Growth of fungi on volatile aromatic hydrocarbons:

Environmental technology perspectives

Promotor:	**prof. dr. ir. W. H. Rulkens** Hoogleraar Milieutechnologie
Co-promotoren:	**dr. ir. J. T. C. Grotenhuis** Universitair Docent Milieutechnologie **dr. ir. J. W. van Groenestijn** Coördinator Water- en Gasbehandeling, TNO-MEP, Apeldoorn
Promotiecomissie:	**prof. dr. ir. S. E. A. T. M. van der Zee** Wageningen Universiteit **dr. T. W. M. Kuyper** Wageningen Universiteit **dr. W. de Boer** NIOO-KNAW, Heteren **dr. M. Grifoll Ruiz** Universitat de Barcelona

Growth of fungi on volatile aromatic hydrocarbons:

Environmental technology perspectives

Francesc X. Prenafeta Boldú

PROEFSCHRIFT

ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van Wageningen Universiteit

Prof. Dr. Ir. L. Speelman,

in het openbaar te verdedigen

op dinsdag 10 december 2002

des namiddags te half twee in de Aula

ISN 1667006

Prenafeta Boldú, F. X.

Growth of fungi on aromatic hydrocarbons: Environmental technology perspectives

Thesis Wageningen University, Wageningen, The Netherlands, 2002 - with summary in Dutch, Spanish, and Catalan.

ISBN: 90-5808-747-6

1. The observation of an axenic fungal culture growing on an inorganic matrix exposed to benzene, added to function as the sole source of carbon and energy, does not provide convincing evidence to conclude utilization of benzene by the fungus.

   This dissertation

   Qi, B., W. M. Moe, and K. A. Kinney 2002. Biodegradation of volatile organic compounds by five fungal species. *Applied Microbiology and Biotechnology* **58**:684-689.

2. The mineralization of volatile aromatic hydrocarbons on fungal biofilters might result in the emission of products that are not as innocuous as previously thought.

   This dissertation

   Devinny, J. S., M. A. Deshusses, and T. S. Webster 1999. *Biofiltration for air pollution control.* Lewis Publishers. Boca Raton, Florida.

3. The variety of substrates that can be assimilated by fungi goes far beyond natural products.

   García-Guinea, J., V. Cardenes, A. T. Martínez, and M. Martínez 2001. Growing of fungal bioturbation paths in a compact disk. *Naturwissenschaften* **88**:351-354

4. The proposal to use genetically modified microorganisms for the biodegradation of environmental pollutants underestimates the complexity both of microbial interactions in the environment and of principles of the human society.

5. The establishment of student exchange programs is an efficient policy for stimulating the European integration, as illustrated by the number of mixed couples that are formed in places like Wageningen.

6. The arrival of a new life is a critical dead-line for finishing a PhD.

7. Patience is the mother of science

   Catalan proverb

Propositions belonging to the thesis "Growth of fungi on volatile aromatic hydrocarbons: Environmental technology perspectives".
Francesc X. Prenafeta Boldú
10th of December, 2002, Wageningen.

als meus pares

Preface

I first came to the Netherlands to carry out a six-month stage at the group of Environmental Technology. I was then far from imagining that those six months would become six years, and that the dissertation you have now in your hands would be written. This research was started at the Division of Industrial Microbiology (IM) but, closing an unforeseen cycle of events, it was finally completed at Environmental Technology. During this time I have encountered many people whose help and support has contributed to the accomplishment of the personal endeavor that this thesis was for me.

I would like to express my gratitude to Jan de Bont for giving me the opportunity to carry out the research presented in this study at the Division of Industrial Microbiology (IM). The sudden announcement of the closure of his group due to the re-organization of the University, one year after my start, truncated the prospects of many ongoing projects, including this one. I am therefore deeply indebted to Wim Rulkens for accepting the responsibility of being my supervisor at that crucial stage. His criticism during the writing of this dissertation and his readiness with the organization of the closing stages were essential. I am also grateful to my co-supervisors, Johan van Groenestijn and Tim Grotenhuis, for the stimulating discussions we had, for their good guidance, and for making everything possible to resume the experimental work at the TNO-MEP institute. My appreciation is also extended to the people from the TNO, who kindly integrated me as another colleague and helped with everything I needed.

I want to thank all my colleagues from Industrial Microbiology, PhDs, post-docs, and regular staff. To all my PhD mates, despite the difficulties we had to face, you have found the good course in obtaining the degree of doctor, and that was very motivating for me. Thanks for your good companionship. I especially want to wish the best to the "last of Mohicans" of IM, Ko, Wout and John, which will promote soon.

The work encompassed in this dissertation is certainly not the product of an individual effort. I want to acknowledge the people who were directly involved in this project: Dion Luykxs, the biochemist of fungal degradation, and my student Nadia Nikolova, who worked on the interactions during the degradation of substrate mixtures. I am also thankful to the people who lent me a hand with different techniques and made of these years a very fruitful learning time. Many thanks to Martin de Wit for solving the troubles with the gas chromatography and to Jacques Vervoort for his assistance with the NMR spectrometer; my thanks also go to Hendrik Ballestedt for his help with the PCR-TGGE technique.

I also want to express my gratefulness to my colleagues from the CBS-KNAW institute: Walter Gams, for the critical reading of this thesis and for his excellent teachings on the field of soil fungi; Richard Summerbell, for checking the correctness of the language in the manuscript; and Hans Josef Schroers, for his help with the microscopy pictures used in the covers.

I want to thank my paranimfs Ana Lopez and John Dijk for their helpfulness in organizing the defense of this dissertation.

Finally, my deepest gratitude is due to you, Rita, for your patience, encouragement, and interest in my work. The evenings and weekends of work have now come to an end and they will be owned by you and by our little Joana.

Table of contents

CHAPTER 1

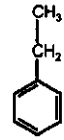
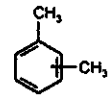
General introduction

1.1 Volatile aromatic hydrocarbons

Since the industrial revolution the use of petroleum and coal derivatives has increased immensely. These products consist of complex mixtures that include a wide range of aliphatic, aromatic, and asphaltic compounds. The intermediate distillate range is rich in monoaromatic hydrocarbons, such as benzene, toluene, ethylbenzene and xylene (BTEX). These chemicals are widely used as solvents, synthesis precursors of other organic chemicals, as well as gasoline components (Table 1.A). Accidental spills and industrial discharges have occurred causing serious pollution of the environment as a consequence of the bulk scale in which BTEX are produced and handled.

From an environmental standpoint the mobility and toxicity of the BTEX are of major concern. Compared with other oil hydrocarbons, BTEX are relatively water-soluble and a significant portion of the spill will move rapidly upon entry into water systems. Gasoline leakage from underground storage-tanks has been identified as one of the main sources of aquifer contamination with BTEX (122). Besides being soil and water contaminants, these compounds are also highly volatile and are subject to air-quality regulations. BTEX hydrocarbons are depressants of the central nervous system and cause damage to liver and kidney. The carcinogenicity of benzene has been established and, in spite of inconclusive evidence, alkylbenzenes are suspected to be long-term carcinogens (82). Besides hazard through direct inhalation, these hydrocarbons are subject to photochemical reactions contributing significantly to smog formation in urban areas (105). Reduction of BTEX content in modern gasoline formulations has been achieved *via* supplementation with methyl-*tert*-butyl ether (MTBE) (83). This additive acts as an octane enhancer and as an oxygenating agent, allowing the elimination of lead-antiknocking compounds. Although MTBE is less toxic than the BTEX, the use of this additive remains controversial due to its high recalcitrance under natural conditions (37).

TABLE 1.A: Properties of the BTEX hydrocarbons

Common name	Benzene	Toluene	Ethylbenzene	*o*-, *m*-, *p*-Xylene
CAS number	71-43-2	108-88-3	100-41-1	95-47-6 108-38-3 106-42-3
Chemical properties (2, 70, 104)				
Structure				
	C_6H_6	C_7H_8	C_8H_{10}	C_8H_{10}
Molecular weight	78.11	92.14	106.17	106.17
Solubility (mg l^{-1})	1800	540	160	170; 160; 180
Vapor pressure (kPa)	12.7	3.8	1.3	0.9; 1.1; 1.2
Water/air partition coefficient	4.58	3.76	3.15	3.55; 3.70; 5.04
Commercial aspects (104)				
World production ($\times 10^9$ l $year^{-1}$)	5.98	3.07	na [b]	3.07
Main uses	Solvents, coating agents, fuel additives, synthesis of medicines, pesticides, dyes, paints, detergents, flavors, plastics, resins, and explosives.			
Environmental aspects (2, 124)				
Air odor threshold (mg m^{-3})	12	2.9	2.3	1.1
IOLV [c] (mg m^{-3})	3.25	150	215	210
MAC in drink water [d] (µg l^{-1})	5.0 [e] – 1.0 [d]	700 [e]	300 [e]	500 [e]

[b] not available
[c] threshold limit value for exposure to airborne contaminants in the work place, weighted for a normal 8 h workday and 40 h workweek
[d] Norm from the European Union
[e] Norm From the World Health Organization

1.2 Biotechnological removal of BTEX pollution

A variety of treatment technologies have been developed in order to meet the regulatory standards concerning pollution with oil hydrocarbons. These techniques strongly differ depending on the chemical nature of contaminants and the environmental compartments where they are found. Historically, the removal of BTEX from ground and groundwater focused on either excavation and disposal or treatment of contaminated soils, or on pumping up the groundwater to

remove dissolved contaminants from the aquifer (16). Both approaches are costly and they interfere adversely with other site activities. To overcome these disadvantages, new developments focused on *in situ* treatment technologies that often take advantage of the metabolic capacity of soil microorganisms to degrade hydrocarbons. Depending on the specific site characteristics, biological activity can be stimulated by addition of nutrients and suitable electron acceptors, by pH amendments, or through the introduction of new microbes, either wild type or genetically modified, that possess an enhanced degradative capacity. The *in situ* aeration of the soil gas phase, named soil bioventing, is one of the most common ways of increasing the BTEX biodegradation capacity in soil (17). With this technique hydrocarbons are removed by a combination of volatilization and aerobic biodegradation, depending on the air flow-rate (66, 77). At high flows, BTEX removal is accomplished mainly by volatilization, a variant that is termed soil vapor extraction (SVE). However, the extracted vapor-phase contaminants usually require further treatment in a separate unit before discharged into the atmosphere (17).

Analogous to the generalization of biological methods for the cleaning-up of water and soil, the need to control air pollution has prompted the development of microbial-based processes for the treatment of gaseous wastes. The biofiltration of air is consolidating as a cost-effective option for the treatment of streams with low concentrations of volatile hydrocarbons that arise from SVE remediation works (67, 94) as well as from a variety of industrial processes (69, 127). The biofiltration of polluted air consists on passing the gas stream through a porous support material that offers a large contact area and immobilizes microbial cultures. Besides inoculation with specific strains, biofilters are biologically open systems to the in-flow air that carries cells of a wide variety of organisms. As biofiltration proceeds, microbes will be enriched according to their abilities to adapt to the biofilter ecosystem.

1.3 Fungi *versus* bacteria in the bioremediation of petroleum hydrocarbons

The use of microorganisms to remove, reduce, or ameliorate pollution from the environment is generally termed bioremediation. Bacteria and fungi constitute the most important part of the microflora present in soils that are polluted with oil hydrocarbons. However, the relative contribution of both microbial groups to bioremediation varies widely depending on their specific degrading capabilities and adaptation to the prevailing physico-chemical conditions.

Bacteria and fungi display a characteristic form of nutrition, often termed osmotrophy or absorbtive tive heterotrophy, which consist on absorbing soluble substrates from the external environment. However, most fungi do not rely solely on soluble, readily absorbed organic compounds for nutrition and they excrete a wide variety of enzymes into their environments that cleave polymeric substances. The soluble breakdown products can be absorbed and further catabolized by internal enzymes. Besides the primary oxidizers, many other microorganisms (fungi and bacteria) benefit from the released substrates. Thus, in nature fungi play a vital role in the recycling of a variety of "recalcitrant" organic compounds, such as lignin, cellulose, chitin, melanin, and keratin. As a result of this biochemical diversity, which combines degradative pathways that resemble those present in prokaryotes as well as in higher organism, the fungal metabolism of xenobiotics is also highly versatile (102). In addition, fungi display a distinctive physiological adaptability. Growth as filamentous hyphae permits translocation over solid materials such as soil particles and wood without the need of a liquid phase. Consequently, many fungi are tolerant towards limited water and nutrient availability, and prefer acidic conditions (131). Instead, soil bacteria typically show a rapid growth when the conditions are favorable and utilize soluble substrates.

The inherent characteristics of fungi make these organisms very suited biocatalysts for solid-state fermentations, where they are used in a wide range of

biotechnological applications (118). This also applies to environmental technology, where the biodegradation of pollutants by fungi has most frequently been investigated in soil systems and packed-bed reactors (Table 1.B). The biodegradation of the poorly available polycyclic aromatic hydrocarbons (PAHs) has extensively been studied for fungi and is summarized in Chapter 2. An important drawback in this process is that biodegradation occurs by co-metabolism. This means that aromatic hydrocarbons do not support growth and are usually degraded partly, sometimes to metabolites that are more toxic than the parent substrate (32, 121). Conversely, biodegradation of low molecular weight monoaromatic hydrocarbons such as BTEX has traditionally focused on bacteria, and the importance of fungi, the subject of this thesis, has been recognized only recently.

TABLE 1.B: Examples of the use of fungi for the degradation of aromatic pollutants in non-sterile environments

System	Pollutant	Extent of degradation[a]	Fungus	Morpho-logy[b]	Ref.
Air biofilter	BTEX	C	*Phanerochaete chrysosporium*	WR	(19)
	Styrene	M/G	*Exophiala jeanselmei*	Y	(35)
	Toluene	M/G	*Cladosporium sphaerospermum*	F	(132)
		M/G	*Exophiala lecanii-corni*	Y	(138)
		M/G	*Scedosporium apiospermum*	F	(54)
	Phenol	M/G	*Candida* sp.	Y	(51)
Soil	Cresols	M/G	*Rhodotorula aurantiaca*	Y	(84)
	PAHs	C	*Cunninghamella echinulata*	F	(40)
		M/C	*Pleurotus* sp.	WR	(80)
		M/C/B	*Penicillium janthinellum*	F	(13)
		M/C/B	*Phanerochaete chrysosporium*	WR	(21)
	Benzo[a]pyrene	M/C	*Marasmiellus troyanus*	WR	(88)
		C	*Irpex lacteus*	WR	(89)
Soil+compost	PAHs	C	*Pleurotus ostreatus*	WR	(50)
Soil+sludge	Benzo[a]pyrene	M/C/B	*Bjerkandera* sp.	WR	(70)

[a] M: mineralized; G: growth substrate for the fungus; C: fungal co-metabolism; B: in association with indigenous bacteria

[b] WR: basidiomycetous white-rot fungus; F: filamentous microfungus; Y: yeast or yeast-like growth

1.4 Outline of this thesis

The earlier observation that a fungus out-competed the bacterial population in a biofilter used for treating toluene-polluted air, and the confirmation that toluene was used as the sole carbon and energy source by a eukaryote opened a new field in the area of microbial metabolism of aromatic hydrocarbons (133). That finding motivated the research presented in this dissertation. The general objective hereby was to gain more insight in the presently poorly understood assimilation of volatile aromatic hydrocarbons by fungi. This knowledge is of importance to evaluate advantages and also the limitations of using fungi in the biotechnological removal of BTEX pollution.

An overview of the present knowledge on the hydrocarbon metabolism by fungi is given in Chapter 2. In Chapter 3, the isolation of fungi capable of growth on volatile aromatic hydrocarbons is described. Different enrichment techniques specifically designed for selecting fungi were used. Fungal isolates were identified and their growth and degradation parameters characterized. Chapter 4 deals with the metabolic pathway for the oxidation of toluene in a variety of fungi, revealing more details about the metabolic diversity involved in the degradation of toluene by fungi. In Chapter 5, the degradation kinetics of BTEX mixtures that were analogous to those resulting from gasoline pollution is described using one selected fungal isolate. The extent of degradation of each BTEX component and the multi-kinetic degradation parameters were determined. These data constitute the basis for Chapter 6, where degradation of BTEX is described in a soil microcosmos containing the fungal inoculum, incubated under different conditions of pH and exposure time to BTEX. A general discussion concludes this dissertation in Chapter 7.

CHAPTER 2

Hydrocarbon metabolism in fungi

2.1 Introduction

The chemical nature of hydrocarbons is extremely diverse, varying from simple saturated aliphatics to the more complex polycyclic aromatics (PAHs). Such a range of carbon substrates can be metabolized by many different micro-organisms using diverse and, sometimes, badly understood degradation pathways. Comprehensive studies have been published encompassing the bacterial catabolism of aliphatics, the monoaromatic BTEX, and PAHs (26, 57, 71, 144). The fungal degradation of aliphatics and PAHs has also been reviewed extensively (31, 74, 86), but the metabolism of the lighter BTEX is less well characterized in the fungi. The present Chapter summarizes the current knowledge on the fungal metabolism of aliphatic and aromatic hydrocarbons.

Despite that fungal growth on hydrocarbons has been reported long ago (144), the interest on hydrocarbonoclastic fungi dramatically increased in the sixties with the advent of the jet aircraft and the shift from gasoline to kerosene-based fuels (97). Some accidents were then caused by fungal mats that clogged the fuel supply to engines. These fungi grew in the oil/water inter-phase that accumulated inside storage tanks, as a result of water condensation, utilizing the soluble hydrocarbons as sources of carbon and energy. The water-soluble fraction of oil-refined fuels contains a mixture of mainly monoaromatic and aliphatic hydrocarbons, but only the latter were found to serve as growth substrates for fungi. Biodegradation of hydrocarbons also resulted in accumulation of organic acids that contributed to corrosion damage, hence increasing the risk of fuel leakage (98). Fuel biodeterioration by fungi is, despite the generalized practice of blending fuels with fungicides, an important economic problem for the petrochemical industry of today (55). Research into the potential biotechnological applications of fuel-growing fungi was initially directed towards

single-cell protein projects and bioremediation of oil pollution (74). These fungi have also been proposed as bioindicators for the prospecting of natural gas (41).

2.2 Aliphatic hydrocarbons

The most frequently isolated fungus from fuel tanks is *Amorphotheca resinae* (anamorph *Hormoconis resinae*, previously named *Cladosporium resinae*). Many other filamentous strains of importance have also been isolated from fuel tanks, mainly *Penicillium*, *Aspergillus*, *Chrysosporium*, *Phialophora*, *Fusarium*, and *Alternaria* species; yeasts typically included *Candida* and *Rhodotorula* strains (55, 62, 75). Studies with these fungi revealed that *n*-alkanes from C_{10} to C_{20}, which are predominant in kerosene and diesel fuels, were preferentially used as carbon and energy sources (74). Though less likely to support fungal growth, *n*-alkanes in the range of gasoline (C_5–C_9) and natural gas (C_1–C_4) were oxidized by *Scedosporium* and *Graphium* spp (41, 93). Unsaturated and branched-chain aliphatics were also assimilated to a lesser degree.

The most common degradation pathway for alkanes reported in fungi starts with the hydroxylation of the terminal methyl group (Fig. 2.1). This reaction is principally catalyzed at the microsomes by cytochrome-P450 monooxygenase NADPH-reductase enzyme complex (107). Following alkane oxidation to a primary alcohol, further oxidation to fatty acids via aldehyde occurs. Although this is the most frequently encountered mechanism, two variants have been described: the diterminal oxidation to form dicarboxylic acids and the subterminal oxidation to secondary alcohols. Fatty acids are incorporated to the central catabolic pathways via β-oxidation, involving the initial activation of the fatty acid to the corresponding acyl-CoA ester.

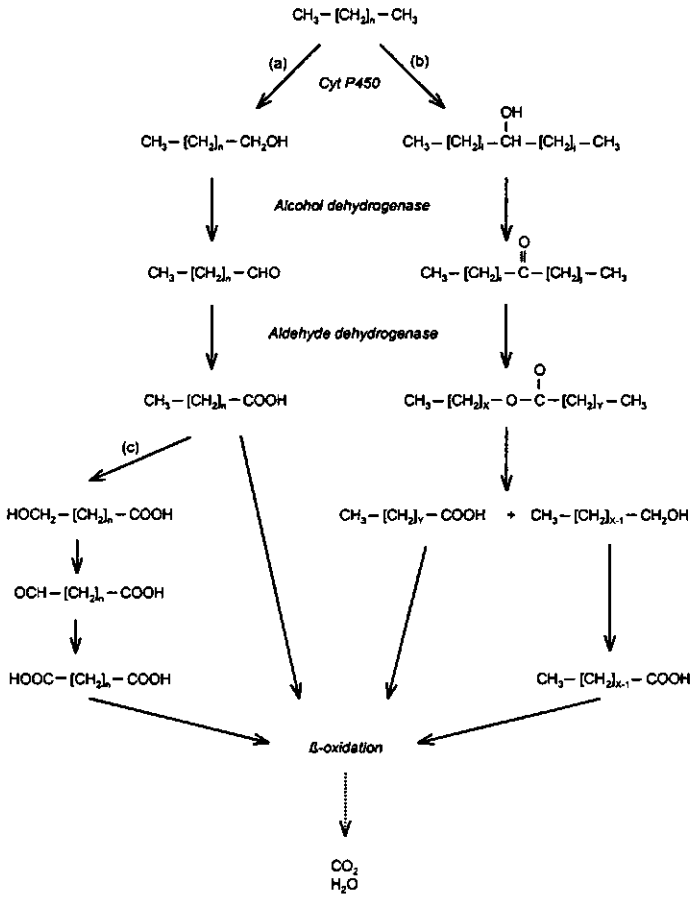


FIGURE 2.1: Metabolic pathways for the assimilation of *n*-alkanes by fungi showing the terminal (a), subterminal (b) and diterminal (c) oxidation variants (22)

2.3 Aromatic hydrocarbons

Degradation of aromatic hydrocarbons that range in size from one to six rings has been demonstrated both for fungi and bacteria (26). However, two general differences have been established between these microbial groups: (i) Bacteria usually oxidize aromatic hydrocarbons as the first step for carbon assimilation and energy-yielding reactions, while in fungi oxidation typically occurs by co-metabolism. (ii) The principal enzymatic mechanism for the oxidation of the aromatic ring in bacteria involves intracellular flavoprotein dioxygenases that form *cis*-dihydrodiols which are then cleaved by other dioxygenases (57); in fungi two very different enzymatic systems have been identified: the cytochrome P450 monooxygenase, and the ligninolytic system. Both fungal pathways are presented schematically in Figure 2.2.

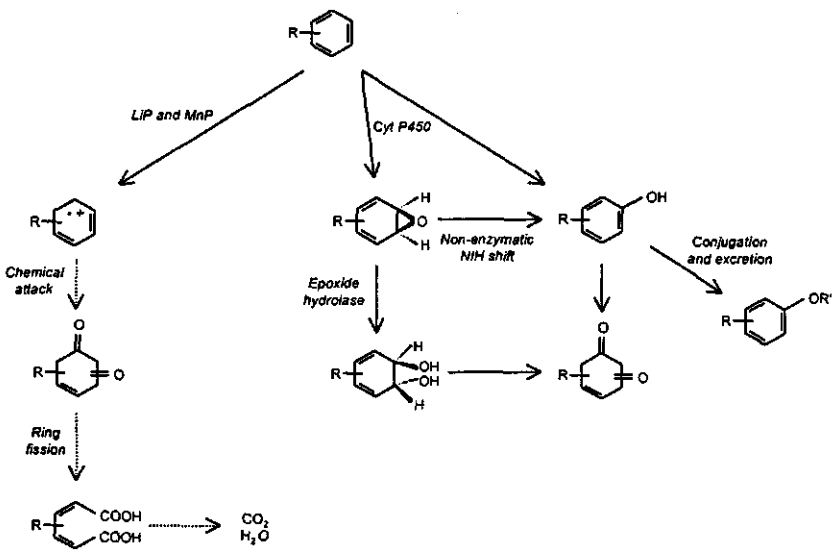


FIGURE 2.2: General metabolic pathways for the ring-oxidation of aromatic hydrocarbons by fungi. R: H, aliphatic, or aromatic substituent. R': methyl, glucoside, glucuronide, sulfate, or xyloside (25, 109)

2.3.1 Cytochrome P450 monooxygenase

Cytochromes P-450 are a special class of heme-containing multicomponent enzymes that are widely distributed in eukaryotes and are also found in certain bacteria (87). These enzymes are coupled to a NADPH-reductase that supplies the electrons for oxidation from the hydrolysis of water. The cytochrome P-450 system plays a central role in the oxidative metabolism of endogenous metabolites, as well as in the detoxification of xenobiotics, including aromatic hydrocarbons (114). Cytochrome P450 catalyzes the ring-epoxidation of aromatic hydrocarbons to form arene oxides, which can either undergo enzymatic hydration by epoxide hydrolase to *trans*-dihydrodiols or else rearrange non-enzymatically to form phenols. Detailed studies on P450-mediated aromatic hydroxylation in eukaryotes, however, indicated that epoxides might not be obligatory precursors of phenolic metabolites (109). Hydroxylation products can undergo further detoxification by O-conjugation to methyl, glucoside, glucuronide, sulfate, or xyloside intermediates, which can be excreted. In general, the later biotransformations are also found in mammals. For this reason fungi, mainly *Cunninghamella* spp., have been proposed as model organisms for studying the detoxification of aromatic hydrocarbons in humans (117, 142).

2.3.2 Ligninolytic enzymes

White-rot fungi are a specialized group of wood-decaying organisms, mainly basidiomycetes and a few ascomycete genera within the *Xylariaceae*, that degrade lignin and give a characteristic bleached appearance to wood. Lignin degradation occurs in order to gain access to cellulose and hemicellulose, substrates that effectively used as carbon and energy sources. Lignin is a highly heterogeneous phenolic polymer that requires extracellular enzymes functioning in a non-specific manner. As a result of this substrate nonspecificity, many other

aromatic compounds, including aromatic hydrocarbons, are also degraded by ligninolytic enzymes. White-rot fungi that have been selected for the degradation of aromatic pollutants include *Phanerochaete*, *Bjerkandera*, and *Trametes* spp. (53, 100). Similarly to lignin and despite that mineralization has been reported in certain cases, aromatic hydrocarbons are only degraded by co-metabolism.

Three different types of enzymes are principally involved in the ligninolytic system: glycosylated heme-containing lignin peroxidases (LiP), Mn-dependent peroxidases (MnP), and copper-containing phenol-oxidizing laccases (Lac). In the presence of endogenously formed H_2O_2, LiP oxidizes veratryl alcohol, an endogenously generated low-molecular mass redox mediator, which in turn carries out one-electron oxidations of non-phenolic aromatics to form aryl cation radicals. These radicals initiate a chain of random oxidative chemical reactions that result in a variety of aliphatic and aromatic products (106). MnP performs an H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+} that oxidizes phenolic compounds. Lac also generates radicals from different low-molecular mass redox mediators in an H_2O_2-independent reaction.

Depolymerization of lignin results in monomeric products, usually phenols, aromatic acids, and their methoxylated or reduced analogues. The latter compounds have a higher water-solubility and are catabolized intracellularly by a wider diversity of fungi, mainly soil saprobes (24). Assimilation of these aromatics occurs through two successive hydroxylations of the aromatic structure previous to the ring fission via dioxygenases. The microbial cleavage of the aromatic ring can take place either at the *ortho* or the *meta* position. However, only the *ortho*-fission has so far been reported for fungi.

2.4 Substituted aromatic hydrocarbons

Two types of oxidative reactions are known in bacteria for the assimilation of *n*-alkylbenzenes: (i) the oxidation of the aromatic ring to form alkyl-catechols and (ii) the oxidation of the alkyl substituent to form aromatic carboxylic acids, which are then dihydroxylated to catechols (57). Assimilation of *n*-alkylbenzenes has also been described in some fungi capable of growth on *n*-alkanes (*Beauveria*, *Verticillium*, *Paecilomyces*, and *Penicillium* spp.) that were isolated from oil-polluted environments (52). Minimum side-chain lengths from C_4 to C_9, depending upon strains, were required for growth. More recently, additional fungal strains were isolated, which were capable of utilizing alkylbenzenes with shorter side-chains (37, 133). In these studies, metabolism of alkylbenzenes was initiated at the alkyl group up to the formation of aromatic acids, which were eventually assimilated.

CHAPTER 3

Isolation and characterization of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source

Francesc X. Prenafeta-Boldú, Andrea Kuhn, Dion M. A. M. Luykx, Heidrun Anke, Johan W. van Groenestijn and Jan A. M. de Bont.

Published in *Mycological Research*, 105(4): 477-484

3.1 Introduction

Aromatic hydrocarbons like benzene, toluene, ethylbenzene and the xylene isomers (collectively known as BTEX) are among the most abundant components from the water soluble fraction of crude oil and refined fuels. Moreover, these compounds are used on a large scale as solvents and for the production of a range of chemicals (72, 122). Besides being relatively water soluble, BTEX compounds are also volatile. BTEX is present in the waste gas of industrial processes and bioremediation works at oil-contaminated sites (6). Due to their toxicity and recalcitrance, aromatic hydrocarbons are regarded as major environmental pollutants and have been subject to stringent environmental regulations (82).

The treatment of gas streams containing volatile pollutants by biofiltration has been proposed as an alternative to other air pollution control technologies, because investment and maintenance costs are relatively low (127). The absence of a mobile water phase simplifies the reactor configuration and it improves the mass transfer of hydrophobic substrates into the active biofilm, where they are degraded. However, control of parameters that strongly affect the microbial activity like pH, water activity and nutrient supply is difficult without free-flowing water (95). Both fungi and bacteria are known to degrade aromatic hydrocarbons (26). Fungi perform oxidation reactions as a prelude to the detoxification and excretion of hydrocarbons, rather than using these compounds as carbon sources for growth (32, 86). Interestingly, Hartmans *et al.* (61) isolated two fungi able to grow on styrene as a sole carbon and energy source. One of those strains, the yeast-like fungus *Exophiala jeanselmei*, was used successfully for treating of styrene-polluted air in a biofilter (37, 39). More recently, the deuteromycete *Cladosporium sphaerospermum* was isolated from a biofilter that had been used to remove toluene from contaminated air. This fungus can use toluene as the sole source of carbon and energy (133). These findings

demonstrate that it is possible to isolate fungi that grow on aromatic hydrocarbons, provided adequate enrichment techniques are used. Compared to most bacteria, fungi adapt more readily to adverse environmental conditions of low moisture and low pH (31, 34, 118). Consequently, these environmental factors are relevant for the specific enrichment of fungi.

The present report describes the use of enrichment techniques to isolate fungi capable of growing on toluene. In addition, a broad range of fungi belonging to the *Basidiomycota*, *Zygomycota* and also deuteromycetes were screened for their ability to grow on toluene. The new isolates were identified and characterized in terms of their growth rates, substrate specificity and toluene degradation kinetics. Emphasis was placed on the toluene assimilation parameters of these fungi in comparison with bacteria.

3.2 Materials and methods

3.2.1 Enrichment and isolation of fungi growing on toluene

Three different enrichment techniques were performed: solid state-like batches, air biofilters and liquid batch cultures. Soil or groundwater samples were used as inoculum.

Solid state-like batches. Serum flasks (250 ml) were filled with 50 ml of perlite granules. Prior to inoculation the perlite was soaked with mineral medium (60). Different unpolluted and BTEX-polluted soils (approx. 1 g) and water samples (1 ml) were used as inocula. The flasks were closed with a cotton-wool and incubated in a desiccator at 30 °C. Toluene, xylenes, benzene, and naphthalene were used individually as carbon sources. Substrates were supplied in the gaseous phase from 5 % (v/v) solutions in dibutyl-phthalate. Naphthalene was added directly in solid form. Humidity was set at 90 % rh using a salt solution (140 g NaCl l^{-1}).

Air biofilters. Four glass columns packed with perlite granules were used as biofilters. The filter bed volume was 1 l in columns A and B and 2 l in columns C and D. The perlite was previously saturated with mineral medium (38) and mixed with soil from a gasoline station (50 g l^{-1} perlite). Humidified air containing 0.1 to 0.55 mg l^{-1} toluene was fed to the biofilters at a constant gas flow rate of 200 l h^{-1}. The pH of the medium in columns A and C was initially set at 4.0 and in columns B and D at 8.0. The influent gas for the biofilters A and B was humidified at 92 % rh and for C and D at 97 % rh. All filters were operated at 25 °C.

Liquid cultures. Soil samples (approx. 5 g) were suspended in 100 ml of liquid medium. Medium composition was (per liter demineralised water): $(NH_4)_2SO_4$, 1 g; $MgSO_4.7H_2O$, 0.5 g; NaCl, 0.1 g; $CaCl_2$, 0.1 g; KH_2PO_4, 0.87 g; $FeSO_4.7H_2O$, 1 g, and 10 ml of mineral trace solution (135). Ground water samples (100 ml) were incubated after adding the components of the mineral medium. In both cases, glucose (0.3 g l^{-1}) was added as an additional carbon source and the pH was adjusted at 5.5. Toluene was supplied by disolving it in dibutyl-phthalate (2 ml, 3 % v/v) from which it evaporated into the incubation vessel. This solution was contained in an open vial attached to the rubber cap. Flasks were incubated at 23 °C on a rotary shaker (130 rpm).

Fungi were isolated by washing samples of the support material or directly from the liquid media. Dilutions from the resulting suspensions were plated and incubated in a dessicator with toluene (3 or 5 % v/v in dibutyl phthalate). Pure fungal cultures were obtained by subsequent transfers to fresh agar plates. Medium composition and incubation temperatures during isolation were identical to those used during enrichment.

3.2.2 Growth experiments on solid culture

260 Fungal strains (from the collection of LB Biotechnology, Kaiserslautern, Germany) were screened for their ability to grow on mineral agar plates

incubated under toluene vapours. The collection of fungi included 165 strains of deuteromycetes and *Zygomycota* previously isolated from polluted soils (polycyclic aromatic hydrocarbons, chlorophenols and nitroaromatic compounds) and 95 strains of *Basidiomycota* representing 80 genera. This second group included white rot and non-white rot fungi from a broad range of habitats. Plates were incubated at 23 °C in dessicators containing a toluene atmosphere (3 % v/v toluene solution in dibutyl phthalate). The composition of the mineral media used in the agar plates was the same as in the enrichments with liquid cultures (see above). Growth was assessed by comparing agar plates incubated with and without toluene.

3.2.3 Growth experiments in liquid culture

Growth kinetics were characterised in 250 ml Boston flasks sealed with Teflon Mininert valves (Phase Separations, Waddinxveen, The Netherlands) to prevent solvent evaporation. Each flask contained 25 ml of buffered (35 mM $K_2HPO_4/NaH_2PO_4.2H_2O$, pH 7) mineral salts medium (60). Hydrocarbons were added up to an initial concentration of 0.5 mM in the liquid media based on reported water/air partition coefficients (2, 76). Substrates with a lower solubility were added in excess up to 50 µmol. Glucose (50 µmol) was used for reference purposes to assess growth on a readily biodegradable and non-inhibitory substrate. Hydrocarbon toxicity was determined by comparing growth in the presence of each pollutant with growth on glucose. Flasks were inoculated with a spore suspension after substrate equilibration. Incubations were performed at 25 °C under static conditions unless stated otherwise. Flasks in which growth was not observed were incubated for a minimum of four months. Growth was evaluated by measuring the consumption of the volatile substrate and the production of carbon dioxide in the headspace. Measurements were related to standards with the same volume of liquid medium and gas phase as the

incubated batches and known amounts of both the hydrocarbon and carbon dioxide.

3.2.4 Preparation of fungal cell suspensions

Higher yields of toluene-grown mycelia were produced in 5 l Erlenmeyer flasks containing 0.5 l of mineral medium (60) and 0.02 % (w/v) of yeast extract. Toluene was supplied via a 5 ml toluene solution (5 % v/v in dibutyl phthalate) as described above. Flasks were incubated under shaken conditions for one week at 20 °C or 30 °C depending on the optimum for growth of each strain. The mycelium was harvested by filtration using filter paper, followed by washing and resuspension in a potassium phosphate buffer solution (50 mM, pH 7.0). Yeast cells were harvested and washed twice by centrifugation (10 000 rpm, r_{av} 8 cm, for 10 min at 4 °C). Cell suspensions were stored at 4 °C until use for up to three days.

3.2.5 Toluene degradation parameters

The rates of toluene degradation and mineralisation by fungal cell suspensions were determined in 250 ml Boston bottles sealed with Teflon valves. At time zero 10 ml of cell suspension (approx. 8 g-dw l^{-1}) and 2 µl of toluene were added, resulting in a toluene concentration of 0.2 mM in the liquid phase. Flasks were incubated at 30 °C under shaking conditions. Toluene consumption and carbon dioxide production in the headspace were monitored during the following hours. Carbon dioxide production was corrected for the endogenous respiration determined in flasks incubated without toluene. All incubations were performed in triplicate.

The effect of the toluene concentration on the oxygen consumption rate was determined by using a biological oxygen monitor. The oxygen uptake by a 4 ml cell suspension was monitored for at least 5 min after the addition of 20 µl of a

toluene stock solution in *N,N*-dimethylformamide. Oxidation rates were corrected for the endogenous respiration rate. The addition of *N,N*-dimethylformamide had no effect on the endogenous respiration. The oxygen consumption rate was measured in triplicate up to a toluene concentration of 0.25 mM.

3.2.6 Analytical methods

Volatile hydrocarbons and carbon dioxide concentrations were determined by injecting 100 µl head-space samples in a HP 6890 Series gas chromatograph (Hewlett Packard). For the hydrocarbons, the stationary phase was a 10 % SE-30 Chromosorb WMP column (Chrompack B.V. Middelburg, The Netherlands). The carrier gas was nitrogen used at a flow of 1.9 ml min^{-1}. The temperature of the column and the flame ionisation detector was 110 and 300 °C respectively. For carbon dioxide a Chrompack Poraplot Q column (Chrompack B.V. Middelburg, The Netherlands) and a thermal conductivity detector were used. Helium at a flow of 3.0 ml min^{-1} was the carrier gas. The column and detector temperatures were respectively set at 70 and 250 °C. Oxygen consumption was measured in a Clark type oxygen electrode (Yellow Springs Instruments Co, Inc., Ohio). Dry weight was determined after drying cell suspensions for 24 h at 105 °C. The latter values were corrected for the salts contained in the suspension buffer.

3.2.7 Chemicals

Hydrocarbons were obtained from Acros Organics (Geel, Belgium), Sigma-Aldrich Chemicals (Steinheim, Germany), Jansen Chimica (Geel, Belgium), Lab-Scan Ltd. (Dublin, Ireland) and Merck KGaA (Darmstadt, Germany). All chemicals were of analytical grade.

3.3 Results

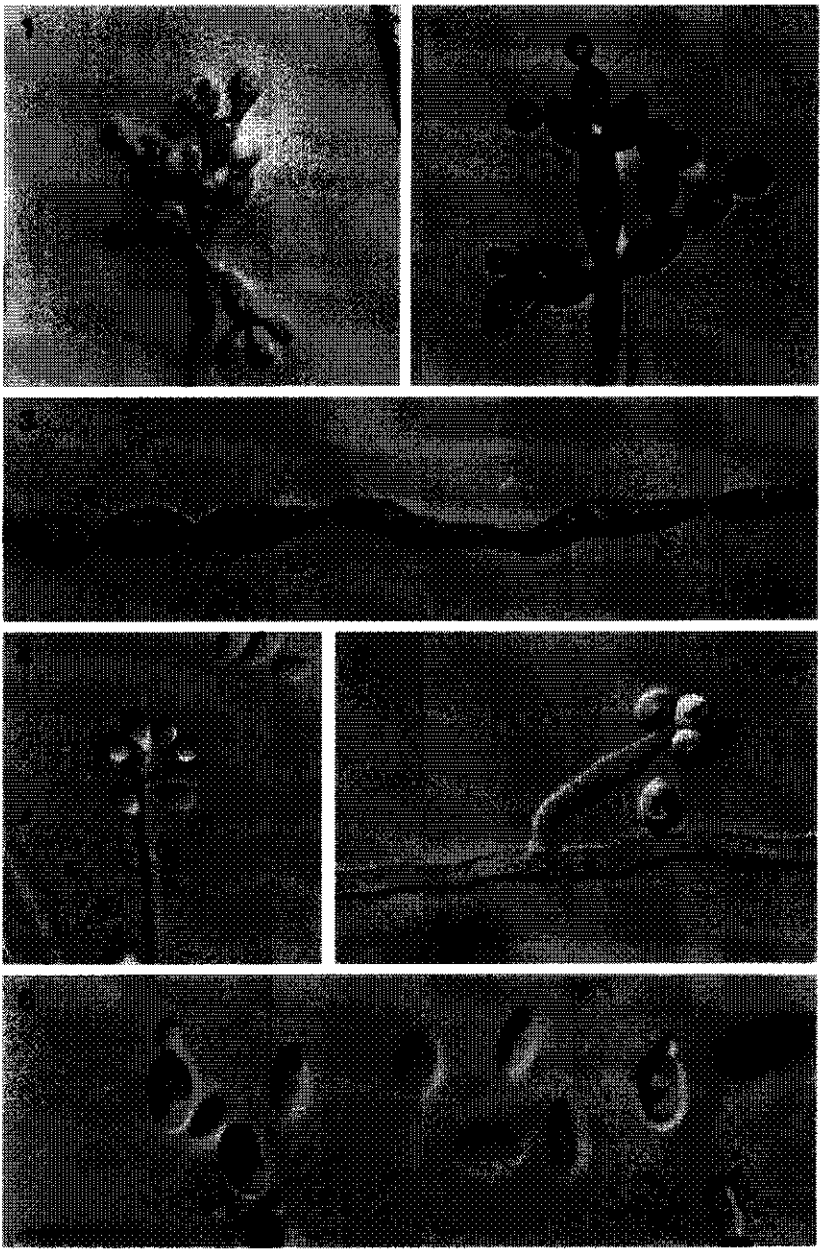
3.3.1 Isolation and identification of the fungi

Three methods were employed for the selective enrichment of toluene-degrading fungi in order to obtain a variety of strains. Soil and water samples of different origin were enriched under conditions of low pH and/or water activity using solid state-like batches, air biofilters and liquid cultures. Fungal growth was only observed in incubations with material from BTEX-polluted sites. Fungi were detected within one month from these sites using all three incubation methods. Five strains, designated T1 to T5, were selected. Attempts to enrich for fungi able to grow on xylenes, benzene, or naphthalene were unsuccessful. Additionally, 260 fungi from a culture collection were screened for their ability to grow under a toluene atmosphere. None of the strains tested was able to grow on toluene as a sole carbon and energy source.

TABLE 3.A: Enrichment conditions and identification of the isolated fungal strains growing on toluene

Source of inoculum	Enrichment Technique	Conditions	Fungus	
			Code	Identification (strain number)
BTEX polluted soil (Bennekom, The Netherlands)	Solid state-like batch	pH = 7.0 rh = 90 %	T1	*Cladophialophora* sp.[a] (CBS 110513, ATCC MYA-2335)
Gasoline station soil (Apeldoorn, The Netherlands)	Air biofilter	pH = 4.0 rh = 97 %	T2	*Cladophialophora* sp.[a] (CBS 110551, ATCC MYA-2336)
			T3	*Pseudeurotium zonatum* sporothrix-like anamorph (CBS 110552, ATCC MYA-2337)
BTEX polluted soil and water (Brandenburg,Germany)	Liquid culture	pH = 5.5	T4	*Exophiala* sp.[a] (CBS 110555)
			T5	*Leptodontidium* sp.[a] (CBS 110554)

[a] Conclusive identification at genus level, further identification not possible



FIGURES 3.1 - 3.6: Photographs of fungi grown on toluene (bar = 10 µm). FIG. 3.1 Conidiophores of *Cladosporium sphaerospermum* T0. FIG. 3.2. Conidiophores of *Cladophialophora* sp. T1. FIG. 3.3. Conidial chain of *Cladophialophora* sp. T2. FIG. 3.4. Sporothrix-like anamorph of *Pseudeurotium zonatum* T3. FIG. 3.5. Conidiophore of *Leptodontidium* sp. T5. FIG. 3.6. Yeast-like growth of *Exophiala sp.* T4 with budding cells

The isolates from BTEX-polluted sites were identified by the Centraalbureau voor Schimmelcultures (CBS), Utrecht (Table 3.A) as hyphomycetes except for T3 for which both the teleomorph, *Pseudeurotium zonatum* and a sporothrix-like anamorph were observed. Ascomata of this fungus were produced on complex agar and on mineral agar with toluene. The previously isolated fungus *Cladosporium sphaerospermum*, here abbreviated as T0, was included in the present investigation. The morphological characteristics of the isolates are shown in Figs 3.1 – 3.6.

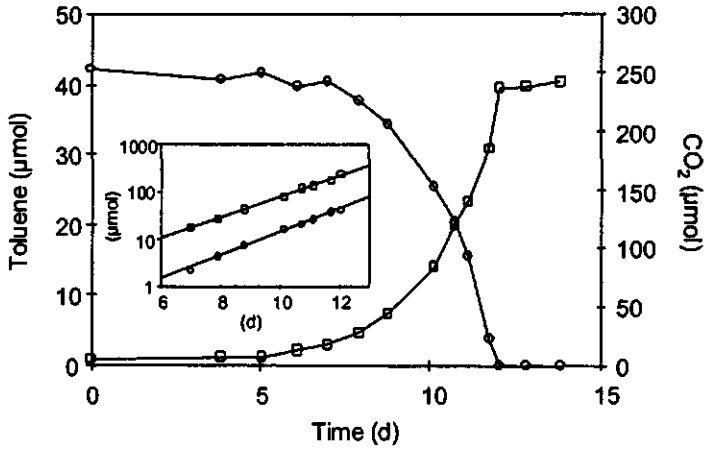


FIGURE 3.7: Toluene degradation (○) and CO_2 production (□) by *C. sphaerospermum* growing on toluene at 30 °C. The inset presents the exponential decrease of toluene and increase of CO_2 plotted on a logarithmic scale

3.3.2 Growth experiments

The ability of the fungal strains T0 – T5 to grow on toluene was further investigated in static submerged cultures. The time course of toluene consumption and carbon dioxide production was followed. After a lag-phase of 5 to 10 days all fungi grew at an apparently constant specific rate until the complete depletion of toluene, as determined from the patterns of substrate utilisation and carbon dioxide production. More than 60 % of the carbon-substrate was recovered as carbon dioxide and 3 – 4 mg-dw of cells were formed. Considering that 26 g-dw of biomass contains about 1 mol of carbon (123), the biomass yield accounted for about the 30 – 40 % of the carbon-substrate. Fig. 3.7 shows as an example of the link between the toluene degradation and growth for *C. sphaerospermum*. Nutrient supplementation of the medium with traces of yeast extract (0.01 % w/v) was essential for the growth of the strain *Cladophialophora* sp. T2. The effect of temperature on the fungal growth is shown in Table 3.B. These results revealed that the fungi differed in their optimum growth temperatures. *Pseudeurotium* sp. T3 and *Leptodontidium* sp. T5 showed good growth at 20 °C but little or no growth at temperatures higher than 30 °C. In contrast, the *Cladophialophora* strains T1 and T2 grew better in the range of 30 to 37 °C.

TABLE 3.B: Influence of temperature on the rate coefficient of carbon dioxide production measured in day $^{-1}$ for fungi growing on toluene ($n \geq 5$, $r^2 \geq 0.98$)

	Temperature (°C)			
Fungus	20	25	30	37
Cladosporium sphaerospermum T0	< 0.10	0.21	0.48	0.16
Cladophialophora sp. T1	0.24	0.31	0.40	0.39
Cladophialophora sp. T2 [a]	0.22	0.21	0.56	0.65
Pseudeurotium zonatum T3	0.27	0.22	0.12	-
Exophiala sp. T4	0.28	0.36	0.40	-
Leptodontidium sp. T5	0.34	0.33	0.13	-

[a] Mineral medium supplemented with yeast extract (0.01 % w/v)
- No growth

The substrate range of the fungi was evaluated using different hydrocarbons as the sole source of carbon and energy (Table 3.C). Besides toluene, ethylbenzene and styrene were used as growth substrates by *C. sphaerospermum* T0 and *Cladophialophora* sp. T1. These strains also grew on the oxygenated aromatic compounds phenol and cresols (data not shown). Ethylbenzene was also assimilated by *Exophiala* sp. T4. Benzene, naphthalene and xylenes did not support growth of any of the fungi. Furthermore, growth on glucose was inhibited by the addition of 2-methylnaphthalene and, in some fungi, by naphthalene exposure. The latter result indicated that these compounds were toxic when saturating the liquid media. The aliphatic hydrocarbons *n*-decane and *n*-hexane served as growth substrates for some of the fungi. Nevertheless, they were catabolized at much lower rates and growth was characterized by low carbon dioxide recoveries.

TABLE 3.C: Rate coefficient of carbon dioxide production, measured in day^{-1} at 25 °C, for the growth of fungi on different hydrocarbons added up to 0.5 mM ($n \geq 5$, $r^2 \geq 0.98$)

	Fungus (strain code)					
Substrate	T0	T1	T2[a]	T3	T4	T5
Toluene	0.21	0.31	0.21	0.22	0.36	0.33
Ethylbenzene	< 0.10	0.28	-	-	0.28	-
Styrene	< 0.10	0.29	-	-	-	-
o-Xylene	-	-	-	-	-	-
m-Xylene	-	-	-	-	-	-
p-Xylene	-	-	-	-	-	-
2-Methylnaphthalene	×	×	×	×	×	×
Benzene	-	-	-	-	-	-
Naphthalene	×	-	×	-	-	-
Cyclohexane	-	±	-	-	-	-
n-Hexane	-	±	-	-	-	-
n-Decane	±	±	±	±	±	-
Glucose	0.31	0.79	0.64	0.67	1.19	0.72

[a] Mineral media supplemented with yeast extract (0.01 % w/v)
± Poor growth, - No growth, × Toxic

3.3.3 Kinetics of toluene degradation

Table 3.D summarizes different parameters for toluene degradation in whole cell suspensions. No biodegradation activity could be measured in strains T3 and T5. Apparently, the latter strains lost their degradation activity during the harvesting procedures. Substrate affinity and toxicity were evaluated from the oxygen consumption rates at different toluene concentrations (data for *C. sphaerospermum* are shown in Fig. 3.8).

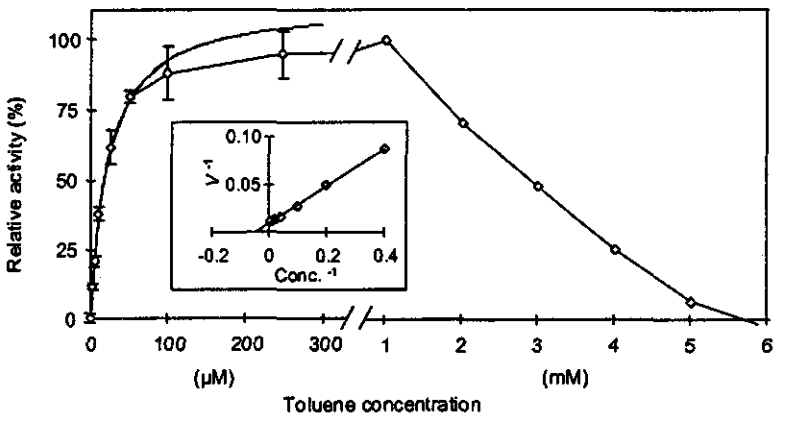


FIGURE 3.8: Kinetics of toluene oxidation by *C. sphaerospermum*. The data are presented as a direct plot and as a Lineweaver-Burk linearisation (inset). Error bars show standard deviations

These data were fitted to the Michaelis-Menten model and the apparent K_m was calculated from the Lineweaver-Burk plot. Oxidation rates progressively decreased at toluene concentrations higher than 1 mM, indicating that toxic levels were reached. Substrate concentrations causing 50 and 80 % of inhibition (IC) relative to the maximum measured rate for oxygen consumption were calculated by extrapolation.

TABLE 3.D: Toluene degradation kinetics by toluene-grown fungal cells. Specific rates are given in μmol g dry-wt $^{-1}$ h^{-1} at 30 °C ($n \geq 6$, $r^2 \geq 0.95$)

Parameter	Fungus (strain code)			
	T0	T1	T2	T4
Specific rates [a]				
Toluene degradation	74 ± 70	81 ± 40	74 ± 60	25 ± 30
CO_2 production [b]	292 ± 12	174 ± 15	227 ± 58	94 ± 14
Transient C-CO_2 recovery (%)	56	31	44	53
Oxidation kinetics				
Max. O_2 consumption rate [b,c]	353 ± 31	73 ± 60	154 ± 11	56 ± 12
K_m (μM) [d]	22	12	5	6
Inhibitory concentration (mM)				
50 % IC	2.9	3.7	3.5	2.1
80 % IC	4.3	4.3	4.7	2.4

[a] Toluene added up to 0.2 mM
[b] Corrected for the endogenous respiration
[c] Measured value
[d] From the Lineweaver-Burk plot ($n \geq 8$, $r^2 \geq 0.98$)

3.4 Discussion

The present paper gives a description of the isolation and characterisation of five fungal strains which are able to grow on toluene as their sole carbon and energy sources. The fungi belong to the following genera: *Cladophialophora*, *Exophiala*, *Pseudeurotium* (anamorph sporothrix–like) and *Leptodontidium*. Previously, *Exophiala* and *Sporothrix* species assimilating styrene were isolated from biofilters used in the treatment of air polluted with styrene (38). *Exophiala* has a high taxonomic affinity with *Cladophialophora* (45). Consequently, with the exception of *Leptodontidium* sp. T5, the new isolates are related to fungi already known to grow on aromatic hydrocarbons. The latter suggests that contrary to the cometabolism of aromatic hydrocarbons, which is widespread among fungi (28), the ability to use these compounds as growth substrates might be restricted to a limited number of fungal genera.

It is interesting to contemplate why, until recently, no fungi have been described with the capacity to grow on aromatic hydrocarbons. The lack of information about hydrocarbon-assimilating fungi may be attributed to the fact that traditional sealed flask enrichments tend to select bacteria rather than slower-growing fungi (31, 34). Nevertheless, our results demonstrate that it is possible to select for fungi by using appropriate enrichment techniques. Interestingly, when enrichments were performed in liquid media, fungi preferentially grew as biofilms attached to the glass walls at the water/air interface. In fact, the development of fungi was effectively favoured in solid state-like fermentations, which might result from their ability to grow under conditions of limited water and nutrient availability. Using an inoculum that originated from a toluene-polluted site increased the chance of obtaining toluene-degrading fungi. Most likely, the polluted soil matrix was already enriched in toluene utilizing fungi.

Due to the inhomogeneous biomass of filamentous fungi, fungal growth is generally correlated to the respiration activity (118). Here we used the rate coefficient of carbon dioxide production (value that was very similar to the rate of substrate utilisation, Fig. 3.1) as an estimate of the specific growth rate. The substrate contribution to maintenance however can not be neglected in slow-growing organisms (123). Therefore, the specific growth rates of our fungal isolates are expected to be lower than the respiration rates given in Tables 3.B and 3.C. From these results it is clear that the fungi grew on toluene with doubling times of at least 2 to 3 days in contrast to their bacterial counterparts, which exhibit doubling times of only 1 to 3 hours (33, 79, 85). The white colony forming strains *Pseudeurotium* sp. T3 and *Leptodontidium* sp. T5 showed poor growth at temperatures of 30 °C. These fungi also lacked degradative activity in washed mycelia. Loss of activity after harvest has been observed in other hydrocarbon-growing fungi (59).

Some of the fungi were also able to assimilate ethylbenzene, and styrene. The substrate specificity was strain or species specific as shown for the two

Cladophialophora isolates T1 and T2, of which only T1 grew either on styrene or ethylbenzene. The same is true for the *Exophiala* strains. Our isolate *Exophiala* sp. T4 was not able to grow on styrene under the test conditions, contrary to the previously studied *E. jeanselmei*, which assimilated styrene but did not grow on toluene (37). The fact that unsubstituted aromatic hydrocarbons did not support growth of any strains suggests that the alkyl side-chain plays an important role in the catabolism of the aromatic structure. Nevertheless, the presence of a second side-chain (e.g. in the xylenes) or an additional fused aromatic ring (e.g. in 2-methylnaphthalene) apparently prevents the catabolism of the methylbenzene structure. In spite of its low solubility in water (0.18 mM according to Mackay & Shiu (76), 2-methylnaphthalene was toxic to all the fungi studied. Naphthalene and its methylated analogues are also highly toxic towards invertebrates (3, 18). In contrast to the aromatic substrates, aliphatic hydrocarbons were poorly utilized. In agreement with our results, low growth rates and biomass yields were obtained in previous studies with filamentous fungi growing on *n*-alkanes (75, 116).

Fungi might be well suited for the treatment of air streams in biofilters, where acidification and drying of the filter bed are likely to occur. In order to avoid clogging problems these systems are ideally operated without net growth, which is the case when the substrate supply equals the energy requirements for maintenance of the biofilm. Since the maintenance coefficient in both types of microorganisms, fungi and bacteria, are known to be comparable (123), the use of bacteria may not be especially advantageous in terms of specific activity for toluene degradation under growth-limited conditions. The biodegradation rates measured with fungal cells (Table 3.D) were of the same order of magnitude as the rates of toluene assimilation measured in a non-growing fed-batch culture of the bacterium *Burkholderia cepacia* G4 (22 μmol g-dw $^{-1}$ h^{-1} at 28 °C; (78)). Fungi were found to be comparable to bacteria in relation to the substrate affinity and toxicity for toluene. The apparent K_m measured in fungal cell suspensions, as determined from the overall toluene oxidation (Table 3.D), fell into the range of

reported Monod constants for toluene degradation by *Pseudomonas* species (0.4 to 43.3 µM; (33, 79, 85)). Furthermore, the 50 % IC values measured for fungi are similar to those reported for most aerobic bacteria, but remain well below the values for solvent-tolerant bacteria (10, 42, 134).

In summary, the newly isolated fungi combine the propensity of many fungi to grow at low water activities and in acidic conditions with an ability to grow on aromatic hydrocarbons. Therefore, the use of fungi in bioremediation processes is very promising when microorganisms must operate under harsh environmental conditions and when near-zero net growth is preferred. Due to the rather narrow substrate specificity of the fungi isolated in this study, their application may be limited to the degradation of a number of compounds that, however, are very important pollutants.

CHAPTER 4

Fungal metabolism of toluene: monitoring fluorinated analogues by ^{19}F NMR spectroscopy

Francesc X. Prenafeta-Boldú, Dion M.A.M. Luykx, Jacques Vervoort and Jan A.M. de Bont.

Published in *Applied and Environmental Microbiology*, 67(3): 1030-1034

4.1 Introduction

In bacteria, five different metabolic pathways for the complete degradation of toluene and its assimilation are known (56, 68, 115, 137, 139). Depending upon the strain, toluene is initially oxidized either at the methyl group or at the aromatic ring. Fungi also can oxidize toluene at both molecular sites. Cultures of *Mortierella isabellina* converted toluene into benzyl alcohol (64). Smith and Rosazza (117) identified two zygomycetes and three deuteromicetes that hydroxylated toluene at the aromatic ring to produce *o*-cresol and, in some cases, *p*-cresol. Mineralization of toluene has been reported for the white-rot fungus *Phanerochaete chrysosporium*, but the metabolic pathway was not determined (141). The fungal degradation of toluene in these cases occurred only by cometabolism and, consequently, it did not support growth. We previously identified and described a *Cladosporium sphaerospermum* strain that can grow on toluene as the sole carbon and energy source (133). Oxygen consumption experiments with whole cells and enzyme activities in cell-free extracts suggest that the initial oxidation of toluene takes place at the methyl group, rather than at the aromatic ring. Recently, we identified five additional fungi that also can assimilate toluene (Chapter 3).

^{19}F nuclear magnetic resonance (^{19}F NMR) has been used previously to characterize the degradation of fluorine-containing aromatic compounds by fungi (11, 99, 126). Fluorine, with its small size, can replace hydrogen in an organic substrate with few steric consequences. It also influences the conversion rate of many enzyme reactions (136). The ^{19}F isotope, with a natural abundance of 100% and a broad chemical shift range, is a very sensitive NMR-active nucleus that can be advantageously used in the identification and quantification of fluorinated intermediates by ^{19}F NMR spectroscopy.

Our objectives were to identify the initial steps for the catabolism of toluene in six previously isolated fungi (101, 133), Chapter 3) ,which are capable

of growth on toluene as a sole carbon and energy source: *C. sphaerospermum* T0, *Cladophialophora* sp. strains T1 and T2, *Pseudeurotium zonatum* T3, *Exophiala* sp. strain T4, and *Leptodontidium* sp. strain T5. The fungi *Cunninghamella echinulata* CBS 596.68 and *Aspergillus niger* CBS 126.48 were included in this study. These two organisms cometabolically hydroxylate the aromatic ring of toluene (7, 117). We used fluorinated toluene isomers as substrate analogs and ^{19}F NMR spectroscopy to characterize the pattern of metabolite accumulation. In particular, we focused on the site of the initial oxidative attack, in order to determine whether fungi can assimilate toluene through pathways as diverse as those used by the aerobic bacteria.

4.2 Materials and Methods

4.2.1 Chemicals

Toluene was purchased from Labscan Ltd. (Dublin, Ireland) 2-, 3-, 4-Fluoro-toluene, and the reference compounds 2-, 3-, 4-fluorobenzyl alcohol, 2-, 3-, 4-fluorobenzaldehyde, 2-, 3-, 4-fluorobenzoic acid, 3-fluorocatechol and 3-fluoro-6-hydroxytoluene were from Acros Organics (Geel, Belgium). 4-Fluoro-3-hydroxytoluene was from ABCR GmbH&Co KG (Karlsruhe, Germany). All chemicals were of analytical grade. 3-Fluoro-4-hydroxybenzoic acid was a gift of Sjef Boeren (Laboratory of Biochemistry, Wageningen University). The purity of all fluorinated compounds was verified by ^{19}F NMR spectroscopy.

4.2.2 Organisms

The fungi capable of growing on toluene used in this study were: *C. sphaerospermum* T0, *Cladophialophora* sp. strains T1 and T2, *Pseudeurotium zonatum* T3, *Exophiala* sp. strain T4, and *Leptodontidium* sp. strain T5. These fungi can be obtained upon request from the culture collection of the Department

of Biotechnology, University of Kaiserslautern (Kaiserslautern, Germany). The strains *C. echinulata* CBS 596.68 and *A. niger* CBS 126.48 were purchased from the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). All organisms were routinely maintained at 4°C on 2% glucose mineral medium (61) agar slants.

4.2.3 Preparation of cell suspensions

Toluene-grown mycelium was obtained as described in Chapter 3. *C. echinulata* and *A. nigra* were grown at 30 °C as shake cultures (120 rpm) in a cotton-plugged 5 l Erlenmeyer flask containing 0.5 l of the following medium (per liter of demineralized water): 20 g glucose, 5 g mycological peptone, 2 g yeast extract, 1 g KH_2PO_4 and 0.5 g $MgSO_4 \cdot 7H_2O$. The medium was inoculated with 1 ml of a spore suspension (approx. 10^7 spores per ml) and incubated for 48 hours. Toluene oxidation activity was induced by replacing the cotton-plug with a rubber cap containing an insert filled with 5 ml of a 5:95 toluene:dibutylphthalate solution. The flask was then incubated for 12 additional hours. Mycelium was harvested with a glass-fiber paper of >1 µm retentivity (Schleicher & Schuell, Dassel, Germany), washed with 500 ml of a 50 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer solution. Cell suspensions were stored at 4 °C and used within three days after preparation.

4.2.4 Incubations with whole cells

(Fluoro)toluene was bioconverted in 250 ml Boston flasks closed with teflon valves (Mininert, Phase Separations, Waddinxveen, The Netherlands). Cell suspensions of toluene-grown fungi (10 ml, approx. 8 g DM l^{-1}) were incubated with the fluorotoluenes (shaking conditions, 120 rpm, 30 °C). Toluene and all three fluorinated isomers, 2-, 3- and 4-fluorotoluene, were added individually up to 2 µl/10 ml culture. T3 and T5 had no degradation activity after harvest, so

these fungi were cultured in Boston flasks containing 25 ml of mineral media (61) and 4.5 µl of toluene (static conditions, 25 °C). After toluene exhaustion, the resulting cultures (approx. 5 g DM l^{-1}) were flushed with non-sterile air and then incubated with a fluorinated toluene (2 µl/25 ml culture). Similarly, toluene induced and non-induced cells of *C. echinulata* and *A. niger* (25 ml, approx. 14 g-dw l^{-1}) were incubated with 2 µl of each fluorotoluene (shaking conditions, 30 °C). We followed fluorotoluene consumption via gas chromatographic analysis of the headspace. Incubations lasted no longer than 48 hours and were stopped before complete substrate depletion. The cell suspension was stored at –20 °C until analyzed. For metabolite determination, samples were thawed and divided in two portions. One was centrifuged (4 °C, 10 min, 13 000 × g) to remove cell debris, and the other was extracted with 1 volume of ethyl acetate. Both the culture medium and the solvent extract were analyzed by ^{19}F NMR.

4.2.5 Identification of fluorinated metabolites

Products of fluorotoluene conversion were identified by comparing their ^{19}F NMR chemical shift values with those of authentic reference compounds whenever available. For compounds not available commercially, the chemical shift was either taken from literature or predicted by using the method of Rietjens *et al.* (110). Comparisons between known and predicted chemical shift values indicated that this approach provides reliable results (not shown). The presence of fluorinated intermediates at trace level (less than 1 % of the total ^{19}F resonance) was confirmed by analyzing new samples from the incubation media.

4.2.6 Analytical methods

Toluene and the fluorinated analogs were measured by injecting 100 µl head-space samples into a HP 6890 gas chromatograph (Hewlett-Packard, Wilmington, Del.) with a 10% SE-30 Chromosorb WMP column (Chrompack B.V.

Middelburg, The Netherlands). The carrier gas was nitrogen at a flow of 1.9 ml/min. The temperature of the column and the flame ionization detector was 110 and 300 °C, respectively. ^{19}F NMR measurements were made with a Bruker DPX 400 MHz NMR as previously described (129). The temperature of the measurement was 7 °C. The sample volume was 2 ml containing 1.8 ml of culture media and 0.2 ml of 0.5 M potassium phosphate buffer (pH 7). Ethyl acetate extracted fractions (2 ml) were assayed directly. 4-Fluorobenzoate (80 μM) was added as internal standard via an insert. ^{19}F chemical shifts (expressed in ppm with respect to $CFCl_3$) and concentrations of the various metabolites were calculated by comparison of their ^{19}F NMR integrals to that of the standard 4-fluorobenzoate. Cell dry weight was determined by weighing dried cell suspensions (24 h at 105 °C).

4.3 Results

4.3.1 Fungal conversion of fluorinated toluenes

The two types of toluene-degrading fungi, those that degrade it by co-metabolism and those that use it as the sole carbon source, both degraded the fluorinated analogs although at very different rates (Table 4.A). The specific degradation activity for the fluorotoluenes was up to one order of magnitude higher in fungi that used toluene for both energy generation and biomass production. These fungi degraded 4-fluorotoluene faster than 3- and 2-fluorotoluene. The latter isomer was not degraded by the strain *Cladophialophora* sp. T2. Recovery of the fluorine label from the consumed substrate as conversion products in the liquid medium was as high as 90% in most of cases. Due to the low specific degradation activity in the fungi cometabolizing toluene, the rates for fluorotoluene conversion were estimated on the basis of the amount of fluorinated products that accumulated in the media in relation to the biomass and the incubation time. The activities presented in Table 4.A for these fungi were

obtained with cells that had been exposed to toluene during growth and were about four times higher than those in non-induced cells.

TABLE 4.A: Specific rates (in μmol h^{-1} g-dw $^{-1}$) for the degradation of toluene and fluorinated toluene analogs by fungi

Fungus	Toluene metabolism[a]	Substrate			
		Toluene[b]	2F-toluene	3F-toluene	4F-toluene
Aspergillus nigra CBS 126.48	C	-	0.012	0.015	0.012
Cuninghamela echinulata CBS 596.68	C	-	0.034	0.041	0.053
Cladosporium sphaerospermum T0	A	74 ± 7	22	39	69
Cladophialophora sp. T1	A	81 ± 4	29	30	59
Cladophialophora sp. T2	A	74 ± 6	ND[c]	35	60
Pseudeurotium zonatum T3	A	2.0 ± 0.4	1.5	1.3	2.1
Exophiala sp. T4	A	25 ± 2	13	8.0	15
Leptodontidium sp. T5	A	4.3 ± 0.9	3.3	2.1	3.5

[a] C: toluene co-metabolism by toluene-induced cells (degradation rates estimated from the amount of accumulated fluorinated products); A: assimilation of toluene by toluene-grown cells (degradation rates measured at the head space, $n \geq 5$, $r^2 \geq 0.98$)
[b] Average and standard deviation of three experiments; - not determined
[c] Not degraded

4.3.2 Identification of fluorinated metabolites

The ^{19}F NMR chemical shift of the various fluorotoluene derivatives were assigned to specific metabolites (Table 4.B). Peak overlap was observed between the ^{19}F NMR signals of 3-fluorobenzyl alcohol and 3-fluorobenzoate, since both compounds have the same chemical shift in the aqueous phase. The fluorine resonance of these compounds differed in ethyl acetate solution. Consequently, the presence of 3-fluorobenzyl alcohol and/or 3-fluorobenzoate was confirmed by analyzing the solvent-extracted fractions.

A small ^{19}F NMR peak at –112.5 ppm resulting from the degradation of 2-fluorotoluene by *C. sphaerospermum* T0 and *P. zonatum* T3 was tentatively

identified as a muconate-derivative due to its proximity to the chemical shift of 2-fluoro-*cis,cis*-muconate. We suggest that this resonance results from a *cis-trans* isomerization product of 2-fluoro-*cis,cis*-muconate or from a fluorinated carboxymuconate arising from the ring opening of fluoroprotocatechuate. This unidentified metabolite had a low signal intensity and limited stability.

TABLE 4.B: Fluorinated products resulting from the degradation of fluorotoluene by eight fungi. The ^{19}F NMR chemical shifts, measured at 7 °C, are assigned in two different solvents: aqueous solution and ethyl acetate

Chemical shift (ppm) [a]		Compound
50 mM potassium phosphate buffer, pH 7	Ethyl acetate	
-124.3 R [b]	-128.1 R	2-fluorobenzyl alcohol
-117.9 R	-121.6 R	3-fluorobenzyl alcohol
-119.5 R	-124.3 R	4-fluorobenzyl alcohol
-120.3 R	-	2-fluorobenzoate
-118.0 R	-120.6 R	3-fluorobenzoate
-114.2 R	-114.9 R	4-fluorobenzoate
-116.4 L	-	2-fluoro-4-hydroxybenzoate
-141.7 R	-	3-fluoro-4-hydroxybenzoate
-141.2 L	-	2-fluoro-3,4-hydroxybenzoate
-126.2 L	-	2-fluoro-4,5-hydroxybenzoate
-140.2 P	-	3-fluoro-4,5-hydroxybenzoate
-140.4 R	-144.6 R	3-fluorocatechol
-111.8 L	-	2-fluoro-*cis,cis*-muconate
-129.7 R	-134.5 R	3-fluoro-6-hydroxytoluene
-120.7 P	-	4-fluoro-6-hydroxytoluene
-123.0 R	-	Free fluorine

[a] Relative to $CFCl_3$
[b] R: reference compound; L: taken from literature (11, 12, 126); P: predicted (110)
- Not detected/not identified

4.3.3 Metabolic pathway for the fluorotoluenes

The fungi co-metabolizing toluene and the toluene-growing strains differed in the nature of the metabolites accumulated (Table 4.C). With 3-fluorotoluene, for example, the main fluorinated product excreted by *C. echinulata* was

TABLE 4.C: Metabolite patterns from the fungal degradation of fluorotoluene analogs as determined by ^{19}F NMR. The relative composition of intermediates is referred to the total amount of fluorinated products other than the parent fluorotoluene, taken as 100 %

Substrate Metabolite	*Fungus (grouped by type of toluene metabolism)*							
	Co-metabolism		Assimilation					
	CBS 126.48	CBS 596.68	T0	T1	T2	T3	T4	T5
2F-toluene								
2F-benzyl alcohol	-	70 *	2 *	83 *	-	-	9 *	37 *
2F-benzoate	100	30	53	-	-	46	7	57
2F-4OH-benzoate	-	-	4	-	-	-	2	-
2F-3,4OH-benzoate	-	-	-	-	-	-	12	-
2F-4,5OH-benzoate	-	-	-	-	-	-	2	-
3F-catechol	-	-	-	-	-	-	1 *	-
2F-*cis,cis*-muconate	-	-	-	Tr	-	-	32	-
Unknown [a]	-	-	2	-	-	6	-	-
Free fluorine	-	-	39	17	-	48	35	6
3F-toluene								
3F-6OH-toluene	-	29 *	-	-	-	-	-	-
3F-benzyl alcohol	-	-	-	51 [b] *	-	-	23 [b] *	92 [b] *
3F-benzoate	83 *	71 *	18 *	*	16 *	65 *	*	*
3F-4OH-benzoate	17	-	54	39	63	15	51	-
3F-4,5OH-benzoate	-	-	Tr	Tr	2	8	-	-
3F-catechol	-	-	-	-	Tr *	-	3 *	-
2F-*cis,cis*-muconate	-	-	-	Tr	Tr	-	-	-
Free fluorine	-	-	28	10	19	12	23	8
4F-toluene								
4F-6OH-toluene	-	7 *	-	-	-	-	-	-
4F-benzyl alcohol	4 *	-	Tr *	41 *	Tr *	-	Tr *	2 *
4F-benzoate	96 *	93 *	99 *	57 *	99 *	100 *	68 *	98 *
Free fluorine	-	-	Tr	2	-	-	31	-

[a] Fluorine resonance at –112.5 ppm tentatively identified as a muconate-derivative

[b] 3F-Benzyl alcohol and 3F-benzoate are quantified together due to peak overlap in water solution

* Identified in the ethyl acetate extracts

Tr Less than 1 % of the total ^{19}F intensity

- Not detected

3-fluorobenzoate (Fig. 4.1). The phenolic metabolite 3-fluoro-6-hydroxytoluene (fluorinated *o*-cresol) also was detected, indicating that this fungus oxidizes toluene both at the methyl group and at the aromatic ring. No fluoride anion was observed and, consequently, the intermediates measured by ^{19}F NMR are the ultimate accumulation products. In contrast, the metabolic profile for *C. sphaerospermum* T0 indicates that 3-fluorotoluene is initially oxidized only at the side chain. The resulting 3-fluorobenzoate is metabolized to 3-fluoro-4-hydroxybenzoate and 3-fluoro-protocatechuate. A relatively high concentration of free fluorine was measured, possibly as a result of the oxidative defluorination of 3-fluoro-4-hydroxybenzoate to protocatechuate.

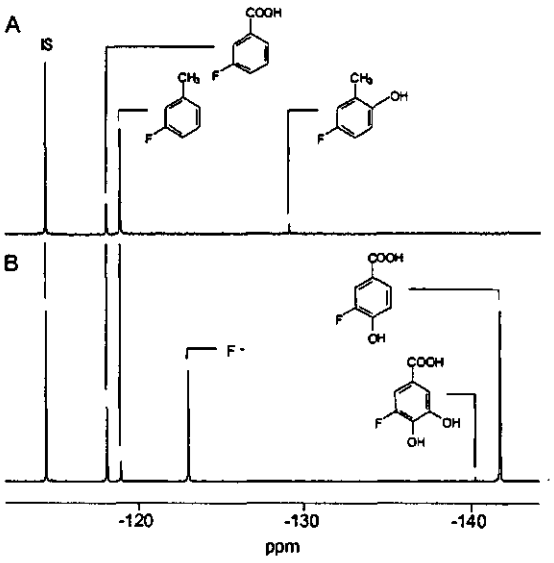


FIGURE 4.1: ^{19}F NMR spectra at 7 °C of the culture supernatant after incubation of whole cells of *C. echinulata* CBS 596.68 (A) and *C. sphaerospermum* T0 (B) with 3-fluorotoluene. The resonance at -114.4 ppm marked as IS is from the standard 4-fluorobenzoate contained in an insert

4.4 Discussion

The initial oxidation of toluene was characterized by using fluorinated analog substrates and identifying the metabolites formed by ^{19}F NMR. Previous attempts to measure and identify intermediates of toluene degradation by using a high-pressure liquid chromatography method were inconclusive (not shown). The fluorine substituent effectively decreased the conversion rate of specific reactions, resulting in the accumulation of intermediates that otherwise would have been rapidly metabolized further. The accumulation pattern depended primarily on the type of toluene oxidation, i.e. co-metabolism versus assimilation, and the position of the fluorine.

Conversion of fluorotoluene was exceptionally low in the co-metabolizing strains *C. echinulata* and *A. niger*. Aryl hydrocarbon hydroxylation by fungi is catalyzed by P-450 monooxygenases, which are usually substrate-inducible enzymes (31). But even cultures that had been exposed to toluene during growth metabolized the fluorinated analogs very slowly. Although both *Cunninghamella* and *Aspergillus* species are reported to hydroxylate the aromatic ring of several aromatic hydrocarbons (31), the two strains we tested both initiated oxidation of the fluorinated toluenes at the side-chain. Only *C. echinulata* also hydroxylated the fluorotoluene structure at the aromatic ring to form fluorinated *o*-cresols. The highest yield of fluorocresol was obtained with the 3-fluorotoluene isomer, which was hydroxylated exclusively at the *para* position in relation to the fluorine to yield 3-fluoro-6-hydroxytoluene. A similar regioselectivity in the related species *C. elegans* was found for the hydroxylation of 1-fluoronaphthalene, in which the fluorine group prevented hydroxylation at the adjacent carbons (30). Fluorinated *o*-cresol also was detected with 4-fluorotoluene but not with 2-fluorotoluene. As before (117), we found no evidence for cresol formation by *A. niger*. Taken together, our results indicate that the presence of a methyl group in the benzene ring channels the fungal oxidative attack towards the side-chain. Preference for

hydroxylation of alkylated aromatic hydrocarbons at the side-chain has been reported for *Cunninghamella* species (27, 29, 63).

Fungi that used toluene for both energy generation and biomass production converted the fluorinated analogs at higher rates and to more oxidized intermediates, which all were products of the side-chain metabolism (Table 4.C). In general, the proximity of fluorine to the methyl group had a negative effect on the degradation rate of fluorotoluene (Table 4.A). This steric effect might result from the change in reactivity caused by the fluorine nucleus towards the side-chain monooxygenase. The extent of fluorotoluene degradation also depended on the fluorine position: 2-fluorotoluene was converted to 2-fluorobenzyl alcohol and/or 2-fluorobenzoate. A significant part of the ^{19}F signal in the liquid media was identified as free fluorine, indicating that these metabolites accumulated transiently, with the aromatic ring being effectively defluorinated at a latter stage. This pattern of substrate conversion was not seen with either *Cladophialophora* sp. T2 or *Exophiala* sp. T4. While the fluorine at the C-2 carbon center of toluene prevented the oxidative attack in the former strain, it induced accumulation of 2-fluoro-*cis,cis*-muconate in the latter. Similar to this, 2-fluoro-*cis,cis*-muconate was the main degradation product of 2-fluorophenol by phenol-grown cells of another *Exophiala* species (11). 3-Fluorotoluene also was metabolized to fluorinated benzoate, but in this case 3-fluoro-4-hydroxybenzoate was often the main product. Besides free fluorine, low concentrations of fluorinated protocatechuate, catechol, and *cis,cis*-muconate were detected with some of the fungi. Apparently, fluorine at C-3 was an important rate-limiting factor for the hydroxylation of 3-fluoro-4-hydroxybenzoate to fluorinated protocatechuate. In contrast to the 2- and 3-fluorotoluenes and with the exception of *Exophiala* sp. T4, the fungi could not cleave the carbon-fluorine bond of 4-fluorotoluene. Consequently, 4-fluoro-benzoate was the end-reaction product of the degradation of 4-fluorotoluene.

In summary, the six toluene-growing fungi converted 2-, 3-, and 4-fluorotoluene to intermediates that matched the toluene metabolic pathway earlier proposed for *C. sphaerospermum* T0 (133). By analogy, we suggest that toluene is assimilated via an initial oxidation of the methyl group by all fungi studied. Thus, toluene is first hydroxylated to benzyl alcohol and then dehydrogenated to benzoate via a putative aldehyde intermediate. Benzoate is the substrate for the hydroxylation of the aromatic ring to 4-hydroxybenzoate and protocatechuate. Detection of fluorinated catechols and fluorinated *cis,cis*-muconate in the *Cladophialophora* sp. strains (T1 and T2) and *Exophiala* sp. strain T4 supports the hypothesis that protocatechuate is decarboxylated to catechol. The aromatic ring of catechol is opened at the *ortho* position to yield *cis,cis*-muconate. This ring cleavage pathway has been reported previously for fungi growing on 4-hydroxybenzoate (24). However, fungi also assimilate 4-hydroxybenzoate via two other alternative ring-fission substrates: protocatechuate and hydroxyquinol (140). All three pathways converge with the formation of 3-oxoadipate. The pattern of ring-cleavage for the metabolism of toluene in the strains *C. sphaerospermum* T0, *P. zonatum* T3 and *Leptodontidium* sp. strain T5 can not be determined from our results.

The toluene catabolic pathway is of special interest because either the benzene nucleus or the aliphatic side-chain may be subject to oxidative attack. Bacteria have evolved both options, but very little is known about the fungal metabolism of toluene. The similarity of the initial oxidative steps in all these strains suggests that fungi may have less metabolic versatility than bacteria for the assimilation of toluene.

CHAPTER 5

Substrate interactions during the biodegradation of BTEX hydrocarbons by the fungus *Cladophialophora* sp. strain T1

F. X. Prenafeta-Boldú, J. Vervoort, J. T. C. Grotenhuis, and J. W. van Groenestijn.

Published in *Applied and Environmental Microbiology*, 68(6): 2660-2665

5.1 Introduction

A considerable amount of gasoline enters the environment as result of leakage from underground storage tanks, accidental spills, or improper waste disposal practices (17). When gasoline is in contact with water, benzene, toluene, ethylbenzene, and the xylene isomers (BTEX) account for as much as 90% of the gasoline components that are found in the water-soluble fraction (113). Consequently, these chemicals are some of the most common contaminants found in drinking water. BTEX are toxic to human and their removal from polluted environments is of special interest (82).

It has been assumed that soil bioremediation of BTEX pollution relies upon indigenous bacterial populations; the significance of fungi has been overlooked (17). Fungi generally withstand harsher environmental conditions than bacteria and could play an important role in the degradation of petroleum hydrocarbons in the soil (15). Nevertheless, fungal degradation of BTEX mixtures has been studied only to a limited extent with white-rot fungi (19, 141). BTEX were mineralized but they did not support fungal growth when supplied as the sole source of carbon and energy. The extracellular lignin-degrading enzymes are capable of oxidize a wide range of aromatic hydrocarbons but they appear not to be involved in BTEX degradation. The low degradation rates and the requirement of an additional carbon source limit the use of white-rot fungi in bioremediation. Interestingly, new non-white rot fungal strains have been isolated and applied successfully for the biofiltration of air contaminated with volatile aromatic hydrocarbons (*i. e.* toluene and styrene), which were metabolized as sole carbon and energy sources (36, 39, 101). When hydrocarbon-degrading microbes are used for bioremediation of gasoline pollution, it is very unlikely that they encounter a sole substrate. Some papers dealing with substrate interactions during the degradation of BTEX mixtures by bacteria have been published (1, 33, 47, 49, 91), but analogous data for fungi are still very scarce.

The objective of the present study was to investigate the degradation pattern of BTEX mixtures by a fungus capable of growth on aromatic hydrocarbons. The deuteromycete *Cladophialophora* sp. strain T1, which grows on toluene, was selected as a model fungus. This strain was isolated previously from a BTEX-polluted soil and showed the best degradative capacity in terms of substrate specificity among the fungal isolates examined (Chapter 3). Special attention was put on the kinetics of utilization multiple substrates and to the extent of degradation of every BTEX component. This information is of importance for devising fungal-based techniques for treatment of BTEX pollution especially under "solid state-like" environmental conditions, such as in air biofilters or acidic soils, which usually favor fungal over bacterial growth (15, 101).

5.2 Materials and methods

Chemicals. BTEX hydrocarbons and reference compounds for intermediate identification were obtained from Acros Organics (Geel, Belgium), Sigma-Aldrich Chemicals (Steinheim, Germany), Jansen Chimica (Geel, Belgium), Lab-Scan Ltd. (Dublin, Ireland) and Merck KGaA (Darmstadt, Germany). All chemicals were of analytical grade. Deuterium oxide (>99.9 % d) was supplied by M. G. Chemicals (Toronto, Canada). Deuterated chloroform (99.8 % d) was from Isotec Inc. (Miamisburg, Ohio).

Fungal strain. *Cladophialophora* sp. strain T1 (ATCC MYA-2335, CBS 110553) was isolated as previously described in Chapter 3. During the present investigation this organism was routinely maintained at 4°C on mineral medium (60) agar slants supplemented with 2 % glucose.

Growth experiments. Tests were performed in 250 ml Boston flasks containing 25 ml of buffered (35 mM $K_2HPO_4/NaH_2PO_4{\cdot}2H_2O$, pH 7) mineral salts medium (60) and sealed with teflon-coated valves (Mininert, Phase Separations,

Waddinxveen, The Netherlands). BTEX compounds were added as the sole carbon and energy source. After water/air substrate equilibration, flasks were inoculated with a fungal spore suspension (containing approx. 10^4 viable spores). Incubations were performed at 25 °C under static conditions. Growth was followed by gas-phase measurements of substrate consumption and CO_2 production against non-inoculated controls. Concentration of BTEX in the water phase was calculated from reported water/air partition coefficients (2, 76). Co-metabolic degradation of the BTEX compounds that individually did not support growth was assayed in combination with toluene. Carbon recovery as CO_2 (C-CO_2) and biomass (C-biomass) was determined after substrate exhaustion and corrected by control flasks inoculated and incubated with no carbon source. The pH of the medium was checked at the end of the incubation for adjustment of the water/air partition of CO_2, but no significant variations were measured. The biomass was collected by filtration and the dry matter determined. C-biomass was calculated considering that 26 g dry-weight contains approx. 1 mol of carbon (123). The culture filtrate was stored at –20 °C for the identification of metabolites. Experiments for carbon mass balance were performed for both, cultures and controls, in triplicate.

Identification of intermediates. The filtrate from the above-described cultures was thawed and divided in two portions of 5 ml each. The first fraction was freeze-dried under vacuum and the residue re-dissolved in 1 ml of deuterium oxide; the second one was extracted with deuterated chloroform (1 ml). Both extracts were analyzed by proton nuclear magnetic resonance (^{1}H NMR). Products of BTEX conversion were identified by comparing their ^{1}H NMR chemical shift values with those of authentic reference compounds, whenever commercially available, and by performing two-dimensional NMR experiments *via* the nuclear Overhauser spectroscopy (2D-NMR NOESY) (65).

Degradation kinetics. A toluene-grown liquid culture of the fungal strain T1 was prepared and harvested as described in Chapter 3. Cells were re-suspended in a

phosphate buffer (25 ml, 50 mM, pH=7) and incubated in 250 ml Boston flasks sealed with teflon valves (120 rpm, 25 °C). The apparent half-saturation constant (K_m) for the degradation of BTEX was determined from the substrate depletion curves. From these data points, the specific substrate consumption rate was calculated at different concentrations and fitted to the Lineweaver-Burk plot. The maximum biodegradation rate (V_{max}) was calculated by linear regression of the data points for which the substrate concentration was more than 5 times higher than the K_m value. The amount of biomass was set for every substrate (between 1 and 6 g dry-matter l^{-1}) in order to obtain a biodegradation activity below the rates of mass transfer between the gas and the aqueous phases for the BTEX, as reported in a similar batch system (33). BTEX adsorption onto the biomass was determined in additional batches containing heat-inactivated cells (25 min, 120° C).

Analytical methods. BTEX and CO_2 were determined by injecting 100 µl headspace samples in a HP 6890 Series gas chromatograph (Hewlett Packard, U.S.A.). For the BTEX, the stationary phase was a 10 % SE-30 Chromosorb WMP column (Chrompack B.V., Middelburg, The Netherlands). The carrier gas was nitrogen used at a flow of 1.9 ml min^{-1}. The temperature of the column and the flame ionisation detector was 110 and 300 °C respectively. A CP-Wax 52CB column (Chrompack B.V., Middelburg, The Netherlands) at a temperature of 50 °C was used to resolve mixtures of ethylbenzene and the three xylene isomers. For CO_2 a CP-Poraplot Q column (Chrompack B.V., Middelburg, The Netherlands) and a thermal conductivity detector were used. Helium at a flow of 3.0 ml min^{-1} was the carrier gas. The column and detector temperatures were respectively set at 70 and 250 °C. ^1H NMR measurements were performed in a Bruker AMX 500 MHz NMR spectrometer. The sample volume was 0.5 ml and the temperature of the measurement was set at 23 °C. Biomass dry weight was determined after filtering and drying (24 h, 105 °C) cell suspensions over a glass-fiber paper of >1 µm retentivity (Schleicher & Schuell, Dassel, Germany). Filters were previously rinsed with de-mineralized water, dried, and weighed.

5.3 Results

Growth experiments. The time-course concentration of every component in a mixture of all BTEX, given as the sole source of carbon and energy, was monitored during growth of *Cladophialophora* sp. strain T1 in a batch system. The benzene concentration remained constant throughout the experiment but toluene and ethylbenzene were exhausted within 17 days of incubation (Fig. 5.1.A). The degradation pattern for the xylenes also differed and only the *ortho* and *meta* isomers were depleted (Fig. 5.1.B). Uptake of these xylenes started after toluene and ethylbenzene had been partially removed. During growth, CO_2 was produced at an exponential rate (0.28 d^{-1}, $r^2 \geq 0.98$) equivalent to a doubling time of 2.5 days (Fig. 5.1.C).

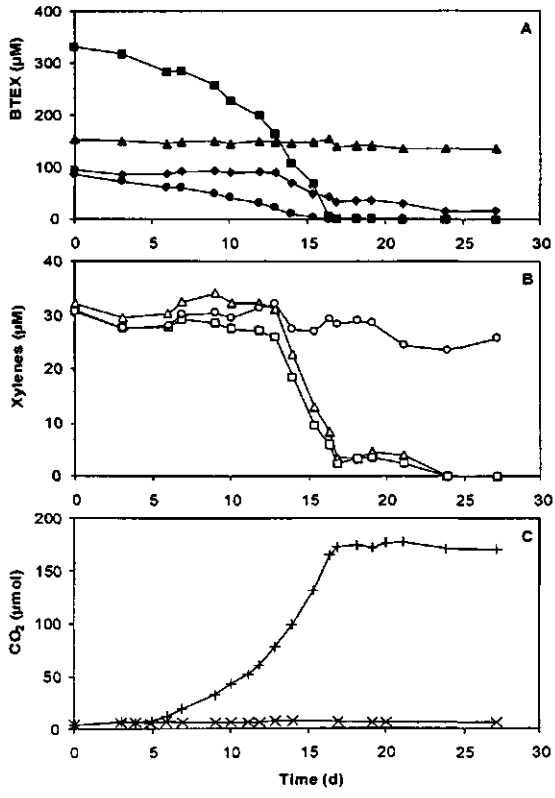


FIGURE 5.1: (A) Degradation of a mixture of benzene (▲), toluene (■), ethylbenzene (●) and xylenes (◆) by a batch-culture of the fungus *Cladophialophora* sp. strain T1 incubated at 25 °C. (B) The degradation curves for the different xylene isomers: *o*-xylene (Δ), *m*-xylene (□) and *p*-xylene (○) are shown separately. (C) Production of CO_2 (+) during growth in comparison with inoculated controls incubated without the BTEX (×)

The type of metabolism for every single BTEX compound, namely assimilation or co-metabolism, was determined in additional growth experiments with single and binary substrate combinations (Table 5.A). Carbon balances were performed by measuring the amount of consumed C-substrate that was recovered as C-CO_2 and C-biomass. Toluene and ethylbenzene were both used for growth and carbon recoveries of about 90% were obtained when these compounds were added either individually or together. Neither benzene nor the xylenes supported growth as single substrates but the latter were successfully co-metabolized in the presence of toluene.

TABLE 5.A: Carbon mass balance after growth of *Cladophialophora* sp. strain T1 on single and binary BTEX combinations. Shown values are the average and the standard deviation of three different experiments

Substrate combination	Substrate (C-µmol)	2nd Substrate depletion (%)	Pattern [a]	CO_2 (C-µmol)	Biomass (C-µmol)	C-Recovery (%)
Ethylbenzene	197		-	132 ± 03	42 ± 04	88 ± 03
Toluene	196		-	131 ± 07	41 ± 04	89 ± 04
Toluene + benzene	196 + 68	<5 [b]	-	135 ± 10	38 ± 05	88 ± 08
Toluene + ethylbenzene	196 + 66	00 ± 00	S	160 ± 23	64 ± 04	86 ± 07
Toluene + *o*-xylene	196 + 67	00 ± 00	S	134 ± 15	45 ± 02	68 ± 07
Toluene + *m*-xylene	196 + 65	00 ± 00	S	138 ± 02	45 ± 06	70 ± 02
Toluene + *p*-xylene	196+65	58 ± 12	D	163 ± 12	58 ± 07	93 ± 04

[a] S: Simultaneous, D: Diauxie
[b] Considered as not degraded

Carbon recoveries in batches containing toluene plus *o*- or *m*-xylene were lower than in those with an identical amount of toluene alone. In contrast to the lack of *p*-xylene degradation observed in BTEX mixtures, about 60% of *p*-xylene was depleted in combination with toluene. Whereas *o*- and *m*-xylene were consumed simultaneously with toluene, degradation of *p*-xylene occurred only after toluene exhaustion. The carbon recovery with mixtures of *p*-xylene and toluene was as high as those measured for toluene and ethylbenzene (Table 5.A).

Identification of intermediates. Extracts from the previously described cultures, grown on single and binary BTEX mixtures, were analyzed by ^{1}H NMR and the resonances from aromatic intermediates assigned (Table 5.B). No aromatic metabolites were detected in cultures incubated with toluene, ethylbenzene, *p*-xylene, and benzene or binary combinations of the latter with toluene. A similar pattern of ^{1}H signals along the aliphatic chemical shift region (0 – 6 ppm) was measured in all cultures (not shown), indicating excretion of downstream metabolites. To address the possibility that volatile intermediates such as aromatic aldehydes, alcohols, phenols, and catechols were lost during the vacuum extraction, we extracted some cultures with d-chloroform. No evidence of these metabolites was found in d-chloroform extracts.

TABLE 5.B: ^{1}H NMR chemical shift values and coupling constants of metabolites formed from xylene degradation. Chemical shifts are determined relative to the HDO signal (4.7 ppm) at 23 °C. Resonances which showed strong coupling were simulated and iteratively fitted onto the measured data

Substrate combination Intermediate	$-CH_3$	$-CH_2OH$	H2	H3	H4	H5	H6
o-xylene + toluene							
4-hydroxy-2-methylbenzoate[a]	2.26	-	-	7.21	-	6.64 $^3J_{H5-H6}$=8.3	6.61
2-methylbenzoic acid	2.25	-	-	7.18 $^3J_{H3-H4}$=7.0	7.12 $^3J_{H4-H5}$=7.0	7.14 $^3J_{H5-H6}$=7.0	7.20
o-phthalic acid	-	-	-	7.36 $^3J_{H3-H4}$=7.0	7.29 $^3J_{H4-H5}$=7.0	7.29 $^3J_{H5-H6}$=7.0	7.36
m-xylene + toluene							
3-methylbenzoate	2.30	-	7.63	-	7.30 $^3J_{H4-H5}$=7.0	7.30 $^3J_{H5-H6}$=7.0	7.60
3-hydroxymethylbenzoate[a]	-	4.65	7.73	-	7.42 $^3J_{H4-H5}$=7.7	7.38 $^3J_{H5-H6}$=7.6	7.71
m-phthalate	-	-	8.17	-	7.87 $^3J_{H4-H5}$=7.6 $^4J_{H4-H6}$=1.3	7.41 $^3J_{H5-H6}$=7.6	7.87

[a] Identified through 2D-NMR NOESY experiments

Degradation kinetics. The effect of a second substrate, present at an increasing concentration, towards growth on toluene was studied (Fig. 5.2). Both *o*- and *m*-xylene negatively affected the growth rate causing complete inhibition when present at a similar concentration as toluene. Under equivalent conditions, ethylbenzene and *p*-xylene inhibited growth moderately whereas benzene did not cause any significant effect. The nature of BTEX inhibitory interactions was characterized by performing degradation activity tests using toluene-grown harvested mycelia and different substrate combinations. Besides toluene, ethylbenzene and the xylenes were degraded without a lag-phase and the Michaelis-Menten kinetic parameters for these substrates were determined (Table 5.C).

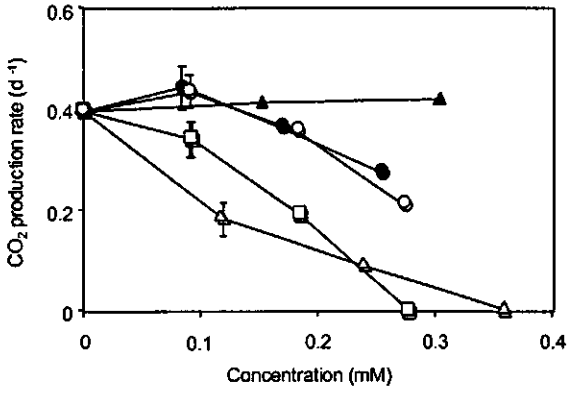


FIGURE 5.2: Effect of benzene (▲), ethylbenzene (●), *o*-xylene (Δ), *m*-xylene (□) and *p*-xylene (○) added at different concentrations in combination with toluene (0.3 mM), on the growth of *Cladophialophora* sp. strain T1. Growth is characterized as the rate-coefficient of CO_2 production at 25 °C ($n \geq 5$, $r^2 \geq 0.98$). When present, error bars indicate the standard deviation of three independent experiments

Additionally, Lineweaver-Burk plots were obtained for the depletion of toluene in the presence of a second substrate (added up to 0.1 mM), as illustrated in Figure 5.3 for the xylenes. The presence of *p*-xylene did not affect the toluene degradation kinetics. However, the K_m for toluene in batches that were supplemented with *o*- or *m*-xylene was respectively 13.3 and 7.8 μM, as determined from Figure 3. These values are significantly higher than that of 2.7±1.0 μM, measured for toluene as the single substrate. It was not possible to generate similar Lineweaver-Burk plots with binary combinations of ethylbenzene and toluene. Ethylbenzene, at 0.1 mM, hindered toluene exhaustion and, if added at a lower concentration, toluene and ethylbenzene were both consumed simultaneously. Controls containing heat-inactivated mycelia showed that adsorption was insignificant in those experiments.

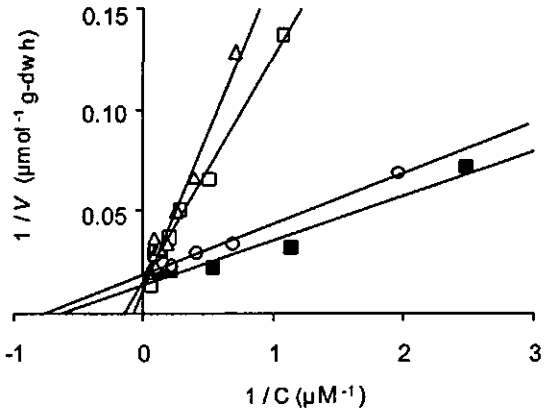


FIGURE 5.3: Lineweaver-Burk plot from toluene-depletion curves by cells of *Cladophialophora* sp. strain T1 incubated with toluene as single substrate (■), and together with 0.1 mM of *o*-xylene (Δ), *m*-xylene (□) and *p*-xylene (○) (T=25 °C, $r^2 \geq 0.98$). The kinetic parameters (V_{max}/K_m) are given in the text

5.4 Discussion

Spores of the soil fungus *Cladophialophora* sp. strain T1 germinated and grew on a complex mixture of BTEX hydrocarbons as the sole carbon and energy source. This BTEX solution was comparable to a real gasoline water-soluble fraction (113). The ability of fungi to grow on the water-soluble fraction of petroleum fuels is well documented, but it was generally believed that only the aliphatic hydrocarbons supported fungal growth (15, 74). Interestingly, this is to our knowledge the first report of a fungus growing on aromatic hydrocarbons from a model gasoline water-soluble fraction, which included all six BTEX components. Most of the previous research in this field has focused on aerobic bacteria growing on simpler BTEX mixtures of only two or three components (1, 33, 47, 49, 91). Although the substrate specificity and the extent of degradation were found to be highly strain specific, co-metabolism and competitive inhibition were the most common substrate interactions. Like bacteria, *Cladophialophora* sp. strain T1 degraded BTEX components by a combination of assimilation and co-metabolism but, particularly, only the alkylated benzenes were metabolized. Toluene and ethylbenzene served as carbon and energy sources whereas the xylenes were co-metabolized. Carbon balance experiments and ^{1}H NMR metabolic profiles revealed that the *o*- and *m*-xylene isomers were partly oxidized to dead-end products. For *p*-xylene, a higher carbon recovery value together with the lack of accumulation of intermediates points to mineralization. However, the low rate of degradation of *p*-xylene seems to be insufficient to sustain growth if this compound is supplied as the single substrate. In BTEX mixtures, *p*-xylene appears to be out-competed by the other substrates.

The Michaelis-Menten model provided a good description of the degradation kinetics in batch experiments in which whole cells were used. The specific affinity (V_{max}/K_m) obtained for every individual BTEX component was consistent with the degradation pattern seen in a complex BTEX mixture, where toluene and ethylbenzene were consumed preferentially, followed by *o*- and

m-xylene, and finally *p*-xylene, which was hardly degraded. Comparisons of the K_m for degradation of toluene, alone and in combination with a second substrate, suggest that competitive inhibition is the main substrate interaction. Competition for substrates that, like *o*- and *m*-xylene, led to neither carbon assimilation nor energy-yielding reactions consequently resulted in growth inhibition (Fig. 5.2). Strong competition might induce a sequential pattern for the utilization of the different substrates, a phenomenon usually referred as diauxie, which makes the treatment of mixtures in a continuous system difficult. Diauxie during BTEX degradation has been described, for example, for a *Rhodococcus* strain (47), where the presence of ethylbenzene blocked the degradation of any additional substrate. In our study, sequential degradation of *p*-xylene (in combination with toluene) was observed, but for all other toluene, ethylbenzene, and xylene components a certain degree of simultaneous uptake occurred (Fig. 5.1).

As reported in our previous study on the assimilation of toluene by *Cladophialophora* sp. strain T1, toluene is first hydroxylated to benzyl alcohol and subsequently converted to benzoic acid, prior to the hydroxylation and cleavage of the aromatic ring (Chapter 4). Here we have shown that *o*- and *m*-xylene were also oxidized at the side-chain to form di-benzoic acids, which were not metabolized further. Considering in addition the likely competitive nature of utilization of multiple substrates, we propose that fungal strain T1 oxidizes the alkylated compounds (toluene, ethylbenzene, and xylenes) at the alkyl side-chain *via* the same monooxygenase enzyme. This conclusion explains the immediate degradation of ethylbenzene and the xylenes observed with toluene-grown mycelia. Conversely, the toluene degradation capacity needs to be induced in glucose-grown mycelia (data not shown). According to this view, the metabolism of benzene requires a very different enzymatic mechanism, apparently absent in our fungus, which is capable of performing the initial oxidation at the aromatic ring.

The results described in this report suggest that fungi with the ability of growing on aromatic hydrocarbons might contribute significantly to the bioremediation of BTEX pollution. At present, a great deal of attention in the field of fungal bioremediation is being paid to the development of fungal biofilters for the treatment of polluted air (36). In relation to this, the low K_m of *Cladophialophora* sp. strain T1 for degradation of TEX makes this organism very suitable for treatment of TEX vapors. The K_m values reported here (Table 5.C) are equivalent to air concentrations below the recommended threshold limit values for exposure to TEX (2).

TABLE 5.C: Michaelis-Menten kinetic parameters for the degradation of single TEX by toluene-grown cells of *Cladophialophora* sp. strain T1 as measured in batch incubations (120 rpm, 25 °C)

Substrate	V_{max} [a] (μmol g-dw $^{-1}$ h $^{-1}$)	K_m [b]		V_{max}/K_m (l g-dw $^{-1}$ h $^{-1}$)
		Water (μM)	Air equivalent (mg m^{-3})	
Toluene [c]	75±5	2.7±1.0	66	30.7
Ethylbenzene	45	5.8	195	7.8
o-Xylene	21	4.2	88	4.4
m-Xylene	19	3.8	112	5.0
p-Xylene	2	1.1	33	1.8

[a] Measured rates ($n \geq 6$, $r^2 \geq 0.99$)
[b] From the Lineweaver-Burk plot linearization ($n \geq 8$, $r^2 \geq 0.97$)
[c] Average and standard deviation of three independent experiments

Regarding the similarities between air biofiltration and soil bioventing, fungi could also be used to enhance the bioremediation of polluted soil. The lack of benzene degradation appears to be the main drawback for application of our strain. Nevertheless, taking into account the high microbial diversity for the metabolism of the BTEX and the environmental variability in field conditions, effective bioremediation is most likely to rely on a consortium rather than on the action of a single microorganism. In support of this hypothesis, an earlier study showed that there is synergism between fungi and bacteria for mineralization of aromatic hydrocarbons in an acidic soil (120). In this study we have shown that

soil fungi possess a metabolic capacity for the degradation of BTEX similar in many aspects to that of bacteria. Fungi thus should not be ignored for the development of more efficient bioremediation strategies.

CHAPTER 6

Biodegradation of BTEX hydrocarbons in soil. Effect of inoculating with the toluene-growing fungus *Cladophialophora* sp. strain T1

F. X. Prenafeta-Boldú, H. Ballested, J. Gerritse, J. T. C. Grotenhuis

Submitted

6.1 Introduction

Gasoline leaking from underground storage tanks, distribution facilities, and various industrial operations represents a prime source of soil and aquifer contamination (122). Among the contaminants present in gasoline, benzene, toluene, ethylbenzene, and xylene (BTEX) are classified as priority pollutants because of their high mobility and toxicity (82). In addition, modern gasoline formulations are commonly supplemented with methyl-*tert*-butyl ether (MTBE) (81). This additive acts as an octane enhancer and as an oxygenating agent, allowing the reduction of BTEX hydrocarbons. Although less toxic than the BTEX, MTBE appears to be more recalcitrant under natural conditions (48). BTEX and MTBE are the most water-soluble components of gasoline and therefore groundwater contaminant plumes from recent accidental gasoline releases often contain mixtures of BTEX and MTBE.

Bioremediation of hydrocarbon pollution relies on the biodegradation activity of soil microorganisms. Bacteria and fungi capable of degrading BTEX and MTBE have been isolated from soil (48, 59, 141). However, while most of the studies have focused in bacteria, little is known on the contribution of fungi to bioremediation of BTEX and MTBE. Fungal degradation of soil pollutants has mainly been assayed with white-rot fungi (100). These fungi oxidize aromatic hydrocarbons by co-metabolism, and significant mineralization can only achieved through the synergic interaction of fungi and bacteria (13, 70). More recently, fungi capable of growing on volatile aromatic hydrocarbons as the sole source of carbon and energy have been isolated from soil (Chapter 3), and have successfully been applied in the biofiltration of air (128).

In this Chapter, the use of fungi for the biodegradation of a mixture of BTEX and MTBE in a soil microcosmos is described. The effect of inoculating with spores of the fungus *Cladophialophora* sp. strain T1 on the degradation rates was evaluated under neutral and acidic conditions. This fungus was

isolated previously from a BTEX-polluted soil (Chapter 3) and was found to be capable of growth on a mixture BTEX hydrocarbons (Chapter 5). In earlier studies, molecular analysis of bacterial 16S ribosomal gene has allowed the detection and identification of specific soil bacteria involved in BTEX bioremediation (103, 143). Here, the presence of this fungus in soil was assessed by analysis of SSU of 18S rDNA.

6.2 Materials and methods

Fungal strain. *Cladophialophora* sp. strain T1 (ATCC MYA-2335, CBS 110553) was isolated as described in Chapter 3. During the present investigation, this organism was routinely maintained at 4°C on mineral medium (60) agar slants supplemented with 2 % glucose.

Soil characteristics. Soil for the experiments was collected from the Agricultural Test Station Kelekamp (Wageningen, The Netherlands), air-dried and stored in the dark at 4°C prior to use. The physico-chemical properties of the soil are shown in Table 6.A. This location had no known history of hydrocarbon exposure.

TABLE 6.A: Physico-chemical properties of the soil

Texture	Grain size distribution (%)		Density ($kg\ m^{-3}$)	f_{oc} ($g\ g^{-1}$)	pH	Porosity ($m^3\ m^{-3}$)	FC (%)
Sandy soil	Sand	93.3	2650	0.028	6.1	0.5	20
	Silt	2.8					
	Clay	3.9					

Soil-batch microcosmos. Soil samples (approx. 30 g dry-weight) were placed into 250 ml boston flasks. The soil was subsequently soaked with sterile de-mineralized water and, after removing the excess by gravity, flasks were sealed with teflon-coated valves (Mininert, Phase Separations, Waddinxveen, The

Netherlands). Sterile control batches were prepared by autoclaving during 50 min at 120°C. A mixture of BTEX and MTBE (10 µl), that contained (in volume) 14 % of benzene, 43 % of toluene, 14 % of ethylbenzene, 5 % of each xylene isomer, and 14 % of MTBE, was introduced in the flasks. A spore suspension of *Cladophialophora* sp. strain T1 (containing approx. 10^4 viable conidia) was used as inoculum. The content of BTEX and MTBE in the headspace was followed in time by chromatographic analysis. Four different treatments were assayed: autoclaved soil as abiotic control; non-inoculated soil containing active indigenous microflora; autoclaved soil inoculated with the fungus; and non-autoclaved soil containing both the indigenous microflora and fungal inoculum. Experiments were performed in triplicate for each treatment. Two additional sets of soil batches were prepared as described previously, in which the soil pH was adjusted by soaking with a phosphate buffer (50 mM, pH=7) or with a phosphoric acid solution (pH=4), respectively. After removal of the excess of liquid, flasks were closed with a cotton plug and placed into desiccators. A volume of 1 ml of the previously described BTEX+MTBE mixture was diluted in 20 ml dibutylphthalate and placed into the desiccators. The water activity was kept to 0.9 by placing a salt solution (140 g NaCl l^{-1}) inside the dessicator. The soil-batches were closed and incubated at 21 °C in darkness. After 60 days, the desiccators were opened and the batches left overnight for aeration. The cotton plugs were aseptically replaced by teflon-coated valves, and 10 µl of the BTEX+MTBE solution was added to the batches. The time course of the concentrations of the substrates was followed in the gas phase and the equivalent concentration in the water phase was calculated from reported water/air partition coefficients (2, 76). The pH of the soil was measured at the end of the experiment.

Analytical methods. The degradation of each component was monitored in the following hours by injecting 100 µl headspace into a HP 6890 Series gas chromatograph (Hewlett Packard, U.S.A.). A CP-Wax 52CB column (Chrompack B.V., Middelburg, The Netherlands) was used as the stationary phase. The

carrier gas was nitrogen used at a flow of 1.9 ml min^{-1}. The temperature of the column and the flame ionization detector was 110 and 300 °C respectively.

DNA extraction and PCR parameters. DNA was extracted from the soil batches and from pure cultures of *Cladophialophora* sp. strain T1 by using a FastDNA Kit (Bio 101, Vista, CA) and purified by using a Wizard Kit (Promega, Madison, WI). Both procedures were carried out according to the standard instructions given by the manufacturers. DNA samples were amplified in 50 µl PCR mixtures containing the following final concentrations or total amounts: 1 to 10 ng of DNA, 50 mM Tris (pH 8.3), 2 mM $MgCl_2$, each deosynucleoside triphosphate (dNTP) at a concentration of 250 µM, 400 nM of forward and reverse primer, and 0.5 U of *Taq* DNA polymerase. All reagents were combined and heated at 94 °C for 4 min, followed by 35 cycles of PCR: 94 °C for 35 s, 55 °C s for 50 s followed by 72 °C for 2 min. The fungal-specific primers nu-SSU-0817-5' (TTA GCA TGG AAT AAT RRA ATA GGA) and nu-SSU-1196-3' (TCT GGA CCT GGT GAG TTT CC) were used, which generated 422 bp amplicons of the fungal 18S rDNA gene (14). A rich GC-clamp was added to the forward primers for TGGE separation. PCR amplification was confirmed in agarose gels (12 g l^{-1}) containing 0.5 µg l^{-1} of ethidium bromide. The gels were run in 1 mM TBE buffer at 80 V for 45 min and visualized using a UV transilluminator.

TGGE and sequence analysis. Each PCR-amplified sample (4 µl) was mixed to 1 µl of loading buffer and loaded onto a TGGE equipment (Bio-Rad, Richmond, CA). An 8% polyacrylamide gel (8 × 8 × 1 mm^3) containing 6 M urea, 1.25 TAE, 0.1% TEMED, 1 g l^{-1} ammonium persulfate, and 5 ml of a solution of 400 g l^{-1} acrylamide/bis was used. TGGE was performed at 130 V for 2 h 30 min, the temperature gradient from 36 to 42°C being run parallel to the migration. A silver staining protocol was applied to the gels.

Relevant bands from TGGE gels were cut out and eluted in 20 µl of PCR buffer for 2 h with intervals of shaking. The eluted fraction (15 µl) was used for re-

amplification and, after purification, the products were sent for sequencing (MWG Biotech AG, Ebersberg, Germany). Obtained sequences were deposited GenBank and homology searches were performed against other available sequences from this database.

6.3 Results

Degradative activity experiments. Biodegradation activity by the indigenous microorganisms from a non-polluted soil was induced in only 3 days after addition of a mixture of BTEX and MTBE (Fig. 6.1). Substrate depletion was complete within 8 days for BTEX, but MTBE was not degraded (not shown). The fungus *Cladophialophora* sp. strain T1 was able to colonize autoclaved soil at the expense of the BTEX hydrocarbons. However, only the alkylbenzenes (TEX) were degraded and a longer time of about 11 days was needed for TEX depletion.

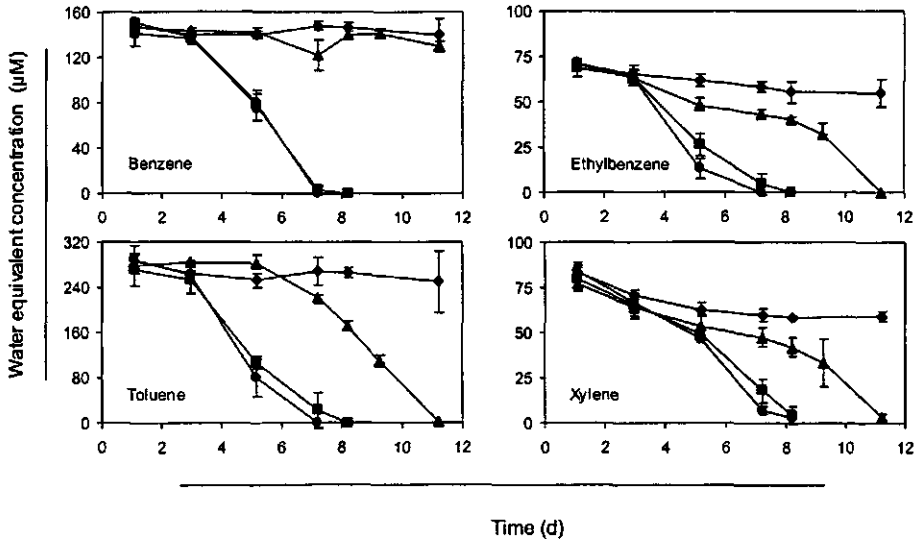


FIGURE 6.1: Degradation of BTEX in soil microcosmos (at 21 °C), after addition of the substrates (Time 0). Untreated soil (■); soil inoculated with the fungus *Cladophialophora* sp. strain T1 (●); autoclaved soil containing fungal inoculum (▲); autoclaved soil without the fungus as abiotic control (◆). Error bars correspond to the standard deviation of three different experiments

After soil had been exposed to a mixture of BTEX and MTBE for a period of two months, degradation of BTEX occurred without a lag-phase and a three- to four-fold increase in the soil-intrinsic biodegradation rates was measured (Table 6.B). The rates observed for the TEX were similar to those measured in autoclaved soil containing the *Cladophialophora* sp. strain T1 inoculum. At the lower soil pH, however, increase of the intrinsic soil degradation activity upon exposure to BTEX and MTBE was not observed. In presence of the fungus, the soil maximum degradation rate appeared to be less dependent on the pH. Filamentous-like growth was macroscopically observed in the acidified soil samples that were inoculated with the strain T1. For all soil treatments, benzene degradation required always the activity of the indigenous soil microflora, while MTBE depletion was very poor in all cases.

TABLE 6.B: Maximal degradation rates (in μmol kg^{-1} h^{-1}, at 25 °C) of gasoline components measured in soil microcosmos incubated at two different pH values, measured at the end of the experiments. The given rate values correspond to the average and standard deviation of three independent experiments

	-N +F		+N -F		+N +F	
Substrate	pH = 6.5	pH = 3.2	pH = 7.1	pH = 4.6	pH = 6.8	pH = 3.7
MTBE	ND	ND	ND	ND	ND	ND
Benzene	ND	ND	12.0 ± 0.7	6.6 ± 1.8	13.2 ± 2.1	7.1 ± 0.2
Toluene	51.8 ±14.0	33.3 ± 6.6	65.7 ± 4.9	11.8 ± 1.1	71.7 ± 6.0	46.3 ±16.9
Ethylbenzene	13.3 ± 0.9	7.9 ± 1.3	9.7 ± 2.4	4.9 ± 1.4	24.2 ± 7.0	9.2 ± 1.6
o-Xylene	4.5 ± 1.7	3.3 ± 0.7	5.2 ± 1.4	3.0 ± 0.5	5.8 ± 2.4	3.9 ± 0.5
m-Xylene	4.9 ± 1.8	3.3 ± 0.5	5.2 ± 0.5	5.0 ± 0.9	9.1 ± 1.5	3.4 ± 0.5
p-Xylene	1.6 ± 0.2	0.3 ± 0.1	4.7 ± 0.4	4.9 ± 0.7	6.7 ± 1.1	4.1 ± 1.0

(-N +F) soil containing the fungal inoculum without native microflora; (+N -F) soil with native microflora; (+N +F) soil containing both the native microflora and the fungal inoculum
ND: not depleted (the gas phase content after 14 days was similar to that of abiotic controls)

TGGE microbial profiles. Amplicons of the expected length, as determined in electrophoretic gels, were generated from the total soil DNA using the universal fungal primers nu-SSU-0817-5' and nu-SSU-1196-3'. Significant PCR-yields were only obtained with the DNA extracts from soils containing *Cladophialophora*

sp. strain T1. The latter produced a band pattern in TGGE gels identical to that from a pure culture of the fungus (Fig. 6.2). Purity of the original culture was subsequently re-assured by performing a new isolation from one single spore. The new culture showed again the same TGGE pattern. Three individual bands of the gel were excised, re-amplified, and successfully sequenced. Bands *a* and *b* showed an identical sequence that was deposited into GenBank under the accession number AY150798; band *c* was also sequenced and deposited under the GenBank number AY150899. Alignment searches with other available sequences in this database showed the highest homology (100 % for *a* and *b*, and 98 % for *c*) with 18S rDNA genes from other fungal strains of the associated teleomorph genus *Capronia*.

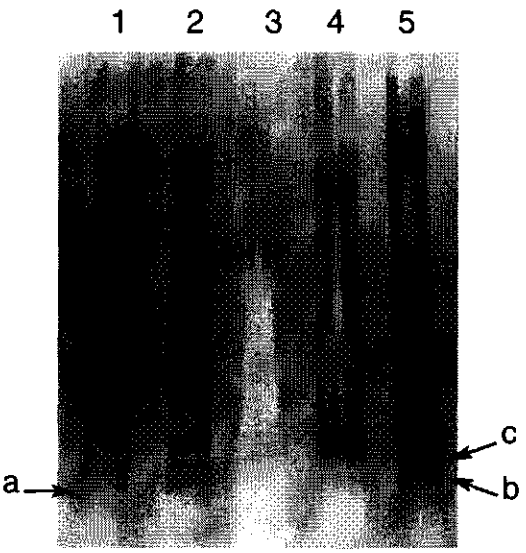


FIGURE 6.2: TGGE for fungal 18S rDNA fragments obtained from the soil samples inoculated with the fungus *Cladophialophora* sp. strain T1: acidified autoclaved soil (Lane 1); acidified and non-autoclaved soil (Lane 2); non-acidified non-autoclaved soil (Lane 3); non-acidified and autoclaved (Lane 4); pure culture of fungal strain T1 (Lane 5). Marked bands are explained in the text

6.4 Discussion

Biodegradation of BTEX hydrocarbons in soil has usually been attributed to the action of bacteria. This study shows that fungi can also degrade BTEX components at significant rates in soil. Biodegradation profiles in autoclaved soil inoculated with the fungus *Cladophialophora* sp. strain T1 were similar to those previously measured using submerged cultures of this fungus (Chapter 5); in which toluene and ethylbenzene served as growth substrates and the xylenes were co-metabolized, while benzene was not biodegraded. The longer lag-phase observed in soil-fungal cultures, in relation to the intrinsic soil biodegradation, is probably the result of the time required for fungal spores to germinate. After long-term exposure of neutralized soil to BTEX, the biodegradation rates by the indigenous soil bacteria and by *Cladophialophora* sp. strain T1 were similar. At acidic pH, however, the intrinsic soil biodegradation rates were lower. Inoculation with the fungus increased significantly the biodegradation rates of TEX, indicating that the fungus appears to be less sensitive to the pH than the original soil degraders. Inactivation of the native soil microbes had little effect on the biodegradation rates of TEX in a soil inoculated with *Cladophialophora* sp. strain T1. Similarly, the presence or absence of fungal inoculum in non-autoclaved soil did not interfere with the biodegradation of benzene, which could only be metabolized by the indigenous soil microflora. The absence of mutual inhibition between both the original soil BTEX degraders and the introduced fungus indicates that the main interaction between these microbes is of commensalistic nature.

Contrary to aromatic hydrocarbons, degradation of MTBE was not observed in the present study. This compound is highly recalcitrant to biodegradation and only very few microbial strains have so far been isolated which were capable to metabolize MTBE under laboratory conditions (48).

Biodegradation of gasoline compounds has usually been assessed using enriched or pure microbial submerged cultures growing under optimal laboratory

conditions. In this situation bacteria usually multiply faster than fungi and hence they have been more often selected and comprehensively studied (Chapter 3). This situation, however, has little to do with the soil where environmental factors that limit microbial growth prevail. A low soil pH has earlier been recognized to be an important parameter that inhibits bacterial growth (9) and acidic soils contaminated with oil hydrocarbons contained large fungal populations (15). The involvement of fungi in the biodegradation of pollutants in acidic soils has previously been demonstrated for toluene and naphthalene (120), and phenol (84).

The nuclear-encoded ribosomal RNA genes (rDNA) of fungi exist as a multiple-copy gene family comprised of highly similar DNA sequences. In this study, the presence of *Cladophialophora* sp. strain T1 in soil was assessed by PCR-TGGE analysis of SSU from 18S rDNA gene amplified using universal fungal primers. Sequence analysis of excised bands showed the highest homology to other known sequences from the genus *Capronia*, which is the holomorph of *Cladophialophora* (124). Species in this genus have recently experienced a very high degree of evolutive diversification and the classification of several closely related species remains puzzling. Consequently, the 18S rDNA gene is unlikely to contain enough sequence variability to allow identification of *Capronia* at species level.

Fungi growing on volatile aromatic hydrocarbons can advantageously be used for the biofiltration of polluted air (128). Our preliminary study indicates that inoculation of acidic soil with fungi might be a viable technique to enhance biodegradation of BTEX pollutants. Further studies are needed at a larger scale in order to assess feasibility of introducing fungal cultures into different soils to enhance bioremediation of gasoline pollution.

CHAPTER 7

General discussion

7.1 Isolation of fungi growing on volatile aromatic hydrocarbons

This study has shown that only a relatively small number of fungi possesses the capacity to assimilate monoaromatic hydrocarbons. These strains could only attack certain alkylbenzene compounds (Chapter 3). The screening of a heterogeneous fungal collection for growth on agar plates under a toluene atmosphere did not yield strains that used toluene as the sole source of carbon and energy in closed liquid cultures. Instead, a more successful strategy for selecting fungi was based on long-term enrichments of polluted environmental samples under growth-limiting conditions, such as acidic pH or low water activity, which favored the development of fungi over the faster multiplying bacteria.

These results contradict previous investigations, which suggested that many more fungi from a wide variety of taxa could grow on BTEX hydrocarbons (90, 112). The latter studies, however, were based only on the observation of fungal growth on agar exposed to these volatiles. Strains that reacted positively were assumed to use the supplied substrates as carbon and energy sources, but further evidence linking growth to biodegradation was not provided. The use of solid media for assessing microbial growth on volatile hydrocarbons usually overestimates the number of micoorganisms that effectively utilize the supplied substrates (104). Impurities in the agar, in the substrate, or traces of volatile compounds from the atmosphere can be used as alternative carbon sources by several microorganisms. This is most likely to be the case with soil fungi, which are commonly of oligotrophic nature (131).

7.2 Taxonomy and ecology of the fungi

In Table 7.A the taxonomic position of the fungi isolated in the present dissertation is compared to that from additional strains that have been mention in recent literature. It can be concluded that utilization of aromatic hydrocarbons

does not appear to be evenly distributed among the fungal system. The qualified isolates belong to the ascomycetes and, remarkably, are often affiliated to the genera *Exophiala* and *Cladophialophora*, both anamorphs of *Capronia* (*Chaetothyriales*), or *Cladosporium* (*Dothideales*) This is especially the case for the assimilation of short-chained alkylbenzenes, like toluene, ethylbenzene, or styrene. The genera mentioned are comprised among the black yeast-like fungi, an heterogeneous group of fungi that encompasses the orders *Dothideales* and *Chaetothyriales*. Members of these orders have a thallus that is melanized throughout, showing a characteristic dark pigmentation. Melanin confers resistance towards hostile environments (23), and black yeasts have often been isolated under conditions of stress in temperature, water availability, oxygen radicals, UV irradiation, electrolyte content, or scarcity of nutrients (46). Dark pigmentation has also been described in species of *Leptodontidium*, *Pseudeurotium*, and *Pseudallescheria*.

Besides a higher resistance to adverse environmental conditions, melanin is also regarded an important virulence factor in pathogenic strains (23). It is therefore interesting to realize that several fungi assimilating aromatic hydrocarbons also occur as human pathogens (43). According to the three-level scale of occupational health-risk, BioSafety Level (BSL)[a], *Cladosporium sphaerospermum* has been placed in BSL-1 as the aetiologic agent of superficial skin mycoses. *Exophiala jeanselmei*, *E. lecanii-corni*, and *Pseudallescheria boydii* are placed into BSL-2; these fungi can cause cutaneous and subcutaneous infections as well as systemic mycoses. *Cladophialophora* contains virulent agents of systemic disease that have been classified into BSL-2 and BSL-3. Since the strains here isolated showed good growth at 37°C (Chapter 3), they must be treated as potential pathogens as well.

[a] BSL-1: Saprobes or plant pathogens occupying non-vertebrate ecological niches, or commensals. Infections are coincidental, superficial, and non-invasive or mild. BSL-2: Species principally occupying non-vertebrate ecological niches, but with a relatively pronounced ability to survive in vertebrate tissue. They may cause deep mycoses in immune-compromised patients. BSL-3: Pathogens potentially able to cause severe mycoses in healthy individuals

TABLE 7.A: Classification of the fungi known to grow on aromatic hydrocarbons

Classification	Anamorph name	Growth substrate[a]	Reference
Division: *Ascomycota*			
Class: *Euascomycetes*			
Order: *Dothideales*			
Family: *Mycosphaerellaceae*			
Mycosphaerella sp.	*Cladosporium* sp.	T	(8)
	C. sphaerospermum T0	T, E, S, P	(133), Chr 3)
Order: *Chaetothyriales*			
Family: *Herpotrichiellaceae*			
Capronia sp.	*Exophiala* sp. T4	T, E	Chr 3
	E. jeanselmei	S	(38, 61)
	E. lecanii-corni	T	
Capronia sp.	*Cladophialophora* sp. T1	T, E, S	Chrs 3, 5
	Cladophialophora sp. T2	T	Chr 3
Order: *Helotiales*			
Family: -	*Leptodontidium* sp. T5	T	Chr 3
Order: *Eurotiales*			
Family: *Pseudeurotiaceae*			
Pseudeurotium zonatum	Sporothrix-like	T	Chr 3
Family: *Trichocomaceae*			
-	*Paecilomyces* sp.	P, H, O, N, D, DD	(52)
-	*Penicillium* sp.	N, D, DD	(52)
Order: *Microascales*			
Family: *Microascaceae*			
Pseudallescheria boydii	*Scedosporium apiospermum*	T	(54)
Order: *Hypocreales*			
Family: -	*Verticillium* sp.	O, N, D, DD	(52)
Family: *Clavicipitaceae*			
-	*Beauveria* sp.	N, D, DD	(52)

[a] T: toluene; E: ethylbenzene ; S: styrene; P: propylbenzene; H: hexylbenzene; O: octylbenzene; N: nonylbenzene; D: decylbenzene ; DD: dodecylbenzene

From an ecological point of view, the fungal isolates described are common and cosmopolitan species that are frequently encountered as soil and plant saprobes, as well as opportunistic pathogens. Interestingly, many of these species and their close relatives have also been reported in environments that

are rich in hydrocarbons or lignin decomposition products: *C. sphaerospermum* has been isolated from a wetland polluted with polycyclic aromatic hydrocarbons (58). Species in *Exophiala* have been found in decaying wood, pulp, and polluted water (111, 125). Furthermore, a survey on 28 yeast-like fungi for growth on 84 oxygenated aromatics (i.e. aromatic acids, phenols, catechols, etc.) revealed that *E. jeanselmei* could utilize the broadest range of substrates (83). The capacity to assimilate short-chain alkanes has also been reported for *E. jeanselmei* (41) as well as in a *Scedosporium* sp. (92). *Pseudeurotium zonatum* has been isolated selectively from wood pulp with a nutrient medium containing the aromatic fungicide *o*-phenylphenol (20). Species of *Leptodontidium* are usually found in rotten wood (44). Degradation of aliphatics in crude oil has been demonstrated for *Pseudallescheria boydii* (4) and this fungus has often been isolated from soil polluted with petroleum (5). *Penicillium* species are among the most commonly isolated species from oil-polluted soil, that grow on aliphatics (96, 119).

The apparent higher frequency of isolation of melanin-producing fungi, particularly from the highly-melanized genera *Cladosporium*, *Exophiala*, and *Cladophialophora*, suggests that these taxa may posses an inherent capacity for the assimilation of volatile aromatic hydrocarbons. It is interesting to hypothesize about a possible biochemical connection between the fungal metabolism of melanin and the assimilation of aromatic hydrocarbons. The most common melanin biosynthesis pathway in fungi starts with the condensation of five acetate residues to form the aromatic structure tetrahydroxy-naphthalene, which then polymerizes. This pathway has been proven for *Exophiala*, and *Cladosporium* (23). However, it might also be possible that melanized fungi are more commonly isolated because of their higher tolerance towards the environmental stress imposed during the enrichment procedures.

7.3 Catabolic pathways

So far, assimilation of volatile aromatic hydrocarbons by fungi has only been proven for single-chained alkylbenzene substrates (Table 7.A). A closer look into the metabolic pathway of toluene in the six strains studied in this thesis revealed that, in all cases, the initial oxidation started at the methyl group resulting in the formation of aromatic acids that were eventually assimilated (Chapter 4). This pathway has also been proposed for the degradation of longer side-chain alkylbenzenes (52). The similarities between the side chain oxidation of alkylbenzenes and the terminal oxidation of *n*-alkanes (Chapter 2) suggest that assimilation of alkylated benzenes might be the result of the simultaneous occurrence of the catabolic pathways for *n*-alkanes and aromatic acids. However, since such a capacity occurs in many more fungal species (83) it is not sufficient to explain assimilation of alkylbenzenes in all cases.

Fungal oxidation of aliphatic hydrocarbons is carried out by cytochrome P-450 (Chapter 2). Similarly, enzymatic studies on some of the fungi growing on alkylbenzenes strongly suggested the involvement of a cytochrome P-450 monooxygenase in the side-chain hydroxylation of alkylbenzenes (37), Luykx *et al.*, in preparation). Fungal toluene monooxygenases displayed a moderate substrate specificity and other alkylated benzenes, such as ethylbenzene and xylene, were also oxidized (Chapter 5, Luyks *et al.*, in preparation). In the case of the xylenes, the presence of an additional alkyl side-chain prevented any subsequent growth when given as the sole carbon and energy source (Chapter 3). Fungal P-450 cytochromes are also involved in the ring-hydroxylation of several aromatic hydrocarbons (Chapter 2). Monoaromatic compounds like toluene can also be oxidized at the aromatic ring by fungi (Chapter 4). The primary ring oxidation of toluene results in cresols, compounds that are readily assimilated by a broad diversity of fungi (Chapter 2). The hydroxylation of the aromatic ring occurs at a rate that is apparently too low to support fungal growth, and therefore occurs only co-metabolically.

The metabolic uniformity observed for the assimilation of toluene by fungi further supports the hypothesis that assimilation of aromatic hydrocarbons is less diversified than in the bacteria, where five different pathways have been identified for the assimilation of toluene (Chapter 4).

7.4 Application of fungi in bioremediation of volatile hydrocarbons

The recent development of air biofilters that are based on fungal metabolic activity has overcome some of the problems found in the conventional biofiltration of hydrocarbons (128). Poor water solubility of volatile aliphatic and aromatic hydrocarbons requires biofiltration to function at relatively low water content. Under these conditions, fungi thrive better than most bacteria and are therefore enriched naturally in air biofilters (Chapter 3). After successful trials in laboratory studies, fungus-based biofiltration is being scaled-up for industrial and bioremediation uses (36). However, a crucial factor affecting the viability of this technology, that has so far been ignored, concerns the potential pathogenicity of suitable fungi to humans. Forced aeration through a fungal biofilm will inevitably result in large amounts of spores being dispersed in the air, which could be hazardous if inhaled. Application of a non-pathogenic strain might not be sufficient to prevent biohazard. Displacement of the originally inoculated cultures by other fungi that coincidentally entered the biofilter was observed in long-term runs (van Groenestijn, pers. comm.). Consequently, safe operation of fungal biofilters will require the inoculation with a competitive and non-pathogenic strain as well as the monitoring of the microbial community that develops during biofilter operation.

The similarities between air biofiltration and soil bioventing (in both techniques a gas phase is blown through a solid porous matrix to which the degrading microbes are attached) means that fungi could be used advantageously in soil bioremediation as well. Biodegradation of BTEX has, in

most cases, been studied using submerged cultures of bacteria. The reason for that might be that bacteria growing on aromatic hydrocarbons are readily enriched from average soils (Chapter 6), while analogous fungi are less ubiquitous and might become only significant under specific environmental conditions that inhibit bacterial growth (Chapter 3). The feasibility of using a specific microbial strain for the biodegradation of a certain pollutant is usually evaluated through the two parameters of the Michaelis-Menten kinetic model: the maximum biodegradation rate (V_{max}) and the half saturation constant (K_m). When grown in liquid cultures with an excess of nutrients and a neutral pH, V_{max} for BTEX are generally higher in bacteria than in fungi (Chapter 3), but this difference can be reversed when more stringent growth conditions and time is given to the more slowly growing fungi to develop (Chapter 6). The K_m value gives information on the residual concentration of pollutant that can be achieved through biodegradation. The values determined here for the degradation of alkylbenzenes in fungi are very similar to those reported for bacteria (Chapters 3 and 5).

In the last decades, the importance of fungi as biodegraders of oil pollutants has been demonstrated for aliphatic and polycyclic aromatic hydrocarbons. The results of this dissertation indicate that the metabolism of BTEX in fungi is not as ubiquitous and metabolically diverse as in bacteria. However, the use of fungi can be an attractive alternative for specific biotechnological applications. The present interest in fungal assimilation of aromatic hydrocarbons is demonstrated by the appearance of new research groups working in this topic and by the increasing number of additional fungal strains that have been isolated since the beginning of the present study. The continuation of this type of research is expected to provide a more complete picture of the taxonomy, ecology, and biochemistry of the fungal assimilation of aromatic hydrocarbons. This information will also contribute to the development of new approaches for environmental biotechnology.

Literature references

1. **Alvarez, P. J. J., and T. M. Vogel** 1991. Substrate interactions of benzene, toluene and *p*-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. *Applied and Environmental Microbiology.* **57**:2981-2985.
2. **Amoore, J. E., and E. Hautala** 1983. Odor as an aid to chemical safety: Odor thresholds compared with threshold limit values and volatilities for 214 industrial chemicals in air and water dilution. *Journal of Applied Toxicology.* **3**:272-290.
3. **Anderson, J. W., J. M. Neff, B. A. Cox, H. E. Tatem, and G. M. Hightower** 1974. The effects of oil on estuarine animals; toxicity, uptake and depuration, respiration, p. 285-310. *In* F. J. Vernbeg, and W. Vernberg (eds), *Pollution and Physiology of Marine Organism.* Academic Press, Inc., New York.
4. **April, T. M., S. P. Abbott, J. M. Foght, and R. S. Currah** 1998. Degradation of hydrocarbons in crude oil by the ascomycete *Pseudallescheria boydii* (Microascaceae). *Canadian Journal of Microbiology.* **44**:270-278.
5. **April, T. M., J. M. Foght, and R. S. Currah** 2000. Hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. *Canadian Journal of Microbiology.* **46**:38-49.
6. **Atlas, R. M., and C. E. Cerniglia** 1995. Bioremediation of petroleum pollutants. *Bioscience.* **45**:332-338.
7. **Auret, B. J., D. R. Boyd, P. M. Robinson, and C. G. Watson** 1971. The NIH shift during the hydroxylation of aromatic substrates by fungi. *Chemical Communications.* **24**:1585-1587.
8. **Auria, R., G. Frere, M. Morales, M. E. Acuna, and S. Revah** 2000. Influence of mixing and water addition on the removal rate of toluene vapors in a biofilter. *Biotechnology and Bioengineering.* **68**:448-455.
9. **Baath, E.** 1998. Growth rates of bacterial communities in soils at varying pH: a comparison of the thymidine and leucine incorporation techniques. *Microbial Ecology.* **36**:316-327.
10. **Blum, D. J. W., and R. E. Speece** 1991. A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. *Research journal of the water pollution control federation.* **63**:198-207.
11. **Boersma, M. G., T. Y. Dinarleva, W. J. Middelhoven, V. B. W. J. H., J. Doran, J. Vervoort, and I. M. C. M. Rietjens** 1998. ^{19}F Nuclear magnetic resonance as a tool to investigate microbial degradation of fluorophenols to fluorocatechols and fluoromuconates. *Applied and Environmental Microbiology.* **64**:1256-1263.
12. **Bondar, V. S., M. G. Boersma, E. L. Golovlev, J. Vervoort, W. J. H. Van Berkel, Z. I. Finkelstein, I. P. Solyanikova, L. A. Golovleva, and I. M. C. M. Rietjens** 1998. ^{19}F NMR study on the biodegradation of fluorophenols by various Rhodococcus species. *Biodegradation.* **9**:475-486.
13. **Boonchan, S., L. Britz Margaret, and A. Stanley Grant** 2000. Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. *Applied and Environmental Microbiology.* **66**:1007-1019.
14. **Borneman, J., and R. J. Hartin** 2000. PCR primers that amplify fungal rRNA genes from environmental samples. *Applied and environmental microbiology.* **66**:4356-4360.

15. **Bossert, I., and R. Bartha** 1984. The fate of petroleum in soil ecosystems, p. 435-473. *In* R. M. Atlas (ed.), *Petroleum Microbiology*. Macmillan Publishing Co., New York.
16. **Bossert, I. D., and G. C. Compeau** 1995. Cleanup of petroleum hydrocarbon contamination in soil, p. 77-125. *In* L. Y. Young, and C. E. Cerniglia (eds), *Microbial Transformations and Degradation of Toxic Organic Chemicals*. Wiley-Liss. Inc., New York.
17. **Bowlen, G. F., and D. S. Kosson** 1995. In situ processes for bioremediation of BTEX and petroleum fuel products, p. 515-542. *In* L. Y. Young, and C. E. Cerniglia (eds), *Microbial Transformations and Degradation of Toxic Organic Chemicals*. Wiley-Liss. Inc., New York.
18. **Boylan, D. B., and B. W. Tripp** 1971. Determination of hydrocarbons in seawater extracts of crude oil and crude oil fractions. *Nature*. **230**:44-47.
19. **Braun-Lüllemann, A., C. Johannes, A. Majcherzyk, and A. Hüttermann** 1995. The use of white-rot fungi as active biofilters, p. 235-241. *In* R. E. Hinchee, G. D. Sayles, and R. S. Skeen (eds), *Biological Unit Processes for Hazardous Waste Treatment*. Battelle Press, Columbus, OH.
20. **Brewer, D.** 1958. Studies on slime accumulations in pulp and paper mills: I. Some fungi isolated from mills in New Brunswick and Newfoundland. *Canadian Journal of Botany*. **36**:941-946.
21. **Brodkorb, T. B., and R. L. Legge** 1992. Enhanced biodegradation of phenanthrene in oil tar-contaminated soils supplemented with *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology*. **58**:3117-3121.
22. **Bulton, C. A., and C. Ratledge** 1984. The physiology of hydrocarbon-utilizing microorganisms. *Topics in Enzyme and Fermentation Biotechnology*. **9**:11-77.
23. **Butler, M. J., and A. W. Day** 1998. Fungal melanins: a review. *Canadian Journal of Microbiology*. **44**:1115-1136.
24. **Cain, R. B., R. F. Bilton, and J. A. Darrah** 1968. The metabolism of aromatic acids by micro-organisms. Metabolic pathways in the fungi. *Biochemical Journal*. **108**:797-832.
25. **Cerniglia, C. E.** 1997. Fungal metabolism of polycyclic aromatic hydrocarbons: Past, present and future applications in bioremediation. *Journal of Industrial Microbiology and Biotechnology*. **19**:324-333.
26. **Cerniglia, C. E.** 1984. Microbial metabolism of aromatic hydrocarbons, p. 99-128. *In* R. M. Atlas (ed.), *Petroleum Microbiology*. MacMillan, New York.
27. **Cerniglia, C. E., P. P. Fu, and S. K. Yang** 1983. Regio- and stero-selective metabolism of 4-methylbenz(a)anthracene by the fungus *Cunninghamella elegans*. *Biochemical journal*. **216**:377-384.
28. **Cerniglia, C. E., R. L. Hebert, P. J. Szaniszlo, and D. T. Gibson** 1978. Fungal transformation of naphthalene. *Archives of Microbiology*. **117**:135-143.
29. **Cerniglia, C. E., K. J. Lambert, D. W. Miller, and J. P. Freeman** 1984. Transformation of 1- and 2-methylnaphthalene by *Cunninghamella elegans*. *Applied and Environmental Microbiology*. **47**:111-118.
30. **Cerniglia, C. E., D. W. Miller, S. K. Yang, and J. P. Freeman** 1984. Effects of a fluoro substituent on the fungal metabolism of 1-fluoronaphthalene. *Applied and Environmental Microbiology*. **48**:294-300.
31. **Cerniglia, C. E., J. B. Sutherland, and S. A. Crow** 1992. Fungal metabolism of aromatic hydrocarbons, p. 193. *In* G. Winkelmann (ed.), *Microbial Degradation of Natural Products*. VCH Verlagsgesellschaft, Weinheim, Germany.

32. **Cerniglia, C. E., G. L. White, and R. H. Heflich** 1985. Fungal metabolism and detoxification of polycyclic aromatic hydrocarbons. *Archives of Microbiology.* **143:**105-110.
33. **Chang, M. K., T. C. Voice, and C. S. Criddle** 1993. Kinetics of competitive inhibition and cometabolism in the biodegradation of benzene, toluene, and *p*-xylene by two *Pseudomonas* isolates. *Biotechnology and Bioengineering.* **41:**1057-1065.
34. **Cooney, J. J.** 1984. The fate of petroleu pollutants in freshwater ecosystems, p. 399-433. *In* R. M. Atlas (ed.), *Petroleum Microbiology.* MacMillan, New York.
35. **Cox, H. H. J.** 1995. Styrene removal from waste gas by the fungus *Exophiala jeanselmei* in a biofilter. PhD thesis. University of Groningen.
36. **Cox, H. H. J., and H. J. Doddema** 1996. Biological filter for the removing volatile hydrophobic compounds from gas emissions.EP-0,710,147 patent.
37. **Cox, H. H. J., B. W. Faber, W. N. M. Van Heiningen, H. Radhoe, H. J. Doddema, and W. Harder** 1996. Styrene metabolism in *Exophiala jeanselmei* and involvement of a cytochrome P-450-dependent styrene monooxygenase. *Applied and Environmental Microbiology.* **62:**1471-1474.
38. **Cox, H. H. J., J. H. M. Houtman, H. J. Doddema, and W. Harder** 1993. Enrichment of fungi and degradation of styrene in biofilters. *Biotechnology Letters.* **15:**737-742.
39. **Cox, H. H. J., R. E. Moerman, S. Van Baalen, W. N. M. Van Heiningen, H. J. Doddema, and W. Harder** 1997. Performance of a styrene-degrading biofilter containing the yeast *Exophiala jeanselmei. Biotechnology and Bioengineering.* **53:**259-266.
40. **Cutright, T. J., and S. Lee** 1994. In situ soil remediation: Bacteria or fungi. *Energy sources.* **17:**413-419.
41. **Davies, J. S., A. M. Wellman, and J. E. Zajic** 1973. Hyphomycetes utilizing natural gas. *Canadian Journal of Microbiology.* **19:**81-85.
42. **de Bont, J. A. M.** 1998. Solvent-tolerant bacteria in biocatalysis. *Trends in Biotechnology.* **16:**493-499.
43. **de Hoog, G. S., and J. Guarro** 1995. *Atlas of clinical fungi.* Centraalbureau voor Schimmelcultures, Baarn/Reus.
44. **de Hoog, G. S., P. Hogeweg, H. L. C. Meuzelaar, and A. C. M. Weijman** 1977. *Rhinocladiella* and allied genera, p. 1. *In* G. S. De Hoog (ed.), *The black yeasts and allied hyphomycetes*, vol. 15. Centrallbureau voor Schimmelcultures, Baarn.
45. **de Hoog, G. S., K. Takeo, E. Goettlich, K. Nishimura, and M. Miyaji** 1995. A human isolate of *Exophiala* (*Wangiella*) *dermatitidis* forming a catenate synanamorph that links the genera *Exophiala* and *Cladophialophora. Journal of Medical & Veterinary Mycology.* **33:**355-358.
46. **de Hoog, G. S., P. Zalar, C. Urzi, F. de Leo, N. A. Yurlova, and K. Sterflinger** 1999. Relationships of dothideaceous black yeasts and meristematic fungi based on 5.8S and ITS2 rDNA sequence comparison. *Studies in Mycology.* **43:**31-37.
47. **Deeb, R. A., and L. Alvarez-Cohen** 1999. Temperature effects and substrate interactions during the aerobic biotransformation of BTEX mixtures by toluene-enriched consortia and *Rhodococcus rhodochrous. Biotechnology and Bioengineering.* **62:**526-536.
48. **Deeb, R. A., H. Y. Hu, J. R. Hanson, K. M. Scow, and L. Alvarez Cohen** 2001. Substrate interactions in BTEX and MTBE mixtures by an MTBE-degrading isolate. *Environmental Science and Technology.* **35:**312-317.

49. **Duetz, W. A., B. Wind, J. G. van Andel, M. R. Barnes, P. A. Williams, and M. Rutgers** 1998. Biodegradation kinetics of toluene, *m*-xylene, *p*-xylene and their intermediates through the upper TOL pathway in *Pseudomonas putida* (pWWO). *Microbiology*. **143:**2331-2338.
50. **Eggen, T.** 1999. Application of fungal substrate from commercial mushroom production - *Pleurotus ostreatus* - for bioremediation of creosote contaminated soil. *International Biodeterioration and Biodegradation*. **44:**117-126.
51. **Ehrhardt, H. M., and H. J. Rehm** 1985. Phenol degradation by microorganisms adsorbed on activated carbon. *Applied Microbiology and Biotechnology*. **21:**32-36.
52. **Fedorak, P. M., and D. W. S. Westlake** 1986. Fungal metabolism of *n*-alkylbenzenes. *Applied and Environmental Microbiology*. **51:**435-437.
53. **Field Jim, A., E. de Jong, G. F. Costa, and A. M. de Bont Jan** 1992. Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white-rot fungi. *Applied and Environmental Microbiology*. **58:**2219-2226.
54. **Garcia Pena, E. I., S. Hernandez, E. Favela Torres, R. Auria, and S. Revah** 2001. Toluene biofiltration by the fungus *Scedosporium apiospermum* TB1. *Biotechnology and bioengineering*. **76:**61-69.
55. **Gaylarde, C. C., F. M. Bento, and J. Kelley** 1999. Microbial contamination of stored hydrocarbon fuels and its control. *Revista de Microbiologia*. **30:**1-10.
56. **Gibson, D. T., M. Henley, H. Yoshioka, and T. J. Mabry** 1970. Formation of (+)-*cis*-2,3-dihydroxy-1-methyl-cyclohexa-4,6,-diene from toluene by *Pseudomonas putida*. *Biochemistry*. **9:**1626-1630.
57. **Gibson, D. T., and V. Subramanian** 1984. Microbial degradation of aromatic hydrocarbons, p. 181-252. *In* D. T. Gibson (ed.), *Microbial Degradation of Organic Compounds*. Marcel Dekker, New York.
58. **Giraud, F., P. Guiraud, M. Kadri, G. Blake, and R. Steiman** 2001. Biodegradation of anthracene and fluoranthene by fungi isolated from an experimental constructed wetland for wastewater treatment. *Water Research*. **35:**4126-4136.
59. **Hardison, L. K., S. S. Curry, L. M. Ciuffetti, and M. R. Hyman** 1997. Metabolism of diethyl ether and cometabolism of methyl *tert*-butyl ether by a filamentous fungus, a *Graphium* sp. *Applied and Environmental Microbiology*. **63:**3059-3067.
60. **Hartmans, S., and J. Tramper** 1991. Dichloromethane removal from waste gases with a trickle-bed bioreactor. *Bioprocess Engineering*. **6:**83-92.
61. **Hartmans, S., M. J. Van Der Werf, and J. A. M. De Bont** 1990. Bacterial degradation of styrene involving a novel FAD-dependent styrene monooxygenase. *Applied and Environmental Microbiology*. **56:**1347-1351.
62. **Hettige, G., and J. E. Sheridan** 1984. Mycoflora of stored diesel fuel in New Zealand. *International Biodeterioration*. **20:**225-227.
63. **Holland, H. L., E. J. Bergen, P. C. Chenchaiah, S. H. Khan, B. Munoz, R. W. Ninniss, and D. Richards** 1987. Side chain hydroxylation of aromatic compounds by fungi: 1. Products and stereochemistry. *Canadian Journal of Chemistry*. **65:**502-507.
64. **Holland, H. L., F. M. Brown, B. Munoz, and R. W. Ninniss** 1988. Side chain hydroxylation of aromatic hydrocarbons by fungi. Part 2: isotope effects and mechanism. *Journal of the Chemical Society. Perkin transaction I*. **1988:**1557-1563.

65. **Jeener, J., B. H. Meier, P. Bachmann, and R. R. Ernst** 1979. Investigation of exchange processes by two-dimensional NMR spectroscopy. *The Journal of Chemical Physics*. **71**:4546-4553.
66. **Johnston, C. D., J. L. Rayner, B. M. Patterson, and G. B. Davis** 1998. Volatilisation and biodegradation during air sparging of dissolved BTEX-contaminated groundwater. *Journal of Contaminant Hydrology*. **33**:377-404.
67. **Jutras, E. M., C. M. Smart, R. Rupert, I. L. Pepper, and R. M. Miller** 1997. Field-scale biofiltration of gasoline vapors extracted from beneath a leaking underground storage tank. *Biodegradation*. **8**:31-42.
68. **Kaphammer, B., J. J. Kukor, and R. H. Olsen** 1990. Cloning and characterization of a novel toluene degradative pathway from *Pseudomonas pickettii* PK01, p. 243, *Abstracts of the 90th Annual Meeting of the American Society for Microbiology*, Washington D. C.
69. **Kennes, C., and F. Thalasso** 1998. Waste gas biotreatment technology. *Journal of chemical technology and biotechnology*. **72**:303-319.
70. **Kotterman, M. J. J., E. H. Vis, and J. A. Field** 1998. Successive mineralization detoxification of benzo(a)pyrene by the white rot fungus *Bjerkandera* sp. strain BOS55 and indigenous microflora. *Applied and Environmental Microbiology*. **64**:2853-2858.
71. **Leahy, J. G., and R. R. Colwel** 1990. Microbial degradation of hydrocarbons in the environment. *Microbial Reviews*. **54**:305-315.
72. **Lee, C. C., W. K. Craig, and P. J. Smith** 1974. Water-soluble hydrocarbons from crude oil. *Bulletin of Environmental Contamination and Toxicology*. **12**:212-216.
73. **Lide, D. R.** (ed.) 2001 *Handbook of Chemistry and Physics*, 82nd ed, Washington.
74. **Lindley, N. D.** 1992. Hydrocarbon-degrading yeast and filamentous fungi of biotechnological importance, p. 905-929. *In* D. K. Arora, R. P. Elander, and K. G. Mukerji (eds), *Handbook of Applied Mycology*, vol. 4. Marcel Dekker, Inc., New York.
75. **Lindley, N. D., J. F. Pedley, S. P. Kay, and M. T. Heydeman** 1986. The metabolism of yeasts and filamentous fungi which degrade hydrocarbon fuels. *International Biodeterioration*. **22**:281-287.
76. **Mackay, D., and W. Y. Shiu** 1981. A critical review of Henry's law constants for chemicals of environmental interest. *Journal of Physical Chemistry Reference Data*. **10**:1175-1199.
77. **Malina, G., J. T. C. Grotenhuis, W. H. Rulkens, S. L. J. Mous, and J. C. M. deWit** 1998. Soil vapour extraction versus bioventing of toluene and decane in bench-scale soil columns. *Environmental Technology*. **19**:977-991.
78. **Mars, A. E., J. Houwing, J. Dolfing, and D. B. Janssen** 1996. Degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 in growth-limited fed-batch culture. *Applied and Environmental Microbiology*. **62**:886-891.
79. **Mars, A. E., T. Prins Gjalt, P. Wietzes, W. De Koning, and B. Janssen Dick** 1998. Effect of trichloroethylene on the competitive behavior of toluene-degrading bacteria. *Applied and Environmental Microbiology*. **64**:208-215.
80. **Martens, R., M. Wolter, M. Bahadir, and F. Zadrazil** 1999. Mineralization of ^{14}C-labelled highly-condensed polycyclic aromatic hydrocarbons in soils by *Pleurotus* sp. Florida. *Soil Biology and Biochemistry*. **31**:1893-1899.
81. **Mays, M. A.** 1989. The use of oxygenates hydrocarbons in gasoline and their contribution to reducing urban air pollution. *Pure and Applied Chemistry*. **61**:1373-1378.

82. **Mehlman, M. A.** 1992. Dangerous and cancer-causing properties of products and chemicals in the oil refining and petrochemical industry: VIII. Health effects of motor fuels: Carcinogenicity of gasoline: Scientific update. *Environmental Research.* **59:**238-249.
83. **Middelhoven, W., J.** 1993. Catabolism of benzene compounds by ascomycetous and basidiomycetous yeasts and yeastlike fungi. *Antonie van Leeuwenhoek.* **63:**125-144.
84. **Middelhoven, W. J., M. Koorevaar, and G. W. Schuur** 1992. Degradation of benzene compounds by yeasts in acidic soils. *Plant and soil.* **145:**37-43.
85. **Mirpuri, R., W. Jones, and J. D. Bryers** 1997. Toluene degradation kinetics for planktonic and biofilm-grown cells of *Pseudomonas putida* 54G. *Biotechnology and Bioengineering.* **53:**535-546.
86. **Muncnerova, D., and J. Augustin** 1994. Fungal metabolism and detoxification of polycyclic aromatic hydrocarbons: A review. *Bioresource Technology.* **48:**97-106.
87. **Munro Andrew, W., and J. G. Lindsay** 1996. Bacterial cytochromes P-450. *Molecular Microbiology.* **20:**1115-1125.
88. **Nemergut, D. R., K. G. Wunch, R. M. Johnson, and J. W. Bennett** 2000. Benzo[a]pyrene removal by *Marasmiellus troyanus* in soil microcosms. *Journal of Industrial Microbiology and Biotechnology.* **25:**116-119.
89. **Novotny, C., P. Erbanova, T. Cajthaml, N. Rothschild, C. Dosoretz, and V. Sasek** 2000. *Irpex lacteus*, a white rot fungus applicable to water and soil bioremediation. *Applied Microbiology and Biotechnology.* **54:**850-853.
90. **Nyns, E. J., J. P. Auquière, and A. L. Wiaux** 1968. Taxonomic value of the property of fungi to assimilate hydrocarbons. *Antonie van Leeuwenhoek.* **34:**441-457.
91. **Oh, Y. S., Z. Shareefdeen, B. C. Baltzis, and R. Bartha** 1994. Interactions between benzene, toluene, and *p*-xylene (BTX) during their biodegradation. *Biotechnology and Bioengineering.* **44:**533-538.
92. **Onodera, M., Y. Endo, and N. Ogasawara** 1989. Utilization of short-chain hydrocarbons and accumulation of methylketones by a gaseous hydrocarbon assimilating mold, *Scedosporium* sp. A-4. *Agricultural and Biological Chemistry.* **53:**1431-1432.
93. **Onodera, M., H. Sakai, Y. Endo, and N. Ogasawara** 1990. Oxidation of short-chain isoalkanes by gaseous hydrocarbon assimilating mold, *Scedosporium* sp. A-4. *Agricultural and Biological Chemistry.* **53:**1947-1989.
94. **Origgi, G., M. Colombo, F. De Palma, M. Rivolta, P. Rossi, and V. Andreoni** 1997. Bioventing of hydrocarbon-contaminated soil and biofiltration of the off-gas: Results of a field scale investigation. *Journal of Environmental Science and Health Part A Environmental Science and Engineering and Toxic and Hazardous Substance Control.* **32:**2289-2310.
95. **Ottengraf, S. P. P.** 1986. Exhaust gas purification, p. 426-452. *In* H.-J. Rehm, and G. Reeds (eds), *Biotechnology*, vol. 8. VCH Verlagsgesellshaft, Weinheim, Germany.
96. **Oudot, J., J. Dupont, S. Haloui, and M. F. Roquebert** 1993. Biodegradation potential of hydrocarbon-assimilating tropical fungi. *Soil Biololgy and Biochemistry.* **25:**1167-1173.
97. **Parberry, D. G.** 1971. Biological problems in jet aviation fuel and the biology of *Amorphotheca resinae*. *Materials and Organisms.* **6:**161-208.

98. **Parberry, D. G.** 1968. The role of *Cladosporium resinae* in the corrosion of aluminium alloys. *International Biodeterioration Bulletin.* **4**:79-81.
99. **Peelen, S., M. C. M. Rietjens Ivonne, G. Boersma Marelle, and J. Vervoort** 1995. Conversion of phenol derivatives to hydroxylated products by phenol hydroxylase from *Trichosporon cutaneum*. A comparison of regioselectivity and rate of conversion with calculated molecular orbital substrate characteristics. *European Journal of Biochemistry.* **227**:284-291.
100. **Pointing, S. B.** 2001. Feasibility of bioremediation by white-rot fungi. *Applied Microbiology and Biotechnology.* **57**:20-33.
101. **Prenafeta Boldú, F. X., A. Kuhn, D. Luykx, H. Anke, J. W. van Groenestijn, and J. A. M. de Bont** 2001. Isolation and characterisation of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source. *Mycological Research.* **105**:477-484.
102. **Raj, H. G., M. Saxena, and A. Allameh** 1992. Metabolism of foreign compounds by fungi, p. 881-904. *In* D. K. Arora, R. P. Elander, and K. G. Mukerji (eds), *Handbook of Applied Mycology*, vol. 4. Marcel Dekker Inc., New York.
103. **Ralebitso, T. K., W. F. M. Roling, M. Braster, E. Senior, H. W. v. Verseveld, and H. W. van Verseveld** 2000. 16S rDNA-based characterization of BTX-catabolizing microbial associations isolated from a South African sandy soil. *Biodegradation.* **11**:351-357.
104. **Randall, J. D., and B. B. Hemmingsen** 1994. A critical evaluation of the fume plate method for the enumeration of bacteria capable of growth on volatile hydrocarbons, p. 400-404. *In* R. E. Hinchee, D. B. Anderson, and G. D. Sayles (eds), *Applied biotechnology for site bioremediation.* Lewis Publishers, Boca Raton (Florida).
105. **Rappendgluck, B., and P. Fabian** 1998. A study of BTEX-ratios in the urban area of Munich/Germany using rapid gas chromatography. *Environmental Science and Pollution Resources.* **5**:65-70.
106. **Reddy, C. A., and T. M. D'Souza** 1994. Physiology and molecular biology of the lignin peroxidases of *Phanerochaete chrysosporium*. *FEMS Microbiology Reviews.* **13**:137-152.
107. **Rehm, H. J., and J. Reiff** 1981. Mechanisms and occurrence of microbial oxidation of long-chain alkanes, p. 175-215. *In* A. Fiechter (ed.), *Advances in Biochemical Engineering*, vol. 19. Springer-Verlag, Berlin.
108. **Relsch, M. S.** 1990. Top 50 chemicals production slowed markedly last year. *Chemical Engineering News.* **68**:11-15.
109. **Rietjens, I. M. C. M., and J. Vervoort** 1992. A new hypothesis for the mechanism for cytochrome p-450 dependent aerobic conversion of hexahalogenated benzenens to pentahalogenated phenols. *Chemical Research in Toxicology.* **5**:10-19.
110. **Rietjens Ivonne, M. C. M., N. H. P. Cnubben, M. van Haandel, B. Tyrakoswka, A. E. M. F. Soffers, and J. Vervoort** 1995. Different metabolic pathways of 2,5-difluoronitrobenzene and 2,5-difluoroaminobenzene compared to molecular orbital substrate characteristics. *Chemical-Biological Interactions.* **94**:49-72.
111. **Rogers, S. O., J. M. McKemy, and C. J. K. Wang** 1999. Molecular assessment of *Exophiala* and related hyphomycetes. *Studies in Mycology.* **43**:122-133.
112. **Rubidge, T.** 1974. A new selective medium for the screening of aircraft fuels for biodeteriogenic fungi. *International Biodeterioration Bulletin.* **10**:53-55.

113. **Saeed, T., and M. Al Mutairi** 1999. Chemical composition of the water-soluble fraction of the leaded gasolines in seawater. *Environment International.* **25:**117-129.
114. **Sariaslani, F. S.** 1991. Microbial cytochromes P-450 and xenobiotic metabolism. *Advances in Applied Microbiology.* **36:**133-178.
115. **Shields, S. M., S. O. Montgomery, P. J. Chapman, S. M. Cuskey, and P. H. Pritchard** 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. *Applied and Environmental Microbiology.* **55:**1624-1629.
116. **Siporin, C., and J. J. Cooney** 1975. Extracellular lipids of *Cladosporium* (*Amorphoteca*) *resinae* grown on glucose or on n-alkanes. *Applied Microbiology.* **29:**604-609.
117. **Smith, R. V., and J. P. Rosazza** 1974. Microbial models of mammalian metabolism. Aromatic hydrocarbons. *Archives of Biochemistry and Biophysics.* **161:**551-558.
118. **Smits, J. P., H. M. van Sonsbeek, A. Rinzema, and J. Tramper** 1998. Solid-state fermentation - a minireview. *Agro-Food-Industry High-Tech.* **9:**29-36.
119. **Snellman, E. A., R. P. Collins, and J. C. Cooke** 1988. Utilization of fuel oils by fungi isolated from oceanic tar balls. *Letters of Applied Microbiology.* **6:**105-107.
120. **Stapleton, R. D., D. C. Savage, G. S. Sayler, and G. Stacey** 1998. Biodegradation of aromatic hydrocarbons in an extremely acidic environment. *Applied and Environmental Microbiology.* **64:**4180-4184.
121. **Sutherland, J. B.** 1992. Detoxification of polycyclic aromatic hydrocarbons by fungi. *Journal of Industrial Microbiology.* **9:**53-61.
122. **Swoboda-Colberg, N. G.** 1995. Chemical contamination of the environment: sources, types, and fate of synthetic organic chemicals, p. 27-74. *In* L. Y. Young, and C. E. Cerniglia (eds), *Microbial Transformations and Degradation of Toxic Organic Chemicals.* Wiley-Liss. Inc., New York.
123. **Tijhuis, L., M. C. M. van Loosdrecht, and J. J. Heijnen** 1993. A thermodynamically based correlation for maintenance Gibbs energy requirements in aerobic and anaerobic chemotrophic growth. *Biotechnology and Bioengineering.* **42:**509-519.
124. **Untereiner, W. A.** 2000. *Capronia* and its anamorphs: exploring the value of morphological and molecular characters in the systematics of the *Herpotrichiellaceae. Studies in Mycology.* **45:**141-149.
125. **Untereiner, W. A., A. H. G. G. van den Ende, and G. S. de Hoog** 1999. Nutritional physiology of species of *Capronia. Studies in Mycology.* **43:**98-106.
126. **van Berkel, W. J. H., M. H. M. Eppink, W. J. Middelhoven, J. Vervoort, and I. M. C. M. Rietjens** 1994. Catabolism of 4-hydroxybenzoate in *Candida parapsilosis* proceeds through initial oxidative decarboxylation by a FAD-dependent 4-hydroxybenzoate 1-hydroxylase. *FEMS Microbiology Letters.* **121:**207-215.
127. **van Groenestijn, J. W., and P. G. M. Hesselink** 1993. Biotechniques for air pollution control. *Biodegradation.* **4:**283-301.
128. **van Groenestijn, J. W., W. N. M. van Heiningen, and N. J. R. Kraakman** 2001. Biofilters based on the action of fungi. *Water Science and Technology.* **44:**227-232.
129. **Vervoort, J., P. A. De Jager, J. Steenbergen, and I. M. C. M. Rietjens** 1990. Development of a fluorine-19 NMR method for studies on the in vivo and in vitro metabolism of 2-fluoroaniline. *Xenobiotica.* **20:**657-670.

130. **VROM** 1999. *Stoffen- en Normen: Overzicht van belangrijke stoffen en normen in het milieubeleid.* Samson, Alphen aan de Rijn, The Netherlands.
131. **Wainwright, M.** 1993. Oligotrophic growth of fungi-stress or natural state?, p. 127-144. *In* D. H. Jennings (ed.), *Stress Tolerance of Fungi.* Marcel Dekker, New York.
132. **Weber, F. J.** 1995. Toluene: biological waste-gas treatment, toxicity and microbial adaptation. PhD thesis. Wageningen University.
133. **Weber, F. J., K. C. Hage, and J. A. M. de Bont** 1995. Growth of the fungus *Cladosporium sphaerospermum* with toluene as the sole carbon and energy source. *Applied and Environmental Microbiology.* **61**:3562-3566.
134. **Weber, F. J., M. W. Schemen, and S. Hartmans** 1995. Toxicity of contaminants in wate gases for microorganisms, p. 61-68, *Toluene: biological waste-gas treatment, toxicity and microbial adaptation*, PhD thesis, Wageningen Agricultural University.
135. **Wedding, R. T., and J. D. Kentrick** 1959. Toxicity of M-methyl-dithiocarbamate and methylisocyanate to *Rhizoctonia solani. Phytopathology.* **49**:557-561.
136. **Whals, C.** 1982. Fluorinated substrate analogs: routes of metabolism and selective toxicity. *Advances of Enzymology.* **55**:197-289.
137. **Whited, G. M., and D. T. Gibson** 1991. Separation and partial characterization of the enzymes of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1. *Journal of Bacteriology.* **173**:3017-3020.
138. **Woertz, J. R., K. A. Kinney, and P. J. Szaniszlo** 2001. A fungal vapor-phase bioreactor for the removal of nitric oxide from waste gas streams. *Journal of the Air and Waste Management Association.* **51**:895-902.
139. **Worsey, M. J., and P. A. Whilliams** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: evidence for a new function of the TOL plasmid. *Journal of Bacteriology.* **124**:7-13.
140. **Wright, J. D., and C. Ratledge** 1991. Isolation of two *Rhodotorula rubra* strains showing differences in the degradation of aromatic compounds. *Applied Microbiology and Biotechnology.* **35**:94-99.
141. **Yadav, J. S., and C. A. Reddy** 1993. Degradation of benzene, toluene, ethylbenzene and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Applied and Environmental Microbiology.* **59**:756-762.
142. **Zhang, D., Y. Yang, J. E. A. Leakey, and C. E. Cerniglia** 1996. Phase I and phase II enzymes produced by *Cunninghamella elegans* for the metabolism of xenobiotics. *FEMS Microbiology Letters.* **138**:221-226.
143. **Zhou, J., V. Palumbo Anthony, and M. Tiedje James** 1997. Sensitive detection of a novel class of toluene-degrading denitrifiers, Azoarcus tolulyticus, with small-subunit rRNA primers and probes. *Applied and Environmental Microbiology.* **63**:2384-2390.
144. **ZoBell, C. E.** 1946. Action of microorganisms on hydrocarbons. *Bacteriological Reviews.* **10**:1-49.

Summary

BTEX hydrocarbons (benzene, toluene, ethylbenzene, and xylene) constitute an important class of pollutants in water, soil, and air environmental compartments (Chapter 1). Recent investigations have shown that certain fungi are capable of growing on BTEX and related compounds. Fungal biodegradation can advantageously be applied for the biofiltration of air containing BTEX. Additionally, the fact that these fungi were isolated from soil indicates that they could play an important role in soil bioremediation. Nevertheless, information on ecology, metabolism, and degradation kinetics of fungi utilizing aromatic hydrocarbons is scarce (Chapter 2).

Fungi generally thrive better than bacteria under growth-limiting conditions, such as low pH and poor water and nutrient availability. Based on this fact, three different enrichment techniques were used for the selective isolation of fungi with the capacity of using volatile substrates as sources of carbon and energy: solid state-like batches, air biofilters, and acidic liquid cultures. Environmental samples were exposed to an atmosphere of volatile aromatic hydrocarbons and incubated under acidic and dry conditions (Chapter 3). Five fungal strains were isolated with toluene as a substrate from soil and groundwater samples from gasoline-polluted environments. The isolates were identified as *Cladophialophora*, *Exophiala* and *Leptodontidium* spp. and the ascomycete *Pseudeurotium zonatum*. Results showed that these fungi grew on toluene with doubling times between 2 and 3 days. The effect of toluene concentration on the respiration rates was also studied in the different fungi. The apparent half-saturation constant (K_m) for toluene oxidation ranged from 5 to 22 μM, depending on the strain. The maximum respiration rates were inhibited by 50% (IC50) at toluene concentrations of 2.4 up to 4.7 mM. Despite repeated attempts, no fungal isolates were obtained growing on benzene, naphthalene, or xylene. Some of the strains isolated on toluene also grew on ethylbenzene and styrene.

Previous studies showed that bacteria possess very diverse metabolic pathways for the assimilation of toluene. Here, metabolic possibilities involved in the oxidation of toluene were studied for the fungi (Chapter 4). Whole cells were incubated with isomeric fluorotoluenes, and metabolites were characterized by ^{19}F nuclear-magnetic resonance spectroscopy. The detected fluorinated metabolites indicated that toluene is oxidized by all toluene-grown fungi at the side-chain to benzoate. The latter is subsequently hydroxylated, first at the *para* position, and then at additional positions so that it forms catecholic intermediates which are eventually assimilated through the 3-oxoadipate pathway. Toluene was also found to be hydroxylated at the aromatic ring in the zygomycete *Cunninghamella echinulata*. However, conversion rate in this latter case was very low and the reaction it was co–metabolic.

Studies on biodegradation of BTEX have focused principally on single substrates, neglecting the fact that pollution with these hydrocarbons often occurs as complex mixtures. The kinetic interactions during degradation of BTEX mixtures were studied with the fungus *Cladophialophora* sp. strain T1 (Chapter 5). This isolate grew well on a mixture of all six BTEX components that was comparable to pollution by the water-soluble fraction of gasoline. Toluene and ethylbenzene were used as sources of carbon and energy, and the xylenes were co-metabolized; *ortho*- and *meta*-xylene were converted to phthalates as end-metabolites; *para*–xylene was not significantly degraded in complex BTEX mixtures but carbon mass-balances suggested that it was mineralized in combination with toluene. Benzene was not degraded with any of the assayed substrate combinations. The metabolic profiles and the inhibitory nature of the substrate interactions indicate that TEX are hydroxylated at the side-chain by the same monooxygenase enzyme.

Biodegradability of BTEX has usually been assessed with submerged cultures growing at optimal conditions. This information can be misleading if extrapolated to field conditions. Growth of the fungus *Cladophialophora* sp. strain

T1 on a mixture of BTEX and the gasoline additive MTBE was studied in sterile and non-sterile soil microcosms (Chapter 6). Comparison of the biodegradation rates measured in soil batches combining presence and absence of indigenous bacteria and the fungal inoculum suggests that the main interaction between indigenous and inoculated BTEX-degrading microorganisms was commensalistic. Alkylbenzenes were all degraded by the fungus, but benzene degradation required the activity of the indigenous soil microflora. MTBE could not be biodegraded. Inoculation with the fungus increased the degradation rates in soil after long exposure to BTEX and a low soil pH. The presence and identity of the fungal inoculum in soil was confirmed by PCR-TGGE analysis of SSU of fungal 18S rDNA.

The results presented in this dissertation are discussed in relation to previous knowledge on fungal assimilation of aromatic hydrocarbons and in view of future potential biotechnological applications (Chapter 7). The use of fungi utilizing aromatic hydrocarbons represent a very promising tool in the bioremediation of BTEX pollution. Fungal biodegradation is similar in kinetic terms to that of bacteria, but the former have the advantage of a higher tolerance to adverse environments. However, fungal assimilation of aromatic hydrocarbons appears to be confined to black yeast-like fungi and allied species some of which can cause mycosis to humans. The potential pathogenicity of fungi also needs to be considered when developing and operating fungal-based biotechnological applications for the bioremediation of BTEX hydrocarbons.

Samenvatting

BTEX koolwaterstoffen (benzeen, tolueen, ethylbenzeen en xyleen) zijn een belangrijke groep verontreinigingen in de milieucompartimenten water, bodem en lucht (Hoofdstuk 1). Recent onderzoek heeft laten zien dat bepaalde schimmels in staat zijn om te groeien op BTEX en aanverwante componenten. Biodegradatie met schimmels kan aantrekkelijk zijn voor de biofiltratie van lucht die verontreinigd is met BTEX. Omdat deze schimmels uit de bodem geïsoleerd zijn, lijkt het waarschijnlijk dat deze schimmels ook een belangrijke rol in de biologische bodemreiniging kunnen vervullen. Tot nu toe is er echter vrij weinig bekend over de ecologie, het metabolisme, de afbraakkinetiek en het gebruik van aromatische verbindingen door schimmels (Hoofdstuk 2).

In het algemeen kunnen onder groeilimiterende omstandigheden zoals lage pH, watergebrek en een tekort aan nutriënten schimmels beter functioneren dan bacteriën. Op basis van deze eigenschappen zijn drie verschillende ophopingstechnieken voor de selectieve isolatie van schimmels met de eigenschap om vluchtige substraten te gebruiken als enige bron voor koolstof en energie. De ophopingstechnieken zijn: vast substraat batches, lucht biofilters en zure vloeistofcultures. Geïnoculeerde organismen werden blootgesteld aan vluchtige verbindingen en geïncubeerd onder zure en droge omstandigheden (Hoofdstuk 3). Uit grond- en grondwatermonsters van een met aromatische koolwaterstoffen verontreinigde locatie werden vijf schimmelstammen geïsoleerd met tolueen als koolstof- en energiebron. De geïsoleerde schimmels werden geïdentificeerd als *Cladophialophora*, *Exophiala* en *Leptodontidium* spp. en de ascomyceet *Pseudeurotium zonatum*. De schimmels vertoonden groei op tolueen als substraat met verdubbelingstijden van 2 tot 3 dagen. Het effect van de tolueen concentratie op de respiratiesnelheid werd eveneens bestudeerd. De schijnbare halfwaarde-verzadigingsconstante (K_m) voor tolueen oxidatie varieerde van 5 tot 22 mM, afhankelijk van de geïsoleerde stam. De ademhalingssnelheid werd voor 50% geremd (IC50) bij

tolueen concentraties variërend van 2.4 tot 4.7 mM. Ondanks herhaalde pogingen konden geen schimmels geïsoleerd worden die in staat waren om te groeien op benzeen, naftaleen of xyleen. Sommige stammen die op tolueen geïsoleerd waren, konden ook groeien op de substraten ethylbenzeen en styreen.

Voorafgaande studies lieten zien dat bacteriën een grote variatie vertonen in metabole afbraakroutes voor de assimilatie van tolueen. In deze studie is de metabole variabiliteit voor de oxidatie van tolueen onderzocht voor de afbraak van tolueen door schimmels (Hoofdstuk 4). Hele cellen werden daarbij geïncubeerd met het isomeer fluortolueen, waarbij de metabolieten werden gekarakteriseerd met 19F magnetische kernspinresonantie spectroscopie. De gedetecteerde fluor bevattende metabolieten lieten zien dat tolueen werd geoxideerd in de methylgroep tot benzoaat in alle op toleen gekweekte schimmels. Vervolgens werd benzoaat gehydroxyleerd, eerst in de para-positie, en daarna tot catechole intermediairen, die eventueel via de 3-oxoadipaat route geassimileerd kunnen worden. Hydroxylering van tolueen kan ook plaatsvinden in de aromaat ring, zoals in de hyphomyceet Cunninghamella echinulata. Echter de degradatiesnelheid was erg laag en was co-metabool.

Voor BTEX afbraak door schimmels is vooral gericht op afzonderlijke substraten, waarbij voorbij wordt gegaan aan het feit dat de vervuiling door koolwaterstoffen meestal in de vorm van complexe mengsels voorkomt. De kinetische interacties gedurende BTEX afbraak zijn bestudeerd voor de schimmel Cladophialophora sp. stam T1 (Hoofdstuk 5). Dit isolaat vertoonde groei op een mengsel van alle zes BTEX componenten van analoge samenstelling als de wateroplosbare fractie van benzine. Tolueen en ethylbenzeen werden gebruikt als koolstof- en energiebron, terwijl de xylenen werden gecometaboliseerd; ortho- en meta-xyleen werden omgezet in phtalaten als eindmetabolieten; para-xyleen werd niet significant afgebroken in de complexe BTEX mengsels, maar uit de koolstofbalans viel af te leiden dat het mogelijk werd gemineraliseerd in

combinatie met tolueen. Benzeen werd in geen enkele combinatie afgebroken. De metabole profielen, samen met de substraat interacties laten zien dat de TEX verbindingen werden gehydroxyleerd in de zijketen door het zelfde monooxygenase enzym.

De biodegradeerbaarheid van BTEX is meestal onderzocht in vloeistofculturen bij optimale condities. Dit gegeven kan misleidend zijn voor extrapolatie naar veldschaal condities. Daarom is de groei van de schimmel Cladophialophora sp. stam T1 op een mengsel van BTEX en MTBE onderzocht in steriele en niet-steriele bodem microcosmossen (Hoofdstuk 6). Vergelijking van de afbraaksnelheden, gemeten in bodembatches, waarbij de aanwezigheid of afwezigheid van endogene bacteriële afbrekers gecombineerd werd met een schimmel inoculum suggereert dat commensalisme de voornaamste interactie was tussen de endogene en geïnoculeerde microorganismen gedurende de biodegradatie van BTEX. Alkylbenzenen werden allemaal door de schimmel afgebroken, maar voor de afbraak van benzeen was de activiteit van de endogene microflora essentieel. Echter MTBE degradatie werd in alle batches met grond waargenomen. Bij een langere blootstelling aan BTEX bij een lage pH leidde dit tot een toename van de biodegradatieactiviteit in de batches met grond die een schimmel inoculum bevatten. PCR-TGGE analyse van schimmel 18S rDNA met verschillende universele schimmel primers gaf aan dat Cladophialophora sp. stam T1 de enige schimmel was die zich ontwikkelde in de batches met grond.

De resultaten van dit proefschrift zijn bediscussieerd in relatie tot kennis die al bekend was over de assimilatie van aromatische koolwaterstoffen door schimmels, evenals de toekomstige biotechnologische toepassingen van dit soort schimmels (Hoofdstuk 7). Biologische afbraak van BTEX door schimmels is in kinetische termen vergelijkbaar met de afbraak door bacteriën, maar schimmels hebben het voordeel van een hogere tolerantie in een meer extreme omgeving. Een mogelijk nadeel van dergelijke schimmels is, dat ze gerelateerd zijn aan

zwarte gistachtige schimmels die bekend zijn om hun vermogen om mycose te veroorzaken bij mensen. Deze potentiële pathogene eigenschappen van schimmels moet nader onderzocht worden bij de ontwikkeling en opschaling van op schimmels gebaseerde biotechnologische toepassingen voor de biologische afbraak van BTEX koolwaterstoffen.

Resumen

Los hidrocarburos aromáticos conocidos con las iniciales BTEX (benceno, tolueno, etilbenceno y xileno) constituyen una fuente importante de polución del suelo, agua y aire (Capítulo 1). Investigaciones recientes han demostrado que, a parte de las bacterias, determinados hongos son capaces de asimilar hidrocarburos monoaromáticos como BTEX. El aprovechamiento de las capacidades degradativas de estos organismos representa una opción interesante de cara a la purificación de aire contaminado con vapores de BTEX. El hecho de que estos hongos fueran aislados a partir de muestras de suelo indica que también podrían jugar un papel importante en la bioremediación de suelo contaminado con BTEX. No obstante, la información disponible al comienzo de esta investigación en relación a la diversidad y así como aspectos cinéticos y metabólicos de los procesos degradativos en relacion a la asimilación de hidrocarburos aromaticos por hongos era muy limitada (Capitulo 2).

De forma genérica se puede afirmar que los hongos se desarrollan mejor que las bacterias en condiciones ácidas, y con poca disponibilidad de agua y nutrientes. Basado en este principio, se desarrollaron tres diferentes métodos para el enriquecimiento selectivo de hongos con la capacidad de utilizar substratos volátiles como fuente de carbono y energía: (i) la fermentación en estado sólido, (ii) biofiltros de aire, y (iii) cultivos líquidos a bajo pH (Capitulo 3). Microorganismos de muestras ambientales fueron expuestos a substratos volátiles e incubados en condiciones de acidez y/o sequedad. Cinco cepas diferentes fueron finalmente aisladas de suelos y acuíferos contaminados con gasolina. Estas fueron identificadas como pertenecientes a los géneros *Cladophialophora*, *Exophiala* y *Leptodontium*, así como el ascomiceto *Pseudeurotium zonatum*. Los resultados demuestran que estos hongos crecen en tolueno doblando su biomasa cada 2 o 3 días, en contraste con las bacterias que lo hacen cada 1 a 3 horas. El efecto de la concentración de tolueno sobre la tasa de respiración fue estudiado. Dependiendo de la cepa, la constante de

saturación (K_m) para la oxidación del tolueno osciló entre 5 y 22 µM. En el otro extremo, la concentración de tolueno que inhibe la tasa de respiración un 50 % (IC50) se situó entre 2.4 y 4.7 mM. A pesar de reiterados intentos no se consiguió aislar hongos con la capacidad de asimilar benceno, naftaleno, o xileno. Sin embargo, etilbenceno y estireno también sirvieron como fuente de carbono y energía en alguna de las cepas aisladas con tolueno.

Las bacterias poseen una alta diversificación en relación a las rutas metabólicas utilizadas para la asimilación del tolueno. En este trabajo, la variabilidad metabólica para la degradación del tolueno fue estudiada para los hongos (Capitulo 4). Cultivos previamente crecidos en tolueno fueron expuestos a diferentes isómeros fluorinados del tolueno y los metabolitos que se formaron fueron identificados mediante la resonancia magnética nuclear del flúor (^{19}F NMR). La oxidación inicial del fluorotolueno tuvo lugar en la cadena de metilo resultando en fluorobenzoato en todos los hongos que asimilan el tolueno. El fluorobenzoato sirvió como sustrato para la hidroxilación del anillo aromático en la posicion *para* y, en función de la posición del flúor, fue metabolizado hasta compuestos catecólicos y muconatos. Estos resultados indican que la utilización del tolueno en los hongos estudiados únicamente ocurre a través de la *para*-hidroxilación del benzoato y la rotura del anillo hacia la ruta del 3-oxoadipato. La oxidación inicial del tolueno en el anillo aromático también es posible en los hongos, tal como fue demostrado en la conversión del fluorotolueno a fluorocresol por el hifomiceto *Cunninghamella echinulata*. No obstante, este último proceso sólo sucedió de forma cometabólica siendo la tasa de conversión muy baja.

La biodegradación de los BTEX se ha estudiado principalmente de forma individual, sin tomar en consideración que cuando estos hidrocarburos causan contaminación ambiental a menudo se encuentran mezclados entre si. La cinética durante la degradación de mezclas de BTEX fue estudiada en el hongo *Cladophialophora* sp. cepa T1 (Capitulo 5). Este hongo fue capaz de crecer en

una mezcla de BTEX análoga a la fracción soluble de la gasolina. Tolueno y etilbenceno sirvieron como fuentes de carbono y energía mientras que los xilenos fueron cometabolizados. El *orto*- y el *meta*-xileno fueron oxidados hasta los respectivos ftalatos como metabolitos terminales. El *para*-xileno, sin embargo, no pudo ser degradado en cantidades significativas en mezclas complejas de BTEX, pero en combinación con tolueno balances de carbono indicaron que fue parcialmente mineralizado. Contrariamente a los compuestos anteriores, el benceno no fue metabolizado en ninguna de las combinaciones de substratos ensayadas. Los patrones de degradación para los diferentes sustratos y la naturaleza competitiva de las interacciones entre ellos indica que los compuestos TEX son hidroxilados en el grupo alquilo mediante la misma monooxigenasa.

La biodegradación de BTEX ha sido estudiada principalmente con cultivos líquidos en condiciones óptimas de crecimiento creadas en laboratorio. Sin embargo, los resultados obtenidos de esta manera suelen ser poco extrapolables al suelo, situación en que los organismos deben afrontar ambientes que limitan su crecimiento. En este estudio, la biodegradación de BTEX y del aditivo de la gasolina MTBE fue evaluada en microcosmos de suelo (Capitulo 6). El efecto de la inoculación del suelo con esporas de *Cladophialophora* sp. cepa T1 sobre las tasas de degradación fue medida en diferentes tratamientos: presencia o inactivación de la microflora original, pH neutro o ácido, exposición previa o no a BTEX. La comparación de las tasas de degradación en presencia y absencia de las bacterias inicialmente presentes en el suelo por un lado, y del hongo por el otro, indican que el comensalismo es la principal interacción entre los degradadores de BTEX indígenos e introducidos. Los alquilbenzenos fueron degradados por el hongo, pero la biodegradación del benzeno requirió siempre de la presencia de la microflora del suelo. El MTBE, no obstante, no fue degradado en ninguno de los casos. La inoculación del suelo con el hongo aumentó las tasa de degradación después de la exposición a BTEX y a un pH acídico. La presencia del hongo en el suelo fue confirmada al final de

los experimentos mediante la amplificación y aislamiento de fragmentos del gen ribosomal 18S.

Los resultados presentados en esta tesis son evaluados con relación a la información disponible en la literatura así como al uso potencial de estos hongos en la biotecnología ambiental (Capítulo 7). El uso de hongos para la bioremediación de la polución causada por BTEX representa una opción muy interesante. La capacidad de los hongos para asimilar BTEX y otros hidrocarburos análogos es similar al de las bacterias en términos cinéticos, con la ventaja que los primeros pueden desarrollarse en medios que son menos favorables para las segundas. Sin embargo, existirían también condiciones que afectan negativamente el uso de los hongos, relacionadas con la posibilidad de causar micosis en humanos, que deben ser tenidas en cuanta durante el desarrollo y operación de aplicaciones biotecnológicas basadas en la actividad de los hongos.

Resum

Els hidrocarburs aromàtics coneguts conjuntament amb les inicials BTEX (benzè, toluè, etilbenzè, i xilè) constitueixen una font important de contaminació del sòl, l'aigua, i l'aire (Capítol 1). En investigacions recents s'ha demostrat que, a part de les bactèries, certs fongs són capaços d'assimilar hidrocarburs aromàtics volàtils, com ara BTEX. L'aprofitament de la capacitat degradativa d'aquest organismes representa una opció interessant de cara al tractament d'aire contaminat amb BTEX. A més a més, el fet que aquest fongs s'hagin aïllat en mostres de sòl indica que també podrien jugar un paper important en bioremediació de sòls contaminats amb BTEX. La informació disponible al començament d'aquesta recerca en relació a la diversitat, metabolisme, i cinètica de degradació en els fongs era escassa (Capítol 2).

De forma genèrica, es pot afirmar que els fongs es desenvolupen millor que les bactèries en condicions que limiten el creixement microbià, com ara un pH baix, i la poca disponibilitat d'aigua i nutrients. Basat en aquest principi, tres mètodes van ser desenvolupats per tal d'enriquir i aïllar fongs amb la capacitat d'utilitzar substrats volàtils com a font de carboni i energia: (i) la fermentació en estat sòl.lid en lots, (ii) biofiltres d'aire, i (iii) cultius líquids acidificats (Capítol 3). Mitjançant aquests sistemes, microorganismes provinents de mostres ambientals van ésser exposats a substrats volàtils en condicions d'acidesa i/o sequetat durant llargs terminis de temps. Cinc cepes amb la capacitat de créixer en toluè van ser finalment aïllades i identificades com pertanyents als gèneres de deuteromicets *Cladophialophora*, *Exophiala* i *Leptodontidium*, així com l'ascomicet *Pseudeurotium zonatum*. En condicions de laboratori, aquest fongs creixen a costa del toluè duplicant llur biomassa cada 2 a 3 dies. Aquests valors contrasten amb el dels bacteris, els quals ho fan cada 1 a 2 hores. L'efecte de la concentració de toluè sobre la taxa de respiració va ser estudiada en els diferents fongs. La constant de saturacio aparent (K_m) va oscil.lar entre 5 to 22 μM, en funció de la cepa estudiada. La toxicitat del toluè, per altra banda,

determinada com la concentració que inhibeix la taxa màxima de respiració en un 50 % (IC50) es va situar entre 2.4 i 4.7 mM. A pesar de repetits intents per tal d'obtenir fongs capacos de créixer en benzè, napthalè, o xilè, aquests van ser infructuosos. Algunes de les cepes aïllades en toluè també utilitzaren l'etilbenzè i l'estirè.

Els bacteris posseixen una grau de diversificació molt elevat en relació a les rutes metabòliques que estan implicades en l'assimilació del toluè. En aquest treball, la variabilitat metabòlica per la degradació del toluè va ser estudiada per als fongs (Capítol 4). Cultius previament crescuts en toluè van ser incubats amb diferents isòmers del fluorotolue. Els metabòlits fluorinats que es van formar a partir de la conversió del fluorotolue van ser caracteritzats mitjançant la resonància magnètica nuclear del ^{19}F. La identificació d'aquests metabòlits permet concloure que la assimilació del toluè s'inicia amb l'oxidació del grup metil en totes les cepes estudiades resultant en benzoat. Aquest compost és subseqüentment hidroxilat, primer a la posició para, i després fins a compostos catecòlics, els quals són finalment assimilats mitjançant la ruta del 3-oxoadipat. La hidroxilació del toluè en l'anell aromàtic també va ésser demostrada en l'hifomicet *Cunninghamella echinulata*. No obstant, la taxa de conversió en aquest darrer cas va ser extremadament baixa i la reacció succeí de forma co-metabòlica.

La biodegradació dels BTEX s'ha estudiat en la majoria dels casos utilitzant compostos de forma individual, ignorant el fet que aquests contaminants sovint es troven com a mescles. Les interaccion cinètiques durant la degradació de mescles de BTEX van ser estudiades en el fong *Cladophialophora* sp. cepa T1 (Capítol 5). Aquest organisme va ser capaç de créixer en una barreja formada pels sis compostos BTEX similar a la fracció soluble de gasolina. Toluè i etilbenzè van ser utilitzats com fonts de carboni i energia, mentres que els xilens van ser co-metabolitzats. Els isomers orto- i meta-xilè van ser oxidats fins als reapectius ftalats; el para-xyle no va ser degradat de forma significativa en

mescles de BTEX, pero balanços de carboni indicaren que en combinació amb toluè va ser mineralitzat. El benzè en canvi no va ser metabolitzat en cap de les combinacions de substrats assajades. Els perfils metabòlics i la naturalesa inhibitòria de les interaccions indica que els TEX són hidroxilats al grup alquil mitjançant la mateixa enzima monooxigenasa.

La biodegradabilitat dels BTEX comunment s'ha estudiat en cultius líquids incubats en condicions òptimes. Els resultats d'aquests experiments solen diferir dels obtinguts en condicions de camp. En aquest treball, la biodegradació de BTEX i de l'aditiu de la gasolina MTBE va ser estudiada en mostres de sòl. L'efecte de la inoculació amb el fong *Cladophialophora* sp. cepa T1 sobre la tasa de degradació va ser estudiada (Capítol 6). La comparació de les tases de biodegradació amb la presència i l'absència dels bacteris inicialment presents en el sòl per una banda, i del fong per un altra indicaren que el comensalisme és la principal interacció entre els degradadors de BTEX indigens i introduits. Els alquilbenzens van ser degradats pel fong, mentres que la degradació del benzè requerí la activitat de la microflora del sòl. El MTBE, però, no va ser degradat en cap dels casos. La inoculació del sòl amb el fong va aumentar les taxes de degradació després d'un temps llarg d'exposició als BTEX i a un pH acídic. La presència i identitat de l'inòcul va ser confirmada mitjançant l'anàlisi de fragments del gen ribosomal 18S.

En el Capítol 7, els resultats presentats en aquesta tesis són evaluats en relació a la informació prèviament disponible en la literatura científica i a l'ús potencial d'aquest organismes en la biotecnologia ambiental. L'aplicació de fongs en la bioremediació de la polució causada per BTEX representa una opció molt interessant. En termes cinètics, la biodegradació de BTEX en fongs i bacteris és similar, però els primers tenen l'avantatge de tolerar millor certes condicions ambientals adverses. No obstant, la assimilació de hidrocarburs aromàtics en fongs està sovint relacionada amb cepes que causen micosis. La capacitat patògena d'aquests fongs vers els humans s'ha de considerar seriosament

alhora de desenvolupar i posar en operació sistemes de bioremediació dels BTEX que estan basats en els fongs.

Curriculum vitae

Francesc Xavier Prenafeta Boldú was born on the 5th of May 1970 in Lleida, Catalonia, Spain. He studied at the Faculty of Agronomy, Lleida University, where he obtained the BSc diploma on Agricultural Sciences in 1994. After one-year civil service he gained his MSc degree on Environmental Sciences at the same university in 1998, after completing a master thesis at the sub-department of Environmental Technology, Wageningen University. The topic of that investigation was the biodegradation of azo dyes under anaerobic and micro-aerofilic conditions. From June to November 1997 he worked at ATO-DLO institute on the environmental aspects of re-circulating the nutrient solutions in greenhouse horticulture. In February 1998 he started his PhD at the Division of Industrial Microbiology, Wageningen University. He investigated the assimilation of aromatic hydrocarbons by fungi. During the last year of the PhD he focused the research toward applied aspects of fungal biodegradation at the TNO-MEP institute. The results from that project are presented in the present dissertation. In April 2002 he is started a post-doc at the Centraalbureau voor Schimmels (CBS) conducting research on the interactions between fungi bacteria in soil.

List of publications

Razo Flores, E., P. Smulders, F. Prenafeta Boldú, G. Lettinga, and A. Field Jim 1999. Treatment of anthranilic acid in an anaerobic expanded granular sludge bed reactor at low concentrations *Water Science and Technology.* **40:**187-194

Tan, N. C. G., F. X. Prenafeta Boldú, J. L. Opsteeg, G. Lettinga, and J. A. Field 1999. Biodegradation of azo dyes in cocultures of anaerobic granular sludge with aerobic aromatic amine degrading enrichment cultures *Applied Microbiology and Biotechnology.* **51:**865-871

Prenafeta Boldú, F. X., A. Kuhn, D. Luykx, H. Anke, J. W. van Groenestijn, and J. A. M. de Bont 2001. Isolation and characterisation of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source *Mycological Research.* **105:**477-484

Prenafeta Boldú, F. X., D. M. A. Luykx, J. Vervoort, and J. A. M. de Bont 2001. Fungal metabolism of toluene: Monitoring of fluorinated analogs by ^{19}F nuclear magnetic resonance spectroscopy *Applied and Environmental Microbiology.* **67:**1030-1034

Prenafeta-Boldú, F. X., J. Vervoort, J. T. C. Grotenhuis, and J. W. van Groenestijn 2002 Substrate interactions during the biodegradation of BTEX hydrocarbons by the fungus *Cladophialophora* sp. strain T1 *Applied and Environmental Microbiology*, 68(6): 2660-2665

Prenafeta-Boldú, F. X..H. Ballested, J. Gerritse, and J. T. C. Grotenhuis Biodegradation of BTEX hydrocarbons in soil. Effect of seeding with the toluene-growing fungus *Cladophialophora* sp. strain T1. Submitted

Prenafeta-Boldú, F. X., and J. W. van Groenestijn Kinetic aspects of toluene biodegradation in fungal biofilters. In preparation

Luykx, D., F. X. Prenafeta Boldú, and J. A. M. de Bont Characterization of a toluene monooxygenase from *Cladosporium sphaerospermum*. In preparation

The research described in this thesis was financially supported by the European Union contract grant BIO4-CT-972295

Cover picture: *Cladophialophora* sp. (CBS 110513, ATCC MYA-2335)

Printing: Universal Press