RESEARCH ARTICLE

Chlorinated biphenyl degradation by wild yeasts pre-cultured in biphasic systems

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Abbreviations: μ: specific growth rate
μ_{max}: maximum specific growth rate
DCB: 2,4-dichlorobiphenyl
GC: gas chromatography
K_i: inhibition constant
K_s: saturation constant
MSM: mineral salts medium
OD: optical density
PCBs: polychlorinated biphenyls
S: substrate level
TCB-3': 2,3',4- trichlorobiphenyl
TCB-4': 2,4',5-trichlorobiphenyl

Environmental biotechnology has developed as an offshoot from sanitary engineering, and only recently the biological component of the ecosystems had been recognized as relevant when bioremediation strategies must be chosen to solve environmental problems. Yeasts were isolated on 2,4-dichlorobiphenyl, 2,3',4- and 2,4',5-trichlorobiphenyl, poorly soluble compounds in water, as carbon sources. *Debaryomyces castelli*, *Debaryomyces maramus* and *Dipodascus aggregatus* composed the mixed culture and represented 72% of the isolates; their

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degradation potential were studied in biphasic and monophasic systems. The biphasic cultures were obtained with phenol as the organic phase and MSM as the aqueous ones, the monophasic medium only with MSM. Both cultures were supplied with 50, 100, 150 and 200 ppm DCB, TCB-3' and TCB-4' as substrate. The growth rates varied with the dispersion degree, agitation rates and cell adhesion to the organic phase. The water-phenolic system improved yeasts selection in pollutant presence with low water solubilities, indeed, the adaptation and degradation were more slowly in the monophasic aqueous medium. Bioremediation is based on the presence of efficient microbial populations and pollutant availability; the tested yeasts and the organic-water system assayed put forward the possibility that hydrophobic substrates could be mineralized in natural habitats by wild yeast consortium.

Successful detoxification of polluted areas is the result of interrelated factors, such as the contaminant toxicity, organism's degradation potential and ecological conditions. The first and major problem in environmental biotechnology is the microbial selection, as the organisms must transform significant amount of toxicants in a short conversion times and produce no-toxic metabolites. Up to date, the research on fungal degradation of pollutants has focused mostly on basidio- and zygomycetes species, but in heavy polluted sites, increasing yeast standing stock had been observed (Oudin et al. 1999; Sietmann et al. 2001; Romero et al. 2004).

This situation leads to consider wild yeasts as a promising organism to clean-up chemicals, and to assess them to develop environmental biotechnologies. Few researches had pointed out these advantages (Sietmann et al. 2002; Romero et al. 2002; Romero et al. 2005).

Polychlorinated biphenyls (PCBs) had been widely used as plasticizers, flame retardants, heat transfer, hydraulic and dielectric fluids, due to their physical-chemical inertness, but they are mutagenic and strongly toxic, so listed as priority pollutants by the US Environmental Protection Agency (USEPA, 2003). Bioremediation is an effective strategy to detoxify many contaminants, and efforts to engineer degradation-systems for PCBs polluted areas had

been studied (Abramowicz, 1990; Swannell et al. 1996; Stringer and Johnston, 2001).

When a low-soluble toxicant, like PCBs congeners, is mixed with an aqueous medium given a byphasic system, the cells in the water-phase are in contact with less xenobiotic concentration than the yeasts adhered to the organic drops, therefore with high pollutant bioavailability (Hammer et al. 1998). Moreover, fungal adaptation is preceded by an acclimation time, requiring the low-soluble substrates longer periods, in these cases the organic presence improve the transformation rates (Havel and Reineke, 1995; Löser et al. 1999). So, the aims of this study were to evaluate the biodegradation of DCB, TCB-3' and TCB-4' by a natural yeast community; to compare their uptake in mono and biphasic cultures, and to assess the removal of poor water-soluble PCBs.

MATERIALS AND METHODS

Yeasts isolation and identification

Surface sediments were taken from natural and artificial channels that drain to Rio de La Plata (Argentina), then decimal diluted samples were assayed for the presence of PCBs-degrading yeasts by standard spread-plate methods, by triplicate. The species were purified by streaking repeatedly on mineral salts medium-agar (MSM, Romero et al. 2002) with 50 ppm DCB, or TCB-3' or TCB-4' as carbon sources. The plates were incubated at 27 ± 1ºC, at 120 rpm, during 45 days; sterile controls were simultaneously incubated. The same PCBs-medium was employed to enumerate the total degrading species, by spread-plate methods, by triplicate, to obtain the studied yeasts percentage.

The yeast community was selected by enriched cultures performed in biphasic, water-phenol, and aqueous monophasic systems, amended with 50 ppm DCB, TCB-3' or TCB-4'. Cultures aliquots were spread on MSM-plates with the xenobiotics and the colony types were isolated. Yeast species were identified using morphological and biochemical tests, additional determinations and growth characteristics were also done (Kurtzman and Fell, 1998).

Table 1. Specific growth rates (μ, h⁻¹) in biphasic systems and 150 ppm of each PCBs.

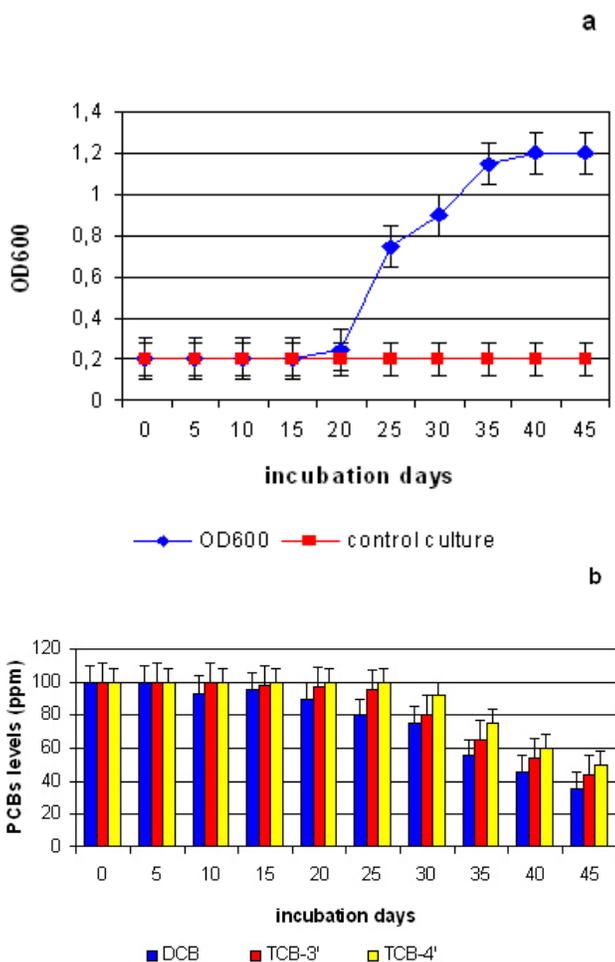|	DCB	TCB-3'	TCB-4'
D. castelli	0.40 ± 0.01	0.30 ± 0.03	0.40 ± 0.01
D. maramus	0.35 ± 0.01	0.46 ± 0.02	0.40 ± 0.01
D. aggregatus	0.40 ± 0.01	0.45 ± 0.02	0.42 ± 0.01
mixed culture	0.75 ± 0.02	0.80 ± 0.01	0.83 ± 0.02

a

b

Figure 1. Growth curves (a) and PCBs concentrations (b) in biphasic system of non-acclimated consortium.

Bioassays

The biphasic system was obtained with phenol as the organic phase and MSM as the aqueous ones, the monophasic medium consisted only of MSM. Both systems were supplied with 50, 100, 150 and 200 ppm 2,4-dichlorobiphenyl; or 2,3',4- or 2,4',5-trichlorobiphenyl as substrates and 50 ml phenol as co-substrate, in the biphasic cultures. The flasks were inoculated with 5×10^5 cells of each species, incubated at 27ºC, 120 rpm and the growth were evaluated by optical density (OD_{600}, Shimadzu UV Photometer).

Growth occurred in both phases, so, the kinetics in the multiphase liquor were described by the Monod-Haldane equation, where: μ_{max} was the maximum specific growth rate; S was the substrate level; K_s was the saturation constant and K_i was the inhibition constant. The specific growth rates were obtained by using the OD_{600}-data and the kinetic parameters were estimated by linear and nonlinear regression analyses:

$$\mu = \frac{\mu_{max}\ S}{K_s + S + S^2 / K_i}$$

Analytical methods

The ration of adhered cells to phenol to cells in the aqueous phase was obtained by centrifuged cultures at 12 000 g for 10 min at 4ºC, the resulting pellets were washed twice in distilled water. The supernatants with phenol and cells were mixed with ethanol-acetone-chloroform (10:10:2, vol/vol/vol), mixed for 15 min and centrifuged again; then the obtained pellets were washed twice. The OD_{600}-pellet suspensions were determined by the spectrophotometer.

Ten-ml cultures were homogenized with Na_2SO_4 and extracted with 400 ml CH_2Cl_2 in a glass Soxhlet apparatus, to analyze the chlorinated compounds. The CH_2Cl_2 extract was reduced and exchanged to hexane using a concentrator, and treated with acid-activated copper powder to remove elemental sulfur. PCBs were eluted with 100 ml hexane,
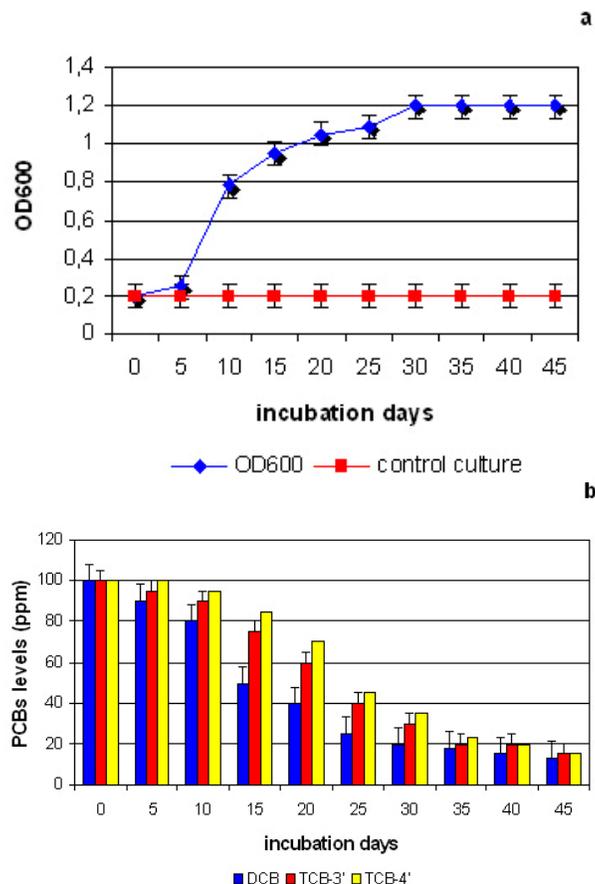
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Figure 2. Growth curves (a) and PCBs concentrations (b) of acclimated consortium.

then these extracts were reduced to 1 ml in a TurboVap (Zymark Corp.) (Kannan et al. 1997).

Extracts were analyzed on Varian 3400CX -GC with electron capture detector (GC/ECD) for primary quantitation, and ion trap mass spectrometric (GC/ITMS) detector to congeners confirmations. The columns (30 m x 0.25 mm) were coated with 0.25 µm DB-5 and DB-XLB for the ECD and ITMS, respectively. The GC/ECD oven was programmed from 120ºC to 260ºC at 2ºC.min^{-1}, and the GC/ITMS oven was programmed from 60ºC to 280ºC at 4ºC x min^{-1}. Both instruments were calibrated by serial dilutions of PCB-standard mixtures and non-chlorobiphenyl congeners. Blanks did not contain any target PCB above the nominal specific detection limit (ca. 1 ng.g^{-1} dry weight) and the mean PCB recovery was ca. 86%.

Growth in chlorinated substrates presence was measured by chloride release with an specific and reference electrode (Microprossesin pMX 2000/ION; Wissenchaftlich-Technische Werkstatten GmbH, Weilheim, Germany), using HCl-solution as standard.

Statistical analysis

Means, standard deviations, regressions and variance analysis (ANOVA) were performed using SPSS/PC + Version 5.0 (Wayne, 1996), and the significance levels were set at $P = 0.01$. Triplicate assays were performed for each set of test conditions.

RESULTS AND DISCUSSION

Debaryomyces castelli Capriotti (1958), *Debaryomyces maramus* di Menna (1954) and *Dipodascus aggregatus* Francke-Grosmann (1952) represented 72% of the isolated yeasts. The mixed culture was composed by equal numbers of these dominant and more efficient species. Indeed, their abilities to grow on each PCBs as carbon sources were evaluated by the µ-values (Table 1).

The DCB, TCB-3' and TCB-4'degradation were observed after 5 and 20 days incubation period for the acclimated and non-acclimated consortium, respectively (Figure 1a and Figure 2a). However, the OD$_{600}$ reached by both communities at 35th days were not significant different (P > 0.05), and the pollutants were transformed to significant low levels in both cultures; although halves concentrations were reached at 40th days in the pre and non-acclimated flasks, respectively (Figure 1b and Figure 2b).
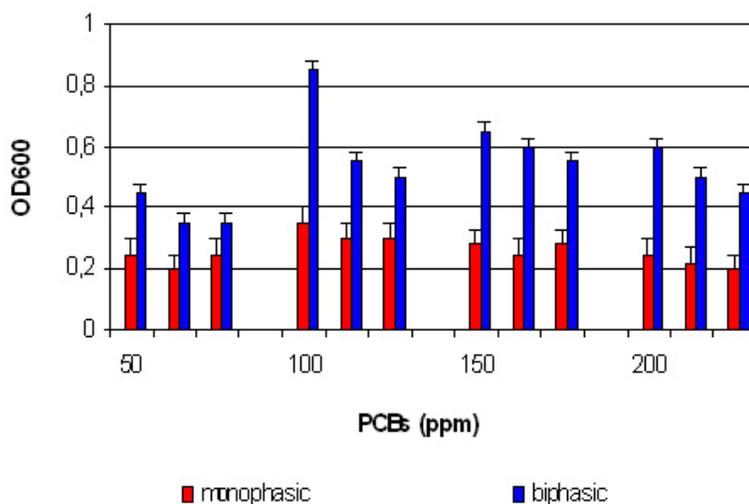
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Figure 3. Specific growth rates (µ, h^{-1}) of the consortium in both systems enriched with PCBs.

The µ-data, specific growth rates, increased from 0.11 to 0.65 h^{-1} in the biphasic medium and from 0.07 to 0.28 h-1 in the monophasic assays, when the yeasts were pre-cultured with PCBs. While µ increased with the xenobiotic levels in the biphasic medium, this parameter showed no significant variability in the monophasic ones (Figure 3). So, the performance of the biphasic was better than the monophasic system, although in both cases the substrate levels had different effects on the yeast activities. It must be pointed out that the surface-fixed cells were important in the degradation of hydrophobic pollutants, so the phenol presence increased the PCBs bioavailability and their uptake.

The µ-values were twice as high in the water-organic assays, with µ = 0.65 h^{-1} in this culture and 0.28 h^{-1} in the aqueous system. Likewise, the inhibition was lower in the biphasic (K$_i$ = 16 mg l^{-1}) than in the aqueous culture (K$_i$ = 8.5 mg l^{-1}), this could be due to the high solubility of the metabolites in phenol presence. Similar saturation constants (K$_s$ = 0.62 mg l^{-1}, K$_s$ = 0.73 mg l^{-1}; P > 0.05) were observed in both experiments.

During the incubation period, the yeasts distribution in the aqueous and phenolic phases were significantly different (P < 0.05), the cells partitioning showed that cells adhered to the organic drops when the shaker stopped. These data and the microscopic observations revealed that yeasts grew in
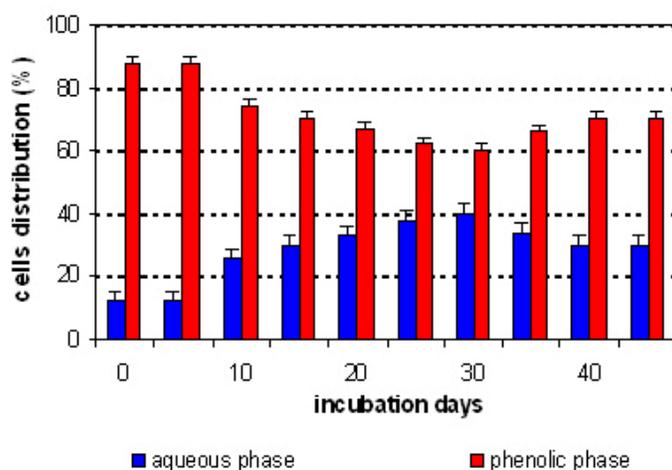

Figure 4. Yeast cells partitioning in the phenolic and aqueous phases.

both phases just like in the liquid-liquid interface (Figure 4).

Significantly better adaptation was observed in the consortium in relation to each species alone in the biphasic assays; moreover, phenol presence incremented the interfacial associated cells and the biodegradation of hydrophobic organic pollutants as was shown by other authors (Köhler et al. 1994; Owen et al. 1997).

In two-phase systems, the compounds diffuse from the organic to the aqueous compartment, yeasts bioconverted the pollutants in the interfacial and water-phase and the metabolites accumulated in the organic ones. So, the relevance of this culture type was based on the avoiding of the substrate inhibition and increasing tolerance to great xenobiotic concentrations in contact with the fungi, finally, the high bioavailability improved the detoxification rates.

Biphasic system with water-heptamethylnonane to evaluate naphthalene degradation by *Artrobacter* spp. (Focht, 1997), and water-silicone oil for di- and trichlorobenzene by bacteria and yeasts spp. (Ascon-Cabrera and Lebeault, 1993; Kim and Picardal, 2001) had been performed. Wild yeasts with *n*-hexadecane and dichloro- and trichlorobenzene as pollutants were assessed (Romero et al. 2002), but in these reports with biphasic assays, other pollutants and species were tested.

Scarce studies were realized on yeast biodegradation potential of biarilic compounds, only *Trichosporon* spp., *Debaryomyces vanrijiae*, *Candida* spp. and *Rhodotorula* spp. had been referred as efficient species (Hammer et al. 1998; Lange et al. 1998; Romero et al. 2001). Indeed, total yeast number and the ratio between red to other yeasts were suggested as pollution indexes (Spencer and Spencer, 1997). In this study, two different *Debaryomyces* species and *Dipodascus aggregatus* were confirmed as effective PCBs-degraders, being the first reference on their conspicuous degradation potential.

The feasibility of bioremediation is based on the presence of efficient microbial populations and pollutant bioavailability (Madsen, 1997; Head, 1998). The tested yeasts and the organic-water system assayed in this research put forward the possibility that hydrophobic substrates could be mineralized in natural habitats by wild yeast consortium (Dighton et al. 2005). This event aimed towards the applications of the results by environmental biotechnologies to detoxify polluted areas (Dighton, 2003; Arora, 2004).

In conclusion, we confirmed that the yeast consortium bio-transformed the substrates and revealed that a significant cell percentage fixed to the organic drops, pointing out the relevance of the interfacial area in the biodegradation processes. The presence of a water-organic system increased the adaptation of degrading yeasts and pollutants biotransformation; so, this system could improve microbial selection and the bioremediation of poorly water-soluble pollutants.

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