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# Selection of autochthonous yeast strains able to degrade biphenyl

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## Summary

In order to assess the role of yeasts in the natural detoxification process of sediments polluted with biaryl compounds, indigenous yeast species able to degrade biphenyl (BP) were isolated and identified. The degradation ability of 24 strains of the genera *Candida* spp., *Cryptococcus* spp., *Pichia* spp., *Rhodotorula* spp., *Trichosporon* spp. and *Yarrowia* spp. was evaluated by the identification of the BP-metabolites, by HPLC analysis. 4-Hydroxybiphenyl was the main derivative in the *Candida krusei*, *C. tenuis*, *C. tropicalis*, *Pichia haplophila*, *Rhodotorula glutinis*, *Trichosporon pullulans* and *Yarrowia lipolytica* cultures. 3-Hydroxybiphenyl was detected in minor amounts in the culture supernatant of *C. tropicalis*, *C. krusei* strains and *R. glutinis*. Further hydroxylation led to 3,4-dihydroxy and 2,3-dihydroxybiphenyl; the former in *C. tropicalis*, *C. krusei* and *R. glutinis* cultures, and the latter only in the *R. glutinis* assays. The cleavage product 4-phenyl-2-pyrone-6-carboxylic acid, was observed in *R. glutinis* and *Y. lipolytica* cultures. The degradation ability of the *R. glutinis* isolates was noteworthy; as four hydrolxylated intermediates and a ring-cleavage product were obtained in both strain cultures. The species studied in this report were dominant in polluted sediments; furthermore, *R. glutinis* had been mentioned as able to degrade other aromatic hydrocarbons and had high relevance in bioremediation experiments.

## Introduction

Biphenyl (BP) and polychlorinated BPs are widespread environmental pollutants because of their extensive industrial and commercial use. BP has been used as a model compound to study the metabolism of higher chlorinated xenobiotics; but most researches have been focused on bacterial metabolism and its mineralization potential (Mohn *et al.* 1997). On the other hand, catabolism of aromatics by yeasts was reviewed by Middelhoven (1993), but detailed biochemical studies have been carried out only recently and they pointed to the synthesis of hydroxylated intermediates (Salvo *et al.* 1990; Oudin *et al.* 1999).

Other studies showed that BP enrichment of cultures enhanced the microbial ability to degrade more toxic contaminants, such as the polychlorobiphenyls, so these microorganisms can be relevant for a practical approach to bioremediation technologies (Chaudhry & Chapalamadugu 1991; Sondossi *et al.* 1992). Although yeasts are the dominant fungi in soils and sediments polluted with aromatic compounds, the biodegradation capacity of the biarylic structure is still uncertain; it is partially due to the scarcity of data on this subject and to the few tested yeast species (Hammer *et al.* 1998).

The biostatic and biocidal properties of BP and its derivatives adversely affected the biodegradation researches. In order to assess the role of yeasts in the natural detoxification process, the objectives of this study were to isolate indigenous yeast species able to degrade BP, and to evaluate this ability by the identification of the intermediate metabolites produced during BP incubation experiments.

## Materials and methods

### *Isolation and identification of yeasts*

Yeasts were isolated from heavy polluted sediments collected from an industrial area, near the oil YPF-Refinery, La Plata, Argentina. Decimal dilutions of the samples were spread on a basal medium (BM) containing per litre of distilled water: $(NH_4)_2SO_4$ 1000 mg, $K_2HPO_4$ 800 mg, $KH_2PO_4$ 200 mg, $MgSO_4 \cdot 7H_2O$ 200 mg, $CaCl \cdot 2H_2O$ 100 mg, $FeSO_4 \cdot 7H_2O$ 5 mg, 2% agar, 0.03 $\mu$g streptomycin, pH 6.8. The plates were incubated in a closed vessel, and 5 g phenol was put in a small open flask inside the bigger one, to provide it as

carbon source in vapour phase, at 27 ± 1 °C during 20 days.

The phenol-tolerant fungi were selected on the basis of their prevailing growth on subsequent platings. Sterile controls with phenol were simultaneously incubated, in triplicate. Yeasts are enumerated by standard spread plate methods on the same medium. Stock cultures of the isolated yeasts were maintained on selective medium at 4 °C and stored in the culture collection at the Instituto Spegazzini (LPSC), Universidad Nacional de La Plata, Argentina.

The yeasts were identified by colony appearance, cell morphologies, pseudo-true hyphae, assimilation and physiological differences (Kurtzman & Fell 1998). D-Glucuronate assimilation in liquid medium, growth on *n*-hexadecane and coenzyme Q-system, were also assesed (Kreisel & Schauer 1987). The results were confirmed by the Yeast identification PC program (Barnett *et al.* 1996).

*Growth and incubation conditions*

Yeasts were precultivated on 40 ml of liquid Sabouraud media, for 48 h at 180 rev/min and 30 °C, for 2 days. For degradation experiments 1 ml of this culture was incubated in 500 ml-shake flasks with 100 ml BM, supplemented with 2% glucose, 1 ml vitamin solution and pH 6.8. After incubation for 3 days at 30 °C and 180 rev/min on a rotary shaker, cells were harvested by centrifugation (5000 × *g*, 5 min), washed twice with sterile BM and the pellets were resuspended to an optical density of 6 (600 nm). BP (10 mg) was added to 100 ml BM with the cell suspensions and incubated as described above.

Additional cultures with 100 ml BM and 1 ml cell suspensions were incubated with 10 mg of 2-hydroxybiphenyl, 3-hydroxybiphenyl and 4-hydroxybiphenyl, to enrich the yield of the other intermediates. The toxic effects of BP and the derivatives were estimated by the decrease on cells densities of cultures with 2% glucose as growth substrate, by comparing the OD of these cultures grown with and without the pollutants.

This methodology was also employed in inhibition experiments, but adding 10 mg BP or 10 mg 4-hydroxybiphenyl as substrates, and 10 mg 4-aminobenzotriazole as enzymatic inhibitor. Cultures with the inoculum of yeast cells in BM, but without substrate, were used as controls; and all the assays were performed in duplicate.

*Metabolism of BP*

For kinetic studies of BP degradation and isolation of metabolites, at different sampling periods, 2 ml of each flask were centrifuged (5000 × *g*, 5 min) and 100 μl of the supernatant were analysed. The BP and metabolite contents were estimated by HPLC analysis (Hewlett-Packard, Bad Homburg, Germany), apparatus 1050 M equipped with a quaternary pump system, a diode array detector 1040 M series I, and an HP Chemstation. The separation was achieved with a LiChroCart 125-4 RP-18 end-capped (5 μm) column (Merck, Darmstadt, Germany). The initial solvent composition was 30% $CH_3OH$–70% $H_3PO_4$ (0.1%), reaching 100% methanol after 14 min at a flow rate of 1 ml/min.

The u.v.–visible absoption spectra of the products were determined in a diode array detector. The monohydroxylated metabolites were identified by comparison of analytical data with commercial standards, the dihydroxylated ones and ring-fission product by other authors data (Lange *et al.* 1998); by their u.v.–visible spectra during detection with a diode array detector. The BP and 2-hydroxybiphenyl were purchased from Merck; 3-hydroxybiphenyl, 4-hydroxybiphenyl, 4-aminobenzotriazole and phenol were obtained from Aldrich-Chemie; all the chemicals and solvents were of the highest purity available.

## Results

To verify that culturable indigenous phenol-tolerant yeasts were able to transform BP, isolation on selective agar plates were performed with sediment samples from chronic polluted sites, with 156 mg BP/l. Although 24 phenol-tolerant yeast strains were isolated, only 10 of them were able to degrade BP, and represented the 41.6% of the isolates.

Three *Candida krusei* strains, *C. tenuis*, *C. tropicalis*, two *Cryptococcus laurentii* strains, *P. Lichia membranaefaciens*, two *P. cactophila* strains, two *P. anomala* strains, *P. haplophila*, two *Rhodotorula mucilaginosa* strains, *R. aurantiaca,* four *R. glutinis*, *R. minuta*, two *Trichosporon pullulans* strains and *Yarrowia lipolytica* were assayed to confirm their ability to grow on BP as carbon source.

BP was not a growth substrate as a decrease in the flask with this pollutant as sole C-substrate was observed in some yeast experiments; though BP was metabolized as two *C. krusei* strains, *C. tenuis*, *C. tropicalis*, *P. haplophila*, two *R. glutinis* strains, two *T. pullulans* strains and *Y. lipolytica* produced BP-metabolites (Table 1).

The degradation of BP resulted in the formation of 4-hydroxybiphenyl and it was the only product in the *C. tenuis*, *P. haplophila* and *T. pullulans* cultures. In *C. tropicalis*, *C. krusei*, *C. tenuis* and *P. haplophila* cultures the 4-hydroxybiphenyl was observed at day 4, though in the *R. glutinis*, *T. pullulans* and *Y. lipolytica* experiments it was detected on day 1 of the incubation time.

In addition to 4-hydroxybiphenyl, four different degradation products were detected in *C. tropicalis*, *C. krusei* strains, *Y. lipolytica* and *R. glutinis* assays. A second monohydroxylated compound was found in minor amounts in the culture supernatant of *C. tropicalis*, *C. krusei* strains and *R. glutinis*, and it was identified as 3-hydroxybiphenyl; 2-hydroxybiphenyl was not detected at all. Further hydroxylation led to 3,4-dihydroxybiphenyl and 2,3-dihydroxybiphenyl, the for-

*Table 1.* Yeast isolates and maximum amounts of each metabolite formed with BP.

| Yeast species | BP metabolites (μg/l) | | | |
|---|---|---|---|---|
| | 3-OH-BP | 4-OH-BP | 3,4-diOH-BP | 2,3-diOH-BP |
| *C. krusei* LPS 589 | 22.8 | 155.0 | 120.0 | – |
| *C. krusei* LPS 590 | 18.4 | 98.0 | 105.0 | – |
| *C. tenuis* LPS 604 | – | 39.5 | – | – |
| *C. tropicalis* LPS 610 | 18.3 | 19.5 | 76.5 | – |
| *P. haplophila* LPS 606 | – | 45.5 | – | – |
| *R. glutinis* LPS 603 | 29.0 | 215.3 | 200.5 | 98.5 |
| *R. glutinis* LPS 597 | 21.0 | 175.5 | 105.0 | 45.3 |
| *T. pullulans* LPS 602 | – | 130.2 | – | – |
| *T. pullulans* LPS 608 | – | 98.5 | – | – |
| *Y. lipolytica* LPS 605 | – | 250.5 | 125.5 | – |

mer in *C. tropicalis*, *C. krusei* strains and both *R. glutinis* cultures and the second metabolite only in the *R. glutinis* assays (Figure 1). The 3,4-dihydroxybiphenyl can be derived from 4-hydroxybiphenyl, and 2,3-dihydroxybiphenyl from 3-hydroxybiphenyl; 3,4-dihydroxybiphenyl was found in *Y. lipolytica*, *C. tropicalis* and *C. krusei* cultures and both metabolites in *R. glutinis* strains.

The ring cleavage product 4-phenyl-2-pyrone-6-carboxylic acid was observed in *R. glutinis* and *Y. lipolytica* cultures (Figure 2). It was detected earlier in the *R. glutinis* assays in respect to the *Y. lipolytica* ones, after 2 and 4 days incubation time, respectively.

To enhance the ability of *R. glutinis* and *Y. lipolytica* strains to transform BP derivatives, in a subsequent experiment they were cultivated under similar conditions with 4-hydroxybiphenyl. This was transformed to 3,4-dihydroxybiphenyl and 4,4′-dihydroxybiphenyl, and a 10.5–12.0% increase in the formation of the former metabolite was detected in each yeast culture. So, under these conditions an increase in the 4-hydroxybiphenyl degradation rate and a different intermediate were obtained.

To assess the toxic effects of BP and the 2-, 3- and 4-hydroxybiphenyl, the OD of the yeast cultures at the stationary phase were controlled in the assays, in 1 week incubation time. BP showed minor effects on growing cells, as a slight decrease of 5–6% (mean values) in the OD of the *R. glutinis* and *Y. lipolytica* cultures were observed. However, the hydroxylated products significantly inhibited the growth of yeasts; the 4-hydroxybiphenyl was more toxic than the other compounds, as a

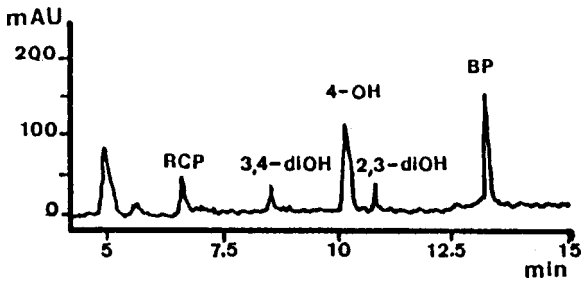


*Figure 1.* HPLC elution profile of the metabolites formed from BP by *R. glutinis*, at the seventh day incubation period (mAU: absorbance units; RCP: ring cleavage product; BP: biphenyl).

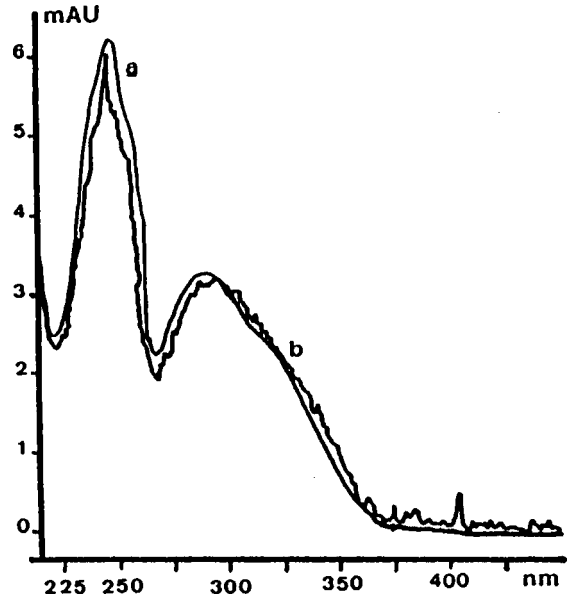


*Figure 2.* HPLC spectrum of the RCP formed from BP by *R. glutinis* (a) library spectrum, (b) 4-phenyl-2-pyrone-6-carboxylic acid spectrum (mAU: absorbance units).

42–48% decrease in the cell densities were obtained with this substrate, in respect to 2- and 3-hydroxybiphenyl (mean values: 12–15 and 22–25%, for *R. glutinis* and *Y. lipolytica* cultures, respectively).

The formation of 4-hydroxybiphenyl as the major metabolite resembled other fungal and mammalian enzyme systems; so, in order to estimate the similarities of these results, enzyme inhibitor assays were performed. The 4-aminobenzotriazole was added as enzymatic inhibitor of the cytochrome *P*-450 systems, to *R. glutinis* cultures enriched with BP or 4-hydroxybiphenyl. As a consequence, the formation of 3,4-dihydroxybiphenyl decreased by 97 and 92% when the enzyme inhibitor was added to each yeast culture.

## Discussion

Screening of polluted sediments for BP degrading yeasts revealed that a significant diversity of yeast strains were able to hydroxylate BP; in fact, *C. krusei*, *C. tenuis*, *C. tropicalis*, *P. haplophila*, *R. glutinis*, *T. pullulans* and

*Y. lipolytica* were confirmed as BP degraders. Indeed, for *C. krusei*, *C. tenuis*, *P. haplophila*, *T. pullulans* and *R. glutinis* it is the first mention. The data available concerning this ability are controversial; Smith *et al.* (1980) suggested that there was no BP transformation in *C. tropicalis* cultures, however, this species was characterized as degraders by Wiseman *et al.* (1975). Furthermore, oxidation of BP by *C. lipolytica* was also shown by Cerniglia & Crow (1981).

The 4-hydroxybiphenyl was the predominant metabolite, as also observed by other researchers (Abramowicz 1990; Mobley 1994). While three different hydroxylated metabolites and a ring-cleavage product were detected in our *R. glutinis* assays, only two of them were found in *Debaryomyces vanrijiae* (Lange *et al.* 1998). Moreover, the same fission product mentioned for *D. vanrijiae* was observed in the *R. glutinis* and *Y. lipolytica* assays.

Fungal aromatic hydroxylation is of interest because it mimics the cytochrome *P*-450-mediated metabolism of xenobiotics in mammals (Parkinson & Safe 1982). A BP-transforming cytochrome *P*-450 had been suggested for *C. tropicalis* (Wiseman *et al.* 1975) and *D. vanrijiae* (Lange *et al.* 1998); therefore, our results on the enzymatic inhibition of cytochrome-*P*-450 bring out the fungal BP catabolism on the subject.

The ability of the *R. glutinis* isolates to degrade BP was noteworthy; in as much as four hydrolxylated intermediates and a ring-cleavage product were obtained, at shorter incubation period in respect to the other yeasts. It must be outstanding, that the species studied in this report were dominant in the fungal population from sediments polluted with aromatic compounds. Furthermore, *R. glutinis* may be of high relevance for a practical approach in environmental biotechnology, due to this species being widely distributed among soil fungi.

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