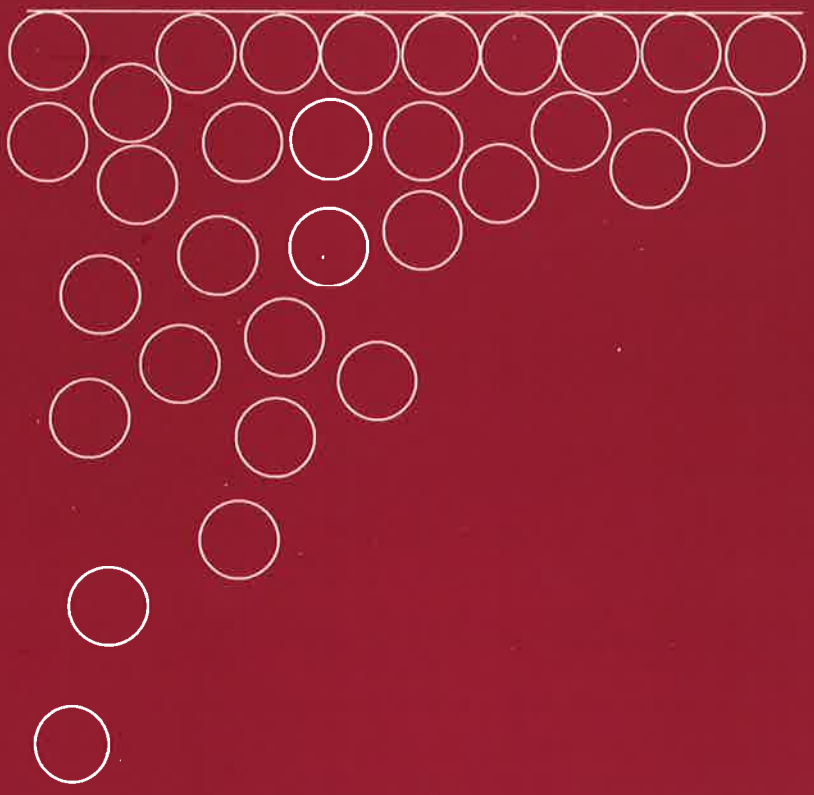
Styrene removal from waste gas by the fungus *Exophiala jeanselmei* in a biofilter



H.H.J. Cox

STYRENE REMOVAL FROM WASTE GAS BY THE FUNGUS *EXOPHIALA JEANSELMEI* IN A BIOFILTER

ISBN 90 367 0556 8

Stellingen

1 Het door Diks *et al.* (1994) waargenomen biologische evenwicht tussen een primaire en secundaire microbiële populatie in een dichloormethaan–afbrekend biotrickling filter is waarschijnlijk het gevolg van nutriënt–limitatie.

Diks, R.M.M., Ottengraf, S.P.P. en Vrijland, S. (1994). The existence of a biological equilibrium in a trickling filter for waste gas purification. Biotechnol. Bioeng. 44: 1279-1287.

2 Ten onrechte wordt vaak aangenomen dat het gebruik van actief kool als drager in biofilters leidt tot een groter massa transport en een grotere beschikbaarheid van verontreinigingen; onder steady–state omstandigheden zijn echter geen voordelen te verwachten.

Liu, P.K.T., Gregg, R.L., Sabol, H.K. en Barkley, N. (1994). Engineered biofilter for removing organic contaminants in air. J. Air Waste Manage. Assoc. 44: 299-303.
Hodge, D.S., Medina, V.F., Islander, R.L. en Devinny, J.S. (1991). Treatment of hydrocarbon fuel vapors in biofilters. Environ. Technol. 12: 655-662.

3 Zonder verificatie dient kanaalvorming in een filterbed niet als verklaring vóór onwelkome resultaten bij biofiltratie–experimenten te worden aangevoerd.

4 De door Ottengraf en Van den Oever (1983) berekende effectieve biofilmdikte in compost biofilters is waarschijnlijk een factor 20–100 te hoog door het negeren van zuurstof–limitatie.

Ottengraf, S.P.P. en Van den Oever, A.H.C. (1983). Kinetics of organic compound removal from waste gases with a biological filter. Biotechnol. Bioeng. 25: 3089-3102.
Shareefdeen, Z., Baltzis, B.C., Oh, Y.-S. en Bartha, R. (1993). Biofiltration of methanol vapor. Biotechnol. Bioeng. 41: 512-524.
Hoofdstuk 5, dit proefschrift.

5 Bij de vergelijking van de activiteit van gesuspendeerde en in een biofilm geïmmobiliseerde cellen dient ook de wateractiviteit te worden betrokken.

Mattiasson, B. and Hahn-Hägerdal, B. (1982). Microenvironmental effects on metabolic behaviour of immobilized cells. A hypothesis. Eur. J. Appl. Microbiol. Biotechnol. 16: 52-55.

6 Biofiltratie–onderzoek dient zich bij voorkeur te richten op het verbeteren van bestaande technieken.

7 Een schot op de paal of lat is geen goed schot.

8 Als 70% van de bevolking moeite heeft met de bediening van video–recorders, lijken files op de electronische snelweg in de nabije toekomst niet waarschijnlijk.

9 De term wachtgelduitkering is voor tweeërlei uitleg vatbaar.

10 Het groeien van gras wordt ten onrechte met traagheid geassocieerd.

Stellingen behorende bij het proefschrift "Styrene removal from waste gas by the fungus *Exophiala jeanselmei* in a biofilter".

Huub H.J. Cox

Delft, 8 december 1995.

RIJKSUNIVERSITEIT GRONINGEN

STYRENE REMOVAL FROM WASTE GAS BY THE FUNGUS *EXOPHIALA JEANSELMEI* IN A BIOFILTER

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus Dr F. van der Woude
in het openbaar te verdedigen op
vrijdag 8 december 1995
des namiddags te 2.45 uur precies

door

Hubertus Henricus Jacobus Cox
geboren op 29 juli 1963
te IJsselstein

Promotor : Prof. Dr. W. Harder

Copromotor : Prof. Dr. L. Dijkhuizen

Referent : Dr. H.J. Doddema

CONTENTS

CHAPTER 1

BIOLOGICAL TECHNIQUES FOR TREATMENT OF WASTE GASES CONTAINING HYDROPHOBIC AND RECALCITRANT COMPOUNDS; STYRENE AS CASE EXAMPLE

On July 2, 1994, the national newspapers in the Netherlands warned for severe smog formation. On this day, temperatures of 30°C and higher were forecasted and wind was minimal. The Dutch government expected atmospheric ozone concentrations of 270 $\mu g.m^{-3}$; people sensitive to air pollution were advised to minimize their outdoor activity during daytime. Apart from these local effects, there is increasing concern on global atmospheric changes due to air pollution. Smog and ozone formation, the greenhouse effect and the hole in the ozone layer are considered serious threats to life on earth, although there is little consensus with respect to causes and consequences.

In order to prevent air pollution, it is a global policy to reduce the emission of volatile and gaseous compounds. Industry is increasingly trying to reduce emissions by process integrated improvement. However, reliable end–of–pipe techniques will still be necessary, at least for the next two decades. Biological techniques, especially biofilters, have proven their utility in odour abatement. Recent legislation also requires reduction of industrial emissions of hydrophobic and/or recalcitrant compounds to the atmosphere. Consequently, biological research on air pollution control concentrates on the treatment of gases containing hydrophobic and/or recalcitrant compounds.

Styrene is an important volatile organic compound (VOC) emitted by styrene monomer/(co)polymer production plants and the reinforced plastics industry. Approximately 1% of the total VOCs emitted by Dutch industry is attributed to styrene with a total emission of 880 tonnes in 1990. At present, reliable biological techniques for treatment of styrene–containing waste gases are not available. It is the objective of this review to describe the state of art of biological waste gas treatment in order to evaluate the possibilities of biological removal of styrene from waste gases. Subjects covered are current biotechniques for waste gas treatment, styrene emission by

industry, Dutch legislation on industrial emissions, the microbiology of styrene degradation and new developments in waste gas treatment.

CURRENT APPLICATION OF BIOTECHNIQUES FOR WASTE GAS TREATMENT

For the purification of industrial waste gases, physical, chemical and biological techniques have been developed. Physical and chemical techniques include incineration (Seifert *et al.*, 1993), catalytic incineration (Herion and Meißner, 1993), chemical and physical absorption in liquids (Mersman and Kutzer, 1993), condensation (Herzog, 1993), chemical oxidation (Menig and Krill, 1993) and adsorption on solids like activated carbon (Krill, 1993). For general reviews on these and other physical and chemical techniques, see Heck *et al.* (1988) and Joziasse and Wiering (1992).

Over the past decades, biological purification of waste gases has become a technique with increasing popularity (Dragt, 1992). Compared to physical and chemical techniques, biotechniques offer two advantages (Ottengraf, 1986). First, biological degradation of waste gas compounds results in the formation of relatively

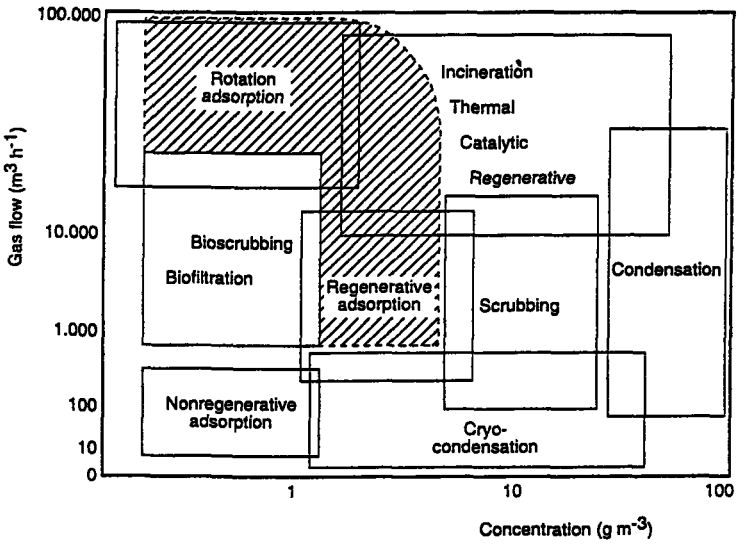


Figure 1.1. Application area of techniques for waste gas treatment (Van Groenestijn and Hesselink, 1993).

Table 1.1. Classification of biotechniques for waste gas purification (Ottengraf, 1987).

Techniques	Microorganisms	Water phase
Bioscrubber	Suspended	Mobile
Biotrickling filter	Immobilized	Mobile
Biofilter	Immobilized	Stationary

harmless end–products (i.e. water, carbon dioxide and biomass), in contrast to physical and chemical techniques, where there may only be transfer of pollutants from the gas phase to another phase. A second advantage of biological treatment of waste gases is that the costs are relatively low because of low energy consumption (Kok, 1992). In general, biological treatment is a competitive technique for waste gases with flows of 1,000–50,000 $m^3.h^{-1}$ and pollutant concentrations up to 1 $g.m^{-3}$ (Dragt, 1992; Kok, 1992). Figure 1.1 presents an indication of the application area of biotechniques in relation to other techniques.

For the biological purification of waste gases three techniques are available: bioscrubbers, biotrickling filters and biofilters. As proposed by Ottengraf (1987), these techniques may be distinguished on the basis of the behaviour of the microorganisms (freely suspended or immobilized on a support material) and the water phase which is either mobile or stationary (table 1.1). Detailed reviews on the design of bioscrubbers, biotrickling filters and biofilters as well as the operation of these installations and the factors that influence their performance are given by Ottengraf (1986), Diks and Ottengraf (1991), Leson and Winer (1991) and Diks (1992). Here, a general outline of the features of these biotechniques will be presented.

The bioscrubber (figure 1.2A) contains two compartments. In the scrub compartment the waste gas is brought in contact with a mobile water phase, resulting in the transfer of pollutants from the gas phase to the water phase. Subsequently, the water phase is transported to a reactor with suspended microorganisms for the degradation of the pollutants. The bioreactor usually contains activated sludge. The addition of nutrients to the bioreactor, aeration and the control of the pH ensures optimal conditions for biodegradation. The biotrickling filter (figure 1.2B) consists of only one compartment in which mass transfer and biodegradation take place simultaneously. The reactor is packed with an inert support material on which a

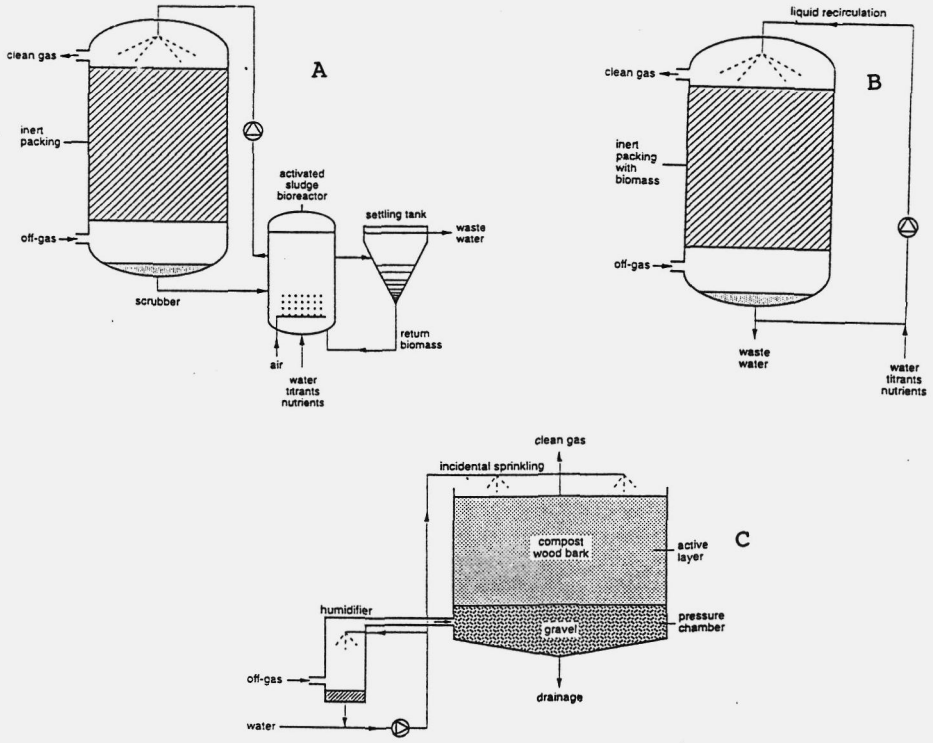


Figure 1.2. Biotechniques for waste gas treatment. A, bioscrubber; B, biotrickling filter; C, biofilter (Van Groenestijn and Hesselink, 1993).

biofilm is present. The waste gas is forced through the packed bed in co– or countercurrent with a mobile water phase. The aqueous phase is continuously recirculated over the packed bed which, as for bioscrubbers, allows for easy control of pH and the level of nutrients. In traditional biofilters (figure 1.2C) the waste gas is forced through a filter bed consisting of natural materials such as compost or peat. The microorganisms, usually originating from the natural flora on the support material, are present in a biofilm which surrounds the particles. Waste gas pollutants diffuse from the gas phase into the biofilm, where they are degraded. In contrast to bioscrubbers and biotrickling filters, there is no mobile aqueous phase in biofilters; this makes control of parameters which influence microbial activity more difficult. Soil biofilters are related to compost/peat biofilters with respect to the mode of operation and the absence of a mobile water phase. These biofilters have been developed by research groups especially in the U.S. (Pomeroy, 1957; Duncan *et al.*, 1982; Prokop

and Bohn, 1985; Bohn and Bohn, 1986). Since soil biofilters show low biodegradation capacities, their use is not widespread (Leson and Winer, 1991).

The application area of bioscrubbers, biotrickling filters and biofilters for waste gas treatment depends on the Henry coefficient (or water solubility) of the pollutants and the concentration in the waste gas (Kok, 1992; Van Groenestijn and Hesselink, 1993). Because of the high specific surface area of compost, biofilters can be used for the removal of pollutants like styrene that are relatively water insoluble (Henry coefficient up to 10). As a rule of thumb, waste gas treatment with bioscrubbers and biotrickling filters is restricted to pollutants with Henry coefficients lower than 0.01 and 1, respectively. Generally, the maximal concentration of pollutants in the waste gas is 0.5 $g.m^{-3}$ for biotrickling filters, 1 $g.m^{-3}$ for biofilters and 5 $g.m^{-3}$ for bioscrubbers.

ENVIRONMENTAL ASPECTS OF STYRENE

Physical and chemical properties

Styrene (CAS 100–42–5; ethenylbenzene) is a colourless viscous liquid to which usually 0.001% tertiary butylcatechol is added to prevent polymerization and oxidation. General properties of styrene are summarized in table 1.2.

The odour threshold of styrene in air is 0.05 (Hellman and Small, 1974) to 0.32 ppm (Amoore and Hautala, 1983). The estimate of the styrene odour recognition threshold, i.e. the minimal concentration required for recognition of the compound by man, is 0.05 (Smith and Hochstettler, 1969) to 0.15 ppm (Hellman and Small, 1974). The odour of styrene has been conflictingly described as unpleasantly sharp/sweet (Hellmann and Small, 1974), pleasantly sweet (RIVM, 1986) and pungent (WHO, 1983).

Production, uses, emission and exposure levels

Styrene is an important product in the chemical industry; the worldwide production in 1977 and 1981 was estimated at 7,000,000 and 8,100,000 tonnes, respectively (Tossavainen, 1978; RIVM, 1986). In the Netherlands, the production of styrene was 810,000 tonnes in 1981 (RIVM, 1986) and 1,250,000 tonnes in 1991 (RIVM, 1993). Part of styrene is exported. Styrene is mainly used for the production

Table 1.2. Physical and chemical properties of styrene (WHO, 1983).

Molecular formula	$C_6H_5CH=CH_2$
Relative molecular mass	104.14
Density (20°C)	0.906
Freezing point	–30.6°C
Boiling point (1 atm)	145.2°C
Vapour pressure (25°C)	0.866 kPa
Water solubility (25°C)	0.32 $g.l^{-1}$
	0.16 $g.l^{-1}$ [a]
	0.28 $g.l^{-1}$ [b]
Octanol/water partition coefficient	$\log K_{ow} = 3.16$ [a]
	$\log K_{ow} = 2.41$ [b]
Conversion factors (25°C, 1 atm)	1 ppm in air = 4.2 $mg.m^{-3}$
	1 $mg.m^{-3}$ in air = 0.24 ppm

a Banerjee *et al.* (1980)
b Yalkowsky *et al.* (1983)

of polymers (table 1.3). In 1990, the industrial emission of styrene accounted for 51% of the total styrene emission in the Netherlands (VROM, 1993[a]). Non–industrial sources mainly concern exhaust gases from traffic vehicles (RIVM, 1993). Over 1981–1990 the industrial styrene emission to air remained constant in volume (table 1.4). In general, styrene is emitted by monomer/(co)polymer production plants and plants involved in the processing of (co)polymers to end–products. Major emission sources are related to the production of styrene polymers, the processing of polystyrene and the use of polyester resins (RIVM, 1993). Styrene–emitting industries can be found in many industrial sectors as shown in table 1.5.

Because of the toxicity of styrene and the large numbers of workers occupationally exposed to styrene, much research has been done on occupational exposure levels (see WHO (1983) and RIVM (1986) for reviews). The styrene

Table 1.3. Use pattern of styrene in the Netherlands in 1981 (RIVM, 1986) and 1991 (RIVM, 1993).

Product	Styrene used (ktonnes/annum)	
	1981	1991[a]
Polystyrene	195	400
Styrene–butadiene–rubber + latex	38	15
Acrylonitrile–butadiene–styrene + styrene–acrylonitrile	45	90
Polyester resins	10	[b]

a Data calculated from the total production of the styrene (co)–polymers in 1991 (RIVM, 1993) and the average styrene content of the styrene (co)–polymers as used by RIVM (1986).

b Data not available.

Table 1.4. Total industrial styrene emission in the Netherlands in 1981-1990 (VROM, 1993[b]).

Year	Styrene emission (tonnes/annum) to	
	water	air
1981–1984	36	790
1985–1987	19	976
1988	16	855
1990	7	880

Table 1.5. Styrene emission to air in 1990 in the Netherlands, classified by type of industry (VROM, 1993[a] and 1993[b]).

Industrial sector	Emission (%)
Chemical feed stock industry	51.2
Polyester resin industry	6.9
Polyester resin processing industry	14.7
Metal products industry	0.3
Automobile industry	0.1
Wholesale trade	25.7
Storage industry	0.7
Rest	0.3

exposure level (8–hour time weight average: 8–h TWA) in styrene production and polymerization plants is relatively low: average 5 ppm but sometimes up to 50 ppm (WHO, 1983). High exposure levels are found in the reinforced plastics industry (Tossavainen, 1978; WHO, 1983; RIVM, 1986). The reported 8–h TWA levels vary between 10 and 300 ppm depending on the type of industry. Especially ship production plants using fibreglass reinforced plastics are notorious (Brooks *et al.*, 1980; Schumacher *et al.*, 1981; Ikeda *et al.*, 1982): in these industrial sectors the styrene exposure level regularly exceeds established limit concentrations (Tomberg, 1992).

Legislation

Removal of styrene from working areas and industrial waste gases is necessary to abate odour nuisance, to warrant safe working conditions and to minimize environmental pollution. As classified by the Dutch government, styrene is a 'priority' compound because of the low odour threshold and the toxicity (VROM, 1994). Priority compounds are those compounds that are considered to be especially harmful to the environment. Styrene back–ground levels are generally well below the target concentration of 8 $\mu g.m^{-3}$ (VROM, 1994). However, odour nuisance may occasionally

occur when styrene–emitting industries are in the vicinity of urban areas and this may force industry to reduce the emission of styrene.

Extensive literature surveys on the toxicity of styrene have been published by WHO (1983), RIVM (1986) and DGA (1989). Generally, it is concluded that 8–h TWA exposure to 20–50 ppm styrene does not cause adverse health effects. The toxicity of styrene is indicated by the maximum allowable concentration (MAC) in working areas as established by Government agencies (table 1.6). In the Netherlands, a MAC–value of 50 ppm has been established in 1994 but a further decrease to 25 ppm is expected in 1996.

Recently, the Dutch government (Anonymous, 1992) published the 'Nederlandse Emissie Richtlijnen' (NER) (Dutch emission guidelines), which are largely based on the 'Technische Anleitung–Luft' (TA–Luft) published in Germany (Anonymous, 1986). According to NER, volatile organic compounds are classified in three categories. Styrene is included in category O–2 containing moderately toxic compounds with MAC–values between 25 and 500 $mg.m^{-3}$. For category O–2 compounds with a total emission of more than 2 $kg.h^{-1}$, the maximal allowable concentration in the off gas is 100 $mg.m^{-3}$.

Apart from the NER, industry is also confronted with the Dutch 'Koolwaterstoffen 2000' Programma (KWS–2000: Hydrocarbon 2000 Programma) (VROM, 1989). Because volatile VOCs contribute to ozone formation (Bos *et al.*, 1977; Derwent and Jenkin, 1991; Japar *et al.*, 1991; Finlayson–Pitts and Pitts, 1993)

Table 1.6. Maximum allowable concentration (MAC, 8-h TWA) of styrene.

Country	Year	MAC	
		ppm	$mg.m^{-3}$
the Netherlands[a]	1994	50	210
	(1996)	25	105
Germany[b]	1992	20	84
USA[c]	1992–1993	50	210

a DGA (1992)
b DFG (1992)
c ACGIH (1992)

in the lower layers of the atmosphere, it is the policy of the Dutch government to reduce the emission of VOCs. The objective is a 50% reduction of the emission in the year 2000 compared to the emission in 1981. Styrene in this respect is an important VOC since approximately 1% of the total VOC–emission (excluding methane according to the VOC definition in KWS–2000) may be attributed to styrene (VROM, 1993[a]). KWS–2000 describes various guidelines and strategies to reduce the emission of VOCs, aiming both at specific VOCs and specific industrial sectors. Biofiltration is considered a possible solution for reducing styrene emissions in the reinforced plastics industry, but the applicability of biofiltration has first to be proven in a demonstration project as recommended by the KWS–2000 group (VROM, 1989).

In the foregoing the necessity for reduction of industrial styrene emission has been illustrated using the situation in the Netherlands and Germany as an example. Existing and future legislation requires action from industry to reduce the total emission of styrene as well as the styrene concentration in waste gases. However, from an inventory of industrial sources and emissions of styrene it is concluded that future styrene emissions are not likely to decrease significantly, partly because of the expected expansion of the styrene industry (RIVM, 1993). In this respect, new techniques for biological removal of styrene from industrial waste gases may contribute to achieve the objectives.

STYRENE BIODEGRADATION

A prerequisite for biological purification of industrial waste gases is biodegradability of the waste gas compounds. Ideally, the waste gas compounds are completely mineralized to carbon dioxide and water without accumulation of metabolic intermediates. Accumulation of intermediates may result in inhibition of microbial activity, and thus in reduction of the biofilter capacity. In this respect, knowledge of styrene metabolism is necessary, but as stated by Smith in 1990, reports on the biodegradation of alkenylbenzenes like styrene are scarce. Because of the toxicity of styrene and the large number of people exposed to styrene, more research has been done on the metabolic fate of styrene in mammals. In the following sections styrene metabolism in microorganisms and mammals as well as the isolation of styrene–degrading microorganisms will be discussed.

Styrene metabolism in mammals

Microorganisms may use xenobiotic compounds to support growth and/or for energy. In case of hydrophobic xenobiotics like styrene, mammalian metabolism generally aims at the conversion of the compounds to more water–soluble metabolites in order to facilitate excretion with urine. Styrene metabolism in mammals has been reviewed by WHO (1983) and RIVM (1986).

The main route in mammalian styrene metabolism (figure 1.3) is thought to proceed via the conversion of styrene to styrene oxide (Ohtsuji and Ikeda, 1971;

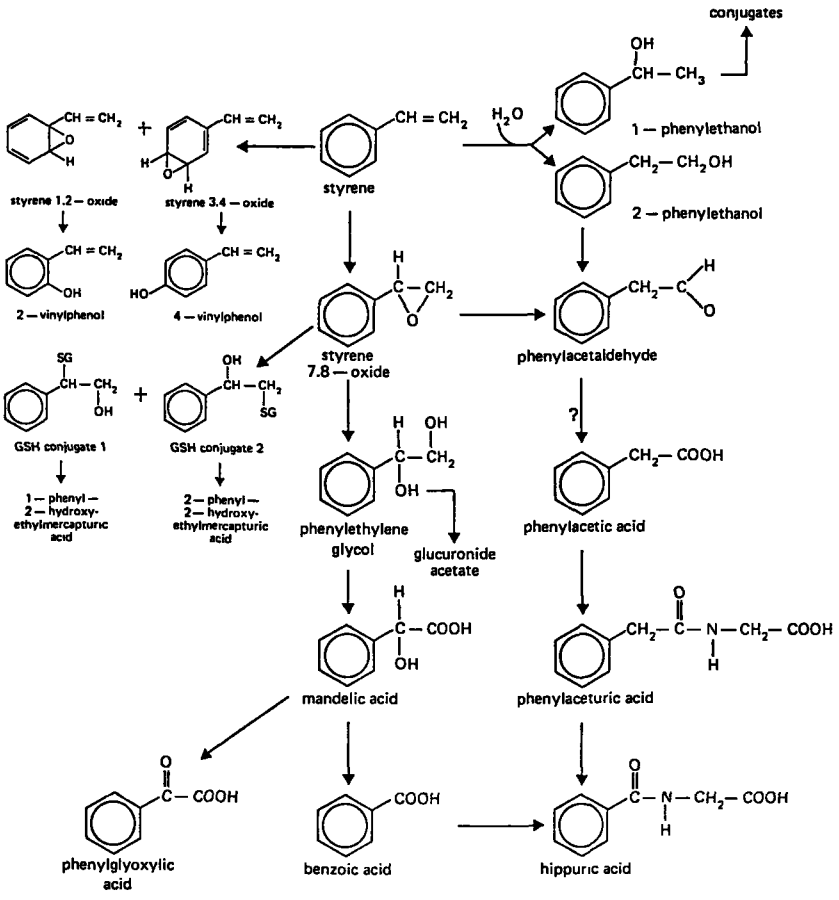


Figure 1.3. Styrene metabolism in mammals (WHO, 1983).

Leibman, 1975; Watabe *et al.*, 1978; Vainio *et al.*, 1982). This oxidation is catalyzed by a cytochrome P–450–dependent monooxygenase as shown by *in vitro* experiments using microsomal preparations of liver homogenates of various mammals (Leibman and Ortiz, 1970; Belvedere *et al.*, 1976; Parkki *et al.*, 1976: Cantoni *et al.*, 1978; Watabe *et al.*, 1981). Subsequently, styrene oxide may be conjugated with gluthatione to form mercapturic acids (Boyland and Williams, 1965; James and White, 1967; Fjellstedt *et al.*, 1973; James *et al.*, 1976; Seutter–Berlage *et al.*, 1978; Delbressine *et al.*, 1981). Approximately, 5 to 10% of styrene administered to test animals was recovered as mercapturic acids in the urine (James and White, 1967; Seutter–Berlage *et al.*, 1978). The conjugation of styrene oxide and glutathione is catalyzed by gluthatione–S–epoxide transferase, present in supernatant preparations of liver (Boyland and Williams, 1965; Fjellstedt *et al.*, 1973; James *et al.*, 1976) and other organs (James *et al.*, 1976).

A divergent route is the addition of water to styrene oxide to form phenylethanediol, catalyzed by epoxide hydratase in microsomal preparations of the liver (Leibman and Ortiz, 1969; Jerina *et al.*, 1970; Oesch and Daly, 1971; Oesch *et al.*, 1971; James *et al.*, 1976; Parkki *et al.*, 1976; Belvedere *et al.*, 1976; Cantoni *et al.*, 1978; Walker *et al.*, 1978; Watabe *et al.*, 1978; Watabe *et al.*, 1981). Phenylethanediol may be conjugated to styrene glycol monoglucuronide. This compound has been found in the urine of styrene–treated rabbits (El Masri *et al.*, 1958) and rats (Ohtsuji and Ikeda, 1971), but the major route proceeds via oxidation to mandelic and phenylglyoxylic acid (El Masri *et al.*, 1958; James and White, 1967; Ohtsuji and Ikeda, 1971; Pantarotto *et al.*, 1978). Mandelic and phenylglyoxylic acid are major metabolites in human styrene metabolism and the concentration of these compounds in urine may be used as biological indicators of occupational exposure to styrene (Härkönen *et al.*, 1974; Engström *et al.*, 1976; Ramsey and Young, 1978; Wolff *et al.*, 1978; Fields and Horstman, 1979; Brooks *et al.*, 1980; Ikeda *et al.*, 1982). Further metabolism may occur to hippuric acid, which was found in urine of styrene–treated rats and rabbits (El Masri *et al.*, 1958; James and White, 1967; Ohtsuji and Ikeda, 1971) and which is formed by conversion of mandelic acid to benzoic acid and subsequent conjugation with glycine (Ohtsuji and Ikeda, 1971).

Minor metabolites found in urine of styrene–treated mammals are 4–vinylphenol (Bakke and Scheline, 1970; Pantarotto *et al.*, 1978), 1– and 2–phenylethanol (Bakke and Scheline, 1970) and phenaceturic acid (Delbressine *et al.*, 1980).

Isolation of styrene-degrading microorganisms

Styrene is emitted to the environment in large quantities, but its presence in nature may also arise from natural processes. Microbial styrene formation from cinnamic acid or cinnamaldehyde has been reported for a *Saccharomyces cerevisiae* mutant strain (Chen and Peppler, 1956), *Aspergillus niger* (Clifford *et al.*, 1969), *Torulopsis candida* (Sato *et al.*, 1988) and *Pichia carsonii* (Shimada *et al.*, 1992). On the assumption that all natural or biogenic compounds are biodegradable, the existence of styrene–degrading microorganisms is to be expected. In addition, the presence of styrene–degrading microorganisms in nature is also illustrated by ^{14}C–styrene decomposition in natural water and soil samples from various origins (Sielicki *et al.*, 1978; Fu and Alexander, 1992).

A few attempts to isolate styrene–degrading microorganisms have been made. Omori *et al.* (1975) used conventional enrichment techniques with styrene as the sole source of carbon and energy at a concentration of 2%. Although they screened 101 soil samples, styrene–degrading microorganisms were not found. Initial attempts of Shirai and Hisatsuka (1979[b]) with 200 soil samples were also unsuccessful, but 31 styrene–degrading bacteria were obtained by using very low (not quantified) styrene concentrations in a soil percolation column. Hartmans *et al.* (1990) isolated 14 styrene–degrading bacteria as well as two fungi from water and soil samples by using styrene as the sole source of carbon and energy in concentrations of 10 to 500 μM. Although tolerance to high styrene concentrations has been reported for two *Pseudomonas putida* strains (Inoue and Horikoshi, 1989; Weber *et al.*, 1993), low styrene concentrations should be used for the isolation of microorganisms from soil and other sources (Hartmans *et al.*, 1990).

Styrene metabolism in bacteria

In general, initial oxidation of the side chain of substituted benzenes is more likely with increasing length of the side chain (Smith, 1990). For styrene, both initial oxidation of the aromatic nucleus (figure 1.4) and initial oxidation of the ethylene side chain (figure 1.5) have been reported.

Oxidation of the aromatic nucleus of styrene was first shown by Bestetti *et al.* (1989), who detected the formation of 1,2–dihydroxy–3–ethenyl–3–cyclohexene in cultures of *Pseudomonas putida* strain MST growing on styrene. Unfortunately, it was not investigated whether this compound was in fact an intermediate in styrene catabolism. Initial oxidation of the aromatic nucleus was also assumed to be present

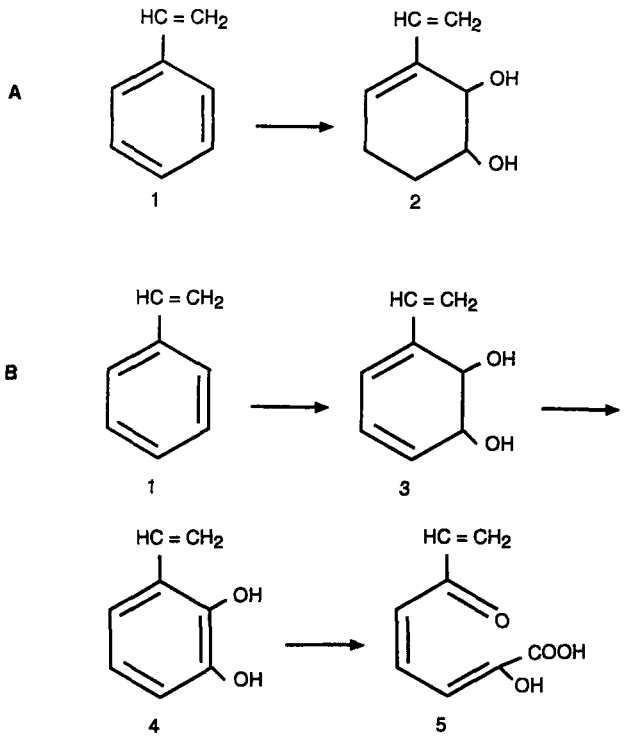


Figure 1.4. Bacterial styrene metabolism involving initial oxidation of the aromatic nucleus. A, Bestetti et al. (1989); B, Warhurst et al. (1994); 1, styrene; 2, 1,2-dihydroxy-3-ethenyl-3-cyclohexene; 3, styrene 'cis'-glycol; 4, 3-vinylcatechol; 5, 2-hydroxy-6-oxoocta-2,4,7-trienoic acid.

in *Xanthobacter* strain 124X (Hartmans *et al.*, 1989), mainly because of lack of evidence for initial oxidation of ethylene side chain. More detailed experiments were done by Warhurst *et al.* (1994). This group showed by product identification and assays of various enzyme activities in extracts of styrene–grown *Rhodococcus rhodochrous* NCIMB 13259 the NADH–dependent dioxygenation of styrene to styrene *cis*–glycol. The latter compound was oxidized to 3–vinylcatechol, followed by *meta*–cleavage by a catechol 2,3–dioxygenase and subsequent conversion of cleavage products to metabolites of the Krebs–cycle.

Initial attack on the ethylene side chain of styrene is more frequently found. A mixed population of bacteria, obtained from landfill soil, formed 2–phenylethanol and

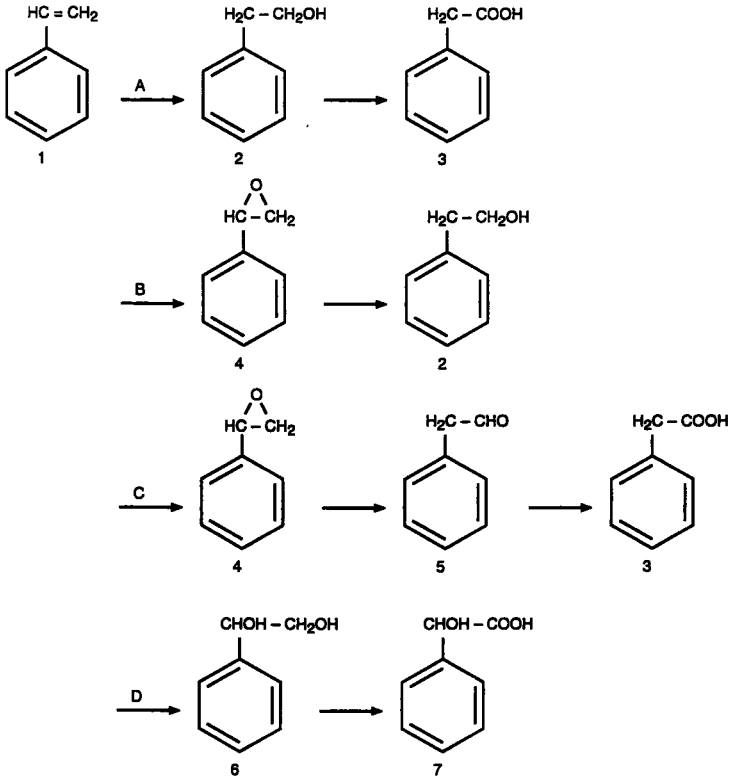


Figure 1.5. Bacterial styrene metabolism involving initial oxidation of the ethylene side chain. A, Sielicki et al. (1978); B, Shirai and Hisatsuka (1979[a]); C, Hartmans et al. (1990); D, Rustemov et al. (1992); 1, styrene; 2, 2-phenylethanol; 3, phenylacetic acid; 4, styrene oxide; 5, phenylacetaldehyde; 6, phenylethanediol; 7, mandelic acid.

phenylacetic acid when 1% styrene was added (Sielicki *et al.*, 1978). Shirai and Hisatsuka (1979[b]) found 2–phenylethanol production by two styrene–assimilating isolates; strain 305–STR–1–4 also produced 2–phenylethanol from styrene oxide, which suggested that styrene oxide is an intermediate in the formation of 2–phenylethanol from styrene (Shirai and Hisatsuka, 1979[a]). Formation of styrene oxide from styrene has been observed in whole cell suspensions of methane–grown *Methylosinus trichosporium* OB 3b (Higgins *et al.*, 1979), glucose–grown *Nocardia corallina* B–276 (Furuhashi *et al.*, 1986) and glucose/toluene–grown *Pseudomonas* strain T–12 (Johnston and Renganathan, 1987), and also in cell–free extracts of

methane–grown *Methylococcus capsulatus* (Colby *et al.*, 1977) and propane–grown *Brevibacterium* strain CRL56 (Hou *et al.*, 1983). However, it is not known whether these styrene oxide–producing bacteria can grow on styrene as the sole source of carbon and energy; the oxygenases involved show a very broad substrate specificity. Baggi *et al.* (1983) propose a pathway of styrene degradation in *Pseudomonas fluorescens* strain ST via phenylacetic acid and homogentisic acid. This pathway appears to be plasmid encoded in this species (Bestetti *et al.*, 1981; Bestetti *et al.*, 1984; Ruzzi and Zennaro, 1989). Utkin *et al.* (1991) report a *Pseudomonas* species converting styrene into 1–phenylethanol, 2–phenylethanol, phenylacetic acid and salicylic acid. From these products, only 2–phenylethanol and phenylacetic acid are considered as intermediates in styrene catabolism (Utkin *et al.*, 1991). From results of the determination of enzyme activities in cell–free extracts and product identification, Hartmans *et al.* (1990) propose a pathway of styrene degradation via styrene oxide and phenylacetaldehyde to phenylacetic acid. In eleven bacterial isolates, a novel FAD–requiring styrene monooxygenase was detected. This enzyme catalyzes the oxidation of styrene to styrene oxide, however, the product could not be detected in cell–free extracts. Conversion of styrene oxide to phenylacetaldehyde and subsequently phenylacetic acid was catalyzed by styrene oxide isomerase and phenylacetaldehyde dehydrogenase, respectively (Hartmans *et al.*, 1990). Styrene oxide isomerase was also present in *Xanthobacter* strain 124X (Hartmans *et al.*, 1989), but styrene monooxygenase activity could not be detected (Hartmans *et al.*, 1990). Therefore, *Xanthobacter* strain 124X probably degrades styrene by initial oxidation of the aromatic nucleus (Hartmans *et al.*, 1990).

Rustemov *et al.* (1992) detected phenylethanediol and mandelic acid in cultures of *Pseudomonas putida* R1 growing on styrene. They propose a metabolic pathway in which styrene is oxidized to mandelic acid via the intermediate phenylethanediol, which is the major route of styrene metabolism in mammals.

Phenylacetic acid appears to be the central metabolite in bacteria degrading styrene initially at the side chain. However, the conversion of phenylacetic acid to central metabolites by styrene–assimilating bacteria has scarcely been investigated. Only Baggi *et al.* (1983) report the conversion of phenylacetic acid to 2–hydroxyphenylacetic acid by a styrene–grown *Pseudomonas fluorescens* strain. Further degradation may proceed via homogentisic acid, maleylacetoacetic acid, acetoacetic acid and fumaric acid (Baggi *et al.*, 1983). Additional literature data are available on the degradation of phenylacetic acid and hydroxylated phenylacetic acids by bacteria grown on these compounds (figure 1.6). Degradation of phenylacetic acid to maleylacetoacetate (ring cleavage product) via homogentisic acid was demonstrated for

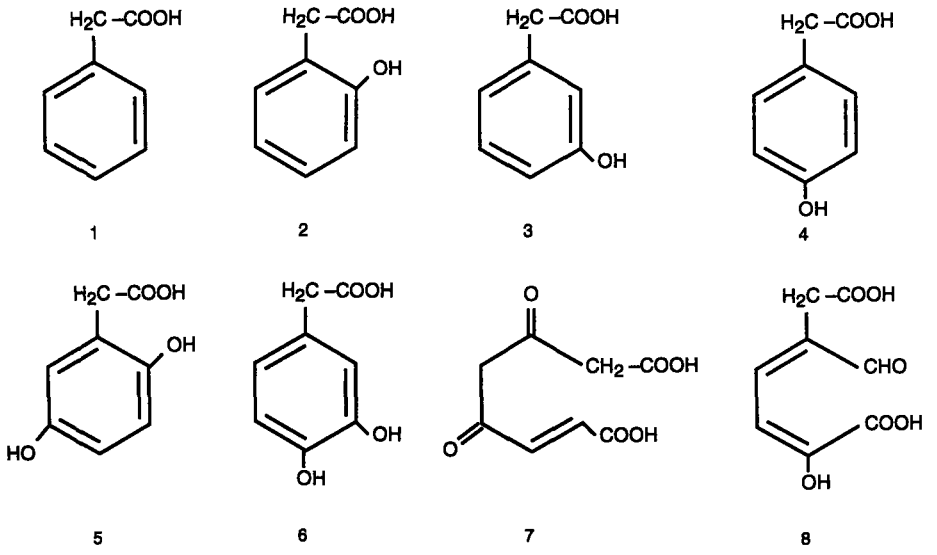


Figure 1.6. Survey of intermediates implicated in bacterial phenylacetic acid metabolism. 1, phenylacetic acid; 2, 2-hydroxyphenylacetic acid; 3, 3-hydroxyphenylacetic acid; 4, 4-hydroxyphenylacetic acid; 5, homogentisic acid; 6, homoprotocatechuic acid; 7, maleylacetoacetic acid; 8, δ-carboxymethyl-α-hydroxymuconic semialdehyde.

a *Vibrio* strain (Chapman and Dagley, 1962). Similarly, the styrene–degrading *Xanthobacter* strain 124X (Hartmans *et al.*, 1989 and 1990) degrades 4–hydroxyphenylacetic acid via homogentisic acid to maleylacetoacetic acid when grown on 4–hydroxyphenylacetic acid (Van den Tweel *et al.*, 1986). The same pathway was shown in 4–hydroxyphenylacetic acid–grown *Pseudomonas acidivorans* (Hareland *et al.*, 1975). Degradation of phenylacetic acid via homogentisic acid and maleylacetoacetic acid was also found in a *Flavobacterium* species (Van den Tweel *et al.*, 1988) and *Nocardia salmonicolor* (Sariaslani *et al.*, 1974), but in these cases 3–hydroxyphenylacetic acid and 2–hydroxyphenylacetic acid were the first phenylacetic acid oxidation products, respectively. On the other hand, Blakley *et al.* (1967) reported the degradation of phenylacetic acid via 4–hydroxyphenylacetic acid, homoprotocatechuic acid and δ–carboxymethyl–α–hydroxymuconic semialdehyde (ring cleavage product). Clearly, the bacterial degradation of phenylacetic acid resulting in ring cleavage may proceed via various pathways. However, it must be emphasized that

these pathways have not been fully investigated in styrene–degrading bacteria.

Styrene metabolism in fungi

Data on styrene metabolism in fungi are limited. Hartmans *et al.* (1990) isolated 2 fungal strains by using styrene as the sole source of carbon and energy in concentrations lower than 500 μM. One of these strains, the yeast *Exophiala jeanselmei*, was studied in our laboratory in more detail (Cox *et al.*, 1993). From substrate specificity and growth experiments it was tentatively concluded that styrene metabolism in *E. jeanselmei* proceeds via the initial oxidation of the ethylene side chain as found in most bacteria. In a review on catabolism of benzene compounds by yeasts and yeast–like fungi, Middelhoven (1993) presents the results of the screening of 28 ascomycetes and basidiomycetes for growth on 84 monoaromatic compounds. It was found that the investigated yeasts could assimilate many monoaromatic compounds; *E. jeanselmei* could assimilate 55 of the 84 compounds tested, among which 2–phenylethanol, phenylacetic acid and hydroxylated phenylacetic acids (Middelhoven, 1993). De Jong *et al.* (1990) report the production of carbon dioxide by *Penicillium simplicissimum* CBS 170.90 when incubated for one month in mineral medium with 9.6 mM styrene, a concentration well above the maximal solubility of styrene in water. However, growth on styrene by this fungus could not be confirmed in our experiments although a similar mineral medium was used (unpublished results).

STYRENE DEGRADATION IN BIOFILTERS

On the basis of the previous section, purification of styrene–containing waste gases using biological techniques must be considered a realistic option. Because of the relatively high Henry coefficient, biological removal of styrene from waste gases has only been studied in biofilters. Van Groenestijn and Hesselink (1993) summarize operating conditions and results obtained with full scale biofilters for the removal of odour and volatile organic compounds at various industrial plants. Flow rates of the waste gases treated varied between 300 and 214,000 $m^3.h^{-1}$. The volumetric load of the biofilters was between 7 and 426 m^3 gas per m^3 biofilter per hour and elimination efficiencies of 49–100% were obtained. A more characteristic parameter describing biofilter performance is the specific elimination capacity, expressed as gram pollutant degraded per m^3 filter per hour. Depending on the biodegradability of the waste gas

compounds, specific elimination capacities may vary between 8 to 200 $g.m^{-3}.h^{-1}$ (Van Groenestijn and Hesselink, 1993).

A few reports on styrene degradation in biofilters have been published, but the provided experimental data are generally insufficient for complete insight into the performance of such filters. Demiriz (1992) reports 65% elimination of styrene from industrial waste gas containing 0.14 $g.m^{-3}$ styrene using a biofilter at a volumetric load of 100 $m^3.m^{-3}.h^{-1}$. From these figures a styrene elimination capacity of 9.1 $g.m^{-3}.h^{-1}$ can be calculated but data on the stability of the biofilter were not provided. Degradation started after an adaptation period of approximately four weeks (Demiriz, 1992). Windsperger *et al.* (1990) report elimination capacities of up to 150 $g.m^{-3}.h^{-1}$ with a laboratory–scale biofilter containing a compost/bark mixture inoculated with adapted bacteria. However, it is not known whether this high elimination capacity could be maintained for longer periods. Pilot plant biofilters at a latex production plant showed 100% degradation at styrene loads of up to 50 $g.m^{-3}.h^{-1}$, but results were unpredictable at higher loads (Windsperger *et al.*, 1990). Sabo *et al.* (1993) investigated various support materials in biofilters for removal of styrene. A mixture of bark/felspar proved to be optimal with an elimination capacity of 12–15 $g.m^{-3}.h^{-1}$ depending on the volumetric load. Majcherczyk *et al.* (1990) used white–rot fungi growing on straw for the removal of styrene, pinene, ammonia, chlorinated phenols and dihydrogen sulphide from gas. Styrene was completely degraded at a concentration of 1.2 $g.m^{-3}$ and a gas flow rate of 0.016 $m.s^{-1}$. This would involve a styrene elimination capacity of 150 $g.m^{-3}.h^{-1}$, but experiments lasted for only 8 days. Braun–Lüllemann *et al.* (1992) report 99% degradation of 0.84–1.68 $g.m^{-3}$ styrene in air by the white–rot fungus *Pleurotus ostreatus* immobilized on straw, but volumetric loads or residence times were not reported. Therefore, the elimination capacity of this biofilter can not be calculated.

Although biological treatment of styrene–containing gas seems to be possible in laboratory experiments or at pilot–plant scale for a short period of time (weeks), the experience with industrial treatment of styrene–containing waste gases in biofilters is less conclusive. Apart from low elimination capacities, complete loss of activity within a few months has regularly been observed (Van Groenestijn *et al.*, 1994). One can only speculate about the causes:

- Styrene may be toxic to the microorganisms present in the biofilters. A TNO investigation on the purification of a waste gas of a styrene polymer–producing plant indicated that the styrene load during the adaptation period should be low to allow the development of styrene degradation activity (Steunenberg, 1987). In this study it was assumed that high styrene loads during the adaptation period

will result in saturation of the biofilter with toxic concentrations of styrene. The saturation concentration of styrene in the water phase of the biofilter – assuming no degradation and equilibrium between the gas and water phase – depends on the concentration in the gas phase and the Henry coefficient. From the data provided by Steunenberg (1987) maximal styrene concentrations in the water phase of 13 $mg.l^{-1}$ or 0.13 mM were calculated. Whether this concentration is toxic is questionable. Studies on styrene degradation by bacteria (Hartmans *et al.*, 1989 and 1990; Warhurst *et al.*, 1994) indicate that styrene in concentrations up to 1 mM may be used as carbon source for growth. On the other hand, the maximal styrene concentration for growth of the yeast *E. jeanselmei* is approximately 0.4 mM (Cox *et al.*, 1993).

- Biological activity in styrene–degrading biofilters may be limited by the availability of nutrients. The addition of nutrients (N or P) to biofilters resulted in higher styrene elimination capacities (Steunenberg, 1987). Since the nutrient availability in compost biofilters is in many applications sufficient without further addition, this would imply a special nutrient requirement of styrene–degrading microorganisms.
- Loss of activity may be caused by acidification of the filter bed, which may result from the transient accumulation of acid intermediates or the accumulation of acid end products. Acidification of the filter bed is a general and major disadvantage encountered with compost biofilters (Ottengraf, 1986), but not specially related to styrene degradation. Furthermore, in styrene–degrading compost biofilters no decline of pH has been observed during several months of operation (Sabo, personal communication).
- Loss of activity may be due to drying out of the filter bed. Severe loss of styrene degradation activity of compost filters by low water contents has been observed (Sabo *et al.*, 1993). In general, the water content of biofilters should be maintained between 40 and 60 weight % (Ottengraf, 1986).
- Styrene degradation in biofilters may be inhibited by other pollutants present in the waste gas. Depending on the source, industrial styrene–containing gases may contain compounds like butadiene, acrylonitrile and α–methylstyrene (Steunenberg, 1987; Van Groenestijn, personal communication). These compounds may be toxic for styrene–degrading microorganisms, thus decreasing the styrene elimination capacity by their presence. As an example, inhibition of hexanal–degrading biofilters by the presence of SO_2 in the waste gas has been shown (Van Langenhove *et al.*, 1989). SO_2 concentrations less than 10 ppm had no effect on the biofilter performance, but 40 ppm SO_2 caused

60% inhibition of hexanal degradation. A concentration of 100 ppm SO_2 resulted in complete and irreversible inhibition of hexanal degradation.

From the above it is clear that many factors may influence styrene degradation in biofilters. Unfortunately, no information is available on the type of microorganisms catalyzing the degradation of styrene in these biofilters and the influence of environmental parameters on the styrene degradation activity. The elimination capacity of styrene–degrading biofilters is generally lower than the elimination capacity of biofilters that degrade easily biodegradable pollutants. However, it is not clear whether the rate of styrene degradation in biofilters is limited by biological activity or the rate of diffusional transport.

NEW DEVELOPMENTS IN WASTE GAS PURIFICATION

Biofilters have traditionally been used for the removal of odours from for example sewage treatment plants and composting facilities (Ottengraf, 1987; Leson and Winer, 1991; Diks and Ottengraf, 1991). In general, these odours consist of easily biodegradable and good water–soluble compounds. Recent developments in biological waste gas treatment focus on the purification of waste gases containing pollutants that are difficult to degrade and/or poorly water–soluble. In this section, the development of new biotechniques and the improvement or adaptation of existing biotechniques is discussed for the removal of xenobiotic and hydrophobic waste gas compounds. In this respect, styrene may be considered a moderately recalcitrant and hydrophobic compound.

Removal of xenobiotic compounds

As indicated by several authors (e.g. Janssen *et al.*, 1989; Engesser, 1992; Hartmans, 1994), there is growing interest in the removal of xenobiotics from industrial waste gases. As a result of scientific research there is an increasing number of strains capable of degrading xenobiotic compounds and xenobiotics formerly thought to be persistent can now be biologically degraded. By using these strains, the application area of biotechniques for waste gas treatment may be broadened. In table 1.7 a survey is presented of the use of specialized microorganisms in biological waste gas treatment. Biological purification of waste gases containing xenobiotics like

aromatics and chlorinated compounds is now or will be in the near future possible although most examples in table 1.7 concern laboratory–scale experiments.

Since biofilters and biotrickling filters are open systems, the fate of specialized microorganisms upon inoculation is an interesting issue. In addition, specialized strains added to biofilters have to compete with the natural flora present on the organic support materials. Support materials commonly used in biofilters may contain about 10^6 – 10^8 microorganisms g^{-1} dry weight depending on the type of material (Eitner, 1989). Samples of biofilters with heather as the support material had bacterial counts of 10^7 to 10^{10} g^{-1} dry weight, whereas the number of yeasts and fungi was between 10^6 and 10^7 g^{-1} (Pearson *et al.*, 1992). The microbial flora in biofilters is very diverse. Bacteria found include coryneforms, endospore formers (e.g. bacilli), actinomycetes (*Streptomyces*) and pseudomonads (Rieneck, 1992). Also fungal species of the genera *Mortierella*, *Rhizopus*, *Penicillium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Trichoderma*, *Alternaria* and *Botrytis* were found.

A compost biofilter inoculated with the bacterial strain S1 for the degradation of 2,4–dichlorophenol and 4–chloro–2–methylphenol showed high degradation efficiency provided that the concentration of chlorophenols was lower than 10 $mg.m^{-3}$ (Fritsche and Lechner, 1992). After 100 days of operation, cell counts indicated decreasing numbers of strain S1 with increasing depth of the biofilter. Furthermore, apart from strain S1 other chlorophenol–degrading strains could be isolated which indicates the development of a mixed population. Gibson *et al.* (1994) inoculated compost biofilters with *Corynebacterium pseudodiphtheriticum* NCIMB 10803, *Rhodococcus rhodochrous* NCIMB 11147 and *Acetobacter aceti* NCIMB 8621 for the removal of the easily degradable compounds ethanol, pentanal and isobutylacetate. Inoculation with this consortium resulted in immediate degradation, but the species could not be detected in pilot–plant biofilters after 5 days of operation. Also, biofilter performance became poor due to acidification of the filter bed. Bronnenmeier and Menner (1992) used two *Pseudomonas* species in a bioscrubber for the simultaneous removal of formaldehyde, methanol and ethylene glycol. Although good degradation results were obtained during 70 days, the pseudomonads could not be detected in the bioreactor. Since microorganisms in bioscrubbers are not immobilized, slower growing microorganisms may be washed out (Kok, 1992). For the removal of the hydrophobic ethene, De Heyder *et al.* (1992) inoculated a biotrickling filter with the ethene–degrading *Mycobacterium* E3. In this filter a mixed population containing the *Mycobacterium* strain developed but growth was poor possibly because of mass transfer problems with ethene. The addition of a second carbon source (vinasse) proved to be useful since it enhanced both growth and filter performance. Reitzig and

Table 1.7. Application of specified strains in the biological treatment of waste gases.

Microorganism(s)	Pollutant(s)	Technique[a]	References[b]
Pseudomonas fluorescens DSM 50090	propionaldehyde, ethylacetate, other VOC's	BTF	1, 2, 3, 4
Aureobacter sp.	naphtalene	BTF	5
Thiobacillus thioparus DW44	hydrogen sulphide, methanethiol, dimethyl–sulphide, dimethyldisulphide	BF	6
Pseudomonas putida DSM 3226	ethylbenzene, xylenes	BTF	7
Pseudomonas sp.	formaldehyde, methanol, ethylene glycol	BS	8
Strain S1	2,4–dichlorophenol, 4–chloro–2–methylphenol	BF	9
Mycobacterium E3	ethene	BTF	10, 11
Mycobacterium E3	ethene	BF	12
Hyphomicrobium GJ21	dichloromethane, 1,2–dichloroethane	BTF	13
Hyphomicrobium DM20	dichloromethane	BTF	14
Pseudomonas putida NCIMB 10015	phenol	BF	15
Methylobacterium sp.	nitrogenmonooxide	MBR	16
Pseudomonas putida	toluene	BTF	17
Corynebacterium pseudodiphteriticum NCIMB 10803, *Rhodococcus rhodochrous* NCIMB 11147, *Acetobacter aceti* NCIMB 8621	ethanol, pentanal, isobutylacetate	BF	18
Mycobacterium aurum	vinylchloride	BF	19
Hyphomicrobium sp.	dimethylsulphide	BF	20
Xanthobacter Py2	propene	MBR	21
Pseudomonas putida	dichloromethane	BF	22

Footnotes on page 24.

Table 1.7 continued.

a BTF, biotrickling filter; BF, biofilter; BS, bioscrubber; MBR, membrane bioreactor.

b References: 1, Kirchner *et al.* (1984); 2, Kirchner *et al.* (1987); 3, Kirchner *et al.* (1992); 4, Kirchner *et al.* (1991); 5, Kirchner *et al.* (1989); 6 Cho *et al.* (1992); 7, Hardes and Werner (1992); 8, Bronnenmeier and Menner (1992); 9, Fritsche and Lechner (1992); 10, De Heyder *et al.* (1992); 11, Van Ginkel *et al.*, (1987); 12, De Heyder *et al.* (1994); 13, Diks (1992); 14, Hartmans (1993); 15, Zilli *et al.* (1993); 16, Hinz *et al.* (1994); 17, Reitzig and Menner (1994); 18, Gibson *et al.* (1994); 19, Meier (1994); 20, Smet *et al.* (1994); 21, Reij and Hartmans (1994); 22, Ergas *et al.* (1994).

Menner (1994) observed increasing biomass formation in biotrickling filters inoculated with *Pseudomonas putida* for the removal of toluene. A mixed population developed which contained *P. putida* and several other toluene–degrading bacteria and yeasts as well as bacterial and fungal strains that could not degrade toluene. The fungi formed a three–dimensional network which was assumed to stabilize the biofilm with an observed thickness of up to 5 mm. As a result of the excessive biomass formation, the void fraction in this biotrickling filter decreased from 88 to 40%. The development of a mixed population was also observed in a biotrickling filter inoculated with *Hyphomicrobium* GJ 21 for the degradation of dichloromethane (Diks, 1992). This filter showed a long–term elimination capacity of approximately 150 $g.m^{-3}.h^{-1}$. According to Diks (1991) a secondary population developed, containing other bacterial strains and higher organisms like flagellates, ciliates and nematodes. The dichloromethane–degrading activity constituted only 12% of the total biological activity present in the biotrickling filter.

Apart from the degradation of xenobiotic compounds, inoculation with specialized microorganisms may also be beneficial for the start–up of biological filters. A compost biofilter inoculated with *Thiobacillus thioparus* DW44 started immediately with the removal of hydrogen sulphide, methanediol, dimethylsulphide and dimethyldisulphide from gas (Cho *et al.*, 1992). Without inoculation an adaptation period of one month was necessary. Similar findings were reported for the degradation of ethanol, pentanal and isobutylacetate in compost biofilters (Gibson *et al.*, 1994). Inoculation with a specialized consortium resulted in immediate degradation, whereas after inoculation with activated sludge an adaptation period of 2–3 weeks was necessary. Kirchner *et al.* (1984) report an adaptation period of two hours for the degradation of propionaldehyde in a biotrickling filter containing adapted *Pseudomonas fluorescens* immobilized on activated carbon.

Removal of hydrophobic compounds

The rate of removal of pollutants from waste gases depends on two processes, i.e. the mass transfer from the gas phase to the liquid phase or the cells in the biofilm and the biodegradability. With hydrophobic pollutants, mass transfer may be the limiting factor for the overall filter performance. As outlined before, problems with mass transfer limitation can be expected for pollutants with Henry coefficients between 0.01 and 10, depending on the type of biotechnique used. Nevertheless, recent developments in biofiltration aim at broadening of the application range to hydrophobic pollutants. Various new techniques and adaptations in existing techniques for improvement of the mass transfer are being studied.

The addition of solid adsorbentia, in particular activated carbon, with high affinity for hydrophobic pollutants has been investigated by several groups. According to Kok (1992), the addition of 15% fine granular activated carbon to the washing liquid of bioscrubbers will also allow efficient removal of pollutants with Henry coefficients between 0.01 and 0.1. Pollutants with Henry coefficients higher than 0.1 would still require large quantities of washing liquid. The use of activated carbon as support material in biotrickling filters has been investigated by Kirchner *et al.* (1987 and 1989). Their experiments with various pollutants and specialized monocultures indicated mass transfer limitation in the liquid phase since the overall reaction rate (at high cell densities) decreased for substances with higher partition coefficients. An important advantage of activated carbon over some other tested materials appeared to be a high affinity for biomass, thus establishing high biomass concentrations and reaction rates (Kirchner *et al.*, 1989). However, these findings indicate limitation by microbial activity rather than limitation by mass transfer. Liu *et al.* (1994) claim 40 to 80 times higher degradation rates of toluene when using activated carbon as the sole support material (instead of for instance compost) in biofilters. Maximal toluene degradation rates of 80 $g.m^{-3}.h^{-1}$ were found, which is, however, comparable to VOC elimination capacities of traditional compost filters. According to Liu *et al.* (1994), the use of activated carbon in biofilters will enable the biological removal of hydrophobic waste gas compounds by improved mass transfer and increased surface concentrations of pollutants. Tiwaree *et al.* (1992) tested various activated carbons and other materials for the use in biofilters and found that a high specific surface area was beneficial for biofilter performance (removal of dimethylsulphide). Ottengraf *et al.* (1986) showed that 5% activated carbon in compost biofilters may improve pollutant removal when the pollutant load of the biofilter is exceeding the elimination capacity. In this case the activated carbon serves as a sink in times of high pollutant

concentrations. However, for the buffering of fluctuating pollutant concentrations a separate activated carbon filter, preceding the biological filter, may be more effective (Weber and Hartmans, 1992).

Comparable to the use of activated carbon is the addition of water–immiscible organic solvents to the washing liquid in bioscrubbers. This technique was developed by Schippert (Schippert, 1989, 1993 and 1994; Poppe and Schippert, 1992), who called it the 'Biosolv–Verfahren'. The organic solvent should meet various requirements, amongst which low vapour pressure and low solubility in water. Furthermore, the organic solvent should not be toxic for microorganisms nor should it be biodegradable. Schippert (1989) suggested di–n–octylphtalate, di–n–nonylphtalate and polydimethylsiloxane as suitable solvents. For styrene it was shown that the partition coefficient between air/organic solvent was 450 times higher than the partition coefficient between air/water. For the purification of 0.8 $g.m^{-3}$ toluene it was shown that increasing concentrations of organic solvent in the washing liquid resulted in increasing removal efficiencies. The maximal toluene degradation was approximately 95% at a organic solvent content of 13%. Using the same principle, Cesario *et al.* (1992) studied the use of organic solvents in a combination of a spray tower and a liquid–impelled loop reactor.

An other method to treat waste gases with hydrophobic pollutants is the use of membrane bioreactors. In these reactors the gas and liquid phase are separated by membranes which are covered with a biofilm on the liquid phase side. By using semipermeable hydrophobic dimethylsilicone as the membrane material, Fischer (1989 and 1992) and Reiser *et al.* (1994) obtained a high permeability for aromatic compounds like styrene and toluene. Elimination capacities of 60 g $styrene.m^{-3}.h^{-1}$ were obtained with a membrane bioreactor with a specific surface area of 30–60 $m^2.m^{-3}$. On the other hand, the rate of methanol and hexane removal was less satisfactory, probably because the solubility of these compounds in dimethylsilicone was considerably less than the solubility of styrene and toluene (Fischer, 1989). Hinz *et al.* (1994) investigated the use of hydrophobic membranes for the removal of NO by *Methylobacterium* species. The oxygen permeability of the membranes appeared to be very important. A high oxygen permeability resulted in rapid biomass growth and clogging of the membrane reactor, and thus in lower elimination capacities. Hydrophobic microporous membranes were investigated by Hartmans *et al.* (1992) and Reij and Hartmans (1994). Best results and high mass transfer rates were obtained with polypropylene membranes with the following characteristics: porosity 70–75%, average pore diameter 0.1 μm, membrane thickness 75–110 μm and specific surface area 250 $m^2.m^{-3}$. Model calculations indicated that membrane bioreactors with

polypropylene membranes had higher elimination capacities than biotrickling filters (dichloromethane as the model substrate), especially at low concentrations when mass transfer limitation can be expected (Hartmans *et al.*, 1992). Experiments with propene showed an elimination capacity of 30 $g.m^{-3}.h^{-1}$, but only at high propene gas phase concentrations (Reij and Hartmans, 1994).

The mass transfer of hydrophobic compounds in biotrickling filters may be improved by intermittent instead of continuous sprinkling as proposed by Wolff (1992) and De Heyder *et al.* (1994). By simulation experiments De Heyder (1994) showed that thick layers of sprinkling water on the biofilm had a negative effect on the removal rate of the hydrophobic ethene in a packed bed containing granular activated carbon. Under dryer conditions (i.e. no sprinkling and low relative humidity of waste gas) ethene removal was twice as high as with continuous sprinkling. However, longer periods of drying resulted in decreasing ethene removal efficiencies which may be caused by decreasing biological activity at low water activity. Wolff (1992) gives two explanations for the lower elimination capacity during humidification. First, during humidification the smaller pores will be filled with water which leaves the microorganisms in these pores without substrate. Secondly, the mass transfer coefficient, defined as the ratio of the diffusion coefficient and the thickness of film, will decrease during humidification.

A new development is the photocatalytic pretreatment of waste gases. Van Groenestijn *et al.* (1994) showed that treatment of styrene–containing gas with UV resulted in the formation of benzaldehyde. Biofiltration experiments (compost filters) with benzaldehyde indicated a three times higher elimination capacity than with styrene. The authors claim that UV–pretreatment of recalcitrant and hydrophobic pollutants generally results in the formation of compounds with higher biodegradability and water solubility, respectively. The experiments of Van Groenestijn *et al.* (1994) were not yet sufficiently conclusive to determine whether the improved biofilter performance was due to the higher biodegradability or the higher water solubility of benzaldehyde.

CONCLUSIONS

Safe working conditions and legislation on industrial emissions require industry to reduce the occupational exposure to styrene and the emission of styrene to the environment. Because the styrene concentration in industrial waste gases is generally

below 1 $g.m^{-3}$, biological treatment may offer an economically feasible method for the purification of these gases. In addition, from the literature it is clear that styrene can be used as sole source of carbon and energy by various types of microorganisms. Also in this respect, biological treatment of styrene–containing waste gases is a realistic option.

Various biological techniques for purification of styrene–containing gases have been studied. New techniques, such as membrane bioreactors and photocatalytic pretreatment, to improve the mass transfer of styrene may be promising but application of these techniques in the near future on an industrial scale is not likely. Biofilters containing organic packings have been shown to degrade styrene. For practical application of styrene–degrading biofilters in industry, these biofilters should maintain high elimination capacities for several years. However, reported styrene elimination capacities differ widely and the long–term stability of styrene–degrading biofilters has not been demonstrated. Data in the literature are not sufficient to determine the factors that influence the elimination capacity and stability of styrene–degrading biofilters.

Research on biofilters degrading styrene and other pollutants is mainly empirical and in many cases only influent and effluent gases are investigated. In our opinion, there is a lack of understanding of the processes that take place inside the biofilter. Since microorganisms are the catalysts responsible for pollutant degradation, research should primarily aim at the microbiology of biofilters. Research should include isolation and identification of the active species in styrene–degrading biofilters, determination of degradation kinetics of the isolated strains and identification and quantification of the factors that influence their activity in biofilters. This way, a high and sustained styrene elimination capacity may be obtained.

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CHAPTER 2

ENRICHMENT OF STYRENE-DEGRADING FUNGI IN BIOFILTERS

INTRODUCTION

Biofilters are being used for odour abatement e.g. at water purification plants and composting facilities (Ottengraf, 1986; Leson and Winer, 1991). New developments in biological waste gas treatment aim at purification of industrial waste gases containing xenobiotic and/or hydrophobic compounds (Van Groenestijn and Hesselink, 1993). Degradation of volatile organic compounds, sulphides, aromatics and chlorinated aromatics in biofilters has been reported (Cho *et al.*, 1992; Bronnenmeier and Menner, 1992; Fritsche and Lechner, 1992; Zilli *et al.*, 1993; De Heyder *et al.*, 1994; Gibson *et al.*, 1994; Smet *et al.*, 1994).

In order to enable degradation of xenobiotics in biofilters or to shorten the adaptation period, inoculation of biofilters with specialized strains may be necessary (chapter 1). Apart from this, the use of specialized strains with known degradation characteristics may facilitate more fundamental research on the microbiology of biofilters. We are interested in the development of styrene–degrading biofilters with fungi displaying high styrene–degrading activity on inert support materials. Various techniques for the selective enrichment of microorganisms with the desired characteristics have been described (Veldkamp, 1970; Cook *et al.*, 1983). Selective enrichments for fungi displaying high and sustained styrene–degrading activity on solid support materials in biofilters at low styrene concentrations thus were necessary. Since poor performance of biofilters is mainly caused by acidification and drying out of the filter bed (Ottengraf, 1986), the fungi to be enriched should also tolerate low pH and low water activity. Therefore, experiments were set up to enrich styrene–degrading fungi in biofilters under conditions representative for industrial waste gas treatment.

MATERIALS AND METHODS

Biofiltration equipment and daily operation

The biofiltration equipment contained 8 biofilters with a height of 0.17 m and a diameter of 0.059 m. The biofilters were operated at ambient laboratory temperature and through each biofilter air with 0.29 $g.m^{-3}$ styrene and a relative humidity of 80–85% was passed at a gas flow of 0.043 $m^3.h^{-1}$. After 110 days styrene in the influent gas was increased to an average concentration of 0.675 $g.m^{-3}$. At different intervals the biofilter contents were humidified to complete saturation by pouring mineral medium on the filter bed. Residual medium was drained off after approximately one hour. The mineral medium contained (per litre demineralized water): 0.5 g K_2HPO_4, 4.5 g KH_2PO_4, 2 g NH_4Cl, 0.1 g $MgSO_4.7H_2O$, 2 ml trace element solution, 2 ml vitamin solution, pH 5.7. The trace element solution contained (per litre demineralized water): 120 mg $FeCl_3$, 50 mg H_3BO_3, 10 mg $CuSO_4.5H_2O$, 10 mg KI, 45 mg $MnSO_4.H_2O$, 20 mg $Na_2MoO_4.2H_2O$, 75 mg $ZnSO_4.7H_2O$, 50 mg $CoCl_2.6H_2O$, 20 mg $AlK(SO_4)_2.12H_2O$, 13.25 g $CaCl_2.2H_2O$ and 10 g NaCl. The vitamin solution contained (per litre demineralized water): 100 mg nicotinic acid, 200 mg Ca–pantothenate, 25 mg cyanocobalamine, 100 mg inositol, 20 mg p–aminobenzoate, 50 mg thiamine.HCl, 25 mg pyridoxine.HCl, 10 mg biotin, 10 mg riboflavin, 10 mg folic acid and 10 mg thioctic acid. The weight of the biofilters was regularly measured to determine the evaporation of water and the absorption of water after the addition of medium. Styrene was determined daily by gas chromatography after sampling of the influent and effluent gas with 50 ml gastight 3–way sample bulbs with Teflon septa. CO_2 in the influent and effluent gas was determined once every two hours using an automatic sampling device connected with an infra–red CO_2–analyzer.

Inoculation of support materials

Eight different support materials were used: six types of activated carbon (donated by Norit NV, Amersfoort, the Netherlands), polyurethane (15x10x5 mm cubes, Linde AG, München, Germany) and perlite (irregular grains with mean diameter of 4.5 mm, Pull BV, Rhenen, the Netherlands). The support materials were saturated with mineral medium and inoculated with a suspension of garden soil in 0.015% (v/v) Tween–80.

Isolation of styrene-degrading microorganisms

Biofilter samples were taken from the inlet and outlet side of the biofilters and plated on mineral medium containing 8 $g.l^{-1}$ agarose. The inoculated plates were placed in desiccators at 25°C, containing gaseous styrene as the sole source of carbon and energy in a concentration of 0.3 $g.m^{-3}$. Styrene was added to the gas phase by equilibrating a solution of 1.5 $g.l^{-1}$ styrene in dibutylphtalate with the gas phase (partition coefficient 2.0 x 10^{-4} $mg.l^{-1}$air/$mg.l^{-1}$dibutylphtalate, Cox *et al.*, 1993). Bacterial and fungal colonies were transferred to new plates and pure cultures were isolated by conventional methods. Fungal strains were identified by Centraalbureau voor Schimmelcultures (CBS, Baarn, the Netherlands).

Analytical methods

Styrene was determined with a Varian model 3700 gaschromatograph (Varian Benelux BV, Amsterdam, the Netherlands) using a WCOT fused silica column with CP–Sil–5CB as the stationary phase (Chrompack, Bergen op Zoom, the Netherlands). Carrier gas was helium at 10 $ml.min^{-1}$, the column temperature was 150°C, FID was at 250°C. The produced CO_2 concentration in the effluent gas was determined using WA–161 autosampler and CO_2–analyzer ADC–225–MK3 from The Analytical Development Co (Hoddesdon, England). The relative humidity was determined with a RH–plus 2250 sensor from Endress + Hauser (Maulberg, Germany).

RESULTS AND DISCUSSION

Growth during the adaptation period

In order to enrich styrene–degrading fungi under dry conditions, the addition of mineral medium to the biofilters in the first period of 75 days was limited to approximately once every 20 days. After the addition of medium, rapid evaporation of water was observed as determined by the loss of weight of the biofilters (for the biofilter containing perlite 10 g water per day when saturated with water). Occasionally, the biofilter weight became constant. In this case an equilibrium between the relative humidity of the influent gas (80–85%) and the water activity of the filter bed (0.80–0.85) was assumed. Addition of medium during the adaptation period,

however, was very limited to allow enrichment at low water activity. Nonetheless, stimulation of growth because of high water contents immediately after the addition of medium may have occurred.

Growth in the biofilters became apparent after 4 to 5 weeks. With perlite as the support material, different types of mycelium were observed on the particle surface. In the biofilter with polyurethane, growth started inside the particles. Microscopic investigations revealed the presence of mycelium inside the pores of the polyurethane cubes. In both these biofilters growth was observed over the entire length of the column. The biofilters with activated carbon also showed growth within 5 weeks, but growth was limited to the inlet side of the biofilters. This may be explained by adsorption of styrene to the activated carbon at the inlet side of biofilter, thus resulting in the absence of styrene at the outlet side of the biofilter. Styrene was not found in the effluent gas of activated carbon biofilters during the adaptation period.

In general, the development of biomass in the adaptation period appeared to be a slow process. CO_2 production was low and styrene degradation was incomplete. Therefore, after 75 days the frequency of medium addition was increased to once every 3–5 days to stimulate growth and styrene degradation activity.

Isolation of styrene-degrading fungi

Styrene–degrading microorganisms were isolated after 40 days incubation of the biofilters, and after 104 days, following a period with more regular humidification. When using soil as the inoculum for the biofilters, a large number of styrene–degrading microorganisms was isolated (table 2.1). Conform these findings, the decomposition of ^{14}C–styrene in natural soil samples from various origins has been demonstrated (Sielicki *et al.*, 1978; Fu and Alexander, 1992). However, despite the apparent presence of styrene–degrading microorganisms in soil, previous attempts by other researchers to isolate these strains were less successful. Omori *et al.* (1975) used conventional enrichment techniques with styrene as the sole source of carbon and energy at a concentration of 2% (192 mM, which is well above the water solubility of styrene, Banerjee *et al.*, 1980). Although they screened 101 soil samples, styrene–degrading microorganisms were not found. Initial attempts of Shirai and Hisatsuka (1979) with 200 soil samples were also unsuccessful, but 31 styrene–degrading bacteria were obtained when using a very low styrene concentration in a soil percolation column. Hartmans *et al.* (1990) isolated 14 styrene–degrading bacterial strains as well as 2 fungal strains from water and soil samples, using styrene concentrations of 10 to 500 μM. Apparently, the styrene concentration in enrichment

Table 2.1. Isolation of styrene-degrading microorganisms (not necessarily different strains) from biofilters.

Support	Number of isolated strains		pH drain[a]
	Bacteria	Fungi	water
Activated carbon[b]	41	5	5.6–5.8[c]
Polyurethane	4	6	2.6
Perlite	1	10	2.7

a The pH of the humidification medium was 5.7.
b Total of 6 biofilters.
c The pH of the water draining from the biofilter containing activated carbon PK1–3 was 3.7.

Table 2.2. Identification of fungal strains isolated from biofilters.

Code	Source	Identification
A–7–1	carbon RB3	*Penicillium* cf. *janthinellum*
A–7–2	carbon RB3	*Penicillium* cf. *janthinellum*
A–19	polyurethane	*Penicillium* cf. *janthinellum*
A–23	perlite	*Penicillium minioluteum* Dierckx
A–24	perlite	*Gliocladium roseum* Bain
A–28	perlite	*Penicillium fellutanum* Biourge
B–15	polyurethane	*Penicillium glabrum* (Wehmer) Westling
B–43	perlite	*Exophiala jeanselmei* (Langeron) McGinnis & Padhye
B–50	perlite	*Penicillium* cf. *miczynskii* Zaleski
B–52–2	perlite	*Penicillium* species

cultures should be sufficiently low to enable growth of microorganisms. In our enriched biofilters this prerequisite was met by applying a styrene concentration in the gas phase of 0.29 $g.m^{-3}$. This corresponds to an equilibrium concentration in the water phase of 25 μM (styrene partition coefficient of 0.11 $mg.l^{-1}$air/$mg.l^{-1}$medium at 19°C, Cox *et al.*, 1993).

As shown in table 2.1, styrene–degrading fungi were readily isolated from the biofilters with polyurethane and perlite as support material, whereas the biofilters with activated carbon mainly contained bacteria. Ten different styrene–degrading fungi were isolated in pure culture, most of them belonging to the genus *Penicillium* (table 2.2). To date, only three fungal species are known to grow on styrene (Hartmans *et al.*, 1990; De Jong *et al.*, 1990). Measurements of the pH in the drain water after the humidification of the filter bed with mineral medium indicated strong acidification in the biofilters with polyurethane and perlite and moderate acidification in the biofilter with activated carbon PK1–3 (table 2.1). Although the pH was not used as a selective factor for enrichment, the results indicate that the low pH in the biofilters with polyurethane and perlite may have favoured the enrichment of acid–tolerant, styrene–degrading fungi. In the biofilters with activated carbon, adsorption of produced acids and buffering of the pH are likely to occur, thus favouring the growth of bacteria. However, apart from the pH and styrene concentration, other factors may have contributed as selective forces for the enrichment of styrene–degrading microorganisms, but these were not investigated.

Performance of enriched biofilters

A characteristic parameter describing the performance of biofilters is the elimination capacity. Depending on the degradability of waste gas pollutants, reported elimination capacities vary between 8 and 200 $g.m^{-3}.h^{-1}$ (Van Groenestijn and Hesselink, 1993). To evaluate the use of the enriched biofilters in the removal of styrene from industrial waste gases, styrene degradation and CO_2 production were determined during a period of 150 days after inoculation of the biofilters on day 0. Figure 2.1 shows the results of the biofilters containing activated carbon RO–3 and perlite as representative examples of biofilters containing bacteria and biofilters containing fungi, respectively. Due to the adsorption of styrene, styrene removal was initially complete in the biofilters with activated carbon (figure 2.1A). Breakthrough of styrene was observed after 55 to 115 days depending on the type of activated carbon which may have been caused by differences in styrene adsorbing properties of the activated carbons used. However, microbial activity in the adaptation period was

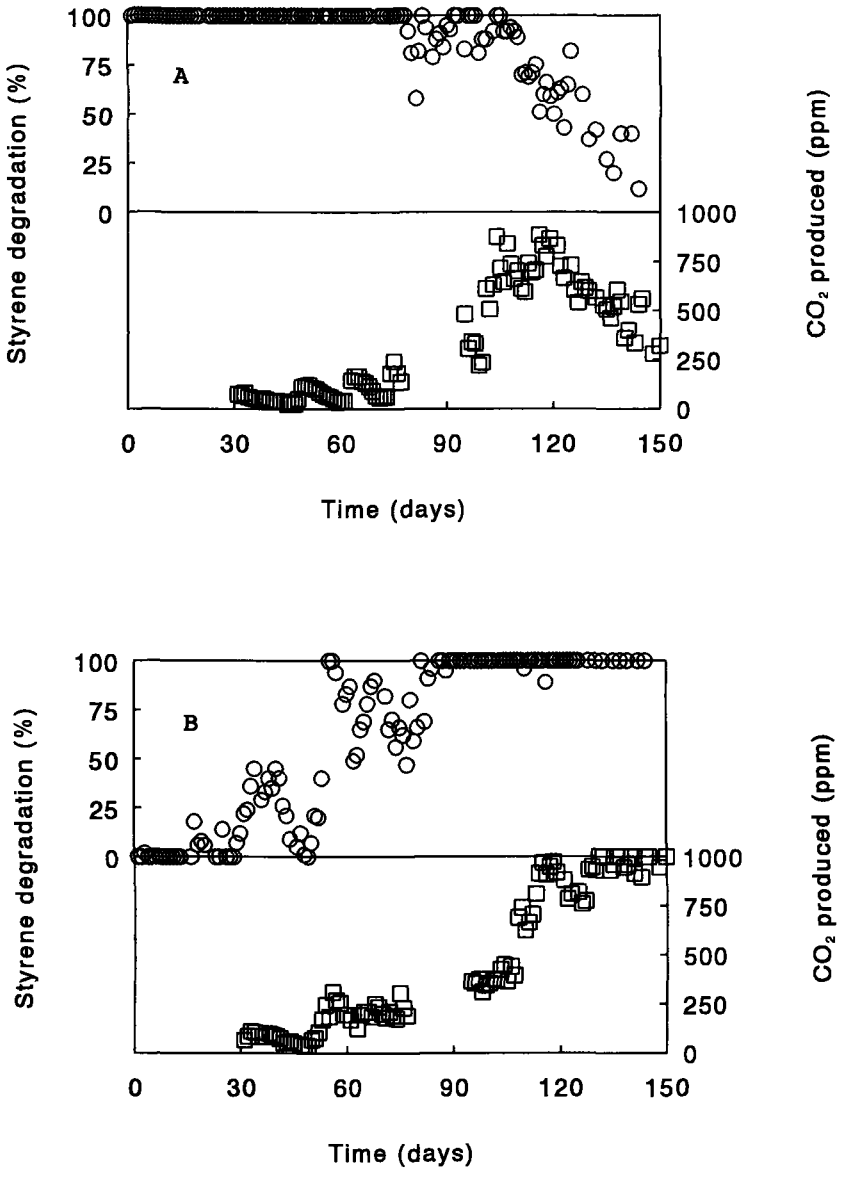

Figure 2.1. Styrene degradation (○) and CO_2 production (□) in enriched biofilters containing bacteria on activated carbon RO-3 (A) and fungi on perlite (B).

Table 2.3. Average styrene degradation and CO_2 production in enriched biofilters at a volumetric load of 93 $m^3.m^{-3}.h^{-1}$ and styrene influent concentrations of 0.290 (day 80-109) and 0.675 $g.m^{-3}$ (day 110-150).

Support[a] material	Influent 0.290 $g.m^{-3}$		Influent 0.675 $g.m^{-3}$	
	Effluent ($g.m^{-3}$)	CO_2 (ppm)	Effluent ($g.m^{-3}$)	CO_2 (ppm)
Carbon RB1	0.110	280	0.392	335
Carbon RB2[b]		355	0.186	400
Carbon RB3[c]		470	0.130	520
Carbon PK1–3	0.039	460	0.200	725
Carbon RBAA1	0.094	340	0.105	705
Carbon RO3	0.020	485	0.283	565
Polyurethane	0	415	0.035	820
Perlite	0	425	0.006	870

a Styrene degradation in activated carbon biofilters was determined after the breakthrough of styrene.
b Breakthrough of styrene at day 115.
c Breakthrough of styrene at day 110.

low in all biofilters as judged from the CO_2 production. Most likely the water content was the limiting factor since more frequent humidification starting from day 75 resulted in an increased styrene degradation and CO_2 production (figure 2.1).

The performance of the enriched biofilters was determined at styrene inlet concentrations of 0.290 and 0.675 $g.m^{-3}$ whilst maintaining high water contents to ensure optimal styrene degradation activity (table 2.3). High styrene degradation activity was observed in the biofilters containing fungi on polyurethane and perlite, whereas the biofilters containing activated carbon showed lower elimination capacities. In some biofilters with activated carbon excessive biomass growth was observed, resulting in clogging of the filter bed and poor performance of the filters. Clogging has been related to high organic loadings (Hartmans, 1994), which may have been the case here. Also, fungal growth has been implied as a cause for clogging (Diks, 1992).

However, clogging in biofilters with fungi on perlite and polyurethane did not occur, despite the high styrene load and the high styrene degradation rate. The biofilter with perlite as the support material showed complete removal of 0.675 $g.m^{-3}$ styrene at a volumetric load of 93 $m^3.m^{-3}.h^{-1}$ with 70% recovery of styrene as CO_2 as expected upon total degradation of removed styrene (table 2.3). Styrene degradation activity in this filter was stable between day 80–150 (figure 2.1B), despite the decrease of the pH to 2.7. A styrene elimination capacity of at least 62 $g.m^{-3}.h^{-1}$ was calculated, whereas the styrene elimination capacity of biofilters with natural packings is 10–50 $g.m^{-3}.h^{-1}$ (Windsperger *et al.*, 1990; Demiriz, 1992; Sabo *et al.*, 1993). Biofilters with fungi on inert support materials, in particular perlite, thus may be applied in the treatment of industrial waste gases containing styrene.

Enrichment of fungi at reduced water activity

The initial aim to enrich fungi at low water activity was left during the experiments described above. It was therefore decided to repeat the experiment, following the same experimental set–up but at reduced and controlled water activity. These experiments were carried out using perlite as the support material that was previously equilibrated with air with a relative humidity of 70–75%. Eight biofilters were inoculated with samples (discharge water, contaminated soil, dust from floors, walls and air filters) from a styrene production plant. After inoculation, air with an average styrene concentration of 0.330 $g.m^{-3}$ was passed through the biofilters at a volumetric load of 70 $m^3.m^{-3}.h^{-1}$. The relative humidity of the air was kept constant at 70–75% to maintain a water activity in the filter bed of 0.70–0.75. Although the inocula contained different types of styrene–degrading fungi as well as xerotolerant fungi capable of growth on solid medium containing 40% sucrose, styrene degradation was not detected during 135 days. Apparently, the inocula did not contain styrene–degrading fungi capable of growth at a water activity of 0.70–0.75. Furthermore, also the strains listed in table 2.2 failed to grow in biofilters containing perlite with a water activity of 0.70–0.75. The tolerance of fungi to low water conditions is well–known. Xerotolerant fungi may grow at a water activity as low as 0.605 (Pitt and Christian, 1968), although the growth rate at low water activity is considerably lower than the maximal growth rate at optimal water activity (Corry, 1987). *Penicillium* species generally require water activities of at least 0.80 (Pitt, 1975), which implies that a water activity of 0.70–0.75 was too low for growth of the *Penicillium* species as well as the other strains listed in table 2.2. It is therefore concluded that operation of biofilters with a sustained and high styrene degradation activity at a water activity of

0.70–0.75 is not a realistic option.

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CHAPTER 3

INFLUENCE OF THE WATER CONTENT AND WATER ACTIVITY ON STYRENE DEGRADATION IN BIOFILTERS

INTRODUCTION

In practice, malfunctioning of biofilters is most often caused by drying out of the filter bed (Heslinga, 1994). Low water contents result in a decreased biodegradation activity and the formation of preferential flow channels (Van Langenhove *et al.*, 1986; Leson and Winer, 1991; Sabo *et al.*, 1993). Therefore, it is recommended to saturate the waste gas with water to prevent drying out. Although the importance of the water content in biofilters has been recognized, a quantitative analysis of the influence of the water content on biofilter performance has scarcely been reported.

The use of fungi in solid–state fermentation is well–known. This type of fermentation has been characterized by the absence of free water (Cannel and Moo–Young, 1980; Aidoo *et al.*, 1982), and, consequently, water availability is one of the key factors influencing microbial activity in these processes (Hesseltine, 1972; Lonsane *et al.*, 1985; Durand and Chereau, 1988). As the availability of water is low, the use of fungi instead of bacteria is advantageous and consequently several studies of solid–state fermentation with fungi at water activities ranging from 0.88 to 1 have been reported in literature (Narahara *et al.*, 1982; Grajek and Gervais, 1987; Oriol *et al.*, 1988; Smits *et al.*, 1993). Since a biofilter may be looked upon as a special case of solid–state fermentation, the use of fungi in biofilters may be advantageous. Previously, we described the enrichment of styrene–degrading fungi in biofilters under conditions representative for industrial waste gas treatment (chapter 2). In this chapter the performance at low water content of styrene–degrading biofilters with fungi on perlite is described. The styrene elimination capacity has been determined at various

water contents, and under drying out conditions. It has been shown that styrene degradation rates are related to water activity, thus examplifying that water activity is a critical parameter for biofilter performance.

MATERIALS AND METHODS

Biofiltration equipment and inoculation of the support material

Most experiments were carried out with biofilters with a volume of 0.41–0.48 l, a diameter of 0.059 m and with 49.5–57.4 g perlite (code 3, particles > 4 mm, Pull BV, Rhenen, the Netherlands). For inoculation, 126 g of an enriched styrene–degrading biofilter (chapter 2) was added to 682 g perlite saturated (62% w/w) with a mineral medium (chapter 2) and distributed over several biofilters. After inoculation, styrene–containing air was passed through the biofilters in a downflow mode. Initially, the biofilters were saturated with mineral medium once every 4–7 days by pouring the medium on top of the filter bed until the void volume was completely filled. Residual liquid was drained off after approximately one hour. Styrene degradation rates were regularly measured to determine whether a high and stable styrene elimination capacity was reached, after which the biofiltration experiments with varying water content were carried out.

Gradients of the water content, water activity, temperature and styrene concentration along the height of the filter bed were determined in a biofilter with a height of 1 m and a diameter of 0.1 m. The operation of this biofilter was comparable to the operation of the smaller ones, but for inoculation a culture of styrene–grown *Exophiala jeanselmei* in mineral medium was used (chapter 5).

Characterization of the microbial population

The number of bacteria, fungi and styrene–degrading microorganisms in the water draining from the biofilters was determined by serial dilution of samples in demineralized water and by plating the dilutions on plate count agar (PCA, total count), oxytetracycline yeast glucose agar (OGGA, fungal count) and a solidified mineral medium (MMS, chapter 2, count of styrene–degrading microorganisms). PCA contained (per litre demineralized water): 5 g tryptose, 2.5 g yeast extract, 1 g glucose and 15 g agar, pH 6.3. OGGA contained (per litre demineralized water): 5 g yeast

extract, 20 g glucose, 20 g agar and 100 ml of 1 $mg.ml^{-1}$ filter–sterilized oxytetracycline, pH 6.3. Serial dilution and plating were done in duplicate. Incubation was at 25°C. MMS–plates were placed in desiccators containing 0.5 $g.m^{-3}$ styrene in the air (chapter 2). The number of bacteria was calculated by subtraction of the cell count on OGGA from the total cell count on PCA.

Determination of desorption isotherms

Desorption isotherms were made with fresh perlite and samples from the filter bed. After saturation with demineralized water, weighed samples (quadruplicate) were placed in desiccators containing saturated salt solutions in demineralized water, or pure demineralized water at 25°C. The salts used were: K_2SO_4 (a_w 0.97), KNO_3 (0.93), $BaCl_2.2H_2O$ (0.90), K_2CrO_4 (0.87), KBr (0.83) and NaCl (0.75) (Smith, 1971; Greenspan, 1977). All salts used were of analytical grade. The samples were regularly weighed to determine whether equilibrium was reached, after which the water content was determined.

Determination of the styrene degradation rate at various water activities

The rate of styrene degradation by *E. jeanselmei* was determined at water activities of 0.90, 0.93, 0.97 and 1. Perlite was saturated with a suspension containing a culture of styrene–grown *E. jeanselmei* in mineral medium and spread on petri–dishes, which were placed in 4 desiccators containing 0.5 $g.m^{-3}$ styrene in air and saturated solutions of $BaCl_2.2H_2O$, KNO_3 or K_2SO_4 in demineralized water or pure demineralized water. The samples were incubated at 25°C and weighed once every two days. When the weight was constant, samples were transferred to flasks sealed with Viton septa. Styrene was added at a concentration of 0.8 $g.m^{-3}$ in air. The rate of degradation of styrene was followed by gaschromatographic analysis of styrene in the gas phase. Experiments were carried out in duplicate. Samples were analyzed for the protein content and the water activity and styrene degradation rates were related to the protein content.

Biofiltration experiments

Experiments were carried out in a thermostatted room at 24–25°C. The maximal styrene degradation rate (i.e. the styrene elimination capacity) was determined in six biofilters, saturated with mineral medium (66% w/w), at a volumetric load of

120 $m^3.m^{-3}.h^{-1}$ and an average styrene inlet concentration of 0.678 $g.m^{-3}$. Styrene degradation rates were determined daily for a period of ten days.

The influence of the water content on biodegradation capacity was determined in three biofilters. Different water contents were obtained by passing styrene–containing air with a relative humidity of 78% at a volumetric load of 137 $m^3.m^{-3}.h^{-1}$, thus allowing for the evaporation of water. The rate of loss of water was varied by adding 1, 2 or 5 ml demineralized water once a day on top of the filter bed. After 19 days, the water content in the biofilters was 22, 37 and 55% (w/w), respectively, and these were kept constant by daily compensation for the evaporation of water (as determined by the decrease of biofilter weight) by the addition of demineralized water. Styrene degradation rates were determined daily at a volumetric load of 146 $m^3.m^{-3}.h^{-1}$ and an average styrene concentration of 0.646 $g.m^{-3}$ for a period of 7 days.

The influence of the relative humidity of the influent gas was determined in six biofilters. Before starting this experiment, the biofilters were saturated (67% w/w) with mineral medium. Styrene–containing gas with average relative humidities of 67% (2 biofilters), 80% (2 biofilters), 91% (1 biofilter) and up to 100% (1 biofilter) were passed through the biofilters at a volumetric load of 133 $m^3.m^{-3}.h^{-1}$. The average styrene concentration in the influent gas was 0.587 $g.m^{-3}$. Styrene removal was determined daily and related to the water content of the filter bed as determined by the weight of the biofilters.

Gradients of the water content, water activity and styrene concentration along the height of the filter bed were determined in a 1 m biofilter. After saturation (69% w/w) with mineral medium, styrene–containing air (1.664 $g.m^{-3}$) with an average relative humidity of 65% was passed through the biofilter at a volumetric load of 64 $m^3.m^{-3}.h^{-1}$. Styrene concentration profiles were determined daily at six positions along the height of the filter bed using a total hydrocarbon analyzer. After twelve days, samples were taken from six heights in the filter bed and analyzed for the water content (triplicate) and water activity (duplicate). Temperature profiles were determined using the same biofilter, though at a volumetric load of 76 $m^3.m^{-3}.h^{-1}$.

Analytical methods

Styrene was determined using a HRGC 5300 Mega series gaschromatograph from Carlo Erba Instruments (Milan, Italy) and a WCOT fused silica column from Chrompack (Middelburg, the Netherlands), stationary phase CP–Sil–5CB, length 25 m, internal diameter 0.53 mm, film thickness 5 μm. Helium was used as the carrier gas at 17 $ml.min^{-1}$, the column and injector temperature were 100°C, FID at 250°C.

Styrene analyses were done by injecting 50 µl samples in triplicate using gastight syringes (Hamilton, Reno, Nev.). Styrene concentration profiles were determined with a model 51 Total Hydrocarbon Analyzer from Thermo Environmental Instruments (Franklin, Mass.) connected to a Sixnet 60–PIB/IO datalogging system (Digitronics Sixnet, Clifton Park, Mass.). The relative humidity of gases was measured with a RH–plus 2250 sensor (Endress + Hauser, Maulberg, Germany). The water content of biofilter samples was determined by drying at 95°C to constant weight. Water contents are expressed as g water.g^{-1} wet sample x 100 (% w/w). The average water content of biofilters during operation was determined by subtraction of the weight of the empty biofilter plus the dry weight of perlite from the total weight of the biofilter. The biofilter was assumed to contain only perlite and water; the weight of adsorbed styrene, metabolites and biomass were considered negligible. Water activity was determined using a ER84/3H/63T hygrometer equipped with en–BSK–4 humidity sensors (Novasina, Pfäffikon, Switzerland) at 25°C. Protein was determined with Coomassie assay reagent from Pierce (Rockford, Ill.) after boiling the samples in 1 M NaOH for 10 min.

RESULTS

Maximal styrene degradation rate (66% water)

At an average styrene concentration of 0.678 g.m^{-3} and a volumetric load of 120 $m^3.m^{-3}.h^{-1}$, the removal efficiency was 96–98%. The average styrene elimination capacity was 79 g.$m^{-3}.h^{-1}$, which is slightly higher than the styrene degradation rate in the enriched biofilter with perlite (chapter 2) that was used as an inoculum.

Microbial population

Cell counting experiments in the water draining from the biofilters after 54 days of operation indicated a dominance of fungi. The number (log N.ml^{-1}) of bacteria, fungi and styrene–degrading microorganisms were 6.3, 8.3 and 8.2, respectively. Four types of yeast–like or fungal species could be distinguished on the OGGA–plates. Based on visual observation, the yeast–like *E. jeanselmei* (identification by Centraalbureau voor Schimmelcultures, Baarn, the Netherlands) attributed for over 90% of the fungal cell count. MMS–plates only contained *E. jeanselmei*, indicating

that this species is responsible for styrene degradation in these biofilters.

Desorption isotherms

Due to the high internal porosity, perlite can adsorb 1.85 g water.g^{-1} perlite (65%). From the desorption isotherms with fresh perlite (figure 3.1) it can be concluded that the major part of adsorbed water is readily available for microorganisms. Limitation of microbial activity by reduced water activity can be expected at water contents lower than 7%. However, the presence of fungal biomass on perlite resulted in a significant stronger binding of water (figure 3.1). Reduced water activities in the filter bed will occur at water contents below 32%.

Styrene degradation by E. jeanselmei at reduced water activity

Samples of *E. jeanselmei* on perlite were equilibrated in the presence of solutions with water activities of 0.90, 0.93, 0.97 and 1. However, the measured water activities of the samples were slightly lower, i.e. 0.88, 0.91, 0.95 and 0.99. Figure 3.2 shows the influence of the water activity on styrene degradation by *E. jeanselmei*. The

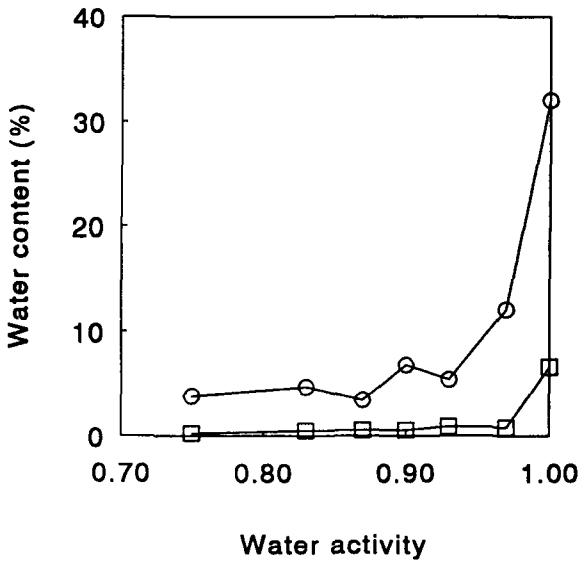


Figure 3.1. Desorption isotherms of fresh perlite (□) and biofilter samples with perlite as the support material (○).

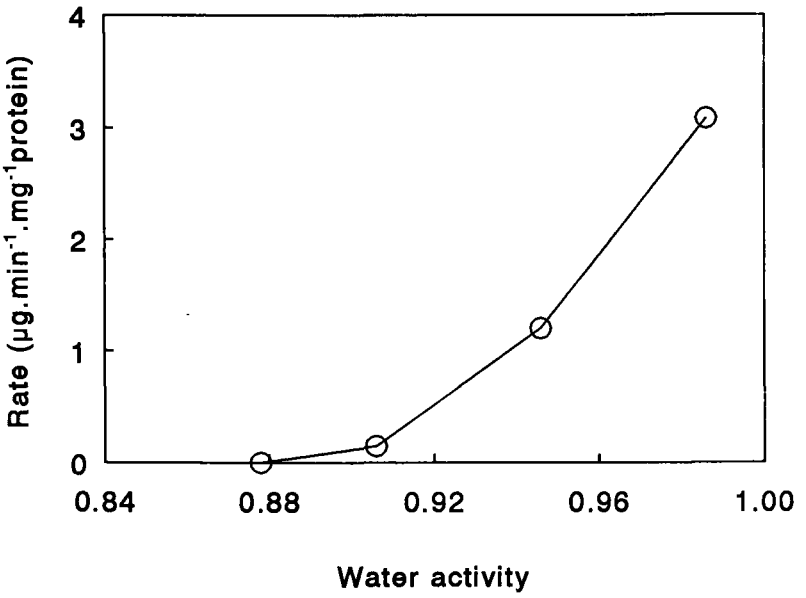


Figure 3.2. Influence of water activity on the styrene degradation rate by Exophiala jeanselmei on perlite.

rate of styrene degradation is highest at a water activity of 0.99, but styrene degradation activity was also observed at a water activity as low as 0.91. These results indicate that styrene degradation at reduced water activity in the filter bed is possible, though at low rates.

Influence of relative humidity on biofilter performance

The relative humidity of the gas phase had a marked influence on biofilter performance (figure 3.3). Due to evaporation of water, the average water content in the biofilter steadily decreased with a rate that depends on the relative humidity of the influent gas. Evaporation of water was accompanied by loss of styrene degradation activity (figure 3.3A–C). Passing air with a relative humidity of 67, 80 or 91% through biofilters resulted in 50% loss of activity after 9, 11 and 18 days, respectively. When biofilters were in equilibrium with gas with a relative humidity of 67 and 80%, styrene degradation stopped. However, the styrene elimination capacity was 9 $g.m^{-3}.h^{-1}$ in a biofilter in equilibrium with air with a relative humidity of 91% (water content

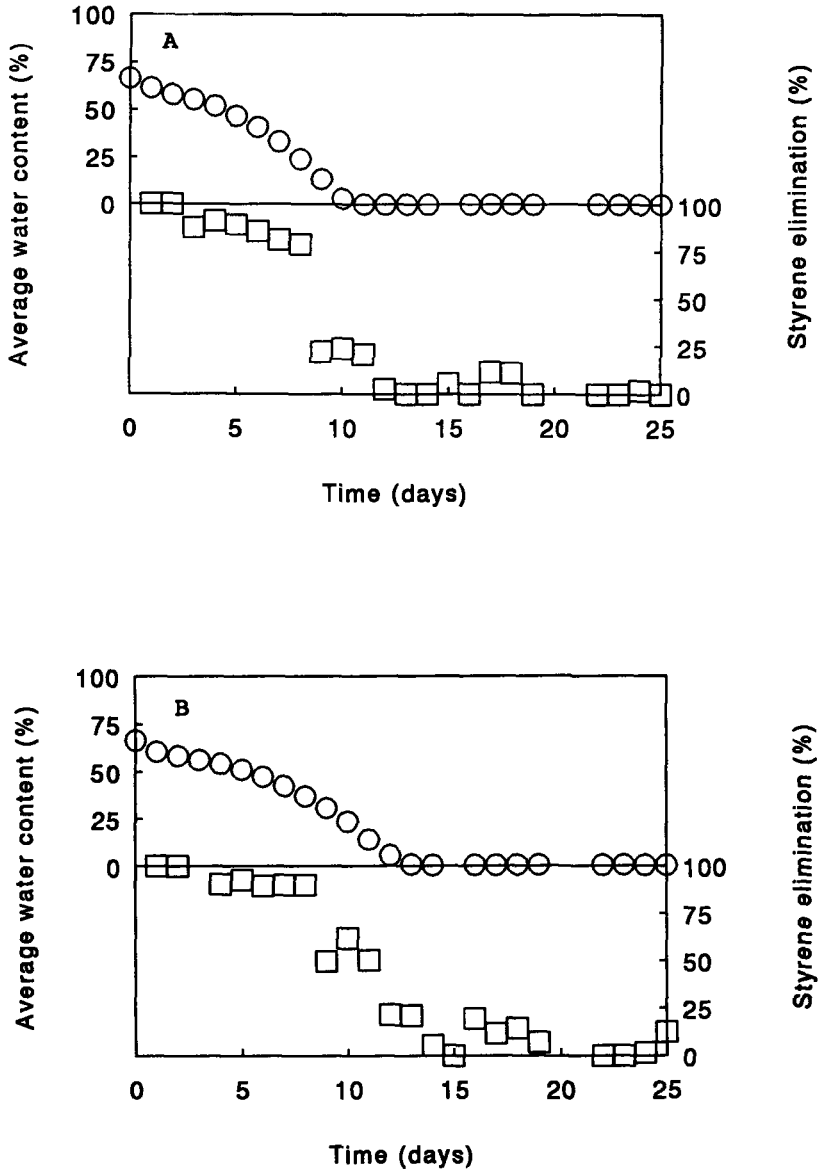
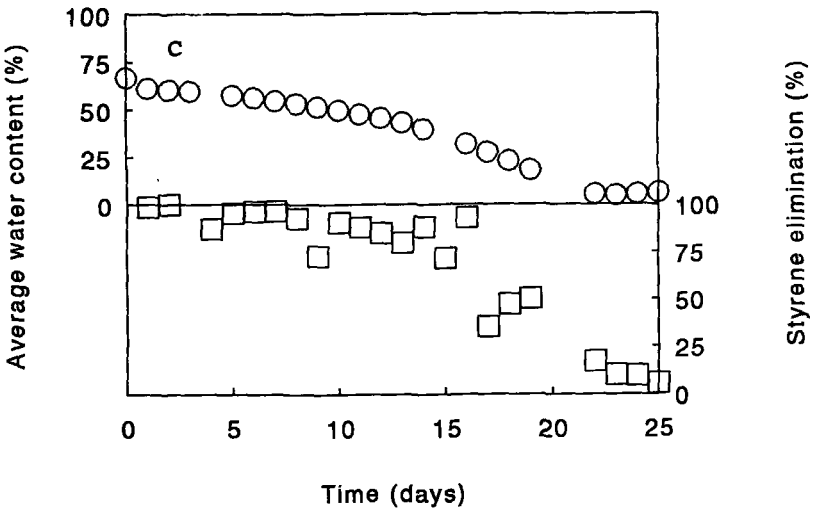


Figure 3.3. Influence of the relative humidity of the feed gas on the average water content of the filter bed (○) and the elimination of styrene (□). A, relative humidity 67%; B, relative humidity 80%.



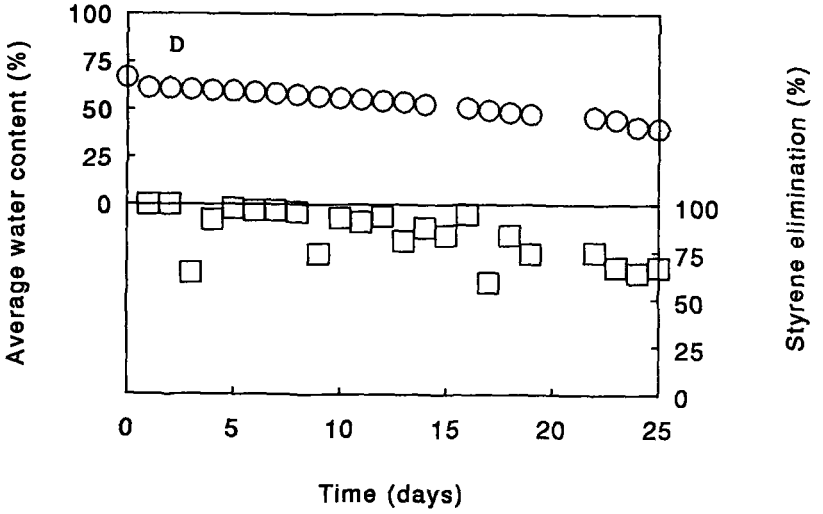


Figure 3.3 continued. C, relative humidity 91%; D, relative humidity 100%.

biofilter 5.8%), which confirms the ability of *E. jeanselmei* to degrade styrene at a water activity of 0.91 (figure 3.2). When water–saturated air was passed through a biofilter, the average water content in the filter bed and the styrene elimination rate decreased slowly (figure 3.3D). Evaporation of water in this biofilter is most likely caused by heat of styrene oxidation (Plas *et al.*, 1994). This implies that for sustained activity additional supply of water is also necessary even when water–saturated air is passed through the filter.

In figure 3.4 the percentage styrene elimination is plotted versus the average water content. Remarkably, a decrease of the average water content in the biofilter from 67 to 30% resulted in about 50% loss of styrene degradation activity although this is not expected from the desorption isotherm (figure 3.1).

Influence of the water content on biofilter performance

Reduction of the average water content in the biofilters resulted in a decrease of the styrene degradation rate as shown in table 3.1. Styrene degradation activity was lowest at 22% water, which can be explained by the reduced water activity. On the

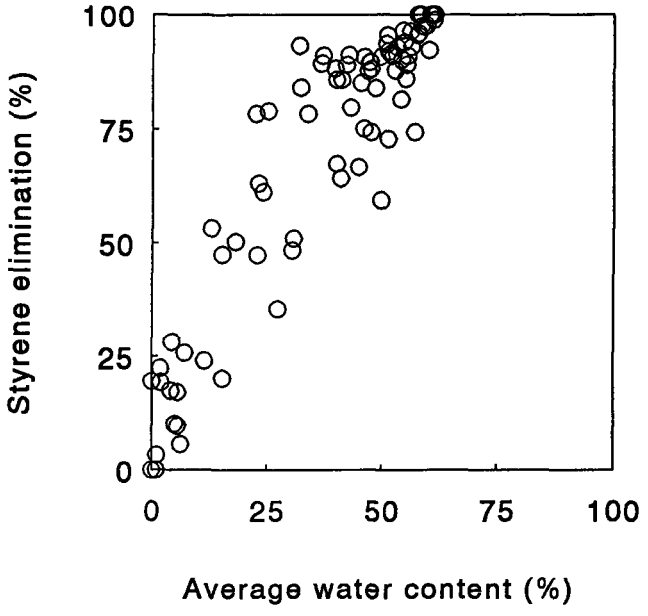


Figure 3.4. Plot of the styrene elimination versus the average water content in biofilters.

Table 3.1. Influence of the average water content on styrene degradation in biofilters with fungi on perlite.

Parameter	Average water content (%)		
	22	37	55
Styrene influent ($g.m^{-3}$)	0.646	0.646	0.646
Styrene effluent ($g.m^{-3}$)	0.550	0.480	0.319
Styrene degradation rate ($g.m^{-3}.h^{-1}$)	14	24	48

other hand, the styrene degradation rates at 37 and 55% water were also considerably lower than the styrene elimination capacity at 66% water, although water contents of 37 and 55% correspond to a water activity of 1 (figure 3.1). Dry weight determinations indicated that the inlet side of the biofilter was dryer than the outlet side. The relatively low elimination capacity at 37 and 55% water may then be explained by low styrene degradation activity in part of the biofilter, i.e. the inlet side. These results imply that compensation for the evaporation of water by the addition of water, thus maintaining a high and constant average water content, is not sufficient when treating air with a relative humidity of 78%.

Determination of gradients

The experiments described above suggest that conditions vary along the filter bed, but the biofilters used were too small for an accurate determination of gradients. Therefore, subsequent experiments were carried out using a biofilter with a height of 1 m. Passing styrene–containing air with a relative humidity of 65% through this biofilter resulted in a decrease of biofilter performance although average water contents were high (figure 3.5). Loss of styrene degradation activity was most prominent at the inlet side of the biofilter, but at the middle and outlet side of the biofilter activity also decreased. Gradients of the water content and water activity along the height of the filter bed clearly show an uneven distribution of water (figure 3.6). Low water activity caused complete loss of styrene degradation activity at the inlet side (0–0.225 m) of the biofilter. Remarkably, styrene degradation rates around the middle part (0.225–0.90 m) were considerably lower than the maximal styrene degradation rate, although the local water activity was near 1. This would imply that,

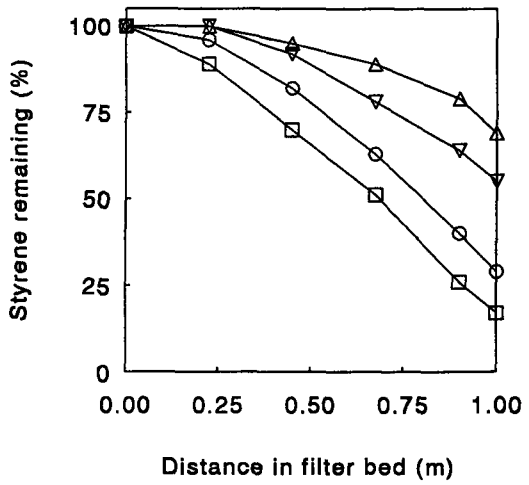


Figure 3.5. Styrene concentration profiles in a biofilter fed with styrene-containing gas (1.4-1.9 g.m^{-3}) with a relative humidity of 65% at a volumetric load of 64 m^{-3}.m^{-3}.h^{-1}. Day 1, average water content 67.6% (□); day 3, average water content 65.9% (○); day 7, average water content 60.4% (▽); day 12, average water content 49.5% (△).

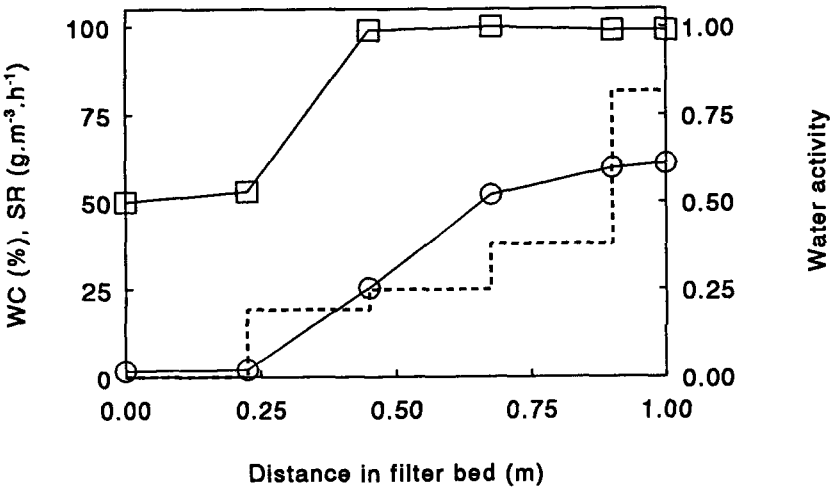


Figure 3.6. Profiles of the water content (WC, ○), water activity (□) and styrene degradation rate (SR, dotted line) in a biofilter after 12 days of operation (styrene 1.4-1.9 g.m^{-3}, relative humidity 65%, volumetric load 64 m^{3}.m^{-3}.h^{-1}).

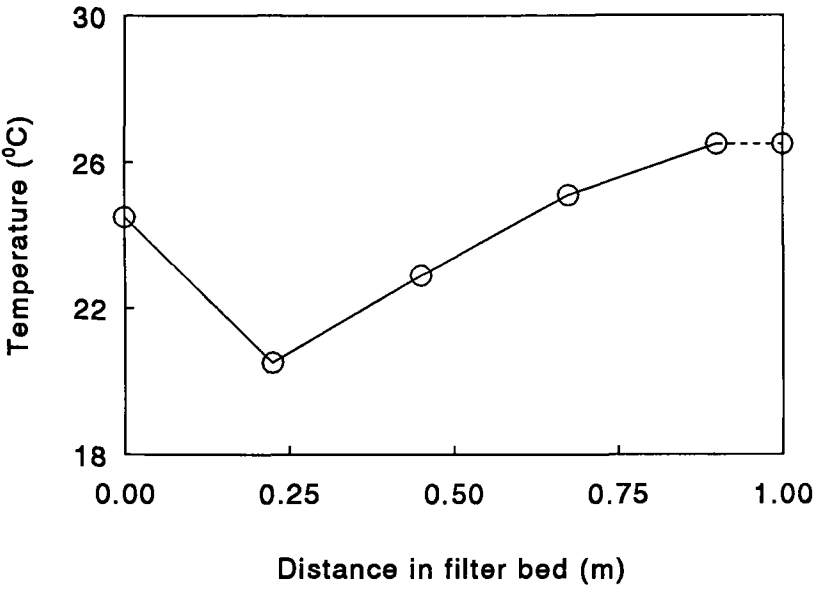


Figure 3.7. Temperature profile in a biofilter treating air with a relative humidity of 65% at a volumetric load of 76 $m^3.m^{-3}.h^{-1}$.

irrespective of the water activity, the styrene degradation rate also depends on the water content. Apart from this, the temperature profile in figure 3.7 shows that temperature may also influence the styrene degradation rate in the filter bed. Heat of styrene oxidation causes an overall increase of temperature from 24.5°C to 26.5°C but at the inlet side of the biofilter the temperature strongly decreases because of the vaporization of water.

DISCUSSION

Biofiltration experiments and batch experiments with *E. jeanselmei* on perlite show that the minimal water activity for styrene degradation is about 0.91. To our knowledge, the influence of the water activity on the performance of compost biofilters has not been investigated as yet. Since bacteria dominate in compost biofilters (Ottengraf, 1986), it is to be expected that by using *E. jeanselmei* an increased tolerance to low water activity of the filter bed may be obtained. Although styrene can

be degraded in biofilters with a water activity of 0.91 corresponding to a water content of 6%, the styrene degradation rate is very low under these conditions. For industrial application, styrene degradation rates should be as high as possible to minimize biofilter size. Humidification of the waste gas and regular additional supply of water to the filter bed to maintain a water content of approximately 60% is therefore necessary. For compost biofilters, water contents of at least 40–65% are required for optimal performance (Ottengraf, 1986: Van Langenhove *et al.*, 1986; Sabo *et al.*, 1993). Since these are average values, a uniform distribution of water over the filter bed is assumed. However, for styrene–degrading biofilters, it is shown that a supply of dry air results in the formation of water content gradients along the height of the filter bed. The presence of dry zones in the filter bed explains the loss of styrene degradation activity at average water contents higher than 32%, i.e. the minimal water content at which the water activity is near 1. Measurement of the average water content, e.g. by weighing of the biofilter, is therefore of little predictive value. Instead, analysis of the filter bed at various depths is necessary to determine water deficiency.

Determination of profiles along the height of the filter bed indicated a loss of styrene degradation activity by decreasing water contents though at a constant water activity of 1. Since it is not the water content, but the water activity which is the critical parameter for microbial activity (Scott, 1957), this would suggest an additional influence of water on styrene degradation in these biofilters. In contrast to our results, improvement of the mass transfer of poorly water–soluble waste gas compounds by reduction of the water content has been shown (De Heyder *et al.*, 1994). However, although styrene is a moderately hydrophobic compound (log K_{ow} = 3.16, Banerjee *et al.*, 1980), we did not observe such an effect because styrene degradation in these biofilters is limited by the reaction rate at concentrations higher than 0.06 $g.m^{-3}$ (chapter 5) rather than by mass transfer. The apparent influence of the water content at a water activity of 1 may be explained by assuming gradients of the water content and water activity in a single particle. Due to the high internal porosity, perlite can adsorb large amounts of water. The styrene–degrading biomass, however, is present at the surface of a biofilm that surrounds the perlite particle (chapter 4). It may thus be hypothesized that supply of dry air to the filter results in drying out of the surface of the biofilm, whereas the internal pores of the particle are still filled with water. Consequently, the water activity as determined with a hygrometer reflects the average water activity in the particle and not that in the biofilm. Because in compost filters the microorganisms are also present in a biofilm surrounding the support material (Ottengraf, 1986), the same may be true for those filters.

In the investigation and modelling of biofilters, a uniform distribution of

microbial activity in the filter bed is usually assumed. Our main finding is that water content, water activity and temperature gradients along the height of the filter bed and probably also gradients of the water content and water activity within single particles are likely to cause variation in microbial activity within the filter bed. Black–box approaches may therefore lead to erroneous results when water is not uniformly present. For biofilters with *E. jeanselmei* on perlite, humidification of the gas to a relative humidity of 90–95% and additional supply of water to the filter bed once every 1–2 weeks is sufficient to maintain high styrene degradation rates at both the inlet and outlet side.

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CHAPTER 4

MICROBIAL POPULATION IN STYRENE-DEGRADING BIOFILTERS

INTRODUCTION

Current research on biofiltration mainly focuses the technical design and operation of biofilters (chapter 1). However, since biofiltration essentially relies on microorganisms as active catalysts, research should also address the microbiology of biofilters. This includes the types of microorganism present, their intrinsic degradation kinetics, the amount and distribution of biomass in the filter bed and the physico–chemical parameters that influence microbial activity in biofilters. The performance of biofilters may be described by a model that is based on pollutant degradation in a biofilm (Ottengraf and Van den Oever, 1983; Ottengraf, 1986). In this model, one of the assumptions is that a biofilm is a smooth homogeneous layer with a thickness that is negligible compared to the diameter of the supporting particles. However, research on biofilms in aqueous systems has shown that biofilms are far from homogeneous. Heterogeneity of the biofilm structure may be related to surface roughness and fluctuating biofilm thickness (Siegrist and Gujer, 1985; Stewart *et al.*, 1993; De Beer *et al.*, 1994; Gjaltema *et al.*, 1994), space competition in multispecies biofilms (Wanner and Gujer, 1985; Rittmann and Manem, 1992), uneven distribution of dead and active biomass (Zhang and Bishop, 1994) and fluctuating cell densities (Zhang and Bishop, 1994; Lawrence *et al.*, 1991). Because biofilm heterogeneities may influence biofilm degradation kinetics (Christensen *et al.*, 1989) and thus biofilter performance, knowledge of the biofilm structure in biofilters is necessary.

In a biofilter with perlite as the support material and inoculated with soil, a mixed population of styrene–degrading fungi was enriched under conditions representative for biofilter operation (chapter 2). This enriched microbial population was the starting inoculum for series of biofiltration experiments. In this chapter, we

report the identification of the species that catalyze the degradation of styrene in biofilters that were designed and used for the investigation of styrene degradation kinetics. Furthermore, results of microscopical investigations of the biofilm structure are presented and discussed in relation to the performance and modelling of styrene degradation in these biofilters.

MATERIALS AND METHODS

Experimental set-up

Biofilters (height 1 m, diameter 0.1 m) filled with 600 g perlite were inoculated with the contents of an old biofilter (chapter 3) and operated for 300 days to study their styrene degradation kinetics. The biofilters were humidified with a mineral medium (chapter 2) approximately once every 2–3 weeks. The procedure of inoculation and operational conditions are described in chapter 5. The microbial population was investigated by cell counting experiments (after 195–216 days of operation of the biofilters) and electron microscopy (after 265 days). The crude lipid content of the filter bed was determined after 300 days.

Microscopy

Samples of the support material containing the biofilm were taken from the inlet and outlet side of the biofilter, stored in closed bottles at room temperature and examined at the day of sampling. For the determination of the biofilm thickness, ten particles from the inlet and outlet side were cut with a razor blade and viewed under a stereo light microscope. The biofilm thickness was determined using a 1 mm scale, divided in steps of 10 μm.

The biofilm structure was investigated using transmission (TEM) and (cryo)scanning electron microscopy (SEM). For TEM, biofilter samples were fixed in 15 $g.l^{-1}$ $KMnO_4$ for 20 min and entrapped in 1.4% (w/v) agar. After overnight poststaining in 10 $g.l^{-1}$ uranyl acetate, samples were dehydrated in a graded ethanol series and embedded in Epon 812. Thin sections were cut with a diamond knife on a LKB Ultratome and examined in a Philips CM10. For SEM, samples were critical point dried as follows: samples were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 2 h at 0^0C, dehydrated in a graded ethanol series and transferred to

amylacetate. Critical point drying was performed at 6°C using CO_2 to remove the amylacetate. Samples were coated with gold and viewed in a Jeol SEM35c scanning electron microscope. For cryo–SEM, samples were dried over silicagel in a desiccator for a few hours to remove excess water. The samples were then frozen in liquid nitrogen, coated with gold as above and viewed at liquid nitrogen temperature in a Jeol SEM35c scanning electron microscope.

Microbial analyses

Samples from the biofilter bed were suspended in 8.5 $g.l^{-1}$ NaCl and vigorously mixed on a magnetic stirrer for 10 min to suspend the cells. The suspension was diluted in 8.5 $g.l^{-1}$ NaCl and samples were plated on plate count agar (PCA), oxytetracycline yeast glucose agar (OGGA) and a solidified mineral medium (MMS). The composition of media is given in chapters 2 and 3. The microbial population was investigated in samples from two identical biofilters. Sampling, diluting and plating were done in duplicate. Incubation was at 25°C. MMS–plates were placed in desiccators containing 0.5 $g.m^{-3}$ styrene in air as the sole source of carbon and energy (chapter 2). Colonies with different morphology, as judged from visual observation, were counted separately and cell counts were related to the dry weight of the samples. Fungal strains were isolated from the OGGA–medium and purified by conventional techniques; identification was done by the Centraalbureau voor Schimmelcultures (CBS, Baarn, the Netherlands).

Determination of the crude lipid content

Biofilter samples were taken at five positions along the height of the filter bed and analyzed in duplicate for the crude lipid content. Crude lipids were extracted from a 4–6 g dried sample with 200 ml 2:1 (v/v) chloroform/methanol in a Soxhlet apparatus for 4 h at 90°C. Chloroform/methanol was removed in a rotary evaporator at 45°C and the amount of crude total lipids was determined by weighing. The average crude lipid content of the filter bed was calculated by taking the mean of the lipid content at different heights. The lipid content of the biomass in the filter bed was calculated assuming that the filter bed (4060 g on day 300) was composed of perlite (600 g), water (2750 g) and biomass dry matter (710 g, by difference). The average water content of the filter bed was determined by drying samples from 5 positions along the height of the filter bed to constant weight at 95°C and averaging the results.

Substrate specificity experiments

Isolated strains were tested for growth on styrene, intermediates in styrene metabolism (chapter 6), crude lipids that were extracted from the biofilter and glucose. Growth was determined in 200 ml mineral medium pH 5.7 (chapter 2) in 1 l serum flasks sealed with a Viton–septum to prevent the escape of volatile substrates. The flasks were inoculated by transferring cells from stock–cultures grown on potato dextrose agar with an inoculation needle. Styrene, styrene oxide, phenylacetaldehyde, 2–phenylethanol, phenylacetic acid, 2–hydroxyphenylacetic acid, homogentisic acid and glucose were added to give a final concentration of 0.25 mM. The concentration of crude lipids was 1 $ml.l^{-1}$. Incubation was stationary at 25^0C. Growth was determined visually over a period of 4 weeks. When growth was absent or doubtful after two weeks of incubation, new substrate was added at 0.25 mM. Growth on 0.5 $g.m^{-3}$ styrene in air was examined using a solidified mineral medium as described for the enumeration of microorganisms.

Pressure drop measurements

These were regularly determined at superficial gas flow velocities between 0.0033 and 0.0569 $m.s^{-1}$ at 5 different heights in the filter bed using a water column.

RESULTS

Styrene degradation started within one day after inoculation of the biofilter. The maximal styrene degradation rate was reached after approximately 35 days. In the following 9 months the gas flow rate (0–1.15 $m^3.h^{-1}$) and the styrene concentration (0–2.4 $g.m^{-3}$) were varied to study styrene degradation kinetics in biofilters (chapter 5). During this period, the styrene load usually exceeded the styrene elimination capacity of 62 $g.m^{-3}.h^{-1}$. Styrene concentration profiles over the filter bed indicated that the amount of styrene degraded around the inlet and the outlet side of the biofilter was comparable. Determination of the water content of the filter bed indicated an approximately even distribution of water. On day 300, the water content in the vicinity of the inlet and outlet side were 64.5 and 70.5% (w/w), respectively. The pH in the filter bed decreased from 5.7 on the day of inoculation to 2.7 on day 40 and then remained constant. The pH at the inlet and outlet side on day 300 were 2.9 and 2.7,

respectively. The water that drained from the biofilter predominantly contained yeasts whereas mycelium–forming cells were less frequently observed. Bacteria were not encountered.

Light microscopy

Low magnification cross–sectional views of particles from the filter bed clearly showed a biofilm surrounding the support particles (figure 4.1). After 300 days of operation of the biofilter, the average thickness of the biofilm at the inlet and outlet side of the biofilter were 240 and 280 μm, respectively. However, large fluctuations in biofilm thickness were observed, both between different particles as well as on single particles, with values ranging between 70 and 600 μm.

Scanning electron microscopy

Figure 4.2 shows the internal structure of perlite itself. The particle contains many internal pores which are separated by thin walls. Although the internal pores are sufficiently large to allow colonization by microorganisms, i.e. up to 50 μm, microbial growth inside the perlite particles in the biofilter was low. The biomass was almost exclusively present in a biofilm on the surface of the perlite particles (figure 4.3).

After a few months of operation of the biofilters, mycelium formation around the middle and outlet side of the biofilter was visible. Microscopical investigation of the biofilm surface at the inlet and outlet side of the biofilter showed marked differences. At the inlet side, the biofilm surface was rough with colonies growing side by side and on top of each other (figure 4.4). Only limited mycelium development was observed at the inlet side of the biofilter. In contrast, the surface of the biofilm at the outlet side of the biofilter showed mycelium formation that initially started in the crevices of the biofilm (figure 4.5). Also mycelium formation that completely covered the biofilm surface was observed (figure 4.6). Higher magnification of the biofilm surface revealed the presence of different types of mycelia with a diameter between 0.75 and 3 μm (figure 4.7). At the outlet side of the biofilter, mycelium formation was most prominent at the surface of the biofilm. Deeper in the biofilm, cells with a diameter up to 10 μm prevailed, presumably yeasts, whereas mycelium was less frequently observed (figure 4.8). Also small cells with a diameter of 1.5 μm were present, which may represent conidia or spores. In the biofilm close to the perlite surface no mycelium was observed but only large cells (figure 4.9).

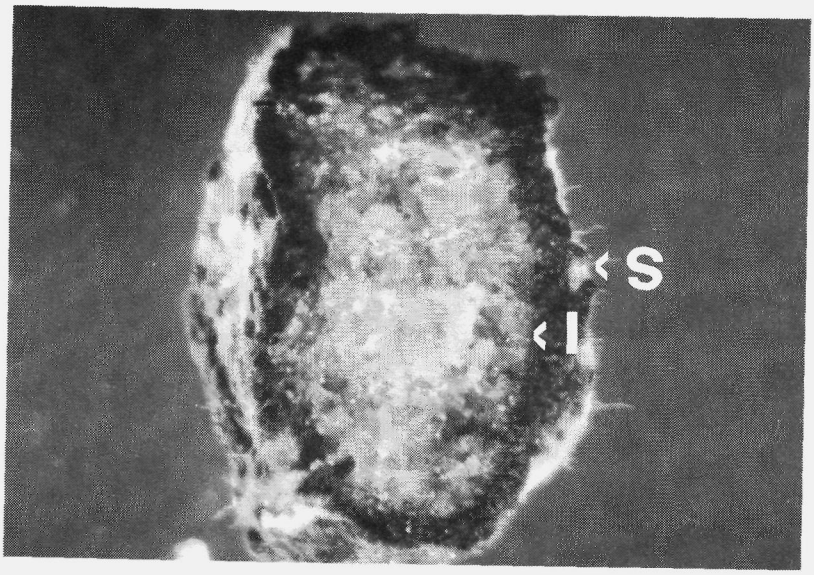

Figure 4.1. Cross-section (light microscopy) of a 1.9x3.5 mm perlite particle surrounded by a dark-coloured biofilm of 180-420 μm; I = perlite/biofilm interface, S = biofilm surface.

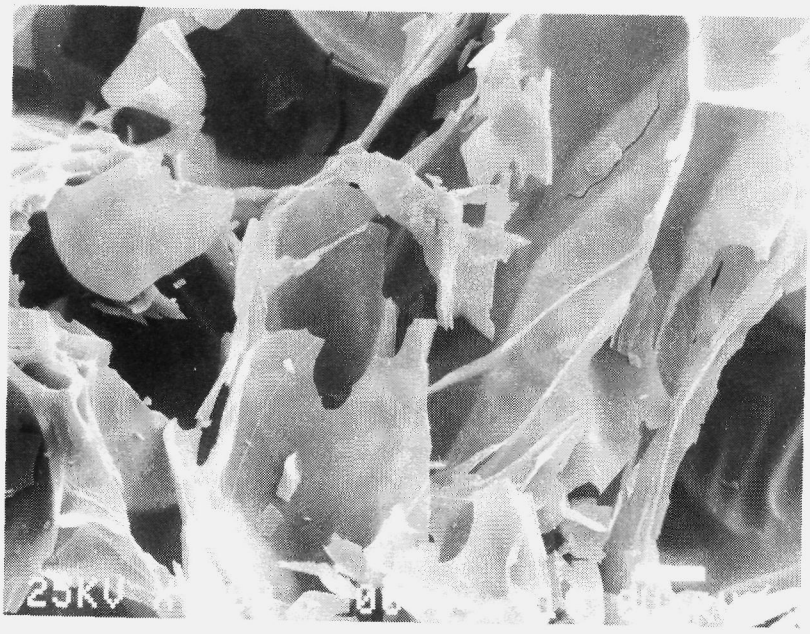

Figure 4.2. SEM of the internal pores of perlite; critical point dried, bar = 10 μm.

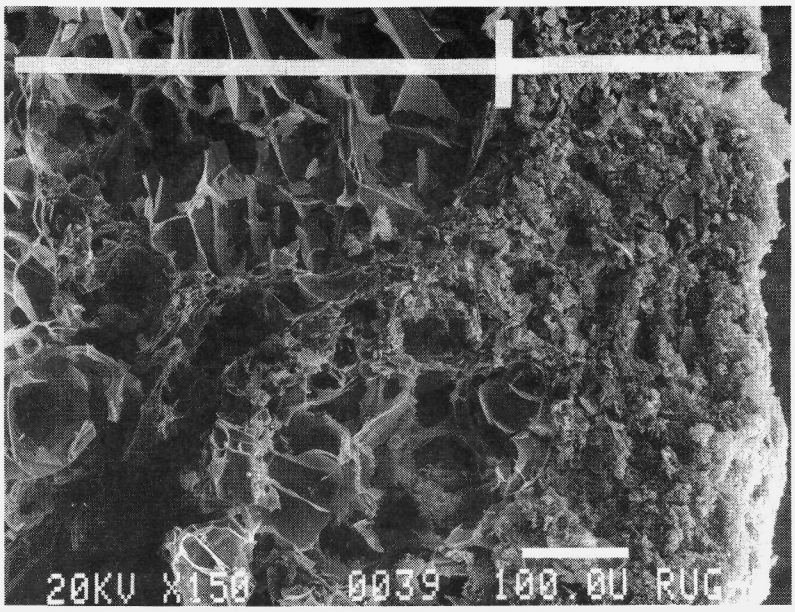

Figure 4.3. SEM of a cross-section of a particle from the inlet side of the biofilter. Biomass is exclusively present in a biofilm of 250 μm (right) whereas the inner pores of the support particle contain no biomass (left); critical point dried, bar = 100 μm.

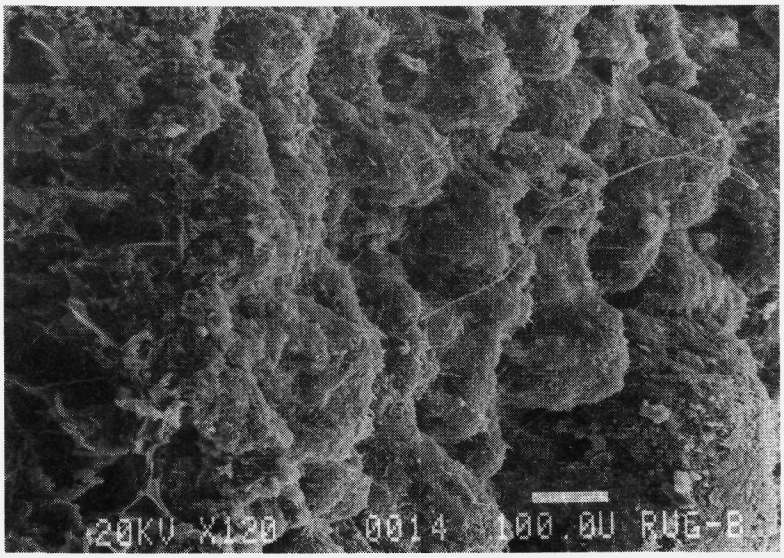

Figure 4.4. SEM of the biofilm surface at the inlet side of the biofilter, showing a mountain-like landscape of colonies; critical point dried, bar = 100 μm.

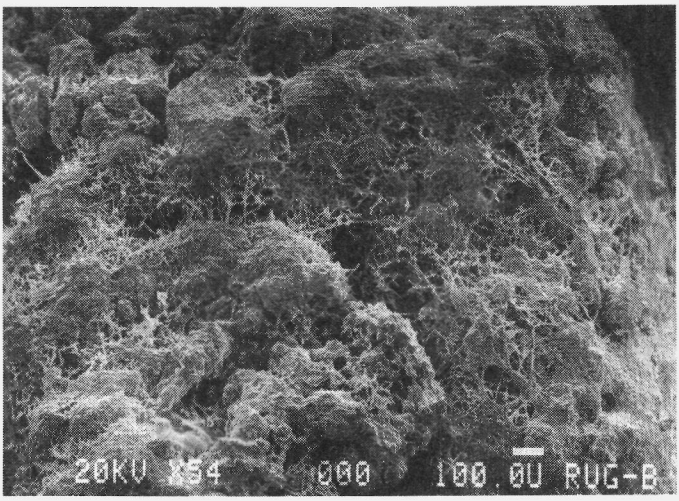

Figure 4.5. Cryo-SEM of the biofilm surface at the outlet side of the biofilter showing mycelium formation in the crevices of the biofilm; bar = 100 μm.

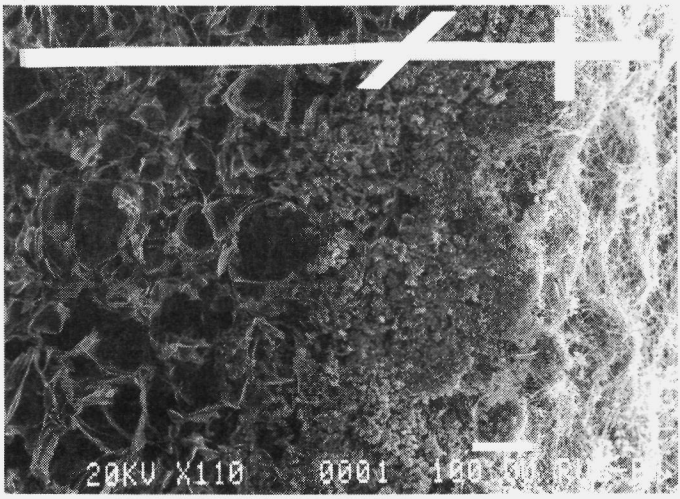

Figure 4.6. SEM of a particle from the outlet side of the biofilter. From left to right: cross-section of the internal pores of the perlite particle, cross-section of the biofilm with a thickness of 150-300 μm and the biofilm surface completely covered with fungal mycelium; critical point dried, bar = 100 μm.

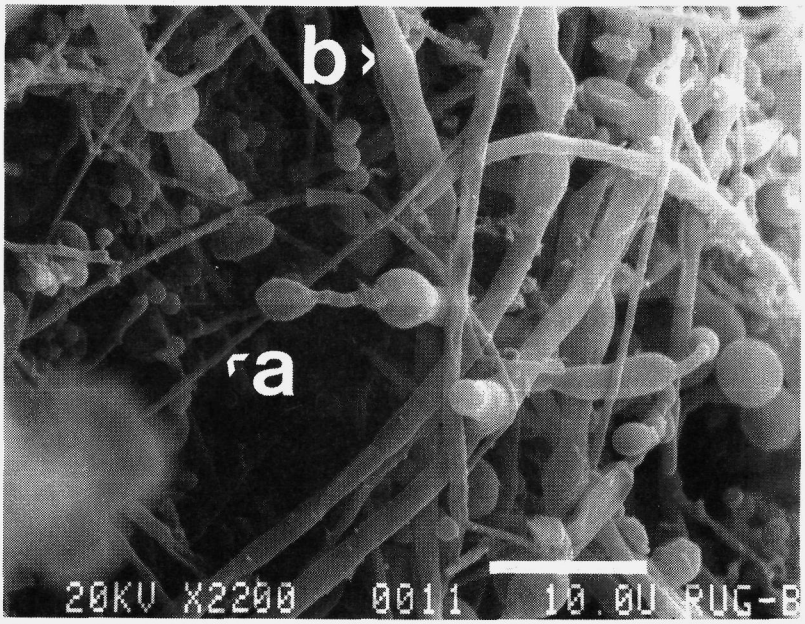

Figure 4.7. SEM of the biofilm surface with mycelium, diameter ranging from 0.75 (A) to 3 μm (B); critical point dried, bar = 10 μm.

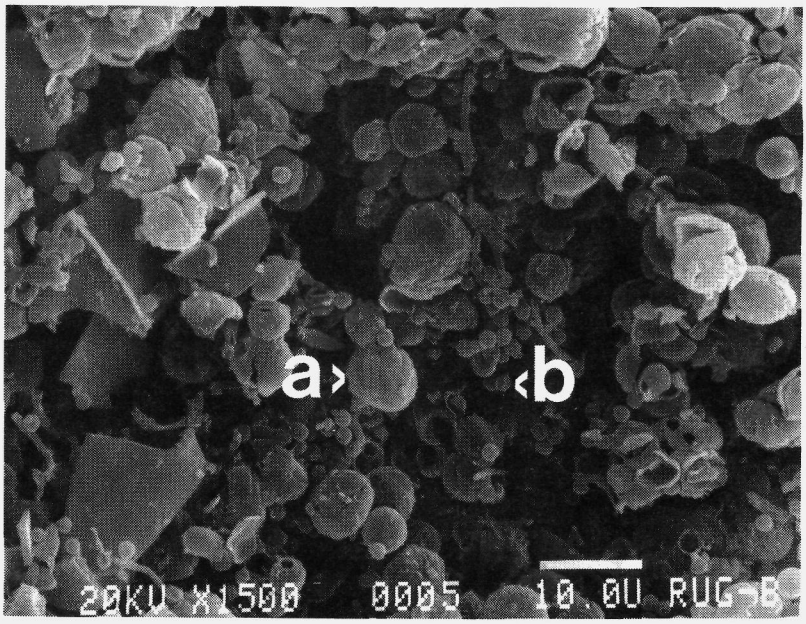

Figure 4.8. SEM of deeper parts of the biofilm at the outlet side of the biofilter with large yeast cells (A) and smaller cells (B), presumably conidia or spores; critical point dried, bar = 10 μm.

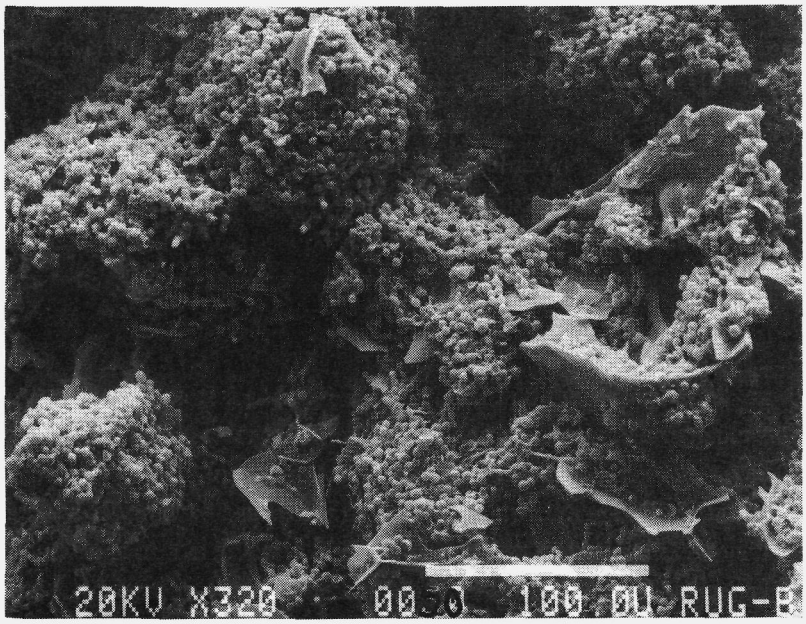

Figure 4.9. Cryo-SEM of the biofilm close to the perlite surface; bar = 100 μm.

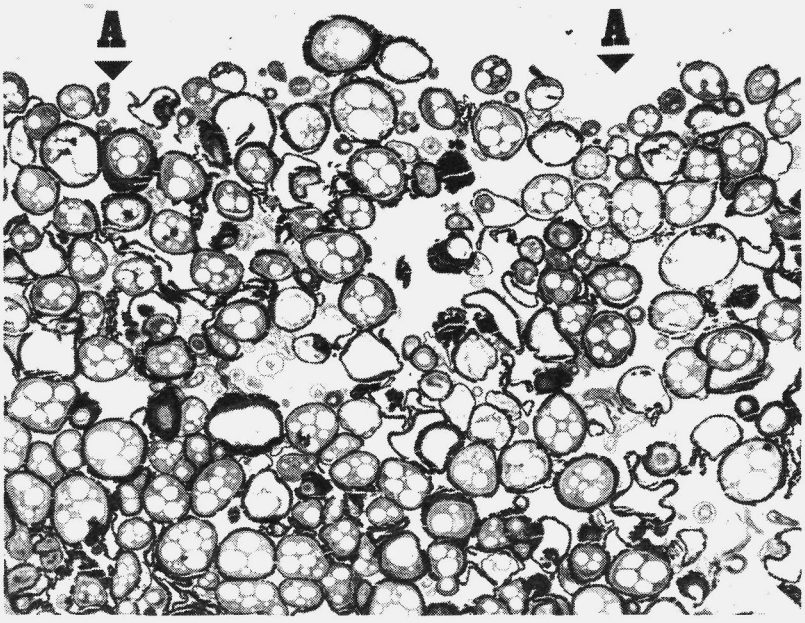

Figure 4.10. TEM of a cross-section of the upper part of the biofilm at the inlet side of the biofilter. At the surface (A), the biofilm is densily packed with large yeast cells but smaller fungal cells are also present.

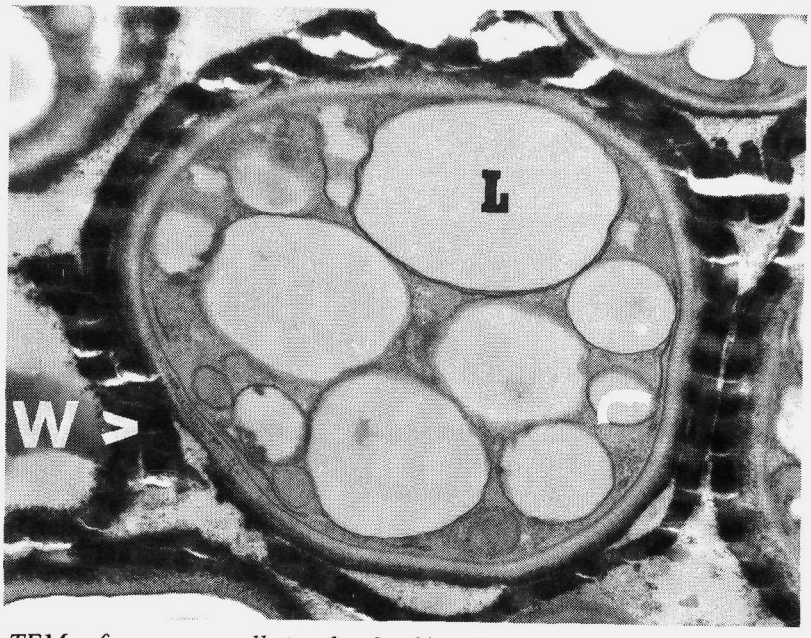

Figure 4.11. TEM of a yeast cell in the biofilm. The cell, probably Exophiala jeanselmei, is thick-walled (W = cell wall) and the cytoplasm contains large bodies (L) that probably contain lipids.

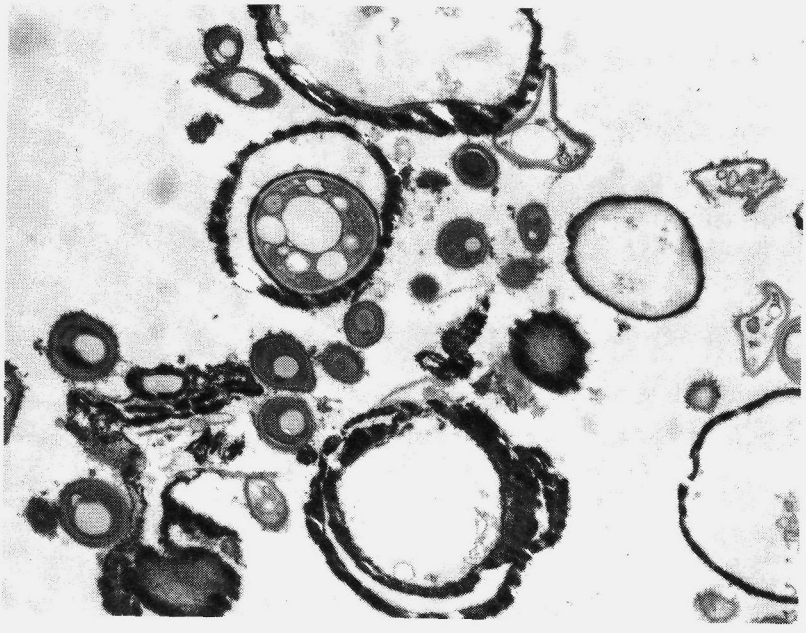

Figure 4.12. TEM of the biofilm surface showing small fungal cells between large yeast cells.

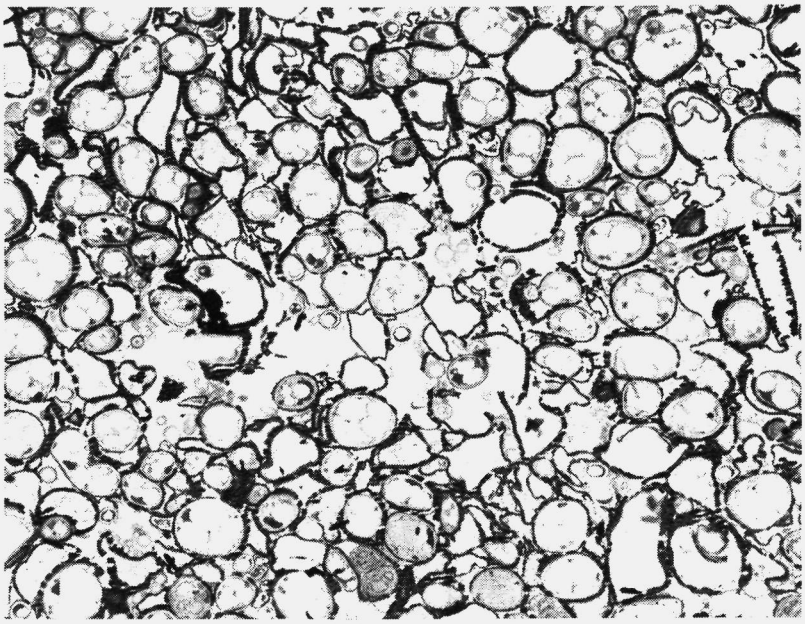

Figure 4.13. TEM of a cross-section of the middle part of the biofilm at the inlet side of the biofilter.

Transmission electron microscopy

TEM studies of the biofilm from samples from the inlet and outlet side of the biofilter show a densily packed biofilm (figure 4.10). Thick–walled yeast cells containing large lipid droplets (figure 4.11) prevailed. Determination of the crude lipid content confirmed the presence of lipid. The lipid content of the filter bed at the inlet and outlet side were 19.2 and 9.4% on dry weight basis, respectively. The average lipid content of the biomass, as present in the filter bed, was 24% on dry weight basis. At the surface of the biofilm, cells with a smaller diameter were observed (figure 4.12). As the SEM–results show mycelium at the biofilm surface (figures 4.6 and 4.7), these smaller cells were identified as cross–sections of fungal mycelium. This is confirmed by the presence of organelles. Differences in TEM views of the outer surface and deeper parts of the biofilm were observed. Whereas the outer surface of the biofilm mainly contained yeast cells with intact cytoplasm (figure 4.10), deeper parts of the biofilm predominantly contained dead cells or cell wall remnants of them (figure 4.13). It appears that the active biomass is mainly localized at the outer surface of the biofilm.

Table 4.1. Identification of fungal strains isolated from styrene-degrading biofilters.

Code	Identification
K1	*Penicillium glabrum* (Wehmer) Westling
K2	*Sporothrix* like[a]
K3	*Cryptococcus hungaricus* (Zsolt) Phaff & Fell
K4	*Exophiala jeanselmei* (Langeron) McGinnis & Padhye
K5	*Stephanoascus ciferii* M.T. Smith
K7	*Sporothrix* like[a]
K15	*Penicillium* species[b]
K17	*Trichoderma harzianum* Rifai

a Conclusive identification not possible.
b Conclusive identification at genus level, further identification not possible.

Identification of microbial species

Samples from the inlet and outlet side of two biofilters after 195–216 days of operation were plated on various media to identify and count the species present in the biofilters. In total eight colony types with different morphology could be distinguished, which were all yeasts and fungi. Bacteria were not detected when samples were plated on PCA or MMS. Strains isolated from OGGA were subcultured to purity and identified as summarized in table 4.1.

Cell counting experiments revealed *Cryptococcus hungaricus*, *Exophiala jeanselmei*, *Stephanoascus ciferii* and the *Sporothrix*–like strain K–2 as the major species present in relatively high numbers (table 4.2). These strains are all yeast or yeast–like species, which confirms the electron micrographs (figures 4.8, 4.9 and 4.10) showing a biofilm mainly containing yeast cells. Results from the duplicate biofilters agree well; some difference was found in the microbial population at the inlet and outlet side of the biofilters. *C. hungaricus* and strain K–7 were detected only at the inlet side. *Penicillium* strain K–15 and *Trichoderma harzianum*, on the other hand, were only detected in samples taken from the outlet side. These mycelium–forming

Table 4.2. Composition of the microbial population at the inlet and outlet side of styrene-degrading biofilters.

Strain[a]	Cell count (log N.g^{-1} dry weight)			
	Filter 1		Filter 2	
	OGGA[b]	MMS[c]	OGGA	MMS
Inlet side biofilter				
K1	+[d]	–	7.1	–
K2	+	–	+	–
K3	8.7	–	+	–
K4	9.0	9.0	8.9	9.0
K5	+	–	9.0	–
K7	–	+	–	7.3
K15	–	–	–	–
K17	–	–	–	–
Outlet side biofilter				
K1	–	–	8.0	–
K2	8.8	8.6	8.2	8.1
K3	–	–	–	–
K4	8.5	8.5	8.7	8.7
K5	9.5	–	7.8	–
K7	–	–	–	–
K15	+	–	+	–
K17	–	–	+	–

a For identification of strains see table 4.1.
b Oxytetracycline yeast glucose agar for enumaration of yeasts and fungi.
c Solidified mineral medium for enumaration of styrene-degrading microorganisms.
d +, log N = 6–7 g^{-1} dry weight; –: log N < 6 g^{-1} dry weight.

Table 4.3. Growth substrate specificity of strains isolated from styrene-degrading biofilters.

Growth substrate[a]	Strain[d]							
	K1	K2	K3	K4	K5	K7	K15	K17
0.5 $g.m^{-3}$ styrene in air[b]	–[e]	+	–	+	–	+	–	–
0.25 mM styrene in liquid	–	+	–	+	–	+	–	–
styrene oxide	+	+	–	+	–	+	–	+
phenylacetaldehyde	+	+	–	+	–	+	+	+
2–phenylethanol	+	+	–	+	±	+	+	+
phenylacetic acid	+	+	–	+	–	+	+	+
2–hydroxyphenylacetic acid	+	–	–	+	–	–	+	+
homogentisic acid	+	+	–	+	–	+	–	–
1 $ml.l^{-1}$ crude lipids[c]	+	+	–	+	+	+	+	+
glucose	+	+	+	+	+	+	+	+

a Growth in liquid cultures with 0.25 mM substrate or as specified.
b Growth on solidified mineral medium.
c Crude lipids extracted from biofilter samples.
d For identification of strains see table 4.1.
e +, growth; ±, poor growth that could not be enhanced by repeated substrate addition; –, no growth.

species are probably those growing on the surface of the biofilm around the middle and outlet side of the biofilm as observed by microscopy (figures 4.6, 4.7 and 4.12). When hypha from the surface of the biofilm were transferred to potato dextrose agar, *Penicillium* strain K–15 and *P. glabrum* but not *T. harzianum* grew.

Sporothrix–like strains K–2 and K–7 and *E. jeanselmei* were detected on MMS which implies that these species can grow on styrene as the sole source of carbon and energy. The other species were only detected on OGGA. The results in table 4.2 suggest that *E. jeanselmei* is a major styrene–degrading species present at both the inlet and outlet side of the biofilter.

Substrate specificity of isolated strains

Confirming the results of the cell counting experiments (table 4.2), only *E. jeanselmei* and the *Sporothrix*–like strains K–2 and K–7 were able to grow on styrene as the sole source of carbon and energy (table 4.3). Potential intermediates in styrene metabolism (chapter 6) could be used as growth substrates by species not capable of growth on styrene, i.e. *P. glabrum*, *T. harzianum* and *Penicillium* strain K–15. All species except *C. hungaricus* could grow on the lipids that were extracted from the filter bed. These results indicate that a mixed population of species has established, with different species capable of growth on styrene and species incapable of growth on styrene but growing on lipids and/or styrene intermediates.

Pressure drop measurements

During the first months, the pressure drop over the filter bed slowly increased. A sharp increase of the pressure drop was observed after approximately 9–10 months of operation of the biofilters (figure 4.14), which coincided with mycelium formation in the filter bed. Pressure drop measurements at different heights of the filter bed indicate that pressure build–up was highest at the outlet side of the biofilter (figure 4.15) where mycelium formation was most prominent.

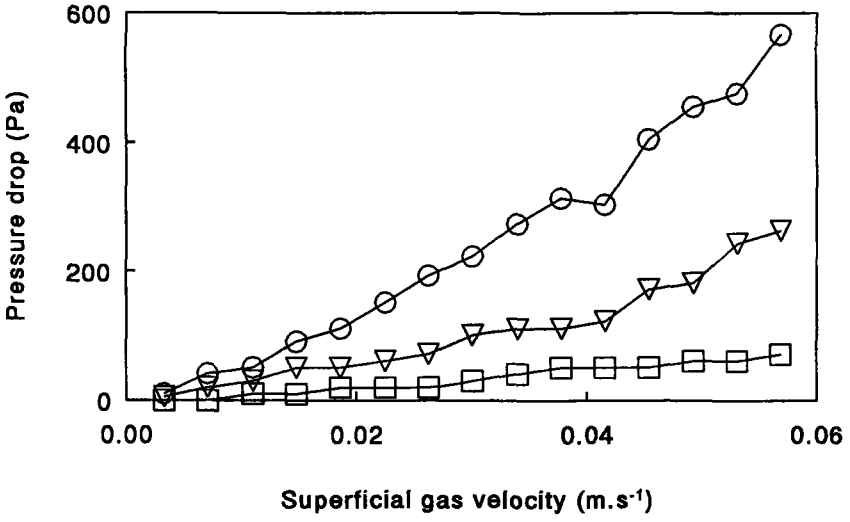

Figure 4.14. Pressure drop measurements over 0.9 m filter bed after 0 (□), 265 (▽) and 294 (○) days.

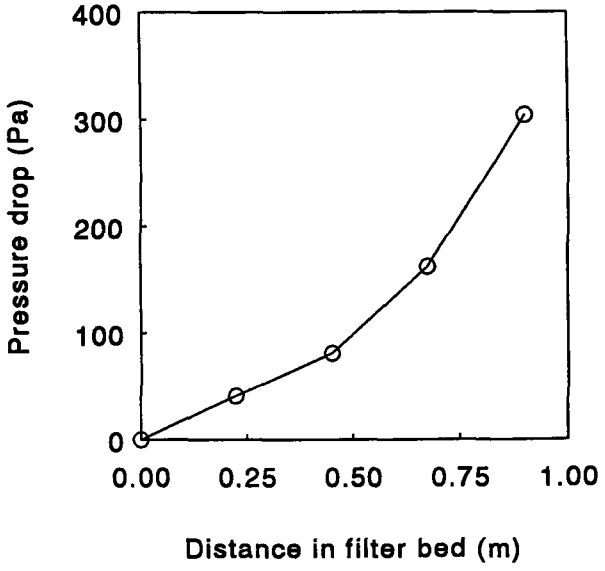

Figure 4.15. Pressure drop profile at a superficial gas velocity of 0.0416 m.s^{-1} after 294 days.

DISCUSSION

Microbial populations in biofilters with support materials as compost contain a wide variety of bacteria and fungi (Ottengraf, 1986; Rieneck, 1992). Biofilters degrading styrene have been reported, but the species catalyzing the degradation have not been identified (chapter 1). In contrast to biofilters with these support materials, the microbial population in styrene–degrading biofilters with perlite as the support material solely consists of fungi. Acidification of the filter bed may have created an environment selective for acid–tolerant fungi.

Cell counting experiments indicate that *E. jeanselmei* is the major styrene–degrading species present at the inlet and outlet side of the biofilter. TEM micrographs show a biofilm that is mainly composed of large yeast cells which are characterized by a thick cell wall and presumably lipid bodies in the cytoplasm. These cells are tentatively identified as *E. jeanselmei*. Electron micrographs of the cells in the biofilm show a similar overall morphology to those of *Exophiala dermatitidis* (Oujezdsky *et al.*, 1973), a species closely related to, and morphologically difficult to distinguish from, *E. jeanselmei* (Tintelnot *et al.*, 1991; De Hoog and Haase, 1993). In addition, *E. dermatitidis* cells contain large lipid bodies that occupy most of the cytoplasm (Oujezdsky *et al.*, 1973); the lipid content in the cells may amount to 33% (Calderone, 1976). This is in agreement with TEM views of the cells in the biofilm and the high lipid content of the filter bed. Because *E. dermatitidis* could not be isolated, we conclude that the major species responsible for styrene degradation in these biofilters is *E. jeanselmei*. Also, *E. jeanselmei* was the only styrene–degrading species that could be isolated in relatively high numbers from the enriched biofilter (chapter 2) as well as all other biofilters investigated. The influence of environmental parameters on the styrene degradation may be investigated more easily with pure cultures of styrene–degrading microorganisms than with biofilters. The present results show that *E. jeanselmei* should be selected for these experiments.

The biomass was exclusively present in a biofilm on the surface of the perlite support particles. The type of biofilm as proposed by Ottengraf and Van den Oever (1983) and Ottengraf (1986) may thus be valid when modelling styrene degradation in these biofilters. However, electron microscopic pictures revealed a heterogeneous biofilm, in contrast to the smooth and homogeneous biofilm as assumed in the models. Possible consequences for the modelling of styrene degradation should therefore be identified.

The specific surface area of the biofilm is usually assumed to equal the specific

surface area of the substratum. However, microscopical investigations revealed a irregular surface with protrusions and crevices and, consequently, the biofilm surface area will be larger than the surface area of the substratum. Depending on the mathematical description of the morphology of the biofilm, the surface area enlargement may be 1.2– to 11–fold (Kugaprasatham *et al.*, 1992; De Beer *et al.*, 1994; Gjaltema *et al.*, 1994). Consequently, substrate flux into the biofilm will be higher than predicted from the specific surface area of the substratum (Siegrist and Gujer, 1985; Kugaprasatham *et al.*, 1992; De Beer *et al.*, 1994; Gjaltema *et al.*, 1994).

Individual measurements of biofilm thickness may vary widely (Siebel and Characklis, 1991; Drury *et al.*, 1993; Stewart *et al.*, 1993), reflecting biofilm surface roughness. The same was found in styrene–degrading biofilters. Although the average biofilm thickness at the inlet and outlet side of the biofilter after 300 days were comparable, i.e. 240 and 280 μm, the actual thickness varied from 70 to 600 μm. It is therefore likely that the thicker layers of the biofilm become substrate–limited, although this is not expected from the average biofilm thickness (Gjaltema *et al.*, 1994).

TEM micrographs of the biofilm suggest that the active biomass was mainly present at the surface of the biofilm whereas the majority of the cells in deeper parts of the biofilm were dead (figures 4.10 and 4.13). The average biofilm thickness as an indication of biomass quantity therefore overestimates the amount of active biomass. Styrene degradation kinetics in biofilters indicate that the active biofilm is about 80 μm thick (chapter 5) which suggests that about 70% of the biofilm is not involved in styrene degradation. For biofilms in aqueous systems, the thickness of the active biofilm is usually related to the penetration depth of substrates such as oxygen (Kornegay and Andrews, 1968; Hoehn and Ray, 1973). The thickness of the active biofilm in styrene–degrading biofilters may also be limited by starvation of the cells in deeper parts of the biofilm, irrespective of the penetration depth of styrene or oxygen. Kinetic studies on styrene degradation in the biofilters and determination of parameters such as the growth rate, death rate and maintenance requirements would be necessary to determine the factors that limit the thickness of the active biofilm.

Substrate specificity experiments indicate the presence of a primary population containing species capable of degrading styrene and a secondary population containing species not capable of degrading styrene. The biofilm may be characterized as a thick layer of styrene–degrading yeasts or yeast–like species, notably *E. jeanselmei*; the surface of this layer is covered with mycelium–forming species not able to degrade styrene. Intermediates of styrene metabolism in *E. jeanselmei* and lipids were identified as possible sources for growth of most of the species of the secondary population. It

is not clear why mycelium formation was not observed at the inlet side of the biofilter. Perhaps the relatively high styrene concentration at the inlet side prevented growth of mycelium–forming species unable to grow on styrene because all other measured parameters were comparable at both sides of the filter bed.

Long–term stability of biofilters is an important requirement for industrial application of biofilters. Continuous biomass formation, growing at μ_{max} as assumed in Ottengraf's model, may result in increasing pressure drops and eventually clogging of the filter bed (Diks, 1992; Hartmans, 1994). Styrene–degrading biofilters with fungi on perlite show a high and constant styrene elimination capacity over at least ten months. However, a sharp increase in the pressure drop was observed after 9–10 months, which was most likely caused by mycelium formation at the middle and outlet side of the biofilter. Although the styrene elimination capacity remained constant, the increasing pressure drop may limit the operational life span of these biofilters. As a rule of thumb, the pressure drop over 1 m filter bed and at volumetric loads of 100–500 $m^3.m^{-3}.h^{-1}$ (0.028–0.139 $m.s^{-1}$) should not exceed 500–1000 Pa (Don, 1985). It may then be predicted that operation of the biofilters under the conditions described in this chapter will eventually result in clogging of the filter bed. For the practical application of styrene–degrading biofilters, both the maximal styrene degradation rate and the long–term operational stability should be considered. Extension of the use of styrene–degrading biofilters may be possible by decreasing the styrene load or by limiting the availability of other essential nutrients to control the rate of biomass formation.

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CHAPTER 5

MODELLING OF STYRENE DEGRADATION IN BIOFILTERS

INTRODUCTION

Styrene–degrading biofilters containing fungi on perlite possess properties that are promising for use in industrial waste gas treatment. Distinct advantages compared to compost biofilters are the high styrene elimination capacity (chapters 2 and 3), the operational stability (chapter 4) and the tolerance to low pH (chapter 2) and drying out conditions (chapter 3). For scaling–up, a mathematical model that describes the performance of these filters is required. According to the classification of biotechniques for waste gas treatment (Ottengraf, 1987), styrene–degrading biofilters with fungi on perlite can be compared with traditional biofilters in which support materials of natural origin, such as compost or peat, are used. Characteristic features of biofilters are: immobilization of microorganisms on support materials, pollutant degradation in a biofilm and, in contrast to biotrickling filters, the absence of a recirculating water phase (chapter 1).

Ottengraf and Van den Oever (1983) and Ottengraf (1986) have developed a general mathematical model for compost biofilters, which is based on the degradation of pollutants in a biofilm. In this model, the biofilm is considered a thin and homogeneous layer of active cells. As in other biofilter models (Tiwaree *et al.*, 1992; Shareefdeen *et al.*, 1993; Ergas *et al.*, 1994; Yang and Allen, 1994; Hodge and Devinny, 1994 and 1995; Deshusses *et al.*, 1995[a] and 1995[b]), it is also assumed that pollutant degradation rates are constant throughout the filter bed, though distinction is generally made between zero– and first–order degradation kinetics. This implies a uniform distribution of biomass in the filter bed and equal conditions with respect to parameters that influence the specific microbial activity. In case of compost biofilters, these assumptions have generally not been validated because of the complexity of the

filter bed (heterogeneous support materials, mixed microbial populations of often unknown composition).

Biofilters with styrene–degrading fungi growing on perlite are well–defined systems with respect to the support material, the microbial population and biofilm characteristics (chapter 4). In the present study, we have attempted to validate the model of Ottengraf and Van den Oever (1983) and Ottengraf (1986) with respect to its possible utility in describing styrene degradation in these filters. Special attention is given to the assumption of a uniform distribution of active biomass in the filter bed as well as in the biofilm.

MATERIALS AND METHODS

Microorganisms and media

Exophiala jeanselmei was maintained on potato dextrose agar at 4^0C; the fungus was cultured for 12–14 days in 4 l mineral medium (chapter 2) in 5 l erlenmeyer flasks at 25^0C. Inoculation of the medium was as previously described (Cox *et al.*, 1993). Styrene–containing gas with a concentration of 0.4–0.6 $g.m^{-3}$ was continuously bubbled through the culture at a rate of about 50 $l.h^{-1}$. For investigation of intrinsic styrene degradation parameters, cells were harvested by centrifugation, washed twice in 50 mM potassium phosphate buffer pH 5.7 and resuspended in this buffer. Biofilters for the determination of styrene degradation kinetics were inoculated with the contents of an old biofilter, containing a mixed population of fungi on perlite (chapter 3). The inoculum contained *E. jeanselmei* as the major species responsible for styrene degradation. The biofilter used for studies of the start–up period was inoculated with a pure culture of *E. jeanselmei*, grown on styrene as described above.

Determination of intrinsic styrene degradation parameters

The rate of styrene degradation by *E. jeanselmei* was determined in duplicate in 0.13 l serum bottles sealed with Viton septa. 9 ml Cell suspension (50–70 $mg.l^{-1}$ protein) and 2 ml 1.5 mM styrene were added at time zero, resulting in 1.6 $g.m^{-3}$ styrene in the gas phase and 0.10 mM styrene in the liquid phase. The serum bottles were vigourously shaken in a water bath at 25^0C. Styrene degradation was followed by GC analysis of 50 µl headspace samples that were taken approximately once every

3 minutes using gastight syringes (Hamilton, Reno, Nev.).

The influence of the pH, temperature and the styrene concentration on the styrene oxidation rate was determined in O_2 uptake experiments using a biological oxygen monitor (Yellow Springs Instrument, Yellow Springs, Oh.). The general procedure was: saturation of 4 ml cell suspension (40–80 $mg.l^{-1}$ protein) pH 5.7 with air at 25°C, measurement of the endogeneous respiration rate for 5 minutes, addition of 100 µl 1.5 mM styrene in demineralized water and measurement of the O_2 uptake for 5 minutes. All experiments were carried out in duplicate; styrene–dependent O_2 uptake rates were corrected for the endogeneous respiration. For determination of the influence of the pH, the pH of the cell suspension was adjusted to 1.0–8.0 with 0.1 M NaOH or HCl. For determination of the influence of the temperature, a circulating water bath was connected to the biological oxygen monitor to control temperatures in the range of 23–42°C. Styrene oxidation rates at low substrate concentration (7.5–40 µM) were determined by adding different amounts of 1.5 mM styrene in demineralized water to 4 ml cell suspension. Styrene oxidation rates at high substrate concentration (25–1000 µM) were determined by adding 50 µl of different concentrations of styrene in dimethylsulfoxide to 4 ml cell suspension. The amount of dimethylsulfoxide added had no effect on the endogeneous respiration rate or the styrene oxidation rate.

Biofiltration equipment, inoculation and daily operation

The biofiltration equipment (figure 5.1) contained 3 biofilters (height 1 m, diameter 0.1 m), each filled with 600 g perlite (sieved fraction 3.45–4.75 mm; Pull B.V., Rhenen, the Netherlands). The void fraction (external pores, i.e. excluding the internal pores in the particles) of the filter bed was 0.41. Styrene–containing air with a relative humidity of 90–95% was prepared by mixing two compressed air streams which were saturated with styrene and water, respectively. The styrene concentration of the feed gas was varied by changing the volume ratio of the two gas streams. The biofilters were operated in a downflow mode. Each biofilter received gas with the same styrene concentration but gas velocities could be varied independently. Styrene in the influent and effluent gas was determined by GC analysis of 50 µl samples using gastight syringes. CO_2 production was followed by measurement of the CO_2 concentration in the influent and effluent gas once every two hours using an automatic sampling device and an infra–red CO_2 analyzer. Styrene concentration profiles along the height of the filter bed were determined using an automatic sampling device connected to a total hydrocarbon analyzer. Samples were taken at 0 (top), 0.225, 0.45, 0.675, 0.90 and 1.0 m (bottom) in the filter bed.

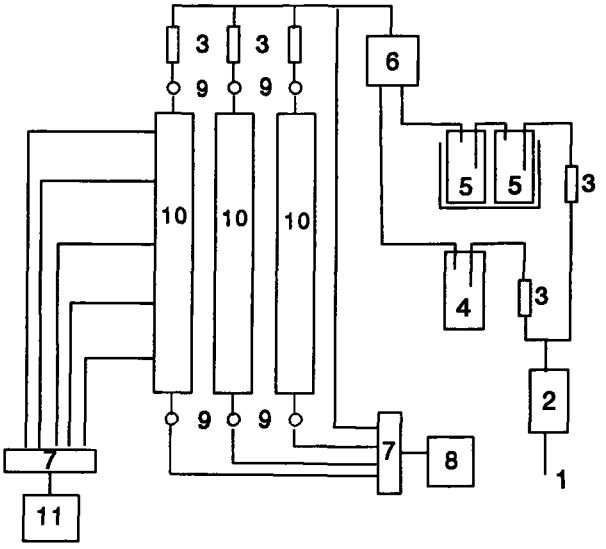

Figure 5.1. Biofiltration equipment. 1, compressed air; 2, activated carbon filter; 3, needle valve with flow meter; 4, gas-washing bottle with styrene; 5, gas-washing bottles with water in water bath set at 31^{0}C; 6, mixing chamber; 7, automatic channel selector; 8, CO_2 analyzer; 9, sampling port for GC analysis; 10, biofilter; 11, total hydrocarbon analyzer.

The biofilters for the investigation of styrene degradation kinetics were inoculated by mixing perlite, mineral medium and the contents of an old biofilter in the ratio 0.28:0.66:0.06 (w/w). The biofilter for the investigation of the start–up period was inoculated by pouring 3 l culture of styrene–grown *E. jeanselmei* on the filter bed. Styrene–containing air was continuously passed through the biofilters over a period of 375 days with varying styrene concentration and gas velocity. The biofilters were regularly weighed to determine the evaporation of water. Mineral medium was added to the biofilters once every 2–3 weeks by pouring approximately 6 l medium on top of the filter bed until complete saturation. After 30–60 minutes, excess medium was drained off, after which the supply of styrene–containing gas was continued. Occasionally, approximately 0.3 l demineralized water was added to the top of the filter bed when the rate of styrene degradation was relatively low and the biofilter weight indicated extensive evaporation of water.

Biofiltration experiments

The biofilter inoculated with a pure culture of *E. jeanselmei* was used to determine the time required to reach the maximal styrene degradation rate. After 35 days, experiments were stopped and the filter bed was analyzed for biomass parameters.

Biofilter performance in the reaction–limited regime was investigated in three identical biofilters 100–300 days after inoculation. Styrene degradation and CO_2 production rates were determined at styrene concentrations of 0.1–2.4 $g.m^{-3}$ and volumetric loads of 39–142 $m^3.m^{-3}.h^{-1}$. Analyses of the influent and effluent gases were performed 1–3 days after a change of the styrene inlet concentration and/or the gas velocity, to ensure stabilization of the filter. A new steady state was normally reached within 12 h. At the end of the experiment, one biofilter was analyzed for the composition of the filter bed. Biofilter performance in the diffusion–limited regime was investigated in one biofilter, 315–375 days after inoculation. Styrene degradation rates were determined at a constant styrene load of 75 $g.m^{-3}.h^{-1}$. Styrene inlet concentrations were varied between 0.022–0.11 $g.m^{-3}$ (i.e., volumetric loads of 684–3322 $m^3.m^{-3}.h^{-1}$). The influence of the oxygen concentration was investigated by determination of styrene degradation and CO_2 production rates at 20 and 40% oxygen in the inlet gas. The styrene concentration was 2.2 $g.m^{-3}$ and the volumetric load was 90 $m^3.m^{-3}.h^{-1}$.

Analysis of the filter bed and analytical methods

The chemical composition of the filter bed was investigated after 300 days of operation; some of the experiments were also carried out with a biofilter operated for 35 days. Samples were taken at five different positions in the filter bed (0–0.11 = inlet, 0.11–0.34, 0.34–0.57, 0.57–0.79 and 0.79–1 m) and analyzed for pH and content of water, ash, protein and crude lipids. Control experiments were carried out with fresh perlite without biofilm. The water content in the filter bed was determined by drying samples to constant weight at 95°C (quadruplicate experiments). The pH was measured after suspending 3–4 g of sample in demineralized water (1:5 w/v) and 15 min vigourously stirring on a magnetic stirrer (quadruplicate). The ash content was determined by combustion of 1–2.5 g of dried sample at 600°C for 45 min (triplicate). Protein (triplicate) and crude lipids (duplicate) were determined as previously described (chapters 3 and 4, repectively). The biofilm thickness was determined microscopically (chapter 4) by examining 20 particles taken from the outlet side of the

biofilter (0.79–1 m).

Styrene concentration profiles and the styrene and CO_2 concentration in the influent and effluent gas were determined as described in chapters 2 and 3. For GC styrene determination, the column and injector temperature were increased to 150°C.

RESULTS

Styrene degradation by Exophiala jeanselmei

Investigation of the microbial population in styrene–degrading biofilters showed the presence of eight fungal strains; *E. jeanselmei* was identified as the major species responsible for styrene degradation (chapter 4). This species was therefore selected to determine the specific styrene degradation activity and to identify kinetic parameters that influence the specific styrene degradation activity in the filter bed.

When styrene was added to closed bottles with a suspension of *E. jeanselmei* cells, the styrene head–space concentration decreased linearly. The maximal styrene degradation rate was 21 $\mu g.mg^{-1}protein.min^{-1}$ at 25°C and pH 5.7. The same rate was found at pH 3.0. However, *E. jeanselmei* immobilized on perlite and without the presence of free liquid (but water activity $\approx$ 1) degraded styrene at a rate of 3.1 $\mu g.mg^{-1}protein.min^{-1}$ (chapter 3). The *E. jeanselmei* specific activities in suspension or immobilized on perlite therefore differ. The styrene oxidation rate in oxygen uptake experiments was maximal at the lowest concentration tested. The K_s for styrene is therefore lower than 7.5 μM, which corresponds to a styrene gas phase concentration of 0.117 $g.m^{-3}$. However, the biofiltration experiments indicate that K_s is even (much) lower (see next section). Styrene has been shown to be toxic for microorganisms (figure 5.2; Hartmans *et al.*, 1990; Cox *et al.*, 1993). Oxidation rates at high styrene concentrations fitted the Haldane equation (Moser, 1981) (figure 5.2). The calculated inhibition constant was 0.21 mM, which corresponds to a styrene gas phase concentration of 3.25 $g.m^{-3}$. Biofiltration experiments were carried out at styrene concentrations of 0.022–2.4 $g.m^{-3}$. The results obtained with *E. jeanselmei* indicate that in this range zero–order degradation kinetics may be assumed. Styrene oxidation rates were approximately constant between 22.5–33°C (figure 5.3) and pH 1.5–8 (figure 5.4). Temperature profiles in the filter bed may exist due to evaporation of water and metabolic heat production (chapter 3). However, the effect of these phenomena on the specific styrene degradation activity in the filter bed is of minor

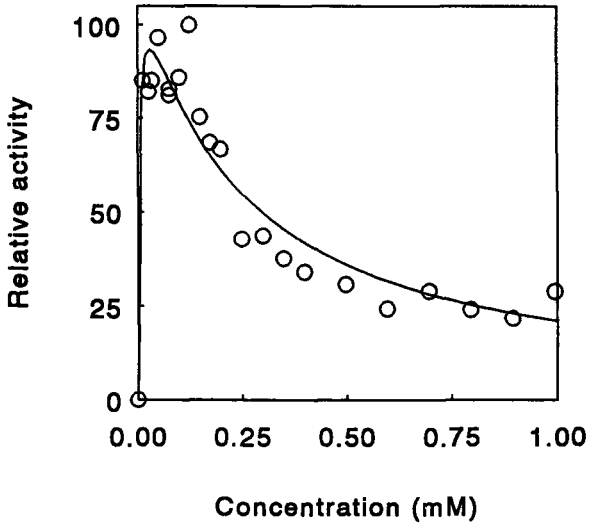

Figure 5.2. Styrene oxidation by Exophiala jeanselmei at high styrene concentrations (100% = 8.6 μg O_2.mg^{-1}protein.min^{-1}). □, experimental results; solid line, Haldane equation with K_i = 0.21 mM.

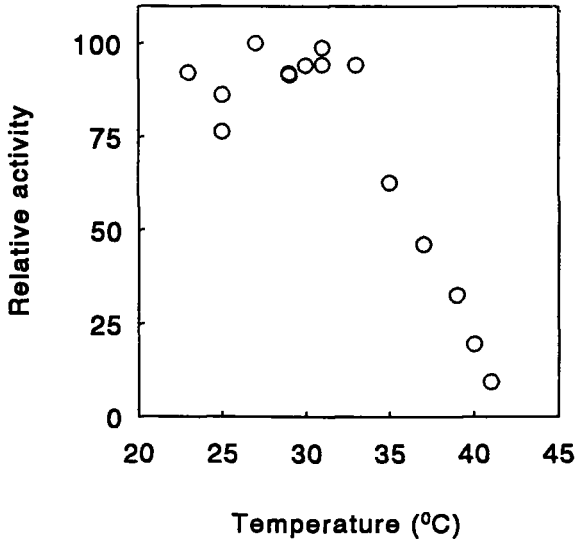

Figure 5.3. Influence of temperature on the styrene oxidation rate of Exophiala jeanselmei (100% = 12.7 μg O_2.mg^{-1}protein.min^{-1}).

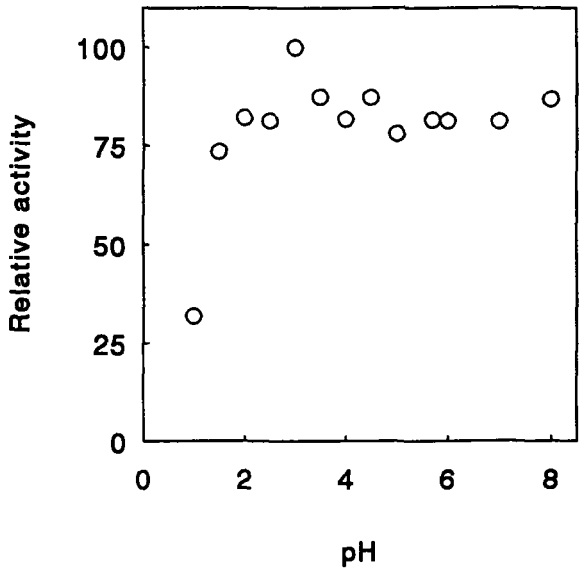

Figure 5.4. Influence of pH on the styrene oxidation rate of Exophiala jeanselmei (100% = 8.7 μg O_2.mg^{-1}protein.min^{-1}).

importance in view of the temperature optimum, the high relative humidity and the low styrene concentration of the feed gas. Analysis of the filter bed indicated that the pH and water content are not likely to cause differentiation of the specific styrene degradation activity along the filter bed (table 5.1). Acidification of the filter bed was rapid (from pH 5.8 to below pH 3 within 35 days), but an adverse effect on biofilter performance was not observed which confirms the tolerance of *E. jeanselmei* to low pH environments (figure 5.4). The water content in the filter bed was generally sufficiently high (table 5.1) to exclude the possibility of inhibition of styrene degradation by dry conditions (chapter 3), although drying out of the inlet side of the filter bed was sometimes observed.

Biomass distribution in the filter bed

Perlite is an inert material and the organic matter or ash content of the filter bed thus reflects the presence of biomass. As judged from the ash content at different

Table 5.1. Chemical analysis of the filter bed, after 300 days of operation of the biofilter, and fresh perlite.

Distance in filter bed (m)	pH	Water (% w/w)	Ash (% w/w)[a]	Protein (% w/w)[a]	Crude lipids (% w/w)[a]	Organic matter excluding lipids (% w/w)[a]
0–0.11	2.9	64.6	54.1	0.35	19.2	26.6
0.11–0.34	2.7	64.4	51.2	0.43	17.1	31.2
0.34–0.57	2.6	68.6	60.9	0.44	9.8	28.7
0.57–0.79	2.6	70.5	60.6	0.38	10.1	28.5
0.79–1	2.7	70.6	58.8	0.36	9.4	30.7
perlite	7.3		98.8	0	0	1.2

a On dry weight basis.

positions in the filter bed after 300 days of operation, more biomass or related products was present at the inlet side of the biofilter (table 5.1). Electron microscopy of the biofilm indicated the presence of storage materials (chapter 4) and analysis of the filter bed showed that lipid accumulation was more pronounced at the inlet side of the biofilter (table 5.1). The distribution of organic matter, excluding lipids, indicate an approximately homogeneous distribution of biomass, as was observed for protein (table 5.1). Moreover, the average biofilm thickness at the inlet and outlet side of the biofilter after 300 days of operation was comparable, i.e. 240 and 280 μm, respectively (chapter 4). Although these parameters were investigated only once after 300 days of operation of the biofilter, a uniform biomass distribution in the filter bed was assumed during the entire experiment. Styrene loadings generally exceeded the styrene elimination capacity, thus providing excess styrene throughout the filter bed.

Biofiltration experiments

The maximal styrene degradation rate was reached after 35 days of operation of the biofilters. All experiments described in this section were performed after this

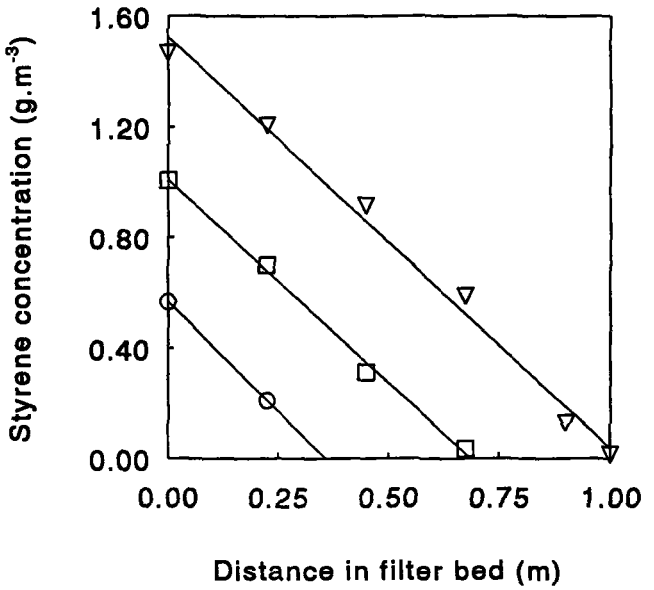

Figure 5.5. Styrene concentration profiles along the filter bed at various styrene inlet concentrations (volumetric load 39 $m^3.m^{-3}.h^{-1}$).

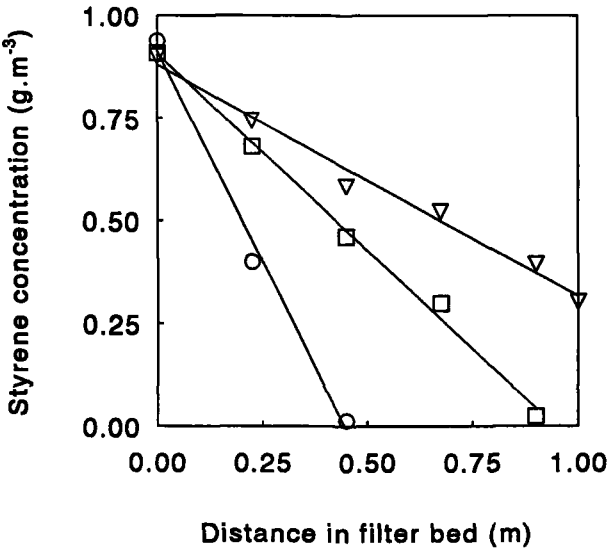

Figure 5.6. Styrene concentration profiles along the filter bed at volumetric loads of 39 (○), 65 (□) and 142 (▽) $m^3.m^{-3}.h^{-1}$.

Table 5.2. The maximal styrene degradation rate at various volumetric loads and the observed and calculated (equation (1), Appendix) styrene concentrations (C) at which the styrene load equals the styrene elimination capacity.

Volumetric load ($m^3.m^{-3}.h^{-1}$)	Rate ($g.m^{-3}.h^{-1}$)	S[a]	C ($g.m^{-3}$)	
			observed	calculated[b]
39	76	14 (4)	2.0–2.2	1.6
51	59	17 (26)	1.3–1.5	1.2
65	72	34 (20)	0.9–1.1	1.0
95	62	20 (42)	0.7–0.8	0.7
122	53	15 (36)	0.5–0.7	0.5
142	62	21 (38)	0.4–0.6	0.4

a Standard deviation and, in parentheses, the number of determinations.
b Assuming a styrene elimination capacity of 62 $g.m^{-3}.h^{-1}$.

start–up period. A uniform distribution of styrene degradation activity in the filter bed is illustrated by the linear decrease of the styrene concentration along the filter bed at various styrene inlet concentrations (figure 5.5) and at various volumetric loads (figure 5.6). Maximal styrene degradation rates were calculated for six volumetric loads as summarized in table 5.2. On average, the styrene elimination capacity was 62 $g.m^{-3}.h^{-1}$, corresponding to a zero–order rate constant (K, Appendix) of 0.0172 $g.m^{-3}.s^{-1}$. A styrene elimination capacity of 62 $g.m^{-3}.h^{-1}$ is comparable to the capacity of the enrichment biofilter with fungi on perlite (chapter 2), but slightly lower than the capacity as found in 0.5 l biofilters (chapter 3). No major differences were found at different volumetric loads, but individual measurements of the maximal styrene degradation rate varied considerably (table 5.2). The same has been observed in compost biofilters (Ottengraf and Van den Oever, 1983) and biotrickling filters (Pedersen and Arvin, 1995). This variability may in part be attributed to fluctuations of the gas flow rate and/or the styrene inlet concentration because CO_2 production rates varied in a similar way (not shown). Also occasional drying out of the inlet side of the filter bed may have caused relatively low styrene degradation rates.

The observed linearity of styrene concentration profiles (figures 5.5 and 5.6) confirms the assumption of a uniform distribution of styrene degradation activity in the filter bed. It also indicates reaction limitation and zero–order degradation kinetics down to a very low styrene concentration (Ottengraf and Van den Oever, 1983; Ottengraf, 1986). When these assumptions are indeed valid, biofilter performance may be described using equation (1) (Appendix). As an example, the influence of the styrene concentration on the styrene removal efficiency at a volumetric load of 142 $m^3.m^{-3}.h^{-1}$ is shown in figure 5.7. Experimental results agree reasonably well with equation (1) using a zero–order rate constant of 0.0172 $g.m^{-3}.s^{-1}$. Furthermore, predicted and observed styrene concentrations at which the styrene load exceeds the styrene elimination capacity are comparable (table 5.2). The performance of the biofilters in the reaction–limited regime could be improved by supplying gas with an increased oxygen concentration. The maximal styrene degradation rate increased to 91 $g.m^{-3}.h^{-1}$ at 40% oxygen in the gas while no effect on the recovery of removed styrene as CO_2 was observed (table 5.3). Apparently, the maximal styrene degradation rate at high styrene concentrations may be limited by the availability of oxygen.

At a constant styrene loading of 75 $g.m^{-3}.h^{-1}$, the styrene degradation rate was maximal down to a styrene concentration of 0.06 $g.m^{-3}$ (figure 5.8). The degradation rate decreased at styrene concentrations lower than 0.06 $g.m^{-3}$, which indicates that at these concentrations biofilter performance was limited by mass transfer of styrene (Ottengraf and Van den Oever, 1983; Ottengraf, 1986). This also confirms the

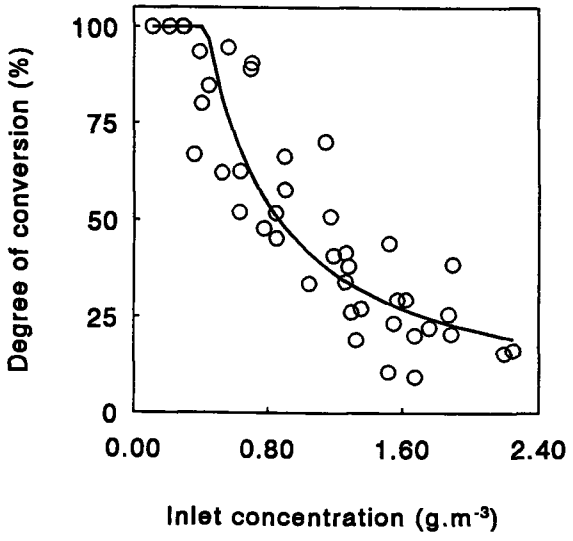

Figure 5.7. Influence of the styrene concentration on the removal efficiency at a volumetric load of 142 $m^3.m^{-3}.h^{-1}$. ○, experimental results; solid line, equation (1) (Appendix) with K = 0.0172 $g.m^{-3}.s^{-1}$.

Table 5.3. Influence of the oxygen concentration in the feed gas on biofilter performance (volumetric load 90 $m^3.m^{-3}.h^{-1}$).

Parameter	Oxygen (%)	
	20	40
Styrene influent ($g.m^{-3}$)	2.18	2.25
Styrene effluent ($g.m^{-3}$)	1.49	1.23
Produced CO_2 concentration (ppm)	960	1455
Recovery as CO_2 (%)[a]	75	78
Styrene degradation rate ($g.m^{-3}.h^{-1}$)	62	91

a Recovery of removed styrene as CO_2 as expected upon complete oxidation of removed styrene.

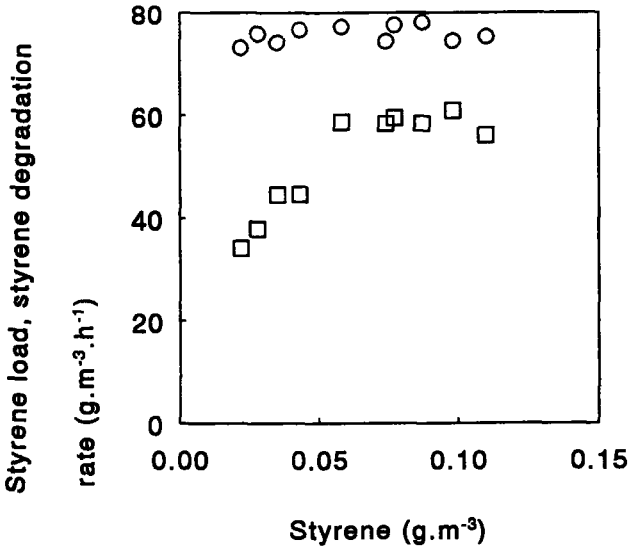

Figure 5.8. Styrene degradation rate (□) at low styrene concentrations at a constant styrene load (○) of 75 $g.m^{-3}.h^{-1}$.

Table 5.4. Parameter estimates.

Parameter	Remarks
$A = 863\ m^2.m^{-3}$	Equation (5)
$C_{crit} = 0.06\ g.m^{-3}$	Figure 5.8
$D = 7.13x10^{-10}\ m^2.s^{-1}$	Perry *et al.* (1984); Christensen and Characklis (1990)[a]
$d_p = 0.0041$ m	Sieved fraction 0.00345–0.00475 m
$H = 1$ m	
$K = 0.0172\ g.m^{-3}filter.s^{-1}$	Table 5.2
$m = 0.15$	At 25°C, extrapolation from Cox *et al.* (1993)
$\varepsilon = 0.41$	

a For estimation of the diffusion coefficient of styrene in the biofilm, the diffusion coefficient of styrene in water, as calculated from the Wilke–Chang relation (Perry *et al.*, 1984), was multiplied by 0.8 (Christensen and Characklis, 1990).

assumption of reaction limitation at styrene concentrations between 0.1–2.4 $g.m^{-3}$ as applied in biofiltration experiments described above. The critical pollutant concentration at the transition point of reaction to diffusion limitation depends on both compound characteristics and biofilter/microorganism characteristics. However, a critical styrene concentration of 0.06 $g.m^{-3}$ is of the same order as critical concentrations of methanol, butylacetate, ethylacetate and toluene as found in compost and peat biofilters (Ottengraf, 1986; Shareefdeen *et al.*, 1993). At this critical concentration, styrene just fully penetrates the biofilm which allows for a calculation of the penetration depth of styrene in the biofilm. Using equation (3) (Appendix) and parameter estimates as summarized in table 5.4, the calculated depth of penetration is 29 μm. Since the actual biofilm thickness at the time of the experiments (day 315–375) was at least ten times higher, it thus appears that only a part of the biofilm is involved in styrene degradation, as also apparent from microscopic evidence (chapter 4).

Determination of biomass parameters

Styrene removal was accompanied by the production of CO_2. Depending on the styrene concentration in the feed gas, the recovery of styrene as CO_2 as expected upon total oxidation of removed styrene was between 40 and 100% (figure 5.9). This

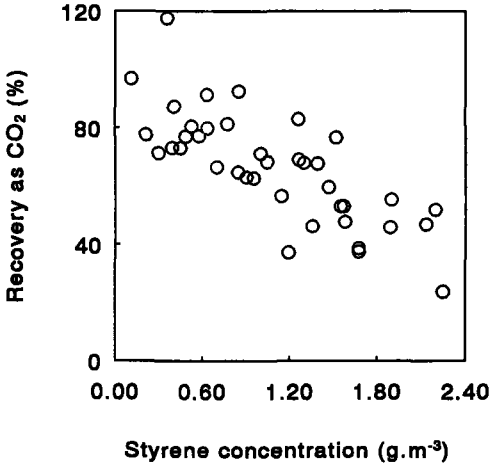

Figure 5.9. Influence of the styrene concentration on the recovery of styrene as CO_2 as expected upon total oxidation of removed styrene to H_2O and CO_2 (volumetric load 122 $m^3.m^{-3}.h^{-1}$).

Table 5.5. Biomass parameters as determined in samples from the outlet side of biofilters operated for 35 and 300 days.

Parameter	Operation time (days)	
	35	300
Biofilm thickness (μm)	80	280[b]
Organic matter (% w/w)[a]	19	41
Protein (% w/w)[a]	0.41	0.36

a Based on the dry weight of the filter bed.
b Chapter 4.

indicates that part of the styrene carbon is used for biomass synthesis. The apparent decrease of the carbon recovery at high concentrations was not investigated.

In table 5.5 relevant biomass parameters are compared for biofilters operated for 35 and 300 days. The increase of the organic matter content and the biofilm thickness clearly indicate microbial growth. However, the styrene degradation rate reached its maximum after 35 days of operation of the biofilter and thereafter remained constant. An average biofilm thickness of 80 μm is required to degrade styrene at the maximal rate in these biofilters. This value is 2.8 times higher than the effective biofilm thickness as calculated from the critical styrene concentration in diffusion–limited experiments. Despite the increase of biomass, the protein content in the filter bed remained approximately constant at prolonged operation of the biofilter (table 5.5).

The average composition of the filter bed was calculated using the results in table 5.1 and assuming that the filter bed contained perlite, water and biomass dry matter. Accumulation of biomass is illustrated by the increase of the dry weight of the filter bed, i.e. 600 g on day 0 to 1310 g on day 300 (table 5.6). Lipids were found to be a major biomass constituent, constituting 24% of the total biomass dry matter after 300 days of operation (table 5.6). Accumulation of lipids in fungi has been related to growth conditions characterized by depletion of nitrogen in combination with excess carbon (Ratledge *et al.*, 1984). The C/N ratio over the total filter bed was roughly estimated to be 500 (on weight basis; 12 l mineral medium added, about 3500 g styrene degraded after 300 days), which illustrates the excess of carbon during these

Table 5.6. Composition of the filter bed after 300 days of operation of the biofilter.

Component	Weight (g)
Filter bed	4060
* Water	2750
* Perlite	600
Ash	593
Organic matter	7
Protein	0
Crude lipids	0
* Biomass dry matter	710
Ash	155
Organic matter	555
Protein	5.1
Crude lipids	172

experiments. The protein content based on biomass dry matter was only 0.7% (table 5.6). Nevertheless, the amount of protein is more than sufficient to account for a styrene elimination capacity of 62 $g.m^{-3}.h^{-1}$. A specific styrene degradation activity of *E. jeanselmei* of 3.1–21 $\mu g.mg^{-1}protein.min^{-1}$ (i.e., on perlite and in suspension, respectively, as determined in batch experiments) implies a styrene elimination capacity of 120–820 $g.m^{-3}.h^{-1}$. This may indicate that part of the biomass in the filter bed is not involved in styrene degradation. Alternatively, the specific styrene degradation activity of *E. jeanselmei* in the filter bed may have been lower than in batch experiments.

DISCUSSION

Biofilter performance is generally described by the volumetric elimination

capacity (Van Groenestijn and Hesselink, 1994). In this view it is implicitly assumed that the filter bed is homogeneous with respect to the distribution of biomass and physico–chemical parameters that influence the specific microbial activity. Our analysis has shown that these assumptions are met in styrene–degrading biofilters with *E. jeanselmei* on perlite. Indeed, styrene concentration profiles along the filter bed were linear at styrene inlet concentrations between 0.1–2.4 $g.m^{-3}$. Styrene degradation in biofilters using bacteria on natural support materials has been investigated by several groups. The styrene elimination capacity of this type of biofilter varies between 10 and 50 $g.m^{-3}.h^{-1}$ (Windsperger *et al.*, 1990; Demiriz, 1992; Sabo *et al.*, 1993), though there is no information about the operational stability of these filters. The present experiments show that a styrene elimination capacity of 62 $g.m^{-3}.h^{-1}$ can be maintained for over 12 months by using *E. jeanselmei* growing on perlite.

Although the biofilm after 300 days of operation may be as thick as 600 μm (chapter 4), degradation of styrene appears only to take place in the upper layers at the biofilm/gas interface. A similarity may be suggested with the biofilm model of Williamson and McCarty (1976), who distinguish between the actual thickness of the biofilm and the effective biofilm thickness in which substrate degradation takes place. In styrene–degrading biofilters, the effective biofilm thickness is 29 (as calculated from the critical styrene concentration) to 80 μm (biofilm thickness at the time of reaching the maximal styrene degradation rate). Though this discrepancy is of minor importance with respect to the actual biofilm thickness, it can be explained by taking the estimate of the biofilm specific surface area into account (chapter 4). Using equation (3) (Appendix), an effective biofilm thickness of 80 μm would result on the assumption of a 2.8 times increase of the biofilm specific surface area as compared to the specific surface area of the support material. Such an increase is not unlikely in view of irregularities of the biofilm surface as has been shown by electron microscopy (chapter 4). We therefore assume an effective biofilm thickness of 80 μm. Comparable values have been reported for substrate degradation in biofilms under submerged conditions (Kornegay and Andrews, 1968; Hoehn and Ray, 1973; Atkinson and Fowler, 1974).

The repeated addition of mineral medium and the continuous supply of styrene allowed for microbial growth, resulting in a more than 100% increase of the filter bed dry weight after 300 days of operation. Despite this increase, the styrene degradation rate reached its maximum after 35 days. It can therefore be concluded that a direct relation between the amount of biomass in the filter bed (as indicated by the biofilm thickness or the organic matter content) and the styrene elimination capacity does not exist. Instead, protein as an indication of the amount of active biomass in the filter bed

may be related to the elimination capacity. The protein content of the filter bed, based on biomass dry matter after 300 days of operation, was considerably less than the protein content of exponentionally growing yeast cells, i.e. 0.7 versus 40–50% (Harrison, 1967; Verduyn, 1991). This may indicate that the biofilm mainly contained non–proteinaceous biomass. The same has been observed by Arcangeli and Arvin (1992), who estimated that active biomass constituted only 5% of the total biomass in a toluene–degrading biofilm. These findings agree with TEM micrographs showing that intact cells were mainly located at the surface of the biofilm whereas deeper parts of the biofilm mainly contained dead cells (chapter 4). Thus it may be suggested that the thickness of the effective biofilm is determined by starvation of cells in deeper parts of the biofilm. Since biofilter performance appeared to be limited by the availability of oxygen, starvation of cells may have been caused by oxygen depletion in the biofilm. In the modelling of biofilters, it is generally assumed that the biofilm is a homogeneous layer of active cells. These experiments with styrene–degrading biofilters show that this condition may only be true in case of thin biofilms that are fully penetrated by the substrate or oxygen. Oxygen limitation has been reported for a peat–perlite biofilter treating methanol (Shareefdeen *et al.*, 1993) and it may be a common phenomenon in biofiltration. Although the hydrophobicity and degradability of waste gas pollutants differ widely and a wide variety of biofilters have been investigated with respect to support materials and microbial populations, the elimination capacity of biofilters is relatively constant between 8 and 200 $g.m^{-3}.h^{-1}$ (chapter 1).

Dutch industry will have to reduce the styrene concentration in waste gases to a concentration under 0.1 $g.m^{-3}$ to comply with legislation (chapter 1). Since this concentration is well above the critical styrene concentration, biofilters with *E. jeanselmei* will in practice be applied in the reaction–limited regime (equation (1), Appendix). The required biofilter volume can therefore be calculated by a direct comparison of the styrene elimination capacity and the absolute amount of styrene to be removed. However, some oversizing of the biofilter volume may be necessary in view of the observed fluctuations of the styrene elimination capacity or if further purification of the waste gas to styrene concentrations lower than 0.06 $g.m^{-3}$ is required. Reduction of the required biofilter volume may be achieved by increasing the styrene elimination capacity. Since the maximal styrene degradation rate appears to be limited by oxygen, possible solutions should not be sought in an increase of the amount of biomass or in strain improvement. The styrene elimination capacity may instead be increased by creating a larger specific surface area to facilitate oxygen availabilty, i.e. by using a support material with a smaller diameter. However, it

should be noted that a higher styrene degradation rate may endanger the operational stability of the biofilter if a limitation of microbial growth is not ensured (chapter 4).

APPENDIX

The theoretical model of Ottengraf (1986) describes biofilter performance. The model is based on pollutant degradation in a biofilm. Assuming zero–order degradation, biofilter performance may be described by equations (1) and (2) for limitation by reaction or diffusion, respectively:

$$(1) \qquad f = \frac{K \cdot H}{C_i \cdot U}$$

$$(2) \qquad \frac{C_e}{C_i} = \{ 1 - \frac{H \cdot A}{U} \cdot \left(\frac{k \cdot D}{2 \cdot m \cdot C_i}\right)^{0.5} \}^2$$

Biofilter performance will be diffusion–limited if the Thiele number (Φ) is equal to or higher than $\sqrt{2}$:

$$(3) \qquad \Phi = d \cdot \left(\frac{k \cdot m}{D \cdot C}\right)^{0.5}$$

C_{crit} is the critical concentration at which $\Phi = \sqrt{2}$. Experimental determination of k is very difficult. This parameter may be estimated with equation (4):

$$(4) \qquad k = \frac{K}{A \cdot d}$$

The specific surface area of the biofilm may be estimated from equation (5), if assuming that the specific biofilm surface area equals the surface area of the

supporting particles and that the filter bed is regularly packed with spheres with an average diameter.

$$(5) \quad A = \frac{6 \cdot (1 - \epsilon)}{d_p}$$

Nomenclature

A Biofilm specific surface area ($m^2.m^{-3}$)
C_i, C_e Styrene inlet and outlet gas concentration ($g.m^{-3}$)
D Effective diffusion coefficient ($m^2.s^{-1}$)
d Biofilm thickness (m)
d_p Particle diameter (m)
f Degree of conversion (–)
H Length filter bed (m)
K Zero–order rate constant ($g.m^{-3}$filter$.s^{-1}$)
k Zero–order rate constant ($g.m^{-3}$biofilm$.s^{-1}$)
m Partition coefficient (–)
U Superficial gas velocity ($m.s^{-1}$)
ε Void fraction (–)
Φ Thiele number (–)

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CHAPTER 6

STYRENE METABOLISM IN EXOPHIALA JEANSELMEI AND THE INVOLVEMENT OF A CYTOCHROME P-450-DEPENDENT STYRENE MONOOXYGENASE

INTRODUCTION

Styrene is emitted in large quantities to air, causing atmospheric pollution as well as occupational exposure to styrene (chapter 1). Optimal application of bioremediation of styrene–containing waste gases requires an understanding of the metabolic route of styrene degradation in microorganisms. Bacterial styrene degradation has recently been reviewed by Warhurst and Fewson (1994), who distinguish between initial oxidation of the ethylene side chain versus initial oxidation of the aromatic nucleus. Bacterial oxidation of the ethylene side chain is most frequently found. In this route, styrene is oxidized to phenylacetic acid via styrene oxide and phenylacetaldehyde (Hartmans *et al.*, 1990). Initial attack of the aromatic nucleus has only been reported for *Rhodococcus rhodochrous* NCIMB 13259; styrene is converted to styrene *cis*–glycol and 3–vinylcatechol, followed by *meta*–cleavage of the nucleus (Warhurst *et al.*, 1994). In mammals, styrene is converted to styrene oxide by a cytochrome P–450 monooxygenase (chapter 1). The main route proceeds via addition of water to the epoxide to form phenylethanediol. The latter compound is converted to mandelic, phenylglyoxylic and hippuric acid, which are major metabolites in urine of mammals exposed to styrene (chapter 1).

By enrichment experiments using biofilters for waste gas treatment, ten new fungal species were obtained, which are capable of utilizing styrene as the sole source of carbon and energy (chapter 2). In the literature, styrene degradation by fungi has not been described. In this chapter the pathway of styrene degradation is described, focusing on *Exophiala jeanselmei* as the major species responsible for the removal of

styrene in biofilters in which fungi grow on perlite (chapter 4). It is also shown that cytochrome P–450 is possibly involved in the oxidation of styrene by *E. jeanselmei.*

MATERIALS AND METHODS

Microorganisms and culture conditions

The fungi listed in table 6.1 were maintained at 4^0C on a solidified mineral medium containing 8 $g.l^{-1}$ agarose; 0.5 $g.m^{-3}$ styrene in air was used as the carbon source (chapter 2). The *E. jeanselmei* species used in this study was obtained from M.J. van der Werf, Department of Industrial Microbiology, Agricultural University, Wageningen, the Netherlands. The species were cultivated at 25^0C in a mineral medium through which styrene–containing air with a concentration of 0.4–0.6 $g.m^{-3}$ was continuously flushed (chapter 5). This corresponds to a styrene concentration in the liquid phase of 25–38 μM. Inoculation was done as previously described (Cox *et*

Table 6.1. List of examined species.

Code[a]	Species
A–7–1	*Penicillium* cf. *janthinellum*
A–19	*Penicillium* cf. *janthinellum*
A–23	*Penicillium minioluteum* Dierckx
A–24	*Gliocladium roseum* Bain
A–28	*Penicillium fellutanum* Biourge
B–43	*Exophiala jeanselmei* (Langeron) McGinnis & Padhye
B–50	*Penicillium* cf. *miczynskii* Zaleski
B–52–2	*Penicillium* species

a Species encoded A–7–2 and B–15 (see table 2.2) were not investigated because biomass yield during growth on styrene was poor.

al., 1993) and cells were harvested after 12–16 days.

Growth substrates were tested by adding 25 µmol of substrate to 100 ml mineral medium in closed 500 ml serum flasks and inoculating the medium with 0.5 ml cell suspension which was prepared from stock cultures (Cox *et al.*, 1993). Incubation was stationary at 25°C. Substrate addition was repeated when growth was absent after two weeks of incubation. Cultures which showed growth within 30 days were transferred (1 ml) to 100 ml fresh medium to which the same amount of substrate was added. Compounds that supported growth in these cultures within 30 days were considered growth substrates.

Cytochrome P–450 was determined spectrophotometrically in microsomal preparations of *E. jeanselmei* grown on styrene, phenylacetic acid (daily addition of increasing amounts to a total concentration of 18.5 mM) and glucose (55.5 mM). Culture conditions were as described above. When phenylacetic acid and glucose were used as substrate, the cultures were continuously flushed with air.

A *Bacillus* species with phenylacetaldehyde dehydrogenase activity (O'Connor *et al.*, 1995) was grown for 7–10 days at 25°C in a mineral medium (Hartmans *et al.*, 1989) to which 1 mM 2–phenylethanol was added repeatedly.

Preparation of cell suspensions and cell-free extracts

Cells were harvested by centrifugation at 500 *g* for 10 minutes (yeast cells) or by filtration (mycelium), washed twice in 50 mM potassium phosphate buffer pH 5.7 and resuspended in this buffer. These suspensions were used for oxygen uptake experiments with whole cells. For the production of intermediates, cell suspensions in mineral medium pH 5.7 were used.

For the determination of styrene monooxygenase activity in cell–free extracts of *E. jeanselmei*, cells were suspended in 100 mM HEPES pH 7.5 containing 0.8 mM dithiothreitol (DTT) and 0.8 mM reduced glutathione (GSH). The cell suspension was mixed with glass beads (1:2 v/w) with a diameter of 0.4–0.5 mm and the cells were disrupted in a bead beater (Biospec Products, Bartlesville, Okl.) for 10x30 s with 30 s intervals under cooling with ice. The homogenate was centrifuged for 10 min at 10,000 *g* (Hermle ZK 401 centrifuge with A8.24 rotor, Berthold Hermle AG, Gosheim, FRG) at 4°C. The cell–free extract was stored on ice and all experiments were carried out within four hours. For the determination of styrene oxide isomerase and phenylacetaldehyde dehydrogenase activity, the cells were suspended in 96 mM potassium phosphate buffer pH 7.5 and disrupted by sonification (cell disruptor B–15, Branson, Danbury, Conn.) for 10 min while keeping the cells on ice. The cell–free

extract was obtained by centrifugation at 10,000 *g* for 10 min. The cell–free extract of the phenylacetaldehyde dehydrogenase–containing *Bacillus* species was prepared in the same way.

Oxygen uptake experiments

These experiments were carried out according to Cox *et al.* (1993).

Determination of intermediates

To 100 ml cell suspension in 1 l serum flasks, sealed with Viton septa, 0.3 mM styrene, styrene oxide, phenylacetaldehyde, 2–phenylethanol, phenylacetic acid, 2–hydroxyphenylacetic acid or homogentisic acid were added. The flasks were placed on magnetic stirrers at 25^{0}C and 2 ml samples were periodically withdrawn through the septum by using syringes. The samples were immediately filtered through 0.22 μm filters, stored at 4^{0}C and analyzed the same day. Depending on the rate of substrate degradation, cell density was varied to obtain complete substrate removal in 40–90 min (0.25 mg protein.l^{-1} for styrene oxide, 75–95 mg protein.l^{-1} for the other substrates). Substrate degradation rates (except for styrene) and the formation of intermediates were determined by HPLC analysis. The styrene degradation rate was determined by GC analysis of the head–space. Intermediate formation from styrene was also investigated in the presence of 5 mM SKF–525A or 2 mM cyclohexene oxide to inhibited cytochrome P–450–dependent styrene monooxygenase and styrene oxide hydratase, respectively.

Enzyme assays

All assays were carried out at 25^{0}C with freshly harvested cells of styrene–grown *E. jeanselmei*. Enzyme activities are expressed as the amount of substrate degraded or product formed per mg protein per min.

Styrene monooxygenase activity was determined in serum flasks of 38 ml sealed with Viton septa. The reaction mixture for the standard assay contained 0.1 ml 5 mM NADPH, 0.1 ml 0.6 mM FAD, 0.1–0.5 ml cell–free extract (total amount of protein 0.5–1 mg) and 100 mM HEPES pH 7.5 in a final volume of 1.5 ml. The reaction was started by the addition of 0.05 ml 1.5 mM styrene, which resulted in styrene liquid and gas phase concentrations at time zero of 11 μM and 0.17 mg.l^{-1}, respectively. After the addition of styrene, the serum flasks were placed in a shaking water bath at

25°C. The styrene degradation rate was determined by periodic sampling of the head–space for GC analysis. Cofactor dependency was investigated by adding 0.33 mM NADH, 0.33 mM NADPH, 0.04 mM FMN and/or 0.04 mM FAD to the reaction mixtures. Styrene degradation under anaerobic conditions was investigated after gentle flushing of the flasks with nitrogen for a few minutes. Inhibition studies were performed with CO, SKF–525–A (1.6 mM), metyrapone (1.0 mM), cytochrome c (1.0 mM) and KCN (1.0 and 10 mM). CO was added by flushing the flasks with pure CO, after which the flasks were flushed with air for a few minutes to create aerobic conditions.

Styrene oxide isomerase activity was assayed spectrophotometrically by measuring the styrene oxide–dependent NADH formation at 340 nm. The reaction mixture contained: 0.1 ml 5 mM NAD^+, 0.16 ml 30 mM KCN, 0.2 ml 10 mM styrene oxide, cell–free extract of the *Bacillus* species (total amount of protein 0.21 mg), cell–free extract of *E. jeanselmei* (total amount of protein 1.5–4.5 μg) and 96 mM potassium phosphate buffer pH 7.5 (total volume 1.5 ml). Control experiments were carried out to determine the KCN concentration required for inhibition of NADH oxidase, the optimal concentration of cell–free extracts of the *Bacillus* species and *E. jeanselmei* and the substrate specificity of the *Bacillus* enzyme.

Phenylacetaldehyde dehydrogenase activity was determined from the rate of NADH formation in a reaction mixture containing 0.1 ml 5 mM NAD^+, 0.16 ml 30 mM KCN, 0.2 ml 10 mM phenylacetaldehyde, cell–free extract (total amount of protein 0.068–0.70 mg) and 96 mM potassium phosphate buffer pH 7.5 (total volume 1.5 ml). The activity of this enzyme was also determined in the presence of 1.1 mM phenazine methosulphate (PMS) with and without 0.33 mM NH_4Cl.

Determination of cytochrome P-450

These experiments were performed with *E. jeanselmei* grown on styrene, phenylacetic acid or glucose. The substrates were not depleted at the time of harvesting of the cells. All steps for the preparation of microsomal fractions were carried out at 4°C. About 3 l of culture was centrifuged at 500 *g* for 10 minutes. The cells were suspended in 100 mM HEPES pH 7.5 containing 1 mM EDTA, 20% (v/v) glycerol, 0.1 mM GSH and 1 mM DTT. The cells were disrupted with glass beads as described above. The microsomal fraction was prepared by filtration over myracloth, followed by centrifugation of the permeate at 5,000 *g* for 10 min. Mitochondria were removed by two centrifugation steps at 16,000 *g* for 20 min. The microsomal fraction was obtained by centrifugation of the resulting supernatant at 105,000 *g* for 1 h

(Beckman L5–65 ultracentrifuge, Palo Alto, Calif.). The microsomes were resuspended using a Potter–tube with a tightly fitting pestle in 1–2 ml of the same buffer as above. CO difference spectra were recorded as described by Omura and Sato (1964) on a SLM Aminco DW2000 spectrophotometer.

Analytical methods

Protein was determined with a Coomassie assay reagent (chapter 3). Glucose was determined with an assay kit from Boehringer (Mannheim, FRG). Styrene head–space concentrations were determined in 50 µl samples by gas chromatography (chapter 5). HPLC was carried out with a Millipore Waters 600S and 616 controller and pump (Milford, Mass.), using an Aromatic Acids column of 10 cm from Chrompack (Bergen op Zoom, the Netherlands). The column temperature was 45^{0}C and the eluent was 5 mM H_2SO_4 at a flow rate of 0.4 ml.min^{-1}. Sample volume was 40 µl, detection was at 215 nm. Under these conditions retention times were (min): homogentisic acid 10.8, homoprotocatechuic acid 12.1, 2–hydroxyphenylacetic acid 15.2, 3–hydroxyphenylacetic acid 16.0, 4–hydroxyphenylacetic acid 17.1, styrene oxide 17.3, phenylacetic acid 22.3, phenylacetaldehyde 26.9, and 2–phenylethanol 37.3.

Chemicals

Styrene, α–methylstyrene, phenylethanediol and benzoic acid were from E. Merck AG (Darmstadt, FRG). Styrene oxide, phenylacetaldehyde, 2–phenylethanol, phenylacetic acid, 1–phenylethanol, acetophenone, benzene, toluene, ethylbenzene, allylbenzene, phenylacetylene, n–butylbenzene and p–xylene were from Janssen Chimica (Geel, Belgium). 2–Hydroxyphenylacetic acid, 3–hydroxyphenylacetic acid, 4–hydroxyphenylacetic acid, homoprotocatechuic acid, homogentisic acid, mandelic acid, phenylglyoxylic acid, hippuric acid, fumaric acid, acetoacetic acid, PMS, HEPES (N–[2–hydroxyethyl]piperazine–N'–[2–ethanesulfonic acid]), DTT, GSH, NADPH, NADH, $NADP^+$, NAD^+, FAD, FMN, cytochrome c (horse heart) and metyrapone (2–methyl–1,2–di–3–pyridyl–1–propanone) were from Sigma Chemical Co. (St. Louis, Mo.). 2–Methylstyrene, 3–methylstyrene, 4–methylstyrene and ß–methylstyrene were from Fluka Chemie AG (Buchs, Switzerland). Phenol and EDTA (ethylenediamine tetraacetic acid) were from J.T. Baker B.V. (Deventer, the Netherlands). Cyclohexene oxide was from Aldrich Chemie (Brussel, Belgium) and SKF–525–A (2–diethylaminoethyl 2,2–diphenylvalerate HCl) was from Calbiochem (San Diego,

Calif.).

RESULTS

Growth experiments

The substrate specificity of *Exophiala jeanselmei* is shown in table 6.2. Both known bacterial and mammalian styrene degradation intermediates could be used as sole source of carbon and energy. However, growth on mammalian intermediates was less profound and relatively long adaptation periods were observed with these compounds. Remarkably, aromatic hydrocarbons resembling styrene could not serve as growth substrate, although *E. jeanselmei* shows a very broad substrate specificity with respect to oxygenated monoaromatic compounds (Middelhoven, 1993).

Previously we reported that *E. jeanselmei* was unable to grow in media containing 1 mM phenylacetaldehyde (Cox *et al.*, 1993). As the present experiments showed growth at a concentration of 0.25 mM, phenylacetaldehyde appears to be toxic for *E. jeanselmei* at higher concentrations. It was also observed that growth of *E. jeanselmei* on 0.25 mM acetophenone, 1–phenylethanol and phenylethanediol is possible, which is contrary to the findings of Middelhoven (1993) who used a slant culture method (Middelhoven *et al.*, 1991). Since the exact substrate concentration in the slant culture method is unknown, this apparent discrepancy may be explained by the toxicity of these compounds at concentrations higher than 0.25 mM.

Oxygen uptake experiments

Glucose–grown *E. jeanselmei* was unable to oxidize the aromatic compounds investigated in this study. After growth on styrene, *E. jeanselmei* oxidized intermediates in bacterial styrene metabolism at a high rate (table 6.3). Intermediates in mammalian styrene metabolism were oxidized albeit at a lower rate, which may indicate that the mammalian route of styrene metabolism is less likely to be present in *E. jeanselmei*. Growth on styrene also induced oxidation of substituted styrenes such as ß–methylstyrene, 3–methylstyrene and 4–methylstyrene, but oxidation rates of other aromatic hydrocarbons were low or undetectable. Apparently, modification of the side chain or the aromatic nucleus of styrene influences the ability of *E. jeanselmei* to oxidize these compounds. There seems to be no apparent correlation between the

Table 6.2. The ability of Exophiala jeanselmei to grow on intermediates implicated in bacterial and mammalian styrene metabolism and styrene-related compounds.

Bacterial intermediates	Growth	*Mammalian intermediates*	Growth	*Other*	Growth
Styrene oxide	+[a]	Phenylethanediol	+	Acetophenone	+
Phenylacetaldehyde	+	Mandelic acid	+	Phenol	+
2–Phenylethanol	+	Phenylglyoxylic acid	+	Allylbenzene	–
Phenylacetic acid	+	Hippuric acid	+	α–Methylstyrene	–
2–Hydroxyphenylacetic acid	+	Benzoic acid	+	Ethylbenzene	–
3–Hydroxyphenylacetic acid	+	1–Phenylethanol	+	Toluene	–
4–Hydroxyphenylacetic acid	+			Benzene	–
Homogentisic acid	+			3–Methylstyrene	–
Homoprotocatechuic acid	+				
Acetoacetic acid	+				
Fumaric acid	–				

a +: growth; –: no growth.

Table 6.3. Oxidation (μg O_2.mg^{-1}protein.min^{-1}) of styrene, styrene-related compounds and intermediates implicated in bacterial and mammalian styrene metabolism in cell suspensions of styrene-grown Exophiala jeanselmei.

Bacterial intermediates	Rate	*Mammalian intermediates*	Rate	*Other*	Rate
Styrene	3.1	Phenylethanediol	1.4	Acetophenone	0.9
Styrene oxide	6.7	Mandelic acid	0.2	α–Methylstyrene	0
Phenylacetaldehyde	6.7	Phenylglyoxylic acid	0.4	ß–Methylstyrene	3.3
2–Phenylethanol	5.0	Hippuric acid	1.8	Allylbenzene	0.7
Phenylacetic acid	5.6	Benzoic acid	0	Phenylacetylene	0.5
2–Hydroxyphenylacetic acid	6.3	1–Phenylethanol	0.7	n–Butylbenzene	0
3–Hydroxyphenylacetic acid	4.3			Ethylbenzene	0
4–Hydroxyphenylacetic acid	3.5			Toluene	0
Homogentisic acid	+[a]			Benzene	0
Homoprotocatechuic acid	3.5			2–Methylstyrene	0
Acetoacetic acid	2.3			3–Methylstyrene	1.7
Fumaric acid	0			4–Methylstyrene	3.4
				p–Xylene	0

a No quantitative results, oxidation was observed.

biodegradability and the structural changes of the substrate molecule.

Identification of styrene intermediates

No accumulation of styrene intermediates in cell suspensions of *E. jeanselmei* was observed when styrene was added at the same concentration (48 μM) as in the oxygen uptake experiments. However, at an initial concentration of 0.35 mM styrene in the medium, styrene was degraded with transient accumulation of phenylacetic acid and 2–hydroxyphenylacetic acid (figure 6.1). Maximal phenylacetic acid and 2–hydroxyphenylacetic acid concentrations of 0.14 and 0.032 mM were produced at the time when styrene was depleted, after which the concentration of both products decreased. This high product yield suggests that phenylacetic acid and 2–hydroxyphenylacetic acid are important intermediates in styrene metabolism of *E. jeanselmei*. Accumulation of intermediates of the mammalian pathway for styrene metabolism was not observed. The addition of 2 mM cyclohexene oxide, which is an inhibitor of styrene oxide hydratase in mammals (Oesch and Daly, 1972; Salmona *et al.*, 1976), did not change the product accumulation as shown in figure 6.1. On the

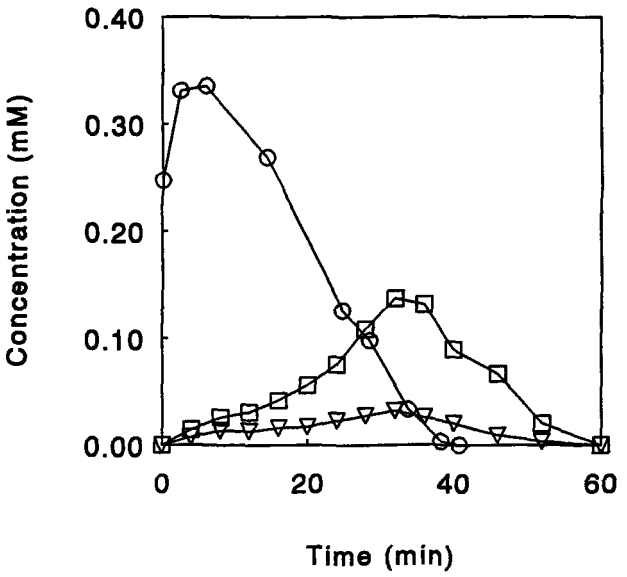

Figure 6.1. Degradation of styrene (○) and the transient accumulation of phenylacetic acid (□) and 2-hydroxyphenylacetic acid (▽) in a cell suspension of Exophiala jeanselmei.

Table 6.4. Degradation of styrene and potential intermediates of styrene metabolism and HPLC-identification of products in cell suspensions of styrene-grown Exophiala jeanselmei.

Substrate	Degradation rate ($\mu mol.mg^{-1}protein.min^{-1}$)	Product	Recovery[a]
Styrene	0.11	Phenylacetic acid	41
		2–Hydroxyphenylacetic acid	10
Styrene oxide	116[b]	Phenylacetaldehyde	60
		2–Phenylethanol	2
		Phenylacetic acid	2
Phenylacetaldehyde	0.15	Phenylacetic acid	20
		2–Hydroxyphenylacetic acid	5
		Homogentisic acid	8
2–Phenylethanol	0.068	Phenylacetic acid	17
		2–Hydroxyphenylacetic acid	4
		Homogentisic acid	9
Phenylacetic acid	0.056	2–Hydroxyphenylacetic acid	4
2–Hydroxyphenylacetic acid	0.048	Homogentisic acid	19
Homogentisic acid	0.029		

a Recovery = (maximal product concentration / initial substrate concentration) x 100%.
b Initial degradation rate.

other hand, 5 mM SKF–525–A completely inhibited styrene degradation and the accumulation of phenylacetic acid and 2–hydroxyphenylacetic acid.

Similar experiments were carried out using styrene oxide, phenylacetaldehyde, 2–phenylethanol, phenylacetic acid, 2–hydroxyphenylacetic acid and homogentisic acid as substrates. Substrate/product combinations suggested that these compounds are involved in the route of styrene degradation in *E. jeanselmei* (table 6.4). This would imply initial oxidation of the ethylene side chain of styrene to phenylacetic acid followed by ring hydroxylation to homogentisic acid. Substrate degradation rates decreased as the substrates were more oxidized (except for styrene oxide), which may explain the accumulation of various products as shown in table 6.4 and the observed product recoveries. Styrene oxide differed in two ways from the other substrates tested. Firstly, the rate of styrene oxide degradation decreased at lower concentrations which may indicate a relatively high affinity constant for this compound. Secondly, the initial rate of styrene oxide degradation was about 1000 times higher than the rate of degradation of other substrates. Chemical conversion of styrene oxide to phenylethanediol and covalent binding of styrene oxide to macromolecules has been reported (Oesch, 1972). However, these processes do not seem to contribute significantly to the styrene oxide degradation rate; the observed accumulation of phenylacetaldehyde (60%) indicates that the enzymatic conversion is the major route.

Determination of enzyme activities

In cell–free extracts prepared by sonification no styrene degradation activity was observed. Cell–free extracts prepared by homogenization with glass beads showed activity, but the addition of DTT and GSH was required. NADPH and FAD were necessary cofactors that could not be replaced by NADH and FMN, respectively (table 6.5). Styrene was not degraded when the cell–free extract was flushed with nitrogen but activity could be fully restored by subsequent flushing with air. Cell–free extracts boiled for ten minutes showed no activity. If stored on ice, styrene monooxygenase activity decreased with 20% after 4 hours. Glycerol, which is normally added to stabilize cytochrome P–450, was not added as this compound interfered in the styrene analysis. The styrene monooxygenase activity measured in cell–free extracts made from cells from different batches varied considerably, i.e. 5–20 $nmol.mg^{-1}protein.min^{-1}$. This was not investigated any further; for each cell–free extract the styrene monooxygenase activity under standard conditions was determined. The results of other experiments with the same extract were compared with this control. Inhibition studies are shown in table 6.6. Styrene monooxygenase activity was

Table 6.5. Cofactor requirement of styrene monooxygenase in the cell-free extract of Exophiala jeanselmei.

Cofactor[a]	Styrene monooxygenase activity ($nmol.mg^{-1}protein.min^{-1}$)
NADH	0
NADH + FMN	0
NADH + FAD	0
NADPH	0
NADPH + FMN	0
NADPH + FAD	16

a NADH and NADPH 0.33 mM, FMN and FAD 0.04 mM.

Table 6.6. Inhibition of styrene monooxygenase in the cell-free extract of styrene-grown Exophiala jeanselmei.

Inhibitor	Concentration (mM)	Inhibition (%)
SKF 525A	1.6	85
Metyrapone	1.0	67
Carbon monoxide		72
Cytochrome c	1.0	100
KCN	1.0	0
	10	0

inhibited by SKF–525A, metyrapone, CO and cytochrome c, but not by KCN. These results indicate a possible involvement of a cytochrome P–450–dependent styrene monooxygenase.

Styrene oxide isomerase activity was determined by oxidation of the formed phenylacetaldehyde with aldehyde dehydrogenase from a *Bacillus* species. After growth on 2–phenylethanol, this species could oxidize phenylacetaldehyde and 2–phenylethanol but not styrene and styrene oxide (oxygen uptake experiments with whole cells). The activity of phenylacetaldehyde dehydrogenase in the bacterial cell–free extract was about 400 nmol.mg^{-1}protein.min^{-1}. NADH oxidase in the cell–free extracts of the *Bacillus* species and *E. jeanselmei* was completely inhibited by the addition of 3.2 mM KCN; this concentration was used in the standard assay. The activity of styrene oxide isomerase in the cell–free extract of *E. jeanselmei* was thus found to be 9,700–12,200 nmol.mg^{-1}protein.min^{-1}. It was ascertained that the amount of *E. jeanselmei* protein in the standard assay was rate–limiting. The styrene oxide isomerase activity is very high if compared to the styrene monooxygenase activity. The same result was found with whole cell suspensions (table 6.4).

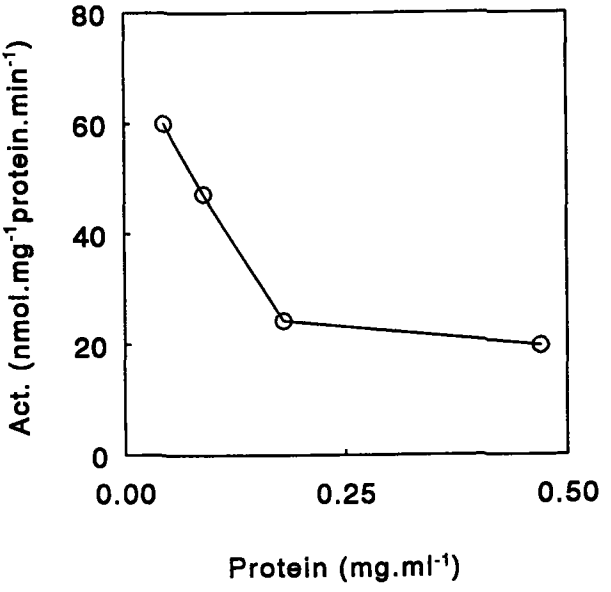

Figure 6.2. Influence of the protein concentration on the phenylacetaldehyde dehydrogenase activity in the cell-free extract of styrene-grown Exophiala jeanselmei.

Phenylacetaldehyde dehydrogenase activity in the cell–free extract of *E. jeanselmei* required the presence of NAD^+. No activity was found with $NADP^+$. Commercial phenylacetaldehyde contains 15% 2–phenylethanol, but this compound was not oxidized under the standard conditions used for the assay of phenylacetaldehyde dehydrogenase. Figure 6.2 shows that the specific phenylacetaldehyde dehydrogenase activity decreased with increasing protein concentration. This was not investigated further; our main objective was to demonstrate the presence of phenylacetaldehyde dehydrogenase. Generally, the activity of this enzyme is in the same range as the activity of styrene monooxygenase. In the reaction mixture phenylacetic acid formation was observed with a product recovery of about 50%. The activity of PMS–dependent phenylacetaldehyde dehydrogenase (Hartmans *et al.*, 1989; O'Connor *et al.*, 1995) was investigated in oxygen uptake experiments. Activity with PMS was higher than with NAD^+, and some stimulation by NH_4Cl was observed, but the results were quantitatively not reproducible because of high rates of oxygen consumption in the absence of cell–free extract. Autooxidation of phenylacetaldehyde in the presence of PMS is a plausible cause (Van den Tweel *et al.*, 1988).

Determination of cytochrome P-450

The reduced CO difference spectrum of the microsomal fraction of styrene–grown *E. jeanselmei* cells shows a characteristic maximum at 450 nm, which indicates the presence of cytochrome P–450 (figure 6.3). The large peak at 420 nm may be due to inactivated cytochrome P–450 (Cerniglia and Gibson, 1978), which may be formed during the preparation of the microsomal fraction. Loss of styrene monooxygenase activity, and probably cytochrome P–450 activity, is also indicated by the difference of the specific styrene oxidation activity in cell suspensions and cell–free extracts. A similar spectrum as in figure 6.3 was found for the microsomal fraction of phenylacetic acid–grown cells. It was previously shown that growth on phenylacetic acid induces oxidation of styrene by *E. jeanselmei* (Cox *et al.*, 1993). With glucose–grown cells, the maximum at 450 nm could not be detected.

Using $\varepsilon = 91\ mM^{-1}.cm^{-1}$ (Omura and Sato, 1964) and the difference in absorption between 450 and 490 nm as an indication of the amount of cytochrome P–450 (Omura and Sato, 1964), the cytochrome concentration in the microsomal fraction of styrene–grown and phenylacetic acid–grown cells was 0.13 and 0.27 $nmol.mg^{-1}$microsomal protein, respectively. Taking the protein content of the initial cell suspensions into account, the cytochrome P–450 level in styrene–grown and

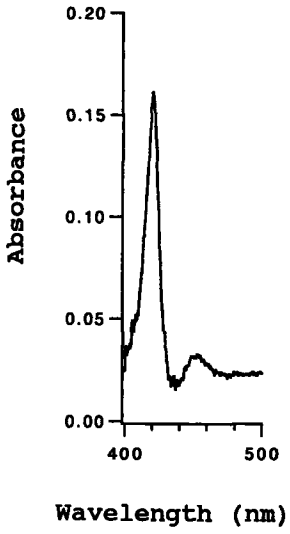

Figure 6.3. Reduced CO difference spectrum of the microsomal fraction of styrene-grown Exophiala jeanselmei.

Table 6.7. The ability of styrene-degrading fungi (excluding E. jeanselmei) to grow on intermediates implicated in bacterial and mammalian styrene metabolism and styrene-related compounds.

Substrate	Species						
	A–7–1	A–19	A–23	A–24	A–28	B–50	B–52–2
Phenylacetic acid	+	+	+	+	+	+	+
Homogentisic acid	+	–	–	+	+	+	–
Phenylethanediol	–	–	–	–	–	–	–
Mandelic acid	–	–	–	–	–	–	–
Allylbenzene	–	–	–	–	–	–	–
α–Methylstyrene	–	–	–	–	–	–	–
Ethylbenzene	–	–	–	–	–	–	–
Toluene	–	–	–	–	–	–	–
Benzene	–	–	–	–	–	–	–
3–Methylstyrene	–	–	–	–	–	–	–

Table 6.8. Oxidation (μg O_2.mg^{-1}protein.min^{-1}) of styrene and intermediates implicated in styrene metabolism via phenylacetic acid by styrene-grown fungi (excluding E. jeanselmei).

Substrate	Species						
	A–7–1	A–19	A–23	A–24	A–28	B–50	B–52–2
Styrene	2.3	3.0	6.0	2.5	7.7	3.9	2.4
Styrene oxide	2.3	2.8	4.5	1.0	4.4	0.4	2.9
Phenylacetaldehyde	2.3	5.4	3.7	6.3	5.9	3.6	3.3
2–Phenylethanol	2.2	3.1	3.6	4.0	3.4	3.1	0.5
Phenylacetic acid	2.3	3.6	3.9	3.8	6.0	3.9	0.8

phenylacetic acid–grown cells was calculated to be 9 and 28 pmol.mg^{-1}protein, respectively. The apparent enrichment of cytochrome P–450 in the particulate fraction that sediments at 105,000 *g* suggests that cytochrome P–450 is located in microsomes (Faber, personal communication).

Growth and oxygen uptake experiments with other styrene-degrading fungi

Of the substrates tested, only phenylacetic acid could be used as a sole source of carbon and energy by all examined species (table 6.7). Homogentisic acid was a growth substrate for four species, whereas intermediates of mammalian styrene metabolism and styrene–related compounds did not promote growth. After growth on styrene, all species could oxidize styrene oxide, phenylacetaldehyde, 2–phenylethanol and phenylacetic acid (table 6.8). These results indicate that the proposed route of styrene degradation in *E. jeanselmei* via phenylacetic acid may also apply for other styrene–degrading fungi.

DISCUSSION

The present results suggest that *E. jeanselmei* degrades styrene via phenylacetic

acid to homogentisic acid (figure 6.4). Evidence for this route is provided by the ability of *E. jeanselmei* to use the proposed intermediates as growth substrates, the induction of intermediate oxidation after growth on styrene, the accumulation of intermediates in whole cell suspensions and the presence of styrene monooxygenase, styrene oxide isomerase and phenylacetaldehyde dehydrogenase in cell–free extracts of styrene–grown *E. jeanselmei*. Accumulation of styrene oxide, phenylacetaldehyde, 2–phenylethanol and phenylacetic acid has been found after addition of styrene to bacterial species (Sielicki *et al.*, 1978; Shirai and Hisatsuka, 1979[a] and 1979[b]; Hartmans *et al.*, 1989; Fruetel *et al.*, 1992). Thus it appears that *E. jeanselmei* degrades styrene via initial oxidation of the ethylene side chain as found in most styrene–degrading bacteria. Preliminary experiments with other fungi isolated from styrene–degrading biofilters suggest that this route may be common among styrene–degrading fungi.

The addition of styrene or styrene oxide to *E. jeanselmei* cell suspensions also resulted in the accumulation of 2–phenylethanol, which would require reduction of styrene oxide or phenylacetaldehyde. Thus, 2–phenylethanol formation from styrene

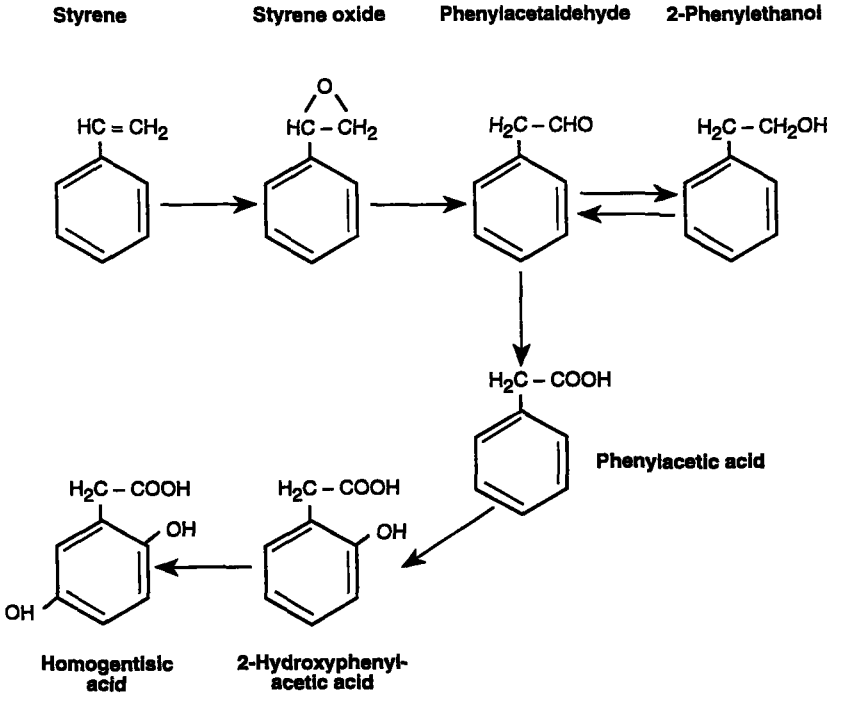

Figure 6.4. Proposed route of styrene degradation in Exophiala jeanselmei.

oxide may be a step in the route of styrene metabolism as suggested by Shirai and Hisatsuka (1979[a]). From our experiments no decisive conclusion can be drawn about the role of 2–phenylethanol in styrene metabolism in *E. jeanselmei*. However, styrene oxide was converted to large amounts of phenylacetaldehyde by whole cell suspensions. In cell–free extracts phenylacetaldehyde was converted to phenylacetic acid with a yield of 50%. We therefore adopt the proposed route of Chapman (1979) and Hartmans *et al.* (1990), in which styrene is metabolized via styrene oxide, phenylacetaldehyde and phenylacetic acid (figure 6.4). 2–Phenylethanol formation may have resulted from phenylacetaldehyde accumulation and the presence of 2–phenylethanol dehydrogenase activity (Hartmans, 1995).

Styrene metabolism via initial oxidation of the aromatic nucleus has been reported for *Rhodococcus rhodochrous* NCIMB 13259 (Warhurst *et al.*, 1994). We did not investigate this route in *E. jeanselmei* because the substrate specificity of both species is quite different. *R. rhodochrous* does not grow on phenylacetaldehyde and phenylacetic acid, whereas styrene metabolism in this organism seems to be closely related to the metabolism of toluene (Warhurst *et al.*, 1994). *E. jeanselmei*, on the other hand, can not grow on toluene nor oxidize this compound after growth on styrene. We therefore assume that initial oxidation of the aromatic nucleus is unlikely to occur in *E. jeanselmei*. This assumption is supported by the high recoveries of intermediates that are involved in the initial oxidation of the ethylene side chain.

Phenylacetic acid catabolism has been widely studied. Generally, degradation of phenylacetic acid may involve the formation of homoprotocatechuic acid via 4–hydroxyphenylacetic acid or the formation of homogentisic acid via either 2– or 3–hydroxyphenylacetic acid (Olivera *et al.*, 1994). After growth on styrene, *E. jeanselmei* could oxidize all mono– and dihydroxylated phenylacetic acids, which may have been caused by gratuitous induction as observed for phenylacetic acid–grown *Nocardia salmonicolor* (Sariaslani *et al.*, 1974) and *Trichosporon cutaneum* (Anderson and Dagley, 1980). However, the identification of accumulating intermediates suggests that styrene–grown *E. jeanselmei* degrades phenylacetic acid via 2–hydroxyphenylacetic and homogentisic acid, though enzyme studies are necessary for unequivocal conclusions. The same route of phenylacetic acid metabolism has been suggested for phenylacetic acid–grown *N. salmonicolor* (Sariaslani *et al.*, 1974) and *Aspergillus niger* (Kluyver and Van Zyp, 1951; Bocks, 1967; Faulkner and Woodcock, 1968) and styrene–grown *Pseudomonas fluorescens* (Baggi *et al.*, 1983).

Styrene transformation in mammals is catalyzed by a cytochrome P–450–dependent styrene monooxygenase (Leibman and Ortiz, 1970; Parkki *et al.*, 1976; Watabe *et al.*, 1978). The present results strongly indicate that styrene oxidation by

E. jeanselmei is also catalyzed by a microsomal cytochrome P–450–dependent monooxygenase. Various observations support this assumption (Smith and Davis, 1980): 1) styrene oxidation is NADPH– and oxygen–dependent, 2) styrene oxidation is inhibited by SKF–525A, metyrapone and CO but not by KCN, 3) the reduced CO difference spectrum of the microsomal fraction of styrene–grown cells shows a characteristic absorption maximum at 450 nm. The presumptive product of styrene monooxygenase is styrene oxide (figure 6.4), which implies monooxygenation; however, we could not detect this compound. Immediate conversion of styrene oxide to phenylacetaldehyde is likely because of the relatively high styrene oxide isomerase activity. Thus it appears that, as in mammals, styrene oxidation in *E. jeanselmei* is catalyzed by a microsomal cytochrome P–450–dependent monooxygenase. However, investigation of styrene oxidation by the purified enzyme is required to unequivocally establish the involvement of cytochrome P–450.

One of the problems in biological treatment of styrene–containing gases could be the formation of toxic or non–degradable intermediates. By using *E. jeanselmei* in biofilters, we do not expect such problems. Accumulation of the toxic styrene oxide is unlikely because of the relatively high rate of styrene oxide degradation. The feasibility of using *E. jeanselmei* in biofilters for styrene removal from waste gases has been demonstrated with respect to the stability of the filter and the high degree of conversion of styrene to CO_2 (chapter 5).

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CHAPTER 7

GENERAL DISCUSSION

Current biofiltration research focuses on the rapid development of biofilters for the treatment of industrial waste gases containing one or more specific pollutants (chapter 1). Biofilters, as compared to other techniques, are especially promising for the treatment of waste gases with flows of 1,000–50,000 $m^3.h^{-1}$ and pollutant concentrations up to 1 $g.m^{-3}$. Though many new types of biofilters are being introduced, the use of biofilters in this application area is still not widespread. Apparently, biofilters are not yet fully recognized as reliable systems that can compete with established techniques, i.e. physical and chemical waste gas treatment. In our opinion, there is a lack of understanding of the microbiological aspects of biofilters. Fundamental and applied research on biofiltration is required to demonstrate the potential use of biofilters in industrial waste gas treatment.

Styrene–degrading biofilters with fungi growing on an inert support material were investigated for two reasons:

- to obtain more general/fundamental knowledge of the processes that take place inside a biofilter;
- to provide industry with an operationally stable and effective biological technique to purify styrene–containing gases.

THE USE OF FUNGI

In nature, fungi typically grow around, in or on solid materials such as soil, wood and leaves, which may result from their ability to grow at a limited water availability. For this reason fungi are preferentially used in for instance solid–state fermentation (Hesseltine, 1977[a] and 1977[b]). Biofiltration is comparable to solid–state

fermentation as the microorganisms grow on a solid surface and not submerged in water. In this view, the use of fungi instead of bacteria in biofilters may also be advantageous. Apart from the utilization of white–rot fungi in biofilters with straw as a support material (Braun–Lüllemann *et al.*, 1991), this study is the first in which fungi are intentionally used for waste gas treatment.

Enrichment of styrene-degrading fungi

Treatment of specific pollutants may require specific microorganisms adapted to the compounds to be degraded. However, growth or permanent activity of selected strains after inoculation of biofilters is in many cases not observed (chapter 1). Instead of selecting strains from culture collections or enriching strains in liquid cultures, we used a different strategy, namely enrichment of fungi in biofilters under conditions that are comparable to industrial waste gas treatment in biofilters. Enrichment in biofilters should lead to species with a high and persistent activity in biofilters. Using soil as an inoculum, a mixed culture of fungi developed, from which ten styrene–degrading fungi were isolated (chapter 2). However, when 'old' biofilters were used to inoculate new biofilters, *Exophiala jeanselmei* became the dominating styrene–degrading species. Obviously, the combined action of low pH, dry conditions, a low styrene concentration and the presence of perlite as support material created an environment favourable for *E. jeanselmei*. These are rather extreme conditions. Interestingly, soil and wood are natural habitats from which *E. jeanselmei* species have been isolated (Henderson, 1961; Dixon *et al.*, 1980; Iwatsu *et al.*, 1981).

Apart from gases containing styrene, enrichment on perlite support material was also successful to obtain biofilters for treatment of gases containing ethene, butadiene, propene, α–methylstyrene (results not published) and a mixture of toluene, ethylbenzene and o–xylene (submitted for publication).

Tolerance to low pH

Acidification of the filter bed may generally be due to the accumulation of acid intermediates or end products. The pH in biofilters with *E. jeanselmei* on perlite decreased from 5.7 to 2.5–3 within some weeks. In this case, acidification of the filter bed most likely resulted from the use of NH_4^+ as a nitrogen source and the absence of a pH–buffering capacity in this filter. Accumulation of intermediates such as phenylacetic acid in the filter bed was not observed (unpublished results). In fact, products from styrene (chapter 6) in batch cultures with *E. jeanselmei* were only

detected when very high styrene concentrations, far exceeding the concentration as supplied to the biofilters, were added. Despite the low pH in the filter bed, the styrene elimination capacity of the biofilter remained high (chapter 5).

An important advantage of the use of (acid–tolerant) fungi in biofilters is that the application range of biofilters may be extended to gases containing compounds which produce acids upon degradation. Degradation of sulfur–containing and chlorinated compounds in biofilters is troublesome with respect to the operational stability of the biofilters and the actions that are necessary to prevent acidification of the filter bed (Tanji *et al.*, 1989; Dolfing *et al.*, 1993; Smet *et al.*, 1994; Ergas *et al.*, 1994; Yang and Allen, 1994). pH control in biotrickling filters is relatively easy, but these filters can not be used for hydrophobic compounds. Biofilters with acid–tolerant fungi, however, may especially be suitable for those hydrophobic, acid–generating compounds, which can not be treated in biotrickling filters.

Tolerance to low water activity

Because *E. jeanselmei* can degrade styrene at a reduced water activity (0.91–1), increased tolerance of the biofilter to drying out conditions is obtained by using this species (chapter 3). However, this is of minor importance from a practical point of view. Humidification of the waste gas to (near) saturation is still required to maintain a high styrene elimination capacity for extended periods (chapter 3). No major long–term improvement is to be expected by using fungi that are more tolerant to low water activity. The lowest water activity for which fungal growth has been reported is 0.605, but at this water activity fungal activity is extremely low (chapter 2). The relative humidity of industrial waste gases from e.g. working areas may well be lower than this value. Therefore, the use of *E. jeanselmei* and other xerotolerant fungi may be more beneficial in case of short periods of drying out, i.e. when the residual water content in the filter bed is still relatively high, rather than for treatment of dry gases.

The water content in compost biofilters has been recognized as a critical parameter influencing microbial activity (chapter 1). Surprisingly, research in this area is very limited. This research has shown that if supply of water is insufficient, a gradient of the water content along the height of the filter bed exists, as well as in single particles. Each biofilter may have different water requirements, which depends both on the water binding capacity of the support material and the demand for water of the microorganisms (chapter 3). General recommendations about the water requirement of biofilters are therefore of limited value.

The high water–holding capacity of perlite provides a large amount of water

which may be available to the microorganisms. This explains why biofilters with *E. jeanselmei* on perlite can effectively treat gases with a relative humidity of 90–95% for 1–2 weeks without adding water to the filter bed. When supplying air with a low relative humidity to the biofilter (± 65%), however, biofilter performance rapidly decreases because the biofilm dries out (chapter 3). Apparently, under these conditions the evaporation of water at the biofilm is not fully compensated by migration of water from the inside of the perlite particle to the biofilm. Biofilter performance under these drying out conditions may therefore be improved by selecting a support material that facilitates the transfer of water from the support material to the biofilm.

Mass transfer of styrene

With respect to the mass transfer of hydrophobic compounds in biofilters, two *a priori* assumptions were made at the beginning of this investigation:

- A less frequent supply of water to the filter bed, as compared to the continuous recirculation of water in biotrickling filters, may result in a thinner water film covering the biofilm and thus in an improved mass transfer. The use of fungi tolerant to dry conditions would be advantageous in this respect.
- Fungi may form mycelium at the biofilm surface, thus improving mass transfer because of a large contact area between the gas phase and the hydrophobic surface of the microorganisms.

To validate the first assumption, determination of biofilter performance at a variable thickness of the water film is necessary. These experiments were not carried out. However, after the addition of mineral medium to the filter bed, styrene degradation rates were relatively low for a few hours (unpublished results), which suggests that excess water in the filter bed may increase the mass transfer resistance of styrene. Recently, Wolff (1992) and De Heyder *et al.* (1994) confirmed this hypothesis for the removal of toluene and ethene, respectively, in biotrickling filters with intermittent sprinkling of the filter bed.

Although mycelium formation at the biofilm surface was observed, *E. jeanselmei* as the dominant styrene–degrading species in the biofilter was only present in the yeast form (chapter 4). A possible improvement of the mass transfer of styrene by mycelium formation could therefore not be demonstrated. This aspect may be investigated by inoculation of biofilters with mycelium–forming fungi (i.e., the *Penicillium* species, see table 2.2). However, it should be noted that limitation of biofilter performance by the diffusion of styrene was only observed at concentrations lower than 0.06 $g.m^{-3}$ (chapter 5). Mass transfer resistance of styrene in biofilters

under industrial conditions is therefore of minor importance.

MODELLING OF BIOFILTERS

Various models describing biofilter performance have been developed (e.g., Ottengraf, 1986; Shareefdeen *et al.*, 1993; Deshusses *et al.*, 1995). Validation is usually done by determination of the removal efficiency at various pollutant concentrations and volumetric loads. It is remarkable that in all models the amount of biomass is not included as an independent parameter. The presence of biomass is only evident from the elimination capacity of the biofilter (expressed per unit of biofilter volume), which would require a certain but unknown amount of biomass. Steady state or quasi–steady state is usually assumed. Models therefore fail to describe possible clogging of the filter bed at high elimination rates and ageing phenomena at long–term operation of biofilters. In this respect a different approach to study biofilters may be advocated. In this approach the time–dependent development of biomass in the filter bed (growth and mineralization), the nutrient availability, the specific activity of the cells in the biofilm and the overall elimination capacity should be correlated, to develop a biofilter with a high elimination capacity for extended periods (years).

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SUMMARY

Styrene is an environmental pollutant, emitted in large quantities to the atmosphere by various industrial sectors. Legislation requires industry to reduce the emission of styrene. One option to purify industrial waste gases is biological treatment in biofilters. Compost biofilters are being used for odour abatement for over 30 years, especially in the Netherlands and Germany. Unfortunately, the results of styrene removal in compost biofilters are poor, although the reason is unclear. The object of the research described in this thesis was to develop a new and more reliable biofilter for the treatment of styrene–containing gases. Basic assumptions prior to this investigation were: 1) the use of fungi instead of bacteria may offer specific advantages with respect to the activity and stability of biofilters, and 2) the use of an inert support material instead of compost may allow for a more fundamental investigation of processes that take place inside biofilters.

To obtain fungi with a high and stable styrene–degrading activity in biofilters, enrichment experiments were carried out using biofilters with various inert support materials and operated under conditions representative for industrial waste gas treatment. In biofilters with activated carbon as support material, mainly styrene–degrading bacteria were enriched. On the other hand, in biofilters with polyurethane or perlite mainly styrene–degrading fungi were enriched. A decrease of the pH in the enrichment biofilters with polyurethane and perlite was probably a decisive factor for this difference. Ten styrene–degrading fungi were isolated: *Gladocladium roseum*, *Exophiala jeanselmei* and eight *Penicillium* species. The styrene degradation rate was highest in the biofilter with perlite and enriched with fungi. The styrene elimination capacity of this biofilter was 62 $g.m^{-3}.h^{-1}$, even after acidification to pH 2.7. This demonstrates an important advantage of using fungi instead of bacteria: acidification of the filter bed does not necessarily results in inhibition of microbial activity in biofilters.

Drying out of the filter bed is a major cause of malfunctioning of compost biofilters. *E. jeanselmei*, immobilized on perlite, degrades styrene at a water activity range of 0.91–1. *E. jeanselmei* may therefore be classified as a xerotolerant fungus. However, for stable operation of biofilters with *E. jeanselmei* growing on perlite, regular supply of water to the filter bed is necessary, even when the relative humidity

of the feed gas is 100%. Treatment of dry gases results in a rapid decrease of the styrene degradation rate because of evaporation of water. However, loss of styrene degradation activity was also observed when the residual water content of the filter bed was still high. This is probably due to an uneven distribution of water: water gradients may exist along the height of the filter bed as well as in single particles. The use of xerotolerant fungi in biofilters may be advantageously when humidification of the waste gas is inadequate for a short period. Stringent control of the water content in the biofilter, however, remains necessary.

In contrast to the simplifying representation of the biofilm as a smooth and homogeneous layer of active cells, the biofilm in styrene–degrading biofilters appeared to be heterogeneous. The microbial population contained eight fungi, of which *E. jeanselmei* was the major styrene–degrading species. The biofilm thickness varied widely (70–600 μm after 265 days of operation) and the surface of the biofilm was far from smooth. Deeper parts of the biofilm contained mainly dead cells, whereas intact cells were mainly observed in the upper layers of the biofilm. After a few months of operation of the biofilters, the surface of the biofilm became covered with mycelium–forming fungi, especially at the outlet side of the biofilter. These fungi were not able to grow on styrene. Extensive mycelium formation causes an increase of the pressure drop over the filter bed, which may endanger the long–term stability of these biofilters.

Biofilter performance can be described with the model developed by Ottengraf. At concentrations of 0.1–2.4 $g.m^{-3}$, styrene degradation was maximal at a rate of 62 $g.m^{-3}.h^{-1}$. The removal of styrene was complete at a lower styrene loading. Styrene degradation takes place only in the upper 80 μm of the biofilm, which probably is caused by oxygen depletion in deeper parts of the biofilm. Although styrene is generally considered a hydrophobic compound, limitation of the degradation rate by the diffusion of styrene was only observed at concentrations lower than 0.06 $g.m^{-3}$. Dutch legislation (NER) requires a maximal styrene concentration of 0.1 $g.m^{-3}$. In this respect no problems are to be expected from the hydrophobicity of styrene and possible mass transfer resistance.

The initial step of styrene degradation by *E. jeanselmei* is oxidation of styrene to styrene oxide. As in mammalian liver cells, this reaction is catalyzed by a cytochrome P–450–dependent monooxygenase, present in the microsomes. Styrene oxide is metabolized to phenylacetic acid via phenylacetaldehyde, catalyzed by a styrene oxide isomerase and a phenylacetaldehyde dehydrogenase. The same pathway is found in most styrene–degrading bacteria. *E. jeanselmei* subsequently hydroxylates phenylacetic acid at the *ortho*–position, after which homogentisic acid is formed.

SAMENVATTING

Styreen is een belangrijke milieuverontreinigende stof die in grote hoeveelheden door de industrie naar de atmosfeer wordt uitgestoten. De wetgeving vereist dat de industriële uitstoot van styreen wordt verminderd. Industriële afgassen kunnen op een biologische wijze in biofilters worden gereinigd. Gedurende 30 jaar worden compost biofilters gebruikt voor de bestrijding van stank, vooral in Nederland en Duitsland. De behandeling van styreen afgassen in compost biofilters is echter weinig succesvol. De reden hiervoor is niet duidelijk. Het doel van het onderzoek van deze dissertatie was de ontwikkeling van een nieuw en meer betrouwbaar biofilter voor de behandeling styreenhoudende gassen. Uitgangspunten bij aanvang van het onderzoek waren: 1) het gebruik van schimmels in plaats van bacteriën kan voordelig zijn met betrekking tot de activiteit en stabiliteit van het biofilter, en 2) met het gebruik van inert dragermateriaal in plaats van compost is wellicht een meer fundamenteel onderzoek mogelijk van de processen die in biofilters afspelen.

Om schimmels met een hoge en stabiele styreenafbrekende activiteit in biofilters te verkrijgen, zijn ophopingsexperimenten uitgevoerd met biofilters gevuld met verschillende inerte dragermaterialen. Deze ophopingsbiofilters werden bedreven onder omstandigheden zoals deze in de praktijk van industriële afgasreiniging voorkomen. In biofilters met verschillende soorten actieve kool als dragermateriaal wordt vooral de ophoping van styreenafbrekende bacteriën waargenomen. In biofilters met polyurethaan en perliet hopen echter vooral styreenafbrekende schimmels op. Waarschijnlijk is een daling van de pH in de biofilters met polyurethaan en perliet de oorzaak van dit verschil. Er zijn tien styreenafbrekende schimmels geïsoleerd: *Gladocladium roseum*, *Exophiala jeanselmei*, en acht *Penicillium* soorten. De styreenafbraak is het snelst in het biofilter met schimmels op perliet. De styreen eliminatiecapaciteit van dit filter is 62 $g.m^{-3}.uur^{-1}$, hoewel een daling van de pH tot 2.7 wordt waargenomen. Dit toont een belangrijk voordeel van het gebruik van schimmels in plaats van bacteriën aan: verzuring van het filterbed leidt niet noodzakelijkerwijs tot een verlies van microbiële activiteit in biofilters.

Een belangrijke oorzaak van het slecht functioneren van compost biofilters is uitdroging van het filter bed. *E. jeanselmei*, geïmmobiliseerd op perliet, breekt styreen af bij een wateractiviteit van 0.91–1. *E. jeanselmei* behoort daarmee tot de groep van

schimmels die onder droge omstandigheden actief kunnen zijn. Voor een hoge en stabiele activiteit van biofilters met *E. jeanselmei* op perliet is echter de toevoeging van water aan het filter bed noodzakelijk, ook wanneer gas met een relatieve luchtvochtigheid van 100% wordt behandeld. De behandeling van droge gassen resulteert in een snelle afname van de styreen eliminatie–activiteit door de verdamping van water. Verlies van activiteit wordt ook waargenomen wanneer het watergehalte in het filterbed nog hoog is. Dit wordt waarschijnlijk veroorzaakt door een ongelijke verdeling van water in het filterbed. Gradiënten van het watergehalte kunnen ontstaan, zowel over de hoogte van het filterbed als in de enkele deeltjes. Het gebruik van schimmels in biofilters kan voordelig zijn wanneer de bevochtiging van het afgas voor een korte periode niet optimaal is. Een strikte controle van het watergehalte in het biofilter blijft echter noodzakelijk.

In tegenstelling tot de vereenvoudigende voorstelling van de biofilm als een gladde een homogene laag van actieve cellen blijkt de biofilm in styreenafbrekende biofilters heterogeen te zijn. De microbiële populatie bestaat uit acht schimmels, waarvan *E. jeanselmei* de belangrijkste styreenafbrekende stam is. Na 265 dagen bedrijfsvoering van het biofilter varieert de dikte van de biofilm tusssen 70 en 600 μm; het oppervlak van de biofilm is ruw. De diepere delen van de biofilm bevatten voornamelijk dode cellen terwijl intacte cellen vooral aan de oppervlakte van de biofilm worden waargenomen. Na een paar maanden treedt groei op van myceliumvormende schimmels op het oppervlak van de biofilm. Deze kunnen styreen niet als groeisubstraat gebruiken. Overmatige myceliumvorming veroorzaakt een stijging van de drukval in het filterbed, hetgeen ongewenst is in verband met de stabiliteit van het filter.

De styreenafbraak in de biofilters kan worden beschreven met het door Ottengraf ontwikkelde model voor compost biofilters. Bij styreenconcentraties tussen 0.1 en 2.4 $g.m^{-3}$ is de maximale styreenafbraaksnelheid 62 $g.m^{-3}.uur^{-1}$. De verwijdering van styreen is 100% indien de belasting van het filter lager is dan deze maximale eliminatie capaciteit. Styreen wordt waarschijnlijk in de bovenste 80 μm van de biofilm afgebroken, mogelijk als gevolg van de uitputting van zuurstof in diepere lagen van de biofilm. Hoewel styreen doorgaans als een hydrofobe verbinding wordt beschouwd, wordt limitatie van het biofilter door de diffusie van styreen alleen waargenomen bij concentraties lager dan 0.06 $g.m^{-3}$. De Nederlandse wetgeving (NER) vereist reiniging van styreenhoudende afgassen tot een concentratie van 0.1 $g.m^{-3}$. In dit opzicht zijn er geen problemen te verwachten met de hydrofobiciteit van styreen en eventuele diffusie–limitatie in het biofilter.

De eerste stap in de afbraak van styreen door *E. jeanselmei* is de oxidatie van

styreen tot styreenoxide. Deze reactie wordt net als in levercellen van zoogdieren gekatalyseerd door een cytochroom P–450–afhankelijk monooxygenase dat in de microsomen aanwezig is. Styreenoxide wordt via fenylacetaldehyde verder afgebroken tot fenylazijnzuur. Dezelfde route wordt bij de meeste styreenafbrekende bacteriën gevonden. *E. jeanselmei* hydroxyleert fenylazijnzuur vervolgens aan de *ortho*–positie, waarna 2,5–dihydroxyfenylazijnzuur wordt gevormd.

NAWOORD

Het mooie van een promotie–onderzoek is dat je (tamelijk) eigenwijs je eigen gang kunt gaan. Maar natuurlijk lukt dat niet alleen. Allen die aan dit onderzoek hebben bijgedragen, met name iedereen van de afdeling Milieubiotechnologie van TNO, wil ik graag bedanken.

Een aantal mensen wil ik in het bijzonder noemen. Allereerst mijn promotoren Wim Harder en Lubbert Dijkhuizen die de grote lijn in de gaten hielden. Hans Doddema was de begeleider van het onderzoek en ook degene die het gebruik van schimmels op inerte dragers in biofilters voorstelde. Het electronen–microscopisch onderzoek (hoofdstuk 4) werd gecoördineerd en uitgevoerd door Marten Veenhuis, Klaas Sjollema en Jan Zwager. Sybe Hartmans gaf mij een introductie in de enzymologie van styreenafbraak (hoofdstuk 6); hij stelde tevens de styreen–afbrekende schimmel *Exophiala jeanselmei* (geïsoleerd door Mariët van der Werf) beschikbaar. Ik sluit niet uit dat het voorkomen van deze schimmel in de ophopingsbiofilters (hoofdstuk 2) wellicht het gevolg van een infectie is geweest. Christian Kennes heeft als post–doc in korte tijd aangetoond dat de strategie van ophoping (hoofdstuk 2) ook succesvol is voor het verkrijgen van biofilters met een hoge capaciteit voor de afbraak van een tolueen/ethylbenzeen/xyleen mengsel. Bart Faber heeft essentiële aanwijzingen gegeven voor het aantonen van het cytochroom P–450–afhankelijke styreen monooxygenase in celvrije extracten van *E. jeanselmei* (hoofdstuk 6).

Een promotie–onderzoek draait echter vooral om het verzamelen van resultaten. Zonder de hulp van José Houtman (hoofdstuk 2), Frank Magielsen (hoofdstuk 3), Richard Moerman (hoofdstuk 5), Erwin van Heiningen (hoofdstukken 5 en 6), Hans Radhoe (hoofdstuk 6) en Sabine van Baalen (hoofstukken 5 en 6) was dit niet gelukt.

Dit onderzoek werd gefinancieerd met doelsubsidie van het Ministerie van Economische Zaken, gericht op de stimulering van de milieubiotechnologie.