Chapter 6
Biodegradation of Mono-Aromatic Hydrocarbons by Fungi

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6.1 Fungal Biodegradation of Pollutants

The biodegradation of organic compounds by fungal strains has been studied for several decades. Fungi have proven to be able to degrade a wide range of different pollutants, including aliphatic, aromatic and polycyclic aromatic compounds, but not necessarily as sole carbon and energy source.

Extensive and detailed studies on fungal assimilation of non-oxygenated mono-aromatic compounds are, however, quite recent. A clear evidence on the use of such substrates as sole carbon and energy source was found for the first time in the early 1990s. Considering the high volatility of such compounds and their relatively common presence in polluted air, much of the research published on this topic is often related to biotechniques for air pollution control (Kennes and Veiga 2004; Kennes et al. 2009).

6.2 Fungal Biodegradation of Aliphatic Compounds

Fungal biodegradation of aliphatic compounds has been studied and reported in the literature for several decades and will, therefore, not be reviewed here extensively. Many different fungal species have been described for their growth on aliphatic substrates including alcohols, alkanes, and aldehydes, among others. Some examples are given in Table 6.1. Often these substrates can be used as sole carbon and energy source, although there are some exceptions, such as, the co-metabolic degradation

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Table 6.1 Examples of fungal strains degrading aliphatic compounds

Fungal species	Substrate	Reference
Paecilomyces variotii	Methanol	Sakaguchi et al. (1975)
Gliocladium deliquescens	Methanol	Sakaguchi et al. (1975)
Paecilomyces variotii	Formaldehyde	Sakaguchi et al. (1975)
Gliocladium deliquescens	Formaldehyde	Sakaguchi et al. (1975)
Acremonium sp.	C_2–C_4 Alkanes	Davies et al. (1973)
Cladosporium resinae	C_6–C_{19} Alkanes	Cofone et al. (1973); Lindley and Heydeman (1983)
Scedosporium sp.	C_1–C_9 Alkanes	Onodera et al. (1989)
Graphium sp.	*n*-Butane	Hardison et al. (1997)
Trichosporon veenshuisii	*n*-Hexadecane	Middelhoven et al. (2000)
Graphium sp.	Di-ethyl-ether	Hardison et al. (1997)
Graphium sp.	Methyl-*tert*-butyl-ether	Hardison et al. (1997)

of the gasoline oxygenate methyl-*tert*-butyl ether (MTBE) by a *Graphium* sp., ATCC 58,400, in the presence of some *n*-alkanes (Hardison et al. 1997).

When dealing with mixtures of hydrocarbons, as present in oil, many yeasts and filamentous fungi easily degrade short-chain hydrocarbons (mainly alkanes), while long-chain hydrocarbons are generally degraded more slowly and aromatic hydrocarbons may even remain undegraded (Lindley and Heydeman 1986a, Lindley et al. 1986b).

Although most studies have been done with mesophilic organisms, some psychrophilic and psychrotolerant alkane-degrading fungi have also been described (Hughes et al. 2007). In these fungi, in the presence of glucose as an additional carbon source, growth rates generally increased if aliphatic hydrocarbons were available but decreased when a mixture of glucose and aromatic compounds was provided.

6.3 Fungal Biodegradation of Polycyclic Compounds

Polycyclic aromatic compounds are hydrophobic pollutants characterized by the presence of more than one benzene ring and found in oil derived products or creosotes together with some other pollutants (Kennes and Lema 1994a). Extensive studies were done on the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in the late 1990s mainly with white-rot ligninolytic fungi, such as *Phanerochaete chrysosporium* or *Trametes versicolor*. Some non-white-rot fungi were also shown to be able to biodegrade and use some PAHs as sole carbon source. This was the case of the fungus *Fusarium solani* degrading benzo[A]pyrene as sole carbon source and partly converting it to carbon dioxide (Rafin et al. 2000). In this organism, the amount CO_2 released decreased in the presence of cytochrome P-450 inhibitors. Fungal strains are generally more efficient than bacteria in degrading polycyclic compounds. It is now known that they can use either the cytochrome P-450 system (Yadav et al. 2006) or extracellular ligninolytic enzymes (Tortella and Diez 2005) such as

lignin- and manganese- peroxidases (known as MnP and LiP) (Hammel 1992) and laccases (Majcherczyk et al. 1998). One of the main roles of lignin-degrading enzymes is to attack lignin. The latter is a complex molecule containing different structures, rings and links between the rings. Ligninolytic enzymes are, therefore, non-specific and highly versatile in their biodegradation capabilities, which allow them to biodegrade many different molecules, such as PAHs. On the other hand, the cytochrome P-450 system is also found in mammals, where PAH degradation follows similar pathways as in fungi. The biodegradation products, with the cytochrome P-450 system, are mainly epoxides and dihydrodiols which may show carcinogenic properties. In the perodixase-linked pathway, quinones are formed (Kennes and Lema 1994a), which are much less harmful. In many cases, these metabolites tend to accumulate as dead-end products.

Reports on the biodegradation of some other compounds with more than one ring, such as biphenyls, by other fungi are also available. Fungal biodegradation and accumulation of soluble metabolites was observed in biphenyl degradation by lygninolytic fungi as well as yeasts, such as *Cunninghamella elegans* (Dodge et al. 1979), *Candida lipolytica* (Cerniglia and Crow 1981), *Saccharomyces cerevisiae* (Wiseman et al. 1975), or *Trichosporon mucoides* (Sietmann et al. 2001). Schauer et al. (1995) reported the ability of the yeast *Trichosporon beigelii* to metabolize and partly degrade diphenyl ether.

6.4 Fungal Biodegradation of Non-Oxygenated Mono-Aromatic Compounds

6.4.1 Biodegradation of Oxygenated Mono-Aromatic Compounds

Although studies on the biodegradation of oxygenated aromatic compounds by fungi were already published more than 40 years ago (Neujahr and Varga 1970), much less research was done at that time on the non-oxygenated mono-aromatic pollutants, such as the benzene-compounds. Most of the research on oxygenated substrates was done with compounds such as phenol and cresols. Some of the researches published over the past 40 years have been summarized in Table 6.2. This is obviously not an exhaustive list, but it illustrates the typical pollutants and fungal species that have been studied. Fungal growth on some other oxygenated benzene compounds, such as benzoate or hydroxybenzoate, is also possible, although these substrates have been less studied (Middelhoven 1993). Again, these pollutants can be degraded either as sole carbon and energy source or co-metabolically. For example, a *Penicillium* strain Bi 7/2 was shown to use phenol as sole source of carbon and energy (Hofrichter et al. 1993). The same was true for *Trichosporon guehoae* CBS 8521 using either phenol or *m*–cresol (Middelhoven et al. 1999). Conversely, *Penicillium* strain Bi 7/2 degraded substituted phenols, such as chlorophenols or cresols, only co-metabolically (Hofrichter et al. 1993, 1995).

Table 6.2 Examples of fungal strains degrading oxygenated mono-aromatic compounds

Fungal species	Substrate	Reference
Trichosporon cutaneum	Phenol	Neujahr and Varga (1970)
Aspergillus japonicus	Phenol	Milstein et al. (1983)
Penicillum spp	Phenol	Scow et al. (1990); Hofrichter et al. (1993)
Phanerochaete chrysosporium	Phenol	Kennes and Lema (1994b)
Trichosporon guehoae	Phenol	Middelhoven et al. (1999)
Trichosporon veenhuisii	Phenol	Middlehoven et al. (2000)
Trichosporon cutaneum	Fluorinated phenols	Peelen et al. (1995)
Trichosporon cutaneum	*o*-, *m*-, *p*-cresols	Hasegawa et al. (1990)
Aspergillus fumigatus	*p*-cresol	Jones et al. (1993)
Phanerochaete chrysosporium	*p*-cresol	Kennes and Lema (1994b)
Penicillium frequentans	*o*-cresol	Hofrichter et al. (1995)
Trichosporon guehoae	*m*-cresol	Middelhoven et al. (1999)
Trichosporon veenhuisii	*m*-cresol	Middlehoven et al. (2000)

6.4.2 Biodegradation of Non-Oxygenated Mono-Aromatic Compounds

Until the end of the past century, the biodegradation of non-oxygenated mono-aromatic compounds seemed to be an unusual capability of fungi (Kennes and Veiga 2004). The first isolated report suggesting minimal growth of a fungal strain on benzene compounds appeared in 1973 (Cofone et al. 1973). However, not enough evidence was obtained at that time to conclude that growth of the *Cladosporium resinae* strain was possible on benzene. Another report appeared several years later (Fedorak and Westlake 1986) showing slow growth of different fungal strains on *n*-alkylbenzenes. The experiments were performed with *Penicillium*, *Beauveria*, *Paecilomyces* and *Verticillium* spp. Compounds with relatively short side chain length, i.e. toluene, ethylbenzene or even benzene, were not degraded. Minimum length of the side chain for the substrates to be degraded, was C_4 or even C_8 or C_9, depending on the strain. Partial removal of those pollutants was detected after four weeks, i.e. 28 days, with transient accumulation of intermediate compounds, mainly aromatic acids. Evolution of end products, such as carbon dioxide, was not reported. It was only in the mid-1990s that a clear evidence for the use of alkylbenzenes as sole carbon and energy source and their complete biodegradation to end products was found in fungi. The use of toluene, as sole carbon and energy source, was reported in 1995 in the fungal strain *Cladosporium sphaerospermum* (Weber et al. 1995), with complete removal of the substrate at relatively high rate and complete conversion to carbon dioxide, water and biomass. According to the following stoichiometric equation, complete substrate biodegradation should yield 6.35 mol of CO_2 per mole toluene degraded (Estévez et al. 2005a) taking biomass growth into account, and with ammonium chloride as nitrogen source:

$$1.55\ C_7H_8 + 12\ O_2 + 0.2\ NH_4Cl \rightarrow CH_{1.8}N_{0.2}O_{0.5} + 9.85\ CO_2 + 5.6\ H_2O + 0.2\ HCl$$

Weber et al. (1995) reported that about 220 μmoles CO_2 was generated from 60 μmoles toluene after ten days, corresponding to a carbon dioxide to substrate ratio of about 3.7, showing that at least 66% of CO_2 was recovered experimentally, compared to the theoretical equation. However, at the end of the ten-day experiment, carbon dioxide release was still increasing exponentially, suggesting that higher product concentrations were presumably released. Toluene biodegradation by *Cladosporium sphaerospermum* seemed to involve Cytochrome P-450, starting initial attack on the side chain (Weber et al. 1995). Earlier, experiments were performed with another benzene-compound, namely styrene (Cox et al. 1993). The black yeast *Exophiala jeanselmei* showed slow growth on styrene, utilizing it as sole carbon and energy source, with an average specific removal rate around 30 nmol/min.mg protein. Potential metabolic intermediates of the biodegradation were suggested to be styrene oxide, phenylacetic acid, 1-phenyl-ethanol, 2-phenyl-ethanol, acetophenone, benzoic acid and phenol, which all supported yeast growth. Around that same period, it was also found that the white-rot fungus *Phanerochaete chrysosporium* was able to biodegrade benzene, toluene, ethylbenzene and xylenes (Yadav and Reddy 1993). However, only a part of the original alkylbenzenes concentration disappeared in the batch culture media. Besides, mass balance calculations also showed that the alkylbenzenes were only partly converted to carbon dioxide.

Non-substituted benzene (benzene as such) is often much more difficult to be degraded by fungal strains (Prenafeta-Boldú et al. 2002), suggesting the importance of the side-chains. Bacteria are known to attack first either the alkyl side chain or the aromatic ring of alkylbenzenes. However, in zygomycetous fungi, both pathways have been observed in case of co-metabolic degradation. Otherwise, only the oxidation of the side chain has been reported, when the removal is not through co-metabolism (Prenafeta-Boldú et al. 2001b). This also confirms the earlier results of Fedorak and Westlake (1986), where benzene compounds with longer side chains were generally more degraded than organic pollutants with shorter side chains. Conversely, the presence of several side-chains, such as in the xylene isomers, does not favour fungal biodegradation. It was shown in one study that in case of mixtures of benzene-compounds, such as benzene, toluene, ethylbenzene and xylene isomers; both toluene and ethylbenzene were used by *Cladophialophora* sp., as sole carbon and energy source, while most of the xylenes were only degraded co-metabolically in presence of other alkylbenzenes (Prenafeta-Boldú et al. 2002). Benzene was also not used by this fungus as carbon source. In some cases, however, benzene was shown to be used as sole carbon source for fungal growth, such as in *Cladosporium sphaerospermim*, *Exophialalecanii-corni*, *Phanerochaete chrysosporium*, or *Paecilomyces variotii* (Qi et al. 2002; García-Peña

Table 6.3 Fungal strains degrading non-oxygenated mono-aromatic compounds

Fungal species	Substrate	Reference
Cladosporium sphaerospermum	Benzene	Qi et al. (2002)
Exophiala lecanii-corni	Benzene	Qi et al. (2002)
Phanerochaete chrysosporium	Benzene	Qi et al. (2002)
Cladosporium resinae	Ethylbenzene	Qi et al. (2002)
Cladosporium sphaerospermum	Ethylbenzene	Qi et al. (2002)
Exophiala lecanii-corni	Ethylbenzene	Qi et al. (2002)
Mucor rouxii	Ethylbenzene	Qi et al. (2002)
Cladophialophora sp.	Ethylbenzene	Prenafeta-Boldú et al. (2002)
Ophiostoma stenoceras	α-Pinene	Jin et al. (2006)
Exophiala jeanselmei	Styrene	Cox et al. (1993)
Cladosporium sphaerospermum	Styrene	Qi et al. (2002)
Exophiala lecanii-corni	Styrene	Qi et al. (2002)
Sporothrix variecibatus	Styrene	Rene et al. (2010a, b)
Cladosporium sphaerospermum	Toluene	Weber et al. (1995)
Cladosporium sphaerospermum	Toluene	Prenafeta-Boldú et al. (2001b)
Cladophialophora sp.	Toluene	Prenafeta-Boldú et al. (2001b)
Pseudeurotium zonatum	Toluene	Prenafeta-Boldú et al. (2001b)
Exophiala sp.	Toluene	Prenafeta-Boldú et al. (2001b)
Leptodontium sp.	Toluene	Prenafeta-Boldú et al. (2001b)
Exophiala oligosperma	Toluene	Estévez et al. (2005b)
Paecilomyces variotii	Toluene	Estévez et al. (2005b)
Cladophialophora sp.	*o*-Xylene	Nikolova and Nenov (2005)
Cladophialophora sp.	*m*-Xylene	Nikolova and Nenov (2005)

et al. 2008). Conversely, the presence of easily metabolized sugars, such as glucose, were shown to inhibit the biodegradation of benzene-compounds, such as styrene, by black yeasts like *Exophiala jeanselmei* (Cox et al. 1993) and white-rot fungi (Braun-Lüllemann et al. 1997).

Many new fungal strains growing on such non-oxygenated mono-aromatic compounds were isolated over the past ten years as reflected in Table 6.3. Studies with these organisms also indicated the effect of physical parameters, such as pH, temperature and the relative humidity on pollutant biodegradation. It is worth mentioning that Table 6.3 only lists results obtained from the use of pure cultures. It is important to mention that many results have been published based on bioreactors inoculated with pure fungal cultures, but operated under open conditions. This does not rule out the possibility of having other microbial strains present in the system. Table 6.3 only summarizes information on fungi or yeast-like fungi using benzene-compounds as sole carbon and energy source. Most of the fungi quoted in Table 6.3 are characterized by quite close taxonomic affinities, suggesting that there may be only a limited number of fungal strains which are able to use these organic pollutants. Other fungi degrade these

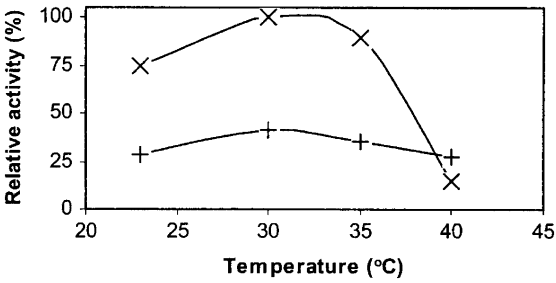

Fig. 6.1 Influence of temperature on the relative activity, in terms of toluene biodegradation rate, in *Pacilomyces variotii* (+) and *Exophiala oligosperma* (×) separately (Estévez et al. 2005b)

compounds co-metabolically, for example, *Aspergillus niger* CBS126.48 and *Cunninghamella echinulata* CBS596.68 (Prenafeta-Boldú et al. 2001b)

6.4.3 *Influence of Environmental Conditions on the Fungal Biodegradation of Non-Oxygenated Mono-Aromatic Compounds*

For most microorganisms, environmental conditions, such as pH, temperature, relative humidity, moisture content of the ecosystem, play a key role on the activity of fungal species degrading mono-aromatic compounds.

6.4.3.1 Temperature

Interestingly, all fungi isolated so far on alkylbenzenes and other related non-oxygenated benzene-compounds are mesophilic organisms. Their optimal temperature is generally around 30°C. Toluene biodegradation by *Exophiala oligosperma* and *Paecilomyces variotii* at different temperatures is reflected in Fig. 6.1 (Estévez et al. 2005b). Another study was done on toluene biodegradation to carbon dioxide by different fungi including *Cladosporium*, *Cladophialophora*, *Pseudeurotium*, *Exophiala*, and *Leptodontium* strains (Prenafeta-Boldú et al. 2001a). For a tested temperature range of 20–37°C, most of the strains exhibited their optimal activity around 30°C, but in two of the strains, namely *Pseudeurotium* and *Leptodontium*, the maximum activity for degradation was recorded at 20°C. In some of our own studies on thermophilic ecosystems, isolations done from mixed cultures, with alkylbenzenes or α-pinene, resulted in the overgrowth of bacteria on plates. However, scanty information is available on thermophilic fungi as compared to thermophilic bacteria, suggesting that fungi tolerating high temperatures are not so common (Maheshwari et al. 2000).

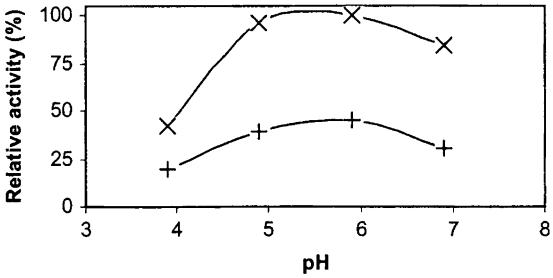

Fig. 6.2 Influence of pH on the relative activity, in terms of toluene biodegradation rate, in *Pacilomyces variotii* (+) and *Exophiala oligosperma* (×) (Estévez et al. 2005b)

6.4.3.2 pH

Compared to bacteria, fungi are generally more tolerant to low pH conditions. The optimal pH of fungi isolated so far on mono-aromatic compounds is often around 5.5–6.0, although this has only been checked for some strains. The effect of pH on toluene biodegradation by *Exophiala oligosperma* and *Paecilomyces variotii* is shown in Fig. 6.2. It appears that biodegradation was possible over all the pH range tested, between 3.9 and 6.9, with optimal conditions around pH 5.9, for cultures grown at a constant temperature of 30°C (Estévez et al. 2005b). Other researchers checked the fungal growth on plates at pH 3.5, 5.0, and 6.5, with different substrates as sole carbon source, including different mono-aromatic benzene compounds (Qi et al. 2002). Although the level of growth was estimated visually, it appeared that optimal conditions were almost in all cases close to pH 5.0, and occasionally pH 6.5, for strains of *Cladosporium*, *Exophiala lecanii-corni*, *Mucor rouxii*, and *Phanerochaete chrysosporium*. For white-rot fungi, there was not much difference between pH 5.0 and 6.5. It is worth mentioning that for white-rot fungi, biodegradation studies are in general performed near pH 4.5 considered as optimal, in the case of BTEX (i.e. benzene, toluene, ethylbenzene, and xylenes) compounds (Yadav and Reddy 1993), but also for other oxygenated mono-aromatic phenolic compounds (Kennes and Lema 1994b).

Fungi have often been inoculated in gas-phase biofilters for the treatment of air polluted with volatile benzene-related compounds (Kennes and Veiga 2004). Such packed-bed bioreactors are open systems and a mixed microbial community may often develop in the long-run. In all cases, simple microscopic observations showed that fungi remained highly dominant species. In such experiments, it was proven that in biofilters in which fungi are dominant, the system can much better withstand pH drops and acidification of the packing material than reactors in which bacteria are dominant. However, in many cases, a higher performance is still observed near neutral or in slightly acidic conditions (Estévez et al. 2005a). Occasionally, it was reported that performance did even improve at lower pH in studies performed at either pH 4.0 or at higher pH 8.0 (van Groenestijn et al. 2001). SEM pictures of some fungi growing on the surface of packing materials in gas-phase biofilters are shown in Fig. 6.3.

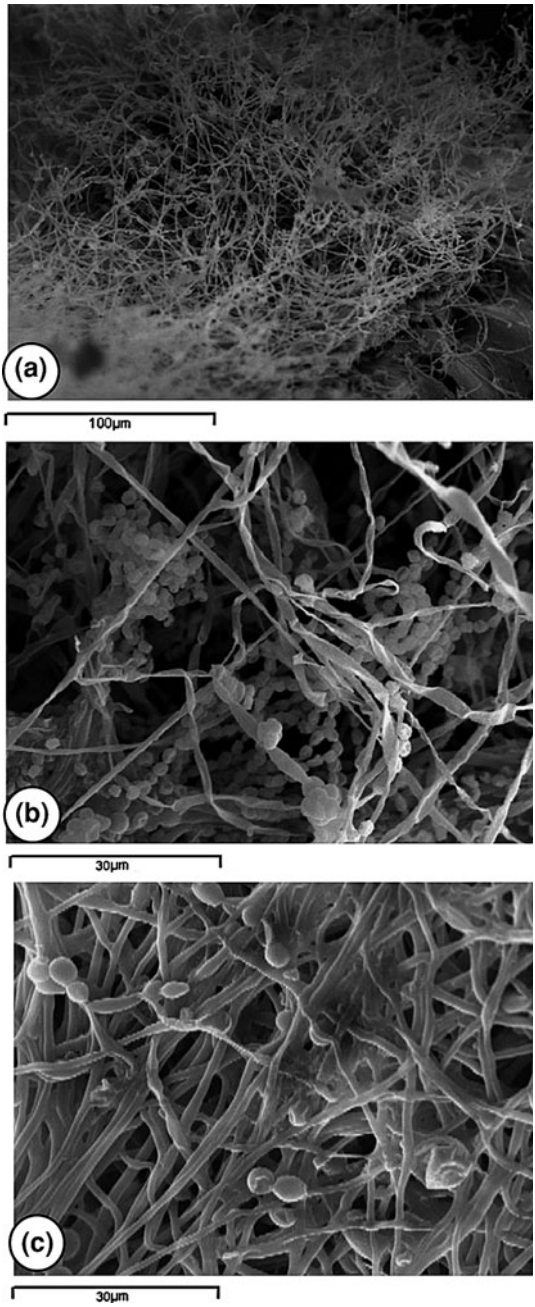

Fig. 6.3 Growth of fungal strains on packing materials, i.e. perlite, in biofilters. **a** *Ophiostoma* sp. growing on α-pinene. **b** *Paecilomyces* sp. growing on toluene. **c** *Exophiala* sp. growing on toluene

6.4.3.3 Relative Humidity and Water Content

As mentioned earlier, mono-aromatic hydrocarbons are commonly found in polluted air. Microorganisms need some moisture to be active and biodegrade pollutants. Compared to bacteria, filamentous fungi usually tolerate better somewhat dryer environments. When polluted air is fed to a packed-bed bioreactor, the air needs a minimum level of relative humidity and the packed-bed needs at least some minimum water content. Both with bacteria as well as with fungi, a relatively high moisture content of the reactor's packing material seems to be favourable in order to reach optimal removal of gas-phase pollutants. Thus, although fungi are more tolerant to dry conditions, their activity will gradually decrease, unless the air's relative humidity is at least 85% (Estévez et al. 2005a), and the packed bed's moisture content should preferably be at least 40–60% with a water activity, a_w, close to one (Cox et al. 1996).

6.5 Conclusions

The biodegradability of aliphatic and polycyclic aromatic pollutants as well as oxygenated mono-aromatic compounds by fungal strains has been studied for several decades. However, a clear evidence of the biodegradability of non-oxygenated mono-aromatic benzene-compounds and their use as sole carbon and energy source by fungi, was only reported for the first time in the 1990s. To the best of our knowledge, all strains isolated so far are mesophilic organisms. In most cases, their optimal pH and temperature are close to 6 and 30°C, respectively. Most fungi tolerate acidic environments, although they exhibit lower growth rates and biodegradation activities at low pH. Several fungal strains have been used in the treatment of air polluted with mono-aromatic benzene-compounds. In packed bed bioreactors, these fungi are less sensitive than bacteria to conditions of low moisture content, although relatively dry environments will decrease reactor performance.

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