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Isolation and characterization of biarylic structure-degrading yeasts: hydroxylation potential of dibenzofuran

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"Capsule": *Autochthonous yeast species hydroxylated dibenzofuran and cleaned the rings.*

Abstract

Yeast communities from heavily polluted sediments that received the discharge from oil refineries and other industries were studied. Yeast species were isolated from these sediments and their ability to degrade dibenzofuran were determined. Twenty-four different yeast strains were isolated and cultured on aromatic medium; two *Candida krusei* strains, *Candida tenuis*, *Candida tropicalis*, two *Pichia anomala* strains, *Pichia haplophila*, two *Rhodotorula glutinis* strains, *Rhodotorula mucilaginosa*, two *Trichosporon pullulans* strains and *Yarrowia lipolytica* were able to hydroxylate dibenzofuran. Three metabolites were identified by HPLC analysis; 3-hydroxydibenzofuran was in all the cases the most abundant isomer, and while 4-hydroxydibenzofuran was also common, 2-hydroxydibenzofuran was detected in very small quantities and with few species. In the *R. glutinis* and *Y. lipolytica* cultures a ring cleavage product was also found. While in the *R. glutinis* assays the hydroxydibenzofuran was detected earlier, at 2 days' incubation time, in the other yeast experiments they were observed at the 4–5th incubation days with the maximum amounts at the 7th day. Our results confirmed the ability of autochthonous yeast species to hydroxylate dibenzofuran and to cleave the rings, and it is the first report for *C. krusei*, *C. tenuis*, *P.anomala*, *P. haplophila* and *R. mucilaginosa*. The ecological relevance of this study is based on the fact that dibenzofuran is a xenobiotic not easily transformed, so the catabolic activities observed in authochonous yeasts contribute to broadening the biodegradable substrate spectrum.

1. Introduction

Dibenzofuran is well known for its strong toxicity and mutagenicity, thus contamination by this compound is a serious environmental problem, because it is formed in the process of producing a variety of halogen-containing aromatics, such as herbicides, and during the combustion of industrial and municipal wastes (Hutzinger, 1985). This xenobiotic is resistant to microbial attack so persist in the environment (Plüss et al., 1988; Ballerstedt et al., 1997).

Although dibenzofuran has been used as substrate in some researches, no considerable microbial growth was observed (Strubel et al., 1989; Fortnagel et al., 1989); the biotransformation of nongrowth compounds is complicated because they did not support cells growth and can only be degraded in the present of a primary substrate (Saéz & Rittmann, 1993; Hyman et al., 1995). Nevertheless, the biodegradation behavior of cometabolized compounds is important for the biological treatment of polluted water, industrial effluents and wastes, because most of the xenobiotics are fungal attacked by cometabolism (Cerniglia et al., 1979; Hammer and Schauer, 1997). Thus, it is interesting to search in nature for indigenous microorganisms able to transform this contaminant. The aims of this study were to isolate and identify yeast species from polluted sediments, and to compare their potential to biotransform dibenzofuran.

2. Materials and methods

2.1. Sampled sites and chemical analysis

Composite samples of the surface sediments were taken from four different natural and artificial channels that drain to the Rio Santiago (Ensenada, Argentina).

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The organic carbon concentrations of the sediment were determined by CHN analyzer (Perkin-Elmer, Norwalk, CT.) and the Macro-Kjeldahl method was employed to measure the amount of total nitrogen. The total hydrocarbon and dibenzouran (DBF) concentrations were analized by a FTIR-Perkin-Elmer, by triplicate; the ultrasonic extraction was realized with Cl_4C. A cell with BrK window, 0.35 mm thick, was employed for this determination.

2.2. Isolation and identification of yeasts

Yeast species were isolated under selective conditions from dilution samples of the sediments, in a mineral medium supplemented with phenol as sole source of carbon (Romero et al., 1998). The isolates were identified by colony, cell morphologies, assimilation and physiological differences; with additional tests, like D-glucuronate assimilation in liquid medium (Kurtzman and Fell, 1998); coenzyme Q-system determination by HPLC were also done (Kreisel and Schauer, 1987). In all the cases, the Yeast identification PC program (Barnett et al., 1996) were used to confirm the results.

2.3. Metabolism of dibenzofuran

Yeasts were precultivated on 40 ml of liquid Sabouroud media, for 48 h at 5 ***g*** and 30 °C, for 2 days. For degradation experiments, 1 ml of this culture was incubated in 500-ml shake flasks with 100 ml of a mineral medium (MM) containing, per liter, 200 mg KH_2PO_4, 800 mg K_2HPO_4, 200 mg $MgSO_4$ 7 H_2O, 100 mg $CaSO_4$, 5000 mg $(NH_4)_2SO_4$, 1 mg $(NH_4)_2$ MoO_4 4 H_2O, supplemented with 2% glucose and 1 ml vitamin solution (van der Walt, 1970), and pH 5.4. After incubation for 3 days at 30 °C and 180 rpm on a rotary shaker, cells were harvested by centrifugation (5000 ***g***, 5 min), washed twice with sterile MM and the pellet was resuspended in MM to an optical density of 6 (600 nm). Dibenzouran (10 000 μg) was added to 100 ml MM with the cell suspensions, and the cultures were incubated as described above.

Additional cultures in 500-ml shake flasks with 100 ml of the cell suspensions (OD 6600 nm) and 150 μg of 2-hydroxydibenzofuran, were incubated to enrich the yield of intermediates. Inoculum of yeast cells without substrate in MM were used as controls, and the assays were made in duplicate.

Periodically, 1 ml of each flask was centrifugated (5000 ***g***, 5 min) and 100 μl of the supernatant were analized. The DBF and metabolites concentrations 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 UV-visible absoption spectra of degradation products were determinated in a diode array detector. The chemicals, dibenzofuran, 2-hydroxydibenzofuran and phenol, were purchased by Aldrich-Chemie, and all of them and solvents were of the highest purity available.

3. Results

Twenty-four different yeast strains were able to grow on the selective medium, and these phenol-induced colonies were used for the degradation experiments. Three *Candida krusei* strains, *Candida tenuis*, *Candida tropicalis*, two *Cryptococcus laurentii* strains, *Pichia membranIfaciens*, two *Pichia cactophila* strains, two *Pichia anomala* strains, *Pichia haplophila*, two *Rhodotorula mucilaginosa* strains, *Rhodotorula aurantiaca,* four *Rhodotorula glutinis*, *Rhodotorula minuta*, two *Trichosporon pullulans* strains and *Yarrowia lipolytica* were cultivated in mineral medium plus DBF as carbon source. Although all the phenol-grown isolates were assayed, only some of them degraded the DBF.

The microscopic observation and carbon utilization spectrum led us to classify the microorganisms; and D-glucuronate was tested to differenciate between *R. aurantiaca* (+) and *R. glutinis* (−). Moreover, *P. anomala* strains and *P. haplophila* were confirmed by the CoQ-system, and the CoQ_7 and CoQ_9 were identified by HPLC for each species, respectively. Coenzyme Q structure is considered of taxonomic and evolutionary values, and similar properties in the yeasts possessing the same CoQ type are expected; even more, nearly all hydrocarbon utilizers possess CoQ_9 and only a few yeast species with CoQ_7 seem to be able to use these compounds. In our case, both *Pichia* species grew on aromatic substrates and biotransformed DBF.

C. krusei strains, *C. tenuis*, *C. tropicalis*, *P. anomala* strains, *P. haplophila*, *Rh. glutinis* strains, *Rh. mucilaginosa*, *T. pullulans* strains and *Y. lipolytica* were able to degrade DBF (Table 1). Though *Cr. laurentii, P. membranifaciens*, *P. cactophila, Rh. aurantiaca* and *Rh. minuta* grew on phenol-medium, their DBF-degradation abilities were not confirmed.

The monohydroxylated DBF were identified by comparing with commercially available standards (2-OH-DBF), synthesized compound (3-OH-DBF) and with data from other authors (4-OH-DBF). The 3-OH-DBF was in all the cases the most abundant isomer; while 4-OH-DBF was also frequent, and 2-OH-DBF was detected in small quantities. The occurence of monohydroxylated-DBF derivates in 54% of the isolates,

Table 1
Isolated yeasts and metabolites produced during dibenzofuran cultures (ug. l^{-1})

Yeast species	Dibenzofuran metabolites		
	2-OH-DBF	3-OH-DBF	4-OH-DBF
Candida krusei LPSC590	–	153.5	28.0
Candida Krusei LPSC589	–	105.0	22.8
Candida tenuis LPSC604	–	28.5	–
Candida tropicalis LPSC610	35.5	199.5	58.8
Pichia anomala LPSC611	–	48.0	21.5
Pichia anomala LPSC601	–	43.5	17.0
Pichia haplophila LPSC606	–	36.5	–
Rhodotorula glutinis LPSC603	44.5	225.5	138.5
Rhodotorula glutinis LPSC597	–	250.0	122.0
Rhodotorula mucilaginosa LPSC596	–	103.5	23.5
Trichosporon pullulans LPSC602	–	95.5	15.5
Trichosporon pullulans LPSC608	–	103.5	–
Yarrowia lipolytica LPSC605	38.5	215.0	173.0

pointed out the presence of active monooxygenase-systems in these natural yeast populations (Cerniglia et al., 1979; Hammer & Schauer, 1997).

The amount of metabolites were correlated to the peak area in the HPLC elution profiles. None of the tested yeasts used DBF as sole source of carbon and energy, and in all the cases a decrease in the viable cell numbers was observed at the end of the experiment. This environmental pollutant could not be used as growth substrate, but it was oxidized and biotransformed to a remarkable degree. *C. tropicalis*, *Rh. glutinis* both strains and *Y. lipolytica* metabolized the 2.9, 4.1, 3.7 and 4.2% of the initial DBF, in 7 days.

In both *R. glutinis* strains and *Y. lipolytica* cultures a ring cleavage product was detected; and the extractable fractions studied by GC–MS and ^{1}H-nuclear magnetic resonance identified this metabolite as 2-(1-carboxy methylidene)-2,3-dihydrobenzo[*b*]-furanylidene glycolic acid. In each case, the cleavage product had a retention time of 5.5 min for both *R. glutinis* strains and 5.3 min for *Y. lipolytica* . Likewise, a small amount of 2,3-dihydroxylated derivates were obtained in both yeast species, at the 14th incubation day.

The incubations with 2-OH-DBF enriched the amounts of the intermediates, and about 82% of this substrate was transformed to 2,3-di OH-DBF; therefore, the further incubations were carried out with this substrate instead of DBF.

In most of the yeast cultures the 2-, 3- and 4-OH-DBF were detected at 4–5th day incubation period, but the maximum amounts were observed at 7th day. It is known that the preculture with a compound with a similar chemical structure so *R. glutinis* was precultured with phenol as sole carbon source to increase the hydroxylation potential (Leahy & Colwell, 1990). In this experiment the metabolites were similar to the ones obtained in the glucose-precultures, even if they were detected earlier, at 24–48 h of incubation. Thus, there was no great difference concerning DBF-degradation between yeast cultured under selective or nonselective conditions; these results pointed out that the enzyme system seemed to be a strain-specific characteristic, but not a species-dependent feature. The hydrocarbon and DBF concentrations of the sampled sediments could also enhance the biotransformation abilities of the tested species (Table 2).

4. Discussion

Screening of polluted sediment samples for suitable microorganisms able to grow with DBF, 13 yeast strains were able to biotransform this pollutant. The degradation of aromatic substances by yeasts had been mentionated (Hofmann and Schauer, 1988; Middelhoven, 1993); however, information about their ability to biotransform aromatic ring in biaryl compounds is scarce.

The 1-OH-DBF was identified as fungal derivates by Cerniglia et al. (1979) in filamentous soil fungi, and 4-OH-DBF and the same cleavage product with the species *T. mucoides* (Hammer et al., 1998); both metabolites were detected in *R. glutinis* and *Y. lipolytica* cultures in this study According to the mentionated authors, the 2-OH-DBF accumulated more than 3- and 4-OH-DBF; on the contrary, in our assays the last metabolites were most abundant in all the yeast cultures.

The hydroxylation reactions of biarylic compounds had been studied in *Debaryomyces vanrijiae*, *T. mucoides* and *T. beigelii* (Lange et al., 1998; Schauer et al., 1995) and in *C. tropicalis*, *Y. lipolytica* and *R. glutinis* (Cerniglia and Crow, 1981; Oudin et al., 1999). We confirmed the ability of other yeast species to biotransform DBF, being the first report for *C. krusei*, *C. tenuis*, *P. anomala*, *P. haplophila* and *R. mucilaginosa*.

Likewise, the ecological importance of these capacities is noteworthy as dibenzofuran is not easily biodegradable,

Table 2
The chemical characteristics of the sampled sediments (TOC total organic carbon (%); TON total organic nitrogen (%); HYD total hydrocarbon and DBF concentrations (ppm)

	TOC	TON	pH	HYD	DBF
Channel Este	20.4	6.0	7.3	1770	80.6
Channel Oeste	4.9	3.0	6.8	890	72.5
Regatas St.	1.3	0.3	6.7	700	25.3
Zanjón St.	0.6	0.15	7.1	460	12.5

and the catabolic activities acquired by yeasts contribute to broadening the biodegradable substrate spectrum. The tested yeasts have relevance for practical approach in natural remediation process, as they are the dominant species in polluted habitats.

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