

## Stable isotope probing as an effective tool for direct identification of active microorganisms in biofilters

André Lipski, Julia Piehl and Michèle Friedrich<sup>a</sup>

Universität Osnabrück, Abteilung Mikrobiologie, Fachbereich  
Biologie/Chemie, 49069 Osnabrück, Germany

<sup>a</sup>Present address: Fachhochschule Flensburg, Institut für Verfahrenstechnik  
und Biotechnologie, Kanzleistr. 91-93, 24943 Flensburg, Germany

**ABSTRACT.** Active styrene and hexane-degrading microorganisms in biofilters were directly characterized without cultivation. For direct probing biofilter material samples were incubated with deuterated substrates. These substrates were rapidly assimilated by the degrading microbial populations and incorporated in chemotaxonomically relevant cell lipids. Phospholipid fatty acids were extracted from the filter material and labeled fatty acids were recognized by gas chromatography-mass spectrometry. The spectrum of labeled fatty acids was compared with the fatty acid profiles of cultivated isolates to assess the relevance of the isolated microorganisms for the degradation process. From styrene treated biofilters we could isolate several styrene degrading strains from various taxonomic groups. However, none of the isolates showed a fatty acid pattern similar to the set of labeled lipids from the filter material. Fatty acid analyses of the enrichment cultures revealed that labeled lipids were rapidly lost during the enrichment procedure. In contrast, for a hexane-degrading biofilter the direct probing approach identified a defined group of isolates as apparent hexane degraders of the full scale bio-filtration process. Isolates with matching fatty acid profiles were identified as members of the genus *Gordonia*. Other isolates with high hexane degrading activities in artificial media were predominantly assigned to the Proteobacteria. According to their deviating fatty acid profiles most of these strains could be excluded from the group of important biocatalysts in the filtration process.

### 1 INTRODUCTION

Most models for the performance of biofilters depend on information about degradation pathways and kinetics of microorganisms isolated from these or similar systems. However, it is well-known that in many complex systems isolation techniques are insufficient for the detection of the catalyzing microorganisms. These techniques are highly selective for fast growing organisms adapted to the enrichment and cultivation medium used. Although the strains are present in the system and have the potential for the degradation of the waste gas compounds, these methods provide no information about the relevance of these strains for the degradation process *in situ*. Slow growing microorganisms, which play a major role in degradation and transformation processes in many habitats, were not recognized as important players of the microbial community of biofilters by isolation techniques. Therefore, it is of central importance to combine

isolation techniques with direct probing methods to validate the relevance of the isolates obtained from enrichment cultures.

For direct probing of biofilter material we incubated filter material samples with deuterated substrates. These substrates were rapidly assimilated by the degrading microbial populations and incorporated in chemotaxonomically relevant cell lipids. Phospholipid fatty acids were extracted from the filter material and labeled fatty acids were recognized by gas chromatography-mass spectrometry. Chemotaxonomically characteristic labelled fatty acids were compared with the fatty acid profiles of bacterial strains isolated from these samples to identify the relevant degraders of a styrene degrading biofilter and a hexane-degrading biofilter.

## 2 MATERIALS AND METHODS

### *2.1 Description of biofilters analysed*

Two full scale biofilters were analysed. One biofilter was used for the treatment of styrene containing waste gas of a varnishing process. The other filter was treated with the waste gas of an oil mill. This waste gas contained hexane as major compound. Tree bark compost and crushed tree roots served as filter material in both biofilters.

### *2.2 Enrichment and characterization of isolates*

Enrichment cultures were started using defined liquid media supplemented with styrene or hexane, respectively, as sole source of carbon and energy. Cultures were transferred periodically into fresh medium. Pure strains from both enrichments were obtained by isolation on agar media. The strains were characterized by fatty acid analyses and sequencing of the 16S rRNA genes as described previously (Lipski and Altendorf, 1997; Timke *et al.*, 2005).

### *2.3 Stable isotope probing*

Filter material samples (10 and 20 g) were supplemented with 20 µl of deuterated styrene or deuterated hexane, respectively, and were incubated in 500 ml screw-cap flasks. The gas phase of the flasks was regularly analysed for concentrations of O<sub>2</sub>, CO<sub>2</sub> and the deuterated substrate to monitor the activity of the samples. When significant degradation of the deuterated compound was detected the samples were subjected to phospholipid fatty acid analysis. Lipid analysis, detection and quantification of the labeled fatty acids were performed as described previously (Alexandrino *et al.*, 2001).

## 3 RESULTS AND DISCUSSION

From both biofilters we could enrich and isolate several strains from various taxonomic groups which grow on the respective compound, styrene or hexane, as the sole source of energy and carbon. The fatty acid profiles of selected isolates which represent groups of similar strains are presented in Table 1 and 3. The identification of the strains is based on their 16S rRNA sequences and their lipid profiles. According to their phylogenetic diversity, the strains showed significant differences in their fatty acid profiles.

Table 1. Fatty acid profiles of styrene degrading strains isolated from the enrichment cultures inoculated with styrene treated biofilter material. Identification is based on fatty acid profiles and 16S rRNA sequences.

Fatty acid [%]	<i>Arthrobacter psychrophilus</i> BS19	<i>Cellulomonas gelida</i> BS27	<i>Rhodococcus erythropolis</i> BS28	<i>Burkholderia multivorans</i> KS12	<i>Sphingomonas</i> spec. B8	<i>Comamonas acidovorans</i> KN3	<i>Pseudomonas citronellolis</i> KN1
10:0 3OH						1	2
12:0						2	4
12:0 2OH							3
12:0 3OH							4
14:0 iso		1					
14:0	1	3	3	4		1	
15:0 iso		1					
15:0 anteiso	67	63					
15:0			2		1	1	
14:0 2OH					13		
14:0 3OH				1			
16:0 iso	6	1					
16:1 <i>cis</i> 9				10	7	8	8
16:1 <i>cis</i> 10/ <i>trans</i> 9			14				
16:0	1	11	44	29	5	46	29
17:0 anteiso	26	20					
17:0 cyclo9-10				10	4	35	11
17:0			2				
18:1 <i>cis</i> 9			21				
18:1 <i>cis</i> 11				34	70	5	23
18:0				1			
18:0 10methyl			15				
19:0 cyclo11-12				11		1	17

The comparison of the lipid patterns of the styrene degrading isolates with the fatty acid profiles extracted directly from the respective filter material revealed that none of the isolates had a fatty acid profile similar to the set of labeled fatty acids from the filter material (Figure 1). The lipid analysis of the filter material showed that the fatty acid 16:1 *cis*11 was intensively labeled as a result of assimilation of the deuterated substrate. This characteristic lipid marker of the styrene degrading microorganisms of the filter could not be detected in any of the strains isolated from this filter. To analyse the effect of the enrichment procedure on the styrene degrading population of the filter material, the microbial communities of the primary enrichment culture and the following transfer cultures were also examined by fatty acid analyses. Table 2 shows that the percentage of the characteristic marker lipid 16:1 *cis*11 decreased drastically during the first enrichment culture and was no longer detectable in the following transfer cultures. From the final transfer culture several strains were isolated and identified as

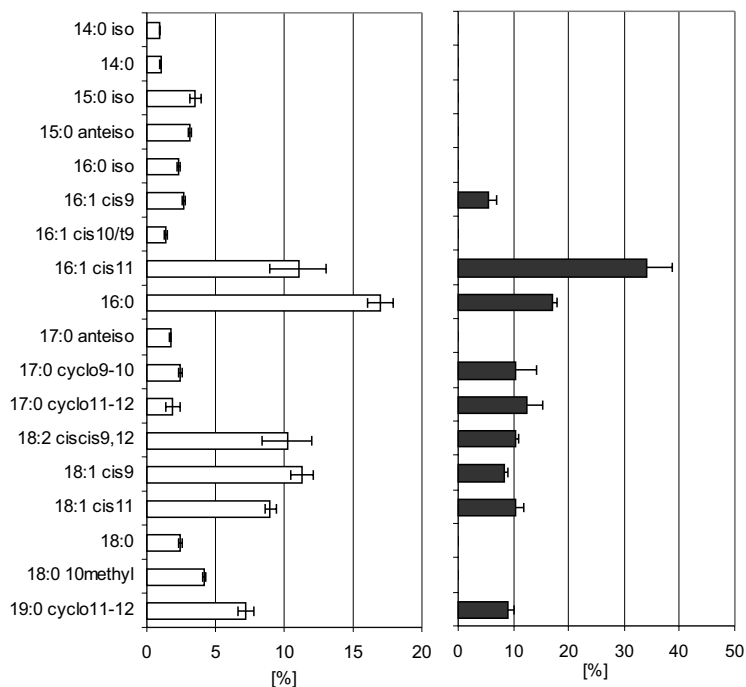


Figure 1. Phospholipid fatty acid profile of a sample from the styrene treated biofilter after incubation for 7 days with deuterated styrene (white bars on the left side). On the right side the labeled fraction of the fatty acids is presented.

*Pseudomonas citronellolis*. The fatty acid profile of this organism dominated the profile of the enrichment culture which showed that the original styrene degrader was quickly outcompeted by the *Pseudomonas* population, which seems to be better adapted to the cultivation conditions used.

In contrast, for the hexane-degrading biofilter the direct probing approach identified a defined group of isolates as apparent hexane degraders of the full scale biofiltration process. Isolates identified as *Gordonia polyisoprenivorans* showed in addition to the ubiquitous palmitic acid (16:0) the characteristic oleic acid (18:1 *cis*9) and tuberculostearic acid (18:0 10methyl) which were identified as the most intensively labeled fatty acids of the filter material by the isotope probing approach (Table 3, Figure 2). Other isolates with high hexane degrading activities in artificial media were predominantly assigned to the Proteobacteria (Table 3). According to their deviating fatty acid profiles most of these strains could be excluded from the group of important biocatalysts in the filtration process.

These examples show that the stable isotope probing technique represents an important complement to enrichment procedures. The method provides a criterion to decide whether or not the cultivated isolates are of central importance for the biofiltration process or mere cultivation artefacts.

Table 2. Shift of an enrichment culture supplemented with styrene after two transfers monitored by fatty acid profiling. For comparison the fatty acid profiles of the filter material and of a *Pseudomonas citronellolis* strain isolated from the final enrichment culture is given.

Fatty acid [%]	Filter-material	Enrichment culture	First transfer culture	Second transfer culture	<i>Ps. citronellolis</i> KN1
10:0 3OH		2	2	3	2
12:0		1	1	1	4
12:0 2OH		2	1	3	3
12:0 3OH		2	1	3	4
15:0 iso	3	1	1	2	
15:0 anteiso	4	1	1	1	
14:0 2OH		1	2	1	
16:0 iso	2	1	1	1	
16:1 cis9	5	11	15	14	8
16:1 cis11	13	1			
16:0	14	22	26	19	29
17:0 cyclo9-10	2	8	10	10	11
17:0 cyclo11-12	2				
18:2 cis9,12	7	1			
18:1 cis9	9	2			
18:1 cis11	16	22	19	19	23
18:0 10methyl	3				
19:0 cyclo11-12	6	12	9	11	17

Table 3. Fatty acid profiles of hexane degrading strains isolated from the enrichment cultures inoculated with hexane treated biofilter material. Identification is based on fatty acid profiles and 16S rRNA sequences.

Fatty acid [%]	<i>Gordonia polyisoprenivorans</i> MN110a	<i>Brevibacillus choshinensis</i> MN47.2a	<i>Leifsonia shinsuensis</i> MN177	<i>Kocuria rhizophila</i> MN59	<i>Pandoraea pnomemusa</i> MN182.2	<i>Burkholderia multivorans</i> MN101.b	<i>Xanthobacter flavus</i> MN45.1	<i>Sphingomonas</i> spec. MN122.2a
8:0	2							
10:0	2							
14:0 iso		15		1				
14:0	2	1	1	1	1	3		
14:0 3OH					2	1		
15:0 iso		20	3	17				
15:0 anteiso		45	25	62				
14:0 2OH								4
16:1 iso 5		4						
16:0 iso		10	11	6				
16:1 cis9					5	14		
16:1 cis10	7							
16:0	37	1	9	1	29	29	1	20
17:0 cyclo9-10					29	5		
17:1 cis11								2
17:0 iso		1	2	1				
17:0 anteiso		3	49	9				
17:0	1						2	
16:0 3OH					2	1		
18:1 cis9	30							
18:1 cis11					10	39	89	63
18:0	6				1	1	2	
18:1 cis11 11methyl								4
18:0 10methyl	13							
19:0 cyclo11-12					21	4	6	7

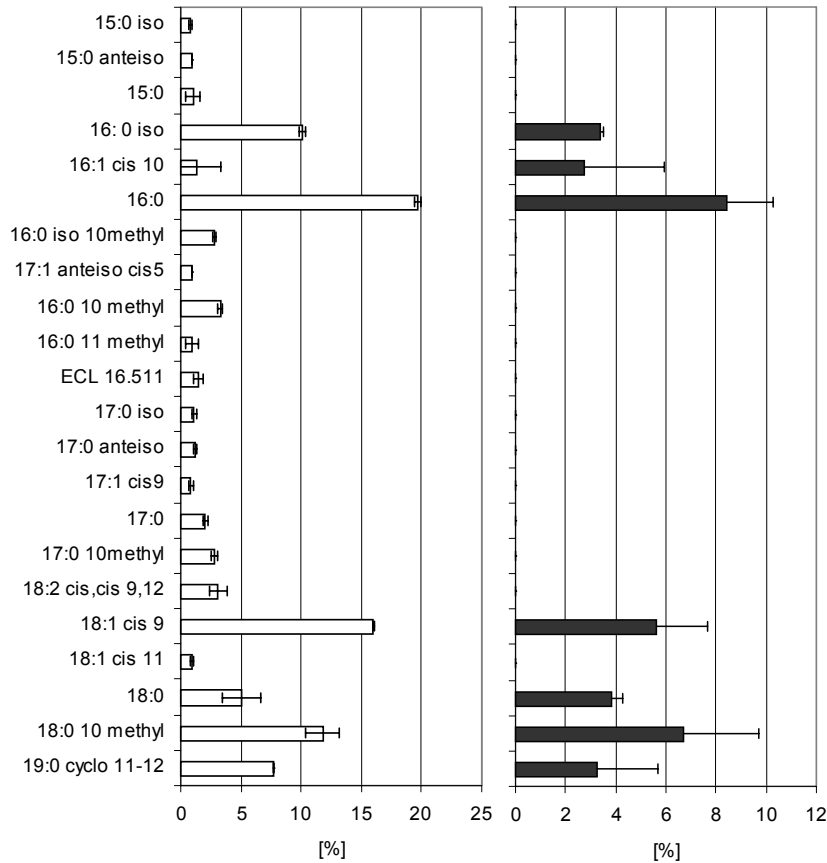


Figure 2. Phospholipid fatty acid profile of a sample from the hexane-treated biofilter after incubation for 9 days with deuterated hexane (white bars on the left side). The labeled fraction of the fatty acids is presented on the right side.

#### 4 REFERENCES

- Alexandrino, M., Knief, C. and Lipski, A. (2001) Stable-isotope-based labelling of styrene-degrading microorganisms in biofilters. *Appl. Environ. Microbiol.* 67: 4796-4804.
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- Timke, M., Wolking, D., Wang-Lieu, N. Q., Altendorf, K. and Lipski, A. (2005) Microbial composition of biofilms in a brewery investigated by fatty acid analysis, fluorescence in situ hybridisation and isolation techniques. *Appl. Microbiol. Biotechnol.* 66: 100-107.