oxidation. Consequently, they proposed that an enzyme-bound cation radical is the mediating species. The results show that veratryl alcohol both can act as a stabilisator of LiP and as a charge-transfer mediator. In experiments it is often impossible to distinguish between both functions.

Veratryl alcohol and the formation of active oxygen species. Before the extracellular ligninolytic enzymes of *P. chrysosporium* were detected, it was speculated that activated oxygen species were important in lignin degradation [247-249]. There is now unequivocal evidence that H_2O_2 plays an important role in lignin degradation [4] as a substrate for the peroxidases but H_2O_2 itself is not a lignin oxidant [3]. Three other activated oxygen species are known to occur in biological systems: superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$). Only $^{\bullet}OH$ and $^1O_2$ are reactive enough to be considered as direct lignin oxidants. Recent research has shown that $O_2^{\bullet-}$ and $^{\bullet}OH$ species can be formed in *in vitro* experiments [134,149,181]. The production of $^1O_2$ by ligninolytic enzymes has not yet been shown.

The production and consumption of activated oxygen species is a complex matter, even in simple model systems. The production of the least reactive species, $O_2^{\bullet-}$ and the involvement of veratryl alcohol in each of the four reactions will be discussed first. In Scheme 1 the different equations leading to activated oxygen species are presented.

1) The unreactive LiP compound III is formed when no reducing substrate, i.e. veratryl alcohol, is present (Figure 6). This compound decays either spontaneously or in the presence of veratryl alcohol (VA) and H_2O_2 to ferric LiP and $O_2^{\bullet-}$ (Scheme 1, eq. 3 and 4) [149].
2) During the oxidation of veratryl alcohol (Scheme 1, eq. 1 and Figure 10) and 4-methoxymandelate by LiP the formation of $O_2^{\bullet-}$ has been suggested [181,230,236]. Further reduction of $O_2^{\bullet-}$ (Scheme 1, eq. 10 and 11) generates a new H_2O_2 molecule. It was found that Mn^{II} strongly stimulates this reduction of $O_2^{\bullet-}$ (Scheme 1, eq. 8), increasing the veratraldehyde/H_2O_2 ratio significantly above 1 [181].

3) In the presence of excess H_2O_2, LiP catalyzes via veratryl alcohol radical cation ($VA^{+\bullet}$) the one-electron oxidation of H_2O_2 to produce $O_2^{\bullet-}$ and veratryl alcohol (Scheme 1, eq. 2 and Fig 9) [250]. Together with equations 10 and 11, a netto production of O_2 and a simultanous decrease of H_2O_2 will be the result. So, this reaction can regulate the extracellular H_2O_2 concentration.

4) Both oxalate and EDTA cause the same non-competitive inhibition of veratryl alcohol oxidation by reducing the veratryl alcohol cation radical [182,251-253]. In this reaction sequence (Scheme 1, eq. 4 and 5 and Fig 9) $O_2^{\bullet-}$ is formed [182,251]. Interestingly, EDTA could not inhibit the oxidation of guaiacol and coniferyl alcohol [253], so phenoxy radicals are not reduced by EDTA. At very low H_2O_2 concentrations also a direct oxidation of oxalate in the absence of veratryl alcohol was noticed [254].

$VA^{\bullet}$ + O_2	→	VAld + $O_2^{\bullet-}$ + H^+	(Eq. 1) [236]
$VA^{+\bullet}$ + H_2O_2	→	VA + $O_2^{\bullet-}$ + $2H^+$	(Eq. 2) [250]
LiP compound III	→	Ferric LiP + $O_2^{\bullet-}$	(Eq. 3) [149]
LiP compound III + VA + H_2O_2	→	Ferric LiP + VA + $O_2^{\bullet-}$	(Eq. 4) [149]
$VA^{+\bullet}$ + Oxalate	→	VA + $CO_2^{\bullet-}$ + CO_2	(Eq. 5) [182,251]
$CO_2^{\bullet-}$ + O_2	→	$O_2^{\bullet-}$ + CO_2	(Eq. 6) [182,251]
$O_2^{\bullet-}$ + Fe^{III}	→	O_2 + Fe^{II}	(Eq. 7) [134,255]
$O_2^{\bullet-}$ + Mn^{II} + $2H^+$	→	H_2O_2 + Mn^{III}	(Eq. 8) [181]
Fe^{II} + H_2O_2	→	Fe^{III} + $^{\bullet}OH$ + OH^-	(Eq. 9) [134]
$O_2^{\bullet-}$ + H^+	⇌	$HOO^{\bullet}$ (pK_a = 4.8)	(Eq. 10)
$2HOO^{\bullet}$	→	O_2 + H_2O_2	(Eq. 11)

Scheme 1

The other species encountered is $^{\bullet}OH$. Its production in biological systems is very difficult to account for because this compound reacts with almost all biological molecules [256]. These radicals may very well be involved in lignin degradation since Gierer et al. [257] showed that $^{\bullet}OH$ can react with both phenolic and non-phenolic lignin model compounds. The most important reactions included oxidative coupling, demethoxylation, hydroxylation and oxidation of methyl groups. The so-called Fenton reaction (Eq. 8) can generate $^{\bullet}OH$, although there is controversy if this reaction occurs under physiological conditions. However, Barr and others [134] have recently shown that in *in vitro* experiments carried out under physiological conditions (pH 5) reactions 5, 6, 7 and 9 leading to $^{\bullet}OH$ formation can take place (Figure 9). *P. chrysosporium* and several brown-rot fungi give chemiluminescence in *in vivo* experiments, indicating

the production $^{\bullet}OH$ during growth on wood or cellulose [258,259]. The $^{\bullet}OH$ production by brown-rot fungi is stimulated by veratryl alcohol addition [258].

We can conclude that LiP can also catalyze several reductive reactions resulting in the formation of $O_2^{\bullet-}$ (Eq. 1,2,3,6) and $^{\bullet}OH$ (Eq. 9) species. In several cases veratryl alcohol acts as a charge-transfer mediator. These results show that activated oxygen species can be generated. Future research should elucidate if these species are formed just by minor side reactions of trivial significance or are indeed important in *in vivo* lignin and xenobiotic degradation.

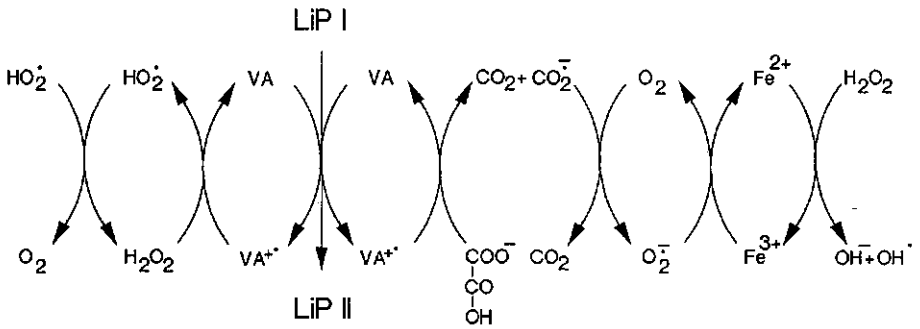


Figure 9. Proposed mechanisms for the production of $HO^{\bullet}$ and the oxidation of excess H_2O_2 to $O_2^{\bullet-}$ by lignin peroxidase. VA = veratryl alcohol, $VA^{\bullet+}$ = veratryl alcohol cation radical (adapted from [134,250]).

The production of H_2O_2 with aryl alcohol oxidase (AAO). Veratryl alcohol is a good substrate for LiP and acts as a stabilisator and charge-transfer mediator. The other common secondary metabolites (Table 1), i.e anisyl, 3-chloro-anisyl and 3,5-dichloro-anisyl alcohol, are not readily oxidized by LiP or other peroxidases [5,105,231] and have apparently another physiological role in the ligninolytic system. All these alcohols are good substrates for extracellular AAO, an enzyme produced by several white-rot fungi (Table 2). Thus, all the aryl alcohols can be used for extracellular H_2O_2-generation.

The oxidases of *P. sajor-caju*, *P. ostreatus* and *P. eryngii* are very similar regarding substrate specifity and affinity for veratryl and anisyl alcohol [124,126,195]. The enzyme has a ten times higher affinity for the latter compound [201]. *P. ostreatus* does not produce significant amounts of veratryl alcohol, however when grown on either synthetic glucose medium or in fungal associated wood samples [32] anisaldehyde could be detected (unpublished results), indicating an active AAO coupled H_2O_2-generating system.

Recently, we have discovered that many wood or forest-litter degrading basidiomycetes produce chlorinated anisyl metabolites (CAM) [32]. Most strains

decolorize Poly R-478, an indicator for ligninolytic activity [101,260,261] and had AAO activity. It was shown that AAO of *Bjerkandera* BOS55 has a much higher affinity for the chlorinated anisyl compounds compared to the non-chlorinated counterparts, anisyl and veratryl alcohol [105]. Another possible advantage of the Cl-groups is the fact that the compounds are not substrates for LiP [105]. The induction of LiP by glucose/peptone medium [102] was correlated with an increased production of veratryl alcohol and not with an increased production of the other aryl alcohols [105].

These results show that some white-rot fungi produce chlorinated aromatic compounds which have an important function in the extracellular H_2O_2 production besides having a possible antibiotic activity [80]. The main function of veratryl alcohol in the ligninolytic system are stabilization and charge-transfer reactions of LiP.

MAINTENANCE OF THE CATALYTIC CYCLE, THE REDUCTION OF ARYL ALDEHYDES

If the aryl alcohols have a truly physiological important role, then the oxidized metabolites must be recycled. These aryl aldehydes, veratraldehyde and (chlorinated) anisaldehydes, must be reduced to again produce the alcohols with their metabolic function as LiP and AAO substrates. It is not permissible to utilize energy for supplying each catalytic cycle with newly biosynthesized aryl alcohols. Already in 1959, Farmer et al. [262] found aromatic acid and aromatic aldehyde reducing activity in *Trametes* (syn. *Polystictus*) *versicolor*. The crude, NAD(P)H-dependent enzyme(s) are active on several aromatic compounds including veratrate. Curiously, it was shown that anaerobic conditions repressed the reducing activity of the fungus. On the contrary, flushing with air or oxygen had a positive effect on the conversion, indicating that anaerobic conditions were not responsible for the reduction of aldehydes and acids [221,262]. Hurst [263] showed that reductive enzymes are common in basidiomycetes and that two different enzymes are involved in the reduction of acid to aldehyde and aldehyde to alcohol. Cultures of the white-rot fungi *P. chrysosporium* [264-266], *P. cinnabarinus* [267] and *T. versicolor* [57,73,222] reduce both non-phenolic (veratrate and veratraldehyde) and phenolic (vanillate and vanillin) compounds to their corresponding aldehydes and alcohols. The reduction of aromatic aldehydes has also been described in strains of *Fomes fomentarius* [268], *P. eryngii* [269], *Trametes hirsutus* [73], *Phlebia radiata* [221] and *Bjerkandera* spp. [105].

Recently an intracellular aryl alcohol dehydrogenase (EC 1.1.1.91) from *P. chrysosporium* was purified and characterized [266]. The enzyme reduced veratraldehyde to veratryl alcohol using NADPH as a cofactor. Both methoxylated and

hydroxylated compounds, including veratraldehyde and anisaldehyde, are substrates for the enzyme. Aromatic ketones, some of which are readily reduced by whole cultures of *P. chrysosporium* [270], are not substrates for this dehydrogenase. High glucose concentrations promotes reduction of aromatic acids to their corresponding alcohols [264]. However, when white-rot fungi are grown in a C-limited instead of N-limited medium the aromatic aldehydes and acids are not reduced to the corresponding alcohols but rapidly metabolized to CO_2 and biomass [135,267,271-273].

METABOLISM OF ARYL ALCOHOLS BY FUNGI

We have seen that non-phenolic aryl alcohols have important physiological functions in the ligninolytic system of white-rot fungi. This means that white-rot fungi must regulate the biosynthesis and metabolization rates of the aryl metabolites, in order to maintain optimal concentrations of those compounds. The biosynthetic routes have been dealt with in section 3 and in this section the degradative routes will be discussed.

A general point of interest in discussing the degradation of aryl aclohols by white-rot fungi is whether or not these fungi are able to grow on such compounds as sole source of carbon and energy. During the biodegradation of lignin and lignin model compounds several monomeric aromatic intermediates, including vanillate and syringate, were detected [20,21,27]. It is now generally accepted that white-rot fungi cannot use lignin as sole source of carbon and energy [3,4]. At present also no clear reports are available demonstrating unequivocally that ligninolytic fungi are indeed able to grow on monomeric aromatic compounds [274-279]. The weak lignin degrader *S. commune* may be an exception in this respect since it grew on sinapyl alcohol as sole carbon and energy source [280]. Fungi from other taxonomic groups, like the hyphomycete *Penicillium simplicissimum*, can readily use veratryl alcohol or vanillate as sole source of carbon and energy [279,282].

However, when an easily metabolized substrate like glucose is available, labelled veratryl alcohol and related compounds are metabolized in CO_2 and biomass [135,211,272,281]. *T. versicolor*, *P. radiata* and *P. chrysosporium* all degrade the phenolic compound vanillate much faster than the non-phenolic compound veratrate [267,283,284], indicating that the non-phenolic compounds are better protected against degradation.

Partial oxidation of aryl alcohols may take place extracellularly by ligninolytic enzymes. Intracellularly, the compounds may be fully oxidized to carbon dioxide and

water. The two degradative patterns will be discussed below.

Aryl alcohols and extracellular ligninolytic enzymes

In the extracellular environment of white-rot fungi, non-phenolic aryl alcohols with a distinct physiological function are present together with several ligninolytic enzymes. To maintain their physiological concentration, it is important that these compounds are not easily degraded by the aspecific extracellular ligninolytic enzymes.

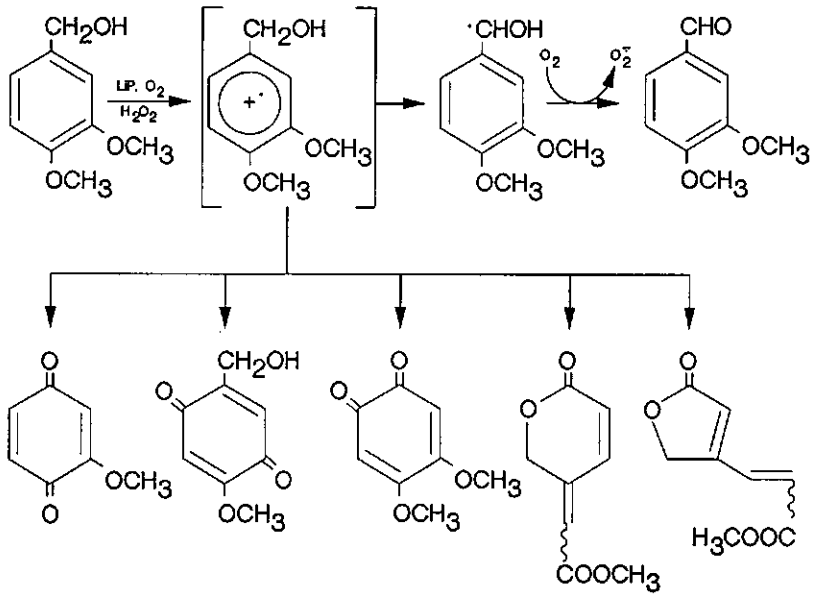


Figure 10. Reaction products of the lignin peroxidase catalyzed veratryl alcohol oxidation (adapted from [236,237,238]).

Non-phenolic compounds: LiP catalyzed reactions. Veratryl alcohol is readily oxidized by LiP, while veratraldehyde and veratrate are not a substrate for this enzyme [222,270]. Initially it was thought that veratryl alcohol was stoichiometrically converted in veratraldehyde [142]. Upon closer examination, a number of other products was identified. In Figure 10 several reaction products of veratryl alcohol by LiP-catalyzed oxidation are presented. Although the major product is veratraldehyde (70 - 90 %), several quinones (10 % yield), and lactones resulting from ring cleavage (up to 20 % yield) are also formed. This product spectrum depends on both the pH and the concentration of Mn [236,238,272]. The formation of the quinones has not yet been studied in detail. Presently it is not known if dioxygen or oxygen from water is

incorporated and if hydroquinones are intermediates. Much more is known about the ring-opening reactions. Shimada et al. [237,285] showed with elegant labeling experiments that one oxygen atom is derived from H_2O and the other atom from O_2 (Figure 11). H_2O reacts at the 3-position of veratryl alcohol radical cation while O_2 [237] or HOO$^{\bullet}$ [236] reacts at the four position. Dioxygen is thus used as an electron acceptor producing HOO$^{\bullet}$ as well as a reagent in the ring cleavage reaction (Figure 10 and 11) [236,286].

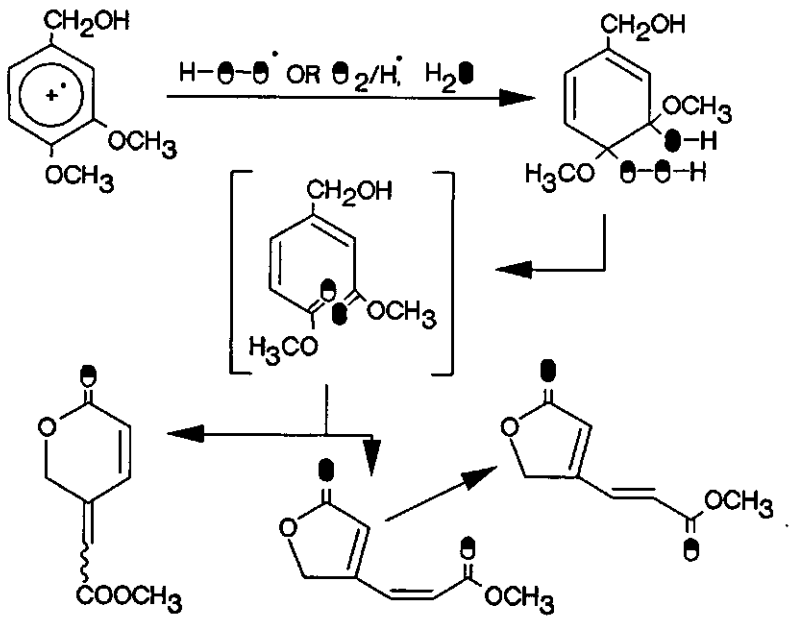


Figure 11. Mechanisms for the formation of aromatic ring cleavage products of veratryl alcohol by lignin peroxidase (adapted from [237,285]).

Addition of Mn^{II} increases the LiP catalyzed oxidation of veratryl alcohol but inhibits the formation of ring-opened products [236]. The scavenging of superoxide anion which results in the formation of H_2O_2 was speculated to be the reason [181]. This result points to HOO$^{\bullet}$ instead of O_2 as the reactive species in the ring cleavage reaction.

Recently, Tuor et al. [287] showed that *cis*-4-hydroxy-6-hydroxymethyl-3-methoxy-cyclohex-2-en-one was a degradation product of LiP-catalyzed oxidation of veratryl alcohol via the intermediate quinone/hydroquinone (Figure 12). It is unclear how this compound is further metabolized. Re-aromatisation in 2-hydroxy-*p*-anisyl alcohol is possible at acid pH's. The latter compound is again substrate for extracellular phenol oxidases, however, the product of this reaction will presumably be

the starting quinone analogous to the LiP-catalyzed oxidation of isovanillyl alcohol [20].

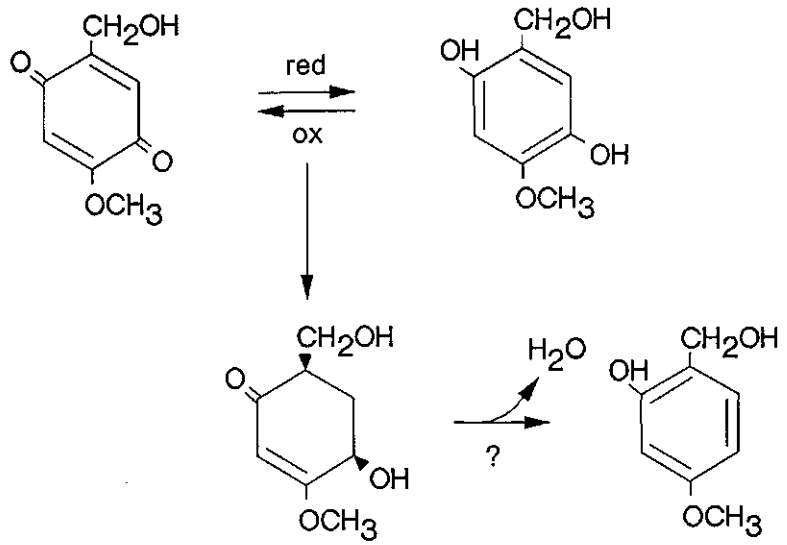


Figure 12. The formation of 4-hydroxy-cyclohex-2-enone and the postulated aromatization reaction from a dynamic reduction equilibrium between quinone and hydroquinone (adapted from Tuor et al. [287]).

It is anticipated that in an *in vivo* situation a pH of around 4.5 [238,272] and charge-transfer reactions prevent the production of significant amounts of quinones and lactones (Figure 8). Consequently, only small quantites of the veratryl alcohol / veratraldehyde couple will be withdrawn from this system. Other *de novo* biosynthesized (chlorinated) anisyl alcohols are poor substrates for LiP. Oxidation of anisyl alcohol only resulted in low yields of anisaldehyde [230,231], while the chlorinated anisyl alcohols were not oxidized at all by LiP [105]. This means that the other couples are very stable against LiP-caused losses.

MnP catalyzed reactions. Non-phenolic aryl alcohols, e.g. veratryl and anisyl alcohol, are not readily oxidized by MnP generated Mn^{III} [93,288]. While under standard conditions (pH 0, 0.1 M ionic strength) Mn^{III} can have a high standard redox potential of 1.5 V, a value comparable with LiP, under a normal physiological situation a redox potential of 0.9-1.2 V would not be high enough to oxidize veratryl alcohol [93,95]. However, the oxidation of small amounts of veratryl alcohol to veratraldehyde with Mn^{III}/PPi, MnP/Mn^{II}/oxalate [96] and Mn^{III}/acetate [97] systems has been reported. Also the results obtained with Mn^{III}/acetate mediated polycyclic aromatic hydrocarbons

(PAH) oxidations show that under certain conditions Mn^{III}/acetate can slowly oxidize the same range of PAH compounds as LiP [289-292].

Forrester et al. [96] reported that the MnP-mediated oxidation of veratryl alcohol to veratraldehyde was strongly stimulated by gluthatione. Wariishi et al. [95] showed that in the presence of reducing compounds, including gluthatione, DTT or other thiol compounds, veratryl alcohol is oxidized in veratraldehyde by MnP/Mn^{II}/malonate, Mn^{III}/malonate or γ-irradiation systems. In the absence of thiol compounds no reaction was seen [93,95]. Anisyl and benzyl alcohol were oxidized at comparable rates, excluding any great influence of the substrates redox potential. It was proposed that thiyl radicals are formed instead of cation radical intermediates. Other peroxidases like horseradish peroxidase can also oxidize veratryl alcohol when thiol compounds are present [293].

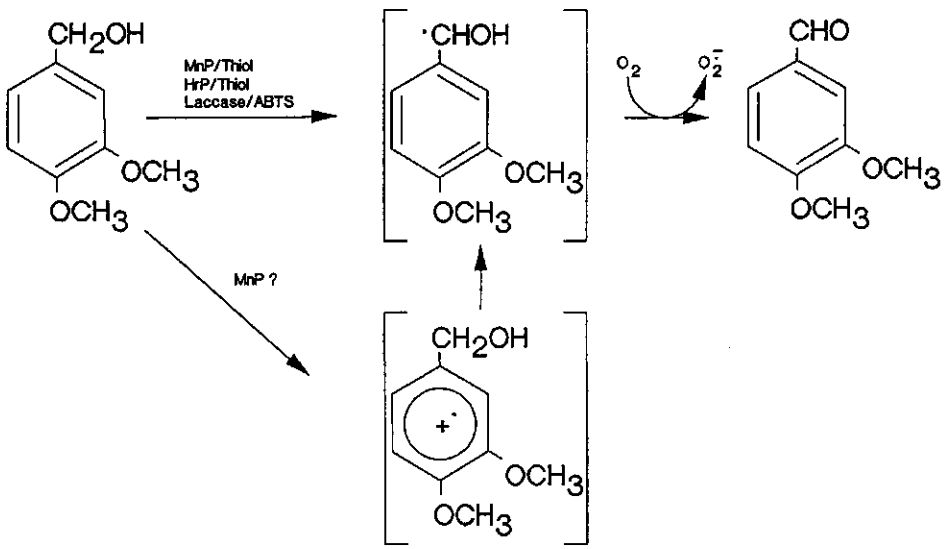


Figure 13. The oxidation of veratryl alcohol by manganese peroxidase/thiol and laccase/ABTS complexes. Possible direct oxidation of veratryl alcohol to veratryl alcohol radical cation is also shown (adapted from [95,96,97,293,294]).

We conclude therefore that two different systems are functioning (Figure 13). There is a direct oxidation of aromatic compound by Mn^{III} influenced by both the redox potential of the substrate, pH and chelator used. Additionally, in the presence of thiol compounds MnP or HRP generated thiyl radicals can abstract a hydrogen from the α-carbon to form a benzylic radical not much influenced by the ionization potential of the substrate. It is at the moment unclear if the Mn^{III} or thiyl catalyzed oxidation of veratryl alcohol is of any significance *in vivo*.

Laccase catalyzed reactions. Recently, Bourbonnais and Paice [294] proposed an expanded role for laccase in lignin biodegradation. They showed that in the presence of the oxidizable substrate 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), *Trametes versicolor* laccase can oxidize veratryl alcohol to veratraldehyde (Figure 13). Also a laccase from *Dichomitus squalens* (syn. *Polyporus anceps*) can oxidize some substrates, as for instance the natural product pesticide rotenone, only in the presence of an electron transfer mediator i.e. chlorpromazine [295]. The laccase/ABTS mixture also cleaved a lignin model dimer [24] and hardwood kraft pulp was bleached to the same extent by a *in vitro* laccase/ABTS mixture compared to whole cultures [296]. However, during the bleaching of hardwood pulp the fungus did not produce a stable physiological equivalent of ABTS. The laccase/syringaldehyde couple can also oxidize veratryl alcohol, although with a lower activity [297]. Syringaldehyde is potentially present under physiological conditions since syringic acid is a common lignin degradation metabolite [27,29] and it is readily reduced by white-rot fungi. Thus syringaldehyde can potentially be considered as a physiological equivalent to ABTS mediated reactions. Future research should reveal if the simultaneous production of laccase and syringaldehyde plays a role in lignin biodegradation.

The exact reaction mechanism is still unknown, but it was shown that the laccase/ABTS couple was the active species and not the $ABTS^{\bullet+}$ cation radical produced [294]. Muheim et al. [94] have re-examined the veratryl alcohol-laccase/ABTS couple and concluded that the veratryl alcohol radical cation was not involved in the reaction sequence. During the oxidation of veratryl alcohol no quinones or ring-opened products were detected, and 1,4-dimethoxybenzene, a good LiP substrate, was not oxidized. They concluded that a mechanism of hydrogen atom abstraction from the Cα-position leading to hydroxy substituted benzyl radicals, and subsequent oxidation to veratraldehyde takes place (Figure 13). The importance of oxidation and cleavage reactions by hydrogen atom abstraction in lignin degradation remains to be elucidated [94].

We propose that also in the LiP/veratryl alcohol catalyzed oxidation of anisyl alcohol and 4-methoxymandelate the charge-transfer reactions can be comparable with those seen with the laccase/ABTS couple [294]. This means that during this oxidation no radical cations but instead hydroxy-substituted benzyl radicals are produced [94]. In his review Schoemaker [20] also proposed the involvement of hydroxy-substituted 4-methoxybenzyl radical explaining the observed oxygen activation. He also postulated that this benzylic radical can serve as an efficient electron donor for the reduction of compound II to native enzyme. This hypothesis was strengthened by the results of Lundell et al. [121] with a β-*O*-4 model dimer.

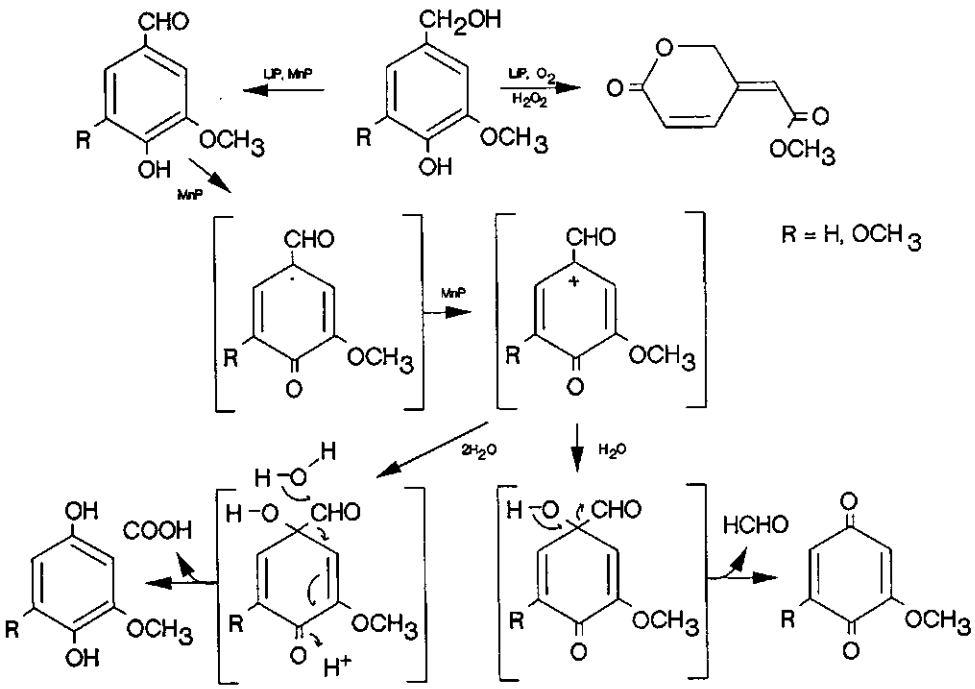


Figure 14. Oxidation of vanillyl alcohol and vanillin by phenol oxidases. Possible mechanism for the oxidation of vanillin/syringaldehyde to methoxyquinone and methoxyhydroquinone by laccase and manganese peroxidase (adapted from [161,288,297,299]).

Phenolic compounds. Phenolic aromatic compounds are common lignin degradation products. It is expected that the different phenol oxidases react in the same way with the phenolic substrates [161,288,298]. With all enzymes, polymerization reactions frequently occur [299,300]. LiP and MnP oxidize phenolic compounds like vanillyl alcohol to aldehydes. Surprisingly, LiP also catalyzes the ring cleavage of this compound to the δ-lactone (Figure 14) [299]. When a compound is adequatly protected, like sterically hindered 4,6-di(tert)butylguaiacol, laccase can also oxidize such compounds to ring cleavage products in high yields instead of polymerizing the phenol [319]. Laccases from *Rhizoctonia praticola* and *Trametes versicolor* react pH-dependent with vanillic and syringic acid. At a high pH of 6.9 the formed phenoxy radicals tend to polymerize while at pH 3.5 substantial amounts of the monomeric quinones are formed (Figure 15) [300]. Both MnP and laccase oxidize syringaldehyde and syringate to 2,6-dimethoxybenzoquinone as the main reaction product [161,288,297,301] (Figure 14). It was proposed that MnP oxidizes syringaldehyde to

2,6-dimethoxybenzoquinone and formaldehyde in a single reaction step [161,288]. However, with laccase also 2,6-dimethoxyhydroquinone was found as a reaction product from syringaldehyde oxidation [297]. The formed hydroquinone is readily oxidized by laccase to the quinone [301]. In Figure 14 the two reaction mechanisms leading to quinone and hydroquinone are presented.

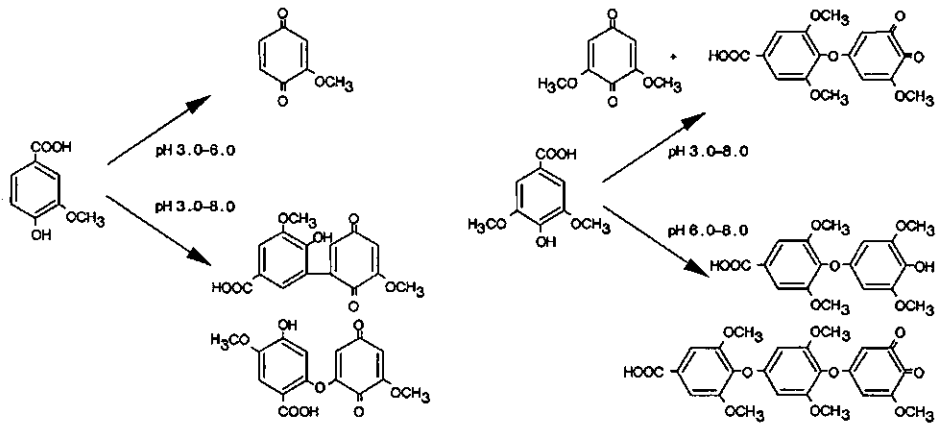


Figure 15. Oxidation of vanillic and syringic acid by *Rhizoctonia praticola* and *Trametes versicolor* laccases at different pH's (adapted from Bollag and Leonowicz [300]).

Metabolism of aryl alcohol metabolites by intracellular enzymes

In the previous section 8.1 it was seen that all the *de novo* biosynthesized non-phenolic aryl alcohols are well protected against the fungus's own extracellular ligninolytic enzymes. The only major products formed extracellularly are the corresponding aryl aldehydes which in turn are readily reduced to the corresponding alcohols. Also several other reports suggest that extracellular phenol oxidases are not important in the degradation of those monomeric aromatic compounds [127,211,264,273,276]. Nonetheless, the fungus can regulate the amount of extracellular aromatic compounds since several intracellular enzymes are acting on aryl alcohols and their degradation products. In this section we will consider alcohol and aldehyde dehydrogenases, hydroxylating enzymes, quinone:oxidoreductases and ring fission enzymes.

At this point it should be stressed that no information is available on active transport mechanisms in white-rot fungi for aryl alcohols / aldehydes. Consequently, we at this stage presume these compounds either diffuse freely into the cells or are selectively taken up by transport systems still to be discovered.

Alcohol and aldehyde dehydrogenases. Basidiomycetes contain several enzymes that reduce aromatic acids and aldehydes to the corresponding aldehydes and alcohols [73,222,263,266,290] as discussed in section 7. However, all those oxidoreductases can also potentially catalyze the reverse reaction from alcohols to acids. There is at the moment not enough information available to determine under which culture conditions and for which substrates (phenolic or non-phenolic) whether oxidation or reduction will predominate.

Hydroxylating enzymes. For most intracellular ring cleavage reactions the aromatic compounds should contain hydroxygroups. Such molecules can be obtained by introducing new hydroxygroups on the aromatic ring, by demethylating already existing methoxygroups or by oxidatively decarboxylating carboxygroups.

Mono-oxygenases and cytochrome P-450 enzymes can introduce hydroxy groups. The hydroxylation of methyl-anisate by *T. versicolor* [302] and the conversion of 4-hydroxybenzoate to protocatechuate by *P. chrysosporium* [248] have been reported. However, the enzymes involved have not been characterized and their relevance in the metabolism of monomeric aromatic compounds is unknown.

Much more is known about the demethylating activity of white-rot fungi, many fungi are capable of demethylating veratrate to vanillate [222,267]. In *P. radiata*, demethylation of ^{14}C-OCH_3 labelled veratrate occurs synchronously with LiP and MnP production [221], however the direct demethoxylation of veratryl metabolites by those enzymes has not yet been reported. This demethylating activity, but not the production of ligninolytic enzymes, is repressed by high glucose concentrations [267,273]. It has been speculated that factors or enzymes other than phenol oxidases may cause this demethylation [267,303], but no intracellular *O*-demethylase activity could be detected [267,281]. *O*-demethylases are very unstable enzymes and only a bacterial enzyme from *Pseudomonas putida* [304] has been thoroughly characterized. Only one report has been published describing the partial purification of a NADH dependent *O*-demethylase from a basidiomycete (*Xeromyces* (syn. *Xerocomus*) *badius*) [305].

Vanillate and other phenolic compounds containing *ortho*-methoxy groups are readily demethylated by phenol oxidases, but the product(s) of the reaction were not analyzed [19]. The conversion of vanillate to protocatechuate, indicating the action of a specific vanillate-*O*-demethylase, has not been found in both *P. chrysosporium* [275], *P. cinnabarinus* [267] and *Gloeoporus* (syn. *Polyporus*) *dichrous* [306].

Another way of introducing hydroxygroups is oxidative decarboxylation. Both laccase, LiP and MnP can catalyze the oxidative decarboxylation of vanillate (Figure 15). Also the oxidative decarboxylation of vanillate to methoxyhydroquinone by mycelial pellets of *G. dichrous* has been reported [306]. An intracellular, NADPH-dependent

vanillate hydroxylase has been purified from *P. chrysosporium* [277,307]. This enzyme also has decarboxylating activity towards protocatechuate (94%) and to a lesser extent veratrate (9%).

Quinone oxidoreductases. During the microbial degradation of lignin [20,21] and also xenobiotics [162,308] various quinones intermediates are formed. Also the oxidation of veratryl alcohol by LiP [238] and of vanillate by LiP, MnP or laccase results in the formation of several different quinones. The rapid metabolism of those quinones was proposed as a mechanism to shift the equilibrium from enzymatic polymerization to the depolymerization of lignin [20,270]. Both intracellular and extracellular quinone reductases are present.

At least two different intracellular NAD(P)H:quinone oxidoreductases (EC 1.6.99.2) are produced by *P. chrysosporium* [270,309,310]. One of the enzymes, a NADH:quinone oxidoreductase was purified [311]. This protein consists of several isozymes with broad substrate specificity and is induced by vanillic acid or 2-methoxy-1,4-benzoquinone.

The extracellular enzyme cellobiose:quinone oxidoreductase is often linked with lignin degradation [312-314]. It was suggested that the reduction of phenoxy radicals formed by phenol oxidases may be important in preventing repolymerization reactions during lignin degradation. For example, the enzyme inhibited the phenol oxidase catalyzed decarboxylation of vanillate [239]. However, conflicting reports about this subject have appeared [239,315]. Recent research has shown that cellobiose:quinone oxidoreductase is a breakdown product of cellobiose dehydrogenase (previously known as cellobiose oxidase) [316-318]. It has been speculated that an important role of cellobiose dehydrogenase is the production of Fenton's reagent [255].

Ring fission. Ring cleavage of the aromatic moiety is necessary for a complete mineralization. In some cases phenol oxidases cause limited ring fission [236-238,299,319]. However, fungi are known to contain at least three intracellular enzymes that cause ring cleavage of protocatechuate, catechol and 1,2,4-trihydroxybenzene, respectively [320]. Fungi only catalyze the *ortho*-cleavage (intra-diol ring fission), whereas bacteria can give both intra- and extra-diol fission [320]. Protocatechuate is a key intermediate in the metabolism of many aromatic compounds both by bacteria and fungi [321]. Many hyphomycetes [279], including *P. simplicissimum* [282] metabolized vanillate via protocatechuate into β-carboxy-*cis,cis*-muconate. Also white-rot fungi are thought to degrade vanillate via the same route [274,322]. Indeed, intracellular protocatechuate 3,4-dioxygenase (EC 1.13.11.3) has been purified from *P. ostreatus* [323,324]. However, protocatechuate could not be detected as an

intermediate in the metabolism of veratrate or vanillate by several basidiomycetes, including *P. chrysosporium* [279]. Kirk and Lorenz [306] found that methoxyhydroquinone is the main intermediate in vanillate metabolism by *G. dichrous*. Also in the culture broth of several other basidiomycetes including *Trametes* sp. [325], *P. cinnabarinus* [267] and *P. chrysosporium* [264,279], methoxyhydroquinone was detected. Intracellular 1,2,4-trihydroxybenzene-1,2-dioxygenase (EC 1.13.11.-) is a common fungal enzyme [314,326], however specific enzymes which convert methoxyhydroquinone in 1,2,4-trihydroxybenzene have not yet been described. Finally, 1,2,4,5-tetrahydroxybenzene was detected as an intermediate of aromatic compound degradation [308]. However, it is not yet known if another specific dioxygenase is involved in this ring cleavage reaction.

CONCLUSIONS

Aryl (veratryl, anisyl and chlorinated anisyl) alcohols are common secondary metabolites of white-rot fungi. These compounds serve physiological functions together with extracellular enzymes involved in lignin biodegradation and their concentration in the extracellular fluid are highly regulated by intracellular enzymes.

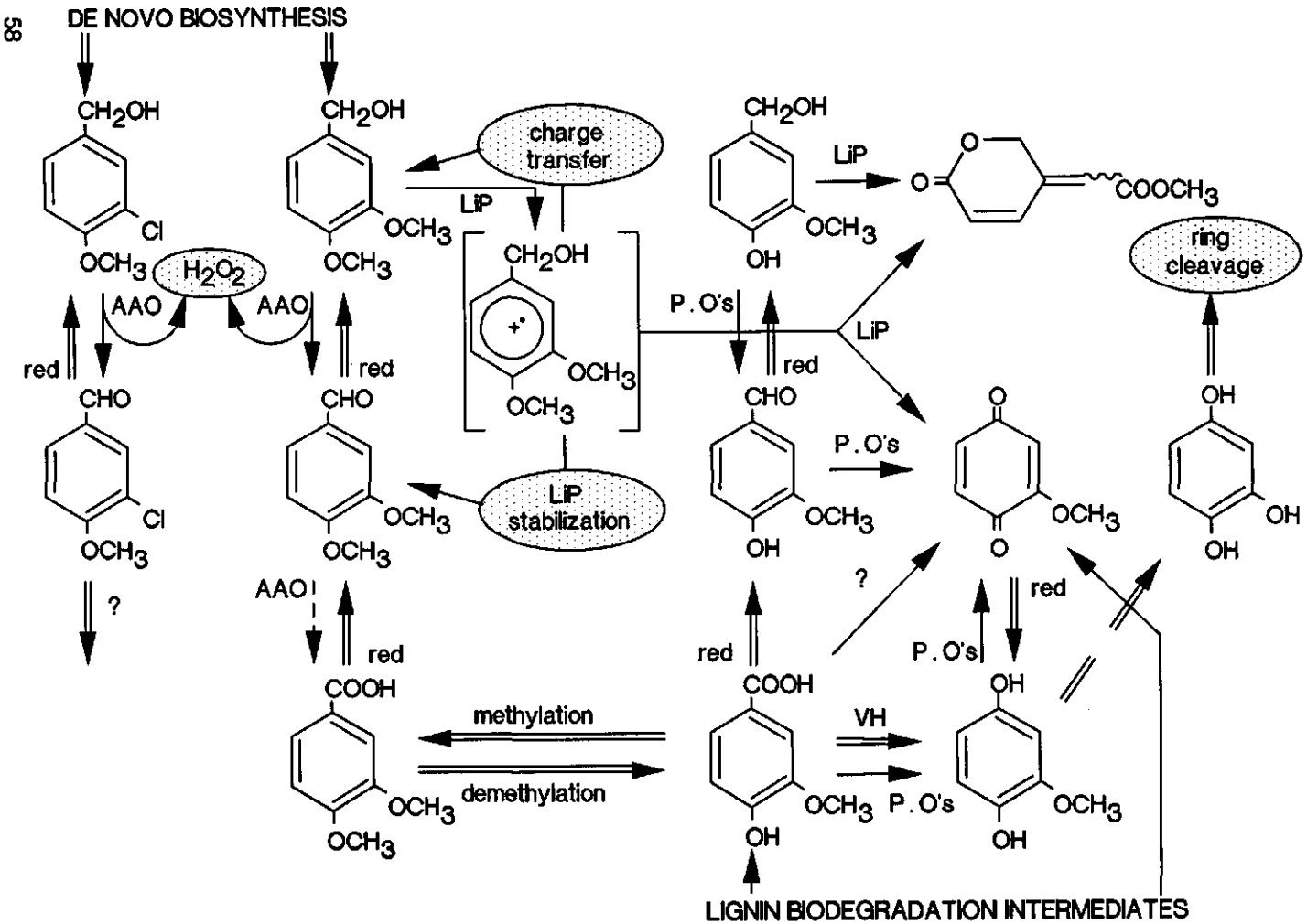
The fungus can produce the aryl alcohols by *de novo* biosynthesis from carbohydrates and by methylation and subsequent reduction of lignin degradation intermediates. It is anticipated that the metabolites are biosynthesized via phenylalanine but most enzymes and intermediates have not yet been characterized. The biosynthesis route of the chlorinated anisyl alcohols is still unclear. In cultures of white-rot fungi no extracellular chloroperoxidase acivity has been found, while chlorinated tyrosines have been detected in other organisms. From these facts, we speculate that it is worthwile to consider chlorinated tyrosines as intermediates in the formation of chlorinated aryl alcohols. It is remarkable that such compounds, generally thought to be mainly of anthropogenic origin, are biosynthesized at high concentrations by white-rot fungi in their natural environment. In our opinion these findings may have an important impact on the norms for chloroaromatics in the environment, and consequently it is necessary to fully determine the ubiquity of this capacity among fungi. The former belief that chloroaromatics were unnatural perhaps has hindered the search for chloroaromatic degrading microbes. Microbiologists will soon learn that such organisms are present in the close vicinity of CAM producing fungi.

The secondary metabolites are produced simultaneously with the ligninolytic enzymes. Veratryl alcohol is an inducer of ligninolytic enzymes, but it is unclear if this is a physiologically important function. More importantly, it stabilizes lignin peroxidase

(LiP) by preventing inactivation and it can act as a charge-transfer mediator both in oxidation of other molecules including lignin and in the reduction of oxygen, generating active oxygen species. Further research is still necessary to attribute more precisely the role of charge-transfer reactions in lignin and xenobiotic biodegradation. Nonethless, it is clear that veratryl alcohol is vital for a proper functioning of LiP. The other aryl alcohols are not involved in stabilizing or mediating peroxidase reactions but they are excellent substrates for extracellular aryl alcohol oxidases (AAO) and generate H_2O_2. This is not the only H_2O_2-generating system available to white-rot fungi, but it may be an important alternative for many fungi.

The aryl alcohol metabolites are stable in the aggressive extracellular ligninolytic system. The only significant conversion of the aryl alcohols is into their corresponding aldehydes. The formed aldehydes are readily recycled through their reduction to the corresponding alcohols by intracellular dehydrogenases. Thus constant alcohol levels are maintained for physiological purposes without the need for energy consuming biosynthesis of additional aryl alcohols. We think the fungus can regulate the levels of the aryl alcohols with the same intracellular enzymes involved in the catabolism of monomeric aromatic compounds released from lignin biodegradation. However, the routes and finetuning of biosynthesis and biodegradation of the aryl alcohols under different culture conditions needs further research.

We have discussed the biosynthesis, physiological roles and biodegradation of aryl alcohols in lignin biodegradation, summarized and depicted in Figure 16. Lignin biodegradation by white-rot fungi is more than just enzymes, it is now clear that aryl alcohol metabolites are also important components involved in this degradation process. This ensures an even more challenging future for scientists involved in the genetic and biochemical characterization of lignin biodegradation.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demethylation
VH
OH
ring cleavage
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LIGNIN BIODEGRADATION INTERMEDIATES

Figure 16. The physiological roles and degradation pathways of aryl alcohols in lignin biodegradation. The physiological roles are shown at the left hand site of the figure shows, while the main degradation routes are shown at the right hand side. All single arrows are extracellular processes while double arrows refer to intracellular enzymes. Dotted arrows refer to proposed reactions. LiP = lignin peroxidase; AAO = aryl alcohol oxidase; P.O's = phenol oxidases (Lignin peroxidase, manganese peroxidase and laccase); VH = vanillate hydroxylase; red = reductive enzymes (alcohol dehydrogenase, aldehyde dehydrogenase and quinone oxidoreductases).

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Many peroxidative fungi that were isolated
from forests,
tropical greenhouses
and several other locations in
the Netherlands are
mushrooms

This is a beautiful pen drawing by
C. Rol
of a dead tree-trunk covered with
honey fungi (*Armillaria mellea*)
and
Trametes versicolor
from the book
"Paddestoelen"
by
Dr. Jac. P. Thijsse,
Verkade's Fabrieken N.V. Zaandam (1929)

At the right sight also
Marasmius rotula
can be seen

CHAPTER 3

ISOLATION AND SCREENING OF BASIDIOMYCETES WITH HIGH PEROXIDATIVE ACTIVITY

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SUMMARY

Sixty-seven Poly R-478 decolorizing basidiomycetes were isolated with a selective medium (containing hemp (*Cannabis sativa*) stem wood, guaiacol and benomyl). Several of the new isolates were promising manganese peroxidase-containing white-rot fungi. Enzyme assays indicated that either glyoxal or aryl alcohol oxidase were present in the culture fluids of peroxidative strains. In contrast, lignin peroxidase was only detected in *Phanerochaete chrysosporium*, despite attempts to induce this enzyme in other strains with oxygen and oxygen / veratryl alcohol additions. A highly significant correlation was found between two ligninolytic indicators: ethene formation from α-keto-γ-methylthiolbutyric acid and the decolorization of a polymeric dye, Poly R. Three of the new isolates had significantly higher Poly R decolorizing activities compared to *P. chrysosporium*. The Poly R decolorization rate is a good assay when trying to optimize culture conditions for peroxidase / H_2O_2 production.

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INTRODUCTION

The possibilities of using white-rot fungi for hemp (*Cannabis sativa* L.) biopulping, biobleaching and wastewater treatment processes are currently being evaluated. Previous work has focused on a limited number of white-rot species, e.g. *Phanerochaete chrysosporium* Burds. and *Trametes versicolor* (L.:Fr.) Pilát (syn. *Coriolus versicolor* (L.:Fr.) Quél.), but the lignin degradation rates and the selectivity with those fungi are low compared to chemical pulping and bleaching. Moreover, lignin degradation by various white-rot fungi is very much dependent on the type of growth substrate (Blanchette et al. 1988). Zadražil (1985) tested eighty five culture collection strains for growth on sterile wheat straw. Maximum lignin degradation after 60 d incubation did not exceed 25 %. Also, the selectivity for lignin (% lignin loss / % weight loss) was, except for one *Phellinus* strain, always much lower than 1. Improvements in lignin degradation rates and selectivity may potentially be achieved by isolating and screening new wild ligninolytic strains.

A selective medium is needed to isolate basidiomycetes from wood or soil samples, and media containing *ortho*-phenylphenol (Russell 1956), benomyl and *O*-phenylphenol (Coggins and Jennings 1975, Carey and Hull 1989), benomyl alone (Maloy 1974) and benomyl plus streptomycin sulfate (Dietrich and Lamar 1990) have been used.

Many researchers have screened wood inhabiting fungi for selective lignin degradation, mainly employing chemical analyses of degraded wood (Setliff and Eudy 1979, Zadražil 1985), ^{14}C-labelled lignin (Trojanowski and Hüttermann 1987) or lignin model dimers (Enoki et al. 1988), but also ultrastructural aspects have been used (Otjen et al. 1987, Blanchette et al. 1988). These studies utilized previously characterized fungi, often from culture collections. A different approach was used by Nishida et al. (1988) to screen newly isolated strains. Their screening was based on the polymerization of guaiacol in wood powder agar plates caused by extracellular phenoloxidases and/or peroxidases excreted by the fungi. A good correlation was found between the formation of coloured zones and ligninolytic activity. Also some quantitative tests correlate ligninolytic activity with the conversion of simple substrates. Preston and McLennan (1948) used simple dyes (neutral red, gentian violet and congo red) to distinguish between brown- and white-rot fungi. Gold et al. (1988) have shown that polymeric dye (Poly R-481) decolorization coincides with lignin degradation by *P. chrysosporium*, manganese peroxidase being at least partly responsible for this decolorization (Kuwahara et al. 1984). Also, Chet et al. (1985) have shown a good correlation between the rate of Poly B-411 decolorization and lignin degradation by eight different white-rot fungi. Polymeric dyes have several advantages in a screening

method; they are easy to use, inexpensive, stable, have a low toxicity and remain extracellular. Another test (Kelley 1988) uses the relationship between ethene formation from α-keto-γ-methylthiolbutyric acid (KTBA), probably caused by hydroxyl radicals, and lignin degradation. Both polymeric dye decolorization and ethene formation assays are specific for peroxidases and show no activity when only laccase is present (Platt et al. 1985, Kelley 1988).

Screening programmes can also make use of direct enzymic assays. A large number of extracellular enzymes involved in lignin degradation are known from studies with *P. chrysosporium* (Kersten and Kirk 1987, Kirk and Farrell 1987), the most important being peroxidases (ligninase and manganese peroxidase) and oxidases (cellobiose oxidase, glyoxal oxidase etc.).

This paper describes the isolation of several basidiomycetes with a selective medium containing hemp stem wood, benomyl and guaiacol. The aim was to select the most potent peroxidative strain, and to compare the different peroxidative tests with the production of extracellular ligninolytic enzymes. The fifteen most active, peroxidative fungi and five reference strains (including *P. chrysosporium* and *T. versicolor*) were quantitatively tested for the decolorization of Poly R-478 and ethene formation from KTBA.

MATERIALS AND METHODS

Organisms and culture conditions. *P. chrysosporium* BKM-F-1767 (ATCC 24725), *P. chrysosporium* SC 26, *T. versicolor* ATCC 20869 (ATCC, Rockville, USA), *Bjerkandera adusta* (Willd.:Fr.) Karsten CBS 595.78, *Trametes villosa* (Fr.) Kreisel (syn. *Polyporus pinsitus* Fr.:Fr.) CBS 678.70 (CBS, Baarn, The Netherlands) and isolated strains were maintained at 4 °C on agar plates containing BIII medium (Kirk et al., 1986) with (0.2%) hemp (*Cannabis sativa* Fibrimon; grown in The Netherlands in 1987) stem wood fibres and (0.02%) Poly R-478 (Sigma, St Louis, USA). Poly R-478 was included to confirm that isolates still contained Poly R decolorizing activity. In all experiments, serum bottles were inoculated with cylindrical agar plugs (6 mm diameter): all taken 3 cm from the initial inoculum, containing the following sterile media:

(1) Hemp stem wood (HSW) medium, containing 2 g l^{-1} 100-1000 μm classified particles of hemp stem wood in 10 mM sodium 2,2-dimethylsuccinate (DMS), at pH 4.5.

(2) BIII medium, prepared according to Kirk et al. (1986).

(3) Kirk medium, containing glucose (1% w/v), DMS (10 mM), BIII, NH_4 tartrate (0.2

g l^{-1}) and thiamin (2 mg l^{-1}) according to Tien and Kirk (1988), veratryl alcohol and extra trace elements being omitted. All components were filter sterilized unless stated otherwise.

Incubation was at 30 °C except with *P. chrysosporium*, which was grown at 37 °C.

Isolation of fungi. A modified method of Nishida et al. (1988) was used for isolation of basidiomycetes. Pieces of rotted wood (about 1x0.5x0.5 cm) or a small amount of soil (about 1g) were placed in the centre of plates containing hemp stem wood (0.2%), guaiacol (0.01%), benomyl (Du Pont Chemical Co., Wilmington, USA, a 50% wettable powder) (15 ppm), agar (1.5%) and sometimes also chloramphenicol (200 ppm). Plates were incubated at either 28, 30 or 45 °C, and subcultures made as soon as browning of agar occurred (indicative of ligninolytic activity, Nishida et al. 1988) until the isolates were pure.

All isolates were qualitatively tested for Poly R decolorization, as described below, 40 decolorizing strains were quantitatively tested so that 15 strains with highest decolorization rates could be selected for further study. The selected strains were identified by the Centraal Bureau voor Schimmelcultures (Baarn, The Netherlands). The following isolates were used: BOS55 a *Bjerkandera* sp.; E39, N11, S33 and probably also B41 a *Trametes* sp.; B47 Agaricales; B15 which is *Polyporus brumalis* (Pers.:Fr.) Fr. or *Polyporus ciliatus* (Fr.:Fr.) Fr.; H2, S54 and S22 which are *Stereum hirsutum* (Willd.:Fr.) Fr. or *Stereum rugosum* (Pers.:Fr.) Fr.; GM2 which is *Daedaleopsis confragosa* (Bolton:Fr.) Schroeter; W23 which is *Chaetomium indicum* Corda, a soft-rot fungus; P30, H37 and B18 whose identity could not be determined.

Poly R-478 decolorization. The method of Gold et al. (1988) was used with some modifications. Poly R-478 (anthrapyridone chromophore) was used since Poly R-481 was no longer available and Poly R-478 was added before inoculation, because it appeared that this had no effect on the growth rate nor on the decolorization velocity of *P. chrysosporium*. Three different media were examined all containing Poly R-478 (0.2 g l^{-1}): HSW, filter sterilized and autoclaved (20 min, 121 °C) Kirk medium. Serum bottles (100 ml), closed with screw caps, containing 15 ml of medium were inoculated with agar plugs. Bottles were incubated statically in the dark at 30 °C (*P. chrysosporium* at 37 °C). During the first two weeks the bottles were flushed with air at least once a day and samples of the extracellular fluid (0.2 ml) were taken, centrifuged (5 min, 13000 **g**) and diluted 10-fold with water. Decolorization was measured by absorbance ratio (A520/A350 nm) decrease (Figure 1). The rates of Poly R decolorization (Table 1) were determined from these slopes. After 30 d incubation, the absorbance ratio (i.e. end value) was also measured. All measurements were done

in triplicate.

Ethene production. The method of Kelley (1988) was used with slight modifications. The fungi were grown in 100 ml serum bottles containing 5 ml HSW medium and closed with a cottonwool plug. Inoculation and incubation was as described above. To estimate peroxidative activity each day three serum bottles were closed with screw caps and α-keto-γ-methylthiolbutyric acid (KTBA) (Sigma, St Louis, USA) (0.5 ml) was added to give a final concentration of 3.3 mM. Ethene production was measured, after 2 h without addition of H_2SO_4, using a Packard 430 (Delft, The Netherlands) gas chromatograph with a Porapak R column (Chrompack, Middelburg, The Netherlands).

Enzyme assays. For each strain, three serum bottles (100 ml) with 15 ml HSW medium and two with HSW medium plus Poly R-478 (0.2 g l^{-1}) were inoculated and closed with cottonwool plugs. When the absorbance quotient of the Poly R bottles was between 1.0 and 0.8, the culture broth of the other 3 bottles was separated from the mycelium by centrifugation at 20000 **g** and assayed for the enzymes listed below. Spectrophotometric assays were done with a Perkin-Elmer (Norwalk, USA) 550A UV-Vis spectrophotometer at 30 °C.

Laccase (Polyphenol oxidase). The oxidation of 2,6-dimethoxyphenol (DMP) to an orange/brown dimer was used to measure laccase activity (ϵ_{468nm} ca 10000 $M^{-1}cm^{-1}$; Paszczynski et al. 1988). The reaction mixture contained up to 750 μl culture broth in 50 mM DMS, pH 4.5, and 1 mM DMP in a total volume of 1 ml.

Aryl (veratryl) alcohol oxidase (AAO). The oxidation of veratryl alcohol to veratraldehyde (ϵ_{310nm} 9300 $M^{-1}cm^{-1}$; Bourbonnais and Paice 1988) was followed. The reaction mixture contained up to 550 μl culture broth in 50 mM DMS, pH 4.5, and 2 mM veratryl alcohol in a total volume of 950 μl.

Lignin peroxidase (LiP). Two methods were used. (1) Following Tien and Kirk (1988), the reaction mixture contained up to 550 μl culture broth in 50 mM sodium tartrate, pH 2.5, and 2 mM veratryl alcohol (vacuum distilled) in a total volume of 1 ml. The reaction was started with 0.4 mM H_2O_2 and the formation of veratraldehyde was monitored. (2) After checking for AAO activity (see above) 0.4 mM H_2O_2 was added in a total volume of 1 ml (pH 4.5).

Manganese peroxidase (MnP). The H_2O_2-dependent formation of an orange/brown product from DMP was used to assay MnP activity. The reaction mixture contained 50 mM Na malonate, pH 4.5 (Wariishi et al. 1989), 1 mM DMP (Paszczynski et al. 1988), 1 mM $MnSO_4$ and up to 650 μl culture broth in a total volume of 1 ml. The reaction was started by the addition of 0.4 mM H_2O_2 and corrected for laccase activity present.

Table 1. Decolorization of the dye Poly R-478 by selected fungi grown on three different media: hemp stem wood, autoclaved and sterilized Kirk medium

	Hemp stem wood medium		Filter sterilized medium		Autoclaved Kirk medium	
Strain	Activity *	End value †	Activity	End value	Activity	End value
Undetermined P30	11.10±0.51	103± 5	9.40±1.49	158± 83	8.59±1.61	157± 87
Trametes E39	10.77±0.78	66± 10	2.96±0.17	337± 87	4.17±0.30	314± 68
Agaricales B47	10.86±0.37	112± 15	4.90±0.62	213± 93	8.51±0.39	126± 18
T. versicolor	8.81±0.51	137± 10	3.04±0.75	203± 7	3.14±0.41	262±122
Trametes B41	8.77±0.49	119± 53	3.60±0.28	215± 64	5.63±0.25	195± 18
Trametes N11	8.96±1.33	95± 49	2.97±0.36	242± 25	5.01±0.45	210± 17
Undetermined H37	8.39±0.36	126± 34	6.42±0.79	187± 54	6.14±0.33	193± 49
Bjerkandera BOS55	8.53±0.68	114± 5	4.78±0.11	190± 13	5.09±0.77	291± 39
P. chrysosporium SC	7.42±1.35	135± 17	6.64±1.45	343±163	7.53±1.03	291± 26
P. chrysosporium BKM	5.71±0.73	370±236	4.69±0.63	282± 65	5.55±1.65	310±106
B. adusta	6.66±0.54	72± 2	5.34±0.47	287± 13	5.43±0.81	180± 27
T. villosa	5.41±1.14	134± 29	1.95±0.36	671±182	1.20±1.01	653±141
D. confragosa GM2	3.71±0.16	147± 11	2.25±0.31	629± 13	1.48±0.54	615±123
Trametes S33	3.66±2.44	496±251	3.34±0.46	317± 71	2.97±0.71	318± 40
Polyporus B15	1.11±0.75	714±251	2.42±0.25	356± 60	3.36±1.55	527±205
Stereum S54	0.31±0.48	893±195	4.08±0.65	321± 33	3.85±0.37	357±107
Stereum S22	2.04±0.24	704± 26	0.02±0.01	1136± 13	0.47±0.73	944±267
C. indicum W23	0.06±0.06	997± 18	0.38±0.05	1009± 45	0.15±0.09	1023± 83
Undetermined B18	<0.01	1057± 25	0.12±0.03	1141± 13	0.03±0.02	1106± 16
Stereum H2	0.17±0.03	1010± 10	0.41±0.10	1019± 36	0.74±0.36	898±124
Blank	<0.01	1081± 16	<0.01	1216± 16	<0.01	1198± 11

* Poly R-478 decolorization rates expressed as absorbance ratio decrease (10^3*A520/A350 hr^{-1}) ± standard deviation.

† End value of Poly R-478 decolorization after 30 days incubation in absorbance ratio (10^3*A520/A350) ± standard deviation.

Glyoxal oxidase (GLYOX). H_2O_2-producing activity of culture broth was determined by the method of Kersten and Kirk (1987). The reaction mixture contained 50 mM DMS pH 4.5, 10 mM glyoxal, 0.01% phenol red, 10 μg horseradish peroxidase (type II) (Sigma, St Louis, USA) and 300 μl culture fluid in a total volume of 1 ml. The reaction was stopped after 1 h by adding 50 μl 2N NaOH, and then the mixture was assayed for phenol red oxidation at 610 nm against an appropriate blank. The H_2O_2-producing activity of culture broth was also assayed with other oxidase substrates including glucose, xylose, cellulose and lactose.

Lignin peroxidase induction. For the induction of LiP production (Tien and Kirk 1988) four serum bottles (100 ml) per strain with 15 ml HSW medium were inoculated, closed with screw caps and flushed with oxygen (10 min, 100 ml min^{-1}). After 3 d veratryl alcohol (4 mM) was added to two bottles. During 2 wks, every day 700 μl medium was alternately withdrawn from two (one with and one without veratryl alcohol) of the four bottles. After sampling the two bottles were flushed (10 min, 100 ml min^{-1}) with oxygen. In this experiment LiP activity was only assayed according to Tien and Kirk (1988).

RESULTS

Isolation of peroxidative fungi. With the HSW-benomyl-guaiacol medium more than 400 plates gave brown-coloured zones but only 127 fungi were isolated to pure cultures. Different enrichment and isolation techniques gave no significant increase in the number of isolated fungi. It was impossible to isolate the colorizing fungi from several plates with brown-coloured zones because fast growing, sporulating fungi interfered. At 45 °C, no peroxidative fungi could be isolated although some plates gave brown-coloured zones. Only 67 of the isolated fungi were capable of Poly R decolorization.

Poly R-478 decolorization. Typical examples of the time course of Poly R decolorization are presented in Figure 1. Although there was variation in the lag phase between replicates, the slopes during the period of maximum activity were similar and remained almost constant for at least 3 d. Based on the Poly R decolorization screening in the HSW medium, three of the newly isolated strains (undetermined P30, *Trametes* E39 and Agaricales B47) showed significantly higher ($P \leq 0.05$) peroxidative activity than the two *P. chrysosporium* strains (Table 1). The strain with the highest decolorization rate, undetermined P30, displayed almost twice as much activity as *P. chrysosporium* BKM in all three media tested. A good correlation was found between

the decolorization rate of the same fungus in filter sterilized and autoclaved Kirk medium (coeffient of determination R^2 = 0.83; $P \leq 0.001$) which indicates that filter sterilization is unnecessary.

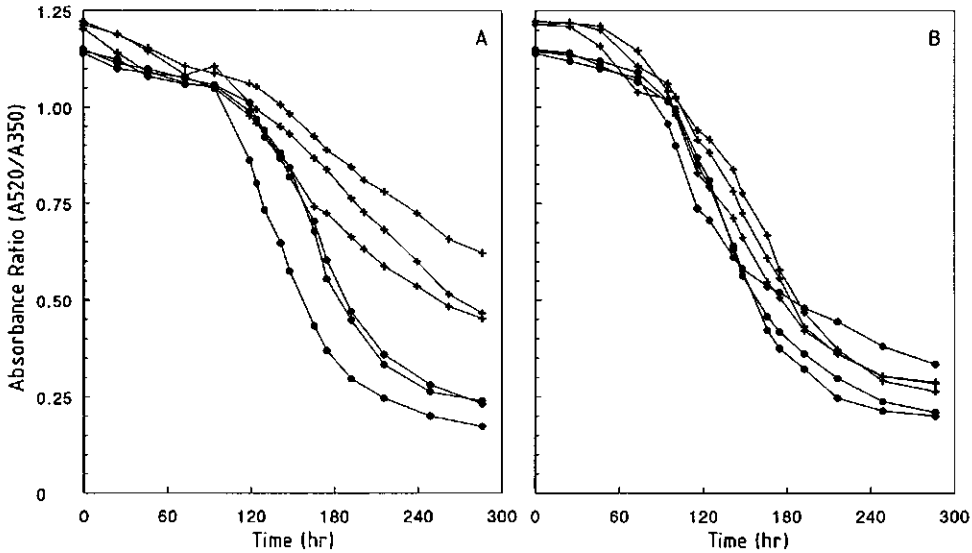

Figure 1. Typical examples of the decolorization of Poly R-478 by fungi. Triplicates of two different media are shown for *Trametes versicolor* (A) and for the undetermined strain H37 (B).
-●- HSW medium and -+- autoclaved Kirk medium.

The decolorization rate in HSW medium was generally twice the rate achieved in Kirk medium. The end values after one month incubation show the same differences between HSW and Kirk medium, indicating that not only the decolorization rate but also the total extent of decolorization was higher in the HSW medium. Although most strains had much higher activity on HSW medium, some strains, namely *Polyporus* B15 and *Stereum* S54, had lower activity on the HSW medium.

Ethene production. Typical examples of the time course of ethene formation are presented in Figure 2, but note that the standard deviations observed were rather large (Figure 2; Table 2). As was the case in the Poly R screening, a number of newly isolated strains had higher levels of ethene production compared to the well characterized white-rot strains (Table 2). The strain with the highest ethene formation rate, Agaricales B47 had almost nine times more activity than *P. chrysosporium* BKM. A highly significant correlation (R^2 = 0.48, $P \leq 0.001$) was found between both ethene production and Poly R decolorization, despite the large variation in the ethene production experiment (Figure 3). The two Poly R negative strains, undetermined B18

and the soft-rot fungus *C. indicum* W23, included in the screening as blanks, showed no ethene formation.

Table 2. The production of ethene from KTBA (ppm hr^{-1} ± standard deviation of a triplicate culture) of fungi grown in HSW medium. Most strains exhibited two maxima during the experiment. The day at which the first maximum occured is given

Strain	Day	First peak (ppm hr^{-1})	Second peak (ppm hr^{-1})	Strain	Day	First peak (ppm hr^{-1})	Second peak (ppm hr^{-1})
Undetermined P30	6	7.46 ± 1.07		*B. adusta*	6	6.12 ± 1.61	
Trametes E39	5	4.73 ± 1.07	5.65 ± 1.67	*T. villosa*	5	1.34 ± 0.28	7.50 ± 2.46
Agaricales B47	6	13.4 ± 3.67	14.2 ± 1.97	*D. confragosa* GM26		4.42 ± 1.45	
T. versicolor	7	5.33 ± 0.72	5.57 ± 2.24	*Trametes* S33	9	6.34 ± 3.08	3.10 ± 0.47
Trametes B41	6	3.59 ± 2.33	5.74 ± 3.80	*Polyporus* B15	9	2.51 ± 1.46	5.90 ± 2.88
Trametes N11	6	4.10 ± 1.69	4.67 ± 3.41	*Stereum* S54	12	0.44 ± 0.09	3.50 ± 1.72
Undetermined H37	11	8.33 ± 4.63		*Stereum* S22	13	1.12 ± 0.37	
Bjerkandera BOS55	6	11.3 ± 1.95	12.7 ± 3.12	*C. indicum* W23		N.D.*	
P. chrysosporium SC6		1.27 ± 0.33	2.46 ± 1.41	Undetermined B18		N.D.*	
P. chrysosporium BKM	7	0.82 ± 0.43	1.65 ± 0.86	*Stereum* H2	13	1.14 ± 0.43	

* N.D. = Not Detected

Extracellular enzymes. The enzyme assays (Table 3) showed that all Poly R decolorizing strains have MnP activity. High laccase levels made the detection of MnP difficult with the assay method utilized, since the laccase activity had to be subtracted from the MnP activity.

When AAO activity is present in the culture broth, veratryl alcohol is not an appropriate substrate for the LiP assay. Both the high blank activity and the H_2O_2 production by AAO make it difficult to distinguish these enzymes. Experiments were carried out to determine the effect of O_2 and veratryl alcohol/O_2 on the LiP production. In both cases, except for *P. chrysosporium*, no extracellular LiP production was detected in the culture broth of the other fungi. In cultures supplemented with veratryl alcohol, significant *in vivo* oxidation (as seen from the absorbance increase of the culture broth at 310 nm) occurred in all strains (not only those containing AAO), except for *P. chrysosporium*, undetermined B18 and *C. indicum* W23 (data not shown). The high starting absorbance at 310 nm in the LiP assay made it impossible to see an inductive role of veratryl alcohol for LiP production by the other fungi.

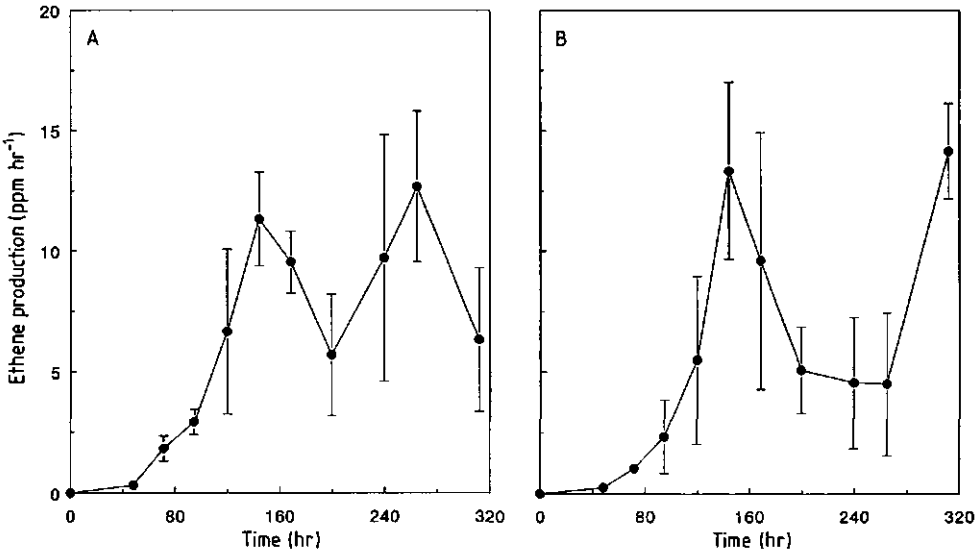

Figure 2. Ethene production rate in time of two newly isolated strains, *Bjerkandera* BOS55 (A) and Agaricales B47 (B) grown in HSW medium. All values reported as means ± standard deviation for three replicate cultures.

The horseradish peroxidase / phenol red assay revealed that mainly GLYOX could be detected in the extracellular fluids. With this assay other H_2O_2 producing oxidases could be detected in the extracellular fluids of three of the fungi. *Trametes* N11, *Trametes* E39 and *Polyporus* B15 had cellobiose oxidase activity on both cellobiose as well as lactose. *Trametes* E39 and *Polyporus* B15 showed oxidase activity with glucose and xylose. GLYOX was also detected in extracellular fluids of *T. versicolor*, *B. adusta* and *D. confragosa* GM2 when grown under an oxygen atmosphere.

The Poly R negative strains, *C. indicum* W23 and undetermined B18 did not produce detectable levels of peroxidases nor oxidases; however small quantities of laccase were detected.

Table 3. Enzyme activities in the culture fluid of the different fungi grown in HSW medium *

Enzymes / Strain	Laccase	Manganese Peroxidase	Lignin Peroxidase	Aryl Alcohol Oxidase	Glyoxal Oxidase
Undetermined P30	+/—	+	—	++	—
Trametes E39	++	++	—	—	+
Agaricales B47	+/—	++	—	+	—
T. versicolor	++	+	—	—	—
Trametes B41	++	++	—	—	+
Trametes N11	++	+	—	—	+
Undetermined H37	+/—	++	—	++	—
Bjerkandera BOS55	+	++	—	+/—	+
P. chrysosporium SC	—	+	++	—	++
P. chrysosporium BKM	—	++	+	—	+
B. adusta	+/—	++	—	+	—
T. villosa	++	+	—	—	+
D. confragosa GM2	+	+	—	—	—
Trametes S33	++	+	—	—	+
Polyporus B15	++	++	—	—	++
Stereum S54	++	++	—	+/—	++
Stereum S22	++	+	—	+	+
C. indicum W23	+/—	—	—	—	—
Undetermined B18	+/—	—	—	—	—
Stereum H2	++	++	—	—	+

* In all cases enzyme activity was found in all or in none of the three replicates. Laccase: ++ > 10 nmol min^{-1} (ml sup)$^{-1}$; + > 1 nmol min^{-1} (ml sup)$^{-1}$; +/- > 0.1 nmol min^{-1} (ml sup)$^{-1}$. Manganese peroxidase (MnP): ++ > 10 nmol min^{-1} (ml sup)$^{-1}$; + > 1 nmol min^{-1} (ml sup)$^{-1}$. Lignin peroxidase (LiP): ++ = 2 nmol min^{-1} (ml sup)$^{-1}$; + > 0.5 nmol min^{-1} (ml sup)$^{-1}$. Aryl alcohol oxidase (AAO): ++ > 2 nmol min^{-1} (ml sup)$^{-1}$; + > 0.5 nmol min^{-1} (ml sup)$^{-1}$; +/- > 0.1 nmol min^{-1} (ml sup)$^{-1}$. Glyoxal oxidase (GLYOX): ++ > 200 mAU hr^{-1}, + > 20 mAU hr^{-1}. For all enzyme assays: - = no activity detected.

DISCUSSION

The isolation and screening method described in this study has revealed several strains of fungi with higher peroxidative activities compared to the known reference strains. Three strains, undetermined P30, *Trametes* E39 and Agaricales B47, when grown on lignocellulosic substrate (HSW medium) were significantly more active in decolorizing Poly R compared to the two *Phanerochaete* strains. The ethene formation assay revealed two strains, *Bjerkandera* BOS55 and Agaricales B47, that were significantly more active than *T. versicolor* and the two *Phanerochaete* strains. The enzyme assays (Table 3) can only be viewed as qualitative because the evolution of the different enzymes was not measured with time. It is well known that the amount of several enzymes produced during secondary metabolism fluctuates considerably during the time course of cultivation (Szklarz et al. 1989). Furthermore, the ligninolytic enzymes in some fungi are strongly associated with the cell wall which gives an apparently low titre in the culture broth (Waldner et al. 1988). All the Poly R decolorizing strains secreted MnP. In contrast, LiP was only found in *P. chrysosporium*. Kuwahara et al. (1984) confirmed that the MnP of *P. chrysosporium* is at least partly responsible for the decolorization of Poly R. However, it is still possible that intracellular or cell wall bound LiP was present. Recently, Wariishi et al. (1991) have shown that MnP of *P. chrysosporium* has extensive lignin degrading capacities. Also, the decolorization of kraft lignin is mainly due to MnP activity (Michel et al. 1991). Many of the MnP secreting Poly R decolorizing fungi were also found to produce laccase. This is in agreement with the results of Nerud et al. (1991), but contrasts with those of Szklarz et al. (1989). Lin et al. (1991) have used DMP as a staining substrate for MnP activity present on the fungal mycelium. However, this method can only be used when the particular fungus studied does not have any laccase activity. Immunogold cytochemistry (Daniel et al. 1990) would distinguish between the different peroxidases both *in vivo* and *in vitro*.

There is no clear correlation between Poly R decolorization or ethene formation (Tables 1 and 2) with enzyme activities (Table 3) assayed. It can be concluded therefore, that the decolorization rate of Poly R and ethene formation from KTBA is not related to any one enzyme in particular but to the 'complete' ligninolytic system (enzyme complex, secondary metabolites, H_2O_2 generation, etc.) (Gold et al. 1988).

Some fungi displayed no activity on any of the oxidase substrates tested although Poly R decolorization did occur. Thus, it seems reasonable to expect that other enzymes, such as methanol oxidase, oxalic acid oxidase or manganese peroxidase (Paszczynski et al. 1988), play a role in the hydrogen peroxide production in those fungi. Both *P. chrysosporium* BKM and SC did not excrete detectable

amounts of cellobiose oxidase or glucose oxidase when grown on HSW medium, even though this enzyme has previously been found in this fungus (Ayers et al. 1978, Kelley and Reddy 1986).

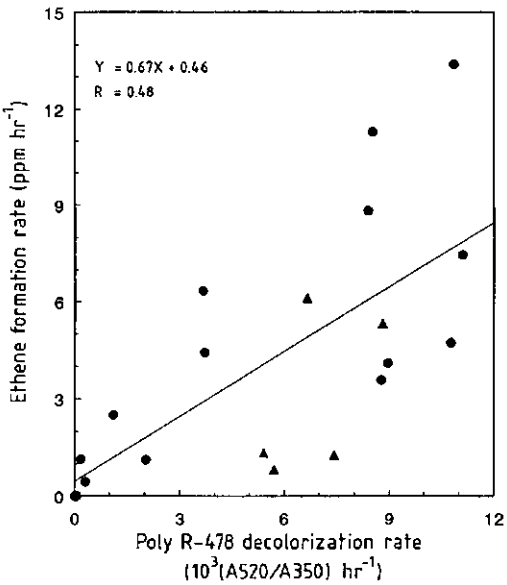

Figure 3. The correlation between the Poly R-478 decolorization rate (delta 10^3*(A520/A350) hr^{-1}) and the ethene production rate (ppm hr^{-1}) of the isolated (●) and reference (▲) strains grown in HSW medium. Both rates are means of triplicates.

A highly significant correlation (R^2 = 0.48; $P \leq 0.001$) was found between Poly R decolorization and ethene formation (Figure 3). Therefore, either is suitable for detecting ligninolytic activity. However, when numerous fungi have to be tested for peroxidative activity, Poly R decolorization is recommended because of simpler methodology, low costs and high reproducibility. Furthermore, the rate of Poly R decolorization (Figure 1) is suitable for optimizing culture conditions. The high fluctuation in the lag periods prior to the first appearance of peroxidative activity (Figure 1) can possibly be reduced by inoculating with a homogeneous suspension of mycelium instead of agar plugs, which in turn can reduce the standard deviation in the ethene formation (Figure 2) significantly.

While the decolorization rate of Poly R was stable for several days, ethene production gave sharp peaks during the time course of the assay. Datta et al. (1991) published analogous results for the peak in peroxidase activity and the linear absorbance increase of aspen pulp extract.

The results indicate that there are roughly two groups of fungi, one producing GLYOX and the other producing AAO. This constitutes yet another example of major differences existing in the ligninolytic system of different white-rot fungi. It also stresses again the importance of studying other white-rot fungi than the well known *P. chrysosporium* and *T. versicolor* to get a better insight in the diversity of the ligninolytic

system occurring in nature.

The newly isolated strains are currently being tested for biopulping, lignin degradation and polycyclic aromatic hydrocarbon oxidation to evaluate the benefits of this isolation and screening procedure for practical applications.

ACKNOWLEDGEMENTS

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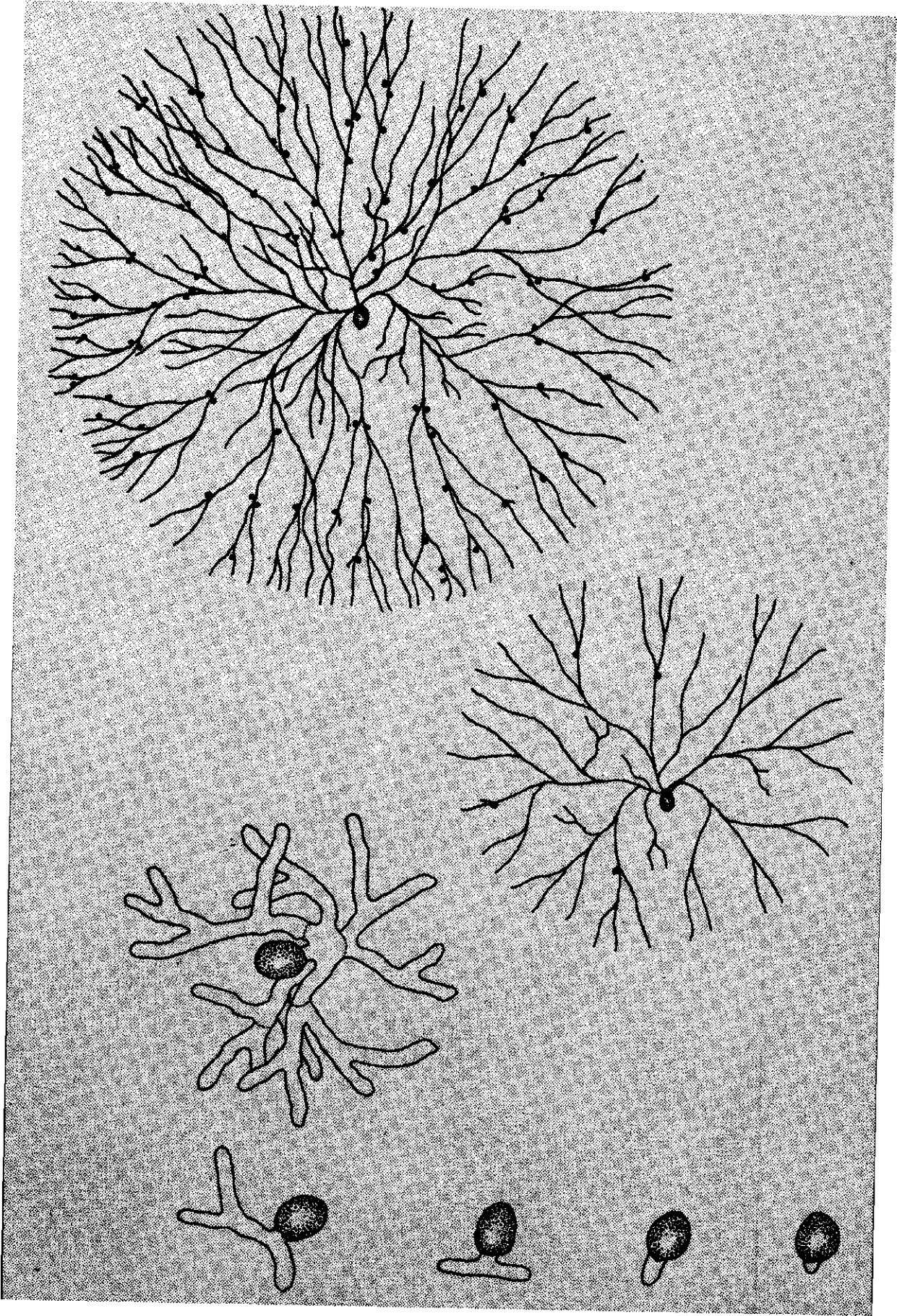
Stages in the germination of a fungal spore to form a circular colony



CHAPTER 4

EVIDENCE FOR A NEW EXTRACELLULAR PEROXIDASE: MANGANESE INHIBITED PEROXIDASE FROM THE WHITE-ROT FUNGUS *BJERKANDERA* SP. BOS55

Ed de Jong, Jim A. Field and Jan A.M. de Bont

SUMMARY

A novel enzyme activity was detected in the extracellular fluid of *Bjerkandera* sp. BOS55. The purified enzyme could oxidize several compounds like phenol red, 2,6-dimethoxyphenol (DMP), Poly R-478, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and guaiacol with H_2O_2 as an electron acceptor. In contrast, veratryl alcohol was not a substrate. This enzyme also had the capacity to oxidize DMP in the absence of H_2O_2.

With some substrates, a strong inhibition of the peroxidative activity by Mn^{II} was observed. Phenol red oxidation was inhibited by 84% with only 1 mM of this metal ion. Because DMP oxidation by this enzyme is only slightly inhibited by Mn^{II}, this substrate should not be used in assays to detect manganese peroxidase. The enzyme is tentatively named "Manganese Inhibited/Independent Peroxidase".

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INTRODUCTION

White-rot fungi are capable of depolymerizing lignin and metabolizing it to CO_2 and H_2O. The ligninolytic enzyme complex of the fungus *Phanerochaete chrysosporium* has been studied most extensively. Two extracellular peroxidases, ligninase (LiP) and manganese peroxidase (MnP), are thought to have an important role in the initial lignin degradation (Kirk and Farrell 1987, Wariishi et al. 1991). Both peroxidases were discovered in the extracellular fluid of this organism (Tien and Kirk 1983, Glenn and Gold 1983, Kuwahara et al. 1984). The mechanism of lignin model compound oxidation by LiP was elucidated in 1985 (Schoemaker et al. 1985), but its exact function in lignin degradation is still a point of dispute (Hammel and Moen 1991, Sarkanen et al. 1991). Also there are conflicting reports on the importance of MnP in the ligninolytic system (Wariishi et al. 1991, Perez and Jeffries 1990).

Waldner et al. (1988) and Nerud et al. (1991) have detected extracellular peroxidase activity in the culture fluids of various white-rot fungi (*Bjerkandera adusta*, *Pycnoporus cinnabarinus* and *Dichomitus squalens*) that cannot be attributed to LiP or MnP. Moreover, the unknown peroxidase activity on ABTS or phenol red was inhibited by Mn^{II}.

In this study, we present the isolation and initial characterization of an manganese inhibited/independent peroxidase from the white-rot fungus *Bjerkandera* sp. BOS55.

MATERIALS AND METHODS

Organism and culture conditions. BOS55 was isolated from forest soil samples with a selective medium containing hemp (*Cannabis sativa*) stem wood (0.2%), benomyl (15 ppm), guaiacol (0.01%) and agar (1.5%). BOS55 was determined as a *Bjerkandera* sp. by the Centraal Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands). The fungus was maintained on malt agar plates at 4 °C. BOS55 was cultivated under static conditions at 30 °C in 5 l erlenmeyer flasks on hemp stem wood medium (500 ml containing 0.2% hemp stem wood in 10 mM dimethylsuccinate pH 4.5). Cultures were inoculated with two cylindrical agar plugs (6 mm diameter) from the agar plates.

Enzyme purification. Medium from 10-12 day old cultures was centrifuged at 20,000 **g** for 30 minutes to remove mycelium. The supernatant was filtered through a 595½ filter (Schleichel & Schuell, Dassel, Germany) and concentrated on DEAE-Sepharose CL-6B column (25 x 2.8 cm). The adsorbed protein was washed with starting buffer

(100 ml) and eluted with a linear gradient from 0 to 1 M NaCl in 10 mM sodium acetate pH 5.5. Active fractions (250-350 mM NaCl) were pooled, washed with 10 mM sodium acetate pH 5.5 and concentrated by ultrafiltration through a PM-10 membrane (Amicon, Rotterdam, The Netherlands). Two ml of the concentrated fraction was purified with gel filtration. Using Sephadex G-100 gel chromatography with 20 mM sodium acetate 0.1 M NaCl pH 5.5 buffer (47 ml $hour^{-1}$, column 65 x 1.7 cm), one peak of activity was obtained. Active fractions were pooled, concentrated and washed as described above. Concentrated enzyme oxidized 2,6-dimethoxyphenol (DMP) with an activity of 0.037 absorbance units min^{-1} (μl supernatant)$^{-1}$.

Native-PAGE was performed with 2 μl of the washed G-100 fraction on a Phast system (Pharmacia, Uppsala, Sweden).

Analytical methods. Peroxidase activity was measured by the oxidation of phenol red at 510 nm or DMP at 468 nm. The reaction mixture contained up to 300 μl culture broth or enzyme solution in 50 mM sodium tartrate pH 4.0 and 0.01% phenol red or 2 mM DMP. The reaction was started by the addition of 0.4 mM H_2O_2 in a total volume of 0.5 ml.

Chemicals. DEAE-sepharose CL-6B and Sephadex G-100 are products of Pharmacia (Uppsala, Sweden). Horseradish peroxidase grade II (HRP), dithiotreitol (DTT) and ABTS were from Boehringer (Mannheim, Germany). All other chemicals were commercially available and used without further purification.

RESULTS AND DISCUSSION

In the extracellular fluids of 10-12 day-old cultures of *Bjerkandera* sp. BOS55, a new peroxidase was found that oxidizes phenol red. The purified peroxidase seems to be different from LiP and MnP. The new peroxidase showed no activity with veratryl alcohol, which is an indicative substrate for LiP. Moreover, the new peroxidase oxidized DMP which is not a substrate of LiP (Paszczynski et al. 1986). Although DMP is oxidized by MnP (Paszczynski et al. 1986), the new enzyme is not stimulated by Mn^{II} addition and moreover, the oxidation of phenol red is actually strongly inhibited by Mn^{II}. For this reason, the new peroxidase is tentatively denominated as manganese inhibited/independent peroxidase (MIP). The fact, that the activity was truly due to an enzyme, was confirmed by observing the loss of all activity after 20 minutes of boiling. MIP was also able to oxidize DMP in the absence of H_2O_2.

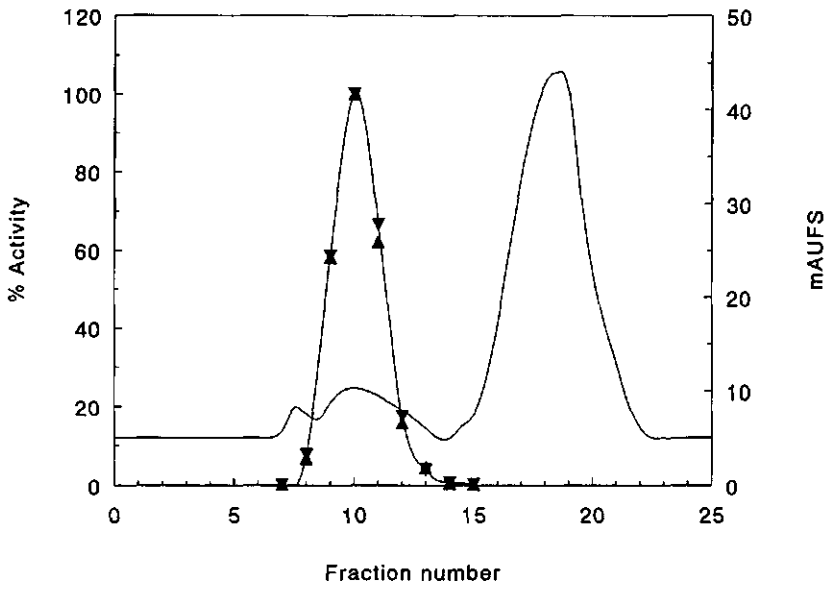
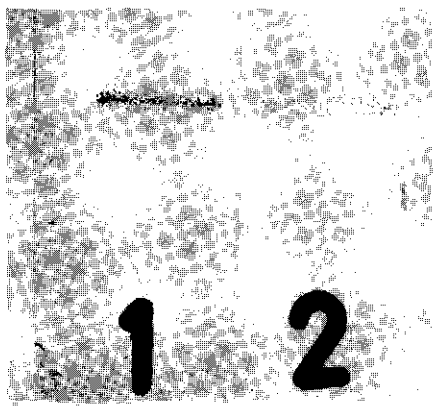


Figure 1. Chromatography of *Bjerkandera* BOS55 extracellular proteins on a Sephadex G-100 gel filtration column. Absorbance at 280 nm (—), oxidase activity with DMP (▲) and peroxidase activity with DMP (▼).

Figure 2. Native-PAGE electrophoresis of G-100 fraction. Enzyme activity was detected by immersing half of the gel in 50 mM sodium tartrate pH 4.0 containing 2 mM DMP and 0.4 mM H_2O_2 (1) and the other half in 50 mM sodium tartrate pH 4.7 containing 2 mM DMP (2).



As shown in Figure 1, the normalized peroxidase and oxidase activities in the different fractions from sephadex G-100 gel chromatography were identical, indicating that the same enzyme was involved. To test this hypothesis, native-PAGE electrophoresis was performed. As can be seen in Figure 2, both oxidase and peroxidase staining with DMP occurred in the same protein band which confirms that the oxidase and the

peroxidase activities originate from the same enzyme.

The occurrence of both oxidase and peroxidase activities in the same enzyme was also noted by Blaich and Esser (1975). Additionally, we found that even HRP displayed limited oxidase activity with DMP as substrate. It is known (McEldoon and Dordick 1991) that HRP has oxidase activity with dihydroxyfumaric acid. With phenol red as substrate, MIP did not have detectable levels of oxidase activity. Extracellular fluid of *Bjerkandera adusta* (Muheim et al. 1990a, Muheim et al. 1990b) has both peroxidase as oxidase activity with veratryl alcohol. In that case two distinct enzymes are involved, lignin peroxidase and veratryl (aryl) alcohol oxidase.

The pH optima for the oxidation of DMP in the absence and presence of H_2O_2 were 4.5 and 3.8, respectively (Figure 3). Almost the same pH optimum for peroxidase activity with phenol red and DMP was found (Figure 4). As can be seen in Figure 3, the oxidase activity at pH 4.0 accounted for only 10% of the total activity with DMP as substrate.

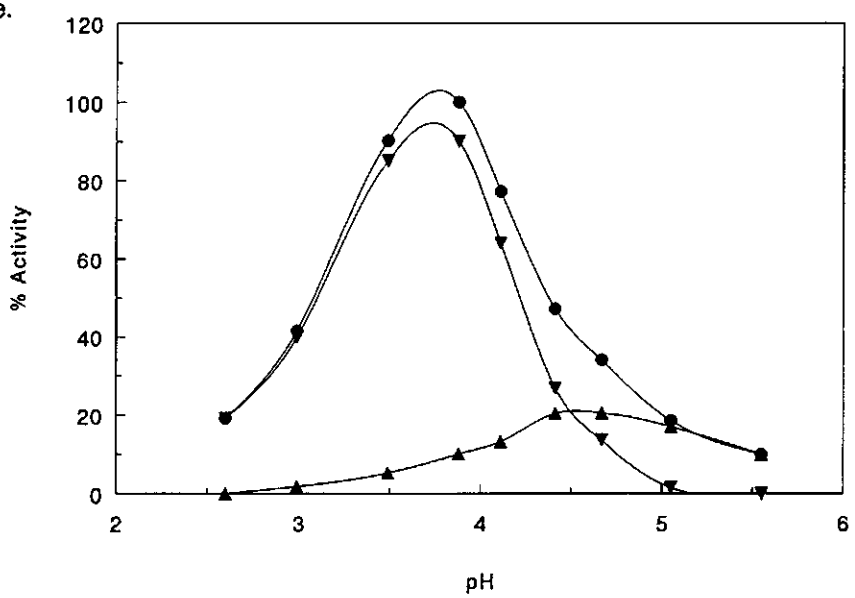


Figure 3. pH-dependency of DMP oxidation rate. Mixtures contained 2 mM DMP in 0.1 M sodium tartrate buffers (Oxidase activity (▲)) or mixtures contained 2 mM DMP and 0.4 mM H_2O_2 in 0.1 M sodium tartrate buffers (Total activity (●)). Peroxidase activity (▼) was calculated by substracting the oxidase activity from the total activity.

Several substrates were tested for oxidation by MIP and HRP, both in the presence and absence of Mn^{II}. It was found that with both enzymes the oxidation of some substrates were inhibited by manganese (Table 1). Because the protein concentration in the purified fraction was too low to assay, only a relative comparison of the MIP and

HRP substrate oxidation rates can be made. The MIP/HRP activity ratios of most of the substrates were comparable within one order of magnitude. However DMP formed an important exception, since the activity ratio on this substrate was from 10 to 49 times higher compared to the other substrates.

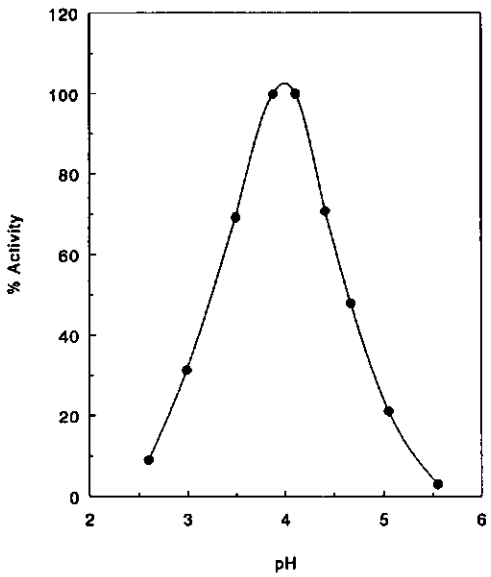


Figure 4. pH-dependency of phenol red oxidation rate. Mixtures contained 0.01% phenol red and 0.4 mM H_2O_2 in 0.1 M sodium tartrate buffers.

The data presented in Table 2 show that DMP oxidation by MIP was markedly inhibited by sodium azide and potassium cyanide, which are both potent inhibitors of hemeprotein catalyzed reactions. On the contrary, 1 mM of EDTA did not cause significant inhibition. In this respect, MIP is quite distinct from both LiP and MnP since several research groups report that these enzymes are highly inhibited by 1 mM EDTA (Kuwahara et al. 1984, Tuisel et al. 1990). Glenn and Gold (1985) did not find inhibition of MnP by 1 mM EDTA when excess manganese was present.

Table 1. Oxidation of various substrates by manganese inhibited peroxidase (MIP) and horseradish peroxidase (HRP) *

		MIP		HRP		MIP/HRP †
Substrate	Wavelength (λ)	Activity ‡ (ΔABS/min•ml)	Mn Inh ɪ (%)	Activity ‡ (ΔABS/min•μg)	Mn Inh ɪ (%)	
ABTS	415	118.0	29.2	252.0	<5.0	0.47
DMP	468	37.0	<5.0	1.6	14.4	23.1
Phenol Red	510	8.0	84.4	5.7	94.8	1.4
Guaiacol	450	8.0	9.4	10.3	<5.0	0.78
Poly R-478 §	520	0.18	N.D.♯	0.074	N.D.♯	2.4

* Rates of oxidation were followed at 30°C at the wavelengths indicated in the table. The complete reaction mixture (0.5 ml) contained 10 μl MIP enzyme (G-100 fraction) or 0.1 μg of HRP, 400 μM H_2O_2 and 100 μg substrate.

† Ratio of the rate of oxidation of substrates by MIP over the rate of oxidation by HRP under the conditions used.

‡ Activity expressed as delta absorbance units per minute with 1 ml of MIP or with 1 μg of HRP

ɪ Inhibition of MIP and HRP by Mn^{II} (1 mM final concentration).

§ In order to observe the reaction, 100 μl MIP and 2.0 μg HRP were added.

♯ Not determined.

The results of this study clearly indicate that aside from LiP and MnP, another peroxidase, MIP, is produced by white-rot fungi. Although we have only demonstrated the presence of MIP in one white-rot strain, the unknown peroxidase activity previously found in the extracellular culture fluids of various white-rot fungi (Waldner et al. 1988, Nerud et al. 1991) has striking similarities since the activity is also inhibited by Mn^{II}. This seems to indicate that MIP is a ubiquitous enzyme of white-rot fungi. At present, it is not clear what role MIP plays in lignin degradation. Therefore, future studies should be directed at elucidating the function that MIP serves in the ligninolytic system of white-rot fungi. In any case, based on the fact that a third peroxidase has been isolated from a white-rot fungus, the standard methods of measuring peroxidase activity in extracellular culture fluids must be reconsidered. This is particularly important for some substrates used for detecting MnP, such as DMP, which is also readily oxidized by MIP even in the presence of Mn^{II}. Phenol red is a better substrate for distinguishing between MnP and MIP, since the oxidation is almost completely inhibited by Mn^{II}. Furthermore, this study stresses once again that some peroxidases have phenol oxidizing activity, which suggests that phenol oxidation in the absence of H_2O_2 is not really a suitable method for distinguishing laccases from peroxidases.

Table 2. *Inhibition of initial DMP oxidation rates* *.

Inhibitor	% Activity remaining
Potassium cyanide (KCN)	14.4
Sodium azide (NaN_3)	1.2
Dithiotreitol (DTT)	0.0
2,2'-azinobis(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt (EDTA)	97.6

* Mixtures contained 10 μl enzyme, 1 mM inhibitor and 2 mM DMP in 50 mM sodium tartrate pH 4.0. After adding 0.4 mM H_2O_2 the total DMP oxidase activity was measured.

ACKNOWLEDGEMENTS

The authors want to thank F.P. de Vries and J.A.C. Oosterhaven (ATO, Wageningen, The Netherlands) for isolating the strain and conducting native-PAGE. Furthermore, we express our gratitude to Dr. H. E. Schoemaker for reviewing the manuscript.

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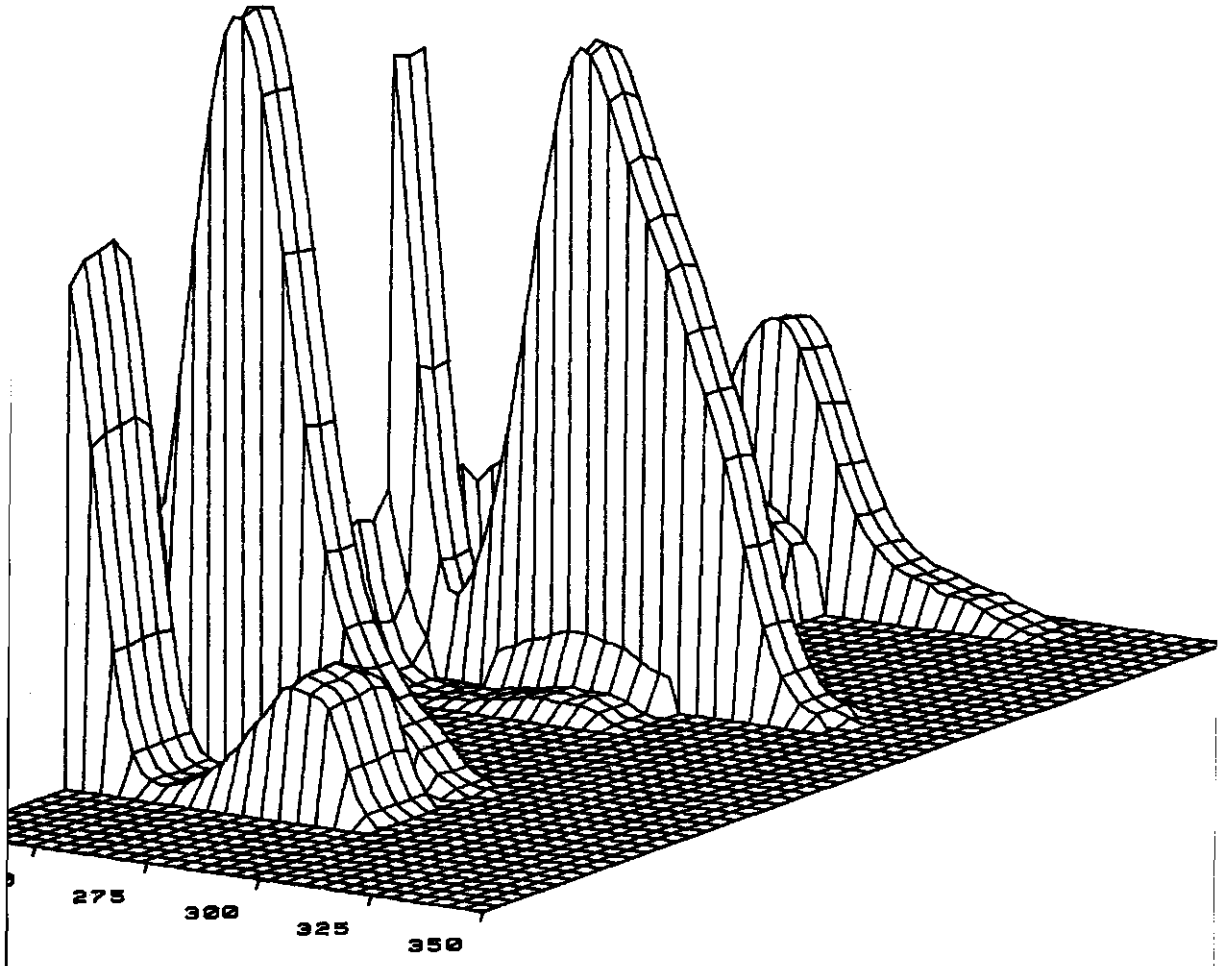
Several
chlorinated anisyl metabolites
(CAM)
are produced
de novo
by common
wood- and forest litter-degrading
fungi

The compounds were separated with a
Hewlett Packard HPLC system with diode
array detection

From left to right a
3-Dimensional (3-D) plot
of
the spectra of
3-chloro-anisyl alcohol,
4-fluorobenzaldehyde (internal standard),
3-chloro-anisaldehyde
and
3,5-dichloro-anisaldehyde can be seen



Wavelength [nm]

Time [min]

CHAPTER 5

DE NOVO BIOSYNTHESIS OF CHLORINATED AROMATICS BY THE WHITE-ROT FUNGUS BJERKANDERA SP. BOS55:
FORMATION OF 3-CHLORO-ANISALDEHYDE FROM GLUCOSE

Ed de Jong, Jim A. Field, Jacobus A.F.M. Dings,
Joannes B.P.A. Wijnberg and Jan A.M. de Bont

SUMMARY

The white-rot fungus *Bjerkandera* sp. BOS55 produced *de novo* several aromatic metabolites. Besides veratryl alcohol and veratraldehyde, compounds which are known to be involved in the ligninolytic system of several other white-rot fungi, other metabolites were formed. These included anisaldehyde, 3-chloro-anisaldehyde and a yet unknown compound containing two chlorine atoms. Additionally GC/MS analysis revealed the production of small amounts of anisyl alcohol and 3-chloro-anisyl alcohol. After 14 days, the extracellular fluid of *Bjerkandera* BOS55 contained 100 μM veratraldehyde and 50 μM 3-chloro-anisaldehyde. This is the first report of *de novo* biosynthesis of simple chlorinated aromatic compounds by a white-rot fungus. Anisaldehyde and 3-chloro-anisaldehyde were also produced by *Bjerkandera adusta* but not by *Phanerochaete chrysosporium*.

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INTRODUCTION

White-rot fungi, which belong to the basidiomycetes, are the most effective lignin degraders in nature. The best characterized white-rot fungus is *Phanerochaete chrysosporium* (Buswell 1992). Besides the complex and heterogeneous aromatic biopolymer lignin, this fungus is also able to degrade a wide range of xenobiotics, including polycyclic aromatic hydrocarbons and pentachlorophenol (Aust 1990; Field et al. 1992). Lignin biodegradation results from the combined action of several enzymes working together in narrow co-operation.

An interesting aspect of the lignin degrading system of *P. chrysosporium*, is the involvement of a aromatic metabolite, veratryl alcohol. Addition of veratryl alcohol to whole cultures of *P. chrysosporium* increases the lignin degrading activity (Buswell 1992). Veratryl alcohol is an important substrate for one of the key enzymes in lignin degradation, lignin peroxidase (Buswell 1992; Tien and Kirk 1988). Furthermore, veratryl alcohol protects this enzyme from inactivation by H_2O_2 (Haemmerli et al. 1986; Valli et al. 1990). Veratryl alcohol is biosynthesized *de novo* by *P. chrysosporium* (Lundquist and Kirk 1978) and various other white-rot fungi including *Bjerkandera adusta* (Berger et al. 1986), *Pycnoporus cinnabarinus* (Hatakka 1985), *Phlebia radiata* (Kantelinen et al. 1986) and *Trametes versicolor* (Kawai et al. 1988). Phenylalanine, 3,4-dimethoxycinnamyl alcohol and veratrylglycerol are intermediates during the biosynthesis of veratryl alcohol by *P. chrysosporium* (Shimada et al. 1981).

Many simple, aromatic compounds derived from intermediates of the shikimic acid pathway are produced by fungi (Turner and Aldridge 1983). However, veratryl alcohol is the only metabolite known to play a definite role in lignin biodegradation. In the present paper we report the *de novo* biosynthesis of other aromatic metabolites, 3-chloro-anisaldehyde and anisaldehyde by white-rot fungi of the genus *Bjerkandera*.

MATERIALS AND METHODS

Microorganisms. *Bjerkandera adusta* (Willd.:Fr.) Karsten CBS 595.78 was obtained from the Centraal Bureau voor Schimmelcultures (CBS, Baarn, the Netherlands). *Phanerochaete chrysosporium* Burds. BKM F-1767 (ATTC 24725) was a kind gift from Prof. T.K. Kirk. *Bjerkandera* sp. BOS55 was isolated and determined as described before (de Jong et al. 1992). Strains were maintained at 4°C on hemp (*Cannabis sativa* L.) stem wood (0.2%) - BIII (Tien and Kirk 1988; de Jong et al. 1992) medium from which they were transferred to malt extract plates (1.5% agar, 0.35% malt extract, 0.5% glucose) and experiments were inoculated with one agar plug as described

before (de Jong et al. 1992).

Culture conditions. The standard basal medium used in the experiments was N-limited liquid BIII medium (Tien and Kirk 1988) with 10 g l^{-1} glucose in 20 mM 2,2-dimethylsuccinate (pH 4.5) buffer. The medium was autoclaved and a filter-sterilized thiamine solution (200 mg l^{-1}) was added (10 ml l^{-1}) afterwards. For measuring the time course of aromatic metabolites production, five-ml aliquots of BIII medium were placed in 30 ml loosely capped serum bottles and experiments were incubated statically (unshaken) under an air atmosphere at 30 °C. For the analysis of mass and ^{1}H-NMR spectra 500 ml aliquots of BIII medium were placed in 5 l erlenmeyers closed with a cotton plug. After inoculation the bottles were incubated statically for 30 days at 30 °C.

Isolation and identification of aromatic metabolites. To measure the amount of aromatic metabolites the entire culture (5 ml) was utilized for the extraction procedure. Consequently, separate cultures were prepared and analyzed in quadruplet on each sampling day. Sterile medium was incubated as an abiotic control. Acetonitrile (10 ml) was added to the culture bottles (5 ml medium, corrected for water evaporation losses), sealed with teflon lined silicon septa and extracted for one hour on a shake table (300 strokes min^{-1}; stroke=2cm). A two ml subsample of the extract was centrifuged (10 min, 13000 rpm) and 50 μL was injected into the HPLC.

For mass and ^{1}H-NMR spectra analysis 500 ml of culture broth was filtered (de Jong et al. 1992). The supernatant was saturated with NaCl and extracted with 2x10 ml dichloromethane. The organic phase was evaporated and this crude fraction was used for total mass spectra analysis. HPLC purified unknown compounds (20 combined injections) were used, after evaporation of the solvent with N_2, for mass and ^{1}H-NMR spectra analysis.

Synthesis of authentic compounds. 3-chloro-anisyl alcohol (3-chloro-4-methoxybenzyl alcohol) was prepared from 3-chloro-4-methoxybenzoic acid by reduction with borane (BH_3) in tetrahydrofuran (Yoon et al. 1973).

^{1}H-NMR ($CDCl_3$) δ (ppm): 3.03 (br s, -OH); 3.82 (s, -OCH_3); 4.46 (s, -CH_2-); 6.81 (d, J = 6.5 Hz, aromatic-H_5); 7.10 (dd, J = 2.0, 6.5 Hz, aromatic-H_6); 7.27 (d, J = 2.0 Hz, aromatic-H_2).

^{13}C-NMR ($CDCl_3$) δ (ppm): 55.92 (q); 63.74 (t); 111.73 (d); 121.99 (s); 126.26 (d); 128.79 (d); 133.79 (s); 154.03 (s).

3-Chloro-anisaldehyde (3-chloro-4-methoxybenzaldehyde) was prepared from 3-chloro-anisyl alcohol by a gentle oxidation with pyridinium dichromate in dry dichloromethane (Corey and Schmidt 1979). Melting point 61-62 °C.

^{1}H-NMR ($CDCl_3$) δ (ppm): 3.96 (s, -OCH_3); 7.02 (d, J = 8.5 Hz, aromatic-H_5); 7.75 (dd, J = 1.9, 8.5 Hz, aromatic-H_6); 7.87 (d, J = 1.9 Hz, aromatic-H_2); 9.82 (s, -CHO).

^{13}C-NMR ($CDCl_3$) δ (ppm): 56.26 (q), 111.44 (d), 123.43 (s), 130.00 (s), 130.29 (d), 130.93 (d), 159.54 (s), 189.42 (d).

Instruments. Supernatant (50 μl) was routinely analyzed on a Hewlett Packard HPLC Chemstation (Pascal series) (Waldbronn, Germany) equipped with a HP1050 pumping station, a HP1040 M series II diode array detector and a HP9000-300 data processor. The column (200 mm x 3 mm) filled with ChromSpher C18-PAH (5 μm particles) was from Chrompack (Middelburg, the Netherlands). Aromatic metabolites were analyzed with the following gradient (0.4 ml min^{-1}, 30°C): 90:10, 0:100 and 0:100 H_2O:CH_3CN at 0, 15 and 20 minutes, respectively. The UV absorbance was monitored at 2 nm wavelength intervals from 210 to 350 nm.

Mass spectra were measured with a Hewlett Packard HP5890 GC with 30 m DB17 column and a HP5970 MSD. Injection temperature was 200°C. The starting temperature of the column was 80°C, gradient 7°C min^{-1}, final temperature 240°C. Helium was used as carrier gas (20 ml min^{-1}).

^{1}H-NMR and ^{13}C-NMR spectra were obtained with a Bruker AC-E200 spectrometer (Rheinstetten-Forchheim, Germany). Synthesized compounds and HPLC purified metabolite were dissolved in $CDCl_3$. Tetramethylsilane was used as an internal standard.

Chemicals. Veratryl alcohol, veratraldehyde, anisyl alcohol (4-methoxybenzyl alcohol), anisaldehyde (4-methoxybenzaldehyde) were from Janssen Chimica (Tilburg, the Netherlands). 3-Chloro-4-methoxybenzoic acid was from Heraeus (Karlsruhe, Germany).

RESULTS

Identification of aromatic metabolites. When grown in a synthetic medium with glucose as sole source of carbon and energy, *Bjerkandera* sp. BOS55 produced several aromatic metabolites. A typical HPLC plot of a 14-day-old supernatant is shown in Figure 1. In the 14-day-old sterile synthetic medium none of the aromatic metabolites were detected. The aromatic metabolites were identified by extracting the supernatant with dichloromethane and analyzing the organic phase on GC/MS. In addition to veratraldehyde and veratryl alcohol, *Bjerkandera* BOS55 produced

anisaldehyde and 3-chloro-anisaldehyde. Furthermore a yet unknown peak was detected (Figure 1) which, based on GC/MS analyses, contained 2 chlorine atoms.

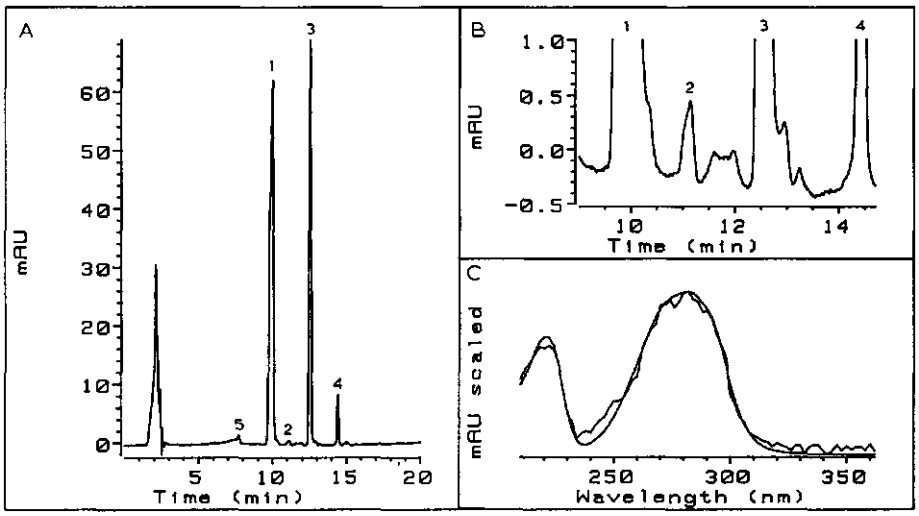
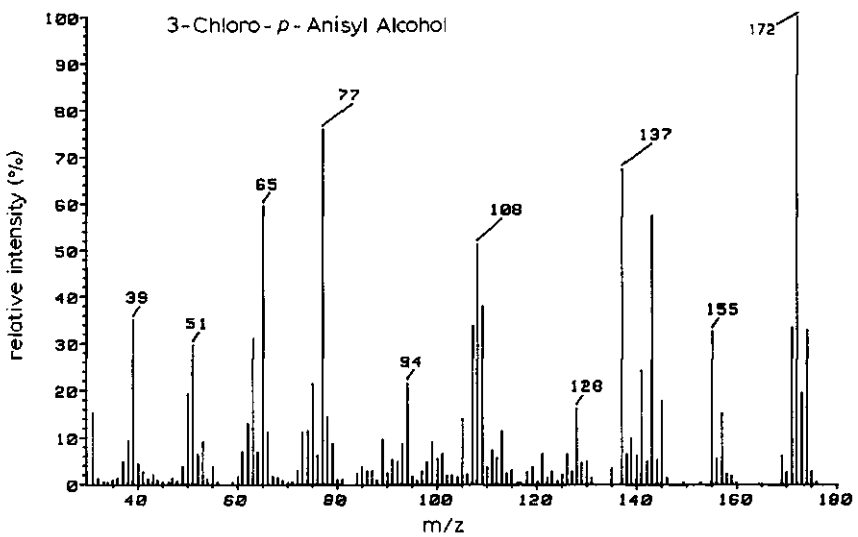


Figure 1. A) Typical HPLC chromatogram (λ 265 nm) of a 14-days old supernatant of *Bjerkandera* BOS55. B) Enlargement of the chromatogram. C) UV spectrum of peak 2 (rough line) and reference spectrum of anisaldehyde (smooth line). Peaks are 1) veratraldehyde, 2) anisaldehyde, 3) 3-chloro-anisaldehyde, 4) unknown compound, 5) veratryl alcohol



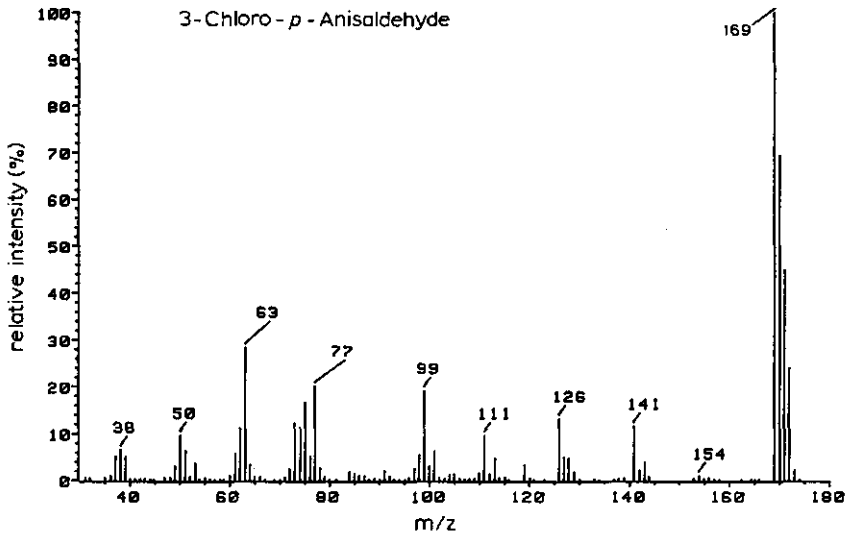


Figure 2. Mass spectra of 3-chloro-anisyl alcohol and 3-chloro-anisaldehyde

With the GC/MS, small amounts of anisyl alcohol and 3-chloro-anisyl alcohol were also detected in 30-day-old cultures. The mass spectra of synthesized 3-chloro-anisyl alcohol and synthesized 3-chloro-anisaldehyde are given (Figure 2). In Figure 3, the normalized UV-spectra of both 3-chloro-anisyl alcohol and 3-chloro-anisaldehyde are presented. For all aromatic metabolites detected with HPLC (Figure 1), the HPLC retention times, the UV spectra, the GC retention times and the mass spectra were identical to authentic compounds. 3-Chloro-anisaldehyde biosynthesized by *Bjerkandera* BOS55 was purified with HPLC. Its ^{1}H-NMR spectrum (^{1}H-NMR ($CDCl_3$) δ (ppm): 3.98 (-OCH_3); 7.03, 7.77, 7.90 ring protons; 9.84 (-CHO)) was identical to the spectrum of the synthesized compound.

Biosynthesis of aromatic metabolites by *Bjerkandera* BOS55. A typical time course of aromatic metabolite production by BOS55 is given in Figure 4. Although the amount of anisaldehyde decreases to very low concentrations after 14 days (Figure 1 and 4) it was still detectable. This is shown with the UV spectrum which fits quite well with the spectrum of the standard (Figure 1C). For most compounds, maximum concentration was reached after 10 (3-chloro-anisaldehyde (50 μM) and anisaldehyde (3 μM)) to 14 (veratraldehyde (100 μM)) days of incubation (Figure 2), only the amount of veratryl alcohol showed a significant increase during the rest of the experiment.

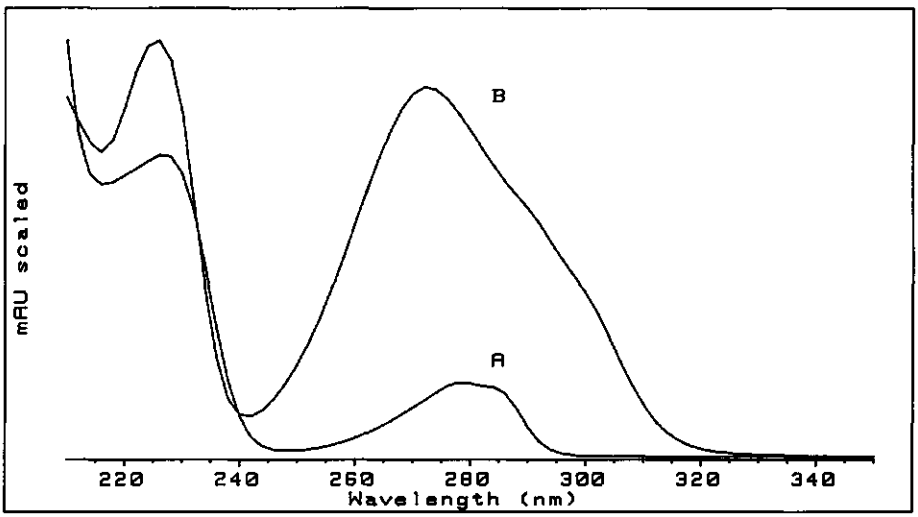


Figure 3. Scaled UV spectra recorded in acetonitrile/H_2O (80:20) of 3-chloro-anisyl alcohol (A) and 3-chloro-anisaldehyde (B)

Biosynthesis of aromatic metabolites by other strains. Formation of the newly detected aromatic metabolites was also investigated in *B. adusta* and *P. chrysosporium*. *Bjerkandera adusta* produced the same aromatic metabolites as *Bjerkandera* BOS55 as judged by HPLC and GC/MS. The metabolites occurred at similar concentrations in both *Bjerkandera* spp., except that the anisaldehyde (20 μM) concentration was higher in the supernatant of *B. adusta*. *P. chrysosporium*, however, only produced veratryl alcohol (0.1 mM after 14 days) as observed by HPLC analysis of the supernatant. GC/MS analysis also revealed trace amounts of veratraldehyde. Anisyl alcohol, 3-chloro-anisyl alcohol or the respective aldehydes, were not detectable by GC/MS.

DISCUSSION

Veratryl alcohol and veratraldehyde are known aromatic metabolites excreted by white-rot fungi which play an important role in lignin degradation (Buswell 1992). In the present article we show that white-rot fungi of the genus *Bjerkandera* produce two more pairs of aromatic metabolites not formed by *P. chrysosporium*: anisyl alcohol and anisaldehyde, 3-chloro-anisyl alcohol and 3-chloro-anisaldehyde. In contrast with *P.*

chrysosporium, *B. adusta* produces an extracellular aryl alcohol oxidase (Muheim et al. 1990), thought to be an important enzyme in the generation of H_2O_2. This enzyme oxidizes both veratryl alcohol and anisyl alcohol, displaying higher activity with the latter (Muheim et al. 1990). Preliminary results indicate that 3-chloro-anisyl alcohol is also a substrate for the aryl alcohol oxidase, suggesting that this alcohol is the precursor to 3-chloro-anisaldehyde.

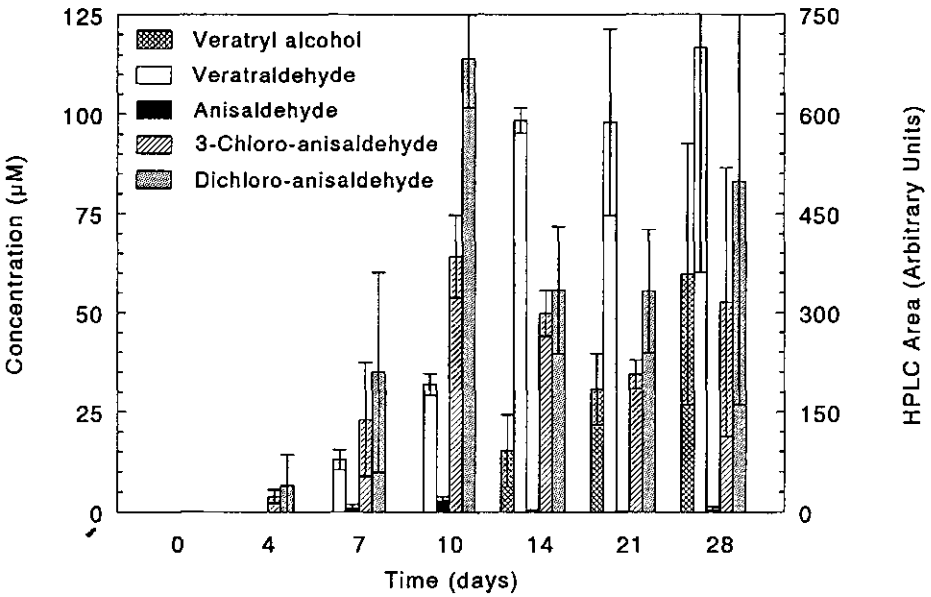


Figure 4. Time course of the *de novo* production of aromatic metabolites by *Bjerkandera* BOS55. The identified compounds are plotted in concentration (μM) units, the unknown compound containing two chlorine atoms is plotted in HPLC area units. The means of quadruplets ± standard deviation are given

As far as we know this is the first report of the *de novo* biosynthesis of 3-chloro-anisyl alcohol by fungi. On the contrary anisaldehyde and 3-chloro-anisaldehyde discovered in the *Bjerkandera* spp. are known aromatic metabolites of other fungi. *Lepista diemii* produces 3-chloro-anisaldehyde (Thaller and Turner 1972), anisaldehyde is produced by several fungi including *Camarophyllus virgineus* (Farrell et al. 1977), *Trametes suaveolens* (Birkinshaw et al. 1944), *Daedalea juniperina* (Birkinshaw and Chaplen 1955) and *Ischnoderma benzoinum* (Berger et al. 1987). Berger et al. (1986) screened many fungi for the production of flavours and fragrances. They found that *B. adusta* biosynthesizes anisaldehyde and veratraldehyde *de novo* and concluded that this fungus is a potential flavour producer. In this work we have demonstrated that under

certain culture conditions *Bjerkandera* spp. also form undesirable chlorinated products.

Biohalogenation is not a rare event in nature, at the moment more than 700 halogenated compounds are known (Neidleman 1975). This is, however, the first report of chlorinated aromatics production by a white-rot fungus.

We conclude that the ligninolytic complexes of *P. chrysosporium* and *Bjerkandera* spp. differ significantly both with respect to enzymes (aryl alcohol oxidase (Muheim et al. 1990) and manganese inhibited peroxidase (de Jong et al. 1992)) and aromatic metabolites produced. Further research in the physiological and ecological role of the above described aromatic metabolites in lignin degradation is therefore necessary.

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Crystals of
pure
3,5-dichloro-anisic acid methyl ether

This is the
first intermediate
in
the chemical synthesis
of
dichlorinated anisyl metabolites

(CAM)

These compounds
are
also biosynthesized
de novo
by
several common
saprophytic fungi

Magnification x 4

CHAPTER 6

PHYSIOLOGICAL ROLE OF CHLORINATED ARYL ALCOHOLS BIOSYNTHESIZED DE NOVO BY THE WHITE-ROT FUNGUS BJERKANDERA SP. BOS55

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SUMMARY

The white-rot fungus *Bjerkandera* spp. BOS55 produces veratryl, anisyl, 3-chloroanisyl and 3,5-dichloroanisyl alcohol and the corresponding aldehydes *de novo* from glucose. All metabolites are produced simultaneously with the extracellular ligninolytic enzymes and have an important physiological function in the fungal ligninolytic system. Both mono- and dichlorinated anisyl alcohols are distinctly better substrates for the extracellular aryl alcohol oxidases compared to veratryl alcohol. The formed aldehydes are readily recycled by reduction by washed fungal mycelium, thus creating an extracellular H_2O_2 production system regulated by intracellular enzymes. Lignin peroxidase does not oxidize the chlorinated anisyl alcohols both in the absence and in the presence of veratryl alcohol. It was therefore concluded that the chlorinated anisyl alcohols are well protected against the fungus's own aggressive ligninolytic enzymes. The relative amounts of veratryl alcohol and the chlorinated anisyl alcohols differ significantly depending on the growth conditions, indicating that the production of veratryl alcohol and the (chlorinated) anisyl metabolites are independently regulated.

We conclude that the chlorinated anisyl metabolites, biosynthesized by the white-rot fungus *Bjerkandera* sp. BOS55, can be purposeful for ecologically significant processes such as lignin degradation.

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INTRODUCTION

White-rot fungi are an important group of microorganisms responsible for the biodegradation of lignin (Kirk and Farrell 1987, Buswell 1992). Extracellular peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), are involved in the initial attack of the complex aromatic lignin polymer (Kirk and Farrell 1987) as well as aromatic xenobiotic compounds (Field et al. 1993). Lignin degradation and ligninolytic enzyme production by the model fungus, *P. chrysosporium* is a secondary metabolic event triggered by nitrogen, carbon or sulfur limitation (Kirk and Farrell 1987, Buswell 1992). Concurrently with the production of extracellular peroxidases, *P. chrysosporium* (Lundquist and Kirk 1978) and several other white-rot basidiomycetes (Gallois et al. 1990, Buswell 1992, de Jong et al. 1992b), produce a secondary metabolite, veratryl alcohol *de novo* from glucose. Since it was observed that the addition of veratryl alcohol to the culture broth increases lignin degradation, LiP production and LiP stability (Faison and Kirk 1985, Leisola et al. 1985, Haemmerli et al. 1986, Leisola et al. 1988), an extensive discussion has been unleashed over which physiological role(s) veratryl alcohol plays in lignin degradation. Four roles for veratryl alcohol in the ligninolytic system have been proposed. Firstly, the addition of exogenous veratryl alcohol was thought to induce lignin degradation and the production of LiP protein (Faison and Kirk 1985, Leisola et al. 1988). However, recent research seems to negate this role (Cancel et al. 1993). Secondly, veratryl alcohol functions to prevent inactivation of LiP by H_2O_2 (Haemmerli et al. 1986, Valli et al. 1990) and to convert inactive LiP compound III back to the native enzyme (Cai and Tien 1992). Thirdly, it has been postulated that veratryl alcohol functions as a charge-transfer mediator between the enzyme and a third substrate (Harvey et al. 1986, Harvey et al. 1992). However, this function has not yet been generally accepted (Valli et al. 1990). Results obtained with monomethoxy-substituted aromatic substrates (Harvey et al. 1986, Fawer et al. 1991), oxalic acid (Akamatsu et al. 1990, Popp et al. 1990) and polymeric dyes (Paszczynski and Crawford 1991) demonstrate that charge-transfer reactions do occur. Furthermore, the formation of activated oxygen species via veratryl alcohol has been confirmed (Haemmerli et al. 1987, Shah et al. 1992). Lastly, veratryl alcohol functions as a substrate for H_2O_2 producing enzymes. Several basidiomycetes, including *Trametes* (Farmer et al. 1960), *Pleurotus* (Bourbonnais and Paice 1988, Guillén et al. 1992) and *Bjerkandera* (Muheim et al. 1990, de Jong et al. 1992c) spp., produce aryl alcohol oxidase. The enzyme oxidizes aryl alcohols, including veratryl and anisyl alcohol, to the corresponding aldehydes with concomitant H_2O_2 formation.

White-rot fungi of the genus *Bjerkandera* are interesting ligninolytic

basidiomycetes because they posses high polycyclic aromatic hydrocarbon (PAH) degrading activity (Field et al. 1992, Field et al. 1993). *Bjerkandera* spp. produce several extracellular ligninolytic enzymes. The production of three peroxidases, LiP, MnP and manganese independent peroxidase (MIP) (Waldner *et al.* 1988, Kimura et al. 1990, de Jong et al. 1992a) and two H_2O_2-generating oxidases, aryl alcohol oxidase (AAO) (Muheim et al. 1990, de Jong et al. 1992c) and glyoxal oxidase (de Jong et al. 1992c) have been reported.

Bjerkandera spp. biosynthesize veratryl alcohol and veratraldehyde *de novo* from glucose together with anisaldehyde, 3-chloro-anisaldehyde and 3,5-dichloro-anisaldehyde, and their respective aryl alcohols (de Jong et al. 1992b, de Jong et al. 1993). In the present study, the physiological role of the chlorinated aromatics is examined. The time dependent production of the chlorinated aromatics and extracellular oxidative enzymes is followed. The reactions of the secondary metabolites with two ligninolytic enzymes, LiP and AAO, are investigated and the proposed purposeful production of the chlorinated aromatics is discussed.

METHODS

Fungal culture and growth conditions. *Bjerkandera* sp. BOS55 was isolated from Dutch forest soil samples (de Jong et al. 1992c) and deposited in the collection of the Centraal Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands). The fungus was routinely grown statically at 30 °C in a glucose/peptone medium as described by Kimura et al. (1990) or exceptionally in low nitrogen (glucose/BIII) medium (de Jong et al. 1992b). The time course of metabolite production and enzyme activity was measured in 30 ml loosely capped serum bottles containing 5 ml medium (de Jong et al. 1992b). Enzyme activity was analyzed in quadruplet, remaining culture fluid was combined and stored at -20 °C for FPLC analysis of extracellular proteins. Metabolite production was also analyzed in quadruplet from separate bottles at each time period since a sacrificed method was utilized for the extraction (de Jong et al. 1992b). For enzyme purification and washed cells experiments, 5 l erlenmeyers containing 500 ml medium were inoculated with *Bjerkandera* sp. BOS55 (Field et al. 1992, de Jong et al. 1992a) and grown for 12-14 days.

Enzyme assays. All enzymes were determined spectrophotometrically (Perkin-Elmer 550A UV-Vis) at 30 °C.

Lignin peroxidase (LiP). A modified method of Tien and Kirk (1988) was used, the reaction mixture contained up to 500 μl culture broth in 50 mM sodium tartrate pH 3.0

and 2 mM veratryl alcohol (vacuum distilled) in a total volume of 1 ml. The reaction was started with 0.4 mM H_2O_2 and the formation of veratraldehyde, (ϵ_{310nm} 9300 M^{-1} cm^{-1}) was monitored and corrected for aryl alcohol oxidase present.

Manganese dependent peroxidase (MnP) / Manganese independent peroxidase (MIP). A combined assay was used to distinguish the enzymes. The oxidation of 2,6-dimethoxyphenol (DMP) to an orange/brown dimer was used to measure laccase, MnP and MIP activity (ϵ_{469nm} 49.6 $mM^{-1}cm^{-1}$, Wariishi et al. 1992). Combined MIP/MnP activity: The reaction mixture contained 50 mM sodium malonate pH 4.5, 1 mM DMP, 1 mM $MnSO_4$ and up to 600 μl culture broth in a total volume of 1 ml (de Jong et al. 1992c). The reaction was started by the addition of 0.4 mM H_2O_2 and corrected for laccase activity present. MIP activity: The reaction mixture contained 50 mM sodium malonate, pH 4.5, 1 mM DMP, 1 mM EDTA and up to 600 μl culture broth in a total volume of 1 ml (de Jong et al. 1992a). The reaction was started by the addition of 0.4 mM H_2O_2 and corrected for laccase activity present. MnP activity is expressed as the combined MIP/MnP activity minus the MIP activity.

Aryl alcohol oxidase (AAO). The oxidation of anisyl alcohol to anisaldehyde (ϵ_{290nm} 15000 $M^{-1}cm^{-1}$, Muheim et al. 1990) was followed. The reaction mixture contained up to 200 μl culture broth in 20 mM potassium phosphate (KPi) buffer, pH 5.7 and 1 mM anisyl alcohol in a total volume of 500 μl.

Glyoxal oxidase. H_2O_2-producing activity of culture broth was determined by a modified method of Kersten and Kirk (1987). 500 ml 12-14 days-old extracellular fluid was washed (50 mM KPi, pH 6.0) and concentrated 10 fold by ultrafiltration (Amicon YM10). The reaction mixture contained 50 mM KPi, pH 6.0, 10 mM glyoxal, 0.01% phenol red, 10 μg horse-radish peroxidase (type II) (Sigma, St Louis, USA) and up to 300 μl culture fluid in a total volume of 1 ml. The reaction was stopped after 1h by adding 50 μl 2N NaOH, and then the mixture was assayed for phenol red oxidation at 610 nm against an appropriate blank. The H_2O_2-producing activity of culture broth was also assayed with other oxidase substrates including glyoxalate, glucose, xylose, galactose, cellobiose, anisyl alcohol, acetaldehyde, methanol, formaldehyde, oxalic acid, *D,L*-glycine, *D,L*-alanine, *D,L*-glutamate and *D,L*-aspartate.

Reduction of aldehydes by washed cells. Suspensions of 12-14 days-old cells (500 ml) were harvested as described before (de Jong et al. 1990), washed with 50 mM KPi buffer, pH 6.0, concentrated 10 fold and resuspended in the same buffer. Aldehydes were added at 250 μM final concentration. Reaction mixture was incubated statically at 30 °C. Samples (800 μl) taken at regular intervals were mixed with 200 μl 2 N HCl and analyzed by HPLC (de Jong et al. 1992b). Protein contents of whole cells were determined according to Bradford (1976).

Enzyme purification. *Time course of enzyme activity experiment:* Frozen samples were thawed and washed with 10 mM KPi, pH 6.0. After washing, enzyme activity was measured and extracellular enzymes were analyzed at 405 nm by anion exchange chromatography using a FPLC system (Mono-Q HR 5/5, Pharmacia). The column was equilibrated with 10 mM KPi, pH 6.0. The enzyme was eluted with a linear salt gradient (0-300 mM KCl in starting buffer) at a flow rate of 1 ml/min and 1 fraction/min during 40 min.

Purification of aryl alcohol oxidase (AAO) and lignin peroxidase (LiP). Aryl alcohol oxidase and lignin peroxidase were purified according to a modified procedure described in literature (Muheim et al. 1990). After filtration and concentration of the enzyme on DEAE-sepharose CL6B (de Jong et al. 1992a), aryl alcohol oxidase and lignin peroxidase were separated from other peroxidases by Mono Q HR 5/5 (FPLC system, Pharmacia) anion exchange chromatography (0-300 mM NaCl in 50 mM KPi pH 6.0, 40 ml, 0.5 ml min^{-1}). LiP and AAO eluted in the same fractions. More than 95% of both aryl alcohol oxidase and lignin peroxidase activity were retained after the anion exchange step. Gel filtration with Superdex 75 HR 10/30 (FPLC system, Pharmacia) (buffer 0.15 M NaCl in 50 mM KPi pH 6.0, 0.5 ml min^{-1}) separated lignin peroxidase from aryl-alcohol oxidase.

Synthesis of authentic compounds. 3-Chloro-anisyl alcohol (3-chloro-4-methoxybenzyl alcohol) and 3-chloro-anisaldehyde (3-chloro-4-methoxybenzaldehyde) were synthesized as described before (de Jong et al. 1992b). The synthesis of 3,5-Dichloro-anisyl alcohol (3,5-dichloro-4-methoxybenzyl alcohol) and 3,5-dichloro-anisaldehyde (3,5-dichloro-4-methoxybenzaldehyde) are published elsewhere (de Jong et al. 1993).

Instruments. Supernatant (50 μl) was routinely analyzed on a Hewlett Packard HPLC Chemstation (Pascal series) (Waldbronn, Germany) equipped with a HP1050 pumping station, a HP1040 M series II diode array detector and a HP9000-300 data processor. The column (200 mm x 3 mm) filled with ChromSpher C18-PAH (5 μm particles) was from Chrompack (Middelburg, The Netherlands). Aromatic metabolites were analyzed with the following gradient (0.4 ml/min, 30 °C): 90:10, 0:100 and 0:100 H_2O:CH_3CN at 0, 15 and 20 minutes, respectively. The UV absorbance was monitored at 2 nm wavelength intervals from 210 to 350 nm.

Chemicals. Mycological peptone was from Oxoid, (Basingstoke, UK). Veratryl alcohol (Janssen Chimica, Tilburg, The Netherlands) was vacuum distilled before use. All other chemicals were commercially available and used without further purification.

RESULTS

Production of enzymes and secondary metabolites. It has been shown that white-rot fungi of the genus *Bjerkandera* produce significant amounts of several aromatic compounds both in the laboratory and in the natural environment (de Jong et al. 1992b, de Jong et al. 1993). In addition to the production of common fungal metabolites, veratryl alcohol and veratraldehyde, the *de novo* biosynthesis from glucose of anisaldehyde, 3-chloro-anisaldehyde and 3,5-dichloro-anisaldehyde, and their respective benzyl alcohols, has been reported. To investigate the physiological role of the chlorinated metabolites, the time-dependent production of ligninolytic enzymes and secondary metabolites was followed in order to confirm their concurrent occurrence. When grown on glucose/BIII medium *Bjerkandera* sp. BOS55 produces MnP, MIP and AAO (de Jong et al. 1992a, de Jong et al. 1992c), but no LiP activity is detectable. On a glucose/peptone medium all enzymes, including LiP, are produced (Fig 1).

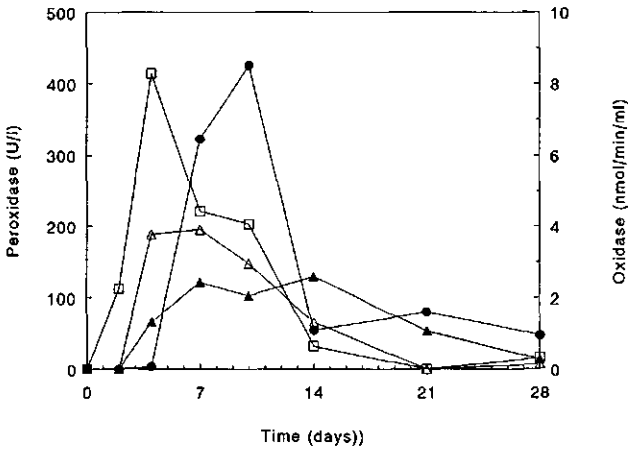

Figure 1. Time course of the production of ligninolytic enzymes by *Bjerkandera* sp. BOS55 grown on glucose/peptone medium. The means of quadruplets are given. (□) aryl alcohol oxidase (AAO); (▲) manganese peroxidase (MnP); (▲) manganese independent peroxidase (MIP); (△) lignin peroxidase (LiP).

Figure 1 and 2A reveal that the production of secondary metabolites coincides with the production of AAO. A remarkable increase in the production of veratryl alcohol in the glucose/peptone medium compared to glucose/BIII medium was observed (Fig 2A and 2B). The increased level of veratryl alcohol coincides with the onset of LiP production (Figure 1 and 2A).

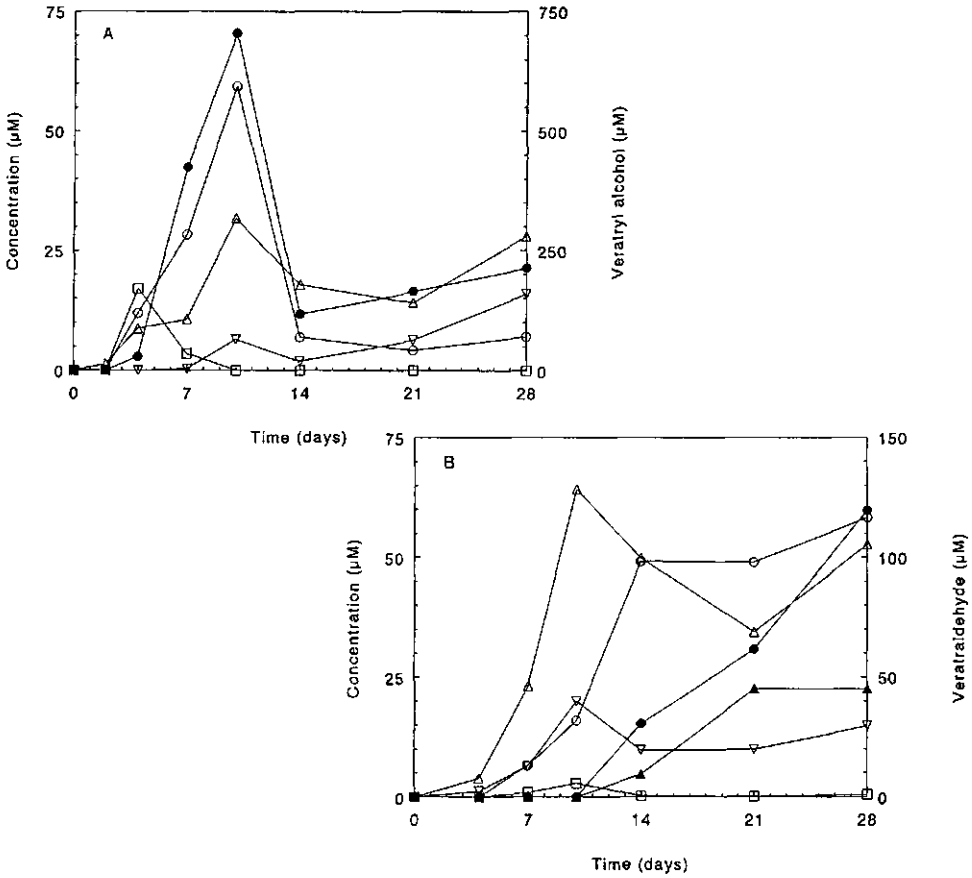

Figure 2. Time course of the *de novo* production of secondary metabolites by *Bjerkandera* sp. BOS55 grown on glucose/peptone (A) and low nitrogen (glucose/BIII) (B) medium. The concentration of veratryl alcohol (Figure 2A) and veratraldehyde (Figure 2B) is given on the right axis, the concentrations for all other metabolites are given on the left axis. The means of quadruplets are given. (●) veratryl alcohol; (○) veratraldehyde; (□) anisaldehyde; (△) 3-chloro-anisaldehyde; (▽) 3,5-dichloro-anisaldehyde.

After 21 days, all enzymes had low activities (Figure 1). To analyze if the proteins were inactivated or had disappeared from the extracellular fluids, the protein profiles were measured. Figure 3 shows that extracellular protein levels decreased dramatically during the time-course. It is possible, there are still intracellular or cell bound ligninolytic enzymes present after 28 days incubation. To check this hypothesis 28 day old cultures were washed with 1 M NaCl or sonified. In both cases no significant increases in any of the enzyme activities was detected.

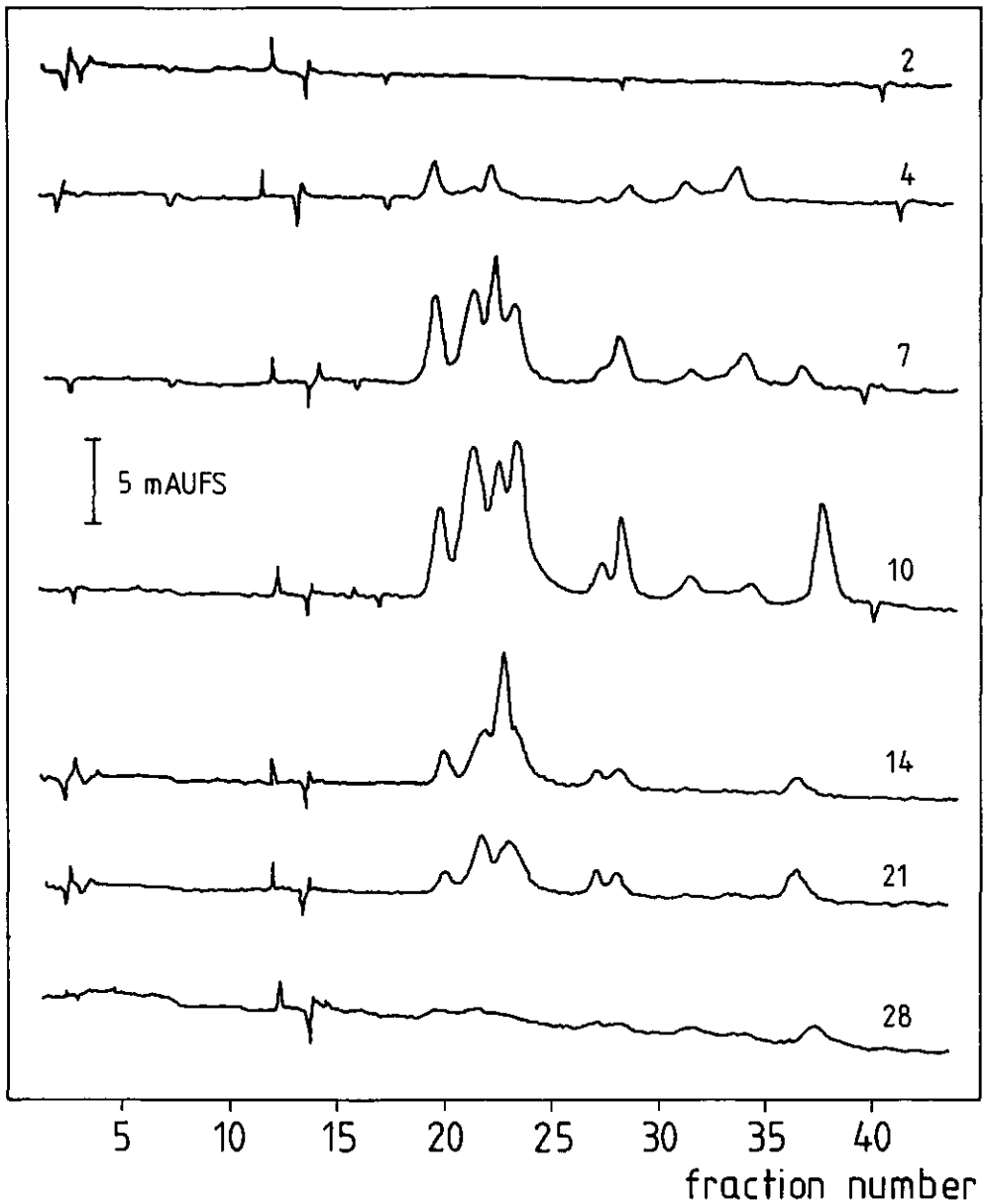

Figure 3. Protein profiles of extracellular fluid of *Bjerkandera* sp. BOS55 analyzed by anion exchange chromatography using a FPLC system (Mono-Q, λ 405 nm). Numbers in the figure represent the incubation time (days). The means of quadruplets are given.

After the removal of low molecular weight compounds by ultrafiltration, LiP activity at day 4 was increased more than 50 times. This effect of LiP activity increased by the ultrafiltration step was only seen with the day 4 sample. However, it should be kept in mind that this inhibiting activity can easily lead to misjudgments about the presence of the LiP protein. To determine if LiP inhibition is caused by compounds already present in the growth medium or by newly produced metabolite(s), washed supernatant containing LiP was incubated with uninoculated sterile glucose/peptone medium and with the dialysate of 4-day-old culture broth after ultrafiltration. The sterile glucose/peptone medium strongly inhibited LiP activity indicating that compounds in the fresh glucose/peptone medium cause the inhibition.

Substrate spectrum of aryl alcohol oxidase (AAO). Since the aryl aldehydes were produced together with AAO, the corresponding aryl alcohols were tested as substrates for this oxidase. To discriminate between peroxidase and aryl alcohol oxidase activity, the AAO enzyme was purified from glucose/peptone medium. It can be seen (Table 1) that all substituted anisyl alcohols are oxidized by AAO producing the corresponding aldehydes and H_2O_2. The Relative Activity/K_m value was almost 300 times higher for 3,5-dichloroanisyl alcohol compared to veratryl alcohol and 6.5 times higher for 3,5-dichloroanisyl alcohol compared to unsubstituted anisyl alcohol. Veratryl alcohol had the highest K_m.

Table 1. Catalytic properties for aryl alcohol oxidase oxidation of substituted benzyl alcohols.

Alcohol	λ * (nm)	ϵ † (mM^{-1} cm^{-1})	K_m ‡ (mM)	Relative Activity (%)⧗	Rel Act (%) /K_m
Veratryl	310	9.3	4.57	42.8	9.4
Anisyl	290	15.0	0.25	100	400
3-Chloro-anisyl	295	8.6	0.13	116	892
3,5-Dichloro-anisyl	255	8.2	0.034	88.5	2603

* Wavelength where the formation of the corresponding aldehyde was measured

† Molar extinction coefficient of the formed aldehyde

‡ The Michaelis-Menten constant K_m was measured in 20 mM KPi pH 5.7 at 30°C. K_m was calculated by a Lineweaver-Burke plot.

⧗ The amount of purified enzyme was not enough for an accurate protein measurement, so only relative activity (Rel act) are given.

Reduction of aldehydes by whole cells. If aryl alcohols are indeed important for the extracellular H_2O_2 production, one would expect that the substituted anisyl aldehydes

must be recycled by reduction to the corresponding alcohols. Whole cells of an 12 day-old culture (500 ml) were washed with buffer and thereafter incubated with the four aldehydes. All the aldehydes were reduced with comparable rates ranging from 2.1 nmol min^{-1} mg $protein^{-1}$ for 3,5-dichloro-anisaldehyde to 1.1 nmol min^{-1} mg $protein^{-1}$ for 3-chloro-anisaldehyde. As an example the stoichiometric conversion of 3,5-dichloro-anisaldehyde to 3,5-dichloro-anisyl alcohol is shown (Figure 4).

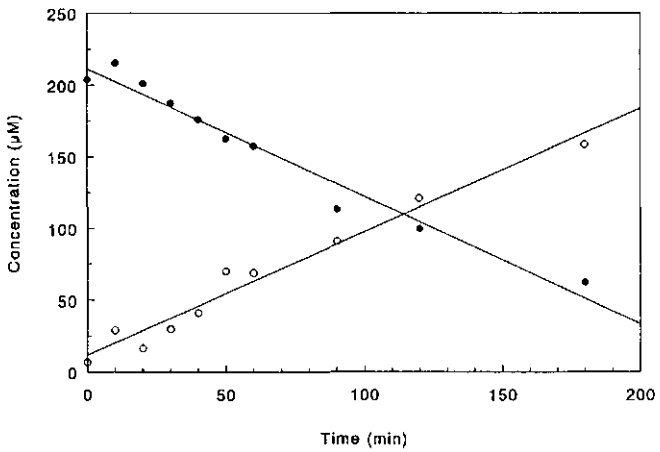

Figure 4. The reduction of 3,5-dichloro-anisaldehyde (●) by washed cells of *Bjerkandera* sp. BOS55 in stoichiometric amounts of 3,5-dichloro-anisyl alcohol (O).

Other extracellular oxidases. To investigate if AAO is the only extracellular enzyme capable of H_2O_2-production, the washed extracellular fluid of a 12 day-old culture (500 ml) was analyzed for other oxidases. With the coupled horseradish peroxidase/phenol red assay for H_2O_2 production, a positive result was found with the four sugars tested. Glucose was oxidized with a rate of 20 nmol min^{-1} ml sup^{-1}. The activities with xylose, galactose and cellobiose (3.4, 0.6 and 0.2 nmol min^{-1} ml sup^{-1}, respectively) were much lower. To compare glucose oxidase activity with AAO activity the 12 day-old extracellular fluid was also analyzed with the phenol red assay for anisyl alcohol oxidating activity. The fluid had an activity of 3.6 nmol min^{-1} ml sup^{-1}. All the other substrates, including glyoxal, did not stimulate H_2O_2 production at all.

Reactions of chlorinated anisyl alcohols with lignin peroxidase. The induction of LiP production only coincides with an increased veratryl alcohol production (Figure 1 and 2A) and not with the (chloro) anisyl alcohols. However, it is still possible that, like

veratryl alcohol, the chlorinated anisyl alcohols are also substrates for LiP. Furthermore, it is known that the oxidation of anisyl alcohol, a poor LiP substrate, is strongly increased by the addition of veratryl alcohol (Harvey et al. 1986, Valli et al. 1990). In Table 2 the results are presented on the LiP catalyzed oxidation of the aryl alcohols in the absence and presence of veratryl alcohol. It can be concluded that LiP does not oxidize the chlorinated anisyl alcohols. Contrary to anisyl alcohol, the oxidation of these chlorinated anisyl alcohols is not stimulated by veratryl alcohol.

Table 2. Oxidation of substituted aryl alcohols by lignin peroxidase (LiP) in the absence and presence of veratryl alcohol.

Substrate	VAlc	AAlc		ClAlc		DClAlc	
	VAld	AAld	VAld	ClAld	VAld	DClAld	VAld
Conditions *	Concentrations (μM)						
LiP + H_2O_2	124.4	16.4		6.3		8.5	
LiP	0.0	7.4		4.1		7.0	
H_2O_2	0.0	1.1		1.8		0.5	
LiP + H_2O_2 + VAlc		49.8	13.5	8.0	13.4	6.7	12.0
LiP + VAlc		6.0	1.7	6.6	1.7	6.1	2.7
H_2O_2 + VAlc		0.0	0.0	0.0	0.0	1.8	0.0

* Reaction mixture (1 ml) contained 20 mM sodium succinate pH 3.0, 1 μM LiP, 100 μM H_2O_2 and 500 μM substrate (VAlc = veratryl alcohol; AAlc = anisyl alcohol; ClAlc = 3-chloro-anisyl alcohol; DClAlc = 3,5-dichloro-anisyl alcohol) in the absence or presence of 20 μM veratryl alcohol. Blanks were without enzyme or H_2O_2. Reactions were initiated by the addition of enzyme and carried out for 60 min at 30 °C. Reactions were stopped by the addition of 50 μl 2 N HCl and products (VAld = veratraldehyde; AAld = anisaldehyde; ClAlc = 3-chloro-anisaldehyde; DClAlc = 3,5-dichloro-anisaldehyde) were analyzed by HPLC as described in the text.

DISCUSSION

This paper has shown that chlorinated anisyl metabolites are not just accidentally produced metabolites. Aside from their possible antimicrobial activity (Pfefferle et al. 1990), they may also fulfil an important physiological function in the ligninolytic system of *Bjerkandera* sp. BOS55.

All metabolites are produced simultaneously with the aryl alcohol oxidase and ligninolytic peroxidases. The dramatic decrease of all ligninolytic enzymes after 10 days

incubation (Figures 1 and 3) is probably caused by proteolytic activity. This has also been described for lignin peroxidase in *P. chrysosporium* cultures (Dosoretz et al. 1990a, 1990b, Cancel et al. 1993). Both chlorinated anisyl alcohols are, compared to veratryl alcohol, excellent substrates for the extracellular aryl alcohol oxidases (AAO). The addition of chlorine atoms to anisyl alcohol substantially increased the affinity of AAO for the alcohols. The AAO enzymes have also been found in cultures of *T. versicolor* (Farmer et al. 1960), *Pleurotus sajor-caju* (Bourbonnais and Paice 1988), *P. ostreatus* (Sannia et al. 1991), *P. eryngii* (Guillén et al. 1992) and *B. adusta* (Muheim et al. 1990). It is interesting to note that the AAO of *Bjerkandera* sp. BOS55 has a much lower affinity for veratryl alcohol compared to AAOs of the *Pleurotus* spp. Thus veratryl alcohol is well protected against unwanted AAO-mediated oxidation and thereby is conserved for its function together with LiP in *Bjerkandera* spp. It has been reported that *Pleurotus* spp. do not produce LiP (Bourbonnais and Paice 1988, Sannia et al. 1990, Guillén et al. 1992) and consequently they do not need to conserve their veratryl alcohol levels.

The experiments reported here demonstrate that LiP production by *Bjerkandera* sp. BOS55 is induced when grown on high nitrogen glucose/peptone medium similar to an earlier report with *B. adusta* (Kimura et al. 1990) but opposite to other white-rot fungi including the model fungus *P. chrysosporium* (Kirk and Farrell 1987). The correspondence in the appearance of LiP and veratryl alcohol may indicate that their production is regulated simultaneously. The relative amounts of veratryl alcohol and the chlorinated anisyl alcohols differ significantly depending on the growth conditions. We therefore conclude that the production of veratryl alcohol and the (chlorinated) anisyl metabolites are independently regulated.

The chlorinated anisyl alcohols would not be useful for extracellular H_2O_2 production if they are destroyed by the fungus's own agressive ligninolytic enzymes. Indeed, it was found that LiP can not oxidize anisyl alcohol derivatives after the addition of electron-withdrawing chlorine atoms. It is known that the addition of veratryl alcohol can increase the oxidation rate of anisyl alcohol (Harvey et al. 1986, Valli et al. 1990). However, the oxidation rate of the chlorinated anisyl alcohols was not improved by the addition of veratryl alcohol. It is anticipated that MnP and other phenol oxidases, which have much lower oxidating capacity (Kersten et al. 1990, Popp and Kirk 1991), react even less with the chlorinated anisyl alcohols. We therefore conclude that the chlorinated anisyl metabolites are very well protected against an unwanted oxidation in the hostile ligninolytic environment and conserved for the intended use by AAO. The minor amounts of aldehyde formed from the chlorinated anisyl alcohols, both in the presence and absence of H_2O_2, is probably a reflection of a small contamination of the LiP enzyme with AAO. With a commercial LiP preparation from

P. chrysosporium (Cultor, Kantvik, Finland), an organism which does not produce AAO and consequently AAO contamination is unlikely, no aldehyde formation was seen in the absence of H_2O_2 (unpublished results).

If the aryl alcohols have a truly important physiological role, then the oxidized metabolites must be recycled. These aryl aldehydes must be reduced to again produce the alcohols with their metabolic function as LiP and AAO substrates. It does not seem permissible to utilize energy for supplying each catalytic cycle with newly biosynthesized aryl alcohols. We demonstrated that all four aldehydes were reduced with comparable rates by washed cells. This indicates that an active H_2O_2 generating system is present. The relative amounts of each alcohol/aldehyde couple (Figure 1) reveals that during the first two weeks of growth intracellular dehydrogenase activity is the rate limiting step in this H_2O_2 production cycle. It is noteworthy that veratryl alcohol, which is the poorest AAO substrate, is always present in high concentrations in the glucose/peptone culture fluid thus capable of performing its other physiological functions as a LiP stabilizer and charge-transfer mediator. Reductive enzymes are common in basidiomycetes (Hurst 1963) and several aromatic acid and aldehyde reducing activities have been described in e.g. *Trametes* (syn. *Polystictus*) *versicolor* (Farmer et al. 1959) and *Phlebia radiata* (Lundell et al. 1990). Recently, an intracellular aryl alcohol dehydrogenase from *P. chrysosporium* was purified and characterized (Muheim et al. 1991). The enzyme reduced veratraldehyde to veratryl alcohol (2 nmol min^{-1} ml sup^{-1}) using NADPH as a cofactor. Also anisaldehyde was substrate for this enzyme.

White-rot fungi posses several systems for extracellular H_2O_2-generation (Kirk and Farrell 1987, Buswell 1992). It was also shown for *Bjerkandera* sp. BOS55, grown in glucose/peptone medium, that at least one other extracellular oxidase active on glucose and several other sugars was produced. Presently, it is not known which glucose oxidase was present. However, the fact that also xylose, galactose and cellobiose were substrates together with their relative activities indicates that the enzyme is a pyranose 2-oxidase (syn. glucose 2-oxidase) (Kelley and Reddy 1986, Volc and Eriksson 1988). Intracellular pyranose 2-oxidase has also been detected in *T. versicolor* (Machida and Nakanishi 1984) and *P. chrysosporium* (Volc and Eriksson 1988). This enzyme has a rather low affinity (K_m around 1 mM) for glucose (Machida and Nakanishi 1984). *P. chrysosporium* produces in addition to several intracellular oxidases (Buswell 1992), extracellular glyoxal oxidase (Kersten and Kirk 1987, Kersten 1990) and excretes some pyranose 2-oxidase (Daniel et al. 1992). Substrates for glyoxal oxidase have K_m's around 1 mM (Kersten 1990). Glyoxal oxidase has also been found in *Bjerkandera* sp. BOS55 grown on hemp stem wood (de Jong et al. 1992c), however in glucose/peptone medium this enzyme was not detected.

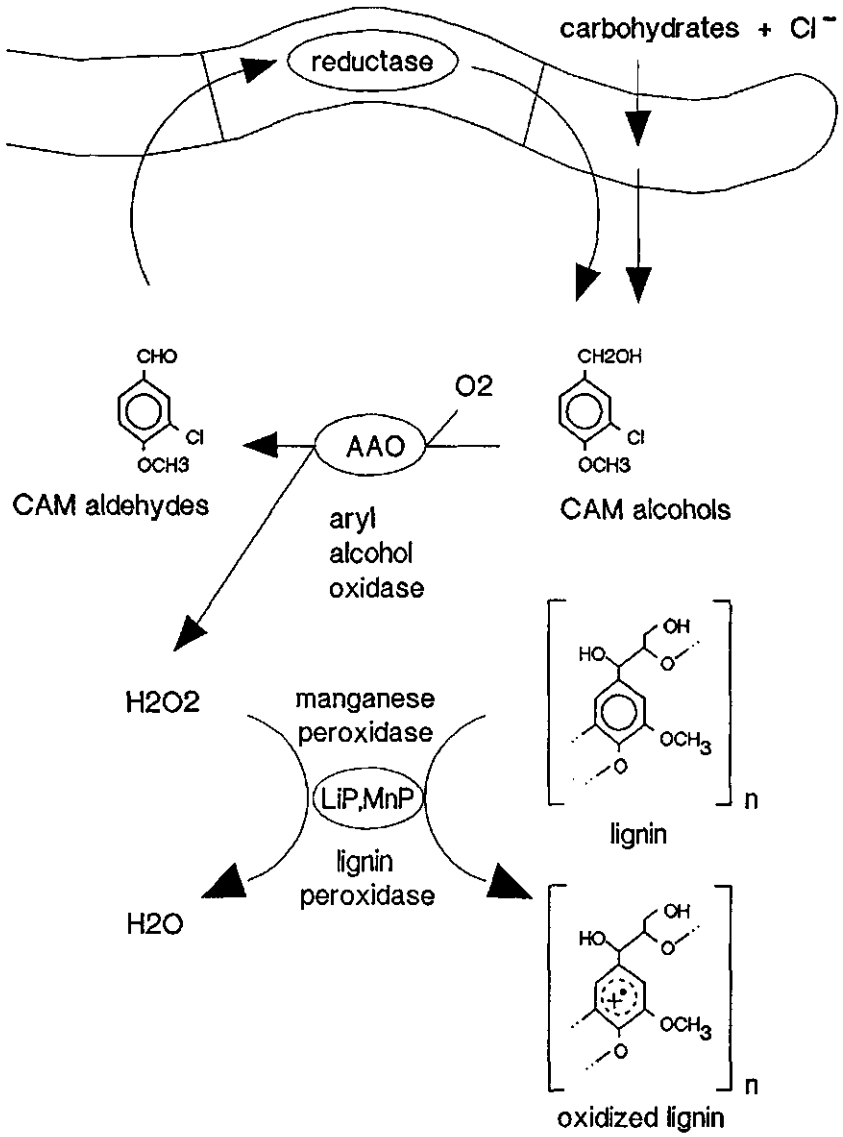

Figure 5. The proposed physiological role for the chlorinated anisyl metabolites (CAM) as substrates of extracellular aryl alcohol oxidase (AAO) generating H_2O_2 in the ligninolytic system of *Bjerkandera* sp. BOS55.

We conclude that the chlorinated anisyl alcohols may have an important physiological function in the ligninolytic system of *Bjerkandera* sp. BOS55. The metabolites are produced simultaneously with the ligninolytic enzymes. In contrast to the four possible roles for veratryl alcohol in the ligninolytic system the chlorinated anisyl alcohols are only good substrates for extracellular aryl alcohol oxidase, generating H_2O_2 for the peroxidases. The formed aldehydes are readily recycled by reduction to the corresponding alcohols. The physiological function of CAM is outlined in Figure 5. The chlorine-substitution makes the aryl alcohols better substrates for AAO, while simultaneously strongly decreasing decay by the aggressive ligninolytic peroxidases. Although AAO are not the only enzymes available for the H_2O_2 generation, CAM may be important under the natural physiological conditions of wood degradation. AAO has an extremely high affinity for these chlorinated compounds compared to other non-chlorinated substrates for the various extracellular fungal oxidases. We were able to detect high CAM concentrations in wood samples in the close vicinity of *B. adusta* and other white-rot fungi fruiting bodies (de Jong et al. 1993). Thus, chlorinated aromatic compounds can be purposeful for ecologically significant processes such as lignin degradation.

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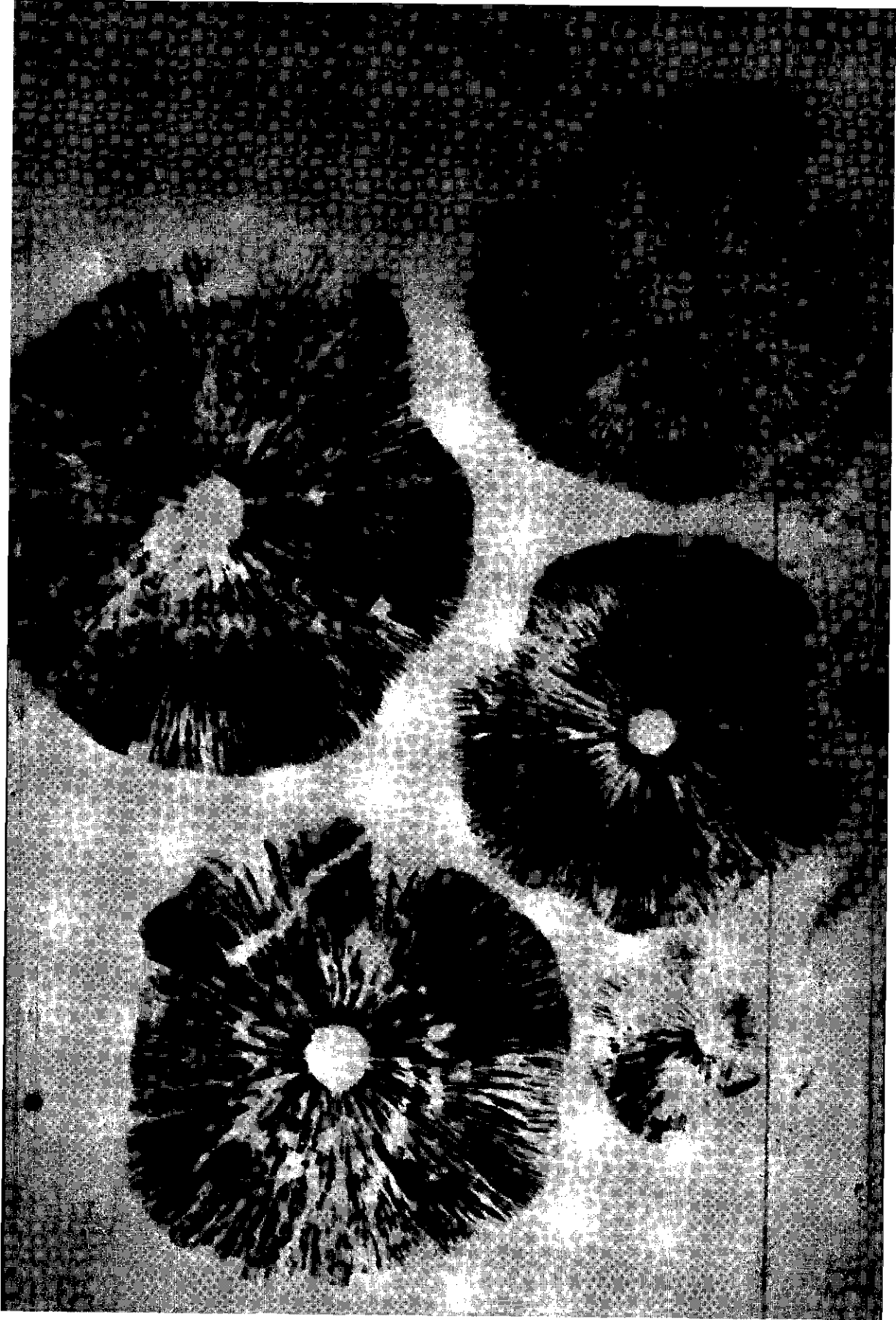
Spore print
of
the sulfur tuft fungus
(*Hypholoma fasciculare*)

This fungus is among
the highest
chlorinated anisyl metabolites
(CAM)
producers in the environment

The cap of the fungus is
sulphur-yellow
with a reddish-brown center,
3-7 cm in diameter

The gills are at first yellowish-green turning
olive-green to chocolate brown

The spores are purplish-brown in mass,
ellipsoid, smooth and have a average size of
6.6 x 4.3 microns



CHAPTER 7

SIGNIFICANT BIOGENESIS OF CHLORINATED AROMATICS BY FUNGI IN NATURAL ENVIRONMENTS

Ed de Jong, Jim A. Field, Henri-Eric Spinnler,
Joannes B.P.A. Wijnberg and Jan A.M. de Bont

SUMMARY

Common wood- and forest litter-degrading fungi produce chlorinated anisyl metabolites (CAM). These compounds, which are structurally related to xenobiotic chloroaromatics, occur at high concentrations of approximately 75 mg CAM kg^{-1} wood or litter in the environment. The ubiquity among common fungi to produce large amounts of chlorinated aromatic compounds in the environment makes us conclude that these kind of compounds can no longer be considered to originate mainly from anthropogenic sources.

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INTRODUCTION

Chlorinated aromatic compounds are largely considered as undesirable man-made pollutants. Adsorbable organic halogens (AOX) measurements are commonly used as a group parameter to monitor pollution in the environment. World-wide industries are required to comply with stringent environmental standards for chloroaromatic substances. However, industry is not necessarily the only source of these "xenobiotic" compounds. Recently, high AOX levels in pristine forest environments have been reported which greatly exceeds diffuse anthropogenic deposition (Asplund et al. 1991). Worldwide the AOX concentrations encountered ranged from 5-350 mg Cl^- kg^{-1} dry weight. A natural origin is plausible since several microorganisms are known to form *de novo* up to 1500 different organohalogen metabolites (Siuda and DeBernardis 1973, Neidleman and Geigert 1986, Gribble 1992). Most wood- and forest litter-degrading fungi are basidiomycetes and several strains from many different genera have been reported to produce chlorinated aromatic compounds in the laboratory. Chlorinated anisyl metabolites (CAM) are biosynthesized by *Lepista*, *Stropharia* and *Bjerkandera* spp. (Thaller and Turner 1972, Pfefferle et al. 1990, de Jong et al. 1992a). Drosophilin A (tetrachloromethoxyphenol) and derivatives are produced by *Psathyrella*, *Fomes*, *Mycena* and *Phellinus* spp. (Anchel 1952, Singh and Rangaswami 1966, Hsu et al. 1971, Butruille and Dominquez 1972, van Eijk 1975). Also the production of other chloroaromatics such as chloroanthraquinones by *Dermocybe* spp. (Gruber 1970), strobilurin B by *Strobilurus* spp. (Anke et al. 1984), mycenon by *Mycena* spp. (Hautzel et al. 1990) and 3-chloro-4-hydroxyphenylacetate by *Marasmius* spp. (Turner and Aldridge 1983) have been reported.

The objectives of this research were to estimate the ubiquity of CAM production among basidiomycetes, to analyze if high CAM concentrations are produced in the laboratory and to investigate if CAM production takes place and is detectable in the natural environment. The results presented in this paper will show for the first time that chlorinated aromatics are widely distributed at high concentrations in natural environments and are produced by many different genera.

MATERIALS AND METHODS

Microorganisms. The following basidiomycetes were tested for CAM production in the laboratory: *Armillaria mellea* (Vahl:Fr.) Kummer UP, *Bjerkandera adusta* (Willd.:Fr.) Karsten CBS 595.78, *Bjerkandera* sp. BOS55 CIMW 1.91, *Ceriporiopsis subvermispora* (Pilát) Gilbertson & Riv. (syn. *Poria subvermispora*) spp. CBS 347.63, DAOM 31816,

DAOM 21398 and DAOM 31817, *Collybia peronata* (Bolton:Fr.) Kummer CBS 426.79, *Daedaleopsis confragosa* (Bolton:Fr.) Schroeter sp. GM2 CIMW 4.91, *Fomes fomentarius* (L.:Fr.) Fr. CIMW 12.92, *Ganoderma applanatum* (Pers.) Pat. CBS 250.61, *Gloeophyllum trabeum* (Pers.:Fr.) Murrill CBS 164.27 and MHN, *Heterobasidion annosum* (Fr:Fr.) Bref. (syn. *Fomes annosus*) spp. CNRF 4, CNRF 38, CNRF 43, CNRF 45 and CNRF 78, spp. CBS 317.29 and MHN, *Hypholoma capnoides* (Fr.:Fr.) Kummer CIMW 5.92, *Hypholoma fasciculare* (Hudsson:Fr.) Kummer CIMW 10.92, *Hypholoma sublateritium* (Fr.) Quélet CIMW 14.92, *Ischnoderma benzoinum* (Wahlenb.:Fr.) Karst. CBS 250.30. *Lentinula edodes* (Berk.) Pegler CBS 833.87 (DSM 2989), *Mycena galopus* (Pers.:Fr.) Kummer CBS 500.79, *Mycena sanguinolenta* (Alb. & Schw.:Fr.) Kummer CBS 518.79, *Oudemansiella mucida* (Schrader:Fr.) Höhnel CIMW 2.92, *Perenniporia subacida* (Peck.) Sacc. (syn. *Poria subacida*) spp. CBS 374.63 and FPRL 104, *Phanerochaete chrysosporium* Burds. BKM F-1767 ATTC 24725, *Phellinus igniarius* (L.:Fr.) Quélet var. *trivialis* CBS 512.85, *Phlebia radiata* Fr.:Fr. CBS 287.73 and DAOM 229.61, *Pholiota squarrosa* (Wiegel:Fr.) Kummer CIMW 1.92, *Piptoporus betulinus* (Bull.:Fr.) Karst. (syn. *Polyporus betulinus*) CBS 378.51, *Pleurotus ostreatus* (Jacq.:Fr) Kummer CBS 342.69, *Pycnoporus cinnabarinus* (Jacq.:Fr.) Karsten sp. CCBAS 595, *Ramaria* sp. 158, *Trametes hirsuta* (Wulf.:Fr.) Pilát DFP 128.14, *Trametes versicolor* (L.:Fr.) Pilát (syn. *Coriolus versicolor*) Paprican 52 (ATTC 20869) and MHN. The basidiomycetes used in this study were obtained from the Centraal Bureau voor Schimmelcultures (CBS, Baarn, The Netherlands), the American Type Culture Collections (ATTC, Rockville, Md), the culture collection of basidiomycetes (CCBAS, Prague, Czechoslovakia), Department of Agriculture (Mycology), Ottawa, Canada (DAOM), Forest Products Research Laboratory, Princes Risborough, Bucks, United Kingdom (FPRL), Division of Forest Products, Commonwealth Scientific and Research Organization, Victoria, Australia (DFP), Centre National de la Recherche Forestière, Nancy, France (CNRF), Université Pierre et Marie Curie, Paris, France (UP), Muséum d'Histoire Naturelle, Paris, France (MHN) and from the Culture Collection of Industrial Microbiology (CIMW, Wageningen, The Netherlands). *P. chrysosporium* and *Ramaria* sp. 158 were kind gifts from T.K. Kirk, Forest Products Laboratory, Madison, Wis. and E. Agosin, Dept. of Chemical Engineering, Catholic University of Chile, Santiago, respectively.

Synthesis of authentic compounds. 3-Chloro-anisyl alcohol (3-chloro-4-methoxybenzyl alcohol) and 3-chloro-anisaldehyde (3-chloro-4-methoxybenzaldehyde) were synthesized as described before (de Jong et al. 1992a).

3,5-Dichloro-anisyl alcohol (3,5-dichloro-4-methoxybenzyl alcohol) was prepared from 3,5-dichloro-4-hydroxybenzoic acid by treatment with dimethyl sulfate and

subsequent reduction with lithium aluminium hydride.

10 ml (105 mmol) of dimethyl sulfate and 13.8 g (100 mmol) of anhydrous potassium carbonate was added to a solution of 8.28 g (40 mmol) of 3,5-dichloro-4-hydroxybenzoic acid in 200 ml of dry acetone. The reaction mixture was heated at reflux temperature for 3 h under a nitrogen atmosphere. After cooling to room temperature, the reaction mixture was filtered through Hyflo supercel, and the filter cake was washed with 200 ml of acetone. The solvent was evaporated under reduced pressure, and the resulting residue was dissolved in 250 ml of ether. The solution was washed successively with two 100-ml portions of 5% aqueous NaOH, one 50-ml portion of 10% aqueous Hcl, one 50-ml portion of saturated aqueous $NaHCO_3$, and one 50-ml portion of brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting solid was recrystallized from petroleum ether (bp 40-60 °C) to give 8.49 g (90 % yield) of methyl 3,5-dichloro-4-methoxybenzoate as white needles, m.p. 82-83 °C (uncorrected). ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.84 (s, -OCH_3); 3.88 (s, -OCH_3); 7.86 (s, 2 aromatic protons). ^{13}C-NMR ($CDCl_3$) δ (ppm): 52.43 (q); 60.67 (q); 127.01 (s); 129.37 (s); 130.04 (d); 155.90 (s); 164.34 (s). MS, calculated for $C_9H_8O_3Cl_2$ (M^+) *m/e* 233.9850, found 233.9849. MS, *m/e* (%): 236 (27); 234 (44); 205 (65); 203 (100); 111 (21); 99 (19); 97 (45); 75 (18); 74 (24); 62 (29); 61 (18).

To a stirred suspension of 0.80 g (21 mmol) of $LiAlH_4$ in 150 ml of dry ether was added dropwise a solution of 4.70 g (20 mmol) of methyl 3,5-dichloro-4-methoxybenzoate in 50 ml of dry ether. When the addition was complete, the reaction mixture was allowed to stir for 1 h at room temperature. The excess $LiAlH_4$ was then quenched by the careful addition of a few drops of saturated aqueous Na_2SO_4. The mixture was dried over anhydrous Na_2SO_4 and filtered through Hyflo supercel. After washing the filter cake with 100 ml of ether, the solvent was evaporated under reduced pressure. The resulting product was flash chromatographed on Merck silica gel 60 (230-400 mesh) (5:1 petroleum ether (bp 40-60 °C)/EtOAc) to give 3.69 g (89 % yield) of 3,5-dichloro-anisyl alcohol as a white solid, m.p. 46-47 °C (uncorrected). ^{1}H-NMR ($CDCl_3$) δ (ppm): 2.98 (br s, -OH); 3.82 (s, -OCH_3); 4.50 (s, -CH_2O-); 7.18 (s, 2 aromatic protons). ^{13}C-NMR ($CDCl_3$) δ (ppm): 60.49 (q); 63.17 (t); 126.74 (d); 128.94 (s); 138.27 (s); 150.85 (s). MS, calculated for $C_8H_8O_2Cl_2$ (M^+) *m/e* 205.9901, found 205.9901.

3,5-Dichloro-anisaldehyde (3,5-dichloro-4-methoxybenzaldehyde) was prepared from 3,5-dichloro-anisyl alcohol by oxidation with barium manganate in dry dichloromethane (Firouzabadi and Ghaderi 1978) in 69 % yield, m.p. 63-65 °C (from petroleum ether (bp 40-60 °C); uncorrected). ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.93 (s, -OCH_3); 7.77 (s, 2 aromatic protons); 9.82 (s, -CHO). ^{13}C-NMR ($CDCl_3$) δ (ppm): 60.75

(q), 129.76 (d), 130.35 (s), 132.84 (s), 156.97 (s), 188.41 (d). MS, calculated for $C_8H_6O_2Cl_2$ (M^+) *m/e* 203.9745, found 203.9743.

Production of chlorinated anisyl metabolites in the laboratory. Fungal production of extracellular CAM was tested with an amino acid medium containing per litre: KH_2PO_4 0.2 g, $MgSO_4$ 0.5 g, glucose 10 g, casamino acids 1 g, tyrosine 1 g, phenylalanine 1 g and yeast extract 1 g. All positive strains were also tested for CAM production with a synthetic (glucose/BIII) low nitrogen medium (de Jong et al. 1992a). Chlorinated metabolites were extracted from the cultures with acetonitrile or dichloromethane and were separated by GC or HPLC (de Jong et al. 1992a). Identifications were based on matching retention times and mass spectra with the chemically synthesized pure compounds.

Production of chlorinated anisyl metabolites in the environment. Sites were sampled between October 28 and December 4, 1992, in The Netherlands (Oranje Nassau Oord and Boswachterij Oostereng both near Renkum, Hoekelumse Bos near Ede, Kaapse Bossen near Doorn, Amelisweerd near Utrecht, Noordhoutse Bos near Maarn, Wageningen and Elspeterbosch near Elspeet), Poland (nearby Gorki within Kampinosky National Park, nearby Palmiry Cementary and Białowieska forest sectors 390-393) and Belgium (nearby Eupen within the Haute Fagne National Forest).

Composite random samples of rotting wood or moldy leaf-litter were taken in all sampling sites. Environmental samples adjacent to fruiting bodies of CAM-producing fungi grown on gymnosperm (pine and spruce) and angiosperm (beech, birch, oak, elm, ash and hornbeam) wood or litter were also analyzed for CAM production. Routinely samples were extracted with dichloromethane and analyzed with GC/MS, but in a few cases samples were extracted with acetonitrile and analyzed by HPLC.

Instruments. Supernatant (50 µl) was routinely analyzed on a Hewlett Packard HPLC Chemstation (Pascal series) (Waldbronn, Germany) equipped with a HP1050 pumping station, a HP1040 M series II diode array detector and a HP9000-300 data processor. The column (200 mm x 3 mm) filled with ChromSpher C18-PAH (5 µm particles) was from Chrompack (Middelburg, The Netherlands). Aromatic metabolites were analyzed with the following gradient (0.4 ml min^{-1}, 30 °C): 90:10, 0:100 and 0:100 H_2O:CH_3CN at 0, 15 and 20 minutes, respectively. The UV absorbance was monitored at 2 nm wavelength intervals from 210 to 350 nm. 3,5-Dichloro-anisaldehyde was purified using a preparative Lichrosorb diol column (250*10mm) in two steps. 1) CH_2Cl_2 (10%) / pentane (90%) to 100% CH_2Cl_2 for 40 min. 2) CH_2Cl_2 (20%) / pentane (80%) to 50% CH_2Cl_2 for 40 min. In both cases flow rate was 3ml min^{-1}, UV detection at 214 nm. 1.1

mg of white thin crystals were isolated.
Mass spectra were measured with a Hewlett Packard HP5890 GC with 30 m DB17 column and a HP5970 MSD. Injection temperature was 200 °C. The starting temperature of the column was 80 °C, gradient 7 °C min^{-1}, final temperature 240 °C. Helium was used as carrier gas (20 ml min^{-1}).
^{1}H-NMR, ^{13}C-NMR spectra were obtained with a Bruker AC-E200 spectrometer (Rheinstetten-Forchheim, Germany). Authentic and synthesized compounds were dissolved in $CDCl_3$. Tetramethylsilane was used as an internal standard.

Chemicals. 3-Chloro-4-hydroxybenzoic acid, 3-chloro-4-methoxybenzoic acid and 3,5-dichloro-4-hydroxybenzoic acid were purchased from Heraeus (Karlsruhe, Germany). Veratryl alcohol, veratraldehyde, anisyl alcohol (4-methoxybenzyl alcohol) and anisaldehyde (4-methoxybenzaldehyde) were from Janssen Chimica (Tilburg, The Netherlands). Barium manganate was obtained from Aldrich (Bornem, Belgium).

RESULTS

Recently, we have shown that white-rot fungi of the genus *Bjerkandera* produce 3-chloro-anisyl alcohol (I) and 3-chloro-anisaldehyde (II) when grown in a glucose medium (de Jong et al. 1992a). In addition to the monochlorinated metabolites, *Bjerkandera* spp. also produce 3,5-dichloro-anisyl alcohol (III) and 3,5-dichloro-anisaldehyde (IV) (Figure 1).

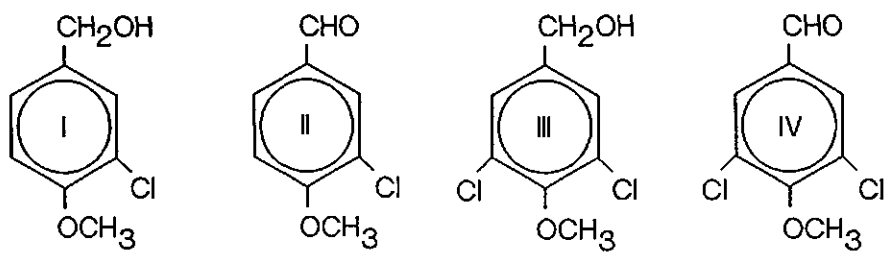

Figure 1. The Chemical structure of the CAM compounds, 3-Chloro-anisyl alcohol (I), 3-chloro-anisaldehyde (II), 3,5-dichloro-anisyl alcohol (III) and 3,5-dichloro-anisaldehyde (IV).

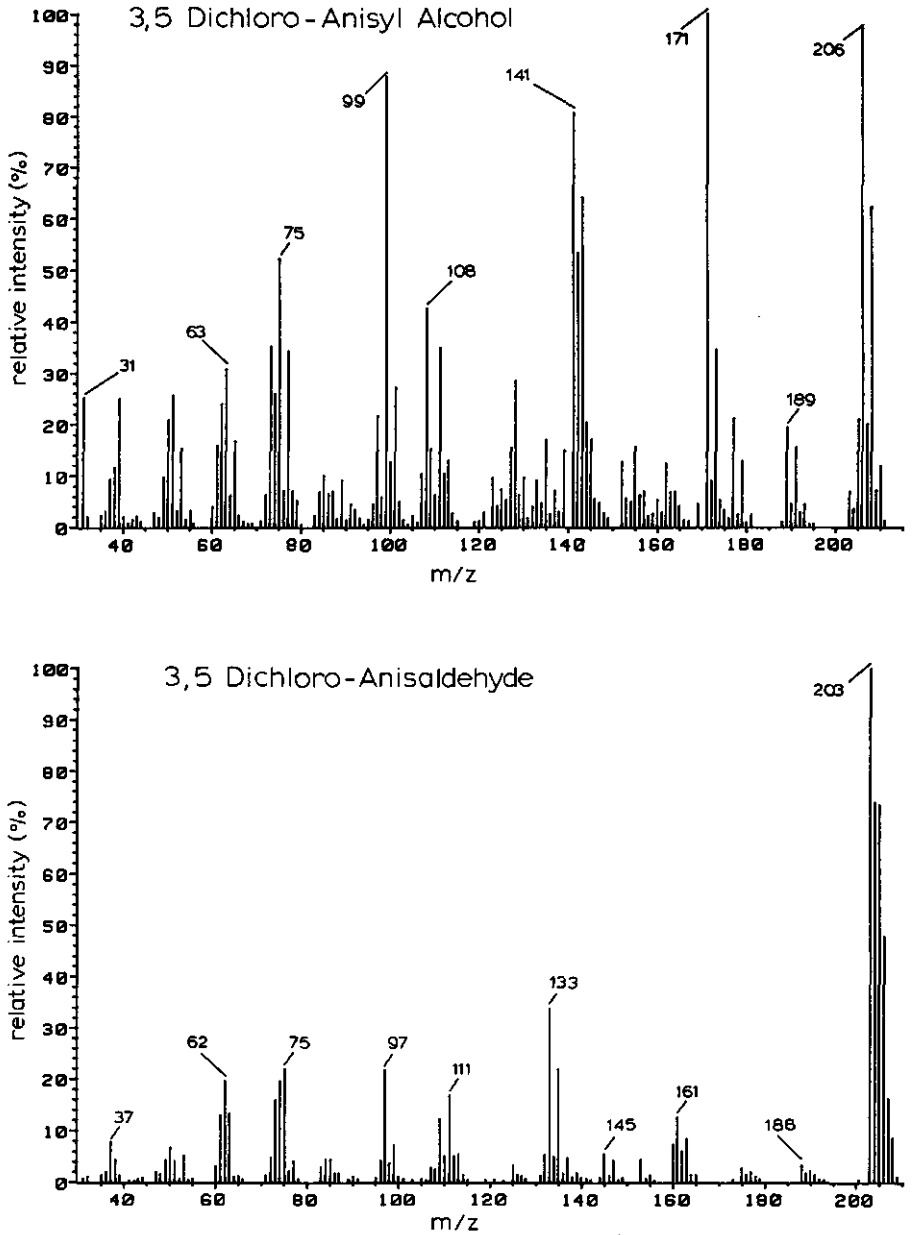

Figure 2. Mass spectra of 3,5-dichloro-anisyl alcohol and 3,5-dichloro-anisaldehyde.

The mass spectra of the dichlorinated compounds are shown in Figure 2. Anisyl alcohol and anisaldehyde, which are the nonchlorinated counterparts of CAM, were only produced in trace amounts by this fungus (de Jong et al. 1992a).
The culture conditions of *Bjerkandera* sp. BOS55 were altered in an attempt to enhance the CAM production. The medium composition, low nitrogen (glucose/BIII) versus amino acid medium, had only a minor effect on the CAM levels. Also the addition of 0.5 mM anisyl alcohol or 0.5 mM anisaldehyde at the beginning of CAM production (day 4) did not stimulate the production of CAM compounds. The production of CAM compounds was also not significantly influenced by a variable Cl^- concentration ranging from 0.4 to 20 mM.

Table 1. The production of chlorinated anisyl metabolites (CAM) by pure cultures of basidiomycetous fungi grown in defined media *.

Fungus	Total CAM ($mg\ l^{-1}$)	Metabolite spectrum †	Peroxidase Activity ‡	AAO ɪ
Bjerkandera adusta	5.4	II, (IV)	+	+
Bjerkandera sp. BOS55	17.3	I, II, IV	+	+
Hypholoma capnoides	37.3	III, (IV)	+	+/-
Hypholoma fasciculare	17.0	III, (IV)	+	+/-
Hypholoma sublateritium	12.3	III, (IV)	+	N.T.
Oudemansiella mucida	29.9	III, (IV)	+	-
Pholiota squarrosa	6.8	IV	+	+
Ramaria sp. 158.	16.3	II, (IV)	+	N.T.
Trametes versicolor	0.8	II	+	+

* CAM production was measured with fungi grown on low nitrogen (glucose/BIII) medium and on amino acid medium. *T. versicolor* only produced CAM when grown on the amino acid medium, all other fungi produced CAM on both media. Basidiomycetous fungi tested for CAM production and the analysis procedure are described in the materials and methods section.

† Roman numerals refer to the numerals given in Figure 1. Brackets indicate only trace amounts detected.

‡ Peroxidase activity as determined by the decolorization of the polymeric dye Poly R-478 (de Jong et al. 1992b).

ɪ AAO = Aryl-alcohol oxidase determined as described by de Jong et al. (1992b). + = extracellular aryl-alcohol oxidase activity measured, +/- = not all samples of quadruplets gave positive results, - = no extracellular aryl-alcohol oxidase activity detected, N.T. = Not tested.

In an extensive screening program, we now have checked 60 basidiomycetous fungi for the production of mono- and dichlorinated anisyl metabolites (CAM) (Table 1). CAM were produced by 15 strains and concentrations were detected up to 37 mg l^{-1}, Table 1 lists 9 organisms which were taxonomically determined. Several other fungi, including *D. confragosa*, *F. fomentarius* and *I. benzoinum* produced small amounts (< 0.8 mg l^{-1}) of 3-chloro-anisaldehyde only when grown on the amino acid medium. Important examples of fungi that did not produce GC/MS detectable amounts of CAM included: *G. applanatum*, *G. trabeum*, *L. edodes*, *P. chrysosporium*, *P. igniarius* var. *trivialis*, *P. radiata* and *P. ostreatus*. This number of positive strains can be a conservative estimate since the amount and spectrum of aromatic compound biosynthesis is very much dependent on the growth medium and the strain used (Gallois et al. 1990). These findings confirm and extend two earlier reports in which basidiomycetes (*Lepista diemmii* and *Stropharia* sp.) were shown to produce CAM (Thaller and Turner 1972, Pfefferle et al. 1990).

Table 2. CAM in environmental samples from forested sites.

Sample *	Number	Fungus †	Average CAM	CAM Range	CAM compounds ‡
			mg CAM kg^{-1} dry weight sample		
Litter	4	composite	0.15	0.02 - 0.25	II, IV
Wood	5	composite	3	1.2 - 4.5	II, III, IV
Wood	7	*B. adusta*	15	7 - 39	II, IV, (I), (III)
Wood	5	*Hypholoma* spp.	75	24 - 180	III, IV
Wood	3	*P. squarrosa*	71	38 - 91	III, IV
Litter	2	*S. aeruginosa*	16	11 - 22	III, IV
Litter	3	*L. nuda*	39	19 - 73	II, III, IV

* Environmental samples were collected in The Netherlands (8 sites), Belgium (1 site) and Poland (3 sites) between October 28 and December 4, 1992.

† Composite random sample of rotting wood or moldy leaf-litter in the sampling site without macroscopically detectable fruiting bodies, species names indicate fruiting bodies of fungus which was colonizing the environmental sample.

‡ Roman numerals refer to the compounds in Figure 1. Brackets indicate only trace amounts detected.

The production of chlorinated aromatic metabolites (CAM) could be an artefact of culture conditions or it could be a purposeful event. The challenging question to be addressed here was if significant production of CAM also occurs in natural

environments. Therfore, we tested to determine if the production of CAM occurs when a CAM-producing basidiomycete is grown on autoclaved lignocellulosic substrates common to forest litter. *Bjerkandera* sp. BOS55 was grown on European beech (*Fagus sylvatica*), European white birch (*Betula verrucosa*), Norway spruce (*Picea abies*) and hemp stem wood (*Cannabis sativa*) in demineralized water all at 30 g l^{-1}, and after 30 days of incubation, significant CAM production was evident on all of these substrates. The total CAM concentration ranged from 12 to 61 mg kg^{-1} dry weight woody material.

If fungal biosynthesis of CAM is a widespread process in nature, then it should be possible to encounter such compounds in the environment. Composite random samples of moldy litter (leaves, twigs, branches and nut husks) and rotting logs were sampled at several natural forested sites. At all sites CAM were detectable and were identified based on matching retention times and mass spectra (Table 2). CAM were not present in control samples such as fresh forest litter or intact wood. Rotting wood or leaf-litter adjacent to the fruiting bodies of CAM producing basidiomycetes consistently contained CAM concentrations high enough to be considered as hazardous wastes according to Dutch environmental regulations on their nonmethylated counterparts, chlorophenols, in soils ($\geq$ 10 mg kg^{-1}) (Stoop and Rennen 1990). We have also examined fruiting bodies of the edible mushroom *L. nuda* for CAM levels. It contained 0.8 mg CAM kg^{-1} dry weight mushroom. No CAM was found in the oyster mushroom (*Pleurotus ostreatus*).

DISCUSSION

In this study, we found that common wood- and forest litter-degrading fungi produce high concentrations of chlorinated anisyl metabolites (CAM) both in the laboratory and in the natural environment. Many of the CAM producers (*B. adusta*, *Hypholoma* spp., *L. nuda*, *P. squarrosa* and *S. aeruginosa*) are widespread in nature (Phillips 1981, Krieglsteiner 1991), and consequently a ubiquitous production of CAM under natural conditions is to be inferred. Future research should determine if other fungal chlorinated aromatic metabolites, e.g. drosophilins (Anchel 1952, Singh and Rangaswami 1966, Hsu et al. 1971, Butruille and Dominquez 1972, van Eijk 1975), are also present at high concentrations in the environment.

Fungal chlorinated aromatic compounds have antibiotic properties (Anchel 1952, Pfefferle et al. 1990, Gribble 1992). In the accompanying article (de Jong et al. 1993), we have shown that CAM alcohols have another physiological role because they are excellent substrates for extracellular aryl alcohol oxidase, generating

extracellular H_2O_2 for the lignin-degrading peroxidases. All CAM producers tested secreted peroxidases and the majority of the strains also produced aryl-alcohol oxidase (Table 1). Future research should elucidate if CAM alcohols are also *in vivo* important as aryl-alcohol oxidase substrates for the other CAM producing strains.

The high fungal production of chlorinated aromatic compounds in natural environments should be compared with the industrial sources of analogous priority pollutants. The results will possibly provide a basis for re-evaluating the strict environmental standards for chloroaromatics in relation to background levels in natural environments. A number of possible environmental fates are outlined in Figure 3. Since the environmental scope of natural "xenobiotic" production seems to be much greater than previously thought, it is important to consider the ultimate fate of CAM released into the soil environment. Although it has been demonstrated that ligninolytic basidiomycetes are able to mineralize chlorophenol compounds (Mileski et al. 1988, Valli and Gold 1991, Joshi and Gold 1993) with peroxidases and laccases causing the initial dechlorinations (Hammel and Tardone 1988, Roy-Arcand and Archibald 1991), we have demonstrated in the companion article that CAM compounds are resistant to ligninolytic attack (de Jong et al. 1993). However, many other microorganisms and microbial consortia are known which are responsible for the complete mineralization of a wide variety of chlorinated aromatic pollutants (Häggblom 1992). Thus part of the CAM may be fully mineralized but another part may become incorporated into humus according to known biotransformations. Partial metabolism of CAM at the methoxy (Nielson et al. 1988, Bernhardt et al. 1988, Brezny et al. 1992) and alcohol/aldehyde (Nielson et al. 1988, Zhang and Wiegel 1992) groups as well as hydroxylation reactions (Crawford et al. 1973) will yield, among others, 2-chlorophenol and 3-chlorocatechol, which in turn are readily copolymerized into humus with phenol oxidizing enzymes as demonstrated both *in vitro* (Bollag et al. 1977, Maloney et al. 1986, Claus and Filip 1990, Dec and Bollag 1990, Roy-Arcand and Archibald 1991) and during soil microcosm studies (Cheng et al. 1983). The resulting chlorohumus polymers are non-toxic (Lyr 1962, Bollag et al. 1988) and poorly biodegraded when incubated in soils (Dec and Bollag 1988). Consequently, CAM should be considered as possible building blocks for the environmentally highly persistent adsorbable organic halogens polymers (AOX) present at high concentrations in pristine forest environments (Asplund and Grimvall 1991).

Recently, the chlorinated aromatic structures in high molecular weight organic materials from unpolluted surface waters and groundwaters have been characterized (Dahlman et al. 1993, Dahlman et al. 1994). Degradation products originating from 3-chloro- and 3,5-dichloro-4-hydroxyphenyl units dominated in samples of unpolluted environments, while bleach kraft mill effluents were most abundant in chlorinated

guaiacyl and syringyl units. These results seem to address chlorinated anisyl metabolites as a potential major source for natural, high molecular weight AOX.

Aside from the above described detoxification reactions, the proposed chlorophenol intermediates can also give rise to biotoxification reactions. It has been demonstrated in *vitro* experiments that peroxidase-mediated oxidation of chlorophenols results in the formation of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (Figure 3) (Maloney et al. 1986, Svenson et al. 1989, Öberg et al. 1990). Recently, also the production of dioxins during waste-water treatments and composting has been reported (Öberg et al. 1993).

We conclude that CAM-production is a very common capacity among wood- and forest litter-degrading fungi. A detailed analysis of subsequent transformation processes of chloroaromatics in different ecosystems is very important to elucidate the relative importance of different environmental fates such as complete mineralization to carbon dioxide, detoxification processes leading to chlorohumus and biotoxification processes generating natural hazardous compounds, including polychlorinated dioxins and dibenzofurans.

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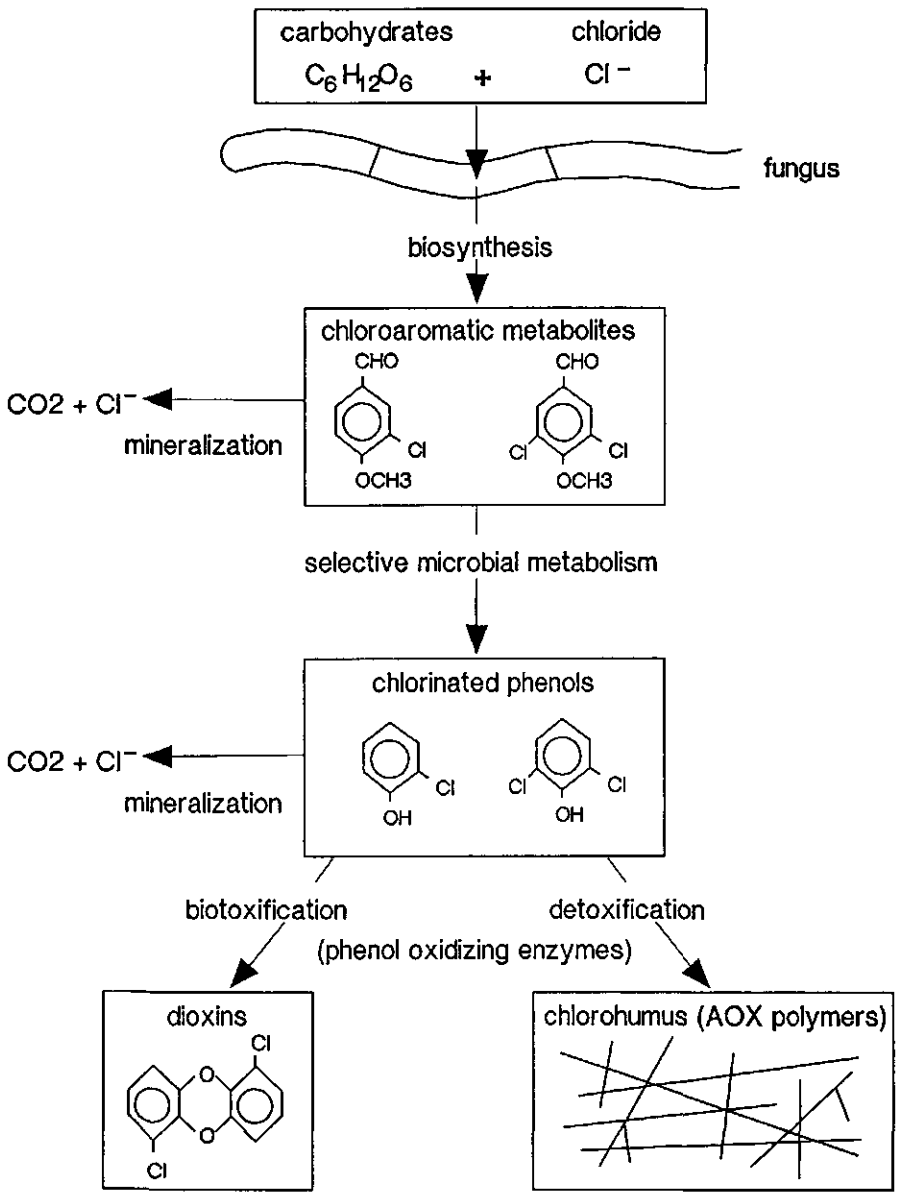

Figure 3. The possible environmental fates of CAM compounds: mineralisation to carbon dioxide, detoxification to chlorohumus and biotoxification to dioxins.

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Scanning
electron
micrographs
(SEM)
of
Penicillium simplicissimum
grown
on
a
malt
agar
plate

Upper plate
(200 x)
shows
invading hyphae

Lower plate
(4000 x)
shows
a conidiophore


KV X200 1737 100.0U 1989
5KV X4000 1747 1.0U 1989

CHAPTER 8

DEGRADATION OF VERATRYL ALCOHOL BY PENICILLIUM SIMPLICISSIMUM

Ed de Jong, Evelien E. Beuling, Rick P. van der Zwan
and Jan A.M. de Bont

SUMMARY

Several bacteria, yeasts and fungi selectively isolated from paper mill waste water grew on veratryl alcohol, a key intermediate of lignin metabolism. *Penicillium simplicissimum* oxidized veratryl alcohol via a $NAD(P)^+$-dependent veratryl alcohol dehydrogenase to veratraldehyde which was further oxidized to veratric acid in a $NAD(P)^+$-dependent reaction. Veratric acid-grown cells contained NAD(P)H-dependent *O*-demethylase activity for veratrate, vanillate and isovanillate. Protocatechuate was cleaved by a protocatechuate 3,4-dioxygenase.

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INTRODUCTION

Lignin is by far the most abundant aromatic substance present in the biosphere. It is a polymer composed of phenylpropanoid units linked through a variety of C-C and C-O-C bonds. Fungi have long been known to metabolize lignin, but the mode of action of these organisms on this difficult to degrade substrate remained obscure for a long time. A breakthrough in the field of lignin biodegradation research occurred in 1983 when extracellular peroxidases were discovered in ligninolytic cultures of *Phanerochaete chrysosporium* (Tien and Kirk 1983, Glenn et al. 1983). These ligninases cause a one-electron oxidation of lignin model compounds via radical cation intermediates, providing a unifying explanation for most of the reactions observed in lignin biodegradation (Schoemaker et al. 1985). However, ligninases alone do not seem to be able to degrade lignin *in vivo*, which indicates that other enzymes are involved in the lignin modification process (Kirk and Farrell 1987).

Veratryl alcohol is a key compound in lignin biodegradation by *Phanerochaete chrysosporium* with several distinct functions. It has possibly an important physiological role in the redox cycle of the lignin peroxidase in converting the oxidized lignin peroxidase to native enzym, it acts as a one-electron transfer mediator between the enzyme and the insoluble polymeric lignin, and it induces the ligninolytic system of *P. chrysosporium*. Veratryl alcohol is also a substrate for the enzyme. The main product is veratraldehyde, but in an oxygen atmosphere also ring cleavage and oxidation/hydroxylation of the aromatic ring occur (Haemmerli et al. 1987, Schmidt et al. 1989, Schoemaker and Leisola 1990). Interestingly, the fungus only grows at the expense of veratryl alcohol or other aromatic compounds when a co-substrate as for instance glucose or cellulose is available.

Our interest in the metabolism of lignin and lignin monomers by fungi originates from environmental problems caused by traditional pulping processes. Because veratryl alcohol is an important compound in lignin degradation, it was decided to study organisms and their enzymes and pathways involved in the catabolism of this aromatic compound. Since *Phanerochaete chrysosporium* does not grow on veratryl alcohol in the absence of co-substrates, several other micro-organisms were isolated that use aromatic compounds as sole source of carbon and energy. In this paper we describe the metabolism of veratryl alcohol by the fungus *Penicillium simplicissimum*.

MATERIALS AND METHODS

Organism and media. *Penicillium simplicissimum* was isolated from paper mill waste

water. The enrichment mineral salts medium contained in 1 l of deionized water: KH_2PO_4, 1.8 g; Na_2HPO_4, 1.0 g; NH_4Cl, 2.0 g; $(NH_4)_2SO_4$, 0.1 g; $MgCl_2.6H_2O$, 0.075g and 0.2 ml of a trace elements solution as described by Vishniac and Santer (1957), carbon sources were added at 0.1% (w/v). Incubations were at pH 5.0 and 30°C. Details of the isolation and characterization are given under Results. The isolate, deposited at (CBS), Centraal Bureau voor Schimmelcultures Baarn, The Netherlands, as CBS 170.90, was maintained on slant agar of 5 g l^{-1} glucose and 3.5 g l^{-1} yeast extract medium to which 15 g l^{-1} Oxoid (Basingstoke, UK) no. 3 agar had been added. It was routinely grown in a mineral salts medium identical to the enrichment medium except that 4.57 g l^{-1} polyacrylic acid (PAA, 63% w/v, Mw 2000) was added. The medium was adjusted to pH 5.2 with NaOH. Unless stated otherwise the carbon sources were added to this mineral medium at 2.0 g l^{-1}.

Culture conditions. Cells were routinely grown fed batch at 30°C in a flat bottomed round flask (2 litre). Aeration was by stirring (600 rpm) with a 5 cm long magnetic stirrer. The flow of the substrate (0.2% (w/v) veratryl alcohol in mineral salts medium) was 24 ml $hour^{-1}$ and air (5 ml min^{-1}) was bubbled continuously through the medium. Cells were harvested daily by withdrawing half (approximately 575 ml) of the culture.

Substrate specificity experiments. The substrate specificity of CBS 170.90 for various aromatic compounds as sole source of carbon and energy was tested in 100 ml serum bottles closed with screw-caps. To 10 ml of the mineral medium various carbon sources (0.1 % (w/v)) were added and CO_2-production was measured after 1 month of incubation. Inoculated bottles containing mineral medium without added carbon source served as blanks. Whenever the CO_2-concentration in a bottle was more than 5 times the concentration in the blank it was concluded that growth had occurred. For concentrations between the blank and five times the blank it was concluded that no growth had occurred. When no CO_2-production at all occurred it was concluded that the substrate was toxic for the organism at 0.1 % (w/v). In those cases experiments were repeated at 0.01% (w/v) substrate concentrations and moreover in a second approach a two phase system was used (Rezessy-Szabo et al. 1987) to reduce the concentration of carbon source in the water phase. In the latter case dibutylphthalate (2 ml) was included as a second phase bringing the total volume to 12 ml. Growth was determined as described above.

Suspensions of washed cells and cell-free extracts. Cells were harvested from the growth medium by filtration over a cheese-cloth, washed with buffer (KH_2PO_4, 1.8 g; Na_2HPO_4, 1.0 g; PAA, 4.57 g) pH 5.2 and resuspended in the same buffer. For the

preparation of cell-free extracts, the cells were washed with potassium phosphate buffer pH 7.2 (50 mM) and disrupted by X-press (Pharmacia, Uppsala, Sweden) disintegration at -25 °C. After slowly thawing, the resulting homogenate was centrifuged at 48000 **g** for 20 min at 4 °C. The supernatant containing 10-20 (mg protein) ml^{-1} was the crude cell-free extract. A cofactor-free extract was obtained by dialyzing the cell-free extract against a 50 mM potassium phosphate solution (pH 7.0) for 12 hours at 4 °C.

Analytical methods. Protein contents of whole cells and cell-free extracts were determined by the method of Bradford (1976) using crystalline bovine serum albumin as a standard. CO_2-production was measured on a Packard 427 (Delft, The Netherlands) gaschromatograph with a Porapak Q (Chrompack, Middelburg, The Netherlands) column. Spectrophotometric assays were done with a Perkin-Elmer (Norwalk, USA) 550A UV-Vis spectrophotometer at 30 °C. Reverse-phase HPLC analysis were performed at room temperature on a ABI Analytical (Kratos division, Foster City, USA) isocratic HPLC system with a ChromSpher C-18 column (200 by 3 mm, Chrompack, Middelburg, The Netherlands). The eluent was a mixture of methanol, Milli-Q H_2O and acetic acid (33:66:1). With an eluent mixture of methanol, Milli-Q H_2O and acetic acid (15:85:1) vanillic acid and isovanillic acid could be separated using the same column as above.

Enzyme assays. ***Veratryl alcohol dehydrogenases***: The activity of a $NAD(P)^+$-dependent alcohol dehydrogenase was assayed spectrophotometrically by following at 340 nm the rate of reduction of NAD^+ and $NADP^+$ in dialyzed cell-free extract in the presence of veratryl alcohol. The reaction mixture (total volume 2.25 ml) contained cell-free extract, 0.5 μmol NAD^+ or $NADP^+$ and 100 μmol Glycine buffer pH 10.0. The reaction was started by adding 0.5 μmol veratryl alcohol.

PMS-(Phenazine methosulfate)-dependent veratryl alcohol dehydrogenase was assayed by measuring by means of HPLC the rate of disappearance of veratryl alcohol. The reaction mixture (total volume 5.0 ml) contained dialyzed cell-free extract, 1.0 μmol PMS, 77.0 μmol NH_4Cl and 200 μmol Glycine buffer pH 10.0. The reaction was started by the addition of 2.5 μmol veratryl alcohol. Samples (0.5 ml), taken at intervals, were mixed with 0.5 ml 2N HCl. The protein free solution was separated and analyzed as described above. Metabolites were identified by comparison of retention times with authentic samples and *in situ* scanning of the UV spectra after the flow had been stopped.

Aryl (Veratryl) alcohol oxidase was measured according to Bourbonnais and Paice (1988) and ***lignin peroxidase*** was assayed for according to Tien and Kirk (1988).

Veratraldehyde dehydrogenase: The enzyme was assayed by measuring by means of HPLC the rate of formation of veratric acid. The reaction mixture (total volume 5.0 ml) contained dialyzed cell-free extract; 2.5 μmol NAD^+ or $NADP^+$ and 150 μmol Glycine buffer pH 10.0. The reaction was started by the addition of 1.25 μmol veratraldehyde. Samples (0.5 ml), taken at intervals, were separated and analyzed as described above.
Veratric acid O-demethylase: The enzyme was assayed with HPLC by measuring the rate of decrease of veratric acid. The reaction mixture (Total volume 1.5 ml) contained cell-free extract (600 μl); 1.5 μmol NADPH and 36 μmol potassium phosphate pH 7.2. The reaction was started by adding 0.15 μmol veratrate. Samples (0.2 ml), taken at intervals, were mixed with 0.2 ml 2N HCl and then diluted with 0.2 ml H_2O. Samples were separated and analyzed as described above.
Oxidative decarboxylase was measured according to Buswell et al. (1979), ***nonoxidative decarboxylase*** was measured according to Kuwahara et al. (1981).
Dioxygenases: All activities were assayed for with dialyzed cell-free extracts containing 2 mM $(NH_4)_2Fe(SO_4)_2$. ***Protocatechuate 3,4-dioxygenase*** (EC 1.13.11.3). The activity of protocatechuate 3,4-dioxygenase was measured spectrophotometrically at 290 nm (Fujisawa and Hayaishi 1968). ***Catechol 1,2-dioxygenase*** (EC 1.13.11.1). The involvement of a catechol 1,2-dioxygenase was investigated spectrophotometrically at 260 nm as described by Nakazawa and Nakazawa (1970). ***Gentisate 1,2-dioxygenase*** (EC 1.13.11.4). Activity of the enzyme was measured spectrophotometrically as described by Crawford et al. (1975). Extradiol cleavage of protocatechuate (EC 1.13.11.8) and catechol (EC 1.13.11.2) were investigated spectrophotometrically at 410 nm (Dagley et al. 1960) and 375 nm (Nakazawa and Nakazawa 1970) respectively.

Chemicals. The biochemicals were from Boehringer, Mannheim, FRG. All the other chemicals were of commercially available analytical grade and were used without further purification.

RESULTS

Isolation of aromatic compounds degrading micro-organisms. Micro-organisms were isolated using as inoculum activated sludge from a waste water purification plant of a thermomechanical pulping paper mill. Particular rapid growth occurred with 3,4-dimethoxycinnamic acid as sole source of carbon and energy and 4 yeast and 4 fungi were isolated on this compound. These organisms also grew on veratryl alcohol. Bacteria were also routinely isolated but these organisms were not further examined. A fungus designated PAZ1 had the shortest doubling time on veratryl alcohol and

furthermore grew on many other aromatic compounds. This organism was used for further studies.

Characterization of strain PAZ1 = CBS 170.90. CBS 170.90 was identified by the CBS as *Penicillium simplicissimum*. The morphology of the isolate accommodate it in the *P. simplicissimum*-complex. Chemical analysis of the secondary metabolites of the fungus supported the outcome of the accommodation, although it has also resemblance to *P. cremeogrisum*.

Table 1. The ability of *Penicillium simplicissimum* CBS 170.90 to grow on various aromatic compounds as sole source of carbon and energy

Aromatic acids		*Aromatic alcohols*	
Benzoic acid	+ *	Veratryl alcohol	+
Veratric acid	+	Vanillyl alcohol	+
Vanillic acid	+	Isovanillyl alcohol	+
Isovanillic acid	+	4-Methoxybenzyl alcohol	
4-Hydroxybenzoic acid	+		
Cinnamic acid	+	*Aromatic aldehydes*	
Nitrobenzoic acid	—	Veratraldehyde	+ †
Aminobenzoic acid	—	Vanillin	+ †
3,4-Dimethoxycinnamic acid	+	3-Ethoxy-4-hydroxybenzaldehyde	+ †
4-Chlorobenzoic acid	—	3,4-Dihydroxybenzaldehyde	+
o-Toluic acid	—		
Syringic acid	—	*Miscellaneous*	
3,5-Dimethoxy-4-hydroxycinnamic acid	+ †	Anisoin	—
		Veratrole	+
Phenolic compounds		Dibuthylphtalate	—
Phenol	+	Polyacrylic acid	—
Gualacol	+	Phenylglycine	+
Methoxyhydrochinon	+	Benzenesulphonic acid	—
Hydrochinon	+	Anisole	—
Catechol	+	Benzene	—
p-Cresol	+ †	Toluene	—
		Tetralin	—
		Styrene	+

+, growth; -, no growth; *) 0.01% (w/v) carbon source; †) 20% Dibutylphthalate

CBS 170.90 grows on various aromatic compounds as sole source of carbon and energy (Table 1). Growth was routinely assessed at 0.1% (w/v) substrate concentrations resulting in several instances in false negative results due to toxicity of the substrates as demonstrated by measuring CO_2 production in the presence and

absence of substrate. In these cases a lower substrate concentration of 0.01% (w/v) was used. Alternatively dibutylphthalate was used as a second liquid phase (see materials and methods section) since this water-immiscible solvent was neither toxic to the fungus nor degraded by the fungus. Using these techniques additional compounds were identified as growth substrate (Table 1).

The doubling time of the fungus with veratryl alcohol as growth substrate was 10 hours and the growth rate constant for growth on Czapek-Malt agar was 165 μm hour^{-1}. The optimum pH for growth on veratryl alcohol was between pH 5.0 and pH 5.5; during fed batch cultivation the pH stays in this range.

Fed batch cultivation of the fungus in flat bottomed round flasks with magnetic stirring gave no pellet formation as contrasted with batch cultivation in agitated 5 l erlenmeyer flasks. Therefore a better reproducibility of enzyme activity in fed batch cultivated cell-free extracts was achieved. Attempts to grow the fungus in a CSTR (Continuously Stirred Tank Reactor) were unsuccesful.

Oxidation and accumulation of metabolites by whole cells. *P. simplicissimum* CBS 170.90, when grown batch-wise on veratryl alcohol, excreted both veratraldehyde and veratric acid in the medium. The organism accumulated vanillic acid when grown fed batch on veratric acid in the flat bottomed round flask. The red color of the growth medium observed correlated to the vanillic acid concentration. This color was never observed while growing the fungus on veratryl alcohol or succinate.

Incubation of veratryl alcohol-grown washed cells with veratryl alcohol resulted in the transient accumulation of veratric acid and vanillic acid (Figure 1a) while no veratraldehyde was detected. Incubation of these washed cells with veratraldehyde resulted in a quick transient accumulation of both veratryl alcohol and veratric acid, with vanillic acid accumulating to a lesser extent (Figure 1b). From veratric acid these cells transiently accumulated vanillic acid (Figure 1c). In a subsequent experiment, both veratryl alcohol and veratric acid grown washed cells were incubated with veratric acid, with vanillic acid and with isovanillic acid. Both types of washed cells metabolized isovanillic acid faster than either vanillic acid or veratric acid (16, 5 and 7 nmol min^{-1} (mg protein)$^{-1}$ by veratryl alcohol-grown cells and 75, 49 and 49 nmol min^{-1} (mg protein)$^{-1}$ by veratric acid-grown cells respectively).

Enzyme activities in crude cell-free extracts. From the above results, veratryl alcohol metabolism by *P. simplicissimum* CBS 170.90 was anticipated to proceed via veratraldehyde and veratric acid. In dialyzed cell-free extracts a NAD^+-dependent veratryl alcohol dehydrogenase activity was detected (Table 2). Activity with $NADP^+$ was slightly lower than with NAD^+. A trace of a PMS-dependent veratryl alcohol

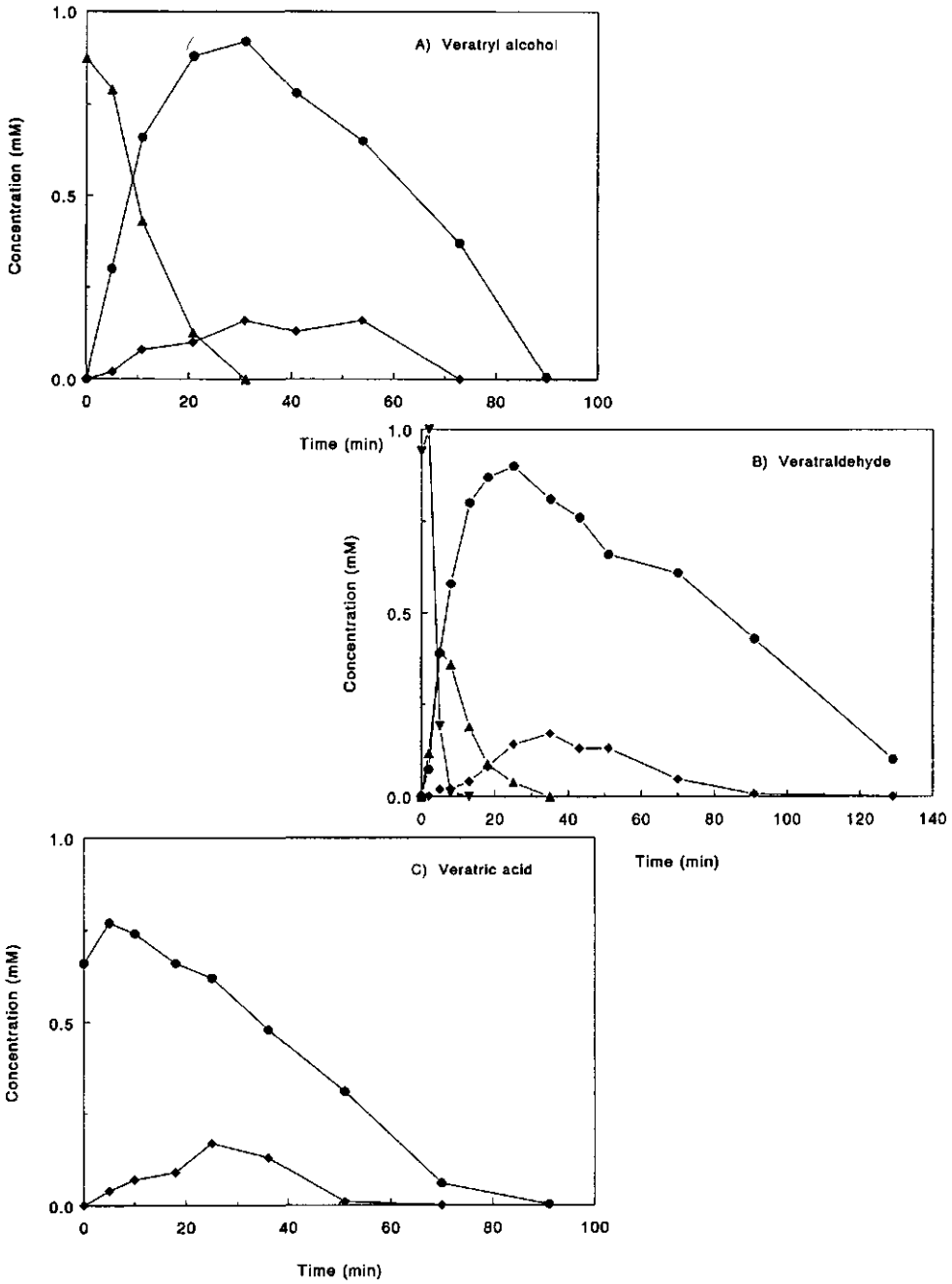

Figure 1. Consumption of veratryl alcohol (**A**), veratraldehyde (**B**) and veratric acid (**C**) and transient accumulation of degradation products by *Penicillium simplicissimum* CBS 170.90. Incubation mixtures (10 ml) contained veratryl alcohol-grown washed cells (32 mg protein) -▲- veratryl alcohol; -▼- veratraldehyde; -●- veratrate; -♦- vanillate.

dehydrogenase was also observed. The PMS-dependent alcohol dehydrogenase activity was stimulated by the addition of NH_4^+-ions. The products of both the NAD^+- and PMS-dependent veratryl alcohol transformation were identified as veratraldehyde by using the authentic compound for comparison of HPLC retention times and absorption spectra. Neither veratryl alcohol oxidase activity nor lignin peroxidase activity could be demonstrated in concentrated supernatant or in cell-free extracts of veratryl alcohol grown cells. It was also possible to detect a $NAD(P)^+$-dependent veratraldehyde dehydrogenase, but no PMS-dependent activity for the aldehyde was observed in dialyzed cell-free extracts. The pH optima of the two alcohol dehydrogenases and the aldehyde dehydrogenase were around pH 10.

A low but distinct *O*-demethylase activity for veratric acid was observed whenever extracts were prepared from veratric acid grown cells (Table 2). Demethoxylation of veratric acid was dependent on the presence of molecular oxygen and NAD(P)H as electron donor. The cell-free extracts from veratric acid grown cells also showed demethylase activity for vanillic and isovanillic acid (Table 2) but not for veratryl alcohol or veratraldehyde. Unfortunately, no *O*-demethylase activity was detected in cells grown on veratryl alcohol, although various techniques for cell disrupture and various incubation conditions were examined. Neither an oxidative decarboxylase nor a nonoxidative decarboxylase activity acting on the three different acids was detected in extracts of both veratryl alcohol and veratric acid grown cells.

Table 2. Specific enzyme activities involved in the veratryl alcohol degradation in *Penicillium simplicissimum* CBS 170.90 grown on various carbon sources

	Specific activity (nmol min^{-1} mg $protein^{-1}$)		
	Growth substrate		
Enzymes	Veratryl Alcohol	Veratric Acid	Succinic Acid
Veratryl alcohol dehydrogenase			
NAD^+-dependent	65	25	6
PMS-dependent	1.4	0.8	>0.1
Veratraldehyde dehydrogenase	13	5	3
Veratric acid-*O*-demethylase	N.D.	0.3	N.D.
Vanillic acid-*O*-demethylase	N.D.	1.5	N.D.
Isovanillic acid-*O*-demethylase	N.D.	0.7	N.D.
Protocatechuate 3,4-dioxygenase	325	875	20
Catechol 1,2-dioxygenase	16	4	4

N.D., not detected; PMS, phenazine methosulphate

Protocatechuate 3,4-dioxygenase activity was induced after growth of CBS 170.90 on both veratryl alcohol and veratric acid (Table 2). Catechol 1,2-dioxygenase was also detected in cell-free extracts, but no significant induction of the enzyme was observed when the fungus was grown on veratryl alcohol or veratric acid. Upon dialysis, addition of $(NH_4)_2Fe(SO_4)_2$ restored full activity of both protocatechuate and catechol dioxygenase. Neither gentisate 1,2-dioxygenase activity nor extra diol cleavage of either catechol or protocatechuate was detected in any of the cell-free extracts.

DISCUSSION

Veratryl alcohol, a natural occurring aromatic compound, was readily degraded by several bacteria, yeasts and fungi. The here described fungus *Penicillium simplicissimum* CBS 170.90 used various aromatic compounds as sole source of carbon and energy (Table 1). This fungus has a rather broad substrate specificity when compared with *Penicillium spinulosum* P6 (Cain et al. 1968) or *Aureobasidium pullulans* (Bourbonnais and Paice 1987). In the case of both *P. spinulosum* P6 and *A. pullulans* substrate toxicity cannot be neglected. Oxidation and reduction patterns for aromatic alcohols and aldehydes by *A. pullulans* grown on rich medium without aromatic carbon sources are very similar to the picture obtained for CBS 170.90 grown on veratryl alcohol (Figure 1). Also non-induced cells of *Methylosinus trichosporium* oxidize immediately several aromatic alcohols including veratryl alcohol (Mountfort et al. 1990).

CBS 170.90 oxidized veratryl alcohol via veratraldehyde to veratrate with a $NAD(P)^+$-dependent enzyme. The very low activity of a PMS-dependent veratryl alcohol dehydrogenase is probably a reflection of a non-specific alcohol dehydrogenase activity. Alternative ways for the oxidation of veratryl alcohol via for instance an oxidase (Bourbonnais and Paice 1988) or a peroxidase (Tien and Kirk 1988) were not detected.

Veratric acid is demethylated to form protocatechuate. It is known from literature (Ribbons 1971, Ander et al. 1988, Bernhardt et al. 1988) that *O*-demethylases are very unstable enzymes. This phenomenon was also experienced in CBS 170.90. It was observed that *O*-demethylase activity in extracts of this fungus was completely lost in less than 8 hours at 4 °C (unpublished results). In cell-free extracts of veratrate grown CBS 170.90 vanillate is demethylated at a higher rate than both isovanillate and veratrate (Table 2). In the experiments with veratryl alcohol grown washed cells incubated with different substrates vanillate accumulated (Figure 1). Both washed cells grown on veratryl alcohol and veratric acid had a higher activity for isovanillate than

for vanillate or veratrate. It is known that some demethylases are specific for either the meta- or the para-methoxyl group while other enzymes act on both substrates but at various rates for the different molecules (Bernhardt et al. 1988). In view of the instability of the demethylating enzyme(s) it has so far not been possible to distinguish whether one or more *O*-demethylases are involved in the degradative route of veratryl alcohol. Presently it is being investigated if conditions can be created to stabilize the veratric acid demethylating enzyme in order to allow a partial purification.

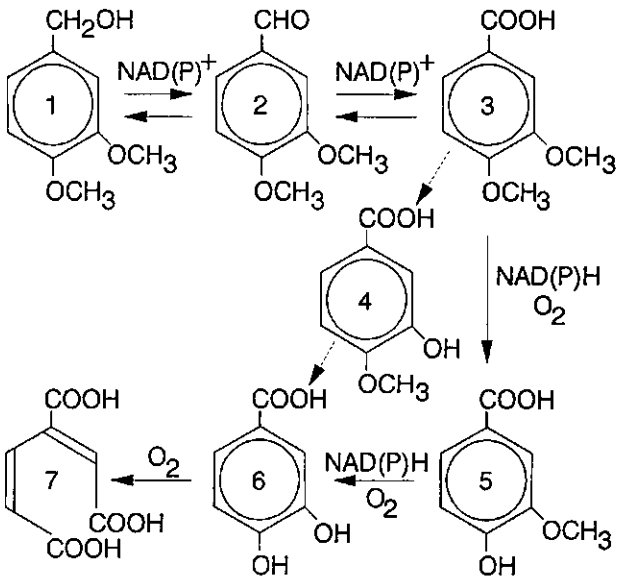

Figure 2. Proposed pathway for the metabolism of veratryl alcohol in *P. simplicissimum* CBS 170.90. *1.* Veratryl alcohol; *2.* Veratraldehyde; *3.* Veratric acid; *4.* Isovanillic acid; *5.* Vanillic acid; *6.* Protocatechuic acid; *7.* ß-carboxy-*cis,cis*-muconnic acid.

There were no indications that nonoxidative decarboxylation (Kuwahara et al. 1981, Pometto III et al. 1981) or oxidative decarboxylation (Buswell et al. 1979) of veratrate, vanillate or isovanillate occurred in CBS 170.90.

From the results obtained it is clear that protocatechuate is an intermediate in the degradation pathway of veratryl alcohol. In contrast to the results of Fujisawa and Hayaishi (1968), Wojtas-Wasilewska et al. (1983) have shown that the protocatechuate 3,4-dioxygenase they purified from *Pleurotus ostreatus* also has a rather high activity for catechol (33%). So it can not be ruled out that only a protocatechuate 3,4-dioxygenase is present in cell-free extracts of CBS 170.90 grown on veratryl alcohol or veratric acid.

From the results obtained it is concluded that a degradation mechanism for veratryl alcohol as described for white-rot fungi as *Phanerochaete chrysosporium*

(Schmidt et al. 1989) does not occur in *P. simplicissimum*. The new inducible pathway for the degradation of this aromatic compound is shown in Figure 2.

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Electron micrograph
(50000 x)
of purified

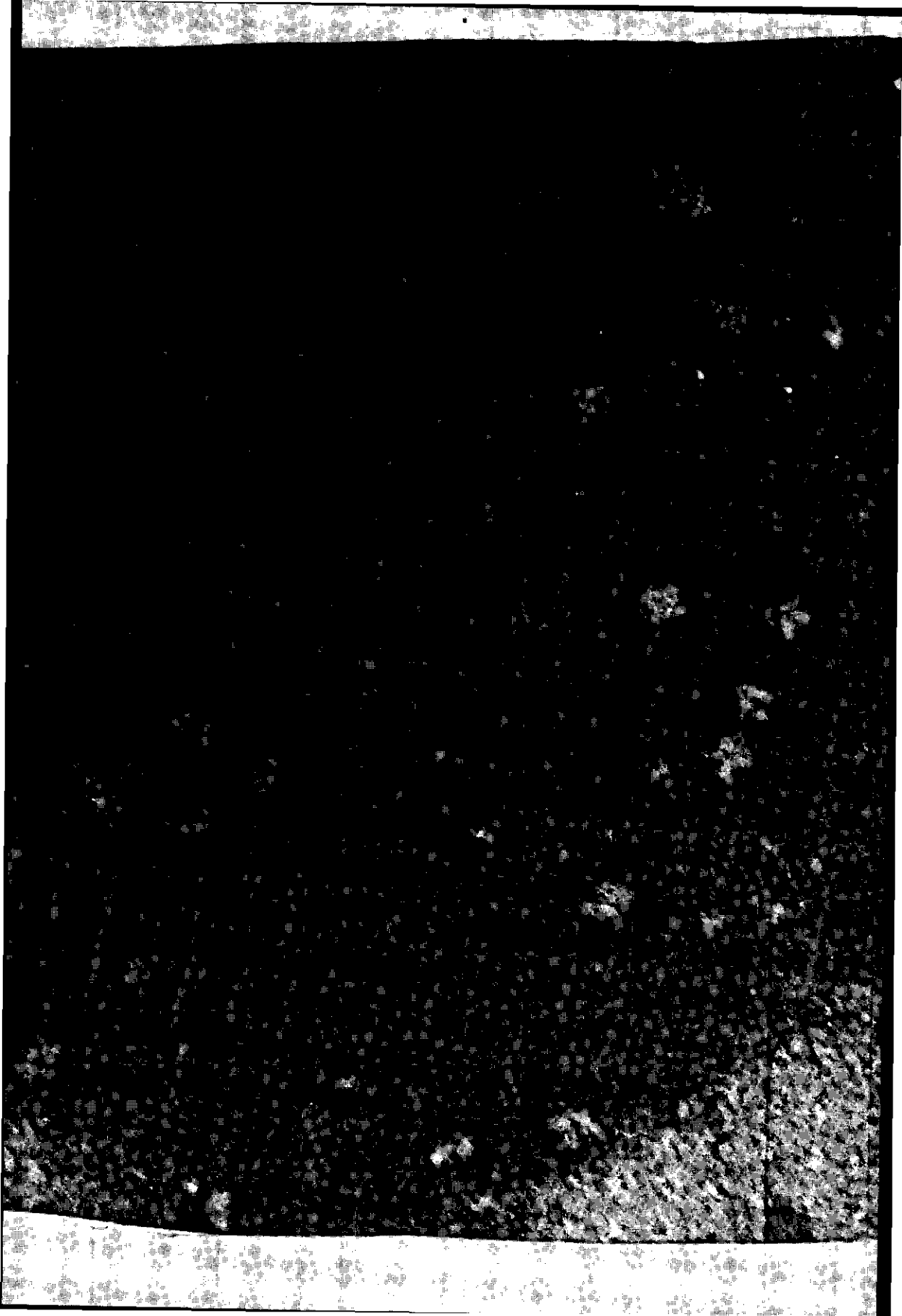
Vanillyl Alcohol Oxidase
(VAO)

octamers isolated from
Penicillium simplicissimum

Enzyme was coloured
with
sodium silicotungstate
(SST)

In the upper left corner
a dissociation of the enzyme
into mono- and dimers
can be seen

Courtesy of
Marco Fraaije



CHAPTER 9

PURIFICATION AND CHARACTERIZATION OF VANILLYL ALCOHOL OXIDASE FROM PENICILLIUM SIMPLICISSIMUM: A NOVEL AROMATIC ALCOHOL OXIDASE CONTAINING COVALENTLY BOUND FAD

Ed de Jong, Willem J.H. van Berkel, Rick P. van der Zwan
and Jan A.M. de Bont

SUMMARY

Vanillyl alcohol oxidase was purified 32-fold from *Penicillium simplicissimum*, grown on veratryl alcohol as its sole source of carbon and energy. SDS-PAGE of the purified enzyme reveals a single fluorescent band of 65 kDa. Gel filtration and sedimentation-velocity experiments indicate that the purified enzyme exists in solution as an octamer, containing 1 molecule flavin/subunit. The covalently bound prosthetic group of the enzyme was identified as 8α-(N^3-histidyl)-FAD from pH dependent fluorescence quenching (pK_a = 4.85) and no decrease in fluorescence upon reduction with sodium borohydride. The enzyme shows a narrow substrate specificity, only vanillyl alcohol and 4-hydroxybenzyl alcohol are substrates for the enzyme. Cinnamyl alcohol is a strong competitive inhibitor of vanillyl alcohol oxidation.

The visible absorption spectrum of the oxidized enzyme shows maxima at 354 nm and 439 nm, and shoulders at 370, 417 and 461 nm. Under anaerobic conditions, the enzyme is easily reduced by vanillyl alcohol to the two-electron reduced form. Upon mixing with air, rapid reoxidation of the flavin occurs. Both with dithionite reduction and photoreduction in the presence of EDTA and 5-deazaflavin the red semiquinone flavin radical is transiently stabilized. Opposite to most flavoprotein oxidases, vanillyl alcohol oxidase does not form a flavin N^5-sulfite adduct. Photoreduction of the enzyme in the presence of the competitive inhibitor cinnamyl alcohol gives rise to a complete, irreversible bleaching of the flavin spectrum.

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INTRODUCTION

Fungal aryl-alcohol oxidases (aromatic alcohol oxidase, E.C. 1.1.3.7) described to date include enzymes from *Polystictus versicolor* (Farmer et al. 1960), *Pleurotus sajor-caju* (Bourbonnais and Paice 1988), *Pleurotus eryngii* (Guillén et al. 1990), *Pleurotus ostreatus* (Sannia et al. 1991), *Bjerkandera adusta* (Muheim et al. 1990) and from *Fusarium* sp. (Iwahara et al. 1980). All the extracellular aryl-alcohol oxidases excreted by the above-mentioned white-rot fungi have a rather broad substrate specificity. It is thought that these aryl-alcohol oxidases play a role in H_2O_2 production, necessary in lignin biodegradation. The aryl-alcohol oxidases of *B. adusta* and *P. ostreatus* are both flavoproteins, containing non-covalently bound FAD as prosthetic group.

The fungus *Penicillium simplicissimum* CBS 170.90 is not ligninolytic but can use a range of aromatic compounds, including veratryl and vanillyl alcohol, as sole source of carbon and energy. Veratryl alcohol plays a crucial role in lignin degradation by the white-rot fungus *Phanerochaete chrysosporium* (Buswell 1992). *P. simplicissimum* degrades veratryl alcohol via NAD(P)$^+$-dependent dehydrogenase(s) and veratric acid *O*-demethylase(s) (de Jong et al. 1990). Recently it was found that this organism, when grown on veratryl alcohol, induces an intracellular H_2O_2-generating oxidase not acting on veratryl alcohol but rather on vanillyl alcohol. In this paper, we describe the production, purification and characterization of this oxidase from *P. simplicissimum*.

MATERIALS AND METHODS

General. Calf intestine phosphatase (grade II, EC 3.1.3.1), hog kidney aminopeptidase M (EC 3.4.11.2), glucose oxidase (grade I, EC 1.1.3.4) and catalase (EC 1.11.1.6) were from Boehringer (Mannheim, FRG). FAD, FMN and *naja naja atra* snake venom (used as a source of phosphodiesterase) were obtained from Sigma (St. Louis, MO). Phenyl-Sepharose CL-4B, Superose 6 and Mono-Q anion exchange column HR 5/5 were products of Pharmacia (Uppsala, Sweden). Hydroxyapatite, Bio-Gel P-6-DG and Bio-Gel P-2 were purchased from Bio-Rad (California, USA). Acrylamide, bisacrylamide, low-molecular-mass calibration kit and Coomassie brilliant blue R250 were from Serva (Heidelberg, FRG). Vanillyl alcohol and vanillin were products of Janssen Chimica (Beerse, Belgium), 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde were products of Fluka AG (Buchs, Switserland). All the other chemicals were of commercially available analytical grade and were used without further purification.

Analytical methods. The activity of vanillyl alcohol oxidase (EC 1.1.3.-) was assayed for spectrophotometrically (Perkin Elmer 550A ultraviolet-visible spectrophotometer) by following at 340 nm the vanillyl alcohol-dependent production of vanillin ($\epsilon_{vanillin}$ = 22.8 $mM^{-1}cm^{-1}$ at pH 10.0). The reaction mixture (total volume 2.4 ml) contained dialysed cell-free extract, 115 mM $(NH_4)_2SO_4$ and 42 mM glycine/NaOH pH 10.0. The reaction was started by adding 1.25 mM vanillyl alcohol. 1 U enzyme oxidizes 1 μmol vanillyl alcohol to vanillin in 1 min at 30 °C and pH 10.0. When measuring the pH optimum of the oxidase, the activity was corrected for the pH dependence of the molar absorption coefficient of vanillin.

Substrate specificity was measured in a YSI model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA) by following oxygen uptake at 30 °C. The reaction vessel contained 150 μmol glycine/NaOH, pH 10.0, 275 μmol $(NH_4)_2SO_4$ and 0.05 U purified enzyme in a total volume of 3.25 ml. The reaction was started by adding 100 μl 10 mM substrate solution. H_2O_2 production was measured in the biological oxygen monitor, under the same conditions as described above, by measuring the oxygen consumption before and after catalase (150 U) addition.

Vanillyl alcohol, vanillin, 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde were determined by HPLC. A Kratos HPLC system (Applied Biosystems B.V., Maarssen, The Netherlands) was used with a Chromspher C-18 reverse phase column (Chrompack, Middelburg, The Netherlands). The eluent was a mixture of methanol, Milli-Q H_2O and acetic acid (33:66:1). The ultraviolet spectra of the different compounds were scanned *in situ* after the flow had been stopped.

Mass spectra were measured with a Hewlett Packard HP 5890 GC with a 30-m DB17 column and a HP 5970 MSD. Sample preparation: the reaction mixture (7.5 ml) containing 0.3 mmol glycine, pH 10.0, 0.8 mmol $(NH_4)_2SO4$, 1 U enzyme and 0.027 mmol substrate (vanillyl alcohol or 4-hydroxybenzyl alcohol) was incubated at 30 °C for 90 min. The reaction fluid was injected without derivatization into GC/MS, and mass spectra of the two products were recorded.

Absorption spectra were recorded at 25 °C on a DW-2000 ultraviolet/visible spectrophotometer (SLM Aminco, Urbana, IL, USA). Enzyme solutions (in 20 mM Tris/HCl, pH 8.5) were made anaerobic by alternate evacuation and flushing with argon. Enzyme reduction by the substrate was performed in the presence of glucose oxidase (10 U) and glucose (310 mM), added to both substrate and enzyme solution to remove remaining trace amounts of oxygen. Photoreduction in the presence of EDTA and 10-methyl-5-deazaisoalloxazine-3-propanesulfonate (5-deazaflavin) as catalyst was performed as described by Massey and Hemmerich (1978) and Duchstein et al. (1979).

50 mM citrate buffer, pH 3.0, and 50 mM sodium phosphate buffer, pH 8.0, both containing equal concentrations of flavin (peptide) (Singer and Edmondson 1980).

RESULTS AND DISCUSSION

Enzyme purification. *P. simplicissimum* was routinely grown fed batch in a flat-bottomed round flask with veratryl alcohol as sole carbon and energy source (de Jong et al. 1990). Under these conditions, an intracellular vanillyl alcohol oxidizing activity was induced. No extracellular activity of the enzyme was detected. Oxidase activity in cell extracts was 75 ± 20 nmol min^{-1} (mg protein)$^{-1}$ compared to 38 nmol min^{-1} (mg protein)$^{-1}$ in extracts of veratrate-grown cells and 18 nmol min^{-1} (mg protein)$^{-1}$ in extracts of succinate-grown cells. Surprisingly, oxidase activity was only 17 nmol min^{-1} (mg protein)$^{-1}$ in extracts of cells grown on vanillyl alcohol. As veratryl alcohol is not a substrate for the oxidase, the physiological role of the enzyme is still unclear. Therefore, and because of, the narrow substrate specificity (see catalytic properties) the enzyme is named vanillyl alcohol oxidase (VAO).

Table 1. Purification procedure for vanillyl-alcohol oxidase

Purification step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Factor	Yield (%)
$(NH_4)_2SO_4$	1122	75.2	0.067	1.0	100
Cell extract	770	63.9	0.083	1.2	85
Phenyl-Sepharose	322	56.4	0.175	2.6	75
Hydroxy apatite	33	51.1	1.57	23.4	68
Mono-Q	17	36.8	2.13	31.8	49

Table 1 summarizes the vanillyl-alcohol oxidase purification procedure. On SDS-PAGE, the different steps of the purification are shown (Figure 1). In some cases, the final step of the purification, using Mono-Q anion-exchange chromatography, resolved the enzyme into several peaks of activity. The different peaks had similar specific activities, molecular masses on SDS-PAGE were also the same. The different peaks after Mono-Q purification were combined without further characterization.

Catalytic properties. Molecular oxygen was required for the oxidation of vanillyl alcohol with vanillin and H_2O_2 formed in stoichiometric amounts. The reaction was

linear for several minutes whenever $(NH_4)_2SO_4$ was included in the reaction mixture. Enzyme assays degassed with nitrogen showed almost no activity. Oxygen (K_m = 12 μM, V = 2.4 U (mg protein^{-1})) could be replaced by phenazine methosulfate (K_m = 43 μM, V = 2.1 U (mg protein^{-1})). With 2,6-dichloroindophenol and potassium ferricyanide as electron acceptors, no reaction was observed.

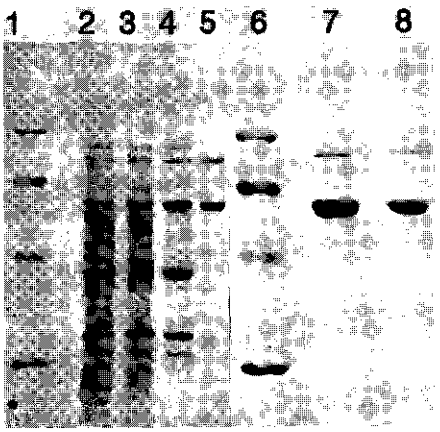
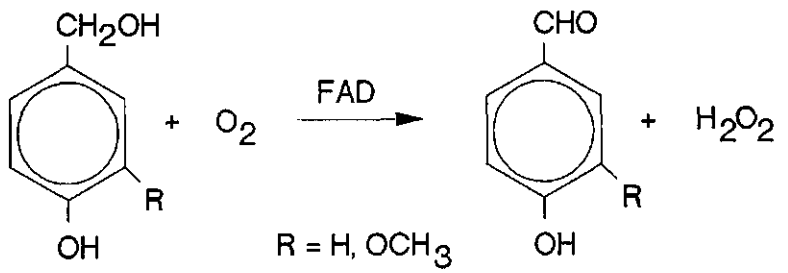


Figure 1. *Purification of vanillyl-alcohol oxidase from Penicillium simplicissimum as followed by SDS/PAGE.*
Lanes 1 and 6, marker proteins (from top to bottom: phosphorylase b, 92.5 kDa; bovine serum albumin, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa). Lane 2, cell extract. Lane 3, $(NH_4)_2SO_4$ precipitation pool. Lane 4, phenyl-Sepharose pool. Lane 5, hydroxyapatite pool. Lanes 7 and 8, Mono-Q pool at two concentrations.

The relative activity of the enzyme with different substrates was determined by measuring the O_2 consumption, as described in Materials and Methods. Apart from vanillyl alcohol (2.4 U (mg protein)$^{-1}$, K_m 0.73 mM), the enzyme only oxidized 4-hydroxybenzyl alcohol (75% of the O_2 consumption rate compared to vanillyl alcohol). Aromatic alcohols not oxidized included 2-bromobenzyl alcohol, 2-hydroxybenzyl alcohol, cinnamyl alcohol, 4-methylbenzyl alcohol, benzyl alcohol, veratryl alcohol, isovanillyl alcohol, 4-hydroxyphenethyl alcohol, 1- and 2-phenethyl alcohols, 3- and 4-methoxybenzyl alcohols. Neither aromatic aldehydes (vanillin, 4-hydroxybenzaldehyde) nor non-aromatic alcohols (methanol, ethanol, allyl alcohol) were oxidized by the enzyme.



Scheme 1. Reactions catalyzed by vanillyl-alcohol oxidase from *P. simplicissimum*.

The identity of the reaction products after oxidation of vanillyl alcohol and 4-hydroxybenzyl alcohol were elucidated in two independent ways.

The enzyme catalyzed oxidation of both substrates was followed by HPLC. Both retention times and *in situ* scanned spectra of the stoichiometric accumulating products were identical with the authentic aldehydes. The mass spectra of the products were recorded. The product of vanillyl alcohol contained the molecular ion at *m/z* (relative intensity) $\{M\}^+$ 152 (95%) and the following diagnostic fragments had more than 15% abundance: 151 (100%), 123 (20%), 109 (22%), 81 (33%), 51 (19%), 52 (19%) and 53 (21%). This spectrum was the same as that of authentic vanillin. The mass spectrum of the product of 4-hydroxybenzyl alcohol contained the molecular ion at *m/z* (relative intensity) $\{M\}^+$ 122 (90%) and there were the following diagnostic fragments more than 15% abundance: 121 (100%), 93 (44%), 65 (45%), 63 (13%) and 39 (43%). This spectrum was the same as that of authentic 4-hydroxybenzaldehyde.

All the substrate analogues tested for activity with the enzyme were tested for competitive inhibition of the oxidation of vanillyl alcohol. Cinnamyl alcohol was a strong competitive inhibitor with a K_i of 84 μM at pH 10.0. 4-Hydroxyphenethyl alcohol also showed some inhibition, other compounds were not inhibitory at 0.3 mM.

The enzyme activity, as a function of pH, is shown in Figure 2. The enzyme has a broad pH optimum for activity, with more than 90% of the maximum activity being observed between pH 9 and 10.5. The optimum temperature for enzyme activity was found to be 38°C. Stability tests showed that the oxidase could be stored at -20°C and at 4°C in 20% $(NH_4)_2SO4$ at pH 7.0 for several weeks without loss of activity. No inactivation occurred after 90 min at 45°C, while 50% of the enzyme activity was lost after 45 min at 60°C (pH 7.0).

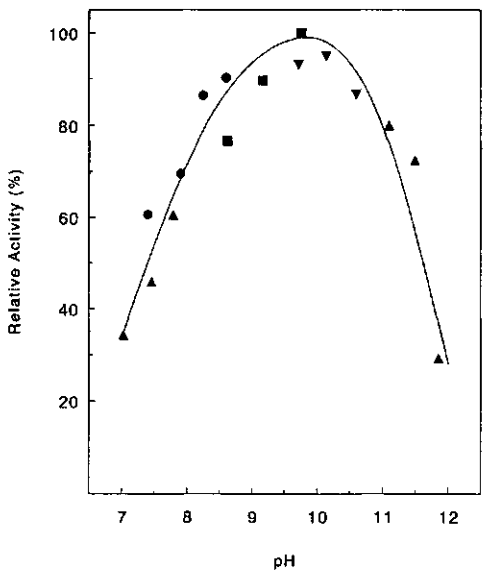
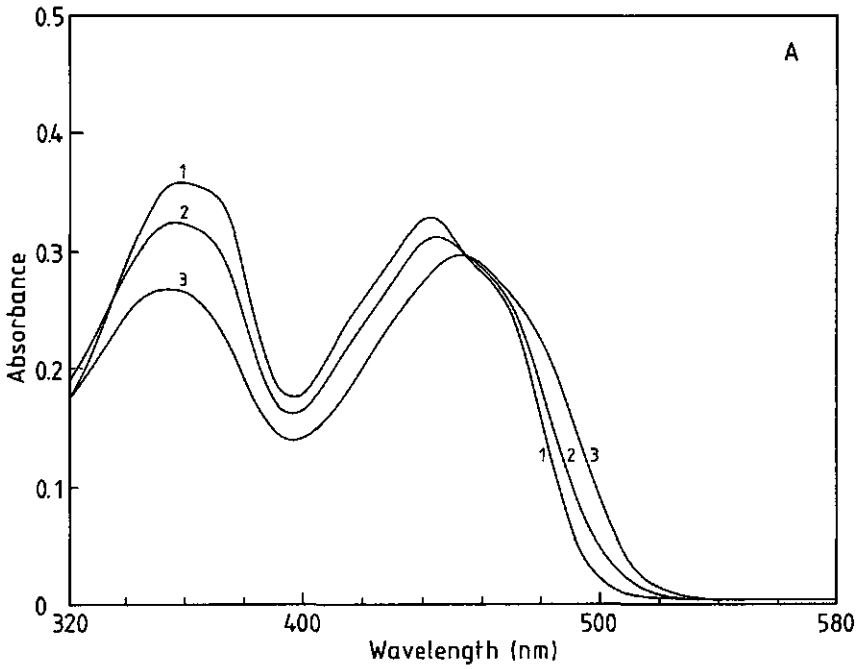


Figure 2. *Influence of pH on the activity of vanillyl-alcohol oxidase.* The activity was determined in buffers of 50 mM containing 1.25 mM vanillyl alcohol and 115 mM $(NH_4)_2SO_4$ at 30°C. (▲) 50 mM potassium phosphate, (●) 50 mM Tris/HCl; (■) 50 mM borate; (▼) 50 mM glycine.

Hydrodynamic properties. Based on the elution pattern of the Superose-6 column, the apparent molecular mass of native vanillyl alcohol oxidase was 520 kDa. The sedimentation coefficient, as determined by ultracentrifugation experiments, also indicated a high molecular mass, yielding a value $S_{20,w}$ = 16.3S. The subunit molecular mass of the enzyme was 65 kDa, as estimated by SDS-PAGE (Figure 1) and indicates that in the native state the enzyme is an octamer composed of eight identical subunits. Both the gel filtration and sedimentation pattern did not indicate the presence of other association states. Comparable quaternary structures have been reported for alcohol oxidase from *Hansenula polymorpha* and *Pichia pastoris* (Kato et al. 1976; Hopkins and Müller 1987). The minor impurity seen with SDS-PAGE (Figure 1) could not be removed by gel filtration on a Sephacryl S300 column. The relative amount of this minor band fluctuated in different purifications, and no second fluorescent band appeared upon ultraviolet illumination (see also below). Therefore, it is concluded that this minor band is an impurity.



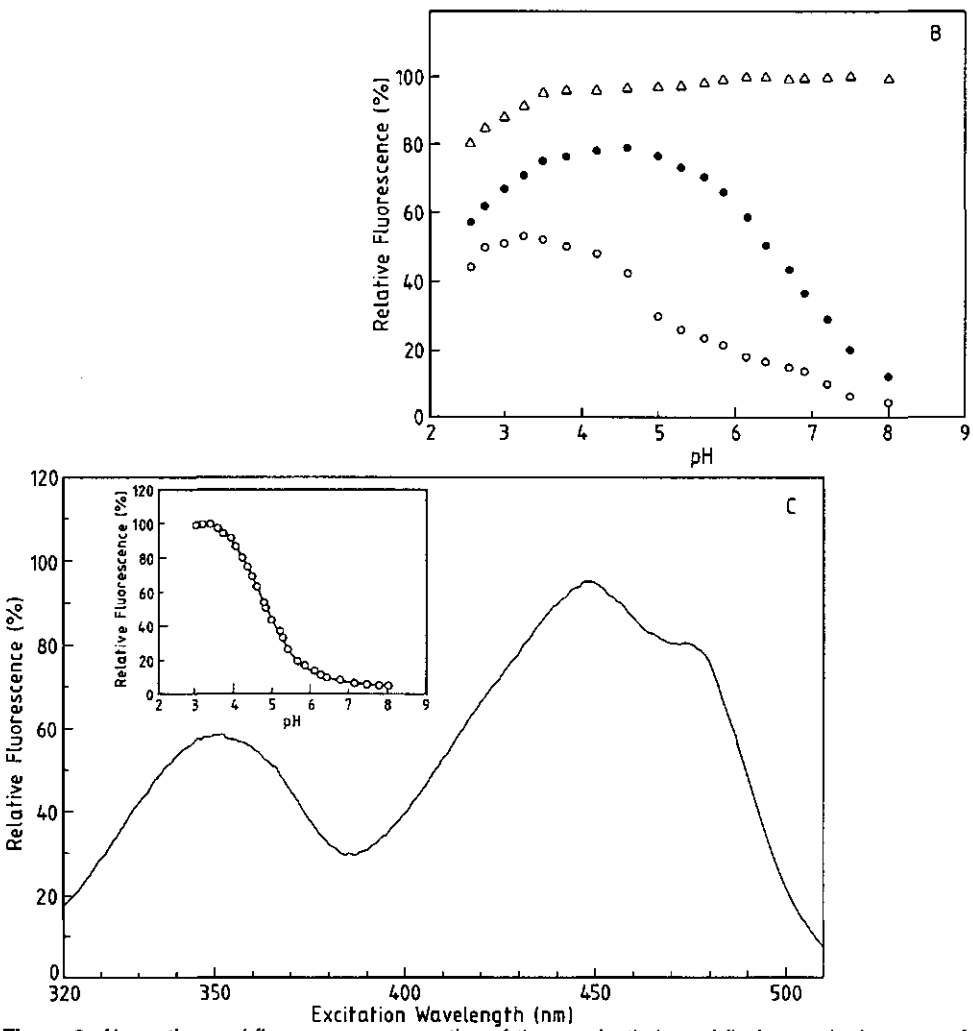


Figure 3. *Absorption and fluorescence properties of the covalently bound flavin prosthetic group of vanillyl-alcohol oxidase from P. simplicissimum.*

Absorption spectra were recorded in 100 mM sodium phosphate, pH 7.0. Fluorescence emission was recorded at 525 nm, using an excitation wavelength of 445 nm. Fluorescence excitation spectra were recorded at 25°C and corrected for dilution. For other details see Materials and Methods.

A) Absorption spectrum of 26.2 μM vanillyl-alcohol oxidase (1); after incubation for 20 min in 0.1% SDS (2) or in 0.5% SDS (3), containing 1 mM dithiothreitol.

B) Fluorescence emission of 0.2 μM SDS-unfolded vanillyl-alcohol oxidase as a function of pH (O); after treatment with *naja naja* snake venom (●); 0.2 μM free FMN as a reference (△).

C) Fluorescence excitation spectrum of 0.1 μM Bio-Gel P-2 purified aminoacylriboflavin preparation of vanillyl-alcohol oxidase. The inset shows the relative fluorescence emission of the same sample as a function of pH.

Prosthetic group. The purified enzyme showed absorption maxima in the visible region at 354 nm and 439 nm, and shoulders at 370, 417 and 461 nm (Figure 3A), indicating that the enzyme contains flavin. The ratio of A_{280}/A_{439} was 13.4 : 1. After precipitation of vanillyl-alcohol oxidase by acidification with 5% trichloroacetic acid or by heat treatment (95 °C, 30 min), the supernatant did not show any absorbance at 439 nm, indicating that the flavin prosthetic group is covalently bound. Upon unfolding of the enzyme in 0.5% SDS, the absorption maxima decreased in intensity and shifted to 352 nm and 450 nm (Figure 3A). The strong hypsochromic shift of the second absorption band, as compared to free FAD or FMN, is a characteristic feature of 8α-substituted flavin (Singer and Edmondson 1980), often found with oxidases containing covalently bound flavin (6-hydroxy-D-nicotine oxidase, Möhler et al. 1972; choline oxidase, Ohta-Fukuyama et al. 1980; cholesterol oxidase, Fukuyama and Miyake 1979; pyranose oxidase, Machida and Nakanishi 1984).

The visible absorption spectrum of free FMN or FAD does not change upon addition of 0.5% SDS (results not shown). If it is assumed that the molar absorption coefficient of the unfolded enzyme (ϵ_{450} = 11.3 mM^{-1} cm^{-1}, Kiuchi et al. 1982) is comparable to that of 8α-substituted FAD (see below), a value (ϵ_{439} = 12.5 mM^{-1} cm^{-1}) is calculated for the molar absorption coefficient of the native enzyme. From this, the quantity of protein/flavin is estimated as 65.1 kg mol^{-1}.

Four types of 8α-substituted flavins (N^1-histidyl, N^3-histidyl, *O*-tyrosyl and *S*-cysteinyl) are known (Singer and McIntire 1984). As firmly established by Singer and Edmondson (1980), 8α-histidylflavins exhibit a characteristic increase in fluorescence below pH 6. SDS-treated vanillyl-alcohol oxidase also showed pH-dependent fluorescence properties (Figure 3B). Upon treatment of the unfolded enzyme with *naja naja* snake venom, there was a threefold increase of fluorescence at pH 6 (Figure 3B). These results indicate that the flavin derivative is present at the FAD level and is covalently bound via a histidyl moiety at the 8α position. Additional evidence for the presence of this type of flavin comes from the observation that upon ultraviolet illumination of unstained SDS-PAGE gels, one strongly fluorescent band (65 kDa) is observed when the protein is fixed in 5% acetic acid (Kvalnes-Krick and Schumann-Jorns 1986).

In order to discriminate between an 8α-(N^1-histidyl) or 8α-(N^3-histidyl) linkage between flavin and protein, aminoacylriboflavin was prepared by proteolytic digestion of the enzyme and successive treatment of the solubilized flavin peptide with phosphodiesterase, alkaline phosphatase and aminopeptidase M (see Materials and Methods). The aminopeptidase-treated flavin peptide eluted on Bio-Gel P-2 as two separate yellow bands, most probably due to incomplete enzymic hydrolysis. Aminoacylriboflavin (about 70% of total flavin yield) eluted in the second strongly

retarded band, as evidenced by TLC and the low ratio A_{280}/A_{450} = 3.0.

Figure 3C shows the excitation spectrum of the aminoacylriboflavin preparation obtained after Bio-Gel P-2. The maximum at 349 nm again is indicative for an 8α-substituted flavin. The fluorescence quantum yield observed at pH 3.8 is in the same range as found for free riboflavin or FMN. In the pH-dependent fluorescence measurements, a tenfold increase in fluorescence was observed at low pH (inset Figure 3C). The observed pK_a of 4.85 is in close agreement with the reported pK_a of 4.7 for 8α-(N^3-histidyl)riboflavin (Edmondson et al. 1976). Unlike the N^3-histidylriboflavin isomer, N^1-histidylriboflavin is easily reduced by sodium borohydride (Edmondson et al. 1976), which leads to a strong decrease in fluorescence. No decrease in fluorescence was observed when the Bio-Gel P-2 purified aminoacylriboflavin preparation was treated with sodium borohydride. Furthermore, and unlike N^1-histidylriboflavin (Edmondson et al. 1978), the aminoacylriboflavin preparation was very stable upon storage at room temperature. It is therefore concluded that vanillyl alcohol oxidase from *P. simplicissimum* contains 8α-(N^3-histidyl)-FAD as a covalently bound prosthetic group. Decker and Brandsch (1991) have recently reviewed the physical properties of flavoproteins with a covalent 8α-(N^3-histidyl)-FAD linkage, including 6-hydroxy-D-nicotine oxidase and choline oxidase.

Spectral properties. Anaerobic reduction of enzyme-bound flavin by vanillyl alcohol resulted in a decrease in absorbance at 440 nm without any detectable semiquinone formation (Figure 4), indicating that the flavin component is functionally involved in the oxidation of vanillyl alcohol. The increase in absorbance at 347 nm is due to the formation of vanillin, as demonstrated by aerobically incubating the enzyme with excess substrate (inset Figure 4). Reoxidation of enzyme with air immediately gave the typical oxidized flavin spectrum at 440 nm (data not shown).

The binding of cinnamyl alcohol, a competitive inhibitor, was investigated. The K_d of cinnamyl alcohol binding to vanillyl alcohol oxidase, as determined with absorption difference spectroscopy, was determined to be 1.6 mM at pH 8.5 (Figure 5) and 127 μM at pH 10.0 (20 mM glycine, data not shown). Cinnamyl alcohol could be resolved from the enzyme-inhibitor complex by gel filtration on Bio-Gel P-6-DG.

Vanillyl alcohol oxidase did not show any reactivity with sulfite. In general, flavoprotein oxidases form an N^5 adduct, with spectral properties resembling those of reduced flavin (Massey et al. 1969). Addition of 11.1 mM sulfite did not result in any spectral changes of vanillyl-alcohol oxidase, neither in 20 mM Tris/HCl, pH 8.5, nor in 20 mM potassium phosphate, pH 7.5. Also, old yellow enzyme and putrescine oxidase (Massey and Hemmerich 1980; DeSa 1972), both producing a red semiquinone flavin radical, do not form a flavin N^5-sulfite adduct.

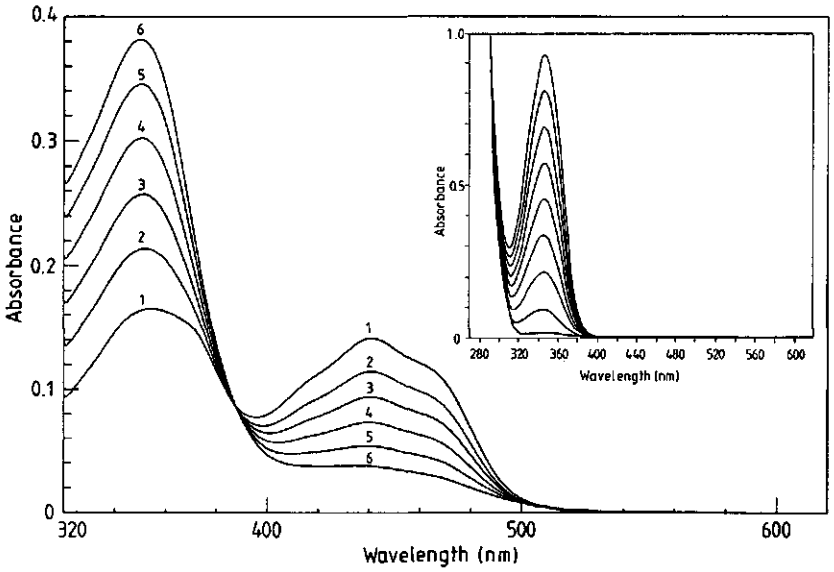


Figure 4. *Anaerobic reduction of enzyme-bound flavin by vanillyl alcohol.*
(1) 11.6 μM vanillyl alcohol oxidase in 0.86 ml 18.6 mM Tris/HCl, pH 8.5, 290 mM glucose and 9.4 U glucose oxidase (added anaerobically). The solution was titrated with a solution containing 1 mM vanillyl alcohol, 310 mM glucose and 10 U glucose oxidase (added anaerobically). The same preparation after addition of vanillyl alcohol (2) 2.32 μM, (3) 4.63 μM, (4) 6.93 μM, (5) 9.22 μM and (6) 11.49 μM. The spectra are not corrected for dilution. Temperature was 18°C.
Inset Figure 4. Spectral modifications caused by the oxidation of vanillyl alcohol by vanillyl-alcohol oxidase. 6.6 mU enzyme, 1.3 mM vanillyl alcohol, 115 mM $(NH_4)_2SO_4$ and 17.4 mM Tris-HCl pH 8.5. The spectra were recorded every minute at a rate of 480 nm/min. Temperature was 30°C.

Another general property of flavoprotein oxidases is the stabilization of the semiquinone anionic radical (Müller 1987). Stabilization does not necessarily imply the occurrence of radical states in the catalytic reaction, but can give some important structural information. The possible stabilization of the anionic radical was investigated with both photoreduction and reduction by sodium dithionite. Photoreduction of the enzyme gave rise to the formation of the red-coloured semiquinone radical (Figure 6A). The predominant formation of the semiquinone is indicated by the formation of three isosbestic points at 357, 406 and 490 nm. On longer illumination, these points are lost, showing the conversion of semiquinone to fully reduced enzyme. Also, anaerobic titration of the enzyme with dithionite gave rise to the formation of the red semiquinone radical (maximal absorbance at 376 nm). Essentially, the same spectrum of (partially)

reduced enzyme was formed, and also the same three isosbestic points (356, 406 and 490 nm) as with photoreduction were observed (data not shown). In both cases complete reoxidation was observed almost immediately on mixing with air.

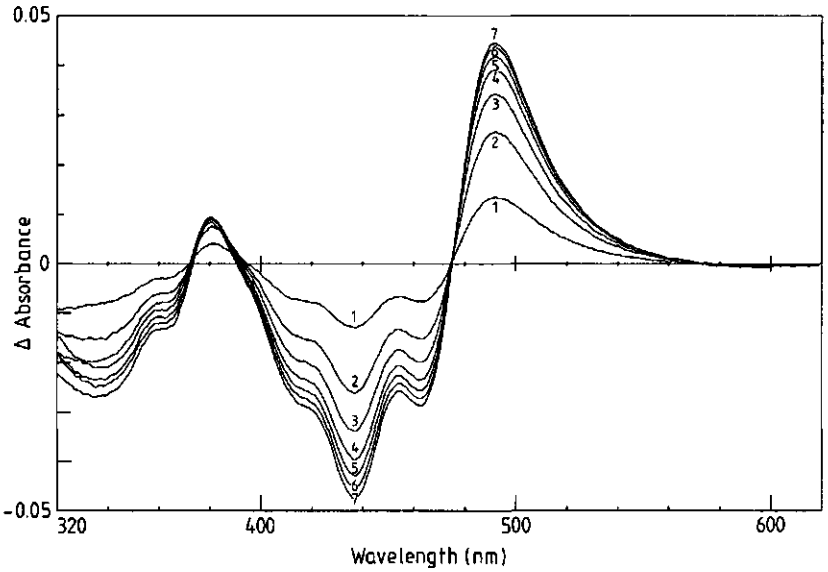


Figure 5. *Reversible effect of cinnamyl alcohol on the visible absorption spectrum of vanillyl-alcohol oxidase.*
(1-7) Aerobic difference spectra with 31.1 μM native enzyme in 0.8 ml 20 mM Tris/HCL, pH 8.5, in the presence of 0.25, 0.50, 0.74, 0.99, 1.23, 1.48 or 1.72 mM cinnamyl alcohol respectively. Aliquots of 2 μl 100 mM cinnamyl alcohol dissolved in 60% ethanol were added. Temperature was 18°C.

Photoreduction of vanillyl alcohol oxidase in the presence of EDTA, 5-deazaflavin and cinnamyl alcohol gave interesting spectral modifications (Figure 6B). No isosbestic points were formed upon illumination, and also formation of the red semiquinone radical was not seen. On mixing with air, no reoxidation of the enzyme was observed. Also gel filtration and addition of vanillyl alcohol did not give rise to reoxidation of the enzyme. Vanillyl alcohol oxidase reduced in the presence of cinnamyl alcohol lost almost all of its activity with vanillyl alcohol. A possible explanation is the formation of a covalent bond between cinnamyl alcohol and the flavin. Singer (1991) reviewed several classes of irreversible inhibitors of monoamine oxidase. Besides flavin adducts also amino acid adducts are possible. Unfolding of photoreduced, cinnamyl alcohol inhibited vanillyl-alcohol oxidase with 0.5% SDS did not give any increase in absorbance at 450 nm. This indicates a covalent bond between cinnamyl alcohol and FAD.

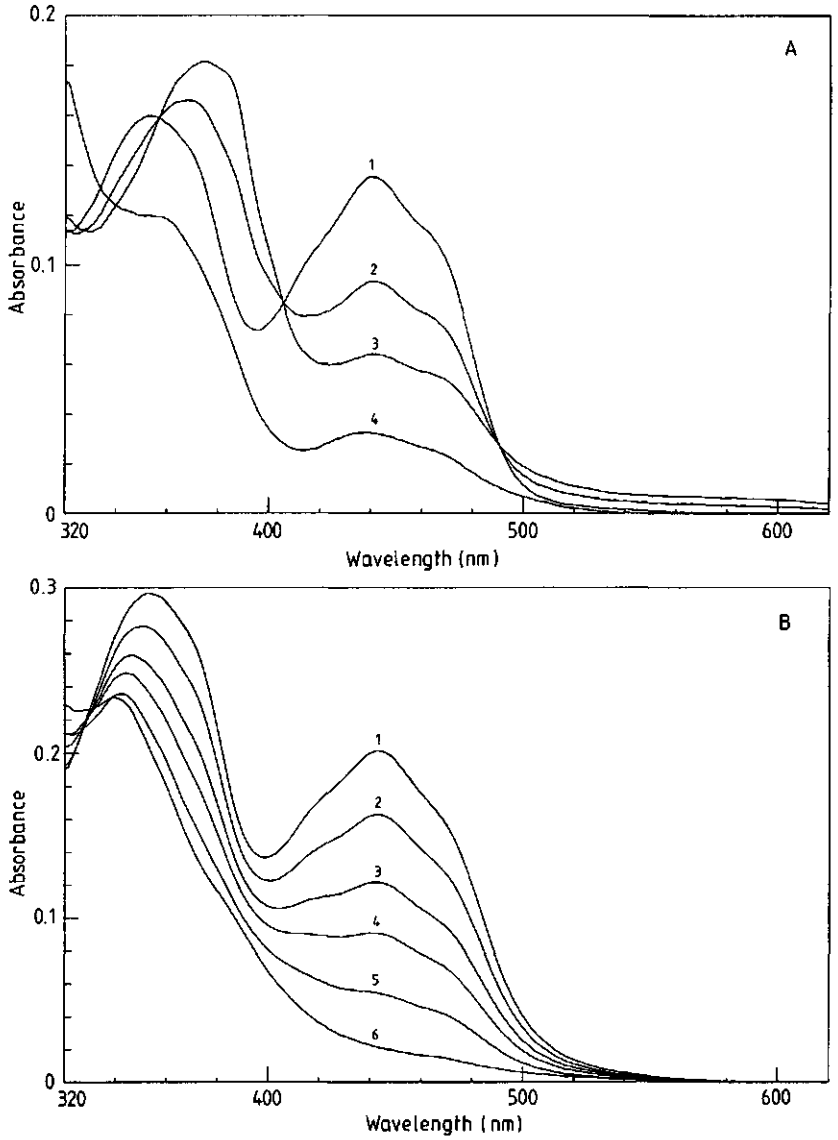


Figure 6. *Anaerobic photoreduction of vanillyl-alcohol oxidase.*

All experiments were performed at 15 °C in 20 mM Tris/HCl, pH 8.5. Selected spectra only are shown.

A) Photoreduction in the presence of 5.7 mM EDTA and 1.9 μM 5-deazaflavin. 11 μM native enzyme (1) and after illumination for 7.5 min (2), 10 min (3) and 36 min (4).

B) Photoreduction in the presence of 5.7 mM EDTA, 1.9 μM 5-deazaflavin and 3.4 mM cinnamyl alcohol. 12 μM native enzyme (1) and after illumination for 2 min (2), 4 min (3), 9 min (4), 13 min (5) and 48 min (6).

In contrast, photoreduction in the presence of vanillin instead of cinnamyl alcohol under the same conditions, as described above, gave a delayed but complete reduction and also one isosbestic point (490 nm) could be observed. The high absorbance of vanillin prevents the observation of the red semiquinone radical and the other two isosbestic points. On mixing with air, complete reoxidation was almost immediately observed (data not shown).

CONCLUSIONS

P. simplicissimum CBS 170.90 produces an intracellular vanillyl alcohol oxidase when grown on veratryl alcohol. The physiological role of the oxidase is not yet clear but it is unlikely that the oxidase plays a significant role in the degradation of veratryl alcohol (De Jong et al. 1990). This intracellular enzyme does not resemble the other described extracellular aryl-alcohol oxidases from *B. adusta* (Muheim et al. 1990), *P. sajor-caju* (Bourbonnais and Paice 1988), *P. ostreatus* (Sannia et al. 1991), *P. eryngii* (Guillén et al. 1990) and *P. versicolor* (Farmer et al. 1960) with respect to substrate specificity, molecular mass and pH optimum.

A main characteristic of vanillyl-alcohol oxidase is the necessity of a hydroxy group at the para position of the benzyl alcohol. The aryl-alcohol oxidases described so far have much broader substrate specificities and show less (*B. adusta, P. ostreatus* and *P. versicolor*) or no activity (*P. sajor-caju*) with compounds containing a hydroxy instead of a methoxy group at the para position.

From the gel filtration and SDS-PAGE experiments, it is concluded that vanillyl-alcohol oxidase contains 1 mol of flavin/mol monomeric enzyme and that in solution the enzyme exists as a multimer, probably an octamer of 520 kDa. Vanillyl-alcohol oxidase from *P. simplicissimum* contains 8α-(N^3-histidyl)-FAD as a covalently bound prosthetic group.

The lack of flavin-adduct formation with sulfite, and the conversion into fully reduced enzyme upon continued illumination, makes it clear that vanillyl-alcohol oxidase is not a typical representative of the class of flavoprotein oxidases (Massey and Hemmerich 1980). Also, the nature of the irreversible bleaching of the flavin spectrum by cinnamyl alcohol remains to be elucidated.

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CHAPTER 10

CONCLUDING REMARKS

Fundamental aspects of lignin biodegradation and the interactions between fungi and aryl alcohols have been addressed in this thesis to investigate the possibilities of white-rot fungi and/or their extracellular ligninolytic enzymes for biopulping of woody materials. In this final chapter three aspects emerging from this thesis are considered: The ligninolytic system of white-rot fungi, the potential of these fungi for biotechnological applications and the implications of chlorinated aromatic compound production in natural environments.

THE LIGNINOLYTIC SYSTEM OF WHITE-ROT FUNGI

Phenol oxidases and the initial oxidation of lignin. In this thesis, the interactions between fungi and aryl alcohols have been addressed. The chapters 3,4,5 and 6 dealt with the isolation and characterisation of a white-rot fungus, *Bjerkandera* sp. BOS55, with a high peroxidative activity. An interesting aspect of *Bjerkandera* sp. BOS55 was its production of the novel enzyme manganese independent peroxidase (MiP), simultaneously with lignin peroxidase (LiP) and manganese peroxidase (MnP) (chapters 3 and 6). This observation is another illustration of the fact that the ligninolytic system of white-rot fungi is more diverse as once thought (chapter 2, Kirk and Farrell 1987). Since the discovery of lignin and manganese peroxidases about 10 years ago, a huge amount of fundamental knowledge about physiological, enzymatic and mechanistic aspects of lignin biodegradation has been acquired. However, from all the evidence available (Kirk and Farrell 1987, Buswell 1992), it is still not clear what the primary agents of lignin degradation in white-rot fungi are (Kirk and Hammel 1992) and if all white-rot fungi basically use the same system. In recent years, the enzymatic

machinery of several white-rot fungi has been examined. LiP was once thought to be an obligatory enzyme in the initial steps of lignin biodegradation. Indeed, several facts indicate that LiP is important in lignin and xenobiotic degradation (chapter 2), and recently it has been proven that the enzyme can depolymerize dilute solutions of lignin *in vitro* (Hammel et al. 1993). However, two major observations make it questionable if LiP is indeed essential in the biodegradation of lignin. Firstly, several fungi do not produce detectable amounts of LiP (chapter 2), but can readily oxidize lignin to CO_2. Secondly, it appears that LiP does not penetrate the lignocellulosic matrix in sound wood (Evans et al. 1991). Although, in some cases, electron micrographs have shown a diffuse delignification throughout the secondary wall. It is possible that LiP is only active as ligninolytic catalysts at the surface of lignin, but it is also possible that low molecular weight compounds, such as Mn^{III} or veratryl alcohol, and subsequent charge-transfer reactions are responsible for the initial steps of lignin biodegradation.

Table 1. Classification of white-rot fungi according to their type of wood decay.

Type of wood decay *		White-rot fungi
1	Selective wood delignification	*D. squalens, C. subvermispora*
2	Simultaneous decay of lignin and polysaccharides throughout the wood	*Trametes versicolor, T. hirsuta*
3	Combination of selective delignification and simultaneous decay within the same substrate	*P. chrysosporium, Lentinula edodes*

* The type of wood decay is determined using both light and electron microscopes. Several white-rot fungi, e.g. *P. chrysosporium* and *Lentinula edodes*, exhibit considerable diversity in delignification capacity among different strains and growth substrates. The discrimination between the groups 1 and 3 is not clear cut.

In chapter 2, it has been shown that almost every possible combination of ligninolytic enzymes (LiP, MnP, MiP and Laccase) has been encountered among the different white-rot fungi investigated. However, the absence of a certain enzyme may be due to the growth conditions rather than to the fungus's inability to produce it. Therefore, the enzyme profiles of white-rot fungi grown on lignocellulosic substrates instead of synthetic media should be analyzed. The extracellular enzymes of newly isolated ligninolytic fungi, grown on hemp stem wood, were investigated to get an indication which enzymes were actually produced (chapter 3). All ligninolytic fungi produced manganese peroxidase and many of these fungi also produced laccase. Lignin peroxidase was only produced in minor amounts by *Phanerochaete chrysosporium*. Similar results were observed by Datta et al. (1991) when *P. chrysosporium* was grown on aspen pulp.

In Table 1, three different types of wood decay are presented. It is still unclear how the different types arise. The white-rot fungi *Ceriporiopsis subvermispora* and *Dichomitus squalens* do not produce LiP and give an extensive and selective delignification combined with little enzyme penetration. Both Kirk and Hammel (1992) and Srebotnik and Messner (1993) have suggested that Mn^{III} is the low molecular weight agent responsible for this kind of delignification. It has been shown that MnP as well as Mn^{III} causes depolymerization of lignin *in vitro* (chapter 2). Future research should elucidate if Mn^{III} is always a prerequisite for extensive lignin-degradation.

Extracellular enzymes producing H_2O_2. Lignin biodegradation research has focused mainly on the regulation, production and action of the different phenol oxidases (Kirk and Farrell 1987, Buswell 1992). It is now also generally accepted that H_2O_2 is an important ligninolytic agent. However, the enzymes and substrates involved in H_2O_2-production and their regulation are poorly understood. Three extracellular oxidases essential for the H_2O_2-generation have been detected. *P. chrysosporium* synthesizes extracellular glyoxal oxidase and pyranose-2-oxidase. *Bjerkandera* and *Pleurotus* spp. also produce an extracellular aryl alcohol oxidase (AAO, chapter 2), for which previously *de novo* biosynthesized veratryl and *p*-anisyl alcohol have been considered as the physiological substrates. This thesis has shown that *Bjerkandera* BOS55 also biosynthesizes *de novo* chlorinated anisyl alcohols, which have a physiological role because they are excellent substrates for AAO and the formed aldehydes are readily recycled by reduction to the corresponding alcohols (chapters 5 and 6). AAO has a much higher affinity for the chlorinated anisyl alcohols compared to veratryl and anisyl alcohol. On the other hand LiP did not catalyze the oxidation of the chlorinated anisyl alcohols indicating the electron withdrawing Cl-groups make the aromatic ring less oxidizable by the fungus's own aggresive ligninolytic enzymes. It is presently unknown if the uptake of the chlorinated aldehydes is energy dependent. Until now, only one report has described the carrier mediated transport of halogenated aromatic compounds (4-chlorobenzoate) in bacteria (Groenewegen et al. 1990), but nothing is known yet about such transport mechanisms in eukaryotes.

Further research should elucidate the *in vivo* extracellular H_2O_2-production rate because it can actually be the rate-limiting step in lignin and xenobiotic biodegradation.

Role of veratryl alcohol/LiP couple. Aryl alcohols are very common secondary metabolites of white-rot fungi produced simultaneously with their ligninolytic enzymes (chapters 2 and 6). In chapter 2 the literature concerning the important physiological functions of veratryl alcohol have been reviewed. Aside from serving as oxidase substrates at least one aryl alcohol, veratryl alcohol, has been shown to be essential

for LiP and has been subject of intense debate. The production of veratryl alcohol and LiP are highly correlated in *Bjerkandera* sp. BOS55 (chapter 6), indicating they are regulated in parallel. A physiological role for veratryl alcohol as a stabilisator of LiP against H_2O_2 inactivation has been established. Addionally, charge-transfer reactions involved in lignin biodegradation have been attributed to the LiP/veratryl alcohol couple. However, future research is still necessary to clarify the extent of these mediated reactions. It is interesting to speculate if the LiP/veratryl alcohol-generated activated oxygen species are partly the cause of the considerable fibre damage seen by Srebotnik and Messner (1993) with *P. chrysosporium*.

POTENTIAL OF WHITE-ROT FUNGI FOR BIOTECHNOLOGICAL APPLICATIONS

Biopulping of wood. One of the objectives of this thesis was to investigate the possibilities of white-rot fungi and/or their extracellular ligninolytic enzymes for the biopulping of woody materials. The recent progress made with the non-LiP producing fungi *C. subvermispora* and *D. squalens* shows that biomechanical pulping is technical feasible for wood and probably also for hemp. The non-LiP producing fungi combine a high degree of delignification with little enzyme penetration. This lack of penetration, even in completely delignified fibres, suggest that delignification occurs without a direct contact between ligninolytic enzymes and lignin. It was suggested that low molecular weight compounds, plausibly Mn^{III}, are the actual active species. These results suggest that a biomimetic delignification with Mn^{III} may be possible. Future research should elucidate if and under which temperature and pH ranges Mn^{III} is an active ligninolytic agent and if other additional low molecular weight compounds and/or enzymes are necessary. Also *Bjerkandera* sp. BOS55 seems to be a good candidate for a selective degradation of lignin, because this organism produces high amounts of manganese peroxidase and no lignin peroxidase when grown on hemp stem wood (chapters 3 and 6). However, the simultaneous production of chlorinated aromatics can be a drawback.

Bioremediation. The random and non-specific nature of the lignin-degrading system also attacks other compounds containing an aromatic structure, such as many xenobiotic compounds. It has been shown with *in vitro* experiments that LiP, MnP and Mn^{III} cause the oxidation of a variety of environmental pollutants and that whole cultures cause extensive mineralization (Field et al. 1993). *Bjerkandera* sp. BOS55 was found to be the best polycyclic aromatic hydrocarbon degrader among a number of white-rot fungi screened (Field et al. 1992). The first promising results with *in situ*

bioremediation of contaminated soils and wood (Lamar and Dietrich 1990) with white-rot fungi have been reported.

Aside from mineralization, ligninolytic enzymes can also be used to polymerize pollutants to non-toxic compounds. The resulting chlorinated compounds should be considered as environmental safe products since also non-toxic, natural chlorohumus occurs at high concentrations (Asplund and Grimvall 1991). This novel approach of copolymerization into chlorohumus may lead to more rapid bioremediation methods.

Flavour and fragrance production. Several white-rot fungi, including *B. adusta* and *Ischnoderma benzoinum* have been proposed as suitable organisms for the production of aromatic compounds. However, the production of desired compounds can be accompanied with the formation of unwanted but physiological important side products, e.g. the production of chlorinated anisyl metabolites (CAM) (chapters 5,6 and 7).

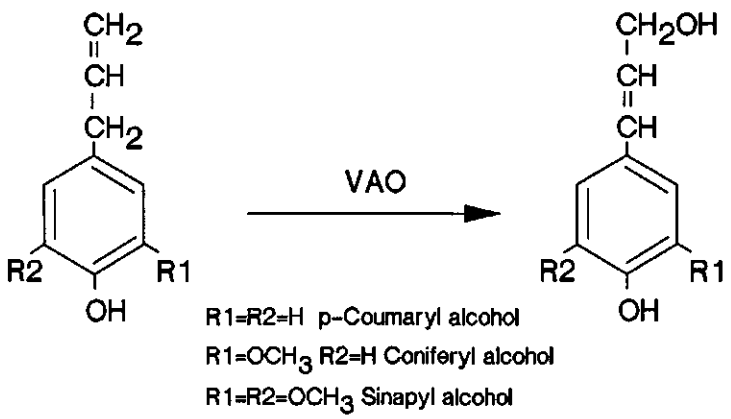

Figure 1. The oxidation of eugenol and chavicol with vanillyl alcohol oxidase (VAO) leading to the production of the lignin precursors coniferyl and coumaryl alcohol. 4-allyl-2,6-dimethoxyphenol, potentially leading to the formation of sinapyl alcohol, was not a substrate for the enzyme.

Also hyphomycetes can be used for flavour and fragrance production. Recent research with vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum* (chapters 8 and 9) has revealed some interesting results regarding the substrate specificity of this enzyme. In addition to vanillyl alcohol also eugenol (4-allyl-2-methoxyphenol) and chavicol (4-allylphenol) are oxidized by the enzyme (van Berkel et al. 1993). The reaction products coniferyl and coumaryl alcohol, which are two of the natural building blocks for lignin (chapter 2), are formed in stoichiometric amounts (Figure 1).

Unfortunately, 4-allyl-2,6-dimethoxyphenol which is the predicted precursor for sinapyl alcohol, was not oxidized by the enzyme, probably because of sterical hinderance. With this unique process, which has been patented (Unilever 1993), the inexpensive production of (labelled) synthetic lignins such as the dehydrogenative polymer (DHP) of coniferyl alcohol and mixtures with coumaryl alcohol will become possible. Future research is directed to solve the intriguing reaction mechanism of VAO. In order to produce all three lignin precursors, attempts will be made to alter the substrate specificity of the enzyme.

PRODUCTION OF CHLORINATED AROMATIC COMPOUNDS IN NATURAL ENVIRONMENTS

Chlorinated aromatic compounds are priority pollutants which were largely considered to be of man-made origin. This thesis has shown that many common occurring wood- and forest litter-degrading basidiomycetes, e.g. *Hypholoma fasciculare*, *H. capnoides*, *Oudemansiella mucida*, *Bjerkandera adusta*, *Pholiota squarrosa*, *Lepista nuda*, produce chlorinated anisyl metabolites (CAM) in the natural environment in concentrations exceeding those permitted by Dutch environmental legislation (chapter 7). We proposed that aside from complete mineralisation to CO_2, CAM intermediates can give rise to detoxification reactions leading to chlorohumus (natural AOX polymers) and biotoxification reactions resulting in the formation of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. Fungal chlorinated aromatic compounds and their natural biotoxification products are probably just as dangerous as their anthropogenic counterparts. Future research should focus on the ubiquity among fungi to produce chlorinated aromatic compounds and relate the natural occurrence of these priority pollutants to existing environmental norms. Moreover, much is to be learned from the transformation/degradation reactions of natural chloroaromatics in the environment, which will give us clues to novel bioremediation methods.

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LIST OF ABBREVIATIONS

AA	:	anisyl alcohol (4-methoxybenzyl alcohol)
AAO	:	aryl alcohol oxidase
ABTS	:	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate)
AOX	:	adsorbable organic halogens
BIII	:	basal III medium
CAM	:	chlorinated anisyl metabolites
cAMP	:	adenosine 3',5'-phosphate
CBS	:	centraal bureau voor schimmelcultures
$CDCl_3$	:	deuterated chloroform
DEAE	:	diethylaminoethyl
DHP	:	dehydropolymerizate of coniferyl alcohol
DMP	:	2,6-dimethoxyphenol
DMS	:	2,2-dimethylsuccinate
ϵ	:	molar absorption coefficient
EDTA	:	ethylenediaminetetraacetate
ESR	:	electron spin resonance
FAD	:	flavin-adenine dinucleotide
FMN	:	riboflavin 5'-phosphate
FPLC	:	fast protein liquid chromatography
GC/MS	:	gas chromatography/mass spectrometry
GLYOX	:	glyoxal oxidase
H_2O_2	:	hydrogen peroxide
HPLC	:	high-performance liquid chromatography
HRP	:	horseradish peroxidase
HSW	:	hemp stem wood
K_d	:	dissociation constant

K_i	:	inhibition constant
K_m	:	Michaelis constant
KTBA	:	α-keto-γ-methylthiolbutyric acid
LiP	:	lignin peroxidase (i.e. ligninase)
MIP	:	manganese independent peroxidase
MnP	:	manganese peroxidase
Mn^{II}	:	manganese^{2+}
Mn^{III}	:	manganese^{3+}
MSD	:	mass selective detector
NADH	:	reduced nicotinamide adenine dinucleotide
NADPH	:	reduced nicotinamide adenine dinucleotide phosphate
NMR	:	nuclear magnetic resonance
$O_2^{\bullet-}$	:	superoxide radical
$^{\bullet}OH$	:	hydroxyl radical
$^1O_2$	:	singlet oxygen
PAA	:	polyacrylic acid
PAGE	:	polyacrylamide gel electrophoresis
PAH	:	polycyclic aromatic hydrocarbon
PMS	:	phenazine methosulphate
P.O.	:	phenol oxidase
Poly R-478	:	polymeric dye with anthrapyridone chromophore (Poly R)
SDS	:	sodium dodecyl sulfate
V	:	rate of enzyme catalyzed reaction at infinite concentration of substrate
VA	:	veratryl alcohol (3,4-dimethoxybenzyl alcohol)
$VA^{\bullet}$	:	veratryl alcohol radical
$VA^{\bullet+}$	:	veratryl alcohol cation radical
VAO	:	vanillyl alcohol oxidase

SUMMARY

The major structural elements of wood and other vascular tissues are cellulose, hemicellulose and generally 20-30% lignin. Lignin gives the plant strength, it serves as a barrier against microbial attack and it acts as a water impermeable seal across cell walls of the xylem tissue. However, the presence of lignin has practical drawbacks for some of the applications of lignocellulosic materials. First, lignin has to be removed for the production of high quality pulps. Second, lignin reduces the digestibility of lignocellulosic materials. High quality pulps can be produced with chemical methods, however the abundant use of chemicals and energy, and the formation of an enormous waste stream has led scientists to investigate the possibilities of biodelignification. White-rot fungi give the most rapid and extensive degradation and have become subject of intensive research. Results obtained with the model organism *Phanerochaete chrysosporium* and other strains have revealed that lignin biodegradation is an extracellular, oxidative and non-specific process. This unique biodegradative potential has been considered for broader applications such as waste water treatment and the degradation of xenobiotic compounds. The research described in this thesis concentrates on the function of aryl alcohols in fungal physiology.

Aryl alcohols in the physiology of white-rot fungi. White-rot fungi have a versatile machinery of enzymes, including peroxidases and oxidases, which work in harmony with secondary aryl alcohol metabolites to degrade the recalcitrant, aromatic biopolymer lignin. In chapter 2 literature concerning the important physiological roles of aryl (veratryl, anisyl and chlorinated anisyl) alcohols in the ligninolytic enzyme system has been reviewed. Their functions include stabilization of lignin peroxidase, charge-transfer reactions and as substrate for oxidases generating extracellular H_2O_2.

The experimental research described in this thesis was initiated to evaluate the

possibilities of white-rot fungi in the biopulping of hemp stem wood. Sixty-seven basidiomycetes were isolated and screened for high peroxidative activity (chapter 3). Several of the new isolates were promising manganese peroxidase-producing white-rot fungi. Enzyme assays indicated that for the production of H_2O_2 either extracellular glyoxal or aryl alcohol oxidase were present. In contrast, lignin peroxidase was only detected in *P. chrysosporium*, despite attempts to induce this enzyme in other strains with oxygen and oxygen/veratryl alcohol additions. A highly significant correlation was found between two ligninolytic indicators: ethene formation from α-keto-γ-methylthiolbutyric acid and the decolorization of a polymeric dye, Poly R-478. Three of the new isolates had significantly higher Poly R decolorizing activities compared to *P. chrysosporium*.

One of the best Poly R decolorizing strains, *Bjerkandera* sp. BOS55 was selected for further characterization. A novel enzyme activity (manganese independent peroxidase) was detected in the extracellular fluid of *Bjerkandera* sp. BOS55 (chapter 4). The purified enzyme could oxidize several compounds like phenol red, 2,6-dimethoxyphenol (DMP), Poly R-478, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and guaiacol with H_2O_2 as an electron acceptor. In contrast, veratryl alcohol was not a substrate. This enzyme also had the capacity to oxidize DMP in the absence of H_2O_2. *Bjerkandera* sp. BOS55 also produced *de novo* several aromatic metabolites. Besides veratryl alcohol and veratraldehyde, compounds which are known to be involved in the ligninolytic system of several other white-rot fungi (chapter 2), other metabolites were formed. These included anisaldehyde, 3-chloro-anisaldehyde, 3,5-dichloro-anisaldehyde and small amounts of the corresponding anisyl, 3-chloro-anisyl and 3,5-dichloro-anisyl alcohol (chapters 5 and 6). This was the first report of *de novo* biosynthesis of simple chlorinated aromatic compounds by a white-rot fungus. These unexpected findings led us investigate the physiological role(s) of the *de novo* biosynthesized chlorinated anisyl alcohols (chapter 6). All metabolites were produced simultaneously with the extracellular ligninolytic enzymes. The mono- and dichlorinated anisyl alcohols appeared to be excellent substrates for the extracellular aryl alcohol oxidases. The formed aldehydes were readily recycled via reduction by washed fungal mycelium, thus creating an extracellular H_2O_2 production system regulated by intracellular enzymes. Lignin peroxidase does not oxidize the chlorinated anisyl alcohols both in the absence and in the presence of veratryl alcohol. It was therefore concluded that the chlorinated anisyl alcohols are well protected against the fungus's own aggressive ligninolytic enzymes. The relative amounts of veratryl alcohol and the chlorinated anisyl alcohols differ significantly depending on the growth conditions, indicating that the production of veratryl alcohol and the (chlorinated) anisyl metabolites are independently regulated.

It was concluded that the chlorinated anisyl metabolites, biosynthesized by the white-rot fungus *Bjerkandera* sp. BOS55, are purposeful for ecologically significant processes such as lignin degradation. These results made us speculate if a significant biogenesis of chlorinated aromatics by fungi occurs in natural environments (chapter 7). Many common wood- and forest litter-degrading fungi were indeed detected that produced chlorinated anisyl metabolites (CAM). These compounds, which are structurally related to xenobiotic chloroaromatics, were present in the environment and occur at high concentrations of approximately 75 mg CAM kg^{-1} wood or litter. The ubiquity among common fungi to produce large amounts of chlorinated aromatic compounds in the environment leads to the conclusion that these kind of compounds can no longer be considered to originate from anthropogenic sources only.

Degradation of aryl alcohols by fungi. In chapter 2 the anabolic and catabolic routes of aryl alcohols by white-rot fungi has been reviewed. These fungi can not use veratryl alcohol as sole source of carbon and energy. However, several bacteria, yeasts and fungi were selectively isolated from paper mill waste water that grew on veratryl alcohol (chapter 8). *Penicillium simplicissimum* was selected for the characterization of the veratryl alcohol degradation route. *P. simplicissimum* oxidized veratryl alcohol via a $NAD(P)^+$-dependent veratryl alcohol dehydrogenase to veratraldehyde which was further oxidized to veratric acid in a $NAD(P)^+$-dependent reaction. Veratric acid-grown cells contained NAD(P)H-dependent *O*-demethylase activity for veratrate, vanillate and isovanillate. Ring-cleavage of protocatechuate was by a protocatechuate 3,4-dioxygenase. An interesting aspect of *P. simplicissimum* is the production of vanillyl alcohol oxidase with covalently bound FAD (chapter 9). The intracellular enzyme was purified 32-fold. SDS-PAGE of the purified enzyme revealed a single fluorescent band of 65 Kda. Gel filtration and sedimentation-velocity experiments indicated that the purified enzyme exists in solution as an octamer, containing 1 molecule flavin/subunit. The covalently bound prosthetic group of the enzyme was identified as 8α-(N^3-histidyl)-FAD from pH dependent fluorescence quenching (pK_a = 4.85) and no decrease in fluorescence upon reduction with sodium borohydride. The enzyme showed a narrow and rather peculiar substrate specificity. In addition to vanillyl alcohol and 4-hydroxybenzyl alcohol, eugenol and chavicol are substrates for the enzyme (chapter 10). The formed products, coniferyl and coumaryl alcohol are the natural precursors of lignin in plants. This reaction has a potential application to produce coniferyl alcohol and subsequent synthetic lignin (DHP) from the inexpensive precursor eugenol.

SAMENVATTING

De drie belangrijkste bouwstenen van hout en andere planten met een vaatweefsel zijn cellulose, hemicellulose en gewoonlijk 20-30% lignine. Lignine, oftewel houtstof, geeft de plant sterkte, beschermt de plant tegen ongewenste microbiële aantasting en zorgt voor een waterondoordringbare afdichting van de celwanden in het xyleemweefsel van planten. Bij het gebruik van hout en houtachtige gewassen levert de aanwezigheid van lignine echter in een aantal gevallen grote problemen op. Ten eerste moet bij de bereiding van het zogenaamde houtvrije papier alle lignine verwijderd worden. Ook bij de bereiding van krantepapier, waar de lignine nog inzit en die daardoor vergeelt, zorgt juist de in het hout aanwezige lignine voor een grote energieconsumptie. Ten tweede verlaagt de aanwezigheid van lignine de verteerbaarheid van planten voor mens en dier. Bij de huidige produktieprocessen voor houtvrij papier wordt de lignine verwijderd met behulp van grote hoeveelheden chemicaliën en bij een hoge temperatuur. Dit proces genereert tevens een grote afvalstroom. De grote praktische voordelen die een biologische in plaats van een chemische verwijdering van lignine geeft, heeft vele wetenschappers er toe aangezet de mogelijkheden van biodelignificatie te onderzoeken.

Lignine wordt door micro-organismen niet makkelijk afgebroken omdat het een complex driedimensionaal molecuul is met op een groot aantal plaatsen allerlei verschillende soorten bindingen. Van alle micro-organismen geven de zogenaamde wit-rot schimmels nog de snelste en grootste afbraak van lignine terwijl de cellulose in eerste instantie grotendeels intact blijft. Onderzoek met het model organisme, *Phanerochaete chrysosporium*, en andere paddestoelen die wit-rot veroorzaken, heeft aangetoond dat de afbraak van lignine een extracellulair, oxidatief en aspecifiek proces is. Hoewel de chaotische struktuur van lignine het niet aannemelijk maakte, bleek toch een extracellulair enzymsysteem verantwoordelijk voor de eerste stappen in het afbraak proces. Dit voor micro-organismen unieke enzymsysteem heeft de aandacht

op een aantal andere toepassingsgebieden gericht waarvan hier de afvalwaterzuivering en de verwijdering van milieuvreemde (xenobiotica) stoffen worden genoemd. Het onderzoek dat in dit proefschrift is beschreven, heeft zich voornamelijk gericht op de rol van aryl alcoholen in de fysiologie van (wit-rot) schimmels.

Aryl alcoholen in de fysiologie van wit-rot schimmels. Bij de afbraak van hout en houtige gewassen produceren wit-rot schimmels een groot aantal extracellulaire enzymen, waaronder peroxidases en oxidases. Deze enzymen werken in nauw verband samen met *de novo* gesynthetiseerde metabolieten, de aryl alcoholen. In hoofdstuk 2 wordt een literatuur overzicht gegeven van de biosynthese van aryl (veratryl, anisyl en gechloreerde anisyl) alcoholen door schimmels uit de klasse van de basidiomyceten. Tevens worden de fysiologische functies van deze verbindingen verduidelijkt. Tot de belangrijkste functies van de aryl alcoholen behoren de stabilisatie van het lignine peroxidase, het overbrengen van lading (mediator) waardoor een oxidatie op afstand van het enzym plaatsvindt, en het fungeren als substraat voor extracellulaire oxidases waardoor waterstofperoxide (H_2O_2) geproduceerd wordt. Daarnaast wordt in hoofdstuk 2 aangegeven hoe deze aryl alcoholen ook weer afgebroken kunnen worden door wit-rot schimmels.

Het onderzoek dat beschreven wordt in dit proefschrift is gestart om de mogelijkheden te inventariseren van wit-rot schimmels bij de biopulping van hennep houtpijp. Er werden 67 basidiomyceten geïsoleerd die vervolgens geanalyseerd werden op hun peroxidatieve vermogens (hoofdstuk 3). Een aantal van de geïsoleerde wit-rot schimmels was veelbelovend, gelet op hun produktie van mangaan peroxidase. Daarentegen werd lignine peroxidase, waarvan algemeen werd aangenomen dat het een noodzakelijk enzym is voor de lignine afbraak, alleen maar bij het modelorganisme *P. chrysosporium* aangetroffen. Extracellulair waterstofperoxide werd geproduceerd met glyoxal oxidase of met aryl alcohol oxidase. Er werd een significante correlatie gevonden tussen de verschillende ligninolytische testen zoals de ontkleuring van een polymere kleurstof Poly R-478 en de afbraak van polycyclische aromatische koolwaterstoffen.

Eén van de stammen met de hoogste Poly R ontkleuringssnelheid werd uitgekozen voor nader onderzoek. *Bjerkandera* sp. BOS55 produceerde naast lignine en mangaan peroxidase een derde klasse van peroxidases, namelijk mangaan onafhankelijk peroxidase (MiP, hoofdstuk 4). Het gezuiverde enzym kan een groot aantal verbindingen oxideren, waaronder phenol rood, 2,6-dimethoxyphenol en Poly R-478. Veratryl alcohol was echter geen substraat voor MiP. Een interessant kenmerk van het enzym is het feit dat een aantal substraten ook in de afwezigheid van H_2O_2 geoxideerd wordt. Naast enzymen produceerde *Bjerkandera* sp. BOS55 ook een groot

aantal secundaire metabolieten. De bekende metabolieten veratryl alcohol en veratraldehyde werden ook door BOS55 gesynthetiseerd. Opmerkelijk was echter dat er tevens 3-chloro-anisyl alcohol en 3,5-dichloro-anisyl alcohol en de corresponderende aldehydes *de novo* gesynthetiseerd werden. Het in de hoofdstukken 5 en 6 beschreven werk toont voor het eerst aan dat een wit-rot schimmel gechloreerde aromaten produceert die een fysiologische rol vervullen in de lignine afbraak als substraat voor het extracellulaire aryl alcohol oxidase. De gevormde aldehydes werden intracellulair gereduceerd tot de corresponderende alcoholen zodat een cyclisch H_2O_2-producerend systeem ontstaat. Lignine peroxidase vertoonde zowel in de aan- als afwezigheid van veratryl alcohol geen enkele activiteit met de gechloreerde anisyl alcoholen. Uit deze resultaten werd geconcludeerd dat de gechloreerde aromaten goed beschermd zijn tegen het agressieve ligninolytische enzymsysteem van de schimmel zelf. Het feit dat de gechloreerde aromaten een fysiologische rol in de afbraak van lignine spelen als H_2O_2-donor en niet makkelijk afgebroken worden door de ligninolytische enzymen gaf aanleiding te onderzoeken of veel verschillende schimmels deze verbindingen maken en of ze ook in het milieu in grote hoeveelheden voorkomen (hoofdstuk 7). Het bleek dat een groot aantal algemeen voorkomende paddestoelen, waaronder de gewone zwavelkop (*Hypholoma fasciculare*), de dennezwavelkop (*Hypholoma capnoides*), de porseleinzwam (*Oudemansiella mucida*), de grijze gaatjeszwam (*Bjerkandera adusta*), de geschubde bundelzwam (*Pholiota squarrosa*) en de paarse schijnridder (*Lepista nuda*), gechloreerde anisyl metabolieten (CAM) maakt. De CAM concentraties liepen op tot 75 mg CAM per kg hout. Uit het algemeen voorkomen van CAM produktie door paddestoelen en uit de hoge concentraties waarin CAM voorkwam in het natuurlijke milieu werd geconcludeerd dat menselijk handelen niet de enige belangrijke oorzaak voor de aanwezigheid van gechloreerde aromatische verbindingen in het milieu is.

Afbraak van aryl alcoholen door schimmels. In hoofdstuk 2 is een overzicht gegeven van de synthese- en afbraakroutes van aryl alcoholen door wit-rot schimmels. Deze schimmels kunnen veratryl alcohol echter niet als enige koolstof- en energiebron gebruiken. Een groot aantal andere micro-organismen kon echter geïsoleerd worden op veratryl alcohol uit het afvalwater van een papierfabriek (hoofdstuk 8). *Penicillium simplicissimum* werd uitgekozen om de afbraakroute van veratryl alcohol op te helderen. *P. simplicissimum* oxideerde veratryl alcohol via twee $NAD(P)^+$-afhankelijke dehydrogenases via veratraldehyde tot veratraat. Wanneer *P. simplicissiumum* op veratraat gekweekt werd, kon een NAD(P)H-afhankelijk veratraat-*O*-demethylase gemeten worden dat ook activiteit vertoonde met vanillaat en isovanillaat. Ringsplitsing van protocatechuaat gebeurde met een protocatechuaat-3,4-dioxygenase. Een

interessant aspect aan *P. simplicissimum* is de produktie van vanillyl alchol oxidase, een intracellulair enzym met covalent gebonden FAD (hoofdstuk 9). Een SDS-PAGE plaatje van het 32-maal gezuiverde enzym liet één fluorescente band van 65 kDa zien. Gelfiltratie en sedimentatie-snelheid toonden aan dat het enzym normaal als een octameer voorkomt, met één flavine molecuul per subunit. De covalent gebonden prosthetische groep werd geidentificeerd als 8α-(N^3-histidyl)-FAD. Het enzym heeft een nauwe maar wel zeer bijzondere substraatspecificiteit. Naast vanillyl alcohol en 4-hydroxybenzyl alcohol werden ook eugenol en chavicol geoxideerd (hoofdstuk 10). De produkten coniferyl en coumaryl alcohol zijn de natuurlijke bouwstenen voor de lignine in planten. Deze enzymatische reactie heeft een potentiële toepassing omdat nu coniferyl alcohol geproduceerd kan worden uit de goedkope, natuurlijke grondstof eugenol, de geurstof van kruidnagelen. Coniferyl alchol kan omgezet worden in vanille en het kan gebruikt worden voor de produktie van synthetisch lignine (DHP).

NAWOORD

Op de voorkant van dit proefschrift staat een close-up opname van de onderkant van de doolhofzwam. Alhoewel er met deze paddestoel geen experimenteel onderzoek verricht is, is dit vruchtlichaam niet alleen vanwege zijn fotogenieke uiterlijk gekozen voor de voorkant. Deze schimmel is namelijk een mooie metafoor voor wetenschappelijk onderzoek in zijn algemeenheid en promotieonderzoek in het bijzonder. Bij het begin van mijn onderzoek leek ik in een doolhof aangeland met tal van mogelijke onderzoekspaden die echter even zo vaak dood bleken te lopen. Zoals blijkt uit de resultaten, weergegeven in dit boekje, waren er echter ook paden die naar een, vaak onverwachte, uitgang leidden. Deze paden heb ik natuurlijk niet alleen gevonden en ik zou dan ook graag een aantal mensen bedanken die mij hierbij behulpzaam zijn geweest.

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CURRICULUM VITAE

Edserd de Jong werd op 31 mei 1962 te Deventer geboren. In 1980 werd het Atheneum B diploma behaald aan het Marnix College te Ede, waarna in hetzelfde jaar werd begonnen met de studie Levensmiddelentechnologie aan de Landbouw Universiteit Wageningen. De doctoraal fase bestond uit afstudeervakken bij Industriële Microbiologie, Proceskunde en Informatica. Het onderzoek bij proceskunde behelsde de mogelijkheden van biopulping van hennep met wit-rot schimmels. Het onderzoek beschreven in dit proefschrift is hier een vervolg op.

In juli 1987 startte de auteur een gecombineerd promotie onderzoek bij de voormalige vakgroep Bosbouwtechniek en de vakgroep Levensmiddelentechnologie, sectie Industriële Microbiologie. De eerste anderhalf jaar als vervangende dienst, daarna als assistent in opleiding (AIO) en de laatste twee jaar als toegevoegd wetenschappelijk medewerker. Deze laatste twee jaar werden uitgevoerd in samenwerking met het agrotechnologisch onderzoeks instituut (ATO-DLO) en gefinancïeerd binnen het Hennepproject. Het resultaat van dit onderzoek staat beschreven in dit proefschrift.

In februari 1993 is aan de auteur de Koninklijke/Shell studiereis prijs uitgereikt.

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