Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics

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cultures and EBV transformation methodologies.

Separating fact from fiction

This conference is the major annual meeting in the field of therapeutic monoclonals and is a 'must' for researchers in this field. It should now progress past methodological descriptions and the need for every sponsor to be given an opportunity to make a presentation. It was tedious to listen to six descriptions of phage technology, and five descriptions of how to construct a single-chain antibody. However, we look forward to the next conference, to continue the progress in separating fact from fiction and to indicate those technologies and targets which are truly leading to the commercialization of mAbs.

Reference

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focus

Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics

Jim A. Field, Ed de Jong, Gumersindo Feijoo-Costa and Jan A. M. de Bont

Woody tissues are composed mainly of three biopolymers: cellulose; hemicellulose; and lignin. Lignin, a highly irregular aromatic polymer which serves to provide strength and structure to the tissue, is synthesized in plants by a random peroxidase-catalysed polymerization of substituted *p*-hydroxy-cinnamyl alcohols. Only a few groups of microorganisms are capable of degrading complex lignin polymers, and they are best exemplified by the white-rot fungi, which cause the greatest degree of mineralization. The white-rot fungus *Phanerochaete chrysosporium* has been used extensively as a model organism to study the physiological requirements and enzymes required for lignin biodegradation (ligninolysis). Lignin cannot be degraded as a sole source of carbon and energy, and ligninolysis only occurs when other readily biodegradable substrates are available; *P. chrysosporium* initiates ligninolysis only after primary growth has ceased due to carbon, nitrogen or sulfur limitation[1,2]. The physiological importance of lignin biodegradation is destruction of the lignin matrix so that the microorganisms can gain better access to the real substrates; hemicellulose and cellulose.

The extracellular machinery involved in lignin degradation by *P. chrysosporium* is composed of lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs), as well as H_2O_2-producing oxidases (Fig. 1). Lignin peroxidases can abstract one electron from a non-phenolic moiety of the lignin molecule, thus creating a cation radical[4] which in turn initiates a random oxidative chemical reaction that finally results in the oxygenation and depolymerization of lignin. Veratryl alcohol, a metabolite synthesized *de novo*, has an important role in stabilizing LiP against inactivation by H_2O_2 (Ref. 5). Manganese-dependent peroxidases function by oxidizing Mn(II) to Mn(III). Mn(III) behaves as a low-molecular-weight mediator that can diffuse to remote regions of the lignin molecule and initiate the oxidation process[6].

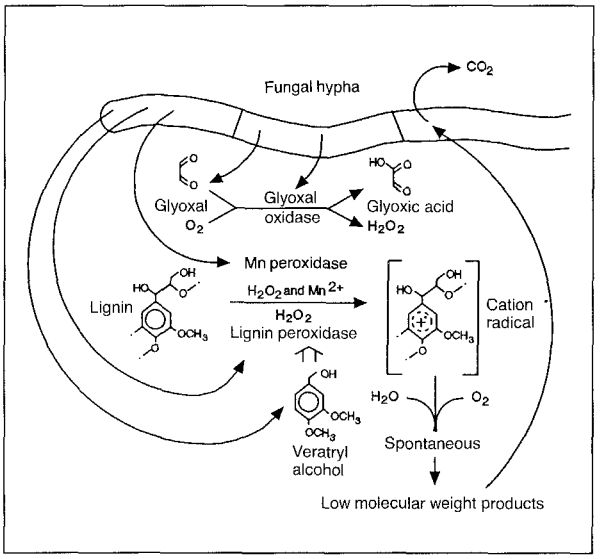

Figure 1
The ligninolytic system of *Phanerochaete chrysosporium*. Redrawn from Ref. 3.

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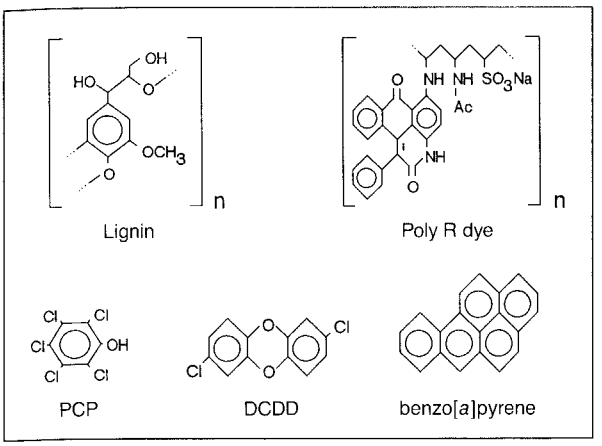

Figure 2
Structural similarities between lignin, Poly R-478 dye and selected xenobiotic compounds. Abbreviations: PCP, pentachlorophenol; DCDD, dichlorodibenzo-*p*-dioxin.

Table 1. Xenobiotic compounds degraded by whole cultures of white-rot fungi during ligninolytic metabolism

Xenobiotic compound	Species[a]	Refs
Chlorinated phenols		
Pentachlorophenol	Pc,Ps,Po,Th,Cs	7–11
Trichlorophenol	Pc	12
Dichlorophenol	Pc	13
Chloroguaiacols	Pc	12
PCB[b]		
Aroclor 1254	Pc,Tv,Pb,Fg	14
Tetrachlorobiphenyl	Pc	15
DDT[b]	Pc	15–17
Dioxin		
Dichlorodibenzo-*p*-dioxin	Pc	18
Tetrachlorodibenzo-*p*-dioxin	Pc	15
PAH[b]		
Fluorene	Pc	19
Anthracene	Pc,Tv,Ba,Nl[b]	20,21,c
Phenanthrene	Pc,Tv,Cl	22–26
Pyrene	Pc	27
Benzo[a]pyrene	Pc,Tv,Ba,Nl[b]	15,20,28
Alkyl halides		
Lindane	Pc	29
Chlordane	Pc	29
Nitrotoluenes		
Trinitrotoluene	Pc	30–32
Dinitrotoluene	Pc	33
Chloroanilines		
Dichloroaniline	Pc,Tv,Cl	23,34
Dyes		
Azo dyes	Pc	35,36
Polymeric dyes	Pc	37
Crystal violet	Pc	38
Chlorolignin	Pc	39

[a]Pc, *Phanerochaete chrysosporium*; Ps, *Phanerochaete sordida*; Po, *Pleurotus ostreatus*; Th, *Trametes hirsuta*; Cs, *Ceriporiopsis subvermispora*; Tv, *Trametes versicolor*; Pb, *Phlebia brevispora*; Fg, *Funalia gallica*; Ba, *Bjerkandera adusta*; Cl, *Chrysosporium lignosum*.
[b]Abbreviations: PCB, polychlorinated biphenyls; DDT, 1,1-*bis*(4-chlorophenyl)-2,2,2-trichloroethane; PAH, polycyclic aromatic hydrocarbons; Nl, new isolates.
[c]S. D. Haemmerli, PhD thesis, Swiss Federal Institute of Technology, Zurich, Switzerland, 1988.

The random nature of the structure of lignin requires lignin degradation to function in a non-specific manner; consequently, other compounds that have an aromatic structure, such as many xenobiotic compounds (Fig. 2), are also highly susceptible to degradation by ligninolytic enzymes. Under culture conditions which favour ligninolysis, many xenobiotics [such as some polycyclic aromatic hydrocarbons (PAHs), chlorinated phenols, polychlorinated biphenyls (PCBs), pesticides, trinitrotoluene (TNT), and industrial dyes] are oxidized and mineralized to varying extents by white-rot fungi (Table 1). The initial attack has, in many cases, been shown to be due to extracellular enzymes of the ligninolytic system (Table 2). Purified preparations of LiP, for example, are able to oxidize a variety of PAH compounds to PAH quinones[5,27]; pentachlorophenol (PCP) to tetrachloro-*p*-benzoquinone[7,41]; and cleave dichlorodibenzo-*p*-dioxin (DCDD) molecules[18]. Manganese-dependent peroxidase can oxidize dichlorophenol to chloro-*p*-benzoquinone[13], and Mn(III) in acetate buffer can oxidize PAH compounds to acetoxylated PAH and PAH quinones[46–48]. However, certain aromatics with a high ionization potential (e.g. phenanthrene and methoxybenzene) are not oxidizable by peroxidases[27,57].

In some white-rot fungi, but not in *P. chrysosporium*, laccases (low-specificity enzymes which act on *o*- and *p*-quinols, and amino-phenols) are also present in significant amounts[1,2]. Laccases were originally thought solely to play a role in the polymerization of phenols, including chlorophenols[49,50,56]. However, it is now known that they also oxidize non-phenolic aromatic compounds as well as Mn(II) in the presence of other oxidizable substrates[58,59].

In a few cases, xenobiotic transformations that occur in culture are not accounted for by the known ligninolytic enzymes. Phenanthrene and DDT [1,1-*bis*(4-chlorophenyl)-2,2,2-trichloroethane] are degraded by nitrogen-limited cultures of *P. chrysosporium* (Table 1), but they are not substrates for peroxidases[16,27,60]. Intracellular systems that are generally present in most fungi, such as cytochrome P450 mono-oxygenases, appear to be involved in phenanthrene degradation during the growth of *P. chrysosporium* under conditions in which nitrogen is not limiting[61]. However, intermediates resulting from phenanthrene degradation in actively ligninolytic, nitrogen-limited cultures could not be attributed to mono-oxygenases[22]. Clearly, other, unidentified enzymes are implicated.

The fact that ligninolytic enzymes and mediators are active extracellularly indicates that white-rot fungi are far better candidates for the bioremediation of highly apolar pollutants compared with non-ligninolytic microorganisms. Degradation of aromatic xenobiotic compounds by non-ligninolytic microorganisms occurs intracellularly, and the consequential limited

Table 2. Xenobiotic substrates of purified ligninolytic enzymes

Enzyme	Xenobiotic compound	Refs
LiP[a]	Chlorophenol	40
	Dichlorophenol	13,41
	Trichlorophenol	41
	Pentachlorophenol	7,40,41
	Chlorocatechol	18
	Anthracene	27,c
	Pyrene	27
	Benzo[a]pyrene	5,27
	Benz[a]anthracene	27
	Perylene	27
	Dibenzo-*p*-dioxin	27
	Dichlorodibenzo-*p*-dioxin	18
	Azo dyes	35,42
MnP[a]	Dichlorophenol	13
	Chlorocatechol	18
	Nitroaminotoluene	33
	Polymeric dyes	43–45
	Chlorolignin	6
Mn(III)[b]	Anthracene	46
	Pyrene	46
	Benzo[a]pyrene	46,47
	Benz[a]anthracene	46
	Dibenzo[a]pyrenes	48
	Anthanthrene	46,48
Laccase	Chlorophenol	49–51
	Chloromethylphenol	52
	Dichlorophenol	49,51,53–55
	Trichlorophenol	49,50,56
	Tetrachlorophenol	49,56
	Pentachlorophenol	49,56
	Chloroguaiacols	49
	Chlorocatechols	49

[a]Abbreviations: LiP, lignin peroxidase; MnP, manganese-dependent peroxidase.
[b]Oxidation by Mn(III) indicates that the xenobiotic is a potential substrate for MnP.
[c]S. D. Haemmerli, PhD thesis, Swiss Federal Institute of Technology, Zurich, Switzerland, 1988.

bioavailability of the compounds (due to the requirement for dissolution and diffusion into cells) is a key factor in the slow rate of biodegradation in these organisms[62,63].

The need for screening

Until recently, research into xenobiotic degradation by white-rot fungi has focused primarily on *P. chrysosporium* (Table 1). However, the practical application of this fungus in waste treatment and bioremediation does not always enable the culture conditions for ligninolysis to be fulfilled (i.e. low nitrogen, high temperature, secondary metabolism and static-culturing conditions). It may therefore be beneficial to screen a variety of white-rot fungi for the ability to degrade xenobiotics under a wide range of environmental conditions. In addition, screening established culture-collection strains and new isolates can also yield strains that are able to degrade xenobiotics more rapidly.

Screening strategies

The initial interest in the industrial application of ligninolytic fungi was their use in biopulping and biobleaching. For such purposes, the fungi should show a greater selectivity for lignin than cellulose[64]. However, screening methods based on selective lignin degradation of lignocellulose cannot be translated directly into the successful identification of microorganisms able to degrade xenobiotics: the selectivity is not required and screening for selectivity may bypass potentially useful biotransformation reactions. Other screening programmes have focused on the amount of lignin that is either mineralized[65,66] or decolorized[67]. Obviously, the complete ligninolytic system necessary for lignin degradation may not be required for xenobiotic oxidation. An initial oxidation of the xenobiotic by white-rot fungi can increase its bioavailability and thus render such compounds more susceptible to further degradation by indigenous microorganisms in waste and contaminated sites. Consequently, screening methods which aim more directly at xenobiotic degradation by measuring elimination or mineralization are preferred. Published examples include screening various strains for PCB- (Ref. 14), PCP- (Ref. 8) and PAH- (Ref. 23) degradation. However, xenobiotic extraction and analysis is time consuming and expensive, in addition to the requirement for special facilities for dealing with toxic compounds. Simpler methods based on the organism's content of specific individual enzymes (e.g. peroxidases, laccases) neglect the fact that the combined action of several enzymes, including oxidases for H_2O_2 production, is necessary for activity *in vivo*. Extracellular peroxidases are intimately associated with the outer hyphal slime layer[68], which indicates that free extracellular enzyme activity would seldom be quantitative. Likewise, important unknown enzymes may be missed. Furthermore, it is known that different fungi have distinct types of peroxidases and/or oxidases[2,65,69]. The ideal screening method should be cheap and based on a relatively non-toxic substrate and should not depend on cumbersome analyses. At the same time, it should show a high correlation with xenobiotic degradation mediated by ligninolytic enzymes.

Use of polymeric dyes in screening programmes

The decolorization of polymeric dyes has been proposed as a useful screening method for ligninolytic activity[8,66,70,71]. The high-molecular-weight dyes cannot be taken up by the organisms and thus provide a specific screen for extracellular activity – a prerequisite for the initial oxidation of lignin. Although correlation between lignin metabolism and polymeric dye decolorization has been claimed, the supporting data are limited. In one study, sufficient data were collected to establish that a correlation between dye decolorization and lignin mineralization does exist in a number of different fungal species (Ref. 66, Fig. 3a). A correlation between PCP degradation and dye decolorization has also been claimed, although only

four strains were tested[8]. To investigate whether the decolorization of polymeric dyes might be used as a general strategy for identifying xenobiotic degraders, we used the dye Poly R-478 for screening new isolates of white-rot fungi.

Correlating xenobiotic degradation with dye decolorization

White-rot basidiomycetes from decaying wood and soil were screened on selective media on agar plates containing powdered lignocellulose as substrate, benomyl as fungicide for non-basidiomycetes, and guaiacol as marker for extracellular oxidative enzymes[72]. Many of the fungal strains isolated were capable of decolorizing Poly R-478 dye. The most promising strains were tested for their ability to degrade specific PAH compounds, such as anthracene (three rings) and benzo[*a*]pyrene (five rings), supplied at 10 mg l^{-1} (Ref. 20). One of the new isolates, *Bjerkandera* sp. strain BOS 55, was clearly the most effective degrader of both anthracene and benzo[*a*]pyrene. After 28 days, 99.2 and 83.1%, respectively, of these compounds had been degraded. The PAH-degrading ability of this strain was superior to all other strains tested, including well-known culture-collection strains such as *P. chrysosporium* BKM-F1767 and *Trametes versicolor* Paprican 52. *Bjerkandera* sp. strain BOS 55 is unusual in that it produces a novel manganese-inhibited peroxidase[69], as well as novel aromatic metabolites (3-chloro-*p*-anisyl alcohol and 3-chloro-*p*-anisaldehyde)[73]. The screen revealed good correlation between PAH degradation and Poly R-478 decolorization rates (Fig. 3b). The coefficient of determination (R^2 values) for anthracene and benzo[*a*]pyrene were 0.91 and 0.76, respectively. The high correlations observed may have resulted from a direct relationship. Manganese-dependent peroxidase (MnP)[43], Mn(III)[44] and manganese-inhibited peroxidase[69], and even horseradish peroxidase[69] are known to be involved directly in polymeric dye decolorization. Poly R-478 decolorization is therefore probably indicative of peroxidative activity. Such activity includes the combined activity of H_2O_2-producing oxidases and peroxidases; mutants of *P. chrysosporium* which lack oxidase are unable to decolorize Poly R-481 (Ref. 74). Peroxidative activity is responsible for the oxidation of PAH compounds with ionization potentials less than 7.35 to 7.55 eV (Refs 27,60). Horseradish peroxidase, LiP and Mn(III) are directly involved in the initial oxidation of many PAH compounds (S. D. Haemmerli, PhD thesis, Swiss Federal Institute of Technology, Zurich, Switzerland, 1988)[5,27,47,48,60]. All dye decolorizing strains used (Fig. 3b) secrete enzymes required for peroxidative activity, peroxidases and oxidases[72]. However peroxidative activity is not the only extracellular system available to white-rot fungi for PAH biodegradation. An uncharacterized degradative system in the extracellular fluids of *P. chrysosporium* eliminates phenanthrene[25], which has an ionization potential (8.03 eV)[60] in excess of the peroxidase substrate spectrum.

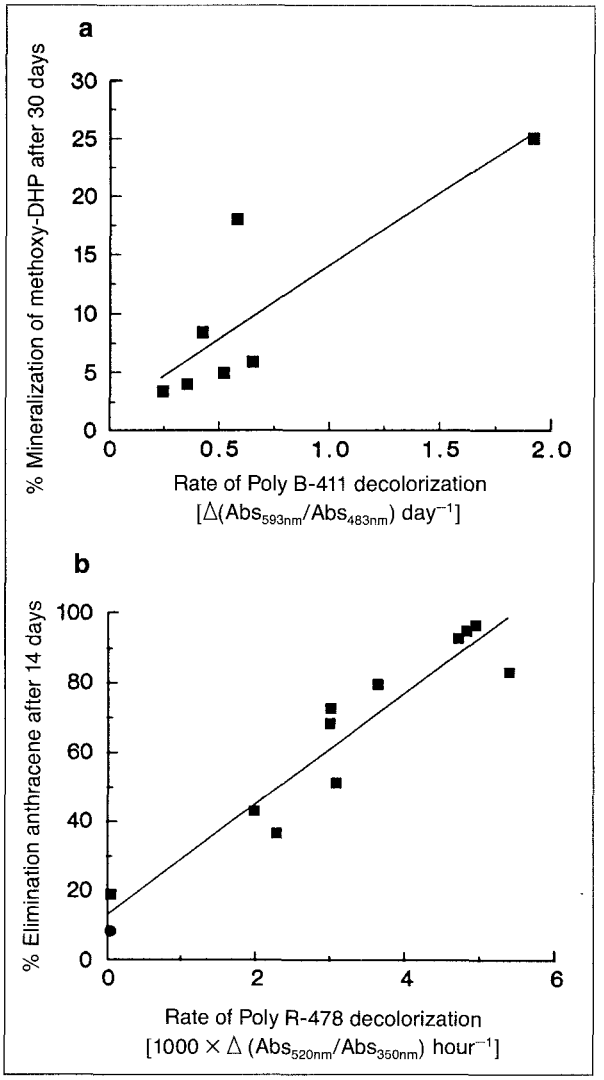

Figure 3

(**a**) The correlation between polymeric dye Poly B-411 decolorization and methoxy-labelled dehydrogenative polymerizate (DHP) lignin mineralization by various wood degrading fungi. The correlation is based on the data presented in Ref. 66. The correlation coefficient, R^2 = 0.721. (**b**) The correlation between polymeric dye Poly R-478 decolorization and anthracene elimination by various strains of newly isolated basidiomycetes and culture collection strains[20]. The correlation coefficient, R^2 = 0.906. Abbreviations: DHP, dehydrogenative polymerizate. •, abiotic controls.

Conclusions

White-rot fungi constitute a promising group of microorganisms for application in the bioremediation of recalcitrant xenobiotic compounds. Most previous research has focused on one fungus, *P. chrysosporium*, and little is known of the xenobiotic degrading capabilities of other lignin-degrading fungi. Decolorization of polymeric dyes has proven to be a good indicator of the initial transformation of xenobiotics mediated by the peroxidative activity of fungi. Although biotransformations mediated by mono-oxygenases might very well be missed by screening programmes using polymeric dyes, peroxidative activity is a component of the lignin degradation system of these fungi, and thus is an appropriate characteristic to screen for. In

addition monitoring dye decolorization is rapid and simple, enabling the handling of a large number of samples in extensive screening programmes.

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feature

Alzheimer's disease – from cause to cure?

Gail Vines

The tools of molecular biology have only been applied to the study of the brain since the early 1980s, but it is already apparent that this approach holds promise for identifying strategies and drugs that are able to modify the activity and function of particular neural pathways. One of the greatest prizes – both for society and commercially – will be the discovery of a safe and effective drug to treat Alzheimer's disease.

The incidence of Alzheimer's disease (AD) is high compared with that of other neurodegenerative diseases, such as Huntington's disease (HD) and motor neurone disease (MND). Throughout the world, ~10% of people in their 70s and 30% of people in their 80s suffer from AD. In the UK alone, this amounts to ~500 000 people – nearly as high as the combined incidence of all forms of cancer. As the proportion of elderly people in the population rises, the incidence of AD will rise, and could be doubled by the year 2050.

A further incentive for finding effective preventative, or therapeutic treatments for AD is the distressing nature of the disease for both the affected individual and their relatives since, as Brian Anderton (Institute of Psychiatry, London, UK) explains; "There is a relentless erosion of a whole range of mental facilities, including memory, and everyday skills like making tea or tying shoelaces. It progresses to a loss of social behaviour and the disappearance of personality". The relentless decline of affected individuals occurs over five to 15 years following the initial onset of the disease.

Identifying therapeutic targets

Attempts to find potential targets for therapeutic intervention need to focus on the primary defect that is responsible for initiating the neural degeneration. Alzheimer's disease is characterized by widespread neuronal dysfunction in the brain (Fig. 1); the appearance of proteinaceous fibrils intraneuronally; and the progressive deposition of amyloid, both intraneuronally and, as amyloid plaques, extracellularly and in blood vessels. There are two characteristic and diagnostic degenerative changes detectable in brain biopsies of individuals who have died of AD. One of these is the presence of 'senile plaques', aggregates of cellular debris with a core composed of β-pleated sheets of amyloid proteins, scattered among the dying nerve cells (Ref. 1; Fig. 2). The second is the presence of neurofibrillary tangles within dying nerve cells. These tangles result from the accumulation of cross-linked fibrils termed paired-helical filaments. While the exact composition of these fibrils remains controversial, one component has been identified as a modified form of the protein tau[2]. In normal nerve cells, tau forms part of the cytoskeleton, a complex network found throughout the cytoplasm of eukaryotic cells, which is involved in coordinating the movement of organelles and molecules within the cell. This transport system fails if the cytoskeleton is disrupted by the formation of paired-helical filaments. The tau protein appears to play a key role in regulating cytoskeletal assembly – a function that depends, in part, on its phosphorylation state. There is some evidence that tau is hyperphosphorylated in sufferers of AD. The disease is also characterized by deficits in the brain of neurotransmitters, enzymes involved in their metabolism and neurotransmitter receptors[3].

What, therefore, is the primary defect? In the past year or so, there has been a growing consensus that amyloid plaques are the cause, rather than the consequence, of neuronal death, with the first step in the developing pathology being the accumulation of β-amyloid protein (β-AP) in neurons. If this is true,

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