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

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Five fungal strains that are able to grow on toluene were isolated from enrichment cultures. Three different techniques were used: solid state-like batches, air biofilters and liquid cultures. Fungal growth in the latter systems was favoured by combining low pH and low water activity. Soil and groundwater samples from gasoline-polluted environments were used as inocula. The isolates were identified as deuteromycetes belonging to the genera *Cladophialophora*, *Exophiala* and *Leptodontium* and the ascomycete *Pseudeurotium zonatum*. The previously isolated toluene-degrading fungus *Cladosporium sphaerospermum* was included in the present study. Results showed that fungi grew on toluene with doubling times of about 2 to 3 days. Some of the strains also grew on ethylbenzene and styrene. The apparent half-saturation constant (K_m) for toluene oxidation ranged from 5 to 22 μM. Degradation activity was inhibited by 50% at toluene concentrations ranging from 2.4 to 4.7 mM. These kinetic parameters are comparable to analogous data reported for toluene-degrading bacteria. The ability of fungi to grow at low water activities and low pH suggest that they may be used for the purification of gas streams containing aromatic hydrocarbons in air biofilters.

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 (Lee, Craig & Smith 1974, Swoboda-Coldberg 1995). Besides being relatively water insoluble, BTEX compounds are also volatile. BTEX is present in the waste gas of industrial processes and bioremediation works at oil-contaminated sites (Atlas & Cerniglia 1995). Due to their toxicity and recalcitrance, aromatic hydrocarbons are regarded as major environmental pollutants and have been subject to stringent environmental regulations (Mehlman 1992).

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 (van Groenestijn & Hesselink 1993). 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 (Ottengraf 1986).

Both fungi and bacteria are known to degrade aromatic hydrocarbons (Cerniglia 1984). Fungi perform oxidation reactions as a prelude to the detoxification and excretion of hydrocarbons, rather than using these compounds as carbon sources for growth (Cerniglia, White & Heflich 1985, Müncnerová & Augustin 1994). Interestingly, Hartmans, van der Werf & de Bont (1990) 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 (Cox & Doddema 1996, Cox *et al*. 1997). 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 (Weber, Hage & de Bont 1995). 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 (Cooney 1984, Cerniglia, Sutherland & Crow 1992, Smits *et al*. 1998). 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 characterised 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.

MATERIALS AND METHODS

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 (Hartmans & Tramper 1991). 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 (Cox *et al*. 1993) 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 °.

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 (Wedding & Kentrick 1959). 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 dissolving 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 ° on a rotary shaker (130 rpm).

Fungi were isolated by washing samples of the support material or directly form 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.

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 nitro-aromatic 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 ° 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.

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 (Hartmans & Tramper 1991). Hydrocarbons were added up to an initial concentration of 0.5 mM in the liquid media based on reported water/air partition coefficients (Mackay & Shiu 1981, Amoore & Hautala 1983). 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 ° 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.

Table 1. Enrichment conditions and identification of the isolated fungal strains growing on toluene.

Source of inoculum	Enrichment technique	Conditions	Fungus	
			Code	Identification
BTEX polluted soil (Bennekom, The Netherlands)	Solid state-like batch	pH = 7.0 rh = 90%	T1	*Cladophialophora* sp.[a]
Gasoline station soil (Apeldoorn, The Netherlands)	Air biofilter	pH = 4.0 rh = 97%	T2	*Cladophialophora* sp.[a]
			T3	*Sporothrix*-like (anamorph) *Pseudeurotium zonatum* (teleomorph)
BTEX polluted soil/water (Brandenburg, Germany)	Liquid culture	pH = 5.5	T4	*Exophiala* sp.[a]
			T5	*Leptodontium* sp.[a]

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

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 (Hartmans & Tramper 1991) 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 1 week at 20° or 30° 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 (10000 rpm, r_{av} 8 cm, for 10 min at 4 °). Cell suspensions were stored at 4 ° until use for up to three days.

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° 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.

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, 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° respectively. For carbon dioxide a Chrompack Poraplot Q column (Chrompack 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 wee respectively set at 70 and 250 °. Oxygen consumption were measured in a Clark type oxygen electrode (Yellow Springs Instruments, Ohio). Dry weight was determined after drying cell suspensions for 24 h at 105 °. The latter values were corrected for the salts contained in the suspension buffer.

Chemicals

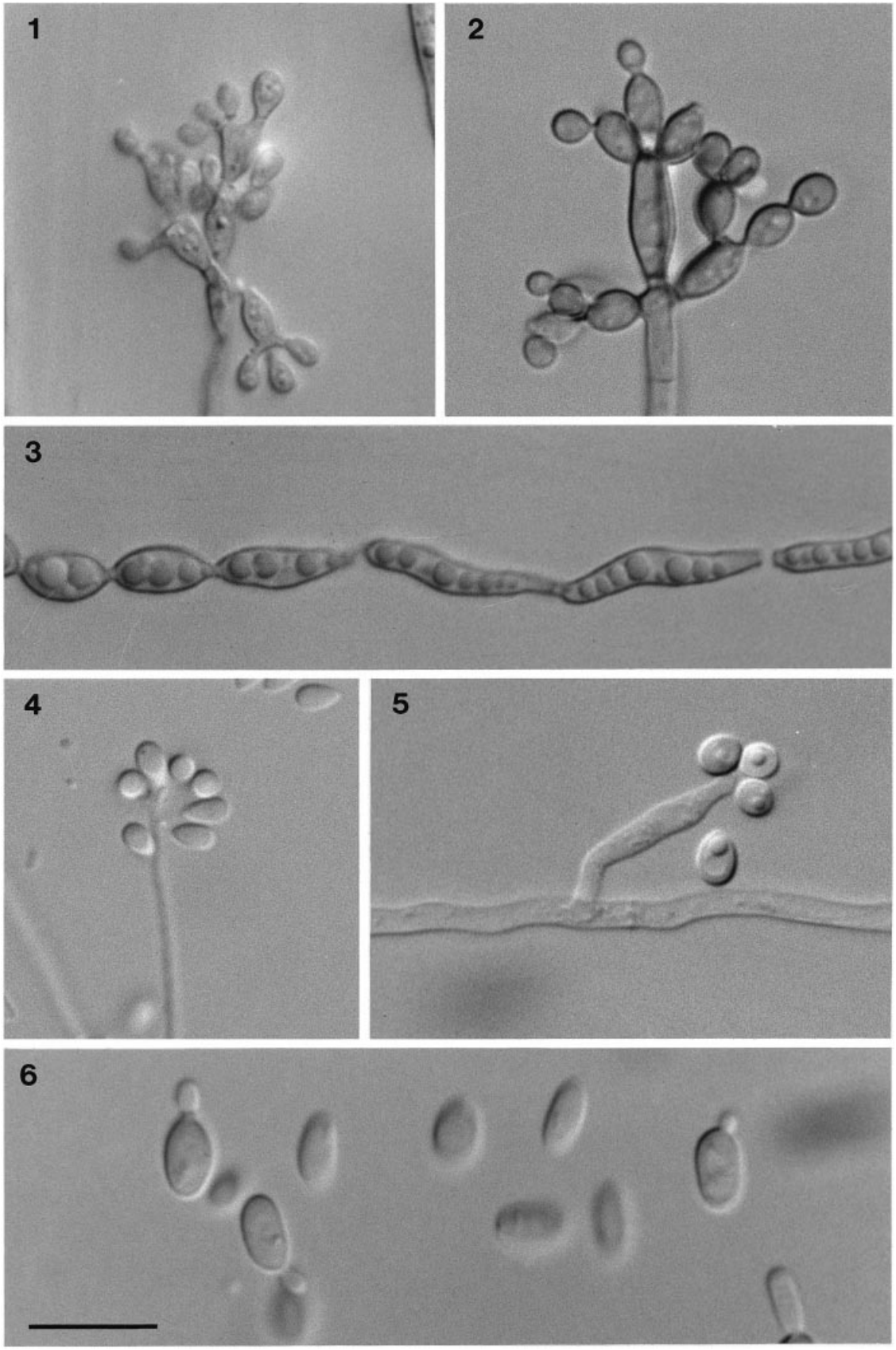
Hydrocarbons were obtained from Acros Organics (Geel, Belgium), Sigma-Aldrich Chemicals (Steinheim, Germany), Jansen Chimica (Geel, Belgium), Lab-Scan (Dublin, Ireland) and Merck KGaA (Darmstadt, Germany). All chemicals were of analytical grade.

RESULTS

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.

The isolates from BTEX-polluted sites were identified by the Centraalbureau voor Schimmelcultures (CBS), Utrecht (Table 1) as hyphomycetes except for T3 for which both the teleomorph, *Pseudeurotium zonatum* and a *Sporothrix*-like ana-



Figs 1–6. Photographs of fungi grown on toluene (bar = 10 μm). **Fig. 1.** Conidiophores of *Cladosporium sphaerospermum* T0. **Fig. 2.** Conidiophores of *Cladophialophora* sp. T1. **Fig. 3.** Conidial chain of *Cladophialophora* sp. T2. **Fig. 4.** *Sporothrix*-like anamorph of *Pseudeurotium zonatum* T3. **Fig. 5.** Conidiophore of *Leptodontium* sp. **Fig. 6.** Yeast-like growth of *Exophiala* sp. T4 with budding cells.

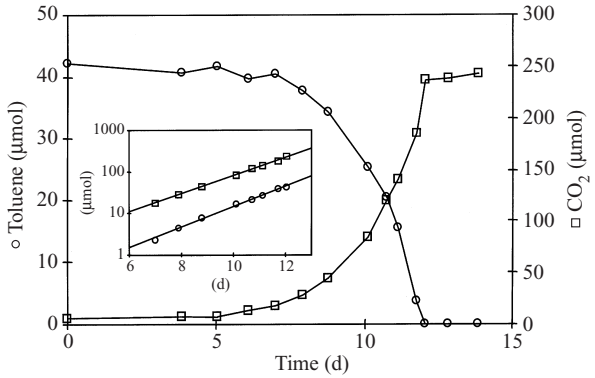


Fig. 7. Toluene degradation and CO_2 production by *C. sphaerospermum* growing on toluene at 30 °C. The inset presents the exponential decrease of toluene and increase of CO_2 plotted on a logarithmic scale.

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

	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 sp. T3	0.27	0.22	0.12	—
Exophiala sp. T4	0.28	0.36	0.40	—
Leptodontium sp. T5	0.34	0.33	0.13	—

[a] Mineral media supplemented with yeast extract (0.01% w/v).
— No growth.

morph 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 1–6.

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 (Tijhuis, van Loosdrecht & Heijnen 1993), the biomass yield accounted for about the 30–40% of the carbon substrate. Fig. 7 shows as an example of the link between the toluene degradation and growth for *Cladosporium 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 2. These results revealed that the fungi differed in their optimum growth temperatures. *Pseudeurotium* sp. T3 and *Leptodontium* sp. T5 showed good growth at 20° but little or no growth at temperatures higher than 30°. In contrast, the *Cladophialophora* strains T1 and T2 grew better in the range of 30 to 37°.

The substrate range of the fungi was evaluated using different hydrocarbons as the sole source of carbon and energy (Table 3). 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

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

	Fungus					
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.

Table 4. Toluene degradation kinetics by toluene-grown fungal cells. Specific rates are given in μmol g DW^{-1} h^{-1} at 30 °C ($n \leqslant 6$, $r^2 \leqslant 0.95$).

Parameter	Fungus			
	T0	T1	T2	T4
Specific rates[a]				
Toluene degradation	74 ± 7	81 ± 4	74 ± 6f	25 ± 3
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 ± 6	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 ($n \leqslant 8$, $r^2 \leqslant 0.98$).

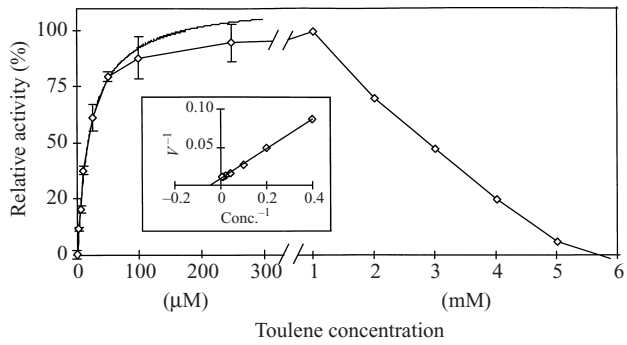


Fig. 8. Kinetics of toluene oxidation by *Cladosporium sphaerospermum*. The data are presented as a direct plot and as a Lineweaver-Burk linearisation (inset). Error bars show standard deviations.

media. The aliphatic hydrocarbons n-decane and n-hexane served as growth substrates for some of the fungi. Nevertheless, they were catabolised at much lower rates and growth was characterised by low carbon dioxide recoveries.

Kinetics of toluene degradation

Table 4 summarises different parameters for toluene degradation in whole cell suspensions. No biodegradation 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 *Cladosporium sphaerospermum* are shown in Fig. 8). These data were fitted to the Michaelis–Menten model and the apparent K_m was calculated form 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.

DISCUSSION

This 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 *Leptodontium*. Previously, *Exophiala* and *Sporothrix* species assimilating styrene were isolated from biofilters used in the treatment of air polluted with styrene (Cox *et al*. 1993). *Exophiala* has a high taxonomic affinity with *Cladophialophora* (de Hoog *et al*. 1995). Consequently, with the exception of *Leptodontium* 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 (Cerniglia *et al*. 1978), 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 (Cooney 1984, Cerniglia *et al*. 1992). 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 utilising fungi.

Due to the inhomogeneous biomass of filamentous fungi, fungal growth is generally correlated to the respiration activity (Smits *et al*. 1998). Here we used the rate coefficient of carbon dioxide production (value that was very similar to

the rate of substrate utilisation; Fig. 1) as an estimate of the specific growth rate. The substrate contribution to maintenance however can not be neglected in slow-growing organisms (Tijhuis *et al*. 1993). Therefore, the specific growth rates of our fungal isolates are expected to be lower than the respiration rates given in Tables 2 and 3. From these results it is clear that the fungi grew on toluene with doubling times of at least 2–3 days in contrast to their bacterial counterparts, which exhibit doubling times of only 1–3 hours (Chang, Voice & Criddle 1993, Mirpuri, Jones & Bryers 1997, Mars *et al*. 1998). The white colony forming strains *Pseudeurotium* sp. T3 and *Leptodontium* sp. T5 showed poor growth at temperatures of 30 °. These fungi also lacked degradative activity in washed mycelia. Loss of activity after harvest has been observed in other hydrocarbon-growing fungi (Hardison *et al*. 1997).

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 (Cox *et al*. 1996). 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 1981), 2-methylnaphthalene was toxic to all the fungi studied. Naphthalene and its methylated analogues are also highly toxic towards invertebrates (Boylan & Tripp 1971, Anderson *et al*. 1974). In contrast to the aromatic substrates, aliphatic hydrocarbons were poorly utilised. In agreement with our results, low growth rates and biomass yields were obtained in previous studies with filamentous fungi growing on n-alkanes (Siporin & Cooney 1975, Lindley *et al*. 1986).

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 (Tijhuis *et al*. 1993), 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 4) 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^{-1} h^{-1} at 28°; Mars *et al*. 1996). 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 4), fell into the range of reported Monod constants for toluene degradation by *Pseudomonas* species (0.4 to 43.3 μM; Change *et al*. 1993, Mirpuri *et al*. 1997, Mars *et al*. 1998). 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 (Blum & Speece 1991, Weber, Schemen & Hartmans 1995, de Bont 1998).

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.

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REFERENCES

Amoore, J. E. & Hautala, E. (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.

Anderson, J. W., Neff, J. M., Cox, B. A., Tatem, H. E. & Hightower, G. M. (1974) The effects of oil on estuarine animals; toxicity, uptake and depuration, respiration. In *Pollution and Physiology of Marine Organism* (F. J. Vernbeg & W. Vernberg, eds): 285–310. Academic Press, New York.

Atlas, R. M. & Cerniglia, C. E. (1995) Bioremediation of petroleum pollutants: diversity and environmental aspects of hydrocarbon biodegradation. *BioScience* **45**: 332–338.

Boylan, D. B. & Tripp, B. W. (1971) Determination of hydrocarbons in seawater extracts of crude oil and crude oil fractions. *Nature* **230**: 44–47.

Blum, D. J. W. & Speece, R. E. (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.

Cerniglia, C. E. (1984) Microbial transformation of aromatic hydrocarbons. In *Petroleum Microbiology* (R. M. Atlas, ed.): 99–128. Macmillan, New York.

Cerniglia, C. E., Hebert, R. L., Szaniszlo, P. J. & Gibson, D. T. (1978) Fungal transformation of naphthalene. *Archives of Microbiology* **117**: 135–143.

Cerniglia, C. E., Sutherland, J. B. & Crow, S. A. (1992) Fungal metabolism of aromatic hydrocarbons. In *Microbial Degradation of Natural Products* (G. Winkelmann, ed.): 194–217. VCH, Weinheim.

Cerniglia, C. E., White, G. L. & Heflich, R. H. (1985) Fungal metabolism and detoxification of polycyclic aromatic hydrocarbons. *Archives of Microbiology* **143**: 105–110.

Chang, M.-K., Voice, T. C. & Criddle, C. S. (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.

Cooney, J. J. (1984) The fate of petroleum pollutants in freshwater ecosystems. In *Petroleum Microbiology* (R. M. Atlas, ed.): 400–433. Macmillan, New York.

Cox, H. H. J. & Doddema, H. J. (1996) Biological filter for the removing volatile hydrophobic compounds from gas emissions. *European Patent EP-0,710,147.*

Cox, H. H. J., Faber, B. W., van Heiningen, W. N. M., Radhoe, H., Doddema, H. J. & Harder, W. (1996) Styrene metabolism in *Exophiala jeanselmei* and involvement of a cytochrome P450-dependent styrene-monooxygenase. *Applied and Environmental Microbiology* **62**: 1471–1474.

Cox, H. H. J., Houtman, J. H. M., Doddema, H. J. & Harder, W. (1993) Enrichment of fungi and degradation of styrene in biofilters. *Biotechnology Letters* **15**: 737–742.

Cox, H. H. J., Moerman, R. E., van Baalen, S., van Heiningen, W. N. M., Doddema, H. J. & Harder, W. (1997) Performance of a styrene-degrading biofilter containing the yeast *Exophiala jeanselmei*. *Biotechnology and Bioengineering* **53**: 259–266.

de Bont, J. A. M. (1998) Solvent-tolerant bacteria in biocatalysis. *Trends in Biotechnology* **16**: 493–499.

de Hoog, G. S., Takeo, K., Göttlich, E., Nishimura, K. & Miyaji, M. (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.

Hardison, L. K., Curry, S. S., Ciuffetti, L. M. & Hyman, M. R. (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.

Hartmans, S. & Tramper, J. (1991) Dichloromethane removal from waste gases with a trickle-bed bioreactor. *Bioprocess Engineering* **6**(3): 83–92.

Hartmans, S., van der Werf, M. J. & de Bont, J. A. M. (1990) Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. *Applied and Environmental Microbiology* **56**: 1347–1351.

Lee, C. C., Graig, W. K. & Smith, P. J. (1974) Water-soluble hydrocarbons from crude oil. *Bulletin of Environmental Contamination and Toxicology* **12**: 212–216.

Lindley, N. D., Pedley, J. F., Kay, S. P. & Heydeman, M. T. (1986) The metabolism of yeasts and filamentous fungi which degrade hydrocarbon fuels. *International Biodeterioration* **22**: 281–287.

Mackay, D. & Shiu, W. Y. (1981) A critical review of Henry's law constants for chemicals of environmental interest. *Journal of Physical Chemistry Reference Data* **10**: 1175–1199.

Mars, A. E., Houwing, J., Dolfing, J. & Janssen, D. B. (1996) Degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 in growth-limited fed-batch culture. *Applied and Environmental Microbiology* **62**: 886–891.

Mars, A. E., Prins, G. T., Wietzes, P., de Koning, W. & Janssen, D. B. (1998) Effect of trichloroethylene on the competitive behaviour of toluene-degrading bacteria. *Applied and Environmental Microbiology* **64**: 208–215.

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.

Mirpuri, R., Jones, W. & Bryers, J. D. (1997) Toluene degradation kinetics for planktonic and biofilm-grown cells of *Pseudomonas putida* 54G. *Biotechnology and Bioengineering* **53**: 535–546.

Müncnerová, D. & Augustin, J. (1994) Fungal metabolism and detoxification of polycyclic aromatic hydrocarbons: a review. *Bioresource Technology* **48**: 97–106.

Ottengraf, S. P. P. (1986). Exhaust gas purification. In *Biotechnology* (H.-J. Rehm & G. Reeds, eds) **8**: 426–452. VCH Verlagsgelsellshaft, Weinheim.

Siporin, C. & Cooney, J. J. (1975) Extracellular lipids of *Cladosporium* (*Amorphotheca*) *resinae* grown on glucose or on n-alkanes. *Applied Microbiology* **29**: 604–609.

Smits, J. P., van Sonsbeek, H. M., Rinzema, A. & Tramper, J. (1998) Solid-state fermentation – a mini review. *Agro-Food-Industry High-Tech* **9**(2): 29–36.

Swoboda-Colberg, N. G. (1995) Chemical contamination of the environment: sources, types, and fate of synthetic organic chemicals. In *Microbial transformations and degradation of toxic organic chemicals* (L. Y. Young & C. E. Cerniglia, eds): 27–74. Wiley-Liss, New York.

Tijhuis, L., van Loosdrecht, M. C. M. & Heijnen, J. J. (1993) A thermodynamically based correlation for maintenance Gibbs energy requirements in aerobic and anaerobic chemotrophic growth. *Biotechnology and Bioengineering* **42**: 509–519.

van Groenestijn, J. W. & Hesselink, P. G. M. (1993) Biotechniques for air pollution control. *Biodegradation* **4**: 283–301.

Weber, F. J., Schemen, M. W. & Hartmans, S. (1995) Toxicity of contaminants in waste gases for microorganisms. In *Toluene: biological waste-gas treatment, toxicity and microbial adaptation* 61–68. PhD thesis, Wageningen Agricultural University.

Weber, F. J., Hage, K. C. & de Bont, J. A. M. (1995) Growth of the fungus *Cladosporium sphaerospermum* with toluene as the sole carbon and energy source. *Applied and Environmental Microbiology* **61**: 3562–3566.

Wedding, R. T. & Kentrick, J. D. (1959) Toxicity of M-methyl-dithiocarbamate and methylisocyanate to *Rhizoctonia solani*. *Phytopathology* **49**: 557–561.

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