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Biotransformation of biphenyl by the filamentous fungus *Talaromyces helicus*

Maria C. Romero[1], Elke Hammer[2,*], Renate Hanschke[2], Angelica M. Arambarri[1] and Frieder Schauer[2]
[1]*Instituto Botanica Spegazzini, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, La Plata, Argentina*
[2]*Institut für Mikrobiologie, Ernst-Moritz-Arndt-Universität Greifswald, F.-L.-Jahn-Str. 15, 17487 Greifswald, Germany*
**Author for correspondence: Tel.: +49-3834-864211, Fax: +49-3834-864202, E-mail: hammer@uni-greifswald.de*

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Summary

The filamentous fungus *Talaromyces helicus*, isolated from oil-contaminated sludge, oxidizes biphenyl via 4-hydroxybiphenyl to the dihydroxylated derivatives 4,4′-dihydroxybiphenyl and 3,4-dihydroxybiphenyl, which, to a certain extent, are converted to glycosyl conjugates. The sugar moiety of the conjugate formed from 4,4′-dihydroxybiphenyl was identified as glucose. Further metabolites: 2-hydroxybiphenyl, 2,5-dihydroxylated biphenyl, and the ring cleavage product 4-phenyl-2-pyrone-6-carboxylic acid accumulated only in traces. From these results the main pathway for biotransformation of biphenyl in *T. helicus* could be proposed to be the excretion of dihydroxylated derivatives (>75%) and their glucosyl conjugates (<25%).

Introduction

Biphenyl and the monohydroxylated derivatives 2-hydroxy- and 4-hydroxybiphenyl are known to be fungistatic substances. These compounds are widely used for the conservation of citrus fruits, even though biphenyl is known for its toxic effects on humans (Hakkinen *et al.* 1973). Furthermore, it has been found that 4-hydroxybiphenyl shows estrogenic effects (Schultz *et al.* 1998).

In contrast to bacteria, complete mineralization of biphenyl has never been found in fungi. However, the transformation pathways for xenobiotics in these organisms are of interest, due to their high similarity to the metabolism of mammalian systems (Smith & Rosazza 1974). The ability to hydroxylate such compounds has been found in some yeasts and filamentous fungi (Dodge *et al.* 1979; Smith *et al.* 1980; Cerniglia 1997) as well as in mammals (Meyer & Scheline 1976). Beside monohydroxylated derivatives, di- and trihydroxylated biphenyls were also found. Generally, the transformation results in the formation of derivatives with better water solubility, that sometimes even exceed the parent compounds in toxicity and accumulate as end products.

Recently, more and more data have accumulated indicating that hydroxylated biphenyl derivatives can also undergo ring cleavage in fungi. Products formed during this biotransformation in yeasts (Lange *et al.* 1998; Sietmann *et al.* 2001) and the filamentous fungus *Paecilomyces lilacinus* (Gesell *et al.* 2001) were identified as phenylmuconic acid derivatives and the corresponding lactones.

Presumably because of its fungistatic activity, few data are available concerning biphenyl metabolism in filamentous fungi. Biphenyl oxidation has only been studied in the deuteromycetes *Aspergillus parasiticus* (Cox & Golbeck 1985; Mobley *et al.* 1993) and *Paecilomyces lilacinus* (Gesell *et al.* 2001) and in the zygomycete *Cunninghamella echinulata* (Seigle-Murandi *et al.* 1991).

The aim of this work was to study the biotransformation of the fungicide biphenyl by an ascomycetous, filamentous soil fungus, isolated from a hydrocarbon-contaminated site, as well as to study the kinetics of the product formation and compare the data obtained with results for other filamentous fungi of the genus *Talaromyces*.

Materials and methods

Growth and culture conditions

Talaromyces helicus was isolated from contaminated sludge of the East Channel, near the YPF-oil Refinery in La Plata, Argentina. Cultures were maintained on malt agar slants at 4 °C. The fungus was identified by colony morphology and morphology of hyphae, conidia as well as ascospores, by scanning electron microscopy

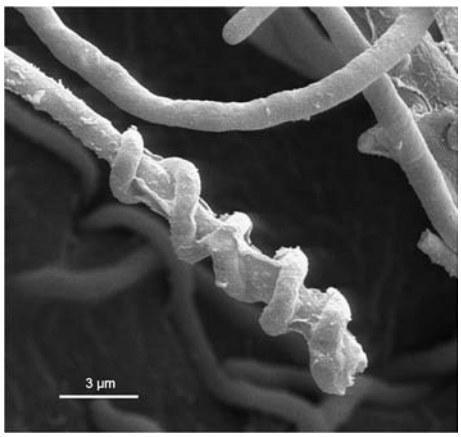

Figure 1. Typical ascogonium with antheridium of the ascomycete *T. helicus*. Scanning electron microscopic study at 5000-fold magnification.

(Figure 1), applying the method of Hanschke & Schauer (1996).

The ability to grow on alkanes or aromatic compounds was tested on a solid mineral salts medium. The substrates were supplemented via the gas phase.

The strain was pre-cultivated in 40 ml Sabouraud (2% glucose, 1% peptone) medium, for 48 h at 180 rev min^{-1} and 30 °C. For degradation experiments 1 ml of this culture was used to inoculate 100 ml of a mineral salts medium (MM: Kaufman & Blake 1973) in 500 ml shake flasks, supplemented with 10 g glucose l^{-1}, pH 5.4. After incubation for 3 days at 30 °C and 180 rev min^{-1} on a rotary shaker, the biomass was harvested by centrifugation (5000 × g, 5 min) and washed twice with sterile MM. The fungal biomass (210 mg dry weight) was resuspended in 100 ml MM and 1 g of biphenyl l^{-1} were added. Additional cultures (500 ml flasks with 100 ml of MM and 0.32 g of mycelium) were incubated with 1 g 4-hydroxybiphenyl l^{-1}, 0.1 g 4,4′-dihydroxybiphenyl l^{-1} or 0.05 g 3,4-dihydroxybiphenyl l^{-1} to enhance the yield of intermediates. Assays with the fungus without substrate in MM as well as biphenyl in MM without biomass were used as controls. All assays were carried out in duplicate. Standard deviation was no more than 10%.

Chemical analysis and identification of metabolites

At different sampling periods, 2 ml of the culture liquid were centrifuged (5000 × *g*, 5 min). Formation of metabolites was followed by analysing 100 µl of the supernatant by reverse phase HPLC according to Hammer & Schauer (1997).

For characterization of metabolites, culture supernatant was separated from the mycelium by centrifugation after 129 h. The supernatant was then extracted twice with ethyl acetate at pH 7, and once again after acidification of the aqueous residue to pH 2. The organic phases were dried over anhydrous sodium sulphate, and the solvent was removed by evaporation. The residues obtained were dissolved in methanol.

The mycelium obtained was washed twice with 5 ml methanol; the extracts were dried over anhydrous sodium sulphate and the solvent was reduced to 1 ml by evaporation.

The three extracts were then analysed by HPLC. The u.v–visible absorption spectra of the degradation products were determined in a diode array detector HP 1040 (Hewlett Packard, Bad Homburg, Germany). Extracts were analysed by GC–MS on a gas chromatograph GC 8000 linked to a mass-selective detector MD 800 (Fisons Instruments, Mainz, Germany) operating at 70 eV, fitted with a 30-m DB5-ms column (0.25 mm by 0.33 µm film; J & W Scientific, Folsom, Calif., USA). The temperature program was : 5 min at 80–300 °C at 10° min^{-1}. Extracts were analysed again after derivatization with diazomethane (formation of methyl derivatives), as described by De Boer & Backer (1956).

Inhibition experiments

The same methodology mentioned above was employed in the inhibition experiments, but in addition to the substrates biphenyl or 4-hydroxybiphenyl, the cytochrome P-450 inhibitor 1-aminobenzotriazole was added in equimolar amounts. Samples were taken and analysed by HPLC as described above.

Deconjugation experiments

Enzymatic deconjugation experiments were carried out with β-glucuronidase and arylsulphatase according to Cerniglia *et al.* (1982) and with xylosidase as described by Sutherland *et al.* (1992). In all assays 50 mg lyophilizate was used. Samples incubated without addition of enzymes served as controls. After enzyme treatment 100-µl samples were analysed by HPLC and another portion was subjected to characterize the sugar moiety of the conjugate. The estimation of glucose was carried out in an enzymatic assay based on glucose oxidase and peroxidase (Bergmeyer *et al.* 1974). This test was shown to be specific for glucose and was negative for xylose, rhamnose, mannose, and glucuronic acid.

Chemicals

Biphenyl, 2- and 4-hydroxybiphenyl were obtained from Merck (Darmstadt, Germany). 3,4-Dihydroxybiphenyl was purchased from Promochem (Wesel, Germany) and 1-aminobenzotriazole from Aldrich (Steinheim, Germany). All other chemicals and solvents were of the highest purity available.

Results

The ascomycete *Talaromyces helicus* was isolated among a number of yeasts and filamentous fungi obtained from oil-contaminated sludge. The strain was identified as *T. helicus* by colonies showing typical yellow reverse,

ascospores, that are only delicately spinulose, and the shaped ascogonia around which thin antheridia coil tightly, soon growing out into a large terminal coil from which the ascogonous hyphae originate (Figure 1).

The fungus was able to use hexadecane as well as phenol supplemented via the gas phase for growth on solid medium. In contrast, biphenyl was not used for growth on solid or in liquid medium. However, incubation of *T. helicus* with biphenyl and analysis of the culture supernatant by HPLC showed six products accumulating, which were not found in control assays (Figure 2). By comparing u.v.-spectral data as well as retention times on the reverse phase HPLC column with those of authentic standards, four of them were identified as 4-hydroxybiphenyl, 2-hydroxybiphenyl, 3,4-dihydroxybiphenyl, and 4,4′-dihydroxybiphenyl. In contrast, four of six other *Talaromyces* strains studied accumulated 4-hydroxybiphenyl as the only product (Table 1).

For kinetic studies, the increase of the metabolites mentioned and the formation of two unknown metabolites, I and II (retention time 4.8 and 5.8, respectively) were determined by HPLC. 4-Hydroxybiphenyl and 4,4′-dihydroxybiphenyl were the major metabolites and reached the maximum values at 48 h (Figure 3). The relatively stable concentrations of all hydroxylated products over the following 100 h analysed, indicated that no further degradation of these products occurred under the incubation conditions used. Metabolites I and II showed u.v. spectra with high similarity to those of 3,4-dihydroxybiphenyl (I) and 4,4'-dihydroxybiphenyl (II), but with lower retention times. Calculated on base of the response factor of the biphenyl derivatives, these unknown metabolites were produced in lower amounts than the hydroxylated products.

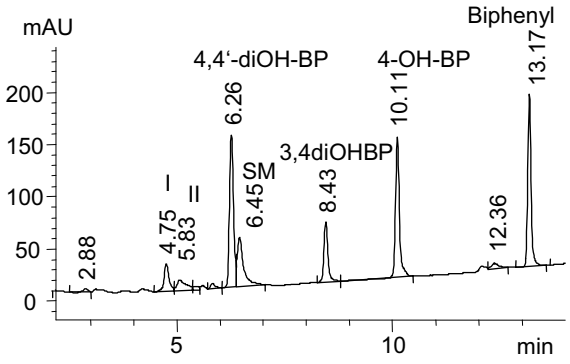

Figure 2. HPLC elution profile of an aqueous culture supernatant after incubation of *T. helicus* with biphenyl (1 g l^{-1}) after 48 h. Six biphenyl transformation products were detectable after separation on a reverse phase column. 2-Hydroxybiphenyl could only be detected by the typical u.v. spectrum next to the 4-hydroxybiphenyl peak. SM: secondary metabolite, 4-OH-BP: 4-hydroxybiphenyl; 3,4diOHBP: 3,4-dihydroxybiphenyl; 4,4'diOHBP: 4,4'-dihydroxybiphenyl; I; II: metabolites I and II.

For further characterization by GC–MS analysis, metabolites were extracted from the culture fluid with ethyl acetate at pH 7 and pH 2, and the methanol mycelium extracts and ethyl acetate extracts of the culture supernatant were analysed. The gas chromatographic and mass spectral data of products adsorbed to the mycelium and extracted from culture supernatant at pH 7 confirmed the data obtained by HPLC-DAD analyses. In the extracts 4-hydroxybiphenyl (170 [M^+], 141 [M]-CHO, 115 [M]-COC_2H_3), 4,4'-dihydroxybiphenyl (186 [M^+], 157 [M]-CHO, 141 [M]-CHO-O, 128 [M]-$C_2H_2O_2$, 115 [M]-COC_2H_3, 77 C_6H_5), 3,4-dihydroxybiphenyl (186 [M^+], 157 [M]-CHO, 128 [M]-$C_2H_2O_2$), and traces of 2,5-dihydroxybiphenyl (186 [M^+], 157 [M]-CHO, 128 [M]-$C_2H_2O_2$) and 2-hydroxybiphenyl (170 [M^+], 141 [M]-CHO, 115 [M]-COC_2H_3) were identified by comparison of chemical data with those of standard compounds.

The main peak found in the acidic extract showed a molecular ion peak at *m/z* 230 ($C_{13}H_{10}O_4$) and main fragment ions at *m/z* 202 ($C_{12}H_{10}O_3^+$, M^+-CO), 171 ($C_{11}H_7O_2^+$, M^+-$COOCH_3$), 115 ($C_9H_7^+$, M^+-$COOCH_3$-2 × CO), which corresponds to the ring cleavage product 4-phenyl-2-pyrone-6-carboxylic acid.

Incubation of the fungus with the hydroxylated biphenyl derivatives 4-hydroxybiphenyl (1 g l^{-1}), 3,4-dihydroxybiphenyl (0.05 g l^{-1}), and 4,4′-dihydroxybiphenyl (0.1 g l^{-1}) showed that these substances were also subject to further transformation. Thus, 4-hydrox-

Table 1. Biotransformation of biphenyl (0.01%) by fungal strains of the genus *Talaromyces*. Product formation after incubation for 14 days.

Strains	Products				
	2-OH-BP[a]	4-OH-BP	3,4diOHBP	4,4′diOHBP	Conjugates
T. flavus SBUG-M892[b]	–	+	–	–	–
T. flavus SBUG-M941	–	+	–	–	–
T. flavus SBUG-M1023	–	+	–	+	–
T. helicus SBUG-M	+	+	+	+	+
T. rotundus SBUG-M1004	+	–	–	–	–
T. stipitatus SBUG-M271	–	+	–	–	–
T. wortmannii SBUG-M410	–	+	–	–	+

[a] 2-OH-BP: 2-hydroxybiphenyl; 4-OH-BP: 4-hydroxybiphenyl; 3,4diOHBP: 3,4-dihydroxybiphenyl; 4,4′diOHBP: 4,4′-dihydroxybiphenyl.
[b] All *Talaromyces* strains except *T. helicus* were obtained from the Strain collection of the Institute for Microbiology of Greifswald University and were isolated from natural habitats.

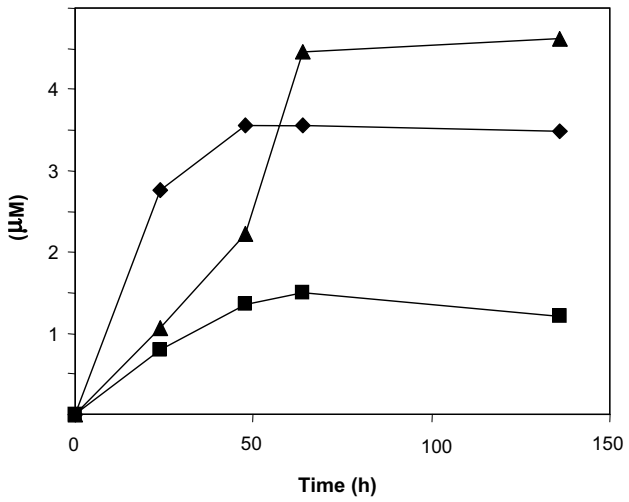

Figure 3. Time-dependent formation of products by glucose-grown cells of *T. helicus* during incubation with biphenyl (1 g l^{-1}). (■) 4-Hydroxybiphenyl; (▲) 3,4 hydroxybiphenyl; (◆) 4,4′-dihydroxybiphenyl.

ybiphenyl was transformed to 3,4-dihydroxybiphenyl (7.5%) and 4,4′-dihydroxybiphenyl (44.5%), within 22 h incubation time. 3,4-Dihydroxybiphenyl was transformed to 4-phenyl-2-pyrone-6-carboxylic acid (>90%) and compound I (traces); 4,4′-dihydroxybiphenyl was slowly transformed to product II (10%).

Identification of products I and II

Both products (I and II) seemed to be more hydrophilic, as indicated by their lower retention times on reverse phase material compared to the parent compounds. Neither of these two products was detectable after extraction of the culture supernatant by ethyl acetate followed by GC–MS analysis. In view of the high similarity of u.v. spectra to those of hydroxylated biphenyls and in comparison to the behaviour of products found after biotransformation of dibenzofuran by *Penicillium canescens* (Hammer *et al.* 2001), these compounds were assumed to be conjugates. This assumption should be proven by deconjugation experiments carried out with glucuronidase and arylsulphatase. Culture supernatant containing products I and II was concentrated by lyophilization, and individual 50-mg-samples were treated with each of the enzymes. Hydrolysis with glucuronidase led to the depletion of the products I and II and produced increasing amounts of 3,4-dihydroxybiphenyl (from product I) and 4,4′-dihydroxybiphenyl (from product II). In contrast, both the control without enzyme and the sample treated with arylsulphatase showed no hydrolysis and both conjugates remained intact. Because of the unspecific reaction of glucuronidase towards several sugar conjugates, the sugar moiety was analysed by a more specific enzymatic assay for glucose with glucose oxidase. After glucuronidase treatment of separated product II, the assay showed accumulation of glucose in the samples, whereas the sugar was not detectable in the controls (medium, buffer, arylsulphatase assay, assay without enzymes). The amount of product I was too low for successful separation and identification of the sugar.

Inhibition experiments and enzyme assays

1-Aminobenzotriazole affected the biphenyl transformation so that only about 50% of the 4-hydroxybiphenyl was formed and no other metabolites could be detected. Biotransformation of 4-hydroxybiphenyl was nearly completely inhibited by 1-aminobenzotriazole. Only traces of 3,4-dihydroxybiphenyl (2%) and 4,4′-dihydroxybiphenyl (8%) were observed.

Discussion

The fungus *T. helicus* cannot use biarylic compounds like biphenyl, dibenzofuran, or naphthalene for growth. However, the fungus is able to co-metabolize these compounds. Cells grown in mineral salts medium with glucose or grown on complex medium in addition, transformed the hydrophobic parent compound to more hydrophilic products: hydroxylated products in high amounts and more hydrophilic sugar conjugates as well as ring-cleavage products in lower amounts.

The fungus produced relatively large amounts of hydroxylated intermediates, which included monohydroxylated and dihydroxylated compounds, indicating the involvement of monoxygenases in the biotransformation pathway. From biphenyl, 4-hydroxybiphenyl was the major product, whereas production of 2-hydroxybiphenyl was rather low. Hydroxylation of the C4- position was also described as the main pathway for other filamentous fungi, yeasts, and mammals (Meyer & Scheline 1976; Smith *et al.* 1980; Golbeck *et al.* 1983; Lange *et al.* 1998; Sietmann *et al.* 2000). A second hydroxylation by *T. helicus* can occur in the same ring as well as in the second ring. As a result, the dihydroxylated derivatives 3,4-dihydroxybiphenyl and 4,4′-dihydroxybiphenyl are produced in a ratio 1:3. Strong accumulation of *para*, *para*′-hydroxylated biphenyl was also observed for *Aspergillus* strains by Mobley *et al.* (1993). In contrast, hydroxylation at the unsubstituted ring of 4-hydroxybiphenyl occurred only in traces or could not be observed at all in yeast. In strains of the genus *Trichosporon* (Sietmann *et al.* 2002) or *Debaryomyces vanrijiae* (Lange *et al.* 1998) the main transformation pathway of biphenyl goes via 4-hydroxybiphenyl and 3,4-dihydroxybiphenyl up to ring cleavage. Formation of considerable amounts of 2,5-dihydroxybiphenyl as described for *Trichosporon mucoides* (Sietmann *et al.* 2000) was not observed in *T. helicus*.

The strong inhibition of hydroxylation reactions in the presence of the cytochrome-P-450-inhibiting substance 1-aminobenzotriazole points to an involvement of such enzymes in the transformation reactions. The differences in the level of inhibition of the first and the second hydroxylation may indicate the existence of different

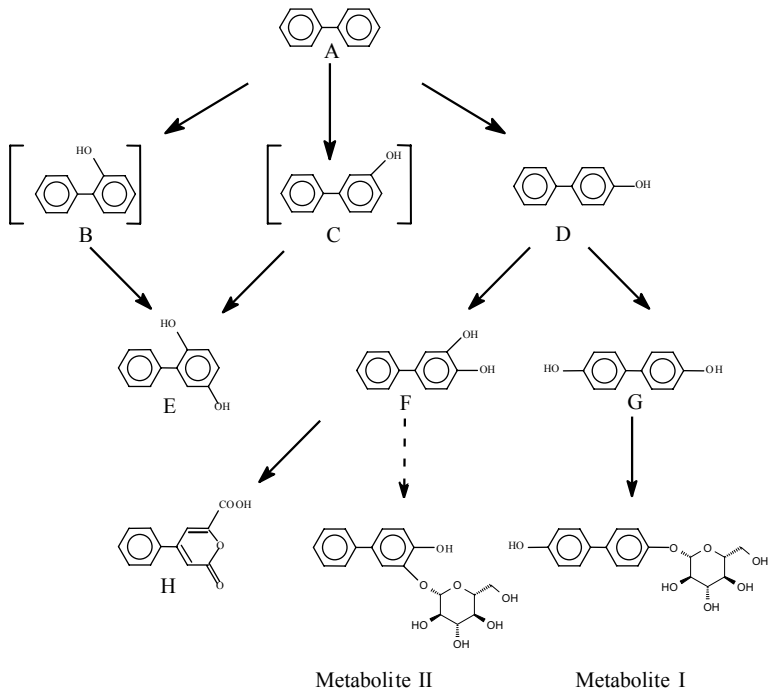

Figure 4. Proposed pathway for the biotransformation of biphenyl by the ascomycete *T. helicus*. (A) Biphenyl; (B) 2-hydroxybiphenyl; (C) 3-hydroxybiphenyl; (D) 4-hydroxybiphenyl; (E) 2,5-dihydroxybiphenyl; (F) 3,4-dihydroxybiphenyl; (G) 4,4′-dihydroxybiphenyl.

monooxygenases in the fungus. Moreover, the substrate specificity of the cytochrome P-450 enzymes involved in unspecific oxidation of hydrophobic compounds in various fungi and yeast seems to be rather different. As mentioned above, the hydroxylation pattern can differ drastically in various eukaryotic organisms.

Although dihydroxylated biphenyl derivatives accumulated in high amounts in the culture supernatant of *T. helicus*, they are not dead-end products, but were further transformed. As found in yeasts – mainly described for *Trichosporon* strains (Sietmann *et al.* 2002) and *Debaryomyces vanrijiae* (Lange *et al.* 1998) but also for the imperfect filamentous fungus *Paecilomyces lilacinus* (Gesell *et al.* 2001), 3,4-dihydroxybiphenyl can be oxidized up to ring cleavage. As a result 4-phenyl-2-pyrone-6-carboxylic acid was formed (Figure 4). In *T. helicus* this reaction only occured to a low extent when biphenyl or 4-hydroxybiphenyl were used as substrates. If 3,4-dihydroxybiphenyl was used as substrate, nearly complete conversion to the pyrone was observed. Perhaps the dihydroxylated biphenyl induces a ring-cleaving enzyme, which does not occur at the low level of 3,4-dihydroxylated biphenyl that accumulated by transformation of biphenyl or 4-hydroxybiphenyl. Furthermore, under these conditions 3,4-dihydroxybiphenyl and 4,4-dihydroxybiphenyl, were partially converted to glucose conjugates. Therefore, detoxification of harmful compounds in *T. helicus* seems to occur via the so-called phase I (hydroxylation)/phase II transformation system (Zhang *et al.* 1996), through which excretion of toxic intermediates becomes possible. In contrast, in yeast detoxification of such compounds is achieved by ring cleavage of the dihydroxylated derivatives produced (Lange *et al.* 1998; Sietmann *et al.* 2000, 2002). Surprisingly, conjugation of 4-hydroxybiphenyl was not observed. Furthermore, it remains unclear why hydroxylated biphenyl derivatives can be excreted partially directly, whereas another part seems to be only excreted after conversion to sugar conjugates. Here, it is proven for the first time that a *Talaromyces* strain isolated from oil- and PAH-contaminated water shows higher potential to tolerate and to transform biphenyl and its derivatives in comparison to *Talaromyces* isolates from natural sites.

Acknowledgements

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