Stellingen

1. Het ontbreken van een duidelijke relatie tussen de gemeten ligninolytische activiteit en de PAK-afbraak in culturen van witrotschimmels is geen bewijs dat ligninolytische enzymen niet zijn betrokken bij de PAK-afbraak.
Dit proefschrift.

2. Bezalel *et al.* negeren met hun bewering, dat PAKs hoogstwaarschijnlijk pas ná intracellulaire pre-oxidatie door de extracellulaire ligninolytische enzymen verder worden geoxideerd, zowel het probleem van de lage biobeschikbaarheid van PAKs als het feit dat PAKs zonder preoxidatie al substraat zijn van ligninolytische enzymen.
Bezalel *et al.* 1996. Appl. Environ. Microbiol. **62**:292-295.

In onderzoek naar de afbraak van PAKs is de keuze voor het gebruik van phenanthreen als model-PAK, gezien de relatief hoge wateroplosbaarheid en vluchtigheid, duidelijk meer ingegeven door meettechnische voordelen dan door wetenschappelijke overwegingen.

4. Bij het financieren van onderzoek naar "zwarte gaten" weet men precies waarheen het geld gaat.

In Nederland is het telen van snijmaïs een vorm van fytoremediatie met tegengesteld effect.

Sinds de laatste epidemie van varkenspest zou de uitdrukking "krokodillentranen" vervangen moeten worden door de uitdrukking "varkensboerentranen".

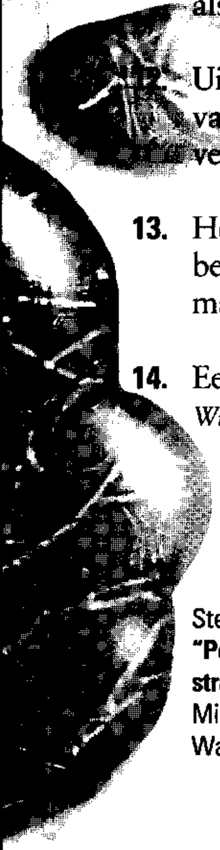
Wie, zoals de VS, gewelddadige groeperingen en dictaturen steunt voor "het goede doel" kan vroeg of laat ook zelf het doelwit worden.

8. In Nederland is een dag zonder wind een dag met smog.

9. De wijze waarop de mens met de aarde omgaat, laat zich goed vergelijken met een bacteriële batchcultuur.

10. Het gebruik van artikel 140 zoals bij de Eurotop in Amsterdam zou niet misstaan in het huidige China.

11. Het uit de VS overgewaaide gevoel van "freedom": totale afhankelijkheid van persoonlijk gemotoriseerd vervoer, laat zich het best vertalen als "vrij dom".



12. Uit het verzet van de plaatselijke vogelbescherming tegen handhaving van het studentencomplex "Droevendaal" in Wageningen blijkt een verbijsterend gebrek aan kennis van zaken.

13. Het aankondigen van de tijd en plaats van verkeerscontroles is een beleid dat niet is gericht op het vergroten van de verkeersveiligheid, maar slechts op het verlagen van de administratieve werkdruk.

14. Een veteraan is iemand met vet eraan.
Wim (44 j), tuinder in ruste te Pijnacker.

Stellingen behorende bij het proefschrift:
"Polycyclic aromatic hydrocarbon degradation by the white rot fungus *Bjerkandera* sp. strain BOS55"
Michiel Kotterman
Wageningen, vrijdag 2 oktober 1998

Polycyclic aromatic hydrocarbon degradation by the white rot fungus *Bjerkandera* sp. strain BOS55

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Polycyclic aromatic hydrocarbon degradation by the white rot fungus *Bjerkandera* sp. strain BOS55

Michiel Kotterman

Proefschrift

ter verkrijging van de graad van doctor op gezag van de rector magnificus van de Landbouwuniversiteit Wageningen, dr. C. M. Karssen, in het openbaar te verdedigen op vrijdag 2 oktober 1998 des namiddags om 13.30 uur in de Aula.

The research described in this thesis has been performed at the Division of Industrial Microbiology, Department of Food Science, Wageningen Agricultural University, the Netherlands, and was funded by IOP project 91219, "Enhancement of PAH bioavailability by white rot fungi", from Senter, an agency of the Dutch Ministry of Economics.

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ISBN: *90-5485-905-9*

Omslag foto: *Microscopische foto van mycelium van* Bjerkandera *sp. strain BOS55 met blauw oplichtende PAK-kristallen*
Druk: *SSN Nijmegen*
Vormgeving: *NMC BV/Max van Poorten*

Aan mijn ouders

Voorwoord

Nu de laatste proefjes echt af zijn, en het proefschrift gereed, kan ik terug kijken op een zeer komplete AIO tijd. Kompleet wat betreft de (bijna) standaard AIO-dips, maar ook de AIO-highs. Een enerverende tijd, waar naast duizenden flessen met kilometers schimmeldraad ook een groot aantal mensen een belangrijke rol speelden.

Een prominente rol in mijn promotie-onderzoek speelden Jan de Bont, mijn promotor en Jim Field, mijn co-promotor.

Jan, bedankt dat je mij de mogelijkheid hebt gegeven om dit onderzoek te verrichten en om het af te ronden met dit proefschrift. Jim, je hebt mij ingewijd in de schimmige wereld van de witrot-schimmels en PAKs, en je voorzag mij van raad en daad als ik even in de wirwar van hyphen kop noch staart kon ontdekken. Een betere begeleider kon ik mij niet wensen; je bent bij het tot stand komen van dit proefschrift van grote waarde geweest.

Veel lab-werk is verricht door mijn studenten; René Wasseveld, Annemarie Hage, Henk-Jan Rietberg en Johan van Lieshout. Ook zij konden het enthousiasme opbrengen om honderden flessen te vullen en wekenlang schimmeltjes te kweken. Welkome hulp bij het labwerk werd geboden door verscheidene personen. Martin de Wit, zijn excellente en, minstens zo belangrijk, snelle assistentie bij materiaalpech of materiaalonkunde van mijn kant heb ik zeer gewaardeerd. Ans Geerling, die mij gastvrij hielp op het radio-isotopenlab bij de vakgroep Microbiologie en ook Tjakko Abee voor de hulp in *"zijn"* radio-isotopenlab in het Biotechnion. Eric Vis die deskundige hulp verleende bij het uitvoeren van mutageniteitstesten.

Geestelijke hulp wat het onderzoek betreft kwam van de leden van mijn begeleidingscommissie en vele personen op het lab: post-docs, AIO's, studenten en gastmedewerkers, met wie ik zinvolle discussies heb gevoerd over het onderzoek. Veel van de personen op het lab waren minstens zo zinvol wat betreft de geestelijke ontspanning. Ook in het weekend of 's avonds waren er altijd wel mensen om mij gezelschap te houden, was het niet op het lab dan wel in de mensa, kroeg of tijdens het rondje puffen. Om niet de plank mis te slaan bij het opnoemen van allerlei personen wil ik simpelweg iedereen die mijn verblijf op Industriële Microbiologie aangenaam heeft gemaakt bij dezen bedanken.

Ondanks het bovenstaande wil ik toch enkele personen noemen.
De generatie nestor-AIO's, Jelto, Gert, Edwin en Jan, waar ik de jongste en ook de laatste van ben. Misschien is het vastroesten van hen, zo tijdens en direct na de promotie, niet eens een slecht idee. Mijn broer Wim voor de correctie van mijn Nederlands, en mijn nieuwe collega's op de 7de verdieping, Nico, Huib, Sybren en Chiel, die de *"stap voorwaarts"* van WP onder DOS naar Word onder Windows aanzienlijk hebben versoepeld.

En dan natuurlijk Liesbeth, die zich verre van dít lab hield, maar wel het leven erbuiten veraangenaamde. Zij was het ook die mij liefdevol oplapte als ik weer eens na een "sportieve uitspatting" in de lappenmand lag.

Kortom, het was goed en het is nog beter dat het af is.

Contents

Chapter 1

General introduction

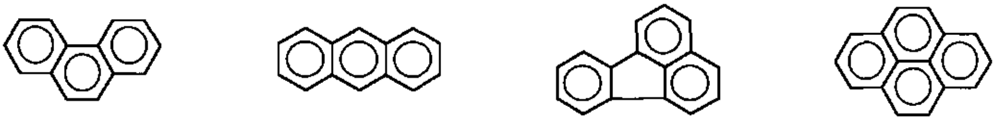
Polycyclic aromatic hydrocarbon contamination of soils

In the Netherlands the contamination of soils with xenobiotic compounds due to industrial activities is widespread. Since many of these pollutants are toxic to human health, clean-up of polluted sites is desirable. Examples of toxic pollutants which have been located in Dutch soils are dioxins, chlorinated hydrocarbons, and polycyclic aromatic hydrocarbons (PAHs). PAHs consist of fused benzene rings, ranging from 2- to up to 7-rings. In ***Figure 1***, the sixteen PAHs selected by the Environmental Protection Agency (EPA) of the United States of America as indicators of PAH pollution are shown. These 16 EPA PAHs include the 10 PAHs selected by the Dutch Ministry of Housing and Environment (VROM). PAH are hydrophobic compounds, the aqueous solubility of PAHs decreases with increasing number of rings to very low values (***Table 1***). PAHs are formed by incomplete burning of fossil fuels and wood. The PAHs in exhaustion gasses can reach the soil via atmospheric deposition. Local and higher levels of PAH contamination are due to industrial activities such as old gasification plants (234 sites in the Netherlands) and wood preserving plants where creosote and anthracene-oil, partial distillates of oil with high concentrations of PAHs, are used (Sims and Overcash, 1983; Wilson and Jones, 1993).

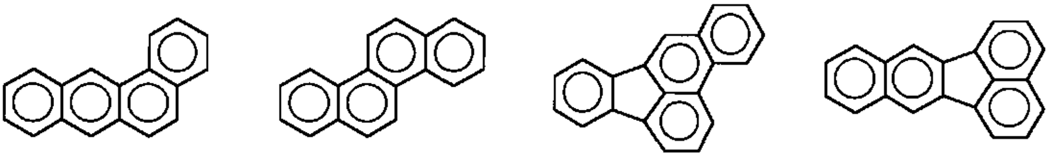
The contamination of soils with PAHs causes a problem since PAHs can have toxic, mutagenic and carcinogenic effects. Some of the smaller PAHs (2- and 3-rings) show acutely toxic effects (Sims and Overcash, 1983), while biotransformation of the larger PAHs by humans and microorganisms can result in metabolites with high carcinogenic activity (***Table 1***). Due to these toxic effects, the use of PAH-polluted soils is restricted by Dutch legislation. Whether clean-up of a specific PAH contaminated site is required depends on the type of soil (percentage of organic matter) and the intended use of the site. In the new guidelines, a soil is considered clean and multifunctional when the concentration of the 10 VROM PAHs is lower than 0.1 mg PAHs kg^{-1} soil (dry weight) x % organic matter (Leidraad bodembescherming, 1995). However, no correction is made for soils with less then 10% organic matter (in these soils, 1 mg PAHs kg^{-1} soil is considered clean), and no correction is made for soils with more than 30% organic matter. When the concentration of the 10 VROM PAHs exceeds 0.4 mg PAHs kg^{-1} soil (dry weight) x % organic matter, the concentration of PAHs has to be reduced. Again, no correction is made for soils with less than 10% organic matter (in these soils, 40 mg PAHs kg^{-1} soil is the upper limit), and no correction is made for soils with more than 30% organic matter. Soils with intermediate PAH contamination levels can be used for industrial purposes only. Taking in account that at many industrial PAH-polluted sites the concentration of PAHs exceeds far 40 mg kg^{-1} soil, high reduction values have to be obtained.

Figure 1. Chemical structures of the 16 EPA PAHs, the 10 PAHs selected by VROM as indicators of PAH pollution in the Netherlands are indicated with an asteriks.

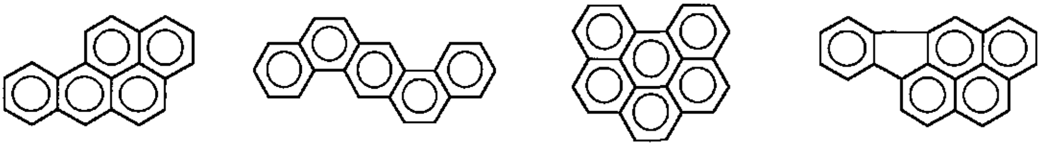
Naphthalene* **Acenaphthylene** **Acenaphthene** **Fluorene**



Phenanthrene* **Anthracene*** **Fluoranthene*** **Pyrene**



Benzo[*a*]anthracene* **Chrysene*** **Benzo[*b*]fluoranthene** **Benzo[*k*]fluoranthene***



Benzo[*a*]pyrene* **Dibenzo[*a,h*]anthracene** **Benzo[*ghi*]perylene*** **Indeno[*123-cd*]pyrene***

Table 1. Physical and chemical properties of the 16 EPA PAHs, the 10 PAHs selected by VROM as indicators of PAH pollution in the Netherlands are indicated with an asteriks.

PAH	Molecular weight	Solubility[a] (mg L^{-1})	Carcinogenic potential[b]	Log K_p[a]
Naphthalene*	128	30		3.37
Acenaphthene	154	3.5		4.33
Acenaphthylene	152	3.9		4.07
Fluorene	166	2.0	±	4.18
Phenanthrene*	178	1.3	±	4.46
Anthracene*	178	$7.3*10^{-2}$	--	4.45
Fluoranthene*	202	$2.6*10^{-1}$	--	5.33
Pyrene	202	$1.4*10^{-1}$	--	5.32
Benzo[*a*]anthracene*	228	$1.4*10^{-2}$	++	5.61
Chrysene*	228	$2.0*10^{-3}$	+	5.61
Benzo[*b*]fluoranthene	252	$1.2*10^{-3}$	++	6.57
Benzo[*k*]fluoranthene*	252	$5.5*10^{-4}$	++	6.84
Benzo[*a*]pyrene*	252	$3.8*10^{-3}$	++	6.04
Dibenzo[*ah*]anthracene	278	$5.0*10^{-4}$	++	5.97
Benzo[*ghi*]perylene*	276	$2.6*10^{-4}$	±	7.23
Indeno[*123-cd*]pyrene*	276	$6.2*10^{-2}$	++	7.66

[a] According to Sims and Overcash (1983)

[b] According to IARC monographs on the carcinogenic risk of chemicals to humans (1983):

++ :	sufficient evidence of causal relationship between the tested agent and human cancer
+ :	limited evidence, causal relationship is likely, but not proven
± :	inadequate evidence, both negative as positive data available
-- :	sufficient evidence to exclude carcinogenity of the tested agent

Remediation of PAH-polluted soils

At present, the main methods for treatment of PAH-polluted soils are thermal combustion and extraction/classification of soils. In the first case, the PAHs are first evaporated from the soil at temperatures around 500°C, and then completely oxidized in a separate incineration step. In the second case, the pollutants are removed from the soil by an extracting agent, *e.g.* water. The soil is separated into clean soil particles (mainly sand) and contaminated extracting agent (sludge). Due to the high costs of these treatments, many soils are not dealt with. Therefore, biological methods to remediate PAH contaminated soils are being investigated.

Biological remediation procedures have been developed for some readily degradable pollutants (*e.g.* fuell-spills at gasstations), and have shown to be successful under field conditions, both in terms of pollutant elimination efficiency and economic feasibility. For example, soils contaminated with mineral oils can be treated by bacterial methods. This treatment is based on the ubiquitous ability of soil microorganisms to degrade oil, as long as adequate environmental conditions are maintained. Conditions favorable for degradation of the pollutant (aeration, moisture, and nutrients) can be created in a low costs system called landfarming. Unfortunately, in the case of PAHs it has been observed that the PAHs are not sufficiently degraded, even under the most favorable landfarming conditions (Bossert and Bartha,1986; Wilson and Jones, 1993).

The low molecular weight PAHs like naphthalene and phenanthrene are relatively easily degraded by many microorganisms. Half-life times in the order of weeks have been observed in soil with PAH-adapted microflora (Wilson and Jones, 1993). In contrast, 4-ring PAHs like pyrene have considerably longer half-life times, in the order of months, while the 5- and 6-ring PAHs like benzo[*a*]pyrene and benzo[*ghi*]perylene, have half-lives of several years or more in soils (Heitkamp and Cerniglia, 1987; Cerniglia, 1992; Wilson and Jones, 1993). These data are confirmed by experiments under laboratory-conditions. So far, only a few microorganisms have been shown to be capable of degrading higher PAHs (4 - 5-rings). However, this degradation only takes place at a slow rate (Heitkamp *et al.*, 1988; Weissenfels *et al.*, 1991; Juhasz *et al.*, 1997), and in most cases these higher PAHs are only degraded in the presence of another substrate (Juhasz *et al.*, 1997; Kanaly *et al.*, 1997). The differences in biodegradability of PAHs can be explained mainly by the aqueous solubility of these PAHs as shown in ***Table 1***. An inverse correlation between the aqueous solubility and the biodegradability has been observed. The water solubility decreases rapidly from 30 mg L^{-1} for naphthalene to as low as 4 µg L^{-1} for benzo[*a*]pyrene (with corresponding decrease of dissolution rates). Many studies have shown that the low PAH dissolution rate limits the PAH uptake and degradation rate by bacteria (Stucki and Alexander 1987; Volkering *et al.*, 1992; Mihelcic *et al.*, 1993). Compounds like PAHs, for which degradation rates are limited by the rate the compounds become available for microbial degradation, are called compounds with low bioavailability.

PAH bioavailability

The intrinsic low bioavailability of PAHs is even further decreased in soil systems. Due to their low solubility and high hydrophobicity, PAHs will occur in soils preferably associated with hydrophobic organic matter (non-aqueous-phase liquids (NAPLs), coal, tar, soil organic matter (SOM)), depending on the source of the PAH contamination. NAPLs, occurring at gasification plants and creosote-applying plants as coal-tar and oil, are especially important with respect to limited PAH degradation. Mass transfer of PAHs out of NAPLs has been described as a very slow process (Efroymson and Alexander, 1994; Yeom *et al.*, 1996; Ramaswami *et al.*, 1997), and aging of the NAPLs decreases the rate even more (Luthy *et al.*, 1993). Some low molecular weight NAPLs (fuels) can serve as cosubstrate for microorganisms and as solvent for the PAHs, which can initially increase the PAH degradation rate by bacteria (Kanaly *et al.*, 1997). However, in time, volatilization and biodegradation of the lower hydrocarbons will turn the oil into a coal-tar like residue, resulting in decreased PAH bioavailability.

The sorption of PAH onto organic matter, either natural soil organic matter (humus) or anthropogenic organic matter introduced with the PAH contamination (soot, coal), also decreases the PAH bioavailability and biodegradability. The current understanding of which processes affect sorption and desorption of contaminants in soils is poor (Pignatello and Xing, 1996; Luthy *et al.*, 1997). Sorption of PAHs to organic matter depends on the hydrophobicity of the PAH involved as well as the concentration, chemical structure of the organic matter, and the distribution of organic matter in the soil matrix. The observation that aged PAH contaminants are degraded more slowly and to a lesser extent than fresh contaminants is generally attributed to sequestration of PAHs in organic matter, followed by slow desorption of the sequestered PAHs (Pignatello and Xing, 1996; Luthy *et al.*, 1997). Additionally, PAHs can be entrapped in aggregates of soil particles with small pore sizes that exclude microorganisms. As a result, PAHs degradation rates are even further decreased by the slow diffusion of the enclosed PAH out of the particle (Crocker *et al.*, 1995; Luthy *et al.*, 1997).

Methods to increase PAH bioavailability

To improve the bacterial degradation rate of high molecular weight PAHs, one should increase the PAH bioavailability. However, methods which can increase the bioavailability of PAHs, such as addition of water miscible solvents or increasing temperatures, still have serious limitations for use in full-scale soil bioremediation.

Research has focused on the use of surfactants to increase PAH bioavailability. Surfactants are molecules with hydrophobic and hydrophilic parts that form micelles above a certain concentration, known as the critical micelle concentration (CMC). PAHs can become solubilized in these micelles, increasing the apparent PAH concentration and, in some cases, the PAH degradation rate (Rousse *et al.*, 1994; Tiehm, 1994; Volkering *et al.*, 1995). However, the PAHs in the micelles are not always bioavailable for microbial attack (Volkering *et al.*, 1995). Moreover, at concentrations above the CMC, surfactants are often toxic towards the PAH-degrading bacteria, while non-toxic surfactants are sometimes preferentially degraded (Tiehm, 1994). Furthermore, some surfactants tend to adsorb to soil, which increases the amount of surfactant needed (Liu *et al.*, 1992).

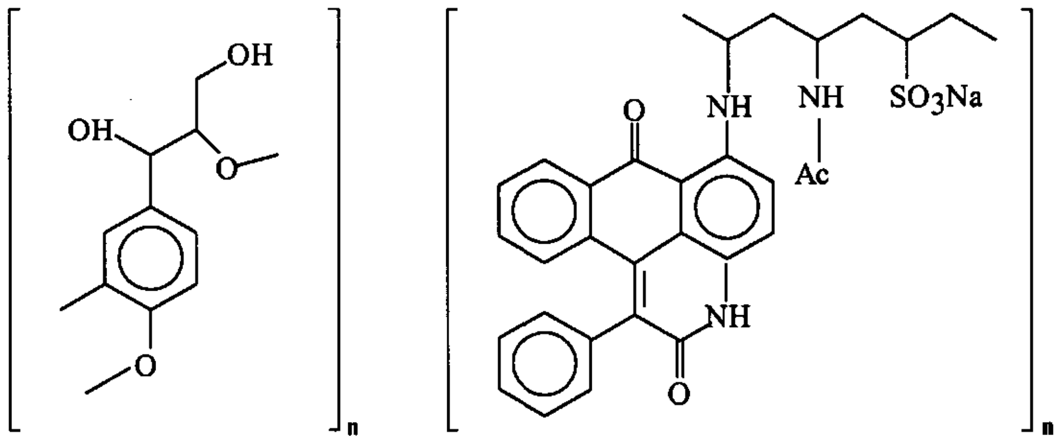
It can be concluded that methods applicable in full-scale bioremediation to increase the bioavailability of PAHs, and thus the biodegradation rate of PAHs to an acceptable level, are not within grasp. An alternative bioremediation method has been sought in the use of white rot fungi. White rot fungi were shown to oxidize PAHs and other related aromatic compounds outside the fungal cell with an extracellular enzyme system (Hammel, 1992; Field *et al.*, 1993). Extracellular enzymes would be expected to oxidize PAH in soil micropores, circumventing the slow process of PAH diffusion out of soil pores towards the microbial cells. This alternative for PAH bioremediation has motivated research for suitable white rot fungal strains and culture techniques for PAH degradation by these fungi.

White rot fungi

Wood inhabiting white rot fungi are virtually the only family of organisms that are capable of extensively oxidizing lignin (Kirk and Farrel, 1987). Lignin is a complex aromatic polymer in wood (wood consists for 20-30% of lignin). Lignin provides wood with water impermeability, strength, and protection of cellulose against bacterial attack. Degradation of lignin by the ligninolytic extracellular enzymes of white rot fungi enables the fungi to gain access to, and to grow on wood polysaccharides, cellulose and hemicelluloses, which are the actual energy and carbon sources (Kirk *et al.*, 1976).

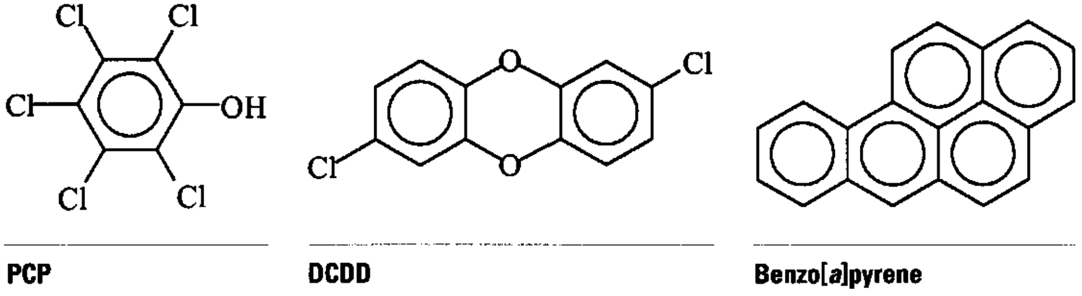
Because of the high molecular weight and the irregular structure, lignin can only be degraded outside the fungal cell with an aspecific extracellular enzyme system. This ligninolytic enzyme system is also responsible for the oxidation of structurally related xenobiotic aromatic compounds such as PAHs, pentachlorophenol (PCP), dioxin, and aromatic dyes such as Poly R-478 (Aust, 1990; Field *et al.*, 1993). The structure formulas of these compounds are shown in ***Figure 2.***

Figure 2. The related structure formulas of lignin, the polymeric dye Poly R-478, PCP, dioxin, and the 5-ring PAH, benzo[*a*]pyrene.



Lignin

Poly R-478



PCP

DCDD

Benzo[*a*]pyrene

The ligninolytic enzyme system

The extracellular ligninolytic enzyme system responsible for the oxidation of lignin and related aromatic compounds is composed of a very complex enzymatic machinery. The mechanism of lignin degradation is based on random oxidation of the aromatic structure by oxidative enzymes. The known ligninolytic enzymes and cofactors required for their activity are shown in *Table 2.*

The oxidative enzymes are either heme based peroxidases or copper containing phenol oxidases (Kirk and Farrel, 1987; Buswell, 1991; De Jong *et al.*, 1994b). Several peroxidases have been observed in white rot fungal cultures; lignin peroxidase (LiP), manganese peroxidase (MnP) and manganese independent peroxidase (MIP).

LiP, the first white rot fungal peroxidase to be discovered, is found in several white rot fungal species (De Jong *et al.*, 1994b). The catalytic cycle of LiP consists of a two-electron oxidation by H_2O_2 of the native (ferric) enzyme to compound I, which returns to the native state via compound II by two separate one-electron oxidations of aromatic substrates (Tien, 1987). Some substrates of LiP, like anisyl alcohol, can only be oxidized by compound I and not by compound II (Koduri and Tien, 1994). Therefore, another substrate that can be oxidized by compound II is required for closing the catalytic cycle. Veratryl alcohol (3,4 - dimethoxybenzyl alcohol), a secondary metabolite produced by many white rot fungi, is a good substrate of compound II and is known to complete the catalytic cycle of LiP (Koduri and Tien, 1995; Schick-Zapanta and Tien, 1997). The reduction of compound II to the native state of LiP also prevents the oxidation of compound II to the relatively inactive compound III by H_2O_2, which results in loss of activity (Wariishi and Gold, 1989). Other compounds such as 1,4-dimethoxybenzene, 3,4-dimethoxytoluene, 3,4,5-trimethoxybenzyl alcohol and

Table 2. The extracellular enzymes of the ligninolytic system and their cofactors.

Enzyme	Cofactor
Peroxidases	
Lignin Peroxidase (LiP)	veratryl alcohol, 2-chloro-1,4-dimethoxybenzene
Manganese Peroxidase (MnP)	MnII, oxalate, lipids, gluthathion
Manganese Independent Peroxidase (MIP)	——
Phenol oxidases	
Laccase	3-hydroxyanthranilate, (N-substituted aromatics)
Oxidases	
Glucose-oxidase	glucose, xylose, mannose
Aryl alcohol oxidase	anisyl, chloroanisyl and veratryl alcohols
Glyoxal oxidase	glyoxal
Cellobiose dehydrogenase	cellobiose

trypthophan have also been shown to be good substrates (Koduri and Tien, 1994; Joshi and Gold, 1996; Collins *et al.*, 1997). Recently, another fungal metabolite, 2-chloro-1,4-dimethoxybenzene, produced *de novo* by *Bjerkandera adusta* and *Lepista nuda*, has also been shown to be a cofactor for LiP (Teunissen *et al.*, 1998). Since high molecular weight substrates such as lignin can not enter the active site of LiP, it has been proposed that the product of LiP-mediated veratryl alcohol oxidation, veratryl alcohol cation radical, could mediate the oxidation of other substrates. Although the life time of the free radical is too short (half-life time 0.5 ms) for the radical to operate as a diffusable oxidator far away from the enzyme (Khindaria *et al.*, 1995; Joshi and Gold, 1996), mediation does occur with certain substrates (Goodwin *et al.*, 1995; Koduri and Tien, 1995). An enzyme-bound veratryl alcohol radical, demonstrated by Khindaria *et al.* (1995), was more stable with a half-life time of 0.5 s and is therefore likely to be more reactive.

MnP is found in most white rot fungi (De Jong *et al.*, 1994b). Like the catalytic cycle of LiP, the catalytic cycle of MnP consists of a two-electron oxidation by H_2O_2 to compound I. MnP differs from LiP that it has an unique Mn binding site (Sundaramorthy *et al.*, 1994, 1997) and compound I can only be reduced back to the native enzyme by oxidation of the cofactor MnII to MnIII (Glenn *et al.*, 1986; Wariishi *et al.*, 1992). When chelated by organic acids such as oxalic acid, the powerful oxidant MnIII can serve as a truly diffusable mediator to oxidize phenolic compounds (Glenn and Gold, 1985; Lackner *et al.*, 1991; Wariishi *et al.*, 1992). Involvement of unsaturated lipids in the MnP reaction (lipid peroxidation) or gluthathion can increase the oxidative power of MnP (Moen and Hammel, 1994; Sack *et al.*, 1997), presumably via the radicals formed from these cooxidants.

MIP is a relatively newly discovered peroxidase, which was first reported in cultures of *Bjerkandera* sp. strain BOS55 (De Jong *et al.*, 1992a). This peroxidase behaves very much like the plant peroxidase, horseradish peroxidase, with respect to its substrate spectrum.

The H_2O_2 independent laccases catalyze the reduction of molecular oxygen to water, while simultaneously they oxidize phenolic aromatic compounds. In the presence of cooxidants, typically N-substituted aromatics such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), non-phenolic compounds of lignin (Bourbonnais and Paice, 1990) and PAHs can also be oxidized (Collins *et al.*, 1996; Johannes *et al.*, 1996). Physiologically occurring cooxidants have only recently been observed in fungal cultures of *Trametes versicolor* as non-identified low molecular weight compounds (Collins *et al.*, 1996) and as 3-hydroxyanthranilate in cultures of *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996).

The hydrogen peroxide in white rot fungal cultures can be generated in two ways. As shown in ***Table 2***, several H_2O_2-generating oxidases have been identified in white rot fungal cultures (Kelly and Reddy, 1986; Kersten, 1990; Muheim *et al.*, 1990; Ander, 1994). These oxidases are typically flavoproteins that oxidize their substrates to the corresponding aldehydes or acids, while oxygen is reduced to superoxide, which dismutates to hydrogen peroxide. The substrates of the aryl alcohol oxidase, which includes chlorinated anisyl alcohols, are produced *de novo* by fungal cultures of many white rot fungal species such as *Bjerkandera* sp. strain BOS55 (De Jong *et al.*, 1994b).

Hydrogen peroxide can also be produced as a result of organic acid oxidation mediated by MnP or LiP activity. Both MnIII and the veratryl alcohol radical cation can oxidize oxalate (Barr *et al.*, 1992; Khindaria *et al.*, 1994; Shimada *et al.*, 1994; Urzua *et al.*, 1998). Oxalate is a metabolite of fungal metabolism found in many white rot fungal cultures (Dutton *et al.*, 1993). Oxalate is oxidized to the formate radical anion ($CO_2^{\cdot-}$) and CO_2. The formate radical anion reduces oxygen to superoxide, dismutation of the superoxide generates hydrogen peroxide (Khindaria *et al.*, 1994; Urzua *et al.*, 1998).

The H_2O_2 generating oxidases can also function as reductases by reducing, instead of oxygen, quinones and phenoxy radicals formed during lignin degradation. This prevents repolymerization of the lignin, and thus contributes to effective depolymerization (Marzullo *et al.*, 1995; Ander and Marzullo, 1997; Guillen *et al.*, 1997).

Induction of the ligninolytic system

The expression of the complex ligninolytic system is regulated by the onset of secondary metabolism by N, C, or S limitation. The relatively low production of enzymes under these starvation conditions is a major disadvantage. Based on studies with the model white rot fungus *Phanerochaete chrysosporium*, high nitrogen culture conditions were believed to repress ligninolytic expression in white rot fungi (Kirk and Farrel, 1987). Recent studies, however, have shown that some white rot fungal species and strains are N-unregulated. The biomass yields and peroxidase production can be highly stimulated by using high-N media (Tien and Myer, 1990; Orth *et al.*, 1991; Kaal *et al.*, 1993).

Mn, the cofactor of MnP, appears to have a profound influence on the white rot fungal physiology. The presence of manganese enhanced the MnP activity in many white rot fungi (Bonnarme and Jeffries, 1990; Brown *et al.*, 1990; Ruttiman *et al.*, 1992), while the LiP activity was decreased (Bonnarme and Jeffries, 1990; Perez and Jeffries, 1992). It was observed that Mn has a regulatory role at the molecular level, since the addition of Mn to Mn-deficient cultures resulted in detectable *mnp* mRNA levels (Gold and Alic, 1993). However, the addition of Mn did not decrease the transcription of *lip* mRNA in *Phanerochaete chrysosporium* (Li *et al.*, 1994), indicating the negative impact of Mn on LiP titers was not regulated at the molecular level. Mester *et al.* (1995) showed that Mn reduces veratryl alcohol biosynthesis. The fact that veratryl alcohol has a well-known role in protecting LiP from inactivation by physiological levels of H_2O_2, was given as the major reason why LiP is apparently regulated by Mn.

Mn is abundantly present in woody tissues, but it has been observed that Mn concentrations decrease during white rot fungal activity due to dismutation of MnIII to insoluble MnO_2 (Blanchette, 1984; Perez and Jeffries, 1993). This causes the soluble Mn concentration to decrease, altering the profile of ligninolytic enzymes, thus accounting for the predominance of LiP during later stages of wood decay.

Oxidation of aromatic compounds by the ligninolytic system

The degradation of lignin is initiated by a one-electron oxidation of the aromatic moiety, forming an instable cation radical. This radical can be spontaneously attacked by water and oxygen, which leads to depolymerization as well as ring opening. Other aromatic compounds like PAHs and PCP are oxidized by the same one-electron oxidation mechanism. LiP has been shown to oxidize PAHs with an ionization potential (IP) up to 7.55 - 8.0 eV (Hammel *et al.*, 1986; Vazquez-Duhalt *et al.*, 1994). The IP is defined as the amount of energy required for a one-electron abstraction. The IP of phenanthrene (8.19 eV) is relatively high compared to other PAHs. Therefore, phenanthrene is not a substrate of LiP. ***Table 3*** shows a brief overview of the spectrum of PAH compounds that are oxidized by ligninolytic enzymes, as well as the identified products.

MnIII, the product of MnII oxidation by MnP, was shown to oxidize compounds with IP values up to 7.8 eV (Cavalieri and Rogan, 1985; Cremonesi *et al.*, 1992). The oxidative power of MnP was therefore believed to be lower than that of LiP. However, recently it was shown that MnP, in combination with unsaturated lipids or gluthathion, can also cause oxidation of phenanthrene (Moen and Hammel,1994; Sack *et al.*, 1997). Laccases, initially thought to oxidize only phenolic aromatics, can oxidize non-phenolic PAH compounds in the presence of an artificial cooxidant, like ABTS or HBT, or in the presence of a natural cooxidant produced by the white rot fungal cultures (Johannes *et al.*, 1996; Collins *et al.*, 1996; Bohmer *et al.*, 1998). The IPs of other PAHs with 3 rings or more are all lower than that of phenanthrene (Vazquez-Duhalt *et al.*, 1994), indicating that these compounds can be oxidized by the ligninolytic enzymes.

Degradation of PAH by white rot fungi

PAHs are not completely mineralized by white rot fungi. The first study monitoring the mineralization of [^{14}C]-labeled benzo[*a*]pyrene by liquid cultures of *Phanerochaete chrysosporium* (Sanglard *et al.*, 1986) observed a recovery of 15% as $^{14}CO_2$. The main effect of the oxidation of PAHs was the formation of unidentified water soluble metabolites (as much as 58% of the label), only 23% was recovered as solvent soluble oxidized metabolites. Similar results, although generally with lower levels of mineralization, have been reported since then for pure cultures of white rot fungi. Mineralization levels ranging from 0.2 to 14% have been found for the oxidation of phenanthrene, anthracene, pyrene, and benzo[*a*]pyrene by cultures of several white rot fungi like *Kuehneromyces mutabilis*, *Trametes versicolor*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, and *Phanerochaete laevis* (Barclay *et al.*, 1995; Bezalel *et al.*, 1996a; Bogan and Lamar, 1996; Sack *et al.*, 1997). The major effect of PAH oxidation by the ligninolytic fungi was the accumulation of more polar compounds.

Only a few PAH metabolites of white rot fungal attack, mainly quinones and aromatic acids, have been identified so far (***Table** 3*). Except for anthraquinone, PAH quinones, if any, are only observed in low yields (Hammel *et al.*, 1986; Bogan and Lamar, 1996). In some white rot fungal cultures, anthraquinone is a dead-end metabolite (Field *et al.*, 1992; Andersson and Henrysson, 1996), whereas in cultures of other species anthraquinone is oxidized further to phthalate or unknown compounds (Hammel *et al.*, 1991, Field *et al.*, 1992, Andersson and Henrysson, 1996). The oxidation of anthracene by ligninolytic cultures of *Phanerochaete chrysosporium* to anthraquinone and phthalate is shown in ***Figure 3***.

The accumulation of oxidized PAH metabolites could be a reason of concern for white rot fungal bioremediation of PAH-polluted soils, since oxidized PAH metabolites can be highly mutagenic compounds. A typical example of bioactivation of PAH is the oxidation of benzo[*a*]pyrene by intracellular monooxygenases and epoxide hydrolases to its ultimate carcinogen dihydroxy benzo[*a*]pyrene-epoxide, which has been observed in mammals, yeasts, and fungi (Sutherland, 1992). These reactive metabolites can be detoxified by sequential processes, such as sulfonation and glucose, xylose, or glucoronic acid conjugation. White rot fungi do have monooxygenases capable of oxidizing PAHs (Masaphy *et al.*, 1996) and in non-ligninolytic white rot fungal cultures, transdihydrodiols of phenanthrene and pyrene were observed as PAH metabolites, indicating that these PAHs were oxidized intracellularly by monooxygenases (Sutherland *et al.*, 1991,1993; Bezalel *et al.*, 1996b; Bezalel *et al.*, 1997). The PAH metabolites observed in ligninolytic white rot fungal cultures, however, suggest that the oxidation of the PAHs was mainly mediated by the ligninolytic enzyme system. The oxidation of benzo[*a*]pyrene to benzo[*a*]pyrene-quinones, the only identified metabolites yet of ligninolytic benzo[*a*]pyrene oxidation (Haemmerli *et al.*, 1986), results in a drastic decrease in carcinogenity towards mice (Thakker *et al.*, 1985). So far, no study has accurately described the effect of PAH oxidation by the ligninolytic system on the mutagenicity.

Table 3. Oxidation of PAH compounds by ligninolytic enzymes *in vitro*.

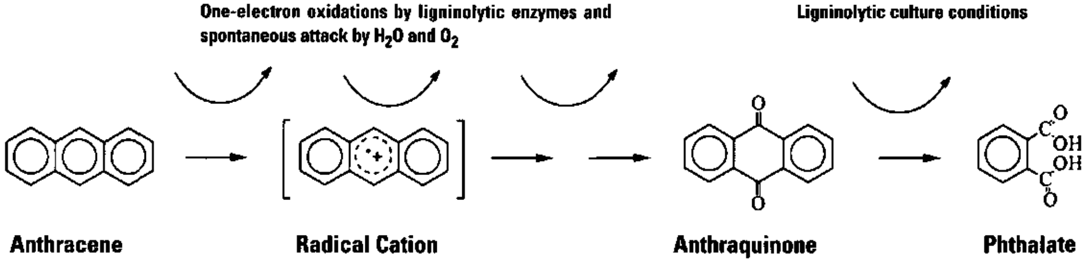
Compound	Enzyme	Identified products	Ionization potential (eV)	References
Phenanthrene	MnP[a]	Phenanthrene-9,10-quinone	8.19	Sack *et al.*, 1997
	MnP[b]	2,2'-diphenic acid		Moen and Hammel, 1994
	Laccase[b,c]			Bohmer *et al.*, 1998
Anthracene	MnP	Anthraquinone	7.43	Field *et al.*, 1996 Sack *et al.*, 1997
	LiP			Hammel *et al.*, 1991 Vazquez-Duhalt *et al.*, 1994
	Laccase[c]			Collins *et al.*, 1996 Johannes *et al.*, 1996
Pyrene	MnP	CO_2	7.50	Sack *et al.*, 1997
	LiP	Pyrene-1,6-quinone Pyrene-1,8-quinone		Hammel *et al.*, 1986 Vazquez-Duhalt *et al.*, 1994
Benzo[*a*]pyrene	MnP[a]	CO_2	7.23	Sack *et al.*, 1997
	LiP	Benzo[*a*]pyrene-quinone (1,6-3,6-6,12)		Haemmerli *et al.*, 1986 Hammel *et al.*, 1986
	Laccase[c]			Collins *et al.*, 1996

[a]: In the presence of gluthathion

[b]: In the presence of unsaturated lipids

[c]: In the presence of cooxidants ABTS or HBT

Figure 3. The oxidation of anthracene by the white rot fungus *Phanerochaete chrysosporium* to anthraquinone and phthalate (according to Hammel *et al.*, 1991).



Bjerkandera sp. strain BOS55

Bjerkandera sp. strain BOS55 was previously isolated from a screening for PAH-degrading fungi and was shown to be an excellent PAH degrader compared to other known PAH-degrading white rot fungal isolates (Field *et al.*, 1992). *Bjerkandera* sp. strain BOS55 has peculiar features. In cultures of this fungus, manganese independent peroxidase (MIP) was discovered for the first time (De Jong *et al.*, 1992a). Next to that, chlorinated aromatics such as 3-chloro-anisaldehyde and 3,5-dichloro-anisyl alcohol and aldehyde were detected in the extracellular culture fluids (De Jong *et al.*, 1992b). Some of these metabolites were shown to be substrates of the aryl alcohol oxidase, providing H_2O_2 for the activity of the peroxidases (De Jong *et al.*, 1994a). Another *de novo* chlorinated metabolite, 2-chloro-1,4-dimethoxybenzene, was shown to be an excellent LiP substrate, capable of enhancing the oxidation of non-phenolic substrates by LiP (Teunissen *et al.*, 1998). Furthermore, the fungus is N-unregulated, producing high extracellular peroxidase titers in N-sufficient medium (Kaal *et al.*, 1993; Mester *et al.*, 1996).

Outline of this thesis

In this thesis the conditions for optimal PAH oxidation by the white rot fungus *Bjerkandera* sp. strain BOS55 were evaluated. In ***Chapter 2***, culture conditions like aeration and cosubstrate concentrations, which influenced the oxidation of the PAH compound anthracene and the ligninolytic indicator dye Poly R-478 by the white rot fungus, were studied. Two parameters were identified as the most important PAH oxidation rate-limiting factors: the hydrogen peroxide production rate by the fungal cultures, needed for full activity of the peroxidases as described in ***Chapter 3***, and the PAH bioavailability as described in ***Chapter 4***. When these rate-limiting parameters were eliminated, extremely high PAH oxidation rates could be observed. In ***Chapter 5***, the oxidation and mineralization of the 5-ring PAH benzo[*a*]pyrene to CO_2 was monitored using ^{14}C-labeled benzo[*a*]pyrene. The accumulated metabolites were subjected to further mineralization by indigenous soil and sediment microflora. The elimination of the highly mutagenic potential of benzo[*a*]pyrene was also monitored. This thesis is concluded in ***Chapter 6***, where the results of this study are discussed in relation to the use of white rot fungi for the bioremediation of PAH-polluted soils.

REFERENCES

Ander, P. 1994. The cellobiose-oxidizing enzymes CBQ and CBO as related to lignin and cellulose degradation - a review. Microbiol. Rev. **13:**297-312.

Ander, P., and L. Marzullo. 1997. Sugar oxidoreductases and veratryl alcohol oxidase as related to lignin degradation. J. Biotech. **53:**115-131

Andersson, B. E., and T. Henrysson. 1996. Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white-rot fungi. Appl. Microbiol. Technol. **46:**647-652.

Aust, S. D. 1990. Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microbiol. Ecol. **20:**197-209.

Barclay, C. D., G. F. Farquhar, and R. L. Legge. 1995. Biodegradation and sorption of polyaromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **42:**958-963.

Barr, D. P., M. M. Shah, T. A. Grover, and S. D. Aust. 1992. Production of hydroxyl radical by lignin peroxidase from *Phanerochaete chrysospori-um*. Arch. Biochem. Biophys. **298:**480-485.

Bezalel, L., Y. Hadar, and C. E. Cerniglia. 1996a. Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. **62:**292-295.

Bezalel, L., Y. Hadar, P. P. Fu, J. P. Freeman, and C. E. Cerniglia. 1996b. Metabolism of phenanthrene by the white rot fungus *Pleurotus ostreatus*. Applied Environ. Microbiol. **62:**2547-2553.

Bezalel, L., Y. Hadar, and C. E. Cerniglia. 1997. Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. **63:**2495-2501.

Blanchette, R. A. 1984. Manganese in wood decay by white rot fungi. Phytopathology **74:**725-730.

Bogan, B. W., and R. T. Lamar. 1996. Polycyclic aromatic hydrocarbon degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. Appl. Environ. Microbiol. **62:**1597-1603.

Bohmer, S., K. Messner, and E. Srebotnik. 1998. Oxidation of phenanthrene by a fungal oxidase in the presence of 1-hydroxybenzotriazole and unsaturated lipids. Biochem. Biophys. Res. Comm. **244:**233-238.

Bonnarme, P., and T. W. Jeffries. 1990. Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white-rot fungi. Appl. Environ. Microbiol. **56:**210-217.

Bossert, I. D., and R. Bartha. 1986. Structure-biodegradability relationships of polycyclic aromatic hydrocarbons in soil. Bull. Environ. Contam. Toxicol. **37:**490-495.

Bourbonnais, R., and M. G. Paice. 1990. Oxidation of non-phenolic substrates. An expanded role for laccases in lignin biodegradation. FEBS Lett. **267:**99-102.

Brown, J. A., J. K. Glenn, and M. H. Gold. 1990. Manganese regulates expression of manganese peroxidase by *Phanerochaete chrysosporium*. J. Bact. **172:**3125-3130.

Buswell, J. A. 1991. Fungal degradation of lignin. In: Arora DK, Rai B, Mukerji KG, Kundsen G (eds) Handbook of Applied Mycology, Vol 1: Soil and Plants. Marcel Dekker, New York, pp 425-480.

Cavalieri, E., and E. Rogan. 1985. Role of radical cations in aromatic hydrocarbon carcinogenesis. Environ. Health Perspect. **64:**69-84.

Cerniglia, C. E. 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation **3:**351-268.

Collins, P. J. J., M. J. J. Kotterman, J. A. Field, and A. D. W. Dobson. 1996. Oxidation of anthracene and benzo[*a*]pyrene by laccase from *Trametes versicolor*. Appl. Environ. Microbiol. **62:**4563-4567.

Collins, P. J., J. A. Field, P. Teunissen, and A. D. W. Dobson. 1997. Stabilization of lignin peroxidases in white rot fungi by tryptophan. Appl. Environ. Microbiol. **63:**2543-2548.

Cremonesi, P., B. Hietbrink, E. G. Rogan, and E. L. Cavalieri. 1992. One-electron oxidation of dibenzo[*a*]pyrenes by manganic acetate. J. Org. Chem. **57:**3309-3312.

Crocker, F. H., W. F. Guerin, and S. A. Boyd. 1995. Bioavailability of naphthalene sorbed to cationic surfactant-modified smectite clay. Environ. Sci. Technol. **29:**2953-2958.

De Jong, E., J. A. Field, and J. A. M. de Bont. 1992a. Evidence for a new extracellular peroxidase: manganese inhibited peroxidase from the white-rot fungus *Bjerkandera* sp. strain BOS55. FEBS Lett. **299:**107-110.

De Jong, E., J. A. Field, J. A. F. M. Dings, J. B. P. A. Wijnberg, and J. A. M. de Bont. 1992b. *De novo* biosynthesis of chlorinated aromatics by the white rot fungus *Bjerkandera* sp. strain BOS55: formation of 3-chloro-anisaldehyde from glucose. FEBS Lett. **305:**220-224.

De Jong, E., A. E. Cazemier, J. A. Field, and J. A. M. de Bont. 1994a. Physiological role of chlorinated aryl alcohols biosynthesized *de novo* by the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. **60:**271-277.

De Jong, E., J. A. Field, and J. A. M. de Bont. 1994b. Aryl alcohols in the physiology of white-rot fungi. Microbiol. Rev. **13:**153-188.

REFERENCES

Dutton, M. V., C. S. Evans, P. T. Atkey and D. A. Wood. 1993. Oxalate production by basidiomycetes, including the white rot species Coriolus versicolor and *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **39**:5-10.

Efroymson, R. A., and M. Alexander. 1994. Biodegradation in soil of hydrophobic pollutants in nonaqueous-phase liquids (NAPLs). Environ. Toxicol. Chem. **13**:405-411.

Eggert, C., V. Temp, J. V. D. Dean, and K. E. L. Erikson. 1996. A fungal metabolite mediates degradation of nonphenolic lignin structures and synthetic lignin by laccase. FEBS Lett. **391**:144-148.

Field, J. A., E. de Jong, G. Feijoo-Costa, and J. A. M. de Bont. 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white-rot fungi. Appl. Environ. Microbiol. **58**:2219-2226.

Field, J. A., E. de Jong, G. Feijoo-Costa, and J. A. M. de Bont. 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol. **11**:44-49.

Field, J. A., R. H. Vledder, J. G. van Zelst, and W. H. Rulkens. 1996. The tolerance of lignin peroxidase and manganese dependent peroxidase to miscible solvents and the *in vitro* oxidation of anthracene in solvent: water mixtures. Enzyme Microbiol. Technol. **18**:300-308.

Glenn, J.K., L. Akileswaran, and M. H. Gold. 1986. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **251**:688-696.

Glenn, J.K., and M. H. Gold. 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **242**:329-341.

Gold, M. H., and M. Alic. 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. **57**:605-622.

Goodwin, D. C., S. D. Aust, and T. A. Grover. 1995. Evidence for veratryl alcohol as a redox mediator in lignin peroxidase-catalyzed oxidation. Biochemistry **34**:5060-5065.

Guillen, F., M. J. Martinez, C. Munoz, and A. T. Martinez. 1997. Quinone redox cycling in the ligninolytic fungus *Pleurotus eryngii* leading to extracellular production of superoxide anion radical. Arch. Biochem. Biophys. **339**:190-199.

Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter. 1986. Oxidation of benzo(a)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*: veratryl alcohol, and stability of ligninase. J. Biol. Chem. **261**:6900-6903.

Hammel, K. E., B. Kalyanaraman, and T. K. Kirk. 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium*. J. Biol. Chem. **261**:16948-16952.

Hammel, K. E., B. Green, and W. Z. Gai. 1991. Ring fission of anthracene by a eukaryote. Proc. Natl. Acad. Sci. USA. **88**:10605-10608.

Hammel, K. E. 1992. Oxidation of aromatic pollutants by lignin-degrading fungi and their extracellular peroxidases. H. Sigel and A. Sigel (eds.), Metal Ions in Biological Systems, Volume 28, Degradation of Environmental Pollutants by Microorganisms and Their Metalloenzymes. Marcel Dekker, Inc., NY, pp. 41-60.

Heitkamp, M. A., and C. E. Cerniglia. 1987. Effects of chemical structure and exposure on the microbial degradation of polycyclic aromatic hydrocarbons in freshwater and estuarine ecosystems. Environ. Toxicol. Chem. **6**:535-546.

Heitkamp, M. A., W. Franklin, and C. E. Cerniglia. 1988. Microbial metabolism of polycyclic aromatic hydrocarbon: isolation and characterization of a pyrene-degrading bacterium. Appl. Environ. Microbiol. **54**:2549-2555.

IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. 1983. **Vol.** 32. Polynuclear aromatic compounds, Part 1, Chemical, Environmental and Experimental data.

Johannes, C., A. Majcherczyk, and A. Hutterman. 1996. Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. Appl. Microbiol. Biotech. **46**:313-317.

Joshi, D. K., and M. H. Gold. 1996. Oxidation of dimethoxylated aromatic compounds by lignin peroxidase from *Phanerochaete chrysosporium*. Eur. J. Biochem. **237**:45-57.

Juhasz, A. L., M. L. Britz, and G. A. Stanley. 1997. Degradation of fluoranthene, pyrene, benzo[*a*]-anthracene and dibenzo[*a,h*]anthracene by *Burkholderia cepacia*. J. Appl. Microbiol. **83**:189-198.

Kaal, E. E. J., E. de Jong, and J. A. Field. 1993. Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. 59:4031-4036.

Kanaly, R., R. Bartha, S. Fogel, and M. Findlay. 1997. Biodegradation of [^{14}C]benzo[*a*]pyrene added in crude oil to uncontaminated soil. Appl. Environ. Microbiol. **63**:4511-4515.

Kelley, R. L. and A. Reddy. 1986. Identification of glucose oxidase activity as the primary source of hydrogen peroxide production in ligninolytic cultures of *Phanerochaete chrysosporium*. Arch. Microbiol. **144**:248-25.

REFERENCES

Kersten, P. J. 1990. Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. Proc. Natl. Acad. Sci. USA. **87:**2936-2940.

Khindaria, A., T. A. Grover, and S. D. Aust. 1994. Oxalate-dependent reductive activity of manganese peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **314:**301-306.

Khindaria, A., D. P. Barr, and S. D. Aust. 1995. Lignin peroxidase can also oxidize manganese. Biochem. **34:**7773-7779.

Kirk, T. K., W. J. Connors, and J. G. Zeikus. 1976. Requirement for a growth substrate during lignin decomposition by two wood-rotting fungi. Applied Environ. Microbiol. **32:**192-194.

Kirk, T. K., and R. L. Farrell. 1987. Enzymatic "combustion": The microbial degradation of lignin. Ann. Rev. Microbiol. 41:465-505.

Koduri, R. S., and M. Tien. 1994. Kinetic analysis of lignin peroxidase. Explanation for mediation phenomenon by veratryl alcohol. Biochemistry **33:**4225-4230.

Koduri, R. S., and M. Tien. 1995. Oxidation of guaiacol by lignin peroxidase. J. Biol. Chem. **270:**22254-22258.

Lackner, R., E. Srebotnik, and K. Messner. 1991. Oxidative degradation of high molecular weight chlorolignin by manganese peroxidase of *Phane-rochaete chrysosporium*. Biochem. Biophys. Res. Comm. **178:**1092-1098.

Leidraad bodembescherming. 1995. Streef- en interventiewaarden voor microverontreinigingen. Afl. 10. Sdu Uitgeverij Koninginnegracht.

Li, D., M. Alic, and M. Gold. 1994. Nitrogen regulation of lignin peroxidase gene transcription. Appl. Environ. Microbiol. **60:**3447-3449.

Liu, Z., D. A. Edwards, and R. G. Luthy. 1992. Sorption of non-ionic surfactants onto soil. Wat. Res. **26:**1337-1345.

Luthy, R. G., G. R. Aiken, M. L. Brusseau, S. D. Cunningham, P. M. Gschwend, J. J. Pignatello, M. Reinhard, S. J. Traina, W. J. Weber, and J. C. Westall. 1997. Sequestration of hydrophobic organic contaminants by geosorbents. Environ. Science Technol. **31:**3341-3347.

Luthy, R. G., A. Ramaswami, S. Ghoshal, and W. Merkel. 1993. Interfacial films in coal-tar nonaqueous-phase liquid-water systems. Environ. Sci. Technol. **27:**2914-2918.

Marzullo, L., R. Cannio, P. Giardina, M. T. Santini, and G. Sannia. 1995. Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and prevents polymerization of laccase-oxidized substrates. J. Biol. Chem. **270:**3823-2827.

Masaphy, S., D. Levanon, Y. Henis, K. Venkateswarlu, and S. L. Kelly. 1996. Evidence for cytochrome P-450 and P-450-mediated benzo[*a*]pyrene hydroxylation in the white rot fungus *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **135:**51-55.

Mihelcic, J. R., R. R. Lueking, R. J. Mitzell, and J. M Stapleton. 1993. Bioavailability of sorbed- and separate-phase chemicals. Biodegradation. **4:**141-153.

Mester, T., E. de Jong, and J. A. Field. 1995. Manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidase. Appl. Environ. Microbiol. **61:**1881-1887.

Mester, T., M. Pena, and J. A. Field. 1996. Nutrient regulation of extracellular peroxidases in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Microbiol. Biotechnol. **44:**778-784.

Moen, M. A., and K. E. Hammel. 1994. Lipid peroxidation by the manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. Appl. Environ. Microbiol. **60:**1956-1961.

Muheim, A., M. S. A. Leisola, and H. E. Schoemaker. 1990. Aryl-alcohol oxidase and lignin peroxidase from the white-rot fungus *Bjerkandera adusta*. J. Biotechnol. **13:**159-167.

Orth, A. B., M. Denny, and M. Tien. 1991. Overproduction of lignin-degrading enzymes by an isolate of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **57:**2591-2596.

Perez, J., and T. W. Jeffries. 1992. Role of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by Phanerochaete chrysoporium. Appl. Environ. Microbiol. **58:**2402-2409.

Perez, J., and T. W. Jeffries. 1993. Role of organic acid chelators in manganese regulation of lignin degradation by *Phanerochaete chrysosporium*. Appl. Biochem. Biotech. **39/40:**227-238.

Pignatello, J. J., and B. Xing. 1996. Mechanisms of slow sorption of organic chemicals to natural particles. Environ. Science Technol. **30:**1-11.

Ramaswami, A., and R. G. Luthy. 1997. Mass transfer and bioavailability of PAH compounds in coal-tar NAPL-slurry systems. 1. Model development. Environ. Sci. Technol. **31:**2260-2267.

Rouse, J.D., D. A. Sabatini, J. M. Suflita, and J. H. Harwell. 1994. Influence of surfactants on microbial degradation of organic compounds. Critical Rev. Environ. Sci. Technol. **24:**325-370.

REFERENCES

Ruttiman, C., E. Schwember, L. Salas, D. Cullen, and R. Vicuna. 1992. Ligninolytic enzymes of the white rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. Biotechnol. Appl. Biochem. **16:**64-76.

Sack, U., M. Hofrichter, and W. Fritsche. 1997. Degradation of polycyclic aromatic hydrocarbons by manganese peroxidase of *Nematoloma frowardii*. FEMS Microbiol. Lett. **152:**227-234.

Sanglard, D., S. A. Leisola, and A. Fiechter. 1986. Role of extracellular ligninases in biodegradation of benzo[*a*]pyrene by *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **8:**209-212.

Schick-Zapanta, L., and M. Tien. 1997. The roles of veratryl alcohol and oxalate in fungal lignin degradation. J. Biotechnol. **53:**93-102.

Shimada, M., D. B. Ma, Y. Akamatsu, and T. Hattori. 1994. A proposed role of oxalic acid in wood decay systems of wood-rotting basidiomycetes. FEMS Microbiol. Rev. **13:**285-296.

Sims, R. C., and M. R. Overcash. 1983. Fate of polynuclear aromatic compounds (PNAs) in soil-plant systems. Residue Reviews. **88:**1-68.

Stucki, G., and M. Alexander. 1987. Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl. Environ. Microbiol. **53:**292-297.

Sundaramorthy, M., K. Kishi, M. H. Gold, and T. L. Poulus. 1997. Crystal structures of substrate site binding mutants of magnanese peroxidase. J. Biol. Chem. **272:**17574-17580.

Sundaramorthy, M., K. Kishi, M. H. Gold, and T. L. Poulus. 1994. The cristall structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06 A resolution. J. Biol. Chem. **269:**32759-32767.

Sutherland, J. B., A. L. Selby, J. P. Freeman, F. E Evans, and C. E. Cerniglia. 1991. Metabolism of phenanthrene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **57:**3310-3316.

Sutherland, J. B. 1992. Detoxification of polycyclic aromatic hydrocarbons by fungi. J. Industrial Microbiol. **9:**53-62.

Sutherland, J. B., P. P. Fu, S. H. Yang, L. S. Von Tungeln, R. P. Casillas, S. A. Crow, and C. E. Cerniglia. 1993. Enantiomeric composition of the trans-dihydrodiols produced from phenanthrene by fungi. Appl. Environ. Microbiol. **59:**2145-2149.

Teunissen, P. J. M., and J. A. Field. 1998. 2-chloro-1,4-dimethoxybenzene as a novel catalytic cofactor for oxidation of anisyl alcohol by lignin peroxidase. Appl. Environ. Microbiol. **64:**830-835.

Thakker, D. R., H. Yagi, W. Levin, A. W. Wood, A. H. Conney, and D. M. Jerina. 1985. Polyclyclic aromatic hydrocarbons: metabolic activation to ultimate carcinogens. In: Bioactivation of foreign compounds (Anders, M.W., ed.), pp. 177-242, Academic Press, Orlando.

Tiehm, A. 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. **60:**258-263.

Tien, M. 1987. Properties of ligninase from *Phanerochaete chrysosporium* and possible applications. Crit. Rev. Microbiol. **15:**141-168.

Tien, M., and S. B. Myer. 1990. Selection and characterization of mutants of *Phanerochaete chrysosporium* exhibiting ligninolytic activity under nutrient-rich conditions. Appl. Environ. Microbiol. **56:**2540-2544.

Urzua, U., P. J. Kersten, and R. Vicuna. 1998. Manganese peroxidase-dependent oxidation of glyoxylic and oxalic acids synthesized by *Ceripo-riopsus subvermispora* produces extracellular hydrogen peroxide. Appl. Environ. Microbiol. **64:**68-73.

Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak. 1994. Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. Appl. Environ. Microbiol. **60:**459-466.

Volkering, F., A. M. Breure, A. Sterkenburg, and J. G. van Andel. 1992. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Appl. Microbiol. Biotechnol. **36:**548-552.

Volkering, F., A. M. Breure, G. van Andel, and W. H. Rulkens. 1995. Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. **61:**1699-1705.

Wariishi. H., and M. H. Gold. 1989. Lignin peroxidase compound III: formation, inactivation and conversion to the native enzyme. FEBS Lett. **243:**165-168.

Wariishi, H., K. Valli, and M. H. Gold. 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium* - kinetic mechanism and role of chelators. J. Biol. Chem. **267:**23688-23695.

Weissenfels, W. D., M. Beyer, J. Klein, and H. J. Rehm. 1991. Microbial metabolism of fluoranthene: isolation and identification of ring fission products. Appl. Microbiol. Biotechnol. **34:**528-535.

Wilson, S. C., and K. C. Jones. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. Environ. Pollut. **81:**689-696.

Yeom, I. T., M. M. Ghosh, C. D. Cox, and K. H. Ahn. 1996. Dissolution of polycyclic aromatic hydrocarbons from weathered contaminated soil. Wat. Sci. Technol. **34:**335-342.

Chapter 2

The physiology of anthracene biogradation by the white rot fungus *Bjerkandera* sp. strain BOS55

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ABSTRACT

A recently isolated white rot strain, *Bjerkandera* sp. strain BOS55, displays high extracellular peroxidase activity, and rapidly degrades polycyclic aromatic hydrocarbons (PAHs). In this study, the culture conditions for the biodegradation of the model PAH compound, anthracene, were optimized with respect to O_2, N, and C. An additional objective was to determine if the decolorization of the polymeric ligninolytic indicator dye, Poly R-478, could be correlated to anthracene biodegradation observed under a wide range of culture conditions. The supply of O_2 was found to be the most important parameter in the biodegradation of anthracene. Increasing culture aeration enhanced the biodegradation of anthracene and the accumulation of its peroxidase-mediated oxidation product anthraquinone. Decolorization of Poly R-478 was less affected by inadequate aeration. Provided that ample aeration was supplied, the degradation of anthracene under different culture conditions was strongly correlated with the ligninolytic activity as indicated by the rate of Poly R-478 decolorization. Concentrations up to 22 mM NH_4^+-N did not repress anthracene biodegradation and only caused a 0%-40% repression of the Poly R-478 decolorizing activity in various experiments. A cosubstrate requirement of 100 mg glucose per mg of anthracene biodegraded was observed in this study.

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INTRODUCTION

White rot fungi are the most extensive degraders of the natural recalcitrant polymer, lignin (Kirk and Farrell, 1987). This capacity is due to the extracellular peroxidase enzymes that initiate the attack of lignin. The ligninolytic system consists of lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP), H_2O_2 generating oxidases, substrates for oxidases, and the secondary metabolite, veratryl alcohol (Kirk and Farrell, 1987; Buswell, 1991; Hammel, 1992). Due to the non-specific character of the radical-mediated reactions of ligninolytic enzymes, a wide variety of xenobiotic compounds, having an aromatic structure like lignin, are also susceptible for attack by these extracellular enzymes (Aust, 1990; Bumpus *et al.*, 1989; Hammel, 1992; Field *et al.*, 1993). LiP has been shown to oxidize polycyclic aromatic hydrocarbons (PAHs) *in vitro* (Haemmerli *et al.*, 1986; Hammel *et al.*, 1986). MnP has been shown to oxidize MnII to MnIII (Glenn *et al.*, 1986), which is a powerful oxidator capable of oxidizing PAHs (Cavalieri *et al.*, 1985; Cremonesi *et al.*, 1992).

The ligninolytic peroxidases of the best-studied white rot fungus, *Phanerochaete chrysosporium*, are induced by the onset of secondary metabolism resulting from N, C or S depletion (Kirk and Farrell, 1987; Bumpus, 1989; Aust, 1990). Commonly, N-limited media are used to promote ligninolysis and ligninolytic enzyme production when culturing *Phanerochaete chrysosporium* and other white rot fungi (Kirk *et al.*, 1978; Buswell, 1991). Likewise, aromatic xenobiotic compounds are also mineralized to a much greater extent in N-limited cultures of *Phanerochaete chrysosporium* compared to N-sufficient cultures (Aust, 1990; Hammel *et al.*, 1991; Hammel, 1992). Among the other culture parameters that are known to influence ligninolysis, aeration is considered to be important. High O_2 levels enhance both the production of ligninolytic enzymes as well as ligninolytic activity (Kirk, 1978; Buswell, 1991; Reid and Seifert, 1982). Neither lignin nor xenobiotic aromatics can serve as sole carbon and energy source; therefore, the degradation of lignin and xenobiotic aromatics depends on readily assimilable cosubstrates such as carbohydrates (Kirk, 1976; Fernando *et al.*, 1989; Morgan *et al.*, 1993).

The ability of various white rot fungal strains to degrade PAHs was found to be highly correlated to the decolorization of the polyaromatic dye Poly R-478 (Field *et al.*, 1992). From a screening with this dye, a new isolate, *Bjerkandera* sp. strain BOS55, was found to be the best PAH degrader. *Bjerkandera* sp. are known to produce MnP (De Jong *et al.*, 1992a; Kaal *et al.*, 1993) and LiP (Kimura *et al.*, 1990; Muheim *et al.*, 1990; Kaal *et al.*, 1993), and to mineralize lignin (Waldner *et al.*, 1988). *Bjerkandera* sp. strain BOS55 has also been shown to produce a novel peroxidase, manganese-independent peroxidase (MIP) (De Jong *et al.*, 1992b).

The objective of this study was to optimize the culture conditions of *Bjerkandera* sp. strain BOS55 for the degradation of PAHs. The three ring PAH anthracene was chosen as the model compound because its oxidation product, anthraquinone, accumulates in the culture fluid (Field *et al.*, 1992). Anthraquinone is a well-known product of anthracene oxidation by purified lignin peroxidase (Haemmerli, 1988; Hammel *et al.*, 1991) and the extracellular fluids of *Bjerkandera* sp. strain BOS55 (Field *et al.*, 1992). Thus anthraquinone accumulation is indicative of the extracellular peroxidative degradation of anthracene.

MATERIALS AND METHODS

Microorganism and inoculum

The organism used, *Bjerkandera* sp. strain BOS55 (CIMW 1.91), was obtained from the Division of Industrial Microbiology culture collection. This strain was maintained on malt extract agar at 4°C, inocula for the experiments were prepared as described by Kaal *et al.* (1993). However, the inoculum used for the C and N limitation experiments was cultivated in a 5 liter erlenmeyer with 350 ml N-limited glucose-BIII medium. After 10 days of incubation at 30°C the mycelium was separated from the medium and washed three times with basal medium without glucose or NH_4^+-N, respectively. The mycelium was milled with a blender (Ystrul, Dottingen, Germany) in 100 ml of BIII medium, 200 µl of this mycelial suspension (800 mg dry weight mycelium liter^{-1}) was added to each experimental culture (5 ml).

Culture conditions

Two types of media were used: BIII medium (Tien and Kirk, 1988), and hemp stem wood (HSW) medium. The standard BIII liquid medium was N-limited with 2.2 mM NH_4^+-N as diammonium-tartrate, and 10 g liter^{-1} glucose or cellulose as primary substrate in a 20 mM pH 4.5 2,2-dimethylsuccinate buffer. After autoclaving, a filter-sterilized thiamine solution (200 mg liter^{-1}) was added at the rate of 10 ml liter^{-1} medium. The standard HSW medium consisted of 10 mM pH 4.5 2,2-dimethylsuccinate buffer, with 2 g liter^{-1} of milled (particle size 100-1000 µm) HSW added.

Aliquots of 5 ml of these media were incubated statically at 30°C in serum bottles of 30-250 ml as described by Kaal *et al.* (1993). In one experiment, 30-ml bottles were shaken with 70 strokes of 2.5 cm min^{-1}.

Poly R-478 decolorization

The rate of Poly R-478 decolorization, at an initial concentration 0.8 g liter^{-1}, was measured as described by Gold *et al.* (1988), except that in most experiments Poly R-478 was added prior to inoculation. Samples of 50 µl were drawn aseptically, diluted in 1 ml demineralized water and centrifuged before analysis. Absorbance readings were performed according to Field *et al.* (1992). The results are shown as the maximum decolorization rate, $1000^*[\Delta(A_{520nm}/A_{350nm})]^*h^{-1}$, generally occurring between days 2 and 6. In one experiment, Poly R-478 was added to cultures at distinct culture ages and analyzed after 18-21 h of incubation.

Carbon dioxide analysis

Carbon dioxide evolution was measured in the headspace by gas chromatography as described previously (Field *et al.*, 1992). The results are shown as the maximum carbon dioxide evolution rate, generally occurring between days 2 and 6.

Enzyme assays

The MnP and MIP activities were measured by the oxidation of 2,6-dimethoxyphenol (DMP) (Kaal *et al.*, 1993). The molar extinction coefficient used for the dimeric DMP oxidation product was 49,600 M^{-1} cm^{-1} as determined by Wariishi *et al.* (1992). LiP activity was not measured since it was not detected in previous work using N-limited culture conditions (Field *et al.*, 1992; De Jong *et al.*, 1992a; Kaal *et al.*, 1993).

Biodegradation of anthracene

Just before inoculation, anthracene was added to the triplicate cultures in a 50 µl aliquot of acetone, providing a final concentration of 10 ml liter^{-1} acetone and either 10 or 50 mg liter^{-1} anthracene. After incubation the whole culture was utilized for extraction; Teflon liners were added to prevent adsorption of anthracene onto the septa, and 10 or 25 ml of acetonitrile were added to 30- and 250-ml serum bottles, respectively. The bottles were then placed in a Branson 5200 sonificator (Danbury, USA) for 15 min and afterwards shaken by 300 2-cm-long strokes min^{-1} for 1 h on a Janke & Kunkel shaking table (Staufen, Germany) in complete darkness. Analysis of anthracene elimination was carried out according to Field *et al.* (1992), using a HPLC for analysis.

Every experimental point described in this paper was run in parallel with 2 sets of triplicate abiotic controls in order to confirm that the elimination of anthracene and the formation of anthraquinone were truly biologically mediated. One set of abiotic controls consisted of incubating sterile media with anthracene, the other set of abiotic controls consisted of incubating anthracene with killed 12-day-old cultures of *Bjerkandera* sp. strain BOS55. The cultures were killed by addition of 2 g liter^{-1} of sodium azide or by autoclaving for 20 min at 120°C. After 12 days, losses of anthracene ranged from 0 to 12% in both the sterile media and the autoclaved killed-fungi controls. Formation of anthraquinone was always less than 1% of added anthracene in these abiotic controls.

In one experiment, anthracene was added at a given culture age and analyzed after 18-21 h of incubation in order to determine the rate of anthracene biodegradation and anthraquinone formation. The results shown are expressed as mg liter^{-1} day^{-1}. In the abiotic controls, which consisted of autoclaved cultures of the same age, the loss of anthracene and accumulation of anthraquinone after incubation was less than 2 and 0.5%, respectively.

Chemicals

Anthracene, 9,10-anthraquinone and 2,6-dimethoxyphenol were obtained from Janssen Chimica (Tilburg, The Netherlands), or Aldrich (Steinheim, Germany). Poly R-478 was obtained from Sigma (St. Louis, USA). Hemp stem wood (*Cannabis sativa* Fibrimon 56) was kindly supplied by the Agrotechnological Research Institute (Wageningen, The Netherlands).

RESULTS

Four liquid media were tested for their suitability to support the biodegradation of 10 mg liter^{-1} anthracene by *Bjerkandera* sp. strain BOS55 in 30-ml serum bottles (deep cultures). N-limited BIII-glucose medium, 2.2 mM NH_4^+-N, was compared with N-limited BIII-cellulose and N-sufficient BIII-glucose, containing 22 mM NH_4^+-N. These media, which contained 10 g liter^{-1} of cosubstrate, were compared with hemp stem wood (HSW) medium containing only 2 g liter^{-1} of natural powdered lignocellulose substrate. No significant differences in the rate of anthracene elimination, the anthraquinone formation and Poly R-478 decolorization was found among the media with 10 g liter^{-1} defined carbohydrate cosubstrates, including the high-N medium (*results not shown*). Only the HSW medium provided an enhanced rate of anthracene biodegradation.

Since the best results were obtained in HSW media, we were not sure if this was due to the type or concentration of cosubstrate. The effect of HSW concentration on the biodegradation of 10 mg anthracene liter^{-1} was tested in deep cultures (***Fig. 1***). Surprisingly, considerable degradation of anthracene occurred in cultures receiving no HSW cosubstrate. The cosubstrate present in the inoculum, provided by the malt extract agar plug, was apparently sufficient to support growth and anthracene cometabolism. The anthracene biodegradation increased up to 2 g HSW liter^{-1}. However, higher concentrations of cosubstrate caused a distinct inhibition in the anthracene biodegradation. In contrast, the average Poly R-478 decolorization rate increased up to 10 g HSW liter^{-1} and was not inhibited by 30 g HSW liter^{-1}. In parallel, the MnP activity, measured on day 5, increased from 5 to 115 nmol DMP oxidized ml^{-1} min^{-1} at HSW concentrations of 2 and 30 g $liter^{-1}$, respectively (*results not shown*). These results indicate that the enzyme production and activity towards the dye was not inhibited by high HSW levels, but rather the inhibition was specific to the anthracene oxidation process.

A possible explanation for the inhibition of anthracene biodegradation at high HSW concentrations could be due to poor aeration or low redox potentials resulting from oxygen uptake for fungal respiration. To test this hypothesis, biodegradation of 10 mg anthracene $liter^{-1}$ in 30 g HSW $liter^{-1}$ medium was monitored in deep and shallow cultures of 30-ml and 250-ml serum bottles, respectively. These cultures were incubated under air, and an 80% O_2 atmosphere (***Table 1***). The anthracene biodegradation as well as the molar yield of anthraquinone per unit of anthracene biodegraded (AQ/A) were remarkably improved in the deep cul-

Figure 1.

The effect of increasing hemp stem wood (HSW) concentration in deep cultures (1600 cm^2 of surface area $liter^{-1}$ culture fluid) on (**A**), the rate of Poly R-478 decolorization; (**B**), the elimination of 10 mg anthracene $liter^{-1}$ (□) and accumulation of anthraquinone (△) as a molar percentage of the initial anthracene concentration after 5 days.

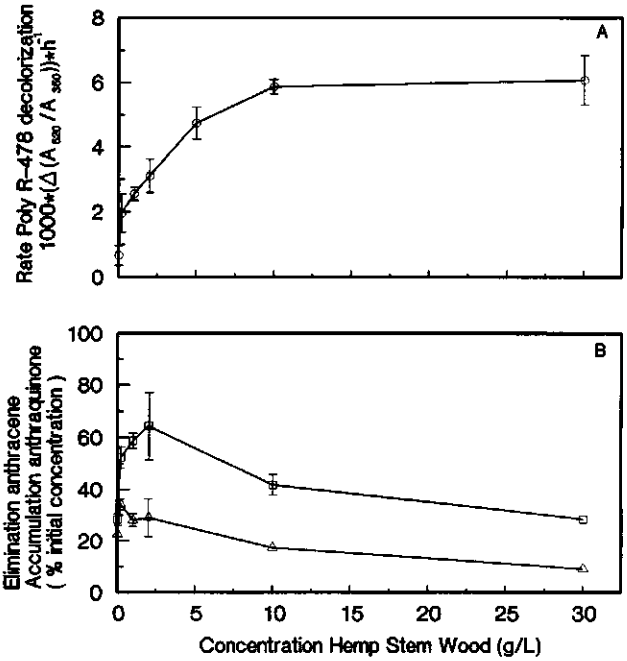
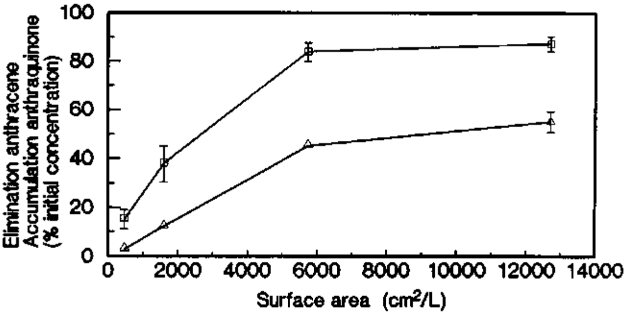


Figure 2.

The effect of aeration surface area in cultures with 30 g HSW $liter^{-1}$ cosubstrate on the elimination of 10 mg $liter^{-1}$ of anthracene (□) and anthraquinone accumulation (△) as a molar percentage of the initial anthracene concentration after 5 days.



tures receiving an 80% O_2 atmosphere. The improvement of these parameters was even more pronounced in shallow cultures, irrespective of the oxygen level. In contrast, the production of MnP and MIP, and the decolorization rate of Poly R-478 were not highly affected (*results not shown*).

The effect of aeration on the biodegradation of anthracene was studied further in the 30 g HSW liter^{-1} media in 15-, 30-, 250- and 1000-ml serum bottles. The results, plotted in terms of effective aeration surface area (***Fig.*** *2*), show that anthracene biodegradation increased up to 5780 cm^2 liter^{-1} (250-ml serum bottle). The AQ/A was again observed to increase with the better aeration. Cultures with 468, 1600, 5780 and 13000 cm^2 liter^{-1} aeration surface area had AQ/A ratios after 5 days of 0.20, 0.32, 0.54, and 0.63, respectively. In cultures of 5780 cm^2 liter^{-1}, 84 and 100% of the anthracene was eliminated after 5 and 12 days, respectively.

A similar experiment was repeated in defined N-limited BIII medium containing 10 g glucose liter^{-1}, and compared with the aeration achieved by shaking deep cultures. The anthracene biodegradation and anthraquinone accumulation obtained in the shaken deep culture were similar to values in the static shallow cultures (*results not shown*). The CO_2 production and the Poly R-478 decolorization were also found to be influenced by the aeration, albeit to a lesser extent. Dye decolorization in the deepest culture was inhibited by only 50%, whereas anthracene degradation was inhibited by 92% compared to shallow cultures.

Table 1. Effect of atmospheric oxygen concentration and aeration surface area on the biodegradation of anthracene (10 mg liter^{-1}) in static cultures after 5 days with 30 g liter^{-1} HSW cosubstrate.

Culture conditions		**Anthracene eliminated (%)**[a]	**Anthraquinone formed (%)**[a,b]	**AQ/A ratio**
Surface area (cm^2 liter^{-1})	Atmosphere (% O_2)			
1600[c]	20	35.4 ± 3.2	14.5 ± 1.2	0.41
1600	80	63.9 ± 3.0	32.8 ± 4.0	0.51
5780[d]	20	84.1 ± 1.8	48.2 ± 5.9	0.57
5780	80	80.5 ± 5.5	47.2 ± 2.1	0.59

[a]: Values are means with standaard deviation (n=3)

[b]: Molar percentage of anthracene added

[c]: Deep culture

[d]: Shallow culture

Figure 3.

The correlation between anthracene elimination on day 5 and the ratio of anthraquinone formed (mol) of anthracene eliminated (mol) (AQ/A) in cultures supplied with a starting anthracene concentration of 10 mg liter^{-1}.
The coefficient of determination (R2) was calculated according the product-moment method for the coefficient of linear correlation. The experimental points contained 1 g liter^{-1} cosubstrate or more so that the anthracene elimination was not limited due to inadequate levels of biocatalyst (*e.g.* biomass).

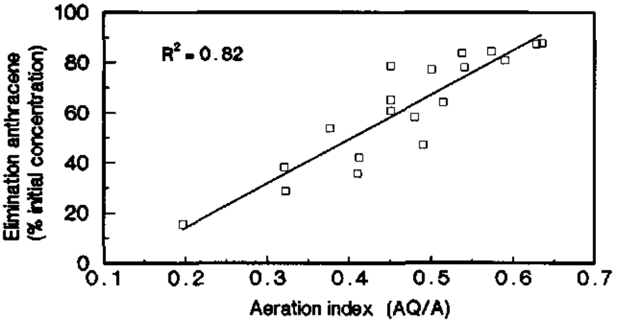
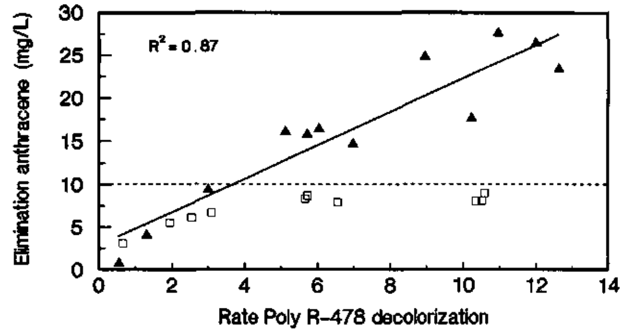


Figure 4.

The correlation between anthracene elimination after 5 days and the rate of Poly R-478 decolorization at two starting concentrations of anthracene;
(□), 10 mg liter^{-1} and (▲), 50 mg liter^{-1}.
The selected points had an AQ/A ratio higher than 0.45. The coefficient of determination (R^2) was calculated for the points corresponding to a starting anthracene concentration of 50 mg liter^{-1}.

The horizontal dashed line indicates the value of 10 mg liter^{-1} anthracene elimination.



Increasing the aeration of culture fluid stimulated both the degradation of anthracene and the molar yield of anthraquinone (AQ/A). ***Figure** 3* shows a strong relationship between the amount of anthracene degraded after 5 days and the AQ/A ratio. Provided that well aerated culture conditions were supplied, the degradation of anthracene showed a strong correlation with the decolorization rate of Poly R-478 (***Fig.** 4*).

An important parameter for the bioremediation of PAH polluted soils by white rot fungi is the amount of cosubstrate required per unit of pollutant. To determine this value, the biodegradation of 50 mg anthracene liter^{-1} was evaluated at different glucose concentrations in N-limited BIII medium placed in 250-ml serum bottles. As described in *Materials and Methods*, special care was taken to obtain a cosubstrate-free inoculum. After 12 days the cosubstrate was depleted in cultures containing up to 2 g glucose liter^{-1} as indicated by the cessation of CO_2 production. Up to this concentration, there was a linear increase in the amount of anthracene degraded. From this line, a cosubstrate requirement of approximately 100 mg glucose mg^{-1} anthracene degraded was calculated.

Anthracene degradation in cultures without glucose (***Fig.** 5*) was not significantly higher than in abiotic controls, confirming that anthracene cannot be degraded as a sole carbon and energy source. The maximum biodegradation of anthracene was obtained with 5 g glucose liter^{-1}; after 5 days of incubation, 27 mg liter^{-1} of anthracene was biodegraded. In parallel, the rate of CO_2 production and Poly R-478 decolorization also reached maximal

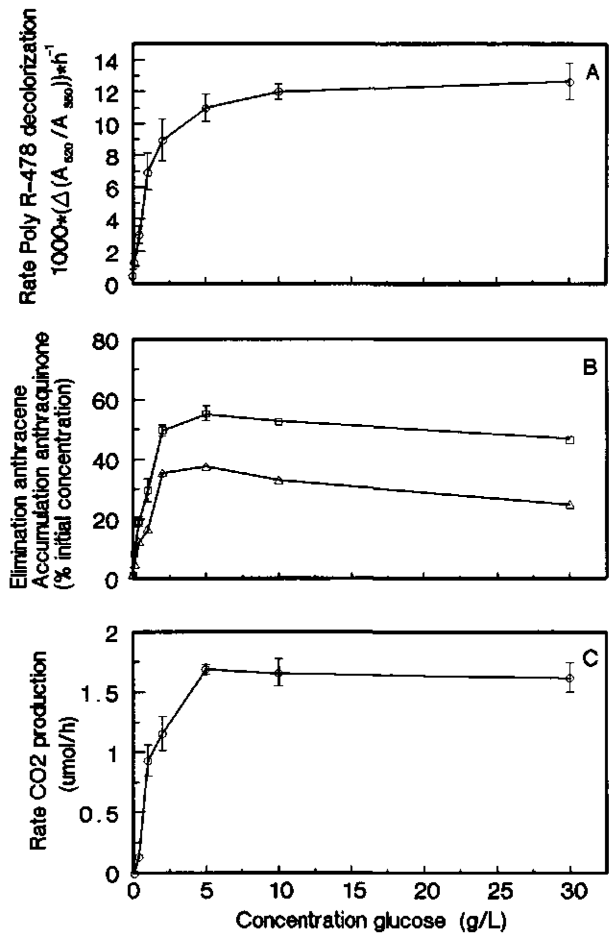


Figure 5.

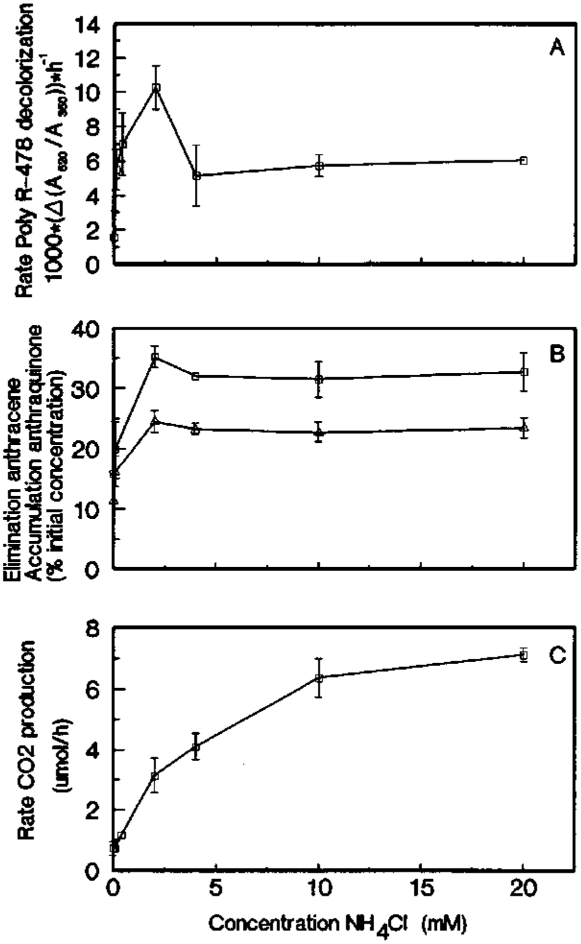
The effect of glucose cosubstrate concentration in shallow cultures (5780 cm^2 surface area $liter^{-1}$ culture fluid) on
(A), the rate of Poly R-478 decolorization,
(B) the elimination of 50 mg anthracene $liter^{-1}$ (□) and anthraquinone accumulation (△) as a molar percentage of the initial anthracene concentration after 5 days, and
(C) the maximum respiration rate as indicated by the maximum rate of CO_2 production.

values at 5 g glucose $liter^{-1}$. Higher glucose concentrations did not inhibit anthracene degradation nor increase the CO_2 production (O_2 uptake) rate. As in the previous experiments, no significant anthracene elimination nor anthraquinone accumulation occurred in the sterile and dead fungus controls, killed by autoclaving. However, dead fungus controls killed by sodium azide, showed a small but significant level of anthracene elimination and anthraquinone accumulation in cultures receiving high levels of cosubstrate. At 30 g glucose $liter^{-1}$, 17 ± 2.0% of the initial concentration of anthracene was degraded, and 7.9 ± 0.6% anthraquinone was accumulated after 12 days, indicating that a heat labile catalyst produced by the fungus was not completely inhibited by sodium azide.

Since the media experiment showed no repression of ligninolytic activity by 22 mM NH_4^+-N, supplied in the form of diammonium tartrate, the influence of the nitrogen concentration, from 0 to 20 mM NH_4Cl, was monitored in shallow cultures with 5 ml of BIII medium containing 10 g glucose $liter^{-1}$, and special NH_4^+-N-free inoculum was used. Anthracene biodegradation increased until 2 mM NH_4Cl and was not repressed by higher concentrations up to 20 mM NH_4Cl (***Fig.*** *6*). The Poly R-478 decolorization rate also increased until 2 mM

Figure 6.

The effect of NH_4^+-N concentration, supplied as NH_4Cl, in shallow cultures (5780 cm^2 of surface area $liter^{-1}$ culture fluid) on (**A**), the rate of Poly R-478 decolorization, (**B**) elimination of 50 mg anthracene $liter^{-1}$ (□) and anthraquinone accumulation (△) as a molar percentage of the initial anthracene concentration after 5 days, and (**C**) the maximum respiration rate as indicated by the maximum rate of CO_2 production.



NH_4Cl; however, partial repression (40%) occurred at higher concentrations although the high-N cultures were still expressing considerable dye decolorizing activity. The rate of CO_2 production increased up to 10 mM NH_4Cl, indicating that below that concentration the medium was truly N-limited. Low but significant levels of biodegradation of anthracene, decolorization of Poly R-478 and CO_2 production occurred in media not supplemented with any NH_4Cl.

The biodegradation of anthracene in the previous experiments was measured after 5 and 12 days. In order to determine the anthracene-degrading activity and the Poly R-478 decolorization rate as a function of culture age, anthracene and Poly R-478 were added to cultures of different ages and incubated for 19-21 h. The respiration rate and the production of MnP and MIP were also monitored at each culture age. The rate of anthracene biodegradation increased rapidly to 10.3 mg $liter^{-1}$ day^{-1} (97% of initial concentration) at day 5 (***Fig.*** *7*). The rate remained high during the rest of the experiment, on day 67, half of the maximal rate was still evident. No significant anthracene degradation occurred in the autoclaved fungi controls. The anthracene-degrading activity paralleled the amount of peroxidase enzymes

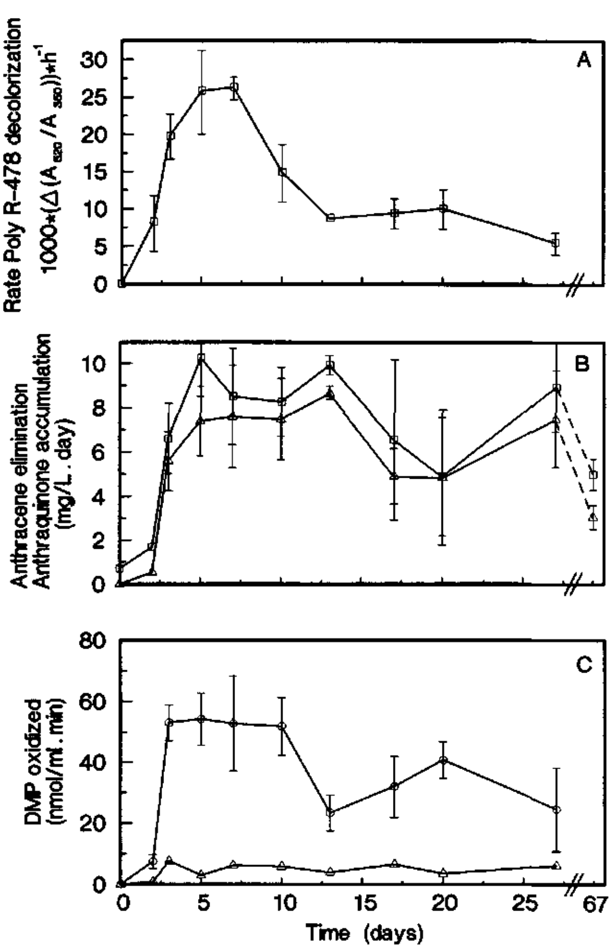


Figure 7.

(A) The Poly R-478 decolorization rate,
(B) anthracene biodegradation rate (□)
and anthraquinone accumulation rate (△),
both expressed as mg liter^{-1} day^{-1},
(C) activity of MnP (○) and
MIP (△) in 30 g liter^{-1} HSW medium in shallow cultures (5780 cm^2 of surface area liter^{-1} culture fluid).

Poly R-478 (0.8 g liter^{-1}) and anthracene (10 mg liter^{-1}) were added to separate triplicate cultures at the indicated times.

present in the culture fluid, and, initially, the Poly R-478 decolorization activity. Adsorption of the dye onto the increasing amount of fungal biomass disturbed the Poly R-478 decolorization measurements. The adsorbed dye was decolorized before the soluble dye became decolorized. Thus the apparent activity in solution measured in the first day after addition, when predominately the adsorbed dye was being decolorized, is lower than the actual activity. The CO_2 production confirmed growth during the experiment, and only a slight decrease in growth rate could be detected towards the end of the experiment (*results not shown*). In a similar experiment, a single dose of anthracene (10 mg liter^{-1}) was added on day zero. By day 5, anthracene was completely eliminated and up to 6 mg liter^{-1} of anthraquinone accumulated. In the following 30 days, only 25% of the anthraquinone was metabolized (*results not shown*).

DISCUSSION

Under the wide variety of culture conditions tested in this study, anthracene was consistently oxidized to anthraquinone in high molar yields (20 to 80%). Anthraquinone has been found earlier as an oxidation product in N-limited whole cultures of *Phanerochaete chrysosporium* and *Bjerkandera* sp. (Hammel *et al.*, 1991; Field *et al.*, 1992). The same oxidation product is found in *in vitro* experiments with purified LiP (Hammel *et al.*, 1986; Haemmerli, 1988; Hammel *et al.*, 1991). Quinones are also formed from the chemical reaction with the one-electron oxidant, MnIII, with various PAHs (Cavalieri *et al.*, 1985; Cremonesi *et al.*, 1992). These observations suggest that peroxidases including MnP are also involved *in vivo*. Anthraquinone was shown to be degraded by *Phanerochaete chrysosporium* to phthalic acid and CO_2 (Hammel *et al.*, 1991). However, as was reported previously (Field *et al.*, 1992), we have again observed that anthraquinone tends to behave as a dead-end metabolite in *Bjerkandera* sp. strain BOS55. Thus anthraquinone levels are a useful indicator for the peroxidative biodegradation of PAH by this fungus.

The most important factor which influenced the biodegradation of anthracene was oxygen. Increasing the effective aeration surface areas of static cultures or increasing the O_2 concentration in the headspace above the culture fluids resulted in higher rates of anthracene degradation. When the degradation of anthracene was optimized by increasing the effective aeration surface area in shallow cultures, no further increase of anthracene degradation could be achieved by supplying higher O_2 tensions in the headspace. So the O_2 transfer into the culture rather than the O_2 partial pressure was the limiting factor. In previous experiments, where the O_2 profiles in stationary cultures of *Phanerochaete chrysosporium* were measured, the importance of O_2 limitation was clearly indicated (Leisola *et al.*, 1983). Even 1 mm into the mycelium mat, O_2 was completely depleted. The O_2 limitation could be overcome by decreasing the culture depth or increasing the O_2 concentration.

In whole cultures of *Bjerkandera* sp. strain BOS55, the yield of anthraquinone per unit of anthracene degraded (AQ/A) increased with better aeration. The AQ/A ratio is also reported to increase with increasing O_2 concentrations during the *in vitro* oxidation of anthracene by LiP (Haemmerli, 1988). Therefore the AQ/A ratio can be viewed as an index for the level of aeration. Moreover, since anthraquinone is only slowly metabolized in our experiments, the ratio would only be susceptible to negligible changes from further metabolism by whole cells. The fact that a strong correlation exists between the AQ/A ratio and anthracene degradation indicates that aeration was an important factor determining the rate of anthracene biodegradation. Hammel *et al.* (1986) demonstrated that the oxygen incorporated into the PAH quinone by LiP mediated oxidation of PAH is provided by H_2O, and not by molecular oxygen. Thus high levels of aeration might be required for maintaining a high redox potential in the culture fluid rather than the supply of molecular oxygen itself.

Our results show that the Poly R-478 decolorization is less repressed by non-optimal aeration than the anthracene degradation. The utilization of Poly R-478 decolorization as an indicator of ligninolytic activity towards compounds as anthracene (Field *et al.*, 1992) is therefore doubtful under low-oxygen. However, provided that the aeration of the culture was adequate, a high correlation between the Poly R-478 decolorization and the degradation of anthracene was found, which is supported by the fact that both PAH and the dye are oxidized by the same ligninolytic enzymes. As discussed previously, anthracene is initially attacked by peroxidases. Ligninolytic peroxidases as well as MnIII have also been shown to be responsible for the decolorization of Poly R-478 (Kuwahara *et al.*, 1984; Glenn and Gold, 1985; Glenn *et al.*, 1986; Paszczynski and Crawford, 1991; De Jong *et al.*, 1992b). In our experiments, MnP and MIP were produced in parallel with the onset of Poly R-478 decolorization and anthracene biodegradation (***Fig.*** *7*).

Fungal growth and H_2O_2 production, which are required for the production and activity of peroxidases, respectively, are dependent on the availability of readily biodegradable cosubstrates (Kirk, 1976; Fernando *et al.*, 1989; Morgan *et al.*, 1993). In this study, we have shown that *Bjerkandera* sp. strain BOS55 was not able to degrade anthracene nor to decolorize Poly R-478 without glucose supplementation. Approximately 100 mg of glucose was needed per mg of anthracene degraded. High HSW cosubstrate concentrations in deep cultures inhibited anthracene biodegradation. The AQ/A ratios in these cultures were low and the inhibition could be overcome by providing better aeration. The inhibition could be due to the poor aeration resulting from the competition between O_2 needed to maintain a high redox potential for anthracene oxidation and O_2 consumed for fungal respiration. Although the maximum O_2 uptake in N-limited cultures had already been reached at 5 g liter^{-1} of glucose, N of ill-defined bioavailability provided by high HSW concentrations (60 mM HSW-N at 30 g liter^{-1} HSW) could have enhanced the respiration rate beyond that of the N-limited cultures. Also it is possible that reducing equivalents, accumulated by the fungus under O_2 limitation, interfere with the anthracene oxidation. The fact that Poly R-478 decolorization was not inhibited at high HSW concentrations indicates that O_2 needed for H_2O_2 producing oxidases was not rate limiting.

Generally, N or C limitation has been considered essential for triggering the onset of secondary metabolism, when the production of ligninolytic enzymes occurs (Kirk and Farrell, 1987; Aust, 1990; Buswell, 1991). However, ligninolytic enzyme activity, degradation of anthracene and decolorization of Poly R-478, were not severely repressed by high-N in *Bjerkandera* sp. strain BOS55. CO_2 production curves indicate that the metabolic activity was N-limited below 10 mM NH_4^+-N. Nonetheless, the cultures were expressing full anthracene degrading activity at the highest NH_4^+-N concentrations tested (20-22 mM). Recently it has been shown that organic N-nutrients up to 34 mM N stimulate MnP and MIP production; moreover, organic N induced LiP, which is usually not detectable in N-limited media nor in the high-NH_4^+-N media of *Bjerkandera* sp. (Kimura *et al.*, 1990; Kaal *et al.*, 1993). Since N-limitation is not required for ligninolytic activity in *Bjerkandera* sp. strain BOS55, future studies should attempt to increase the PAH degradation rate by organic N-supplements, which are known to stimulate peroxidase production.

REFERENCES

Aust, S. D. 1990. Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microb. Ecol. **20:**197-209.

Bumpus, J. A. 1989. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **55:**154-158.

Bumpus, J. A., T. Fernando, M. Jurek, G. J. Mileski, and S. D. Aust. 1989. Biological treatment of hazardous wastes by *Phanerochaete chrysosporium*. In: Lewandowski G, Armenante P, Baltzis B (eds) Biotechnology applications in hazardous waste treatment, Engineering Foundation, New York, pp 167-183.0

Buswell, J. A. 1991. *Fungal degradation of lignin. In: Arora DK, Rai B, Mukerji KG, Kundsen G (eds)* Handbook of Applied Mycology, Vol 1: Soil and Plants. Marcel Dekker, New York, pp 425-480.

Cavalieri, E., and E. Rogan. 1985. Role of radical cations in aromatic hydrocarbon carcinogenesis. Environ. Health Persp. **64:**69-84.

Cremonesi, P., B. Hietbrink, E. G. Rogan, and E. L. Cavalieri. 1992. One-electron oxidation of dibenzo[*a*]pyrenes by manganic acetate. J. Org. Chem. **57:**3309-3312.

Fernando, T., S. D. Aust, and J. A. Bumpus. 1989. Effects of culture parameters on DDT[1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] biodegradation by *Phanerochaete chrysosporium*. Chemosphere **19:**1387-1398.

Field, J. A., E. de Jong, G. Feijoo-Costa, and J. A. M. de Bont. 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl. Environ. Microbiol. **58:**2219-2226.

Field, J. A., E. de Jong, G. Feijoo-Costa, and J. A. M. de Bont. 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol. **11:**44-49.

Glenn, J. K , and M. H. Gold. 1985. Purification and characterization of an extracellular MnII-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **242:**329-341.

Glenn, J. K., L. Akileswaran, and M. H. Gold. 1986. MnII oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **251:**688-696.

Gold, M. H., J. K. Glenn, and M. Alic. 1988. Use of polymeric dyes in lignin biodegradation assays. Methods Enzymol. **161:**74-78.

Haemmerli, S. D. 1988. Lignin peroxidase and the ligninolytic system of *Phanerochaete chrysosporium*. PhD thesis, Swiss Federal Institute of Technology, Zurich, Switzerland.

Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter. 1986. Oxidation of benzo[*a*]pyrene by extracellular ligninase of *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**6900-6903.

Hammel, K. E. 1992. Oxidation of aromatic pollutants by lignin degrading fungi and their extracellular peroxidases. In: Siegel H, Siegel A (eds) Metal Ions in Biological Systems, vol 28. Degradation of Environmental Pollutants by Microorganisms and Their Metalloenzymes , Marcel Dekker, New York, pp 41-60.

Hammel, K. E., B. Kalyanaraman, and T. K. Kirk. 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]dioxins by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. **36:**16948-16952.

Hammel, K. E., B. Green, and W. Z. Gai. 1991. Ring fission of anthracene by a eukaryote. Proc. Natl. Acad. Sci. USA **88:**10605-10608.

De Jong, E., F. P. de Vries, J. A. Field, R. P. van der Zwan, and J. A. M. de Bont. 1992a. Isolation and screening of basidiomycetes with high peroxidative activity. Mycol. Research **96:**1098-1104.

De Jong, E., J. A. Field, and J. A. M. de Bont. 1992b. Manganese-inhibited peroxidase from the white rot fungus *Bjerkandera* sp. BOS55. FEBS Lett. **299:**107-110.

Kaal, E. E. J., E. de Jong, and J. A. Field. 1993. Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. **59:**4031-4036.

Kimura, Y., Y. Asada, and M. Kuwahara. 1990. Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl. Microbiol. Biotechnol. **32:**436-442.

Kirk, T. K., and R. L. Farrell. 1987. Enzymatic "combustion": The microbial degradation of lignin. Ann. Rev. Microbiol. **41:**465-505.

Kirk, T. K., E. Schultz, W. J. Conners, L. F. Lorenz, and J. G Zeikus. 1978. Influence of culture parameters on lignin degradation by *Phanerochaete chrysosporium*. Arch. Microbiol. **117:**277-285.

REFERENCES

Kuwahara, M., J. K. Glenn, M. A. Morgan, and M. H. Gold. 1984. Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett. **169:**247-250.

Leisola, M., D. Ulmer, and A. Fiechter. 1983. Problem of oxygen transfer during degradation of lignin by *Phanerochaete chrysosporium*. Eur. J. Appl. Microbiol. Biotechnol. **17:**113-116.

Morgan, P., S. A. Lee, S. T. Lewis, A. N. Sheppard, and R. J. Watkinson. 1993. Growth and biodegradation by white rot fungi inoculated into soil. Soil. Biol. Biochem. **25:**279-287.

Muheim, A., M. S. A. Leisola, and H. E. Schoemaker. 1990. Aryl-alcohol oxidase and lignin peroxidase from the white rot fungus *Bjerkandera adusta*. J. Biotechnol. **13:**159-168.

Paszczynski, A., and R. L. Crawford. 1991. Degradation of azo compounds by ligninase from *Phanerochaete chrysosporium*: involvement of veratryl alcohol. Biochem. Biophys. Res. Comm. **178:**1056-1063.

Reid, I. D., and K. A. Seifert. 1982. Effect of an atmosphere of oxygen on growth, respiration, and lignin degradation by white rot fungi. Can. J. Bot. **60:**252-260.

Tien, M., and T. K. Kirk. 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. **161:**238-248.

Waldner, R., M. S. A. Leisola, and A. Fiechter. 1988. Comparison of ligninolytic activities of selected white rot fungi. Appl. Microbiol. Biotechnol. **29:**400-407.

Wariishi, H., K. Valli, and M. H. Gold. 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium* - kinetic mechanism and role of chelators. J. Biol. Chem. **267:**23688-23695.

Chapter 3

Hydrogen peroxide production as a limiting factor in xenobiotic compound oxidation by nitrogen-sufficient cultures of *Bjerkandera* sp. strain BOS55 overproducing peroxidases

Michiel J.J. Kotterman, René A. Wasseveld, and Jim A. Field

ABSTRACT

The overproduction of ligninolytic peroxidases in the N-unregulated white rot fungus *Bjerkandera* sp. BOS55 under nitrogen-sufficient conditions had no noteworthy effect on improving the oxidation of anthracene or the decolorization of the polymeric aromatic dye, Poly R-478 in 6-day-old cultures. Only when the endogenous production of H_2O_2 was increased by addition of extra oxygen and glucose could a 2.5-fold increase in anthracene oxidation rate and a 6-fold increase in Poly R-478 decolorization rate be observed in high-N cultures with 10- to 35-fold higher peroxidase activities than N-limited cultures. Further increase of the H_2O_2 generation rate in high-N cultures with glucose oxidase led to an additional 3.5-fold increase in anthracene oxidation rate (350 mg liter^{-1} day^{-1}), and a 10-fold increase in Poly R-478 decolorization rate. These results indicate that xenobiotic compound oxidation by white rot fungi cannot be improved by overproducing peroxidases without increasing the endogenous production of H_2O_2. The absence of Mn, which decreased the manganese peroxidase titers and increased the lignin peroxidase titers, was associated with up to 95% improvements in the anthracene oxidation rate. The simultaneous presence of Mn and veratryl alcohol was observed to have a synergistic negative effect on the oxidation of anthracene and the decolorization of Poly R-478.

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INTRODUCTION

White rot fungi produce extracellular lignin peroxidase (LiP), and manganese dependent peroxidase (MnP), which are responsible for the oxidation of the complex aromatic polymer lignin in wood (Kirk and Farrell, 1987). These enzymes are also able to oxidize a variety of high-priority aromatic pollutants, such as polycyclic aromatic hydrocarbons (PAH) (Haemmerli *et al.*, 1986; Hammel *et al.*, 1986), chloroaromatics and polyaromatic dyes (Aust, 1990; Field *et al.*, 1993). Therefore white rot fungi are being considered for the bioremediation of contaminated soils and wastes (Aust, 1990; Field *et al.*, 1993).

The expression of the ligninolytic peroxidases in white rot fungi is regulated by the presence of nutrients such as nitrogen and manganese. The ligninolytic enzyme system of the best studied model organism, *Phanerochaete chrysosporium*, is triggered in response to N, C, or S limitation (Kirk and Farrell, 1987; Aust, 1990). Consequently, N-limited conditions are typically used to study the production of peroxidases (Kirk *et al.*, 1978) as well as the degradation of aromatic pollutants (Aust, 1990; Hammel *et al.*, 1991; Hammel, 1992; Field *et al.*, 1993). The use of nutrient-limited media, however, results in low biomass and low enzyme titers. Recently, N-deregulated mutants and strains of *Phanerochaete chrysosporium* as well as wild type *Bjerkandera adusta* strains have been reported (Kimura *et al.*, 1990; Orth *et al.*, 1991). Organic N nutrients stimulated LiP and MnP production by the strain *Bjerkandera* sp. BOS55 (Kaal *et al.*, 1993; Mester *et al.*, 1995). The presence of manganese is known to induce the production of MnP in many white rot fungi (Bonnarme and Jeffries, 1990; Ruttiman *et al.*, 1992). On the other hand, Mn lowers LiP titers (Bonnarme and Jeffries, 1990; Perez and Jeffries, 1992).

The white rot fungal strain used in this study, *Bjerkandera* sp. strain BOS55, was selected as an outstanding PAH-degrading white rot fungal isolate from a previous screening (Field *et al.*, 1992). It was also shown that anthracene oxidation by this isolate was not repressed by high NH_4^+-N (Kotterman *et al.*, 1994). In this study, large changes in its extracellular peroxidase titers and profiles were obtained by varying N and Mn concentrations, respectively. The objective was to determine whether increased peroxidase production or changes in the enzyme profiles would result in an enhanced oxidation of the model compound PAH, anthracene, and the ligninolytic indicator dye, Poly R-478.

MATERIALS AND METHODS

Microorganism and inoculum

The organism used, *Bjerkandera* sp. strain BOS55 (CIMW 1.91), was obtained from the culture collection of the Division of Industrial Microbiology (Wageningen, The Netherlands). This strain was maintained on peptone yeast agar (Kimura *et al.*, 1990) at 4°C. The experiments were inoculated with agarplugs as described previously (Kaal *et al.*, 1993).

Culture conditions

The standard medium used was modified from the work of Tien and Kirk (1988). This N-limited medium contained 2.2 mM NH_4^+-N as diammonium tartrate and 10 g liter^{-1} glucose and was buffered at pH 6 with a combined 20 mM dimethylsuccinate and 40 mM phosphate buffer. High-N media were obtained by adding 3 g liter^{-1} amino acid mixture (AA-N) (Mester *et al.*, 1995) or 5 g mycological peptone (PEP-N) liter^{-1} to this medium, both resulting in 33 mM N. Manganese-free media were obtained by omitting 33 µM Mn from these media. Media were autoclaved for 30 min at 115°C, except for the AA-N medium, which was filter sterilized. After sterilization, 10 ml of a filter-sterilized thiamine solution (200 mg liter^{-1}) liter^{-1} was added. Aliquots of 5 ml of these media were transferred to sterile 250-ml serum bottles, incubation took place for 6 days statically at 30°C.

Additions to 6 day-old cultures. An oxygen atmosphere was supplied by flushing the cultures for 5 min with pure oxygen. Glucose and veratryl alcohol were added as a 100 x concentrated solution, increasing the glucose concentration by 5 g liter^{-1} and veratryl alcohol concentration by 2 mM. In some experiments, 19 or 38 units of glucose oxidase liter^{-1} was added.

Culture fluid experiments. The culture fluid was first separated from the biomass and then filtered through a Schleicher & Schuell FP 030/3 filter with pore size of 0.4 µm. Aliquots of 1.5 ml were transferred to 30-ml serum bottles.

Oxidation of anthracene

Anthracene was added to 6-day-old cultures or culture fluids in a 50- or 15-µl aliquot of acetone, respectively, resulting in a final concentration of 10 ml acetone liter^{-1} and 50 to 75 mg anthracene liter^{-1}. After incubation, each culture was completely utilized for extraction, teflon liners were added to prevent adsorption of anthracene onto the septa, and 25 or 8 ml acetonitrile was added to the whole cultures or culture fluids, respectively. The bottles were then placed in a 5200 sonicator (Branson, Danbury, Conn) for 15 min and afterwards shaken by 300 2-cm-long strokes min^{-1} for 1 h on a shaking table (Janke & Kunkel, Staufen, Germany) in complete darkness. Analysis of anthracene elimination was carried out as described by Field *et al.* (1992) by using high-pressure liquid chromatography. Each experiment described in this paper was run in parallel with a triplicate abiotic control, which consisted of autoclaved 6-day-old cultures, in order to confirm the biotic oxidation of anthracene. After 24 h of incubation, less than 2% of the initial concentration of anthracene was lost in the abiotic controls. The initial concentration of anthracene, obtained by extraction of a triplicate abiotic control directly after addition of anthracene, was used for the calculation of anthracene elimination by fungal cultures.

Poly R-478 decolorization

The rate of Poly R-478 decolorization, at an initial concentration of 0.8 or 1.6 g liter^{-1}, was measured described by Gold *et al.* (1988) in 6-day-old cultures. Samples of 50 µl were drawn, diluted in 1 or 2 ml demineralized water and centrifuged before analysis. Readings of A_{350nm} and A_{520nm} were performed as described by Field *et al.* (1992). The results are shown as the maximum decolorization rate, $1000^*[\Delta(A_{520nm}/A_{350nm})]^*h^{-1}$, generally measured between 1 and 5 h after the addition of Poly R-478.

Enzyme assays

The Mn-dependent and Mn-independent peroxidase activities were measured by the oxidation of 2,6-dimethoxyphenol and corrected for the laccase activity in the absence of H_2O_2 (Kaal *et al.*, 1993). The molar absorption coefficient used for the dimeric dimethoxyphenol oxidation product was 49,600 M^{-1} cm^{-1} as determined by Wariishi *et al.* (1992). LiP was measured by the oxidation of veratryl alcohol and corrected for veratryl alcohol oxidase activity in the absence of H_2O_2 (Kaal *et al.*, 1993).

H_2O_2 measurements

H_2O_2 production rate was measured with the H_2O_2-dependent oxidation of phenol red. The 6-day-old cultures were supplied with an excess of phenol red (0.02%) and horse radish peroxidase (10 mg liter^{-1}) and incubated in a 30°C water bath. At time intervals, 200 µl samples were drawn and the reaction was stopped by the addition of 100 µl 4N NaOH. Phenol red oxidation was then measured by the increase in A_{610nm}. The increase in A_{610nm} by addition of known concentrations of H_2O_2 to the cultures was used to calculate the H_2O_2 production rate.

Analytical methods

Carbon dioxide evolution was measured in the headspace by gas chromatography as described previously (Field *et al.*, 1992). Glucose concentration in the culture fluid was determined with a glucose oxidase-peroxidase enzyme assay, Peridochrom glucose GOD-PAP from Boehringer (Mannheim, Germany). Mycelial dry weight was determined by filtering the cultures over tarred glass fiber filters (Schleicher & Schuell GF50), and washing twice with demineralized water. After drying overnight at 104°C the mycelial dry weight was determined.

Chemicals

Anthracene, anthraquinone, 2,6-dimethoxyphenol, and veratryl alcohol were obtained from Janssen Chimica (Tilburg, The Netherlands). Horseradish peroxidase (GradII), and glucose oxidase (GradI) were obtained from Boehringer (Mannheim, Germany). All other chemicals were commercially available and were used without further purification.

Statistical procedures

All experiments were carried out in triplicate. The values reported are means with standard deviation.

Table 1. The effect of N and Mn nutrients on the peroxidative activity in 6-day-old cultures.

Medium	Presence of Mn	Activity (nmol ml^{-1} min^{-1})[a] of		
		MnP	MIP	LIP
NH$_4^+$-N (2.2 mM N)	-	1.7 ± 1.0[a]	1.8 ± 0.6	14.3 ± 4.0
	+	77.0 ± 9.0	5.8 ± 1.3	7.8 ± 3.3
AA-N (33 mM N)	-	66.1 ± 17.3	60.9 ± 16.7	294.5 ± 43.0
	+	471.8 ± 60.6	29.9 ± 4.6	55.1 ± 11.1
PEP-N (33 mM N)	-	32.3 ± 7.3	65.0 ± 8.0	472.4 ± 104.7
	+	304.8 ± 51.1	56.6 ± 14.4	71.1 ± 28.5

[a]: Values are means with standard deviation (n=3)

RESULTS

The effects of high-N media and the absence of Mn on enzyme titers and other physiological parameters were investigated. The addition of organic N in the form of an amino acid mixture or peptone had large effects on enzyme titers. As shown in ***Table 1***, the peroxidase titers were dramatically increased on day 6. In high-N cultures, the peroxidase titers were 10- to 35-fold higher compared to N-limited cultures. Omitting Mn from the media had a large impact on the enzyme profile; LiP activity was increased while MnP activity was not induced.

Surprisingly, the higher peroxidase titers obtained by N sufficiency had no beneficial effect on anthracene oxidation. In cultures containing Mn, approximately 30% of the anthracene (50 mg liter^{-1}) was converted when incubated in 6-day-old cultures for 22 h, irrespective of the N nutrient regime. In the absence of Mn, however, the conversion of anthracene was increased to approximately 40% and the yield of anthraquinone was significantly increased. The Poly R-478 decolorization rate was only slightly stimulated by N sufficiency and was not affected by Mn (*results not shown*).

The biomass and cumulative CO_2 production were stimulated by approximately 5-fold by N sufficiency. The absence of Mn had no effect on biomass and only a small effect on the cumulative CO_2 production (*results not shown*). Consequently, it can be assumed that glucose and oxygen were more rapidly consumed in the high-N media. The residual glucose concentrations on day 6 in the PEP-N, AA-N, and basal N-limited media were 0.1, 2.5 and 6.0 g liter^{-1}, respectively. Therefore, the oxidation of anthracene and the decolorization of Poly R-478 may have been limited by the lack of sufficient O_2 and glucose in the high-N cultures.

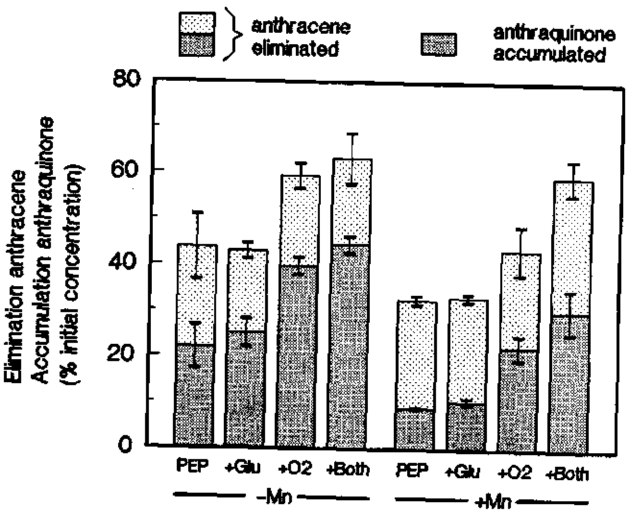

Figure 1.

The effect of glucose and oxygen additions to 6-day-old cultures of PEP-N media on the anthracene elimination (initial concentration, 50 mg liter^{-1}), and the molar yield of anthraquinone after 22 h of incubation.

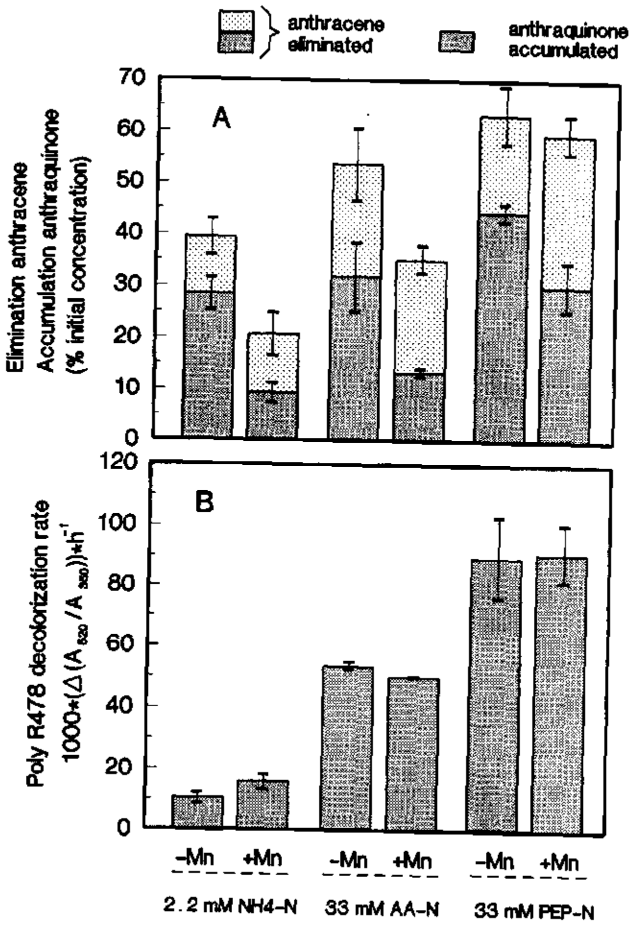

Figure 2.

The effect of N and Mn nutrients on the elimination of anthracene (initial concentration, 50 mg liter^{-1}), and the molar yield of anthraquinone after 22 h of incubation (**A**) and the rate of decolorization of Poly R-478 (0.8 g liter^{-1}) (**B**) when the cultures were supplied with glucose and oxygen.

As shown in ***Figure 1*** for the example of cultures grown on PEP-N medium, improvement of anthracene oxidation could be obtained by the addition of oxygen, and by the addition of both oxygen and glucose. The same effects were observed with the decolorization of Poly R-478 (*results not shown*). The addition of glucose and oxygen to the 6-day-old cultures did not remarkably change the enzyme profiles or enzyme titers on day 7, compared to control cultures not receiving glucose and oxygen (*results not shown*).

The effect of N and Mn nutrients on the anthracene oxidation and Poly R-478 decolorization rate after supplementation of media with extra oxygen and glucose on day 6 is shown in ***Figure 2***. Poly R-478 decolorization rates were up to 6-fold higher in the N-sufficient cultures. Anthracene oxidation was also significantly increased by N sufficiency, but the highest increase obtained was 2.5-fold. Supplementation of oxygen, glucose, or the combination of both did not improve the anthracene oxidation nor the Poly R-478 decolorization rate in the N-limited media. Mn deficiency was again seen to markedly stimulate anthracene oxidation and the yield of anthraquinone. The Poly R-478 decolorization rate, however, was not affected.

Mn was also shown to have an effect on the anthracene oxidation and the anthraquinone yield when added simultaneously on day 6 with anthracene to Mn-deficient 6-day-old cultures. Addition of 33 µM Mn to low-N Mn-deficient cultures decreased anthracene oxidation and anthraquinone accumulation by 8 and 34%, respectively, compared to control Mn-deficient cultures (*results not shown*). In a parallel experiment, the anthracene oxidation and anthraquinone accumulation in 6-day-old Mn-sufficient cultures, containing Mn from day 0, were 34 and 49% lower than in the Mn-deficient cultures, respectively.

Ligninolytic activity towards anthracene could potentially be limited due to the fact that one or more essential components of the ligninolytic system, such as the endogenous H_2O_2 production or the veratryl alcohol concentration, were not equally stimulated by N sufficiency. The H_2O_2 production rate was not greatly affected by the N and Mn nutrients. In all cultures, the rate was low and varied between 1.2 and 3.4 µmol liter^{-1} min^{-1}. As was reported previously (Mester *et al.*, 1995), the veratryl alcohol concentrations were consistently increased by the absence of Mn and were approximately 40 µM in the Mn-sufficient and 280 µM in the Mn-deficient cultures. The addition of glucose and oxygen on day 6 to high-N cultures was accompanied by an approximately 2-fold increase in the H_2O_2 production rate, up to 5.1 µmol liter^{-1} min^{-1} in PEP-N cultures.

In order to determine if H_2O_2 production was still limiting in high-N media when extra glucose and oxygen were supplied, glucose oxidase was added to 6-day-old whole cultures and extracellular culture fluids of the AA-N medium, as shown in ***Figure 3***. Glucose oxidase addition alone and in combination with veratryl alcohol enhanced the anthracene oxidation up to 30%. The same effects were observed in extracellular culture fluids itself, demonstrating that the extracellular peroxidases were largely responsible for the anthracene oxidation. In the experiment illustrated in ***Figure*** *3*, anthracene was incubated for 13 h in the whole culture. A similar experiment in which anthracene was incubated for 48 h was conducted. In this case, glucose oxidase and veratryl alcohol supplements improved the anthracene oxidation by 90% compared to non-supplemented controls (*results not shown*). The beneficial effect of glucose oxidase was even more drastic for the Poly R-478 decolorization rate, improvements of 4- to 10-fold were achieved in both whole cultures as well as in the culture fluids by adding glucose oxidase (*results not shown*). The addition of veratryl alcohol alone had no effect on anthracene oxidation nor the Poly R-478 decolorization rate in the Mn-deficient medium; however, it significantly decreased both the anthracene oxidation and Poly R-478 decolorization rate in the medium with Mn.

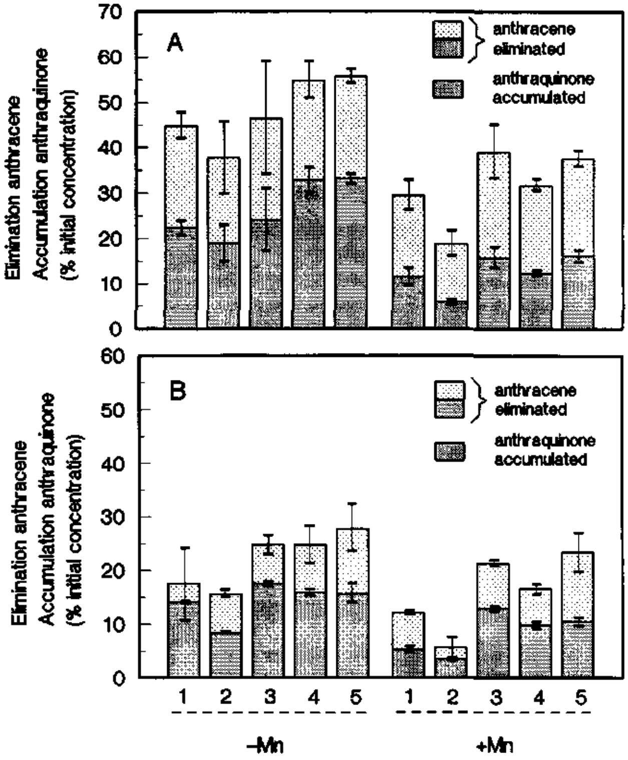

Figure 3.

The effect of veratryl alcohol and glucose oxidase additions to 6-day-old cultures of AA-N media supplied with glucose and oxygen on the anthracene elimination (initial concentration, 70 mg liter^{-1}) and the molar yield of anthraquinone in whole cultures (**A**) and extracellular culture fluids (**B**) after 13 h of incubation.

Additions to the medium are indicated as follows:

1: None (control)

2: + Veratryl alcohol (2 mM)

3: + Glucose oxidase (19 units liter^{-1})

4: + Veratryl alcohol (2 mM) and glucose oxidase (19 units liter^{-1})

5: + Veratryl alcohol (2 mM) and glucose oxidase (38 units liter^{-1})

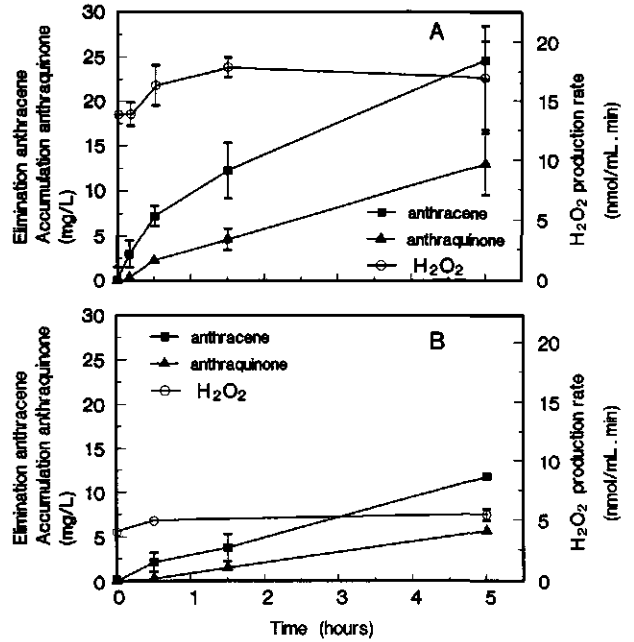

Figure 4.

The effect of the addition of veratryl alcohol and glucose oxidase to 6-day-old cultures of Mn-deficient AA-N medium supplied with glucose and oxygen on the anthracene oxidation rate (initial concentration, 75 mg liter^{-1}) and the anthraquinone accumulation rate.

(**A**) Cultures receiving veratryl alcohol and *glucose oxidase*

(**B**) Control cultures

Table 2 presents the optimum anthracene oxidation and Poly R-478 decolorization rates obtained in each of the 6 media with respect to the dosage of glucose oxidase and veratryl alcohol supplements. At least one of the combinations of glucose oxidase and veratryl alcohol improved the anthracene and Poly R-478 decolorization in all of the N-sufficient media as well as in the N-limited Mn-sufficient medium. When the compound oxidation caused by the N-sufficient cultures is compared with that of the N-limited cultures under conditions of optimal glucose oxidase concentration, it can be seen that N-sufficiency improved anthracene oxidation up to 60% and improved Poly R-478 decolorization rates up to 37-fold in the whole cultures. A similar comparison in 6-day-old extracellular fluids revealed that N sufficiency improved anthracene oxidation up to 2.1-fold and Poly R-478 decolorization up to 14-fold (*results not shown*).

The results clearly indicate that Poly R-478 decolorization responded to a much greater degree to the increased peroxidase titers in N-sufficient medium compared to anthracene oxidation. On the one hand, this difference can be attributed to the large differences in compound bioavailability. Poly R-478 is completely soluble in water, whereas anthracene is a highly nonpolar compound with a maximum aqueous solubility of 70 µg liter^{-1} (Walters and Luthy, 1984). Only 0.1% of the anthracene added was present in the soluble form suggesting that there may have been rate limitations due to the dissolution of insoluble anthracene precipitates. On the other hand, Poly R-478 decolorization was measured in the 1- to 5-h period following addition while anthracene oxidation was measured after 13 to 15 h of incubation. Therefore, we also monitored the effects of glucose oxidase addition on the initial

Table 2. The effect of veratryl alcohol and glucose oxidase additions on the anthracene elimination and the Poly R-478 decolorization rate in 6-day-old cultures.

Medium	Presence of Mn	Optimal comb[a]	Anthracene elimination (mg L^{-1})[b,c]	Comb	Poly R-478 decolorization rate[c,d]
NH_4^+-N (2.2 mM N)	-	1	23.9 ± 4.4	1	10.8 ± 1.7
	+	4	19.9 ± 1.7	4	110.5 ± 3.7
AA-N (33 mM N)	-	4	40.7 ± 3.0	5	403.2 ± 12.2
	+	3	29.0 ± 4.3	3	207.0 ± 1.1
PEP-N (33 mM N)	-	4	28.3 ± 5.1	4	252.8 ± 26.3
	+	3	31.7 ± 3.4	5	390.7 ± 6.0

[a]: Combination: 1, control (no addition); 3, +glucose-oxidase (19 units liter^{-1}); 4, +veratryl alcohol (2 mM) and glucose oxidase (19 units liter^{-1}); 5, +veratryl alcohol (2mM) and glucose oxidase (38 units liter^{-1})

[b]: Initial concentrations of anthracene were between 72.5 and 74.4 mg liter^{-1}. Incubation times of anthracene for NH_4^+-N, AA-N, and PEP-N were 13.5, 14.5, and 13.5 hours, respectively

[c]: Values are means with standard deviations (n=3)

[d]: Poly R-478 (1.6 g liter^{-1}) was sampled after 1 h. Values were calculated as $1000*[\Delta(A_{520nm}/A_{350nm})]*h^{-1}$

rate of anthracene oxidation. ***Figure 4*** clearly shows that a 3.5-fold increase in the anthracene oxidation rate could be achieved by glucose oxidase addition. Calculated over the first 30 minutes of incubation, the anthracene oxidation rate was 350 ± 52.8 mg liter^{-1} day^{-1}, corresponding to 85 mg of anthracene g biomass^{-1} day^{-1}. During this experiment, the decreases in peroxidase titers and veratryl alcohol concentration were less than 20%. The H_2O_2 production rate was approximately 3.5-fold higher in the glucose oxidase amended cultures compared to the control cultures (***Fig.*** *4*). Similar rates of anthracene oxidation, 354.1 ±49.6 and 100.1 ±42.2 mg liter^{-1} day^{-1}, were obtained in the extracellular culture fluids with addition of glucose oxidase and veratryl alcohol and the nonsupplemented control, respectively (*results not shown*).

DISCUSSION

Organic nitrogen enhances the peroxidase titers in the white rot fungus *Bjerkandera* sp. strain BOS55 (Kaal *et al.*, 1993; Mester *et al.*, 1995). Since both purified MnP and LiP proteins were shown to oxidize anthracene (Haemmerli, 1988; Hammel, 1992; Field *et al.*, 1995), and the polymeric dye Poly R-478 *in vitro* (Field *et al.*, 1992; Ollikka *et al.*, 1993), it has been assumed that if the peroxidase titers were increased this could be translated into a higher xenobiotic oxidation rate (Orth *et al.*, 1991; Kotterman *et al.*, 1994). However, the initial experiments reported here show that although peroxidase titers could be increased 10- to 35-fold with high-N media, no substantial benefit for the anthracene and Poly R-478 oxidation rates resulted. Either the free extracellular peroxidases are simply not involved, or another physiological factor aside from the peroxidase titer, is limiting in the N-sufficient media. The main oxidation product of anthracene found in our fungal cultures is anthraquinone. This result indicates the involvement of peroxidases as anthraquinone is the main product identified from the *in vitro* oxidation of anthracene by either LiP (Haemmerli, 1988; Hammel *et al.*, 1991; Field *et al.*, 1995) or MnP (Field *et al.*, 1995). Furthermore, since the rates of anthracene oxidation in the extracellular culture fluids were similar to those in the whole cultures, the role of intracellular oxygenases can be excluded. Consequently, the involvement of peroxidases in the oxidation of anthracene and Poly R-478 is implicated.

The complete ligninolytic system of white rot fungi is composed not only of peroxidases, but also of H_2O_2 producing oxidases and secondary metabolites. Several oxidases, such as glucose oxidase, aryl alcohol oxidase, glyoxal oxidase, and cellobiose dehydrogenase have been identified in white rot fungi (Kelley and Reddy, 1986; Kersten, 1990; Muheim *et al.*, 1990; Ander, 1994). Secondary metabolites such as veratryl alcohol also play an important role in the ligninolytic system (De Jong *et al.*, 1994). The results of this study clearly demonstrated that although high-N concentrations caused peroxidase overproduction, they did not stimulate endogenous H_2O_2 production and veratryl alcohol concentration. Supplying 6-day-old high-N cultures with extra oxygen or extra oxygen and glucose resulted in an increased endogenous H_2O_2 production, which enhanced the anthracene oxidation 2.5-fold and the Poly R-478 decolorization rate 6-fold compared to low-N media.

In order to test if H_2O_2 production was still rate limiting in glucose- and oxygen-supplemented cultures, these cultures were further supplemented with glucose oxidase to enhance the H_2O_2 production rate even more. Veratryl alcohol was also added together with glucose oxidase to protect LiP from being inactivated by the higher-than-physiological levels of H_2O_2

(Tonon and Odier, 1988; Valli *et al.*, 1990). The glucose oxidase addition increased the rate of H_2O_2 production by 3.5 -fold which corresponded to a 3.5 fold increase in the anthracene oxidation rate. Consequently, we must conclude that the peroxidative activity towards xenobiotic oxidation in the high-N media was limited by low endogenous H_2O_2 production. The H_2O_2 supply could be improved by nutritional factors, such as glucose and oxygen or by simply adding more oxidase enzymes into the culture. By taking these measures, cultures of *Bjerkandera* sp. strain BOS55 could degrade anthracene at extremely high rates of 350 mg liter^{-1} day^{-1}, corresponding to 85 mg g biomass^{-1} day^{-1}.

The significance of our findings is that strain improvements or physiological improvements of white rot fungi for the degradation of xenobiotic compounds should be sought in increasing the production of H_2O_2-generating oxidases. This contrasts with the general approach currently used, which is directed at improving peroxidase production (Orth *et al.*, 1991; Kaal *et al.*, 1993). No benefit from the overproduced peroxidases can be expected unless the endogenous H_2O_2 production is also improved.

In this study, Mn was found to influence the oxidation of anthracene. The impact of Mn may be due to its effect on the types of peroxidases expressed by white rot fungi (Bonnarme and Jeffries, 1990), including *Bjerkandera* sp. strain BOS55 (Mester *et al.*, 1995). Mn clearly induces the expression of MnP (Gold and Alic, 1993), whereas it has no effect on genetic transcription of LiP (Li *et al.*, 1994). Nonetheless, the titers of LiP are generally severely lowered by addition of Mn. In the case of *Bjerkandera* sp. strain BOS55, this was shown to be due to the fact that the presence of Mn represses veratryl alcohol biosynthesis (Mester *et al.*, 1995). In this study, it was consistently observed that omission of Mn from the medium increased the veratryl alcohol concentration and LiP titers, resulting in an improved anthracene oxidation and an increased anthraquinone yield. The Poly R-478 decolorization rate, however, was not affected by the presence or absence of Mn. These results might reflect the fact that LiP is a more potent enzyme than MnP towards oxidizing high ionization potential substrates such as anthracene. Previous studies have shown that LiP can oxidize substrates with higher ionization potentials than MnP (Popp and Kirk, 1991). However, the impact of Mn is not solely due to its effect on enzyme expression and veratryl alcohol biosynthesis. Simultaneous addition of Mn and anthracene to 6-day-old Mn-deficient cultures overproducing LiP, as well as simultaneous additions of veratryl alcohol and anthracene to 6-day-old Mn-sufficient cultures overproducing MnP resulted in decreased anthracene oxidation. A similar synergistic negative effect of the simultaneous presence of Mn and veratryl alcohol in the medium has been observed for the degradation of melanoidin pigments by *P. chrysosporium* (Guimaraes *et al.*, 1994). Manganese was shown to interfere with the LiP mediated oxidation of veratryl alcohol (Schmidt *et al.*, 1989; Lundell and Hatakka, 1994; Khindaria *et al.*, 1995). All these results suggest that Mn in combination with veratryl alcohol directly affects the reaction chemistry of peroxidases towards anthracene. Consequently, the negative impact of Mn on compound oxidation often observed in white rot fungi cultures (Muheim *et al.*, 1990; Vares *et al.*, 1994; Sayadi and Ellouz, 1995) may be due to the fact that veratryl alcohol is used as a standard supplement in the media.

REFERENCES

Ander, P. 1994. The cellobiose-oxidizing enzymes CBQ and CBO as related to lignin and cellulose degradation - a review. Microbiol. Rev. **13:**297-312.

Aust, S. D. 1990. Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microbiol. Ecol. 20:197-209.

Bonnarme, P., and T. W. Jeffries. 1990. MnII regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white rot fungi. Appl. Environ. Microbiol. **56:**210-217.

Field, J. A., E. de Jong, G. Feijoo-Costa, and J. A. M. de Bont. 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl. Environ. Microbiol. **58:**2219-2226.

Field, J. A., E. de Jong, G. Feijoo Costa, and J. A. M. de Bont. 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol. **11:**44-49.

Field, J. A., R. H. Vledder, J. G. van Zelst, and W. H. Rulkens. 1995. The tolerance of lignin peroxidase and manganese dependent peroxidase to miscible solvents and the *in vitro* oxidation of anthracene in solvent:water mixtures. Enzyme Microbiol. Technol. **18:**300-308.

Gold, M. H., and M. Alic. 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. **57:**605-622.

Gold, M. H., J. K. Glenn, and M. Alic. 1988. Use of polymeric dyes in lignin biodegradation assays. In: Wood, W. A. and Kellogg, S. T. (eds) Methods of Enzymology. Volume 161B. Lignin, Pectin and Chitin. Academic Press Inc., San Diego, USA, pp. 74-78.

Guimaraes, C. B. C., L. S. M. Bento, and M. G. Mota. 1994. Effect of manganese and veratryl alcohol on the colour removal from sugar refinery effluents by *Phanerochaete chrysosporium*. Proceedings of Sugar Processing Research Conference. Helsinki, Finland, pp. 103-117.

Haemmerli, S. D. 1988. Degradation of polycyclic aromatic hydrocarbons. Chapter 3.2 in Lignin Peroxidase and the Ligninolytic System of *Phanerochaete chrysosporium*, PhD Dissertation, Swiss Federal Institute of Technology (Diss. ETH No. 8670), Zurich, pp. 49-61.

Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter. 1986. Oxidation of Benzo[*a*]pyrene by extracellular ligninases of *Phanerochaete chrysosporium*: veratryl alcohol, and stability of ligninase. J. Biol. Chem. **261:**6900-6903.

Hammel, K. E. 1992. Oxidation of aromatic pollutants by lignin-degrading fungi and their extracellular peroxidases. H. Sigel and A. Sigel (eds.), Metal Ions in Biological Systems, Volume 28, Degradation of Environmental Pollutants by Microorganisms and Their Metalloenzymes. Marcel Dekker, Inc., NY, pp. 41-60.

Hammel, K. E., B. Green, and W. Z. Gai. 1991. Ring fission of anthracene by a eukaryote. Proc. Natl. Acad. Sci. USA. **88:**10605-10608.

Hammel, K. E., B. Kalyanaraman, and T. K. Kirk. 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**16948-16952.

De Jong, E., J. A. Field, and J. A. M. de Bont. 1994. Aryl alcohols in the physiology of white rot fungi. Microbiol. Rev. **13:**153-188.

Kaal, E. E. J., E. de Jong, and J. A. Field. 1993. Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. **59:**4031-4036.

Kelley, R. L., and A. Reddy. 1986. Identification of glucose oxidase activity as the primary source of hydrogen peroxide production in ligninolytic cultures of *Phanerochaete chrysosporium*. Arch. Microbiol. **144:**248-25.

Kersten, P. J. 1990. Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. Proc. Natl. Acad. Sci. USA. **87:**2936-2940.

Khindaria, A., D. P. Barr, and S. D. Aust. 1995. Lignin peroxidase can also oxidize manganese. Biochem. **34:**7773-7779.

Kimura, Y., Y. Asada, and M. Kuwahara. 1990. Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl. Microbiol. Biotechnol. **32:**436-442.

Kirk, T. K. and R. L. Farrell. 1987. Enzymatic "combustion": The microbial degradation of lignin. Ann. Rev. Microbiol. **41:**465-505.

Kirk, T. K., E. Schultz, W. J. Connors, L. F. Lorenz, and J. G. Zeikus. 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117:**227-285.

REFERENCES

Kotterman, M. J. J., E. Heessels, E. de Jong, and J. A. Field. 1994. The physiology of anthracene biodegradation by the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Microbiol. Biotechnol. **42:**179-186.

Li, D., M. Alic, and M. Gold. 1994. Nitrogen regulation of lignin peroxidase gene transcription. Appl. Environ. Microbiol. **60:**3447-3449.

Lundell, T., and A. Hatakka. 1994. Participation of MnII in the catalysis of laccase, manganese peroxidase and lignin peroxidases from Phlebia radiata. FEBS Lett. **348:**291-296.

Mester, T., E. de Jong, and J. A. Field. 1995. Manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidase. Appl. Environ. Microbiol. **61:**1881-1887.

Muheim, A., M. S. A. Leisola, and H. E. Schoemaker. 1990. Aryl-alcohol oxidase and lignin peroxidase from the white rot fungus *Bjerkandera adusta.* J. Biotechnol. **13:**159-167.

Ollikka, P., K. Alhonmaki, V. M. Leppanen, T. Glumoff, T. Raijola, and I. Suominen. 1993. Decolorization of azo, triphenyl methane, heterocyclic, and polymeric dyes by lignin peroxidase isoenzymes from *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. **59:**4010-4016.

Orth, A. B., M. Denny, and M. Tien. 1991. Overproduction of lignin-degrading enzymes by an isolate of *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. **57:**2591-2596.

Perez, J., and T. W. Jeffries. 1992. Role of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. **58:**2402-2409.

Popp, J. L., and T. K. Kirk. 1991. Oxidation of methoxybenzenes by manganese peroxidase and by Mn^{3+}. Arch. Biochem. Biophys. 288:145-148.

Rüttimann, C., E. Schwember, L. Salas, D. Cullen, and R. Vicuña. 1992. Ligninolytic enzymes of the white rot basidiomycetes Phlebia brevispora and Ceriporiopsis subvermispora. Biotechnol. Appl. Biochem. **16:**64-76.

Sayadi, S., and R. Ellouz. 1995. Roles of lignin peroxidase and manganese peroxidase from *Phanerochaete chrysosporium* in the decolorization of olive mill wastewaters. Appl. Environ. Microbiol. **61:**1098-1103.

Schmidt, H. W. H., S. D. Haemmerli, H. E. Schoemaker, and M. S. A. Leisola. 1989. Oxidative degradation of 3,4-dimethoxybenzyl alcohol and its methyl ether by the lignin peroxidase of *Phanerochaete chrysosporium.* Biochemistry **28:**1776-1783.

Tien, M. and T. K. Kirk. 1988. Lignin peroxidase of *Phanerochaete chrysosporium.* In: Wood, W. A. and Kellogg, S. T. (eds) Methods of Enzymology. Volume 161B. Lignin, Pectin and Chitin. Academic Press Inc., San Diego, USA, pp. 238-248.

Tonon, F., and E. Odier. 1988. Influence of veratryl alcohol and hydrogen peroxide on ligninase activity and ligninase production by *Phanerochaete chrysosporium.* Appl. Environ. Microbiol., **54:**466-472.

Valli, K., H. Wariishi, and M. H. Gold. 1990. Oxidation of monomethoxylated aromatic compounds by lignin peroxidase: Role of veratryl alcohol in lignin biodegradation. Biochemistry **29:**8535-8539.

Vares, T., O. Niemenmaa, and A. Hatakka. 1994. Secretion of ligninolytic enzymes and mineralization of ^{14}C-ring-labelled synthetic lignin by three Phlebia tremellosa strains. Appl. Environ. Microbiol. **60:**569-575.

Walters. R. W., and R. G. Luthy. 1984. Equilibrium adsorption of PAH from water onto activated carbon. Environ. Sci. Technol. 18:395-403.

Wariishi, H., K. Valli, and M. H. Gold. 1992 . Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium* - kinetic mechanism and role of chelators. J. Biol. Chem. **267:**23688-23695.

Chapter 4

Polycyclic aromatic hydrocarbon oxidation by the white rot fungus *Bjerkandera* sp. strain BOS55 in the presence of nonionic surfactants

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ABSTRACT

The effect of nonionic surfactants on the polycyclic aromatic hydrocarbon (PAH) oxidation rates by the extracellular ligninolytic enzyme system of the white rot fungus *Bjerkandera* sp. strain BOS55 was investigated. Various surfactants increased the rate of anthracene, pyrene and benzo[*a*]pyrene oxidation by 2- to 5-fold. The stimulating effect of surfactants was found to be solely due the increased bioavailability of PAH, indicating that the oxidation of PAH by the extracellular ligninolytic enzymes is limited by low compound bioavailability. The surfactants were shown to improve PAH dissolution rates by increasing their aqueous solubility and by decreasing the PAH precipitate particle size. The surfactant Tween 80 was mineralized by *Bjerkandera* sp. strain BOS55, and as a result both the PAH solubilizing activity of Tween 80 and its stimulatory effect on anthracene and pyrene oxidation rates were lost within 24 h after addition to 6-day-old cultures. It was observed that the surfactant dispersed anthracene precipitates recrystallized into larger particles after Tween 80 was metabolized. However, benzo[*a*]pyrene precipitates remained dispersed, accounting for a prolonged enhancement of the benzo[*a*]pyrene oxidation rates. Since the endogenous production of H_2O_2 is also known to be rate limiting for PAH oxidation, the combined effect of adding surfactants and glucose oxidase was studied. The combined treatment resulted in anthracene and benzo[*a*]pyrene oxidation rates as high as 1450 and 450 mg liter^{-1} day^{-1}, respectively, by the extracellular fluid of 6-day-old fungal cultures.

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INTRODUCTION

Bacterial degradation of PAH is rate limited by the low bioavailability of these compounds (Bossert and Bartha, 1986; Heitkamp and Cerniglia, 1987; Stucki and Alexander, 1987; Mihelcic *et al.*, 1993). Interestingly, lignin degrading white rot fungi can rapidly oxidize and partially mineralize polycyclic aromatic hydrocarbon (PAH) compounds (Aust, 1990; Field *et al.*, 1993; Bezalel *et al.*, 1996; Bogan and Lamar, 1996). With the accumulating evidence that the extracellular ligninolytic enzymes of these fungi are involved in the initial oxidation of PAH, it is believed that the PAH degradation rate by white rot fungi is less limited by the low bioavailability. Direct oxidation of PAH by *in vitro* incubations with lignin peroxidase (LiP) (Hammel *et al.*, 1986; Vazquez-Duhalt *et al.*, 1994), and with manganese peroxidase (MnP) (Field *et al.*, 1996) has been reported. LiP can oxidize PAH substrates with an ionization potential (IP) up to 7.55-8.0 eV (Hammel *et al.*, 1986; Vazquez-Duhalt *et al.*, 1994). The oxidized cofactor of MnP, MnIII, has also been shown to oxidize PAHs with IP values up to 7.8 eV (Cavalieri and Rogan, 1985; Cremonesi *et al.*, 1992). MnP can also oxidize PAHs with higher IP values such as phenanthrene (8.2 eV) when coincubated with unsaturated lipids via a lipid peroxidation mechanism (Moen and Hammel, 1994).

Bjerkandera sp. strain BOS55 was previously identified as an outstanding PAH degrading white rot fungal isolate (Field *et al.*, 1992). This fungus has several peculiar features, aside from LiP and MnP, it also produces manganese independent peroxidase (MIP), and it is N-unregulated, producing high extracellular peroxidase titers in N-sufficient medium (Kaal *et al.*, 1993; Mester *et al.*, 1995). We have observed that the PAH oxidation rates were similar in extracellular culture fluids as in whole cultures of *Bjerkandera* sp. strain BOS55 (Kotterman *et al.*, 1996), clearly indicating a major role of the extracellular enzymes in PAH metabolism.

In attempts to optimize the PAH degradation by *Bjerkandera* sp. strain BOS55, it was shown that physiological conditions such as high-N that greatly enhance the LiP, MnP, and MIP titers were not associated with a significantly increased PAH oxidation rate. The PAH oxidation rate in high-N cultures was only enhanced when the H_2O_2 generation rate was improved by the addition of extra glucose and oxygen, or exogenous glucose oxidase (Kotterman *et al.*, 1996), indicating that the endogenous H_2O_2 production rate was an important rate limiting factor. However, exogenous glucose oxidase improved the oxidation rate of water-soluble substrates, *e.g.*, the polymeric dye Poly R-478, to a much greater extent than the poorly water soluble PAH. The aqueous solubility of the PAH compounds anthracene, pyrene, and benzo[*a*]pyrene is as low as 70, 135 and 3.8 µg liter^{-1}, respectively (Dzombak and Luthy, 1984). This observation indicated that the low bioavailability of PAH could very well be an important rate-limiting factor as well. The low bioavailability is generally considered to be due to slow dissolution kinetics. Key factors influencing the dissolution rate are both aqueous solubility and particle surface area of PAH (Tiehm, 1994; Vazquez-Duhalt *et al.*, 1994; Field *et al.*, 1996). Consequently, methods of increasing PAH bioavailability are expected to enhance PAH oxidation rates.

Early attempts to increase the PAH bioavailability for the white rot fungus *Bjerkandera* sp. strain BOS55 were done with water-miscible solvents. Acetone had a beneficial effect on both the PAH dissolution and the oxidation rate, but at dosages higher than 5-10%, acetone was acutely toxic to the fungal cells (Field *et al.*, 1995). Another means to improve the solubility of PAH compounds is through the use of surfactants. Surfactants are known to increase the solubility of PAHs when the concentration of the surfactant is above the critical micelle concentration (CMC). However, the addition of surfactants to improve PAH degradation by bacteria has given mixed results. Aside from enhancement of PAH degradation, it was also observed that certain surfactants, at concentrations just above the CMC, can actually inhibit PAH degradation by bacteria (Rouse *et al.*, 1994).

The purpose of this study was to determine if nonionic surfactants can be utilized to increase PAH bioavailability and PAH oxidation rates by *Bjerkandera* sp. strain BOS55. The toxicity, the biodegradability as well as the physiological effects of the surfactants on the ligninolytic enzyme system were also investigated.

MATERIALS AND METHODS

Microorganism and inoculum

Bjerkandera sp. strain BOS55 (ATCC 90940) was maintained on peptone-yeast agar slants (Mester *et al.*, 1995) at 4°C. The experiments were inoculated with agar plugs as described previously (Kaal *et al.*, 1993).

Culture conditions

The standard high nitrogen (N), manganese (Mn) free medium used was described before (Kotterman *et al.*, 1996). This medium contained 33 mM N as mycological peptone (5 g liter^{-1}), 10 g liter^{-1} glucose, and was buffered at pH 6 with a combined 20 mM 2,2-dimethylsuccinate and 40 mM phosphate buffer. Manganese-containing medium was obtained by addition of 33 µM MnII (as $MnSO_4$) to the medium. The mineralization of surfactants was tested in a modified medium, the peptone was replaced by 20 mM NH_4Cl. The media were autoclaved for 30 min at 115°C. After sterilization, 10 ml liter^{-1} of a filter-sterilized thiamine solution (200 mg liter^{-1}) was added. Aliquots of 5 ml medium were placed in sterile 250-ml serum bottles, incubation took place for 6 days statically at 30°C with loosely capped bottles in complete darkness.

Additions to 6 day-old cultures. At day 6, all cultures were supplied with an oxygen atmosphere by flushing the cultures for 5 min with pure oxygen. Extra glucose was added as a 100-times concentrated solution, increasing the glucose concentration by 5 g liter^{-1}. Surfactants were added as 100-times concentrated solutions, PAH was added in acetone, resulting in a final concentration of 10 ml acetone liter^{-1} and 50 to 100 mg benzo[*a*]pyrene, pyrene and anthracene liter^{-1}. In some experiments, 20 or 40 units liter^{-1} of glucose oxidase were added together with 2 mM veratryl alcohol.

Extracellular culture fluid experiments

In certain experiments, PAH compounds were incubated with extracellular culture fluids. The extracellular culture fluid was prepared by separating the biomass, followed by 20 min of centrifugation in a Sorvall RC26 plus centrifuge (Dupont, Newton, USA) at 16,200 *g*. Aliquots of 1.5 ml were transferred to 30-ml serum bottles.

Surfactants

Five non-ionic surfactants were used in this study. Tween 80, Tween 85, and Triton X-100 were obtained from Merck (Darmstadt, Germany), Brij 35 and polyoxyethylene 10 lauryl ether (PLE) from Sigma (St. Louis, USA). All surfactants were used without any further purification.

Oxidation of PAH

After incubation of PAH compounds, each culture was completely utilized for the extraction with acetonitrile as was described earlier by Kotterman *et al.* (1996). Analysis of PAH elimination was carried out using high-pressure liquid chromatography equipped with a diode array detector for analysis, according to a method modified from Field *et al.* (1992). All PAH were analyzed under isocratic conditions; anthracene and pyrene with 85:15 acetonitrile/water, benzo[*a*]pyrene with 100% acetonitrile. The wavelengths used for integrating peak areas were 250, 240 and 295 nm for anthracene, pyrene, and benzo[*a*]pyrene, respectively. Each experiment in this study was run with triplicated cultures in parallel with a triplicated abiotic control consisting of autoclaved 6-day-old cultures. The PAH elimination by fungal cultures was calculated from the initial concentration of PAH, obtained by extracting a triplicate abiotic control directly after addition of PAH. No more than 2% PAH elimination was found in triplicated abiotic controls incubated in parallel with the fungal treatment in the presence or absence of surfactants.

Measurement of anthracene dissolution rate

Anthracene in a stock solution of acetone was added to water as in PAH degradation experiments. The precipitate was centrifuged (16,200 *g*, 15 min), resuspended in 1 ml of water and transferred to an eppendorf vial. The precipitate was washed, spun down (15 min) and resuspended in 0.5 ml water. To bottles containing 1.5 ml of water 50 µl of the resuspended precipitate was added (final concentration 40 mg liter^{-1}). Afterwards samples to measure dissolved anthracene were taken at selected time intervals. The aqueous concentration of these samples was measured by filtration through a Millex LCR13 0.2 µm pore-size filter from Millepore (Bedford, USA). Anthracene concentration was then measured with the HPLC. The saturation concentration of anthracene in the presence of Tween 80 was measured after a 24 h incubation period.

Measurement of PAH particle size

The PAH particle size was measured by using a microscope (Leitz Laborlux S, Germany), using a ocular with micrometer display.

Poly R-478 decolorization

The rate of Poly R-478 decolorization in 6-day-old cultures, at an initial concentration of 0.8 g liter^{-1}, was measured described by Kotterman *et al.* (1996). Samples of 50 µl were drawn, diluted in 1 ml demineralized water and centrifuged before analysis. The results are shown as the maximum decolorization rate, $1000*[\Delta(A_{520nm}/A_{350nm})]*h^{-1}$. Generally, this was measured between 5 to 24 hours after addition of Poly R-478.

Enzyme assays

The MnP and MIP activities were measured by the oxidation of 2,6-dimethoxyphenol and corrected for the laccase activity in the absence of H_2O_2 (Kaal *et al.*, 1993). The molar absorption coefficient used for the dimeric dimethoxyphenol oxidation product was 49,600 M^{-1} cm^{-1} as determined by Wariishi *et al.* (1992). LiP was measured by the oxidation of veratryl alcohol and corrected for veratryl alcohol oxidase activity in the absence of H_2O_2. The molar absorption coefficient used for the veratryl aldehyde product was 9300 M^{-1} liter^{-1} (Bourbonnais and Paice, 1988).

Analytical methods

H_2O_2 production rate was measured with the H_2O_2-dependent oxidation of phenol red by horseradish peroxidase as described earlier (Kotterman *et al.*, 1996). Carbon dioxide evolution was measured in the headspace by gas chromatography as described previously (Field *et al.*, 1992). Glucose concentration in the culture fluid was determined with a glucose oxidase-peroxidase enzyme assay, Peridochrom glucose GOD-PAP from Boehringer (Mannheim, Germany). Protein concentration in extracellular culture fluids was measured according to the method of Bradford (1976).

Chemicals

Anthracene, anthraquinone, pyrene, benzo[*a*]pyrene, 2,6-dimethoxyphenol, and veratryl alcohol were obtained from Janssen Chimica (Tilburg, The Netherlands).

Horseradish peroxidase (GradII), and glucose oxidase (GradI) were obtained from Boehringer (Mannheim, Germany). All other chemicals were commercially available and were used without further purification.

Statistical procedures

All experiments were carried out in triplicate. The values reported are means with standard deviation.

RESULTS

In this study, five non-ionic surfactants were tested in cultures of *Bjerkandera* sp. strain BOS55 cultivated in N-sufficient, Mn-deficient medium. Surfactants were added to 6-day-old cultures and their effects were monitored during the 3-4 d period that followed.

Physiological effects

First, the effects of surfactants on the ligninolytic activity of the fungus were monitored by the decolorization of the water-soluble ligninolytic indicator dye Poly R-478, and by measuring changes in the titers of extracellular ligninolytic peroxidases. The rate of dye decolorization was negatively affected by all surfactants used, as shown in ***Table 1***. Tween 80 had the least inhibitory effect (only 13%), while Brij 35 caused the strongest inhibition (55%).

Most surfactants completely repressed LiP activity after 54 h of incubation, while in control cultures receiving no surfactant some LiP activity was still present (approximately 50 nmol ml^{-1} min^{-1}). Tween 80 and PLE had no significant effect on the MnP and MIP activity, which were approximately 200 and 70 nmol ml^{-1} min^{-1}, respectively. On the other hand, Brij 35 and Tween 85 decreased the MnP titers by up to 50%, and totally decreased MIP after 54 hours. Surprisingly, Triton X-100 stimulated both MnP and MIP titers by about 3-fold; whereas, LiP titers were not affected.

The metabolic activity of the fungal cultures in the days that followed the addition of surfactants, measured as the CO_2 evolution rate (*results not shown*), was not inhibited by any of the surfactants tested. The viability of the fungal mycelium, however, was visually severely affected by Brij 35 and Triton X-100.

The effect of Tween 80 and PLE on the H_2O_2 production rate was also monitored. No increase was observed in either whole cultures (±4 µmol $liter^{-1}$ min^{-1}) or culture fluids (±1 µmol $liter^{-1}$ min^{-1}) by the addition of PLE or Tween 80 (2.5 g $liter^{-1}$), after 0 and 24 h of incubation.

Effect on PAH solubility

The effect of Tween 80 on the apparent aqueous solubility of anthracene was monitored. A linear relationship between measured anthracene solubility in water and Tween 80 concentration was observed. The apparent anthracene solubility increased from 70 µg $liter^{-1}$ to 246, 1440, and 4000 µg $liter^{-1}$ at 0, 0.25, 1.0, and 2.5 g $liter^{-1}$ Tween 80, respectively. The addition of Tween 80 to 6-day-old extracellular culture fluids had the same beneficial effect on anthracene solubility (*results not shown*). The addition of surfactants was also accom-

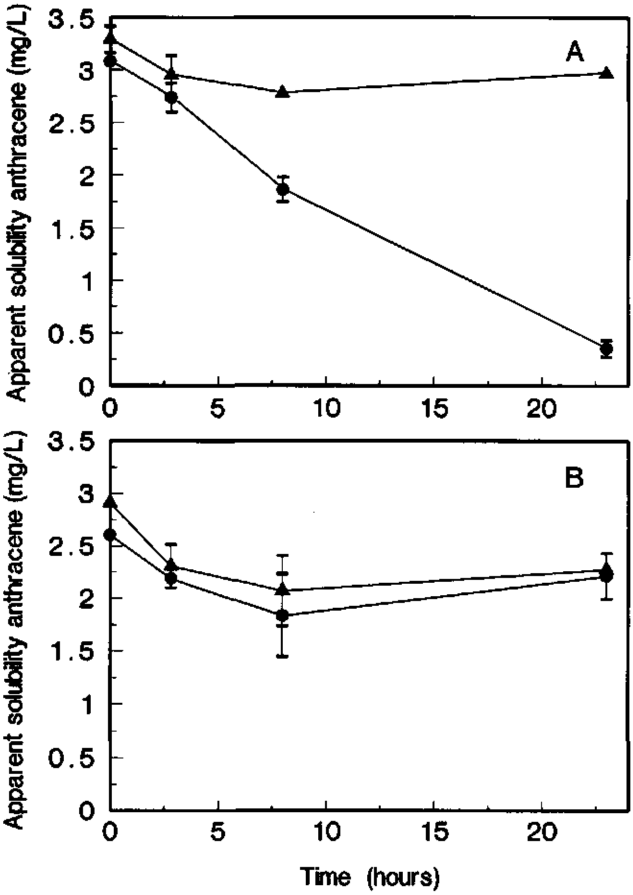

Figure 1.

The effect of Tween 80 (**A**), and PLE (**B**) on anthracene solubility after incubation in 6-day-old cultures of *Bjerkandera* sp. strain BOS55, and in autoclaved 6-day-old cultures as a control.
At selected timepoints, extracellular fluid was taken from the cultures, boiled for 5 min, and anthracene was added (50 mg liter^{-1}).
After equilibration, the aqueous anthracene concentration was measured.

Symbols:
(●) Live cultures
(▲) Dead control cultures

panied by a dramatic decrease in PAH particle size. In our experiments, anthracene was present as a non-uniformly dispersed precipitate with a wide particle size distribution ranging from 0.8 µm to aggregates as large as 3 mm. Upon addition of surfactant, most particles rapidly decreased in size to 0.4 µm and smaller and aggregates larger than 30 µm were no longer detectable. Furthermore, the precipitate appeared as a well dispersed milky white colloidal suspension. In contrast to anthracene, benzo[*a*]pyrene was already present as a colloidal suspension before adding the surfactant, with particle sizes ranging for the most part between 0.8 to 10 µm. Addition of Tween 80 resulted in a further decrease in particle size to 0.2-4 µm. Due to the presence of very small benzo[*a*]pyrene particles, a very high apparent aqueous solubility (800 µg liter^{-1}) was observed, which was further increased up to 10 mg liter^{-1} by the addition of 2.5 g liter^{-1} Tween 80. In these experiments, the apparent anthracene dissolution rate was also measured. Upon addition of anthracene (40 mg liter^{-1}) into the culture medium, the aqueous saturation concentration of 70 µg liter^{-1} was reached within 5 min. Addition of Tween 80 (2.5 g liter^{-1}) resulted in a rapid increase of the soluble anthracene concentration with an initial rate corresponding to 1200 mg liter^{-1} day^{-1}. The new apparent aqueous saturation concentration of 4 mg liter^{-1} was reached within 30 min.

Surfactant degradation

During incubation in 6-day-old cultures, the ability of Tween 80 to enhance the apparent solubility of anthracene rapidly decreased, while PLE was active throughout the entire 24 h incubation period considered (***Fig. 1***). The loss in the anthracene solubilizing activity of Tween 80 appeared to be biologically mediated since Tween 80 retained anthracene solubilizing activity in autoclaved fungal controls. When Tween 80 and PLE were added as sole energy and carbon source (1, 2.5 and 10 g liter^{-1}) to a basal N-sufficient (20 mM NH_4^+-N) mineral medium, Tween 80 was found to be a better substrate for *Bjerkandera* sp. strain BOS55 than PLE. After 17 d of incubation, 57% of the Tween 80 carbon was recovered as CO_2, regardless of the initial Tween 80 concentration; whereas, only 13% of the PLE carbon was recovered as CO_2 in the cultures with 1 g liter^{-1} PLE, higher concentrations repressed growth (*results not shown*).

After degradation of Tween 80 in 6-day-old cultures of *Bjerkandera* sp. strain BOS55, the initial effect of Tween 80 on the anthracene particle size was counteracted. Anthracene recrystallized into somewhat larger and more globular shaped particles (2-30 μm) as was observed by microscopy, while no change in particle size and shape could be observed in control cultures not receiving Tween 80. In the case of benzo[*a*]pyrene precipitates, no changes in the particle size of Tween 80 dispersed precipitates were observed after degradation of Tween 80.

Table 1. Effect of surfactants (2.5 g liter^{-1}) on the decolorization rate of the ligninolytic indicator dye, Poly R-478, in 6-day-old cultures of *Bjerkandera* sp. strain BOS55.

	Treatment	Poly R-478 decolorization rate[a,b]	Normalized to control[c]
Experiment 1	None	35.1 ± 1.4	100
	Brij 35	15.9 ± 1.2	45
	Triton X-100	20.0 ± 7.3	57
	Tween 80	30.6 ± 3.3	87
Experiment 2	None	23.7 ± 5.3	100
	Tween 85	13.1 ± 0.8	55
	PLE	12.2 ± 1.2	52

[a]: Values were calculated as $1000*[\Delta(A_{520nm}/A_{350nm})]*h^{-1}$

[b]: Values are means with standard deviation (n=3)

[c]: Set at 100% of the control (no surfactants added)

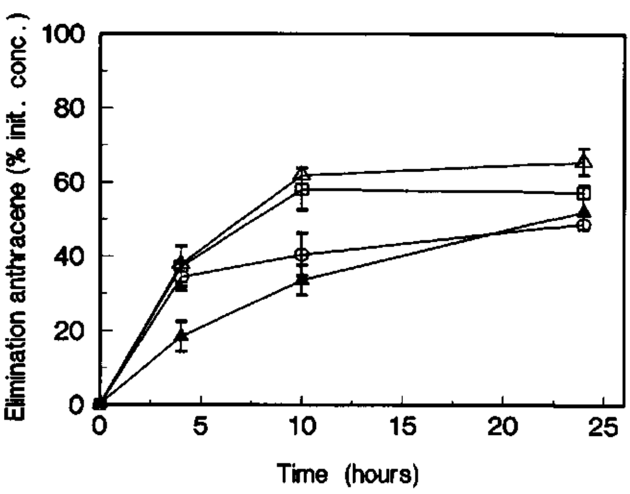

Figure 2.

The effect of Tween 80 concentrations on the time course of anthracene oxidation in 6-day-old whole cultures.

The initial anthracene concentration was 100 mg liter^{-1}.

Symbols:
(▲) Control,
(○) + 0.25 g liter^{-1} Tween 80
(□) + 1.0 g liter^{-1} Tween 80
(△) + 2.5 g liter^{-1} Tween 80

PAH oxidation

The effect of five surfactants on anthracene oxidation was tested by adding 0.25 or 2.5 g liter^{-1} of each surfactant to 6-day-old cultures of *Bjerkandera* sp. strain BOS55. The results for the addition of 2.5 g liter^{-1} are shown in ***Table** 2*. Clearly, all surfactants except Triton enhanced the anthracene oxidation rate significantly in the initial period (6.5 h). The yield of anthraquinone accumulated per unit of anthracene oxidized was also increased by all surfactants. Tween 80, Tween 85, and PLE were the most beneficial. No increase in the oxidation rate of anthracene was observed between 6.5 and 32 h, compared to the cultures not receiving surfactant (*results not shown*). The effects of 0.25 g liter^{-1} surfactant on the anthracene oxidation rates were similar but less pronounced (*results not shown*). Tween 80 was chosen for further study because Tween 80 showed the lowest toxicity to the ligninolytic system and because it caused a high enhancement of the anthracene oxidation rate.

The effect of Tween 80 concentration on the time course of anthracene, pyrene and benzo[*a*]pyrene oxidation was monitored. As shown in ***Figure** 2*, increasing Tween 80 concentrations only had large effects on stimulating the initial oxidation rate of anthracene. Tween 80 at a concentration of 0.25 g liter^{-1} stimulated anthracene oxidation for only 4 h, and at a concentration of 1-2.5 g liter^{-1}, anthracene oxidation was stimulated for 10 h. After this time, the rate of anthracene oxidation was even lower in cultures previously receiving Tween 80, as compared to control cultures. All Tween 80 concentrations were beneficial for pyrene oxidation over a 24 h period, thereafter, the rate was equal to the control cultures. The initial rate of pyrene oxidation was proportional to the surfactant concentration, the oxidation rate was increased 1.5-, 2-, and 2.5-fold by 0.25, 1, and 2.5 g liter^{-1} Tween 80, respectively. Tween 80 stimulated the rate of benzo[*a*]pyrene oxidation during the entire experimental period of 100 h, even in the case of the lowest Tween 80 concentration utilized (***Figure** 3*). The oxidation rate enhancement was again proportional to the surfactant concentration used. Tween 80 also stimulated anthracene and benzo[*a*]pyrene oxidation rates in 6-day-old extracellular culture fluids in a fashion similar to that observed in the whole cultures (*results not shown*).

Figure 3.

The effect of Tween 80 concentrations on the time course of benzo[*a*]pyrene oxidation in 6-day-old whole cultures.

The initial benzo[*a*]pyrene concentration was 50 mg liter^{-1}.

Symbols:
(▲) Control,
(○) + 0.25 g liter^{-1} Tween 80
(□) + 1.0 g liter^{-1} Tween 80
(△) + 2.5 g liter^{-1} Tween 80

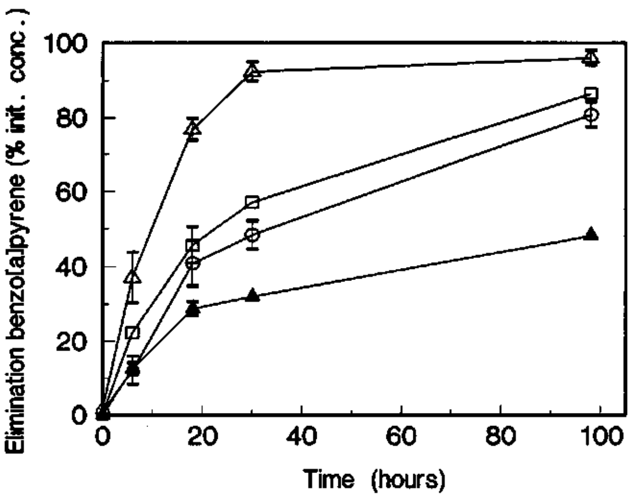

Table 2. The effect of surfactants (2.5 g liter^{-1}) on the anthracene oxidation and the molar yield of anthraquinone per mole of anthracene eliminated in 6-day-old cultures after 6.5 h of incubation. The initial anthracene concentration was 100 mg liter^{-1}.

	Treatment	**Elimination anthracene (%)**[a]	**Normalized to control**[b]	**AQ/A**[c]
Experiment 1	None	35.7 ± 1.4	100	0.47
	Brij 35	49.0 ± 5.0	137	0.83
	Triton X-100	36.2 ± 10.9	101	0.68
	Tween 80	64.1 ± 2.8	180	0.70
Experiment 2	None	23.2 ± 5.4	100	0.49
	Tween 85	38.4 ± 7.3	165	0.54
	PLE	45.5 ± 4.8	196	0.69

[a]: Values are means with standard deviation (n=3)

[b]: Set at 100% of the control (no surfactants added)

[c]: The ratio of the molar yield of anthraquinone accumulated and the amount of anthracene eliminated

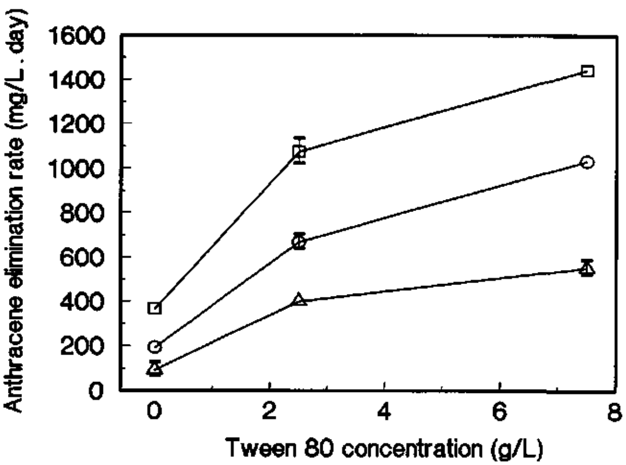

Figure 4.

Combined effect of the addition of Tween 80 and glucose oxidase on anthracene oxidation rates in 6-day-old extracellular culture fluids determined after one h of incubation.
The H_2O_2 production rates were measured in a parallel experiment not receiving anthracene during the first 30 min.

Production rates of H_2O_2 in control (no glucose oxidase added), gluox1 and gluox2 were 1.7, 9.5, and 17 μmol liter^{-1} min^{-1}, respectively.

Symbols:
(△) Control
(○) Gluox1
(□) Gluox2

Recently, Moen and Hammel (1994) presented data indicating that MnP can cause a lipid peroxidation mediated oxidation of PAH in the presence of unsaturated lipids and Mn. The stimulating effect of surfactants containing unsaturated fatty acids, such as Tween 80 and Tween 85, could therefore also be due to lipid peroxidation. However, no evidence was found for the involvement of lipid peroxidation since PLE stimulated anthracene oxidation to similar extents, and this surfactant is a fully saturated lipid. Furthermore, the stimulation of anthracene oxidation by Tween 80 was also not favored by Mn containing media, in contrast to the requirement of lipid peroxidation for Mn. In fact, the presence of Mn lowered the anthracene oxidation rate by 45%, confirming previous observations (Kotterman *et al.*, 1996).

H_2O_2 production

Previously it was shown that the endogenous H_2O_2 production rate is a limiting factor for the oxidation of anthracene in high-N cultures of *Bjerkandera* sp. strain BOS55 (Kotterman *et al.*, 1996). Consequently, increasing the H_2O_2 production rate by exogenous addition of glucose oxidase resulted in a higher PAH oxidation rate. Therefore, in this study the combined effect of Tween 80 and glucose oxidase was tested in 6-day-old extracellular culture fluids. As shown in ***Figure 4***, increased bioavailability by the use of Tween 80 improves the anthracene oxidation rate up to 5-fold. The increased H_2O_2 generation rate improved the anthracene oxidation rate up to 4-fold. The combination of both resulted in a 14-fold increase to a very high rate of anthracene oxidation of 1450 mg liter^{-1} day^{-1}, corresponding to 30 mg anthracene mg protein^{-1} in the extracellular culture fluid day^{-1}. The anthraquinone production rate (*results not shown*) showed parallel trends as that shown for anthracene elimination. Under these conditions, the benzo[*a*]pyrene oxidation rate increased to 450 mg liter^{-1} day^{-1} with the combined treatment.

DISCUSSION

The rate of PAH degradation by bacteria, especially of the high molecular weight PAH, is limited by the low bioavailability of these highly nonpolar compounds (Bossert and Bartha, 1986; Heitkamp and Cerniglia, 1987; Mihelcic *et al.*, 1993). Therefore, the bioavailability of PAH should be enhanced in order to increase the degradation rate. The bioavailability of PAH depends on the dissolution rate, which in turn depends on the surface area and the aqueous solubility (Volkering *et al.*, 1992; Tiehm, 1994; Volkering *et al.*, 1995; Field *et al.*, 1996). Enhancement of the bioavailability by increasing the specific area by utilizing PAH particles of a smaller size (Volkering *et al.*, 1992; Tiehm, 1994), or by using surfactants (Rouse *et al.*, 1994; Volkering *et al.*, 1995; Grimberg *et al.*, 1996), stimulated PAH degradation rates. The former increases the dissolution rate, the latter increases both the solubility and the dissolution rate. However, the use of surfactants is not always beneficial. Some surfactants show toxicity to the bacterial cells in concentrations just above CMC, and PAH present in surfactant micelles is not always available to the bacteria. Furthermore, preferential use of the surfactant as substrate decreases both the solubilizing activity of the surfactants as well as the degradation of the target compound (Rouse *et al.*, 1994; Tiehm, 1994; Grimberg *et al.*, 1996; Guha and Jaffe, 1996).

Whereas bacteria depend on intracellular enzymes to oxidize PAH compounds, white rot fungi can oxidize PAH extracellularly with ligninolytic enzymes (Hammel, 1992; Field *et al.*, 1996; Kotterman *et al.*, 1996). The slow process of PAH diffusion towards microbial cells is thus circumvented. However, in previous studies evidence was found that even PAH oxidation by the ligninolytic enzyme system of *Bjerkandera* sp. strain BOS55 was limited by the low bioavailability of PAH (Field *et al.*, 1995; Kotterman *et al.*, 1996). The objective of this study was therefore to enhance the bioavailability of PAH by surfactants in order to increase the PAH oxidation rate.

The surfactants used in this study were shown to increase the bioavailability of PAH. The apparent aqueous solubility of anthracene was increased from 70 µg liter^{-1} to 4000 µg liter^{-1} by the addition of 2.5 g liter^{-1} Tween 80 or PLE. Additionally, the particle size of PAH precipitates was decreased, which provides a greater specific surface area of the PAH particles. These two effects were associated with a higher dissolution rate of PAH, accounting for the increased bioavailability. Surfactants stimulated the rate of PAH oxidation in the extracellular culture fluids in the same fashion as in the whole cultures, emphasizing that the improved bioavailability was beneficial for PAH oxidation by the ligninolytic enzymes. The rates of anthracene, pyrene and benzo[*a*]pyrene oxidation were enhanced up to 5-fold. This enhancement could truly be attributed to the increase in bioavailability, since no possible physiological effects of surfactants, which were likely to affect the PAH oxidation rate, were observed. The activity of the ligninolytic enzyme system was not enhanced by the addition of surfactants. In fact, the decolorization rate of the polymeric dye Poly R-478, which is shown to be a very appropriate indicator of ligninolytic activity (Field *et al.*, 1992; Kotterman *et al.*, 1994), was even partially inhibited by the addition of surfactants. Likewise, no beneficial effects of surfactants on the H_2O_2 production rate or enzyme titers were observed. Finally, no evidence of any significant involvement of Tween 80 in lipid peroxidation mediated PAH oxidation was found. As mentioned earlier, surfactants can be toxic to microorganisms. Although the PAH oxidation by *Bjerkandera* sp. strain BOS55 was enhanced by high surfactants loads, toxicity was observed. Apparently, the oxidation of PAH is extremely limited by the low bioavailability of PAH so that the partial loss in biocatalytic activity was far less important.

The extracellular oxidation of aromatic compounds by white rot fungal cultures is the net effect of peroxidases and endogenous H_2O_2 production. We have shown previously that in nitrogen-sufficient cultures of *Bjerkandera* sp. strain BOS55 overproducing peroxidases, the rate of both Poly R-478 and anthracene oxidation was limited by the endogenous H_2O_2 production rate (Kotterman *et al.*, 1996). At any bioavailability level of PAH obtained by supplying surfactant, the addition of glucose oxidase greatly enhanced the PAH oxidation rates. So bioavailability and H_2O_2 production were shown to be two independent rate limiting factors. The effect of additional peroxidative activity obtained by the addition of glucose oxidase, however, is more pronounced on the decolorization of the water soluble dye Poly R-478 or with PAH in the presence of surfactants compared to PAH alone. Notably, the maximum anthracene oxidation rate (1450 mg liter^{-1} day^{-1}) obtained in this study was in the same order of magnitude as the apparent dissolution rate of anthracene (1200 mg liter^{-1} day^{-1}) in the presence of surfactant.

Some surfactants are easily degraded by microorganisms (Rouse *et al.*, 1994; Tiehm, 1994), while others are not. Little is known about the degradation of nonionic surfactants by basidiomycetes. The hydrolysis of Tween 80 in cultures of *Phanerochaete chrysosporium* and the accumulation of fatty acids has been reported by Asther *et al.* (1986). In our study, Tween 80 was mineralized by *Bjerkandera* sp. strain BOS55, and the anthracene solubilizing activity of Tween 80 was lost within 24 h when added to 6-day-old cultures. In the case of anthracene, this rapid loss of solubilizing activity was associated with a lower PAH oxidation rate compared to control cultures not receiving Tween 80. Microscopic observations showed that recrystallization of dispersed anthracene to larger particles occurred, which is probably the reason the bioavailability of anthracene decreased after Tween 80 was metabolized. However, the rate of benzo[*a*]pyrene oxidation in the Tween 80 amended cultures remained higher than in the control cultures after Tween 80 was degraded. Because no recrystallization of benzo[*a*]pyrene particles was observed, the enhanced bioavailability of benzo[*a*]pyrene could be attributed to the decreased particle size of the dispersed benzo[*a*]pyrene. Surfactant degradation is therefore not necessarily a disadvantage for their use in bioremediation processes, as long as the initial contact of surfactant with PAH results in a decrease in particle size, thereby increasing the surface area for dissolution.

This study shows that the beneficial effect of surfactants on the oxidation rate of PAH was solely due to an increased PAH bioavailability. This implies that PAH oxidation by the extracellular ligninolytic enzyme system of the white rot fungus *Bjerkandera* sp. strain BOS55 is rate limited by the low bioavailability of PAH. Therefore, methods which improve the bioavailability of PAH are likely to enhance not only the rate of PAH degradation by bacteria but also by white rot fungi.

REFERENCES

Asther, M., G. Corrieu, R. Drapon, and E. Odier. 1986. Effect of Tween 80 and oleic acid on ligninase production by *Phanerochaete chrysosporium* INA-2. Enzyme Microb. Technol. **9:**245-249.

Aust, S. D. 1990. Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microbiol. Ecol. **20:**197-209.

Bezalel, L., Y. Hadar, and C. E. Cerniglia. 1996. Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. **62:**292-295.

Bogan, B. W., and R. T. Lamar. 1996. Polycyclic aromatic hydrocarbon degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. Appl. Environ. Microbiol. **62:**1597-1603.

Bossert, I. D., and R. Bartha. 1986. Structure-biodegradability relationships of polycyclic aromatic hydrocarbons in soil. Bull. Environ. Contam. Toxicol. **37:**490-495.

Bourbonnais, R., and M. G. Paice. 1988. Veratryl alcohol oxidases from the lignin degrading basidiomycete Pleurotus sajor-caju. Biochem. J. **255:**445-450.

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254.

Cavalieri, E., and E. Rogan. 1985. Role of radical cations in aromatic hydrocarbon carcinogenesis. Environ. Health Perspect. **64:**69-84.

Cremonesi, P., B. Hietbrink, E. G. Rogan, and E. L. Cavalieri. 1992. One-electron oxidation of dibenzo[*a*]pyrenes by manganic acetate. J. Org. Chem. **57:**3309-3312.

De Jong, E., J. A. Field, and J. A. M. de Bont. 1992. Evidence for a new extracellular peroxidase: manganese inhibited peroxidase from the white rot fungus *Bjerkandera* sp. strain BOS55. FEBS Lett. **299:**107-110.

Dzombak, D. A., and R. G. Luthy. 1984. Estimating adsorption of polycyclic aromatic hydrocarbon on soils. Soil Science **137:**292-308.

Field, J. A., F. Boelsma, H. Baten, and W. H. Rulkens. 1995. Oxidation of anthracene in water/solvent mixtures by the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Microbiol. Biotechnol. **44:**234-240.

Field, J. A., E. de Jong, G. Feijoo-Costa, and J. A. M. de Bont. 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl. Environ. Microbiol. **58:**2219-2226.

Field, J. A., E. de Jong, G. Feijoo Costa, and J. A. M. de Bont. 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotech. **11:**44-49.

Field, J. A., R. A. Vledder, J. G. van Zelst, and W. H. Rulkens. 1996. The tolerance of lignin peroxidase and manganese dependent peroxidase to miscible solvents and the *in vitro* oxidation of anthracene in solvent:water mixtures. Enzyme Microbiol. Technol. **18:**300-308.

Grimberg, S. J., W. T. Stringfellow, and M. D. Aitken. 1996. Quantifying the biodegradation of phenanthrene by Pseudomonas stutzeri P16 in the presence of a nonionic surfactant. Appl. Environ. Microbiol. **62:**2387-2392.

Guha, S., and P. R. Jaffe. 1996. Biodegradation kinetics of phenanthrene partitioned into the micellar phase of nonionic surfactants. Environ. Sci. Technol. **30:**605-611.

Hammel, K. E. 1992. Oxidation of aromatic pollutants by lignin-degrading fungi and their extracellular peroxidases. H. Sigel and A. Sigel (eds.), Metal Ions in Biological Systems, Volume 28, Degradation of Environmental Pollutants by Microorganisms and Their Metalloenzymes. Marcel Dekker, Inc., NY, pp. 41-60.

Hammel, K. E., B. Kalyanaraman, and T. K. Kirk. 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**16948-16952.

Heitkamp, M. A., and C. E. Cerniglia. 1987. Effects of chemical structure and exposure on the microbial degradation of polycyclic aromatic hydrocarbons in freshwater and estuarine ecosystems. Environ. Toxicol. Chem. **6:**535-546.

Kaal, E. E. J., E. de Jong, and J. A. Field. 1993. Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. **59:**4031-4036.

Kotterman, M. J. J., E. Heessels, E. de Jong, and J. A. Field. 1994. The physiology of anthracene biodegradation by the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Microbiol. Biotechnol. **42:**179-186.

Kotterman, M. J. J., R. A. Wasseveld, and J. A. Field. 1996. Hydrogen peroxide production as a limiting factor in xenobiotic compound oxidation by nitrogen-sufficient cultures of *Bjerkandera* sp. strain BOS55 overproducing peroxidases. Appl. Environ. Microbiol. **62:**880-885.

REFERENCES

Mester, T., E. de Jong, and J. A. Field. 1995. Manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidase. Appl. Environ. Microbiol. **61:**1881-1887.

Mihelcic, J. R., R. R. Lueking, R. J. Mitzell, and J. M. Stapleton. 1993. Bioavailability of sorbed- and separate-phase chemicals. Biodegradation. **4:**141-153.

Moen, M. A., and K. E. Hammel. 1994. Lipid peroxidation by the manganese peroxidase of *Phanerochaete chrysospo-rium* is the basis for phenanthrene oxidation by the intact fungus. Appl. Environ. Microbiol. **60:**1956-1961.

Rouse, J. D., D. A. Sabatini, J. M. Suflita, and J. H. Harwel. 1994. Influence of surfactants on microbial degradation of organic compounds. Critical Rev. Environ. Sci. Technol. **24:**325-370

Stucki, G., and M. Alexander. 1987. Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl. Environ. Microbiol. **53:**292-297.

Tiehm, A. 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. **60:**258-263.

Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak. 1994. Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. Appl. Environ. Microbiol. **60:**459-466.

Volkering, F., A. M. Breure, A. Sterkenburg, and J. G. van Andel. 1992. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Appl. Environ. Microbiol. **36:**548-552.

Volkering, F., A. M. Breure, G. van Andel, and W. H. Rulkens. 1995. Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. **61:**1699-1705.

Wariishi, H., K. Valli, and M. H. Gold. 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium* - kinetic mechanism and role of chelators. J. Biol. Chem. **267:**23688-23695.

Chapter 5

Successive mineralization and detoxification of benzo[*a*]pyrene by the white rot fungus *Bjerkandera* sp. strain BOS55 and indigenous microflora

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ABSTRACT

White rot fungi can oxidize high molecular weight polycyclic aromatic hydrocarbons (PAH) rapidly to polar metabolites, but only limited mineralization takes place. The objectives of this study were to determine if the polar metabolites can be readily mineralized by indigenous microflora from several inocula sources, such as activated sludge, forest soils and PAH-adapted sediment sludge, and to determine if such metabolites have a decreased mutagenicity compared to the mutagenicity of the parent PAH. ^{14}C-radiolabeled benzo[*a*]pyrene was subjected to oxidation by the white rot fungus *Bjerkandera* sp. strain BOS55. After 15 days, up to 8.5% of the [^{14}C]-benzo[*a*]pyrene was recovered as $^{14}CO_2$ in fungal cultures, up to 73% was recovered as water soluble metabolites, and only 4% remained soluble in dibutyl ether. Thin-layer chromatography analysis revealed that many polar fluorescent metabolites accumulated.

Addition of indigenous microflora to the fungal cultures with oxidized benzo[*a*]pyrene on day 15 resulted in an initially rapid increase in the level of $^{14}CO_2$ recovery to a maximal value of 34% by the end of the experiments (>150 days), and the level of water-soluble label decreased to 16% of the initial level. In fungal cultures not inoculated with microflora, the level of $^{14}CO_2$ recovery increased to 13.5%, while the level of recovery of water-soluble metabolites remained as high as 61%. No large differences in $^{14}CO_2$ production were observed with the several inocula, showing that some polar metabolites of fungal benzo[*a*]pyrene oxidation were readily degraded by indigenous microorganisms, while other metabolites were not. Of the inocula tested, only PAH-adapted sediment sludge was capable of directly mineralizing intact benzo[*a*]pyrene, albeit at a slower rate and to a lesser extent than the mineralization observed after combined treatment by white rot fungi and indigenous microflora.

Fungal oxidation of benzo[*a*]pyrene caused a rapid and almost complete elimination of its highly mutagenic potential as observed in the *Salmonella typhimurium* revertant test with strain TA100 and TA98. Moreover, no direct mutagenic metabolite could be detected during fungal oxidation. The remaining weak mutagenic activity of fungal cultures containing benzo[*a*]pyrene metabolites towards strain TA98 was further decreased by subsequent incubations with indigenous microflora.

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INTRODUCTION

Bioremediation of polycyclic aromatic hydrocarbon (PAH)-polluted soil is severely hampered by the low rate of degradation of the higher (PAH), particularly the four- and five-ring PAH (Bossert and Bartha, 1986; Wilson and Jones, 1993). These higher PAH have very low water solubility and are often tightly bound to soil particles. This results in a very low bioavailability for bacterial degradation. The observation that white rot fungi can oxidize PAH rapidly by their extracellular ligninolytic enzyme systems has therefore raised interest in the use of these organisms for bioremediation of PAH-polluted soils (Aust, 1990; Field *et al.*, 1993).

Although PAH are extensively oxidized by white rot fungi, the degree of mineralization to CO_2 is always limited. In various studies evaluating the degradation of the potent carcinogen benzo[*a*]pyrene by several white rot fungal species, from 0.17 up to 15% of the radiolabeled PAH was recovered as $^{14}CO_2$ (Sanglard *et al.*, 1986; Bezalel *et al.*, 1996; Bogan and Lamar, 1996). The major products of the oxidation were both nonpolar and polar metabolites. The accumulation of such metabolites could be a reason for concern, since mammalian and fungal monooxygenases can oxidize benzo[*a*]pyrene to epoxides and dihydrodiols, which are very potent carcinogens (Thakker *et al.*, 1985; Sutherland, 1992). However, peroxidase-mediated extracellular oxidation of benzo[*a*]pyrene in cultures of white rot fungi results initially in benzo[*a*]pyrenediones, which show weak mutagenic activity (Thakker *et al.*, 1985). These primary metabolites are rapidly metabolized further to unidentified metabolites by *Phanerochaete laevis* and *Phanerochaete chrysosporium* (Sanglard *et al.*, 1986; Bogan and Lamer, 1996). Furthermore, the oxidized benzo[*a*]pyrene metabolites have a higher aqueous solubility. Since the low bioavailability of PAH is a major rate-limiting factor in the degradation of these compounds by bacteria (Stucki and Alexander, 1987; Volkering *et al.*, 1992), the increased bioavailability of oxidized PAH metabolites suggests that these compounds can be more easily mineralized by bacteria.

The aim of this study was to investigate the degradation and mineralization of the five-ring PAH benzo[*a*]pyrene, by the white rot fungus *Bjerkandera* sp. strain BOS55 and the subsequent mineralization of the metabolites by natural mixed cultures of microorganisms. During the oxidation and mineralization of benzo[*a*]pyrene, the decrease in mutagenicity of the metabolites was monitored. The white rot fungal strain *Bjerkandera* sp. strain BOS55 was used because of its outstanding ability to rapidly oxidize PAH (Field *et al.*, 1992; Kotterman *et al.*, 1994) and because extensive information concerning its physiology is available (De Jong *et al.*, 1992; Kotterman *et al.*, 1994, Mester *et al.*, 1995; Kotterman *et al.*, 1996; Mester *et al.*, 1996).

MATERIALS AND METHODS

Organisms used

The white rot fungus *Bjerkandera* sp. strain BOS55 (ATCC 90940) was maintained on peptone-yeast agar slants (Kimura *et al.*, 1990) at 10°C. Malt-extract plates were inoculated 5 days prior to the experiments, fungal cultures were inoculated with an agar plug as described previously (Kaal *et al.*, 1993).

Natural mixed populations of microorganisms were retrieved from different sources. Activated sludge was obtained from a municipal wastewater treatment plant (Bennekom, The Netherlands) (concentration of volatile suspended solids, 5 g liter^{-1}), and was used without further treatment. Samples of two soils were collected from forests from the litter layer (Forest 1) (pH 4.5) and from a decomposed beech log (Forest 2) (pH 4.8). The forest soils were diluted 1:1 (wt:wt) with water to prepare slurries and were filtered through cheese cloth. A 50-year-old PAH-polluted sediment sludge dredged from Rotterdam harbor was diluted 10 times in buffer (pH 7) containing 0.02% yeast extract and incubated for 10 days at 21°C in a shake flasks before it was used as an inoculum. An enrichment culture on 2,2'-diphenic acid, an intermediate of phenanthrene oxidation by *Phanerochaete chrysosporium* (Hammel *et al.*, 1992), was prepared from activated sludge (from a municipal waste water treatment plant in Delft, The Netherlands), containing four different bacteria (as determined on the basis of morphology on yeast-glucose agar plates). This enrichment culture was incubated for 10 days in a pH 7 buffer containing 1 g 2,2'-diphenic acid liter^{-1} at 21°C before it was used as an inoculum.

In all cases, approximately 2-5*10^{8} CFU was added to each fungal culture on day 15 in a volume of 5 to 10 ml.

Culture conditions

Fungal cultures were cultivated on the standard high nitrogen, manganese-free medium, modified from the medium described by Tien and Kirk (1988). This medium contained 33 mM N as mycological peptone (5 g liter^{-1}) and 10 g glucose liter^{-1} buffered at pH 6 with 40 mM phosphate. Manganese-containing medium was obtained by addition of 66 µM Mn (as $MnSO_4$). The media were autoclaved for 30 min at 115°C. After sterilization, 10 ml of a filter-sterilized thiamine solution (200 mg liter^{-1}) liter^{-1} was added. Sterile serum 250-ml bottles containing 5 ml of medium were loosely capped to facilitate aeration during the incubation for 6 days at 30°C. Preparations containing bacteria were incubated in a shaking water bath (80 rpm) at 21°C.

Additions to fungal cultures

Benzo[*a*]pyrene was added on day 6 as a 100 x concentrated stock solution in acetone, which resulted in a final benzo[*a*]pyrene concentration of 20 mg liter^{-1} and an acetone concentration of 1%. Glucose (5 g liter^{-1}) and Tween 80 (2.5 g liter^{-1}) were added as 100 x concentrated stock solutions in water. The bottles were then tightly capped, and an oxygen atmosphere was supplied by flushing the bottles for 5 min with pure oxygen. This was repeated once every 2 or 3 days.

The microbial inocula from soils, sludge or sediments were added 15 days later, bottles were placed in a shaking water bath at 21°C. The bottles were tightly capped, and each headspace was flushed once every 2 or 3 days with air. Control experiments indicated that the spent fungal medium was not toxic towards the microorganisms; in fact, the levels of CO_2 production and plate counts increased when spent medium was added, indicating that organic matter in the spent fungal culture medium was metabolized.

[^{14}C]benzo[*a*]pyrene experiments

In the ^{14}C-experiments, 80,000-100,000 dpm of [7,10-^{14}C]benzo[*a*]pyrene (Specific activity 2.24 MBq mmol^{-1}, Amersham, Amersham, United Kingdom) was added to each culture together with 20 mg benzo[*a*]pyrene liter^{-1}. Production of $^{14}CO_2$ was measured by flushing the headspace once every 2 or 3 days for 8 min through a three-stage trap in which each stage contained 6 ml of 2 M NaOH. The $^{14}CO_2$ production values for triplicate cultures were pooled. Control experiments revealed a CO_2-trapping efficiency of more than 99%. For detection of volatile metabolites, a fourth trap containing acetonitrile was added. The total recovery of label remaining in the culture medium was measured by adding 3 volumes of acetone to each bottle; the bottles were then shaken for 1 h. The recovery of benzo[*a*]pyrene in autoclaved fungal controls, as evidenced by a high-performance liquid chromatography (HPLC) analysis of unlabeled benzo[*a*]pyrene, was more than 98% when this method was used. The distribution of label remaining in the culture medium between the water-soluble and organic compound-soluble phases was measured by adding 10 ml of dibutyl ether. After dibutyl ether was added, the cultures were vigorously shaken for 1 h. After phase separation, the amount of label in both the water phase and solvent phase was measured. The amount of water-soluble label was sometimes also measured quickly by filtering the culture fluids through hydrophilic Schleicher & Schuell type FP 030/3 0.2-μm-pore-size filters, which resulted in levels of recovery that were 5 to 10% higher than those obtained after dibutyl ether extraction. The amount of label associated with fungal biomass was measured by incubation of acetone-extracted fungal biomass with Soluene (Packard). One milliliter of the partly homogenized biomass was then transferred to a microvial with 5 ml of scintillation cocktail. Quenching by the biomass was corrected by adding a known amount of label to control vials with and without equal amounts of Soluene treated biomass.

All samples (volume, up to 1 ml) were added to 5 ml of scintillation-cocktail (Ultima Gold, Packard) in microvials, and the radioactivity was measured in a liquid scintillation analyzer (Tri-carb 1600 TR, Packard) with appropriate controls.

Salmonella typhimurium **revertant test (Ames test)**

To monitor the mutagenic potential of benzo[*a*]pyrene metabolites, the *Salmonella typhimurium* revertant plate test, first described by Ames *et al.* (1975) was used. Experiments were basically performed according to the plate incorporation test described by Maron and Ames (1983). Both strain TA-98 for point mutations and strain TA-100 for frameshift mutations were used. Overnight cultures were inoculated directly from a -80°C stock, and the genotypes were tested after preparation of the -80°C stock. To mimic liver biotransformation, a rat liver homogenate (S-9 mix) was used (Boehringer Mannheim, Germany) from Araclor-induced rats. Benzo[*a*]pyrene dissolved in acetone was used as a positive control in experiments performed with S-9 mix, and 4-nitroquinoline-N-oxide was used as a positive control in experiments performed without S-9 mix. A preincubation step (10 min, 37°C) included in initial tests resulted in no significant increase in the mutagenic response and was therefore omitted in the subsequent experiments.

The culture fluids were separated from the fungal biomass by filtration through cheese cloth. The biomass in samples containing the indigenous microflora (which contained no insoluble benzo[*a*]pyrene anymore) was removed by filtration through a Schleicher & Schuell type FP 030/2 0.45-μm-pore-size filter. All samples were treated aseptically and were boiled for 5 min before use. Both 100-μl samples and 400-μl samples were used, and the data shown below were obtained with 400-μl samples. Inoculated plates were incubated at 37°C for 48 hours. The samples taken at the start of the benzo[*a*]pyrene incubation had relatively low mutagenic activities compared to the mutagenic activities of the benzo[*a*]pyrene standards (they had 40-50% of the expected response). This was due to retention of large benzo[*a*]pyrene precipitates in the filtration step, and was not due to adsorption of benzo[*a*]pyrene to the fungal biomass. This was of no concern, since we were mainly interested in the mutagenic activity of the free available water soluble benzo[*a*]pyrene metabolites in the extracellular culture fluids, which were not retained by the filtration step.

Analytical methods

For the analysis of residual unlabeled benzo[*a*]pyrene, triplicate cultures were sacrificed by adding 3 volumes of acetone to each bottle. These bottles were then sealed with Teflon liners, shaken for 1 h, and samples were centrifuged in an eppendorf centrifuge (10 min 13,000 *g*). The analysis was conducted with a HPLC equipped with diode-array detector as described previously (Field *et al.*, 1992). Dead fungal controls, consisting of autoclaved 6-day-old fungal cultures were used to monitor abiotic losses of benzo[*a*]pyrene. The abiotic losses never exceeded 2% of benzo[*a*]pyrene added.

For the extraction of the polar benzo[*a*]pyrene metabolites from the water phase, the culture fluid was first separated from the biomass by filtration through cheese cloth. The filtrate was then acidified with HCl, saturated with NaCl, and extracted six times with 0.5 volume of ethyl acetate. The ethyl acetate was evaporated at 40°C under a nitrogen atmosphere, and the residue was dissolved in acetone. The extracts were analyzed on thin-layer chromatography (TLC) plates (Silica gel F_{254}, Merck), developed with chloroform-methanol (97:3, 75:25, and 65:35), petroleum ether 40-60-ethyl acetate (2:1), and ethyl acetate-methanol (65:35). The metabolites were detected by UV illumination and by autoradiography on Kodak X-Omat film.

The numbers of CFU were determined on yeast-glucose plates.

Chemicals

All chemicals used were commercially available and of analytical grade or higher. Solvents used for TLC were chromatography grade. All chemicals were used without further treatment.

Statistical procedures

The data shown, unless indicated otherwise, are the means of triplicate cultures with standard deviation.

RESULTS

Degradation of benzo[*a*]pyrene by the white rot fungus *Bjerkandera* sp. strain BOS55

In the first experiments, the degradation of benzo[*a*]pyrene was monitored in both Mn-sufficient and Mn-deficient cultures in the presence of the surfactant Tween 80, since the presence of Mn has been shown to affect the PAH oxidation by *Bjerkandera* sp. strain BOS55 (Kotterman *et al.*, 1996). For Mn-sufficient cultures (***Fig.*** *1*), the level of recovery of $^{14}CO_2$ after 15 days was 8.4% of the initial amount of label added; in manganese-deficient cultures, a slightly lower level of recovery of $^{14}CO_2$ (7.9%) was observed (*results not shown*). The distribution of the ^{14}C-label in the culture fluid in the hydrophobic solvent and water phases was monitored in Mn-sufficient cultures (***Fig.*** *1*). The amount of ^{14}C-label extractable with dibutyl ether decreased very rapidly, while water-soluble polar metabolites quickly accumulated. After 15 days of incubation, the major products of fungal benzo[*a*]pyrene oxidation were water-soluble products, which accounted for 68% of the initial amount of label, as shown in ***Figure*** *1*. In separate experiments, the water-soluble products at day 15 accounted for 67 to 73% of the initial amount of label. HPLC analysis of simultaneously grown cultures contain-

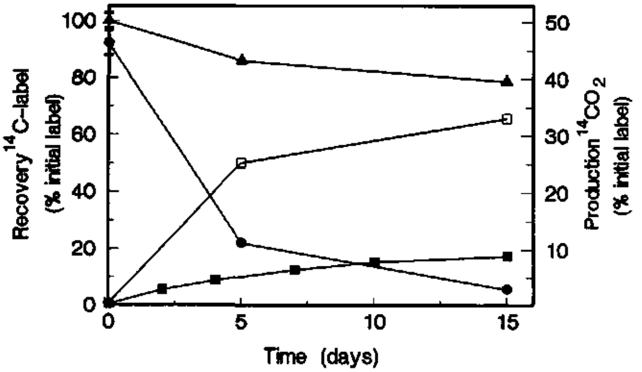


Figure 1.

The oxidation of [^{14}C]benzo[*a*]pyrene by Mn-sufficient fungal cultures.
Day 0 is the time of benzo[*a*]pyrene (20 mg liter^{-1}) addition to 6-day-old fungal cultures.

The distribution of ^{14}C-label from benzo[*a*]pyrene in dibutyl ether (●) and water phase (□), the total recovery of label in the culture medium by addition of acetone (▲), and the mineralization to $^{14}CO_2$ (■).

ing unlabeled benzo[*a*]pyrene showed that benzo[*a*]pyrene (20 mg liter^{-1}) was eliminated for 91, 96 and 100% after 1, 5 and 15 days, respectively. The total amount of ^{14}C-label recovered in the gas and water phases decreased slightly with time, whereas the amount of label associated with the fungal biomass increased from zero at the start of the experiment to around 8% at day 15. Volatile compounds other than CO_2 were not observed. In the Mn-deficient cultures, polar water-soluble products were also found to be the major products, accounting for 70% of the label (*results not shown*). After 15 days, no $^{14}CO_2$ production was detected in the autoclaved fungal controls; the level of recovery of label with acetone was 100%, and only 0.1% of the label was recovered from the water phase.

Characterization benzo[*a*]pyrene metabolites

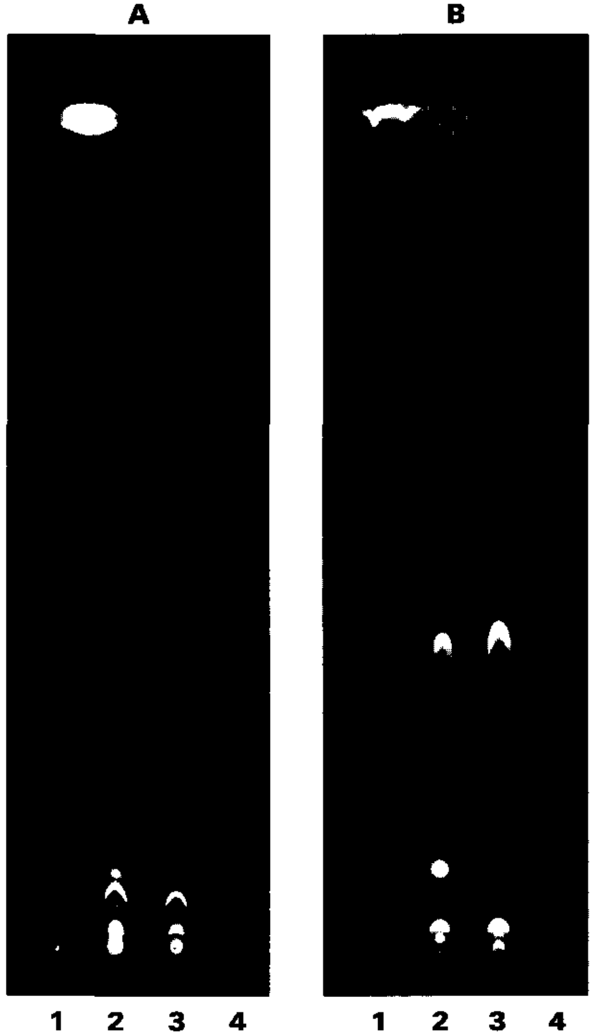
The spectrum of oxidized benzo[*a*]pyrene products produced by Mn-sufficient cultures of *Bjerkandera* sp. strain BOS55 was investigated further. When the ethyl acetate-extracted metabolites were examined by using TLC with nonpolar eluents, such as petroleum ether 40-60-ethyl acetate (3:1) (not shown) and chloroform-methanol (97:3), most of the metabolites remained in the origin, while benzo[*a*]pyrene exhibited significant migration (***Fig.** 2A*). A more polar eluent, such as chloroform-methanol (75:25) was required for any significant migration of the polar metabolites (***Fig. 2B***). Use of the eluents chloroform-methanol (65:35) and ethyl acetate-methanol (65:35) caused migration of all the metabolites, but the resolution was very poor (*results not shown*). Clearly, extensive oxidation of benzo[*a*]pyrene to polar metabolites took place during the first day of incubation. After 15 days no intact benzo-[*a*]pyrene was detected, and some metabolites present on day 1 were apparently oxidized further. Ethyl acetate extracts of 15-days-old control fungal cultures (without added benzo-[*a*]pyrene) produced only two faint blue spots under UV light (*not visible in Fig. 2*), which were tentatively identified as the secondary metabolites veratryl alcohol and veratraldehyde. Identical benzo[*a*]pyrene metabolite profiles were observed after TLC with an autoradiogram of ^{14}C-labeled benzo[*a*]pyrene metabolites (*results not shown*). Acidification of the culture fluids with HCl was necessary for a high level of recovery of the metabolites by ethyl acetate extraction. Still, the extraction efficiency decreased during the incubation period; around 15% of the water-soluble label was not extractable after 15 days. Extensive degradation of benzo[*a*]pyrene and accumulation of polar metabolites was also observed when benzo[*a*]pyrene was incubated for 1 day in extracellular culture fluids of 6-day-old fungal cultures (*results not shown*).

Figure 2.

TLC profiles of benzo[*a*]pyrene metabolites during the incubation in fungal cultures, visualized by UV illumination.

Panel **A** was developed twice with chloroform/methanol 97:3,
Panel **B** shows the metabolites which were retained nearby the origin in panel **A** after an additional run with chloroform/methanol 75:25.

Lane **1:** Time 0
Lane **2:** 1 Day
Lane **3:** 15 Days after addition of benzo[*a*]pyrene
Lane **4:** Control fungal culture not receiving benzo[*a*]pyrene



Mineralization of benzo[*a*]pyrene by *Bjerkandera* sp. strain BOS55 and indigenous microflora

The mineralization of benzo[*a*]pyrene metabolites by natural mixed cultures of microorganisms not previously adapted to PAH from four different inocula sources was examined. The inocula included activated sludge, two acidic forest soils, and an enrichment culture grown on 2,2'-diphenic acid. The results of this kind of experiment is shown in ***Figure** 3*; in this experiment [^{14}C]benzo[*a*]pyrene was oxidized for 15 days by Mn-sufficient cultures of *Bjerkandera* sp. strain BOS55 before activated sludge was added to the fungal cultures. Just before the activated sludge was added, the fungal cultures had already mineralized 8% of the benzo[*a*]pyrene. Just after the activated sludge was added, the level of mineralization rapidly increased to 20% in a few days, and thereafter it increased slowly to 27% by day 56 of the experiment. Adding 5 ml of fresh activated sludge and 0.02% yeast extract at this point had no positive effect on the $^{14}CO_2$ production. In the control fungal cultures that did not receive

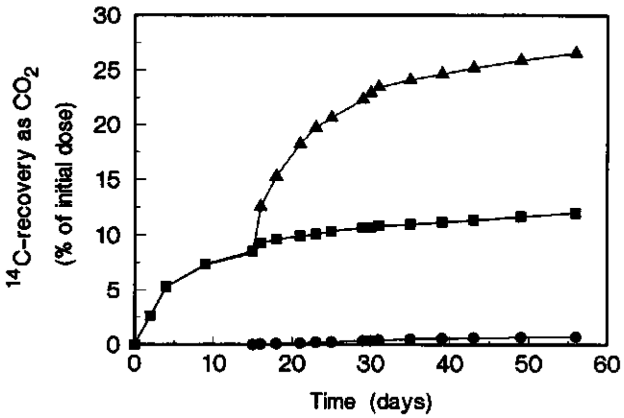


Figure 3.

Mineralization of $[^{14}C]$benzo[*a*]pyrene by fungal cultures, by activated sludge, and by the combination of both.

Day 0 is the time of benzo[*a*]pyrene addition to the 6-day-old fungal cultures, at day 15 the activated sludge is added.

Symbols:
(■) Fungus
(▲) Fungus + activated sludge
(●) Dead fungus + activated sludge + intact $[^{14}C]$benzo[*a*]pyrene

activated sludge, the level of mineralization was only 12% on day 56. Adding autoclaved sludge to the fungal cultures had no effect on $^{14}CO_2$ production (*results not shown*).

Similar results were obtained with the other natural inocula as well, as shown in ***Table 1***. The maximum extent of benzo[*a*]pyrene mineralization was somewhat lower when the 2,2'-diphenic acid enrichment culture was used.

In control cultures in which these inocula were directly incubated with intact benzo[*a*]pyrene in the presence or absence of an autoclaved fungal culture, very low levels (only 0.4-1%) of benzo[*a*]pyrene mineralization were observed at the end of the experiment, as shown in ***Figure 3*** for activated sludge in the presence of an autoclaved fungal culture. Also, incubation of benzo[*a*]pyrene with the various inocula resulted in no significant decreases in benzo[*a*]pyrene concentration.

The levels of recovery of water-soluble label in the culture fluids were also monitored. On day 15 of the experiment, 72% of the ^{14}C-label was present in water-soluble metabolites in the fungal cultures. In the cultures not receiving indigenous microflora, 69% of the label was still recovered in water-soluble metabolites 29 days later. On the other hand, only 48 and 37% of ^{14}C-labeled water-soluble metabolites remained in the cultures supplied with activated sludge and the forest soils, respectively. As shown in ***Table 1***, the experiment was extended to day 215, and at that time the $^{14}CO_2$ production rate was virtually zero. The levels of recovery in water-soluble metabolites in the fungal cultures were still as high as 61%, whereas in the indigenous microflora-inoculated cultures the levels of recovery were much lower. In the culture with the highest level of recovery of $^{14}CO_2$, 34% (Forest 2), only 16% of the label was recovered in water-soluble metabolites at the end of the experiments. In all cultures the total levels of recovery of label in the culture fluids by acetone extraction were slightly higher than the levels of recovery in water-soluble metabolites alone. Between 1 and 2% of the label was extractable with dibutyl ether (*results not shown*). The amount of label associated with the biomass was not determined in this experiment; by assuming a normal conversion of the metabolites to CO_2 (60%) and biomass (40%), mass balances ranging from 80 to 90% were obtained.

The mineralization of benzo[*a*]pyrene metabolites by a natural mixed culture previously adapted to PAH pollution was also examined in a similar experiment. As shown in ***Figure 4***, addition of this PAH-adapted culture had an effect on the $^{14}CO_2$ production similar to the effect of the addition of non-adapted microorganisms (***Fig. 3***). A rapid increase in

$^{14}CO_2$ production was observed during the first days, and then the rate slowed down, resulting in a total level of mineralization of 26% by day 105. In this experiment, the recovery in water-soluble metabolites had decreased to 46%.

In contrast to the results obtained with the nonadapted mixed cultures, incubation of intact benzo[*a*]pyrene with the PAH-adapted sludge resulted in fast mineralization after a lag period of 5 days. However, mineralization by cultures stopped at a lower level (17%) than mineralization of benzo[*a*]pyrene by the fungus and adapted microflora combined. On day 105, 3% of the label was recovered in water-soluble metabolite in these cultures, and in parallel experiments without label HPLC analysis showed that 60% of the initial amount of benzo[*a*]pyrene was still present in these cultures.

Mutagenicity studies

Ames tests were performed to monitor the changes in the mutagenic potential of benzo[*a*]pyrene during its oxidation and mineralization. In the experiments performed with *Salmonella typhimurium* strain TA100, no increase in the number of revertants was observed in the fungal culture broth itself in the absence of rat-liver activation mix (S-9 mix) compared to the spontaneous number of revertants (65 revertants per plate). However, the fungal culture broth did cause a small increase (10 to 20 revertants) in the presence of S-9 mix throughout the experiment compared to the number of spontaneous revertants in the presence of S-9 mix (72 revertants per plate). Addition of benzo[*a*]pyrene (20 mg liter^{-1}) to the fungal cultures resulted in high mutagenic activity only when the S-9 mix was added, and this mutagenic ac-

Table 1. The effect of addition of mixed cultures to fungal cultures with oxidized [^{14}C]benzo[*a*]pyrene at day 15 on the recovery of label from [^{14}C]benzo[*a*]pyrene as $^{14}CO_2$, water soluble metabolites, and the total recovery of label in culture medium by acetone.

Treatment	Recovery label as $^{14}CO_2$ (%)		Recovery label on day 215	
	Day 56	Day 215	By acetone (%)	In water phase (%)[a][b]
Fungus	12.0	13.5	66	61
+ Act. Sludge[c]	26.6	28.7	39	33
+ Forest 1	25.8	32.0	25	18
+ Forest 2	26.9	34.0	24	16
+ Dip. culture[d]	20.4	21.7	45	40

[a]: Duplicate measurement

[b]: Measured by filtration through hydrophilic filter

[c]: Activated sludge

[d]: Enrichment culture on 2,2'-diphenic acid

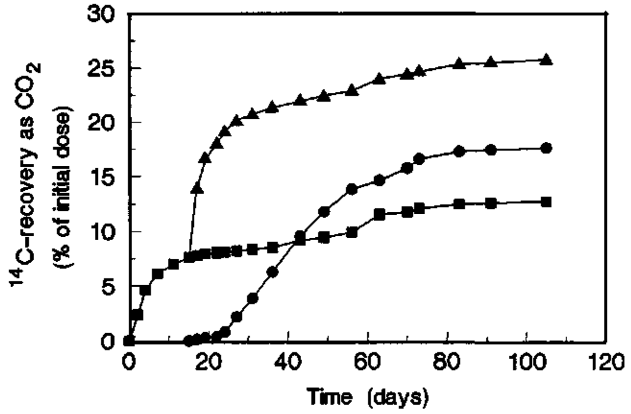


Figure 4.

Mineralization of [^{14}C]benzo[*a*]pyrene by fungal cultures, by PAH-adapted sludge, and by the combination of both.

Day 0 is the time of benzo[*a*]pyrene addition to the 6-day-old fungal cultures, at day 15 the adapted sludge is added.

Symbols:
(■) Fungus
(▲) Fungus + PAH-adapted sludge
(●) Dead fungus + PAH-adapted sludge + intact [^{14}C]benzo[*a*]pyrene

tivity was reduced to background levels within 15 days when incubated with *Bjerkandera* sp. strain BOS55 (***Fig.*** *5*). No decrease in the mutagenic potential of benzo[*a*]pyrene was caused by autoclaved fungal cultures (*results not shown*).

In the case of strain TA98, no increase in the number of revertants was observed by the fungal culture broth compared to the average number of spontaneous revertants (14 revertants per plate). In the presence of S-9 mix, the fungal culture broth caused an increase of 15 to 20 revertants per plate throughout the experiment compared to the number of spontaneous revertants in the presence of S-9 mix (28 revertants per plate). Again, benzo[*a*]-pyrene-containing cultures displayed high mutagenic activity only in the presence of S-9 mix. Although this high mutagenic activity was quickly reduced by fungal activity, it remained slightly higher than the mutagenic activity of the fungal culture itself. Addition of activated sludge on day 15 to the cultures containing oxidized benzo[*a*]pyrene metabolites further reduced the number of revertants in the presence of S-9 mix. The addition of activated sludge reduced the number of revertants in the fungal culture broth in the presence of S-9 mix to background levels (*results not shown*).

DISCUSSION

In this study, mineralization of the recalcitrant pollutant benzo[*a*]pyrene was investigated by successively incubating of [^{14}C]benzo[*a*]pyrene with the white rot fungus *Bjerkandera* sp. strain BOS55 and natural mixed cultures of microorganisms from soil, sediment and sludge. *Bjerkandera* sp. strain BOS55 can oxidize PAH very rapidly in high-nitrogen cultures, supplied with adequate H_2O_2 for maximal peroxidase activity and the surfactant Tween 80 to improve PAH bioavailability. Under such conditions, benzo[*a*]pyrene supplied at a concentration of 50 mg liter^{-1} was oxidized at rates up to 450 mg liter^{-1} day^{-1} (Kotterman *et al.*, 1998). In the experiments performed here with ^{14}C-labeled benzo[*a*]pyrene, we found that with a level of recovery of 8% $^{14}CO_2$, at an initial mineralization rate of 0.3 mg liter^{-1} day^{-1}, water-soluble metabolites accounting for up to 73% of the label accumulated after 15 days. These results correlate well with the results of other studies in which oxidation of PAH was examined, although the results depend strongly on the white rot species and strain used. For [^{14}C]benzo[*a*]pyrene, levels of $^{14}CO_2$ recovery between 0.17 and 15% have been reported in studies with different species (Sanglard *et al.*, 1986; Bezalel *et al.*, 1996; Bogan and Lamar, 1996). The initial level of accumulation of water-soluble metabolites observed in this study is high compared to the levels found in other studies. This difference can be attributed to both the species used and the definition of the term water-soluble.

The metabolites of peroxidase-mediated benzo[*a*]pyrene oxidation have not been identified yet. The initial oxidation products have been identified as benzo[*a*]pyrenequinones (Haemmerli, 1988), but these quinones are rapidly further oxidized to more water-soluble products with unknown structure by cultures of *Phanerochaete laevis* and *Phanerochaete chrysosporium* (Sanglard *et al.*, 1986; Bezalel *et al.*, 1996, Bogan and Lamar, 1996). Peroxidase-mediated oxidation of the low-molecular-weight PAH, such as anthracene and phenanthrene, also results in quinones. Depending on the strain used, anthraquinones are either accumulated as metabolites (Field *et al.*, 1992; Andersson and Henrysson, 1996) or are further oxidized to unidentified products (Field *et al.*, 1992; Andersson and Henrysson, 1996) or phthalate (Hammel *et al.*, 1991). Oxidation of phenanthrenequinone to 2,2'-diphenic acid

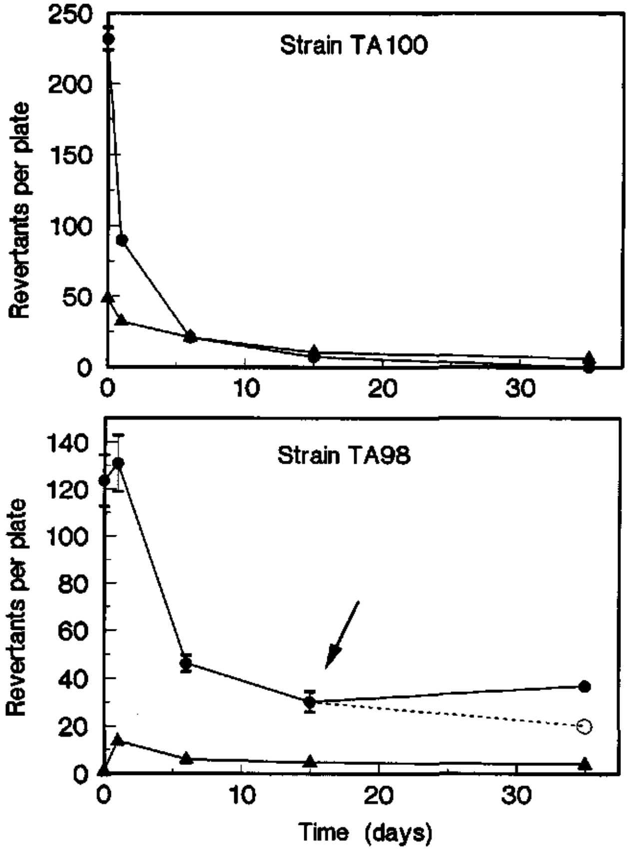


Figure 5.

Mutagenic activity of benzo[*a*]pyrene and benzo[*a*]pyrene metabolites towards *Salmonella typhimurium* strains TA100 and TA98.

At time 0, benzo[*a*]pyrene (20 mg liter^{-1}) was added to 6-day-old Mn-sufficient fungal cultures. At selected time points samples were taken and tested. The number of spontaneous revertants in the controls is subtracted from the data shown.
The arrow indicates the time of activated sludge addition.

Symbols:
(●) Fungal cultures + benzo[*a*]pyrene + S-9 mix
(▲) Fungal cultures + benzo[*a*]pyrene
(○) Effect of activated sludge addition

by ligninolytic cultures of *Phanerochaete chrysosporium* has been reported (Hammel *et al.*, 1992). The observation in this study that all benzo[*a*]pyrene metabolites detected by autoradiography also were highly fluorescent under UV light indicates that the polyaromatic structure of benzo[*a*]pyrene was not completely destroyed. The high water solubility of these metabolites can presumably be attributed to the presence of carboxyl and/or hydroxyl groups. The presence of carboxyl groups is suggested because the acidification of the culture media greatly increased the efficiency of extraction of these metabolites with ethyl acetate.

The interest in white rot fungi for PAH degradation is mainly fueled by the slow bacterial PAH breakdown. It has been reported many times that low bioavailability of PAH is the main factor limiting bacterial PAH degradation (Stucki and Alexander, 1987; Volkering *et al.*, 1992), and up till now, the attempts to improve PAH bioavailability and degradability by use of surfactants have not been very successful (Rouse *et al.*, 1994). Oxidation of PAH by white rot fungi to more water-soluble products with greater bioavailability could therefore result in rates of mineralization of these metabolites by bacteria higher than the rates of mineralization of the parent PAH compounds. A study performed with the 3-ring PAH anthracene has confirmed that all known oxidation products of this compound are mineralized by activated sludge more rapidly than anthracene itself is mineralized (Meulenberg *et al.*, 1997).

Addition of undefined microbial inocula, irrespective of the source, to oxidized benzo[*a*]pyrene metabolites clearly resulted in initially rapid mineralization rates, comparable to 1.0 mg of benzo[*a*]pyrene liter^{-1} day^{-1}. Benzo[*a*]pyrene not previously subjected to fungal oxidation was not mineralized at all by the inocula not adapted to PAH, whereas PAH-adapted sludge mineralized intact benzo[*a*]pyrene at a rate of only 0.1 mg of benzo[*a*]pyrene liter^{-1} day^{-1}. This confirms that fungal preoxidation of PAH increases the rate of mineralization by bacteria. A similar synergistic effect of a combination of white rot fungi and soil microorganisms has also been observed for pyrene mineralization with *Pleurotus* sp. and *Dichomitus squalens* (In der Wiesche *et al.*, 1996). Andersson and Henrysson (1996) showed that the dead-end metabolites of both anthracene oxidation and benzo[*a*]anthracene oxidation by *Phanerochaete chrysosporium* were slowly further degraded in the presence of nonadapted soil microorganisms.

By the end of the successive mineralization experiments, the maximum yields of $^{14}CO_2$ were between 39 and 47% and at least 16% of the initial label remained in water-soluble metabolites. The lack of mineralization of this fraction could not be attributed to toxicity of the metabolites, lack of trace elements or vitamins. This suggests that not all of the fungus-oxidized metabolites were easily mineralized by indigenous microflora. If these metabolites are cometabolically degraded by bacteria, as has been described for higher PAH, such as benzo[*a*]pyrene and dibenzo[*ah*]anthracene (Heitkamp and Cerniglia, 1987; Juhasz *et al.*, 1997), the absence of a suitable cosubstrate could explain the absence of complete mineralization. However, the addition of glucose, autoclaved spent fungal medium or activated sludge as a cosubstrate did not have any significant effect. This suggests that the remaining metabolites have structures that are poorly degradable.

Degradation of PAH like benzo[*a*]pyrene is desired since oxidation by eukaryotic monooxygenases can result in metabolites with high carcinogenic activity (Sutherland, 1992). The lack of complete mineralization of benzo[*a*]pyrene after sequential treatment by fungi and bacteria made it necessary to monitor the mutagenic activity during this treatment. The high mutagenic activity of S-9 mix-activated benzo[*a*]pyrene towards *Salmonella typhimurium* TA100 and TA98 rapidly decreased during fungal incubation without any significant accumulation of direct or indirect mutagens. This suggests that either the intracellular monooxygenases were not involved in the oxidation of benzo[*a*]pyrene or their oxidation products did not accumulate. In any case, involvement of the extracellular peroxidases, which previously have been shown to rapidly oxidize anthracene and benzo[*a*]pyrene (Kotterman *et al.*, 1998), was in this study demonstrated by the extensive oxidation of benzo[*a*]pyrene in the extracellular culture fluids of 6-day-old cultures of *Bjerkandera* sp. strain BOS55.

This research showed that benzo[*a*]pyrene is quickly oxidized into polar, water-soluble compounds by the white rot fungus *Bjerkandera* sp. strain BOS55. Most of these metabolites could be mineralized by non-PAH-adapted microbial indigenous communities under aerobic conditions. The lack of complete mineralization is not a serious setback for the use of white rot fungal techniques in PAH bioremediation, since the highly mutagenic potential of the parent compound was eliminated.

REFERENCES

Ames, B. A., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research. **31:**347.

Andersson, B. E., and T. Henrysson. 1996. Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white-rot fungi. Appl. Microbiol. Technol. **46:** 647-652.

Aust, S. D. 1990. Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microbiol. Ecol. **20:**197-209.

Bezalel, L., Y. Hadar, and C. E. Cerniglia. 1996. Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. Appl. Environm. Microbiol. **62:**292-295.

Bogan, B. W., and R. T. Lamar. 1996. Polycyclic aromatic hydrocarbon degrading capabilities of Phanerochaete leavis HHB-1625 and its extracellular ligninolytic enzymes. Appl. Environ. Microbiol. **62:**1597-1603.

Bossert, I. D., and R. Bartha. 1986. Structure-biodegradability relationships of polycyclic aromatic hydrocarbons in soil. Bull. Environ. Contam. Toxicol. **37:**490-495.

De Jong, E., J. A. Field, and J. A. M. de Bont. 1992. Evidence for a new extracellular peroxidase: manganese inhibited peroxidase from the white-rot fungus *Bjerkandera* sp. strain BOS55. FEBS Lett. **299:**107-110.

Field, J. A., E. de Jong, G. Feijoo-Costa, and J. A. M. de Bont. 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white-rot fungi. Appl. Environ. Microbiol. **58:**2219-2226.

Field, J. A., E. de Jong, G. Feijoo Costa, and J. A. M. de Bont. 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol. **11:**44-49.

Haemmerli, S. D. 1988. Degradation of polycyclic aromatic hydrocarbons. Chapter 3.2 in Lignin Peroxidase and the Ligninolytic System of *Phanerochaete chrysosporium*, PhD Dissertation, Swiss Federal Institute of Technology (Diss. ETH No. 8670), Zurich, pp. 49-61.

Hammel, K. E., W. Z. Gai, B. Green, and M. A. Moen. 1992. Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**1832-1838.

Hammel, K. E., B. Green, and W. Z. Gai. 1991. Ring fission of anthracene by a eukaryote. Proc. Natl. Acad. Sci. USA. **88:**10605-10608.

REFERENCES

Heitkamp, M. A., and C. E. Cerniglia. 1987. Effects of chemical structure and exposure on the microbial degradation of polycyclic aromatic hydrocarbons in freshwater and estuarine ecosystems. Environ. Toxicol. Chem. **6:**535-546.

In der Wiesche, C., R. Martens, and F. Zadrazil. 1996. Two-step degradation of pyrene by white-rot fungi and soil microorganisms. Appl. Microbiol. Biotechnol. **46:**653-659.

Juhasz, A. L., M. L. Britz, and G. A. Stanley. 1997. Degradation of fluoranthene, pyrene, benzo[*a*]anthracene and dibenzo[*a,h*]anthracene by *Burkholderia cepacia*. J. Appl. Microbiol. **83:**189-198.

Kaal, E. E. J., E. de Jong, and J. A. Field. 1993. Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. **59:**4031-4036.

Kimura, Y., Y. Asada, and M. Kuwahara. 1990. Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl. Microbiol. Biotechnol. **32:**436-442.

Kotterman, M. J. J., E. Heessels, E. de Jong, and J. A. Field. 1994. The physiology of anthracene biodegradation by the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Microbiol. Biotechnol. **42:**179-186.

Kotterman, M. J. J., R. A. Wasseveld, and J. A. Field. 1996. Hydrogen peroxide as a limiting factor in xenobiotic compound oxidation by nitrogen-sufficient cultures of *Bjerkandera* sp. strain BOS55 overproducing peroxidases. Appl. Environ. Microbiol. **62:**880-885.

Kotterman, M. J. J., H-J. Rietberg, A. Hage, and J. A. Field. 1998. Polycyclic aromatic hydrocarbon oxidation by the white-rot fungus *Bjerkandera* sp. strain BOS55 in the presence of nonionic surfactants. Biotechnol. Bioeng. **57:**220-227.

Maron, D. M., and B. R. Ames. 1983. Revised methods for the Salmonella mutagenicity test. Mutation research. **113:**173-215.

Mester, T., E. de Jong, and J. A. Field. 1995. Manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidase. Appl. Environ. Microbiol. **61:**1881-1887.

Mester, T., M. Pena, and J. A. Field. 1996. Nutrient regulation of extracellular peroxidases in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Microbiol. Biotechnol. **44:**778-784.

Meulenberg, R., H. H. M. Rijnaarts, H. J. Doddema, and J. A. Field. 1997. Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. Fems Microbiol. Letters. **152:**45-49.

Rouse, J. D., D. A. Sabatini, J. M. Suflita, and J. H. Harwell. 1994. Influence of surfactants on microbial degradation of organic compounds. Critical Rev. Environ. Sci. Technol. **24:**325-370

Sanglard, D., S. A. Leisola, and A. Fiechter. 1986. Role of extracellular ligninases in biodegradation of benzo[*a*]pyrene by *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **8:**209-212

Stucki, G., and M. Alexander. 1987. Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl. Environ. Microbiol. **53:**292-297.

Sutherland, J. B. 1992. Detoxification of polycyclic aromatic hydrocarbons by fungi. J. Industrial Microbiol. **9:**53-62.

Thakker, D. R., H. Yagi, W. Levin, A. W. Wood, A. H. Conney, and D. M. Jerina. 1985. polycyclic aromatic hydrocarbons: metabolic activation to ultimate carcinogens. In: Bioactivation of Foreign Compounds (Anders, M.W., ed.),pp. 177-242, Academic Press, Orlando.

Tien, M. and T. K. Kirk. 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods of Enzymology. **161B:**238-248.

Volkering, F., A. M. Breure, A. Sterkenburg, and J. G. van Andel. 1992. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Appl. Microbiol. Biotechnol. **36:**548-552.

Wilson, S. C., and K. C. Jones. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a Review. Environm. Pollution **81:**229-249.

Chapter 6

Summary and concluding remarks

An alternative approach for bioremediation of PAH polluted soils has been investigated in this thesis. The approach is based on the ability of white rot fungi to oxidize PAHs with an extracellular oxidative enzyme system. In this chapter, the main results of this study are discussed and compared to literature data.

First, this chapter will show the evidence that PAHs are oxidized extracellularly by the ligninolytic enzymes of the white rot fungus *Bjerkandera* sp. strain BOS55. The parameters which influence the PAH degrading capacity of the white rot fungus are then discussed. The results concerning the degradation of PAHs with respect to mineralization, accumulation of metabolites and effect on mutagenicity are summarized. Finally, the future prospects of white rot fungal bioremediation of PAH polluted soils are discussed briefly.

PAH degradation by white rot fungi

Soon after the initial observation that PAHs are oxidized by white rot fungi, the involvement of the extracellular ligninolytic enzyme system was indicated. Direct oxidation of PAH by the extracellular ligninolytic enzymes was first observed for LiP (Haemmerli, 1986; Hammel *et al.*, 1986), and later for MnP (Moen and Hammel, 1994; Field *et al.*, 1996b; Sack *et al.*, 1997) and laccase (Collins *et al.*, 1996; Johannes *et al.*, 1996).

In this study, further evidence was obtained demonstrating that extracellular oxidation by peroxidases is the main mechanism of PAH metabolism in white rot fungi (***Chapter 2, 3, 4 and 5***). The rate of PAH oxidation was similar in extracellular culture fluids as in the whole cultures. Furthermore, the same metabolite of anthracene oxidation, anthraquinone, was observed in similar yields in both extracellular culture fluids and whole cultures. Anthraquinone is a well-known metabolite of anthracene oxidation by ligninolytic enzymes (Haemmerli, 1988; Hammel *et al.*, 1991, Field *et al.*, 1996b; Sack *et al.*, 1997). Additionally, the oxidation of the ligninolytic indicator dye Poly R-478 was found to be correlated to PAH oxidation. The aromatic polymer Poly R-478 is a substrate of peroxidases and can only be oxidized extracellularly due to its excessive size. The involvement of peroxidases was also indicated by the stimulation of PAH and Poly R-478 oxidation by increasing the hydrogen peroxide production rate.

Oxidation of PAH by non-ligninolytic cultures of white rot fungi has also been reported (Sutherland *et al.*, 1991). The PAH metabolites observed, trans-dihydrodiols, suggested intracellular oxidation by P450 monooxygenases. White rot fungi have cytochrome P450 monooxygenases, which are capable of oxidizing PAH under physiological conditions when ligninolytic enzymes were not expressed (Masaphy *et al.*, 1996; Bezalel *et al.*, 1997). In this study, no significant participation of intracellular monooxygenases in the oxidation of PAH by ligninolytic cultures of *Bjerkandera* sp. strain BOS55 could be demonstrated.

Enhancement of the PAH oxidation by white rot fungi

In this study, the effect of several parameters, both physiological and non-physiological, on the PAH oxidation by *Bjerkandera* sp. strain BOS55 was monitored.

Physiological Parameters Limiting PAH Oxidation

Biomass

Since PAHs are not sole E- or C-sources for white rot fungi, a suitable cosubstrate is required for biomass production and ligninolytic activity. Consequently, oxidation of PAHs by white rot fungi is only observed in the presence of a cosubstrate (Aust, 1990; Morgan *et al.*, 1993). Both biomass production and mineralization of the 5-ring PAH benzo[*a*]pyrene in soil by several white rot fungi was stimulated by increasing concentrations of carbon sources like wood chips and wheat straw (Morgan *et al.*, 1993). Likewise, the PAH degradation by the white rot fungus *Bjerkandera* sp. strain BOS55 also depended on a suitable cosubstrate. This cosubstrate could either be a complex lignocellulose substrate such as hemp-stem-wood (HSW) or a simple substrate such as glucose (*Chapter* 2). The biomass production, the anthracene degradation as well as the decolorization of the ligninolytic indicator dye Poly R-478 increased with the glucose concentrations in the culture medium from 0 to 5 g liter^{-1} (containing only 2.2 mM nitrogen). Above these concentrations, no increase in biomass nor ligninolytic activity was observed.

Peroxidase titers

Stimulation of the degradative capacity of white rot fungi is often sought in selection of strains or culture conditions with higher peroxidase production (Orth *et al.*, 1991; Kaal *et al.*, 1993). Initially, N-limited media were thought to be a necessity for ligninolytic activity, since high-N repressed both the production of ligninolytic enzymes as well as the PAH degradation by the model white rot fungus *Phanerochaete chrysosporium* (Aust, 1990; Hammel 1992). The disadvantage of these media is the poor production of biomass and ligninolytic enzymes. *Bjerkandera* sp. strain BOS55, however, was shown to be N-unregulated, high nutrient nitrogen concentrations did not repress ligninolytic enzyme production nor PAH degradation (*Chapter* 2). Instead, the use of high organic N-nutrients even dramatically improved the peroxidase titers (Kaal *et al.*, 1993; Mester *et al.*, 1996). Although the peroxidase titers were improved up to 30-fold in high organic N media compared to the N-limited media in this study, the rate of anthracene oxidation was not remarkably increased (*Chapter* 3). Clearly the low peroxidase titers in the N-limited media were not the rate limiting factor in the oxidation rate.

Peroxidase profile

Manganese has an important impact on enzyme profiles and ligninolytic activity in white rot fungal cultures. The MnP titers in many white rot fungi are strongly stimulated by the presence of Mn (Bonnarme and Jeffries, 1990; Brown *et al.*, 1990), and Mn can also severely decrease LiP titers (Bonnarme and Jeffries, 1990; Perez and Jeffries, 1992). In the absence of Mn, LiP titers were also increased in *Bjerkandera* sp. strain BOS55; whereas, the presence of Mn stimulated the MnP titers and partially repressed LiP titers (Mester *et al.*, 1995). These different enzyme profiles clearly affected the anthracene oxidation rate (*Chapter* 3). In the absence of Mn, the anthracene oxidation rate was improved by up to 95%. However, no difference in the Poly R-478 oxidation rate was observed. These results suggest that LiP is a better anthracene oxidizing enzyme than MnP.

Addition of Mn to 6-day-old Mn-deficient cultures simultaneously with anthracene decreased the anthracene oxidation as well as the anthraquinone accumulation (*Chapter 3*), suggesting Mn, in some way, also affected the activity of the existing ligninolytic enzymes. Mn can scavenge reduced oxygen radicals like superoxide (Bono *et al.*, 1990; Rotschild *et al.*, 1998), which might be disadvantageous for the oxidation of anthracene.

Oxygen transfer

In static, low-nitrogen liquid cultures of *Bjerkandera* sp. strain BOS55, poor oxygen transfer into the cultures negatively affected the PAH oxidation rate (*Chapter 2*). Increasing the aeration by either increasing the surface area of the culture or applying an oxygen atmosphere dramatically improved the PAH oxidation rates. In high-N cultures, the PAH oxidation was limited by the oxygen transfer even in shallow cultures with high aeration surfaces (*Chapter 3*). Addition of an oxygen atmosphere to these high-N cultures enhanced the PAH oxidation rate up to 2.5-fold, resulting in an anthracene oxidation rate of 100 mg liter^{-1} day^{-1}. Apparently, the oxygen uptake rate by the metabolic activity of the fungus media interfered with the oxygen needed for PAH oxidation. High oxygen levels were shown previously to enhance the production of ligninolytic enzymes as well as ligninolytic activity (Reid and Seifert, 1982; Buswell 1991). During the short term experiments in this study, no effect of oxygen on the peroxidase titers was observed. Our study showed that improved aeration increased the endogenous hydrogen peroxide production rate in the fungal cultures, upon which the peroxidases are dependent for their oxidizing activity (*Chapter 3*).

H_2O_2 production rate

The effect of the hydrogen peroxide production rate on the PAH oxidation rate was further investigated. The endogenous hydrogen peroxide production rate in high-N cultures was enhanced 2.5-fold by improved aeration, which resulted in a 2.5-fold increase in PAH oxidation rate. A further 3.5-fold increase in the hydrogen peroxide production rate by an extra addition of glucose oxidase resulted in a 3.5-fold increase in anthracene oxidation rate up to 350 mg liter^{-1} day^{-1} (*Chapter 3*). Even in adequately aerated low N cultures with very low peroxidase titers, a small increase in hydrogen peroxide production rate by a small addition of glucose oxidase resulted in higher PAH oxidation rates. Apparently, the H_2O_2 production rate was more rate limiting than the peroxidase titers under both N-limiting and non-limiting culture conditions. This could have physiological significance, since it is well known that peroxidases can be inactivated by high H_2O_2 levels (Wariishi and Gold, 1989; Cai and Tien, 1992).

The results of our study clearly show that of the physiological parameters that improve PAH oxidation by *Bjerkandera* sp. strain BOS55, the hydrogen peroxide production rate is the most important parameter.

Non-physiological Parameters Limiting PAH Oxidation

The most appealing feature of white rot fungi is the ability to degrade poorly bioavailable high molecular weight PAHs, with their extracellular enzyme system (Hammel *et al.*, 1986; Field *et al.*, 1993). However, during the optimization of physiological parameters for PAH oxidation, a discrepancy was observed between the increase in anthracene oxidation rate and the increase in the oxidation rate of the ligninolytic indicator Poly R-478. The oxidation rate of the water-soluble dye Poly R-478 was consistently improved to a greater extent than the oxidation rate of the poorly water-soluble PAH anthracene, which was present as colloidal suspension. This led us to believe that the oxidation rate of anthracene was limited by the low aqueous solubility of anthracene (low bioavailability).

The hypothesis that the oxidation rate of PAHs by the ligninolytic enzymes was limited by the low bioavailability was confirmed by the 5-fold increase in the PAH oxidation rate when the bioavailability of the PAH was increased by the addition of surfactants to adequately aerated high-N cultures. The surfactants had no positive effect on the ligninolytic activity, instead, toxicity was observed. However, the partial loss of biocatalytic activity was clearly overcompensated by the increased bioavailability of PAH (*Chapter 4*).

The role of surfactants in increasing the bioavailability was shown to be due to their effect on decreasing the particle size of the PAH precipitates and on increasing the apparent aqueous solubility of the PAHs. Both factors are shown to increase PAH bioavailability (Volkering *et al.*, 1992; Rouse *et al.*, 1994; Tiehm, 1994; Volkering *et al.*, 1995; Field *et al.*, 1996b). The addition of surfacants often fails to stimulate bacterial degradation of PAH, since many surfactants can have toxic effects above the CMC (Rouse *et al.*, 1994) or preferential degradation of the surfactant occurs (Tiehm, 1994; Grimberg *et al.*, 1996). Tween 80, a commonly used surfactant that showed both high PAH solubilizing activity as well as low toxicity towards *Bjerkandera* sp. strain BOS55, was degraded rapidly by the fungus. The stimulatory effect of Tween 80 on the benzo[*a*]pyrene oxidation rate, however, was still observed after degradation of the surfactant. Apparently, the reduction in benzo[*a*]pyrene particle size by Tween 80 accounted for the increased bioavailability and hence the oxidation rate (*Chapter 4*).

The large effect of the two PAH oxidation rate limiting factors identified in this study, the hydrogen peroxide production rate and the PAH bioavailability, was demonstrated in extracellular culture fluids of high-N cultures of *Bjerkandera* sp. strain BOS55. By enhancing the hydrogen peroxide production rate with exogenous glucose oxidase and by enhancing the PAH bioavailability with surfactants, the anthracene oxidation rate could be increased 4- and 5-fold, respectively. The combination of both effects led to a 14-fold increase to a very high rate of anthracene oxidation of 1450 mg liter^{-1} day^{-1}. Under these conditions, the 5-ring PAH benzo[*a*]pyrene was oxidized at a rate of 450 mg liter^{-1} day^{-1}.

Fate and environmental impact of PAH degradation by white rot fungi

In this study, the degradation of the 5-ring PAH benzo[*a*]pyrene by *Bjerkandera* sp. strain BOS55 was monitored in several ways. The extent of mineralization to CO_2 and the accumulation of metabolites, as well as the effect of oxidation on the highly mutagenic potential of benzo[*a*]pyrene was investigated (*Chapter 5*).

PAH mineralization

White rot fungal degradation of PAHs by the ligninolytic enzyme system does not result in complete mineralization, in general the main effect is the accumulation of more polar products (Sanglard *et al.*, 1986; Bumpus *et al.*, 1989; Bogan and Lamar, 1996). In this study, benzo[*a*]pyrene was only mineralized to a maximum of 13%, but was oxidized for up to 73% to water-soluble products by *Bjerkandera* sp. strain BOS55. These water-soluble metabolites showed strong fluorescence when illuminated with UV light, indicating a polyaromatic structure still existed. Since the recovery of these metabolites by solvent extraction was enhanced by acidification of the medium, the increased solubility of these metabolites is tentatively attributed to the presence of carboxyl groups. Carboxyl groups have been identified previously in PAH metabolites after white rot fungal oxidation. For example, 2,2'-diphenic acid and phthalate have been observed after oxidation of phenanthrene and anthracene, respectively (Hammel *et al.*, 1991; Moen and Hammel, 1994). Metabolites of benzo[*a*]pyrene oxidation by white rot fungi other than quinones have not yet been identified (Haemmerli *et al.*, 1986). In whole cultures, more polar, unidentified metabolites of benzo[*a*]pyrene accumulate (Sanglard *et al.*, 1986; Bogan and Lamar, 1996).

PAH detoxification

The beneficial effect of white rot fungal biodegradation of PAHs has been questioned, since oxidized PAH metabolites accumulate. PAHs are well known examples of compounds that can be activated into mutagens by intracellular monooxygenases (Sutherland, 1992), and white rot fungi have monooxygenases capable of oxidizing PAHs (Masaphy *et al.*, 1996; Bezalel *et al.*, 1997). Therefore, the accumulation of PAH metabolites is considered not to be desirable. So far, the effect of white rot fungal oxidation of PAHs on the mutagenicity has not been studied in detail. The mutagenicity of benzo[*a*]pyrene-quinones, the only identified metabolites of white rot fungal benzo[*a*]pyrene oxidation so far, is largely reduced compared to the parent PAH (Thakker *et al.*, 1985). This study indicated that the ligninolytic oxidation of the infamous PAH benzo[*a*]pyrene, a notorious example of a PAH which can be activated by intracellular monooxygenases to highly mutagenic metabolites, did not result in mutagenic activation. On the contrary, the highly mutagenic potential of benzo[*a*]pyrene was

Table 1. The mineralization of PAH by indigenous microflora, by white rot fungi and the combination of both.

PAH	**Mineralization (% of initial concentration)**			**White rot fungus**	**References**
	Microflora	White rot fungi	Combination		
Phenanthrene	20	1	38	*Phanerochaete chrysosporium*	Brobkorb and Legge, 1992
Pyrene	N.D[a]	28	44	*Dichomitus squalens*	In der Wiesche *et al.*, 1996
	N.D	37	50	*Pleurotus* sp.	In der Wiesche *et al.*, 1996
	27	5	48	*Kuehneromyces mutabilis*	Sack and Fritsche, 1997
Benzo[*a*]pyrene	0-1	13	34	*Bjerkandera* sp. strain BOS55	Kotterman *et al.*, 1998

[a]: Not determined

drastically decreased, and no direct mutagenic activity of the metabolites was observed in the *Salmonella typhimurium* revertant test (AMES test) (***Chapter 5***).

Successive mineralization

To minimize the risks of accumulated PAH metabolites, further metabolism of the metabolites is desired. The PAH metabolites have higher aqueous solubilities than the parent PAH, therefore, these metabolites are likely to be better available for degradation by other microorganisms. This synergistic effect has been illustrated with anthraquinone, a well-known dead-end metabolite of anthracene oxidation by some white rot fungi (Field *et al.*, 1992, Anderson and Henrysson, 1996). Meulenberg *et al.* (1997) showed that this metabolite was degraded faster by non-adapted activated sludge and soil microflora than anthracene.

In this study, the addition of natural occurring microflora in soils, sediment sludge and activated sludge to cultures of *Bjerkandera* sp. strain BOS55 with oxidized [^{14}C]-radiolabeled benzo[*a*]pyrene resulted in a rapid increase in mineralization. A 21% higher recovery of $^{14}CO_2$ was observed upon addition of the natural occurring microflora, confirming that some of the metabolites had high bioavailability and biodegradability. These results also showed that the benzo[*a*]pyrene metabolites were mineralized faster and to a higher level by the added microflora than by the fungal culture itself. ***Table 1*** summarizes the main findings of this study together with other studies in the literature concerning the combined action of white rot fungi and indigenous microflora.

The results taken as a whole clearly show that the white rot fungal oxidized PAH metabolites are mineralized faster by indigenous microflora of different sources than their parent PAH. The metabolites of fungal oxidation are also better substrates for bacteria than for the white rot fungi themselves. The highest stimulatory effect of fungal "pre-oxidation" on the mineralization of PAHs is observed with benzo[*a*]pyrene, which also has the lowest bioavailability and degradability of the PAHs tested. Consequently, a faster and higher level of PAH mineralization is obtained by sequential treatment than by bacteria or fungi alone and the observed accumulation of white rot fungal PAH metabolites in pure cultures is therefore not likely to occur under non-sterile conditions, *e.g.* creosote contaminated soils.

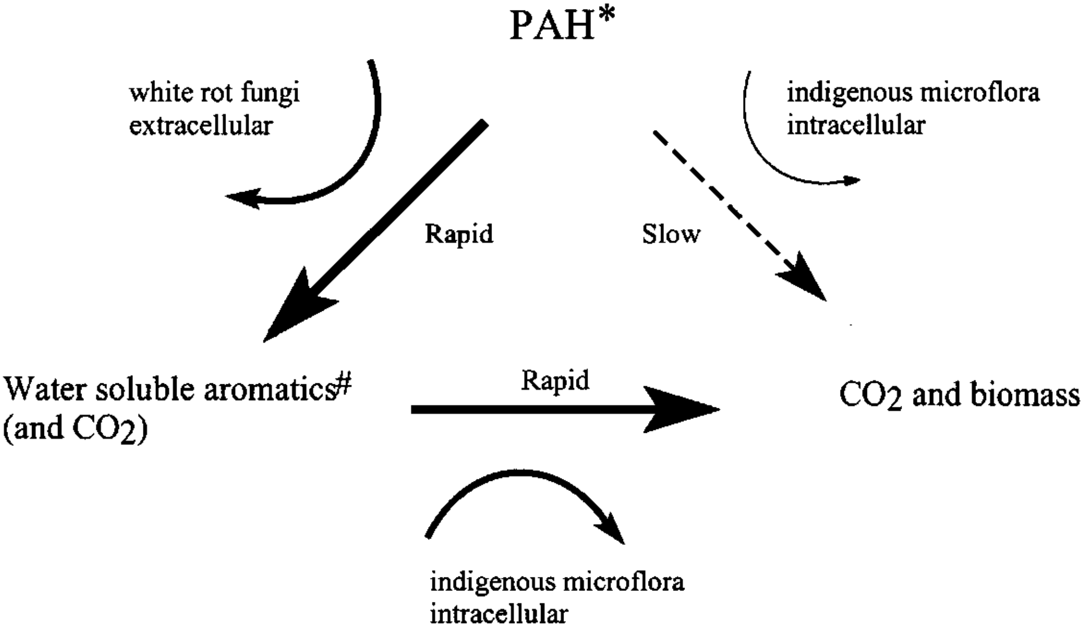
The results of this study are also depicted in ***Figure 1***, showing that both the mineralization as well as the detoxification of PAH can be accelerated by the use of white rot fungi.

Future prospect of white rot fungi in the bioremediation of PAH-contaminated soils

The effect of white rot fungi on the PAH removal in aged, industrially PAH-contaminated soils has been monitored. These studies show an increased elimination of in particular the 4-ring PAHs like pyrene compared to the degradation by the indigenous microflora alone, but little or no increase in the elimination of 5- and 6-rings PAHs (Davis *et al.*, 1993; Lamar *et al.*, 1994; Field *et al.*, 1996a). In spite of the improved overall PAH elimination, still high residual concentrations of PAHs were observed in these studies. Field *et al.* (1996a) showed that the recalcitrance of PAHs in soils from an old creosote-facility was due to the low PAH bioavailability; pretreatments that increased the bioavailability (presoaking of the soil in acetone and subsequent rapid evaporation of the acetone) increased the PAH degradation. Weissenfels *et al.* (1992) also observed higher PAH degradation by PAH adapted bacteria in a soil from a tar-oil refinery after a similar pretreatment. In contrast, *Bjerkandera* sp. strain BOS55 can rapidly degrade PAH in artificially contaminated soils (Field *et al.*, 1995) for which only low residual concentrations were observed.

The large difference between the PAH bioavailability in artificially contaminated and aged, industrially contaminated soils is probably mainly caused by the method of soil contamination. In artificially contaminated soils, PAHs are added to the soil dissolved in solvents. After evaporation of the solvent, PAH are likely to be present as precipitated particles. The degradation rate of this artificially added benzo[*a*]pyrene by *Bjerkandera* sp. strain BOS55 appeared to be influenced mainly by the particle size of these precipitates and not by the presence or absence of organic matter (non published results). Generally, the recalcitrance of PAH in soil systems is attributed partly to adsorption of PAH to adsorbents such as organic matter (Pignatello and Xing, 1996; Luthy *et al.*, 1997). In contaminated soils at gasification sites and creosote-facilities, the PAH spills are associated with non-aqueous-phase liquids (NAPLs) such as coal-tar and mineral oils. Due to the high hydrophobicity, PAHs are sequestered by the NAPLs, resulting in lower aqueous concentrations than in the absence of NAPLs (Efroymson and Alexander, 1995). Only very low mass transfer of PAH out of NAPLs has been observed (Efroymson and Alexander, 1994; Yeom *et al.*, 1996), which can be decreased even more by weathering and hardening (Luthy *et al.*, 1997), severely decreasing the bioavailability.

Figure 1. Accelerated mineralization and detoxification of PAHs by the use of white rot fungi.



(*): Low water solubility, low bioavailability, and highly mutagenic potential

(#): Increased water solubility, increased bioavailability, and decreased mutagenic potential

Whether PAH-contaminated soils can be bioremediated successfully in the near future strongly depends on the bioavailability of PAH in the specific soil, and therefore on methods that can possibly increase this bioavailability. The use of surfactants, which have been successfully applied in liquid cultures (***Chapter** 4*), also resulted in higher PAH degradation by *Bjerkandera* sp. strain BOS55 in both artificially contaminated as aged industrially contaminated soils (*non published results*). The use of non-toxic, relatively persistent surfactants could therefore increase the potential of biological remediation of PAH-contaminated soils.

REFERENCES

Andersson, B. E., and T. Henrysson. 1996. Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white-rot fungi. Appl. Microbiol. Biotechnol. **46:**647-652.

Aust, S. D. 1990. Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microbiol. Ecol. **20:**197-209.

Bezalel, L., Y. Hadar, and C. E. Cerniglia. 1997. Enzymatic mechanisms involved in phenanthrene degradation by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. **63:**2495-2501.

Bogan, B. W., and R. T. Lamar. 1996. Polycyclic aromatic hydrocarbon degrading capabilities of Phanerochaete leavis HHB-1625 and its extracellular ligninolytic enzymes. Appl. Environ. Microbiol. **62:**1597-1603.

Bonnarme, P., and T. W. Jeffries. 1990. Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white-rot fungi. Appl. Environ. Microbiol. **56:**210-217.

Bono, J., P. Goulas, J. Boe, N. Portet, and J. Seris. 1990. Effect of Mn(II) on reactions catalyzed by lignin peroxidase from *Phanerochaete chrysosporium*. Eur. J. Biochem. **192:**189-193.

Brodkorb, T. S., and R. L. Legge. 1992. Enhanced biodegradation of phenanthrene in oil tar-contaminated soils supplemented with *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**3117-3121.

Brown, J. A., J. K. Glenn, and M. H. Gold. 1990. Manganese regulates expression of manganese peroxidase by *Phanerochaete chrysosporium*. J. Bact. **172:**3125-3130.

Bumpus, J. A. 1989. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **55:**154-158.

Buswell, J. A. 1991. Fungal degradation of lignin. In: Arora D. K., B. Rai, K. G. Mukerji, G. Kundsen (eds) Handbook of applied mycology, vol 1. Soil and plants. M Dekker, New York, pp 425-480.

Cai, D., and M. Tien. 1992. Kinetic studies on the formation and decomposition of compounds II and III. Reactions of lignin peroxidase with H_2O_2. J. Biol. Chem. **267:**11149-11155.

Collins, P. J. J., M. J. J. Kotterman, J. A. Field, and A. D. W. Dobson. 1996. Oxidation of anthracene and benzo[*a*]pyrene by laccase from *Trametes versicolor*. Appl. Environ. Microbiol. **62:**4563-4567.

Davis, M. W., J. A. Glaser, J. W. Evans, and R. T. Lamer. 1993. Field evaluation of the lignin-degrading fungus *Phanerochaete sordida* to treat creosote-contaminated soil. Environ. Sci. Technol. **27:**2572-2576.

Efroymson, R. A., and M. Alexander. 1994. Biodegradation in soil of hydrophobic pollutants in nonaqueous-phase liquids (NAPLs). Environ. Toxicol. Chem. **13:**405-411.

Efroymson, R. A., and M. Alexander. 1995. Reduced mineralization of low concentrations of phenanthrene because of sequestering in nonaqueous-phase liquids. Environ. Sci. Technol. **29:**515-521.

Field, J. A., E. de Jong, G. Feijoo-Costa, and J. A. M. de Bont. 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white-rot fungi. Appl. Environ. Microbiol. **58:**2219-2226.

Field, J. A., E. de Jong, G. Feijoo Costa, and J. A. M. de Bont. 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol. **11:**44-49.

Field, J. A., H. Feiken, A. Hage, and M. J. J. Kotterman. 1995. Application of a white rot fungus to biodegrade benzo[*a*]pyrene in soil. In: R. E. Hinchee, J, Fredrickson and B. C. Alleman (ed), Bioaugmentation for site remediation, Batelle press, Columbus, Ohio, USA, pp. 165-171.

Field, J. A., J. T. A. Van Veldhoven, B. Weller, C. Soeter, R. Wasseveld, J. T. C. Grotenhuis, and W. Rulkens. 1996a. Degradation of polycyclic aromatic hydrocarbons in polluted soil by the white rot fungus *Bjerkandera* sp. Strain BOS55. Proceedings of the Eight International Congress for Culture collections, Veldhoven, The Netherlands. R.A. Samsom, J.A Stalpers, D. van der Mei and A.H. Stouthamer (eds). pp 336-343.

Field, J. A., R. H. Vledder, J. G. van Zelst, and W. H. Rulkens. 1996b. The tolerance of lignin peroxidase and manganese dependent peroxidase to miscible solvents and the *in vitro* oxidation of anthracene in solvent:water mixtures. Enzyme Microbiol. Technol. **18:**300-308.

Grimberg, S. J., W. T. Stringfellow, and M. D. Aitken. 1996. Quantifying the biodegradation of phenanthrene by *Pseudomonas stutzeri* P16 in the presence of a nonionic surfactant. Appl. Environ. Microbiol. **62:**2387-2392.

Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter. 1986. Oxidation of benzo[*a*]pyrene by extracellular ligninases of *Phanerochaete chrysosporium*: veratryl alcohol, and stability of ligninase. J. Biol. Chem. **261:**6900-6903.

REFERENCES

Haemmerli, S. D. 1988. Degradation of polycyclic aromatic hydrocarbons. Chapter 3.2 in Lignin Peroxidase and the Ligninolytic System of *Phanerochaete chrysosporium*, PhD Dissertation, Swiss Federal Institute of Technology (Diss. ETH No. 8670), Zurich, pp. 49-61.

Hammel, K. E., B. Kalyanaraman, and T. K. Kirk. 1986. Oxidation of polycyclic aromatic hydrocarbons and dibenzo[*p*]-dioxins by *Phanerochaete chrysosporium*. J. Biol. Chem. **261**:16948-16952.

Hammel, K. E., B. Green, and W. Z. Gai. 1991. Ring fission of anthracene by a eukaryote. Proc. Natl. Acad. Sci. USA. **88**:10605-10608.

Hammel, K. E. *1992. Oxidation of aromatic pollutants by lignin-degrading fungi and their extracellular* peroxidases. H. Sigel and A. Sigel (eds.), Metal Ions in Biological Systems, Volume 28, Degradation of Environmental Pollutants by Microorganisms and Their Metalloenzymes. Marcel Dekker, Inc., NY, pp. 41-60.

In der Wiesche, C., R. Martens, and F. Zadrazil. 1996. Two-step degradation of pyrene by white-rot fungi and soil microorganisms. Appl. Microbiol. Biotechnol. **46**:653-659.

Johannes, C., A. Majcherczyk, and A. Hutterman. 1996. Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. Appl. Microbiol. Biotech. **46**:313-317.

Kaal, E. E. J., E. de Jong, and J. A. Field. 1993. Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. **59**:4031-4036.

Kotterman, M. J. J., E. H. Vis, and J. A. Field. 1998. Successive mineralization and detoxification of benzo[*a*]pyrene by the white rot fungus *Bjerkandera* sp. Strain BOS55 and indigenous microflora. Appl. Environ. Microbiol. **64**:2853-2858.

Lamar, R. T., M. W. Davis, D. D. Dietrich, and J. A. Glaser. 1994. Treatment of a pentachlorophenol- and creosote-contaminated soil using the lignin-degrading fungus *Phanerochaete sordida*: a field demonstration. Soil Biol. Biochem. **26**:1603-1611.

Luthy, R. G., G. R. Aiken, M. L. Brusseau, S. D. Cunningham, P. M. Gschwend, J. J. Pignatello, M. Reinhard, S. J. Traina, W. J. Weber, and J. C. Westall. 1997. Sequestration of hydrophobic organic contaminants by geosorbents. Environ. Science Technol. **31**:3341-3347.

Masaphy, S., D. Levanon, Y. Henis, K. Venkateswarlu, and S. L. Kelly. 1996. Evidence for cytochrome P-450 and P-450-mediated benzo[*a*]pyrene hydroxylation in the white rot fungus *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **135**:51-55.

Meulenberg, R., H. H. M. Rijnaarts, H. J. Doddema, and J. A. Field. 1997. Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. FEMS Microbiol. Lett. **152**:45-49.

Mester, T., E. de Jong, and J. A. Field. 1995. Manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidase. Appl. Environ. Microbiol. **61**:1881-1887.

Mester, T., M. Pena, and J. A. Field. 1996. Nutrient regulation of extracellular peroxidases in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Microbiol. Biotechnol. **44**:778-784.

Moen, M. A., and K. E. Hammel. 1994. Lipid peroxidation by the manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. Appl. Environ. Microbiol. **60**:1956-1961.

Morgan P., S. A. Lee, S. T. Lewis, A. N. Sheppard, and R. J. Watkinson. 1993. Growth and biodegradation by white-rot fungi inoculated into soil. Soil Biol. Biochem. **25**:279-287.

Orth, A. B., M. Denny, and M. Tien. 1991. Overproduction of lignin-degrading enzymes by an isolate of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **57**:2591-2596.

Perez, J., and T. W. Jeffries. 1992. Role of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysoporium*. Appl. Environ. Microbiol. **58**:2402-2409.

Pignatello, J. J., and B. Xing. 1996. Mechanisms of slow sorption of organic chemicals to natural particles. Environ. Science Technol. **30**:1-11.

Reid, I. D., and K. A. Seifert. 1982. Effect of an atmosphere of oxygen on growth, respiration, and lignin degradation by white-rot fungi. Can. J. Bot. **60**:252-260.

Rotschild, N., A. Levkowitz, Y. Hadar, and C. G. Dosoretz. 1988. Manganese deficiency can substitute high oxygen level needed to induce lignin peroxidase formation by *Phanerochaete chrysosporium*. Accepted for publication in Appl. Environ. Microbiol.

REFERENCES

Rouse, J.D., D. A. Sabatini, J. M. Suflita, and J. H. Harwell. 1994. Influence of surfactants on microbiol degradation of organic compounds. Critical Rev. Environ. Sci. Technol. **24:**325-370.

Sack. U., and W. Fritsche. 1997. Enhancement of pyrene mineralization in soil by wood-decaying fungi. FEMS Microbiol. Ecology **22:**77-83.

Sack, U., M. Hofrichter, and W. Fritsche. 1997. Degradation of polycyclic aromatic hydrocarbons by manganese peroxidase of *Nematoloma frowardii.* FEMS Microbiol. Lett. **152:** 227-234.

Sanglard, D., S. A. Leisola, and A. Fiechter. 1986. Role of extracellular ligninases in biodegradation of benzo[*a*]pyrene by *Phanerochaete chrysosporium.* Enzyme Microb. Technol. **8:**209-212.

Sutherland, J. B., A. L. Selby, J. P. Freeman, F. E Evans, and C. E. Cerniglia. 1991. Metabolism of phenanthrene by *Phanerochaete chrysosporium.* Appl. Environ. Microbiol. **57:**3310-3316.

Sutherland, J. B. 1992. Detoxification of polycyclic aromatic hydrocarbons by fungi. J. Industrial Microbiol **9:**53-62.

Thakker, D. R., H. Yagi, W. Levin, A. W. Wood, A. H. Conney, and D. M. Jerina. 1985. Polyclyclic aromatic hydrocarbons: Metabolic activation to ultimate carcinogens. In: Bioactivation of foreign compounds (Anders, M.W., ed.), pp. 177-242, Academic Press, Orlando.

Tiehm, A. 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. **60:**258-263.

Volkering, F., A. M. Breure, A. Sterkenburg, and J. G. van Andel. 1992. Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Appl. Microbiol. Technol. **36:**548-552.

Volkering, F., A. M. Breure, G. van Andel, and W. H. Rulkens. 1995. Influence of nonionic surfactants on bioavailability and biodegradation of polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. **61:**1699-1705.

Wariishi, H., and M. H. Gold. 1989. Lignin peroxidase compound III: formation, inactivation and conversion to the native enzyme. FEBS Lett. **243:**165-168.

Weissenfels, W. D., H-J Klewer, and J. Langhoff. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. Appl. Environ. Biotechnol. **36:**689-696.

Yeom, I. T., M. M. Ghosh, C. D. Cox, and K. H. Ahn. 1996. Dissolution of polycyclic aromatic hydrocarbons from weathered contaminated soil. Wat. Sci. Technol. **34:**335-342.

Samenvatting en afsluitende opmerkingen

In deze studie is een alternatieve biologische saneringsmethode voor met PAK verontreinigde grond onderzocht. De methode is gebaseerd op het vermogen van witrot-schimmels om PAKs te oxideren met een extracellulair oxidatief enzymsysteem. In dit hoofdstuk worden de belangrijkste resultaten bediscussieerd en vergeleken met literatuurwaarden. Allereerst wordt aangetoond dat PAKs door de extracellulaire ligninolytische enzymen van de witrotschimmel *Bjerkandera* sp. stam BOS55 worden geoxideerd. Daarna worden de factoren belicht die de mate van PAK-afbraak door de schimmel beïnvloeden. Als laatste is de afbraak van PAKs bestudeerd wat betreft mineralisatie, ophoping van metabolieten en de mutageniteit van deze metabolieten. Daarnaast wordt kort ingegaan op de mogelijke toepassing van witrot-schimmels in de biologische reiniging van PAK-vervuilde grond.

PAK-afbraak door witrot-schimmels

Al snel na de ontdekking dat PAKs door witrot-schimmels kunnen worden geoxideerd, werd de betrokkenheid van de extracellulaire, ligninolytische enzymen aangetoond. Oxidatie van PAKs door de ligninolytische enzymen is voor het eerst aangetoond voor lignine peroxidase (LiP) (Haemmerli *et al.*, 1986; Hammel *et al.*, 1986), en daarna voor mangaan peroxidase (MnP) (Moen en Hammel, 1994; Field *et al.*, 1996b; Sack *et al.*, 1997) en laccase (Collins *et al.*, 1996; Johannes *et al.*, 1997).

In dit onderzoek is wederom aangetoond dat de extracellulaire afbraak van PAKs het belangrijkste mechanisme is van PAK-metabolisme in witrot-schimmels (***Hoofdstuk 2, 3, 4, en 5***). PAKs werden even snel geoxideerd in de extracellulaire cultuurvloeistof als in de hele cultures (inclusief de schimmel-biomassa). Onder deze omstandigheden werd dezelfde metaboliet van anthraceen-oxidatie, anthraquinon, waargenomen in een identieke opbrengst. Anthraquinon is een bekend metaboliet van anthraceen-oxidatie door ligninolytische enzymen (Haemmerli, 1988, Hammel *et al.*, 1991; Field *et al.*, 1996b; Sack *et al.*, 1997). Daarnaast was de oxidatie van de kleurstof Poly R-478, een indicator van ligninolytische activiteit, gerelateerd aan de PAK-oxidatie. De aromatische polymeer Poly R-478 is een substraat van peroxidases en kan door zijn grootte alleen buiten de cel geoxideerd worden. De betrokkenheid van de peroxidases werd ook bevestigd door de stimulatie van PAK- en Poly R-478-oxidatie door een verhoogde waterstofperoxide-productiesnelheid.

Oxidatie van PAKs door niet-ligninolytische cultures van witrot-schimmels is ook waargenomen (Sutherland *et al.*, 1991). Echter, de geobserveerde PAK-metabolieten, transdihydrodiolen, suggereerden de betrokkenheid van de intracellulaire P450 mono-oxygenases. Witrot-schimmels bezitten cytochroom P450 mono-oxygenases die in staat zijn om PAKs te oxideren wanneer de ligninolytische enzymen niet tot expressie gebracht zijn (Masaphy *et al.*, 1996; Bezalel *et al.*, 1997). In dit onderzoek is echter geen significant aandeel van intracellulaire PAK-afbraak waargenomen in de ligninolytische cultures van *Bjerkandera* sp. stam BOS55.

Stimulatie van de PAK-oxidatie door witrot-schimmels

Het effect van verschillende parameters, zowel fysiologische als niet-fysiologische, op de afbraak van PAKs door *Bjerkandera* sp. stam BOS55 is bestudeerd in dit onderzoek.

Fysiologische Parameters die de PAK-afbraak Limiteren

Biomassa

Omdat PAKs niet als enige koolstof- en energie-bron kunnen dienen voor witrot-schimmels is een geschikt cosubstraat nodig voor zowel de productie van biomassa als voor ligninolytische activiteit. De oxidatie van PAKs wordt daarom alleen waargenomen in de aanwezigheid van een cosubstraat (Aust, 1990; Morgan *et al.*, 1993). Zowel de biomassaproductie als de mineralisatie van de 5-ring PAK benzo[*a*]pyreen door verschillende witrot-schimmels in grond werd gestimuleerd door toenemende koolstofbronconcentraties van houtsnippers en tarwestro (Morgan *et al.*, 1993). De PAK-afbraak door de witrot-schimmel *Bjerkandera* sp. stam BOS55 was ook afhankelijk van een geschikt cosubstraat. Dit cosubstraat kon zowel een complex ligninocellulose-substraat zijn als hennep houtpijp als een eenvoudig substraat als glucose (***Hoofdstuk* 2**). De biomassa-productie, de anthraceen-oxidatie en de Poly R-478 ontkleuring, een indicator van ligninolytische activiteit, namen toe met de verhoging van de glucoseconcentratie in het medium van 0 tot 5 g liter^{-1} (stikstof-concentratie 2.2 mM). Boven deze concentratie namen zowel de biomassa als de ligninolytische activiteit niet meer toe.

Peroxidase-titers

Stimulatie van de oxidatieve capaciteit van witrot-schimmels is vaak gezocht in de selectie van stammen of kweekcondities met hogere peroxidase-titers (Orth *et al.*, 1991; Kaal *et al.*, 1993). In eerste instantie werd het gebruik van stikstof-gelimiteerde kweekomstandigheden noodzakelijk geacht voor ligninolytische activiteit, want hoge stikstof-concentraties remde zowel de productie van ligninolytische enzymen als de PAK-afbraak door de model witrot-schimmel *Phanerochaete chrysosporium* (Aust, 1990; Hammel, 1992). Het nadeel van deze media is de lage productie van biomassa en ligninolytische enzymen. *Bjerkandera* sp. stam BOS55 is echter niet stikstof-gereguleerd; de productie van ligninolytische enzymen en de afbraak van PAKs werden niet geremd door hoge stikstof-concentraties (***Hoofdstuk* 2**). Het gebruik van hoge concentraties organische stikstof verhoogde de peroxidase-titers juist enorm (Kaal *et al.*, 1993). Ofschoon in dit onderzoek de peroxidase-titers tot 30-voud hoger waren in de organische stikstof-rijke media dan in de stikstof-gelimiteerde media werd de oxidatiesnelheid van anthraceen niet aanzienlijk verhoogd (***Hoofdstuk* 3**). De lage peroxidase-titers in de stikstof-gelimiteerde media waren dus duidelijk niet de snelheidsbeperkende factor.

Peroxidase-profiel

Mangaan (Mn) heeft een belangrijk effect op de enzym-profielen en ligninolytische activiteit in cultures van witrot-schimmels. De MnP-titers zijn in veel witrot-schimmels sterk verhoogd in de aanwezigheid van Mn (Bonnarme en Jeffries, 1990; Brown *et al.*, 1990) en Mn kan de LiP-titers sterk verlagen (Bonnarme and Jeffries, 1990; Perez and Jeffries, 1992). De afwezigheid van Mn verhoogde de LiP-titers in *Bjerkandera* sp. stam BOS55 sterk, terwijl de aanwezigheid van Mn de MnP-titers verhoogde en de LiP-titers gedeeltelijk represseerde

(Mester *et al.*, 1995). Deze verschillende peroxidase-profielen hadden een duidelijke uitwerking op de anthraceen-oxidatiesnelheid (***Hoofdstuk** 3*). In de afwezigheid van Mn was de anthraceen-oxidatiesnelheid tot 95% hoger. Er werd echter geen verschil in de Poly R-478 oxidatiesnelheid waargenomen. Deze resultaten suggereren dat LiP beter anthraceen kan oxideren dan MnP.

De toevoeging van Mn, tegelijkertijd met anthraceen, aan 6 dagen oude, Mn-deficiënte cultures, verlaagde zowel de anthraceen-afbraaksnelheid als de anthraquinon-ophoping (***Hoofdstuk** 3*). Dit suggereerde dat Mn op de een of andere manier ook de activiteit van de ligninolytische enzymen beïnvloedde. Mn kan gereduceerde zuurstof-radicalen zoals superoxide wegvangen (Bono *et al.*, 1990; Rothschild *et al.*, 1998), wat negatief zou kunnen zijn voor de afbraak van anthraceen.

Zuurstofoverdracht

In de statische, stikstof-gelimiteerde cultures van *Bjerkandera* sp. stam BOS55 wordt de PAK-oxidatie negatief beïnvloed door een slechte zuurstofoverdracht naar de cultuurvloeistof (***Hoofdstuk** 2*). Het verhogen van de beluchting door vergroting van het cultuurvloeistof-oppervlak danwel het toevoegen van een pure zuurstofatmosfeer stimuleerde de PAK-afbraak sterk. In stikstof-rijke cultures was de PAK-oxidatie zelfs in ondiepe cultures met een groot vloeistofoppervlak geremd door de zuurstofoverdracht (***Hoofdstuk** 3*). Het toevoegen van een zuurstofatmosfeer aan deze stikstof-rijke cultures stimuleerde de PAK-oxidatiesnelheid tot 2.5 keer, resulterend in een anthraceen-oxidatiesnelheid van 100 mg liter^{-1} dag^{-1}. Blijkbaar beïnvloedde het zuurstofverbruik door het schimmelmetabolisme de PAK-afbraak. Het was eerder aangetoond dat hoge zuurstofconcentraties de productie van ligninolytische enzymen en de ligninolytische activiteit bevorderden (Reid and Seifert, 1982; Buswell, 1991), tijdens de korte duur van de experimenten in dit onderzoek is echter geen effect van verhoogde zuurstofconcentraties op de peroxidase-titers waargenomen. Deze studie heeft laten zien dat de endogene waterstofperoxide-productiesnelheid, nodig voor de activiteit van de peroxidases, werd verhoogd door de verbeterde beluchting (***Hoofdstuk** 3*).

Waterstofperoxide-productiesnelheid

Het effect van de waterstofperoxide-productiesnelheid op de PAK-oxidatiesnelheid is verder onderzocht. De endogene waterstofperoxide-productiesnelheid in de stikstof-rijke cultures werd 2.5 keer door de verbeterde beluchting, wat resulteerde in een 2.5 keer hogere PAK-oxidatiesnelheid. Een verdere 3.5-voudige verhoging van de waterstofperoxide-productiesnelheid door een extra toevoeging van glucose-oxidase resulteerde in een 3.5-voudige verhoging van de anthraceen-oxidatiesnelheid tot 350 mg liter^{-1} dag^{-1} (***Hoofdstuk** 3*). Zelfs in goed beluchte, stikstof-gelimiteerde cultures werd de anthraceen-oxidatiesnelheid gestimuleerd door een lage dosis glucose-oxidase. Blijkbaar was de waterstofperoxide-productiesnelheid meer snelheidslimiterend dan de peroxidase-titers in zowel stikstof-gelimiteerde als stikstof-rijke kweekcondities. Dit kan van fysiologisch belang zijn, omdat de inactivatie van peroxidases door hoge waterstofperoxide-concentraties een bekend fenomeen is (Wariishi and Gold, 1989; Cai en Tien, 1992).

De resultaten van dit onderzoek laten duidelijk zien dat, van de fysiologische parameters die de PAK-afbraak door *Bjerkandera* sp. stam BOS55 verhogen, de waterstofperoxide-productiesnelheid de meest belangrijke is.

Niet-fysiologische parameters die de PAK-afbraak limiteren

De meest in het oog springende eigenschap van witrot-schimmels is het vermogen om PAKs met hoog-moleculair gewicht met een lage biologische beschikbaarheid af te breken met hun extracellulaire enzymsysteem (Hammel *et al.*, 1986; Field *et al.*, 1993). Tijdens de optimalisatie van de fysiologische parameters voor PAK-afbraak werd echter een diskrepantie waargenomen tussen de toename in de anthraceen-oxidatiesnelheid en de toename in de oxidatie van de ligninolytische indicator Poly R-478. De oxidatiesnelheid van de wateroplosbare kleurstof Poly R-478 werd consequent meer verhoogd dan de oxidatie-snelheid van de slecht oplosbare PAK anthraceen, welke aanwezig was als een colloïdale suspensie. Dit deed het vermoeden rijzen dat de oxidatiesnelheid van anthraceen gelimiteerd werd door de lage wateroplosbaarheid (lage biologische beschikbaarheid).

In goed beluchte, stikstof-rijke cultures werd de oxidatiesnelheid van PAKs tot 5 keer verhoogd als de biologische beschikbaarheid van de PAK werd verhoogd door de toevoeging van surfactants. Dit bevestigde dat de PAK-afbraaksnelheid door de ligninolytische enzymen ook werd beperkt door de lage biologische beschikbaarheid. De surfactants hadden geen positief effect op de ligninolytische activiteit; de surfactants vertoonden zelfs toxiciteit. Het gedeeltelijke verlies van biokatalytische activiteit werd echter meer dan gecompenseerd door de verhoogde biologische beschikbaarheid van de PAK (***Hoofdstuk** 4*).

Het mechanisme waarmee de surfactants de biologische beschikbaarheid verhoogden was zowel door verkleining van de PAK-deeltjesgrootte als door de verhoging van de schijnbare wateroplosbaarheid van de PAK. Voor beide factoren is aangetoond dat deze de biologische beschikbaarheid van PAKs verhogen (Thiem, 1994; Volkering *et al.*, 1992; Rouse *et al.*, 1994; Volkering *et al.*, 1995; Field *et al.*, 1996). De toevoeging van surfactants stimuleert de afbraak van PAKs door bacteriën vaak niet; veel surfactants vertonen toxiciteit boven de critische micelconcentratie (Rouse *et al.*, 1994), of de surfactant wordt preferent afgebroken (Grimberg *et al.*, 1996; Thiem, 1994). Tween 80, een algemeen gebruikt surfactant dat zowel een hoge PAK-solubiliserende werking als een lage toxiciteit voor *Bjerkandera* sp. stam BOS55 vertoonde, werd snel afgebroken door de schimmel. Het stimulerende effect van Tween 80 op de benzo[*a*]pyreen-afbraaksnelheid werd echter nog waargenomen nadat de surfactant was afgebroken. Blijkbaar had de verkleining van de benzo[*a*]pyreen-deeltjes nog steeds een positief effect op de biologische beschikbaarheid en dus de oxidatiesnelheid (***Hoofdstuk** 4*).

Het grote effect van de twee PAK-afbraaksnelheid limiterende factoren die in dit onderzoek zijn geïdentificeerd, de waterstofperoxide-productiesnelheid en de biologische beschikbaarheid van PAK, werd gedemonstreerd in de extracellulaire kweekvloeistof van stikstof-rijke cultures van *Bjerkandera* sp. stam BOS55. Door verhoging van de waterstofperoxide-productiesnelheid met extra glucose-oxidase en door verhoging van de biologische beschikbaarheid met surfactants werd de anthraceen-oxidatiesnelheid met respektievelijk 4- en 5-voud versneld. De combinatie van beiden resulteerde in een 14-voudige toename van de oxidatiesnelheid tot een erg hoge anthraceen-oxidatiesnelheid van 1450 mg liter^{-1} dag^{-1}. Onder deze omstandigheden werd de 5-ring PAK benzo[*a*]pyreen geoxideerd met een snelheid van 450 mg liter^{-1} dag^{-1}.

Resultaat en milieu-impact van PAK-oxidatie door witrot-schimmels

De afbraak van de 5-ring PAK benzo[*a*]pyreen door *Bjerkandera* sp. stam BOS55 is gevolgd op verschillende manieren in dit onderzoek. De mate van mineralisatie tot CO_2, de accumulatie van metabolieten en het effect op de hoge mutagene potentie van benzo[*a*]pyreen is onderzocht (*Hoofdstuk 5*).

PAK-mineralisatie

Afbraak van PAKs door witrot-schimmels leidt niet tot complete mineralisatie, in het algemeen treedt accumulatie van meer polaire producten op (Sanglard *et al.*, 1986; Bumpus *et al.*, 1989; Bogan en Lamar, 1996). In dit onderzoek werd benzo-[*a*]pyreen slechts voor een maximum van 13% gemineraliseerd en werd tot maximaal 73% geoxideerd tot wateroplosbare producten door *Bjerkandera* sp. stam BOS55. Deze wateroplosbare metabolieten vertoonden sterke fluorescentie onder UV-belichting, wat suggereerde dat deze verbindingen nog steeds een polyaromatische structuur bevatten. Omdat de recovery van deze metabolieten door extractie met organische oplosmiddelen werd verhoogd door aanzuring van het kweekmedium is de verhoogde wateroplosbaarheid van deze metabolieten waarschijnlijk te danken aan de aanwezigheid van carboxyl-groepen. Carboxyl-groepen zijn eerder aangetoond in PAK-metabolieten na oxidatie door witrot-schimmels; 2,2-difeenzuur en ftalaat zijn waargenomen na de oxidatie van respektievelijk anthraceen en fenanthreen (Hammel *et al.*, 1991; Moen en Hammel, 1994). Er zijn geen andere metabolieten van de oxidatie van benzo[*a*]-pyreen door ligninolytische enzymen dan quinonen geïdentificeerd (Haemmerli *et al.*, 1986). In complete cultures met schimmel-biomassa hopen andere, meer polaire metabolieten zich op (Sanglard *et al.*, 1986; Bogan en Lamar, 1996).

PAK-detoxificatie

Het gunstige effect van PAK-oxidatie door witrot-schimmels wordt soms in twijfel getrokken, omdat geoxideerde metabolieten ophopen. PAKs zijn algemeen bekende voorbeelden van stoffen die door intracellulaire mono-oxygenases kunnen worden geactiveerd tot sterk mutagene en carcinogene stoffen (Sutherland *et al.*, 1992) en witrot-schimmels hebben mono-oxygenases die in staat zijn tot PAK-oxidatie (Masaphy *et al.*, 1996; Bezalel *et al.*, 1997). De ophoping van geoxideerde metabolieten is daarom niet gewenst. Tot nog toe is het effect van PAK-oxidatie door witrot-schimmels niet gedetailleerd bestudeerd. De mutageniteit van benzo[*a*]pyreen-quinonen, de enige geïdentificeerde metabolieten van benzo[*a*]pyreen-oxidatie door witrot-schimmels tot nog toe, is voor een groot deel geëlimineerd in vergelijking tot de oorspronkelijke PAK (Thakker *et al.*, 1985). Dit onderzoek liet zien dat de ligninolytische oxidatie van benzo[*a*]pyreen, een berucht voorbeeld van een PAK die kan worden geactiveerd door intracellulaire mono-oxygenases tot sterk mutagene en carcinogene metabolieten, niet resulteerde in activering van mutagene activiteit. In tegendeel, de hoge mutagene potentie van benzo[*a*]pyrene was drastisch verminderd en geen directe mutagene metabolieten werden waargenomen in de *Salmonella typhimurium* revertant test (AMES test) (***Hoofdstuk** 5*).

Successieve mineralisatie

Om de risico's van geaccumuleerde PAK-metabolieten te minimaliseren is verder metabolisme van de metabolieten gewenst. De PAK-metabolieten zijn beter wateroplosbaar dan de oorspronkelijke PAKs en daardoor zijn deze metabolieten waarschijnlijk beter beschikbaar voor afbraak door andere micro-organismen. Zo'n synergistisch effect is aangetoond met anthraquinon, een bekend dead-end metaboliet van anthraceen-oxidatie door sommige witrot-schimmels (Field *et al.*, 1992; Anderson en Henrysson, 1996). Meulenberg *et al.* (1997) toonden aan dat deze metaboliet sneller werd afgebroken door niet-geadapteerde slib- en bodem-micro-organismen dan anthraceen zelf.

In dit onderzoek leidde de toevoeging van natuurlijke microflora uit grond, sedimentslib en aktief slib aan cultures van *Bjerkandera* sp. stam BOS55 met geoxideerd [^{14}C]-gelabeld benzo[*a*]pyreen tot een snelle toename van mineralisatie. Tot 21% meer CO_2 werd teruggevonden na de toevoeging van de natuurlijke microflora, wat aantoonde dat sommige metabolieten goed biobeschikbaar en afbreekbaar waren. Deze resultaten lieten ook zien dat de benzo[*a*]pyreen-metabolieten sneller en in hogere mate werden gemineraliseerd door de toegevoegde microflora dan door de schimmelcultures zelf. ***Tabel 1*** vat de belangrijkste resultaten van dit onderzoek en van andere onderzoeken over de gecombineerde afbraak door witrot-schimmels en natuurlijke microflora samen.

De resultaten in hun geheel genomen laten duidelijk zien dat de PAK-metabolieten, ontstaan door witrot-schimmeloxidatie, sneller door de endogene microflora van verschillende bronnen worden gemineraliseerd dan de oorspronkelijke PAKs. De metabolieten van schimmel-oxidatie zijn ook betere substraten voor bacteriën dan voor de witrot-schimmels

Tabel 1. De mineralisatie van PAKs door endogene microflora, door witrot-schimmels en door de combinatie van beiden.

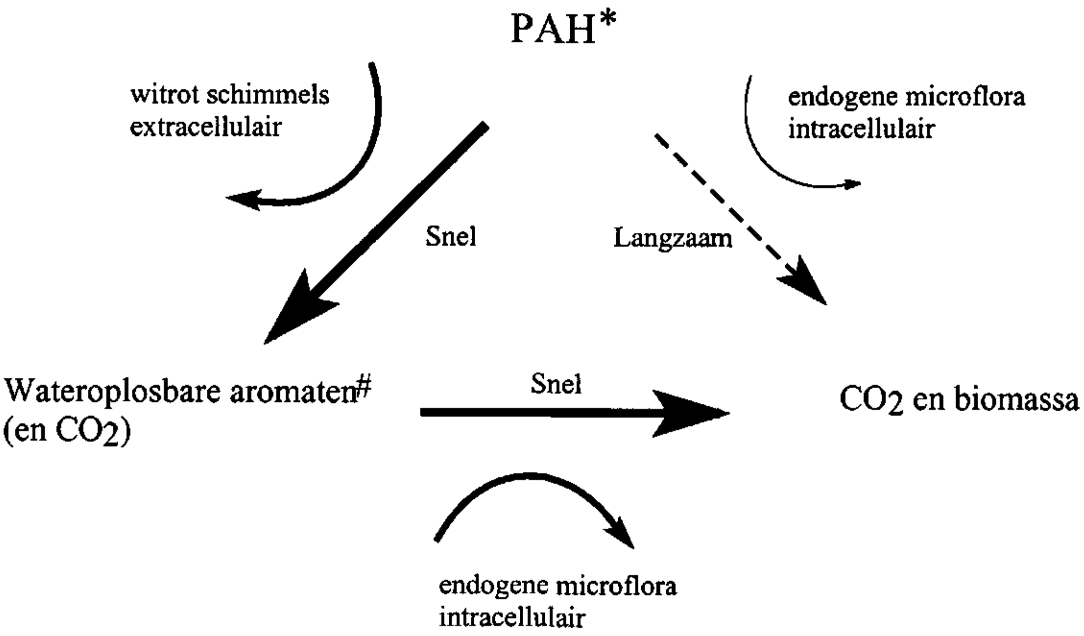
PAH	**Mineralisatie (% van initiële concentratie)**			**Witrot-schimmel**	**Referentie**
	Microflora	Witrot-schimmel	Combinatie		
Fenanthreen	20	1	38	*Phanerochaete chrysosporium*	Brobkorb en Legge, 1992
Pyreen	N.D[a]	28	44	*Dichomitus squalens*	In der Wiesche *et al.*, 1996
	N.D	37	50	*Pleurotus* sp.	In der Wiesche *et al.*, 1996
	27	5	48	*Kuehneromyces mutabilis*	Sack en Fritsche, 1997
Benzo[*a*]pyreen	0-1	13	34	*Bjerkandera* sp. stam BOS55	Kotterman *et al.*, 1998

[a]: Geen gegevens

zelf. Het hoogste stimulerende effect van "schimmel-pre-oxidatie" op de mineralisatie van PAKs is waargenomen met benzo[*a*]pyreen, dat ook de laagste biologische beschikbaarheid en afbreekbaarheid heeft van de geteste PAKs. Een snellere en hogere mate van mineralisatie van PAKs wordt daarom bereikt door sequentiële afbraak, vergeleken met de afbraak door bacteriën of witrot-schimmels alleen. De waargenomen accumulatie van PAK-metabolieten in reincultures van witrot-schimmels zal daarom niet voorkomen in praktijkomstandigheden, bv creosoot-verontreinigde bodems.

De resultaten van dit onderzoek zijn ook schematisch weergegeven in ***Figuur 1***, die laat zien dat zowel de mineralisatie als de detoxificatie van PAKs versneld kan worden door het gebruik van witrot-schimmels.

Figuur 1. Versnelde mineralisatie en detoxificatie van PAKs door het gebruik van witrot-schimmels



(*): Lage wateroplosbaarheid, lage biobeschikbaarheid en sterk mutagene potentie

(#): Verhoogde wateroplosbaarheid, verhoogde biobeschikbaarheid en zwak mutagene potentie

Vooruitzichten van de toepassing van witrot-schimmels in biologische sanering van PAK-vervuilde grond

Het effect van de toepassing van witrot-schimmels op de PAK-afbraak in verontreinigde gronden is onderzocht. Deze onderzoeken vertonen een toename in de afbraak van vooral de 4-ring PAKs zoals pyreen, vergeleken met de afbraak door alleen de endogene microflora, maar slechts weinig of geen afbraak van de 5- en 6-ring PAKs (Davis *et al.*, 1993; Lamar *et al.*, 1994; Field *et al.*, 1996). Ondanks de toename in PAK-afbraak werden toch hoge restconcentraties PAKs waargenomen. Field *et al.* (1996) toonden aan dat de recalcitrantie van PAKs in grond van een oude creosoterings-fabriek werd veroorzaakt door een lage biologische beschikbaarheid van de PAKs. Een voorbehandeling die de biologische beschikbaarheid verbeterde (voorweken van de grond in aceton, gevolgd door een snelle verdamping van de aceton) verhoogde de PAK-afbraak. Weissenfels *et al.* (1992) namen ook een verhoogde PAK- afbraak waar door PAK-geadapteerde bacteriën in grond van een teer-olieraffinaderij na een soortgelijke voorbehandeling. In tegenstelling tot deze resultaten was *Bjerkandera* sp. stam BOS55 in staat tot snelle afbraak van PAKs in kunstmatig gecontamineerde grond (Field *et al.*, 1995), waarbij slechts lage restconcentraties werden waargenomen.

Het grote verschil tussen de biologische beschikbaarheid van PAKs in kunstmatig en industrieel gecontamineerde grond is waarschijnlijk voornamelijk veroorzaakt door de methode van contaminatie. In de kunstmatig gecontamineerde gronden worden PAKs opgelost in een oplosmiddel toegevoegd aan de grond. Na de verdamping van het oplosmiddel zijn de PAKs waarschijnlijk aanwezig als geprecipiteerde deeltjes. De afbraaksnelheid van deze benzo[*a*]pyreen-deeltjes scheen voornamelijk te worden beïnvloed door de deeltjesgrootte van het precipitaat en niet door de aan- of afwezigheid van organische stof (niet gepubliceerde resultaten). In het algemeen wordt de recalcitrantie van PAKs in grond voor een groot deel toegeschreven aan adsorptie van PAKs aan sorbens zoals organische stof (Pignatello en Xing, 1996; Luthy *et al.*, 1997). In verontreinigde grond van gasfabrieken of creosoteringsfabrieken zijn de PAK-verontreinigingen geassocieerd met non-aqueous-phase liquids (NAPLs) als koolteer en minerale oliën. Als gevolg van de hydrofobiciteit van PAKs verblijven deze voornamelijk in de NAPLs, wat resulteert in PAK-concentraties in de waterfase die lager zijn dan in de afwezigheid van NAPLs (Efroymson en Alexander, 1995). Het transport van PAKs uit de NAPLs is erg laag (Efroymson en Alexander, 1994; Yeom *et al.*, 1996) en dit transport kan nog verder vertraagd worden door verwering en verharding van de NAPLs (Luthy *et al.*, 1997), wat de biologische beschikbaarheid ernstig vermindert.

Of PAK-verontreinigde grond in de toekomst succesvol gesaneerd kan worden met biologische methoden hangt sterk af van de biologische beschikbaarheid van de PAKs in de betreffende grond en daarom ook van methoden die de biologische beschikbaarheid eventueel kunnen verhogen. Het gebruik van surfactants, die met succes gebruikt zijn in vloeibare culturen (***Hoofdstuk** 4*), hebben ook geleid tot een verbeterde afbraak van PAKs in zowel kunstmatig gecontamineerde grond als industrieel vervuilde gronden (niet gepubliceerde resultaten). Het gebruik van niet-toxische, relatief persistente surfactants zou daarom de potentie van biologische sanering van PAK-vervuilde grond kunnen verhogen.

Curriculum vitae

Michiel Kotterman is op 1 maart 1967 geboren in Alkmaar, NH. Na het VWO in Alkmaar ging hij in 1985 Moleculaire Wetenschappen studeren aan de toenmalige landbouwhogeschool in Wageningen (LH). In 1991 kreeg hij daar de titel landbouwkundig ingenieur na welgeteld één practicum waarin een landbouwgewas enige rol van betekenis speelde.

In 1992 aanvaardde hij de aanstelling als Assistent in Opleiding bij de vakgroep Levensmiddelentechnologie, sectie Industriële Microbiologie, Landbouwuniversiteit Wageningen (LUW). De resultaten van het bij deze sectie verrichte onderzoek staan in dit proefschrift beschreven. In 1996 was hij tijdelijk toegevoegd onderzoeker bij deze sectie, maar eind 1997 zocht hij het hogerop binnen hetzelfde Universiteitsgebouw en werd toegevoegd onderzoeker bij het departement Agro-, Milieu-, en Systeemtechnologie, sectie Milieutechnologie, Wageningen Universiteit en Researchcentrum (WUR).

List of Publications

De Bruin, W.P., M.J.J. Kotterman, M.A. Postumus, G. Schraa, and A.J.B. Zehnder. 1992. Complete biological reductive transformation of tetrachloroethene to ethane. Appl. Environ. Microbiol. **58:**1996-2000.

Kotterman, M.J.J., E. Heessels, E. de Jong, and J.A. Field. 1994. Physiology of anthracene biogradation by the white-rot fungus *Bjerkandera* sp. strain BOS55. Appl. Microbiol. Biotechnol. **42:**179-186.

Kotterman, M.J.J., R.A. Wasseveld, and J.A. Field. 1995. Influence of nitrogen sufficiency and manganese deficiency on anthracene degradation by the white-rot fungus *Bjerkandera* sp. strain BOS55. In: R.E. Hinchee (ed), *In situ* and on site bioreclamation, Lewis Publ., Boca Raton, Florida, pp. 189-194.

Kotterman, M.J.J., R.A. Wasseveld, and J.A. Field. 1995. Hydrogen peroxide production as a limiting factor in xenobiotic compound oxidation in nitrogen-sufficient cultures of *Bjerkandera* sp. strain BOS55 overproducing peroxidases. Appl. Environ. Microbiol. **62:**880-885.

Collins, P.J., M.J.J. Kotterman, J.A. Field, and A.D.W. Dobson. 1996. Polycyclic aromatic hydrocarbon oxidation by laccases from *Trametes (Coriolus) versicolor*. Appl. Environ. Microbiol. **62:**4563-4567.

Kotterman, M.J.J., H-J. Rietberg, A. Hage, and J.A. Field. 1998. Polycyclic aromatic hydrocarbon oxidation by the white-rot fungus *Bjerkandera* sp. strain BOS55 in the presence of nonionic surfactants. Biotechnol. Bioeng. **57:**220-227.

Kotterman, M.J.J., E.H. Vis, and J.A. Field. 1998. Successive mineralization and detoxification of benzo[*a*]pyrene by the white-rot fungus *Bjerkandera* sp. strain BOS55 and indigenous microflora. Appl. Environ. Microbiol. **64:**2853-2858.