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Mycoremediation of high concentrations of polychlorinated biphenyls with *Pleurotus sajor-caju* LBM 105 as an effective and cheap treatment

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**Title: Mycoremediation of high concentrations of polychlorinated biphenyls with *Pleurotus sajor-caju* LBM 105 as an effective and cheap treatment**

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**Highlights**

- *P. sajor-caju* LBM 105 could remove more than 90% of PCBs after 35 days.
- PCBs modified hyphal diameter, biomass, glucose, proteins and laccase secretion.
- The mycoremediation treatment of PCB with GA costed 0.69 \$ g<sup>-1</sup>d<sup>-1</sup>.

**Abstract**

Polychlorinated biphenyls (PCBs) are exceptionally stable organic pollutants. An effective and economically feasible process is essential for their removal. Mycoremediation using white-rot fungal strains proved as a highly effective approach that has not been thoroughly investigated. The aim of this work was to evaluate PCBs remediation efficacy applying *Pleurotus sajor-caju* LBM 105. Axenic cultures of the fungus, either in complex or N-limited liquid media, were spiked with 217 mg L<sup>-1</sup> of a technical mixture of Aroclor 1242, 1254 y 1260 in oil transformer. PCBs removal capacity, toxicity reduction, changes in fungal morphology and laccase expression caused by pollutant addition and the cost-effectiveness of the remediation treatment were assessed when growing the fungus under two different nutritional conditions. *P. sajor-caju* LBM 105 could remove 97.7% and 91.7% of the PCBs mixture and reduced the toxicity in complex and mineral media respectively after 35 days of incubation. The addition of PCBs to *P. sajor-caju* LBM 105 culture media resulted in an increase in hyphal diameter, changes in biomass, glucose consumption, proteins and laccase secretion. A 3-fold rise in laccase activity was detected in both media at 28 day. Laccase mRNA augmented 16% and 91% at 21 and 28 days respectively when PCBs were added to the N-limited liquid media. Mycoremediation of PCBs with *P. sajor-caju* LBM 105 cultivated in N-limited liquid medium is an effective low-cost (0.69 \$ g<sup>-1</sup>d<sup>-1</sup>) alternative technique for PCBs treatment.

**Key words: Polychlorinated Biphenyls; Mycoremediation; Laccase; Morphology; Tolerance**

## 1. Introduction

Polychlorinated biphenyls (PCBs) are persistent organic pollutants that were applied as dielectric and coolant fluids in transformers and electric motors in the twentieth century [1]. Although their use and production were banned in the last decades, they persist in the environment and represent a serious problem due to their recalcitrance and hazardous effects on wild life and human health [2]. They remain in soils and water bodies for many years, and because of their lipophilic nature, they bioaccumulate and biomagnify through the food chain [1]. Consequently, the clean-up of PCBs-contaminated sites has become a priority due to the teratogenic, carcinogenic and endocrine-disrupting features of these xenobiotics [3, 4]. Several methods have been applied for *in situ* and *ex situ*

remediation/destruction of PCBs and other toxic substances: landfilling, landfarming, incineration, thermal desorption, chemical dehalogenation, catalytic hydrogenation, ultrasonic technology, advanced oxidation processes, etc. [5, 6, 7, 8]. Biological strategies were implemented as a result of the limitations associated with the traditional methods. These methodologies use the microorganism's metabolic capabilities for transforming the pollutants into smaller molecules or for affecting the functional groups involved in their toxicity. They demand longer treatment periods than physicochemical processes, but are easy, low-cost and eco-friendly [9]. Mycoremediation using white-rot fungal strains proved as a highly effective remediation approach that has not been thoroughly investigated [10]. These fungi are capable of mineralizing even xenobiotic compounds (e.g., chlorophenols, polycyclic aromatic hydrocarbons, dioxins, furans, etc.) due to the nonspecific nature of their extracellular oxidative ligninolytic system; and they can be applied in solid, liquid and gaseous media [11, 12, 13, 14, 15]. As white-rot fungi grow by hyphal extension, they can easily penetrate across environmental matrices, reach pollutants in soil and act reaching as dispersion vectors [11]. They employ a variety of enzymes in pollutant degradation including intracellular cytochrome P450 monooxygenases and their extracellular ligninolytic system consisting of several enzymes, among them the phenoloxidase laccase (Lac, E.C. 1.10.3.2) and various peroxidases, such as lignin peroxidase (LiP, E.C. 1.11.1.14), manganese peroxidase (MnP, E.C., 1.11.1.13) and versatile peroxidase (VP, E.C. 1.11.1.16). These fungi are also capable of degrading PCBs, although the exact role of the enzymatic system has not been fully elucidated yet [10, 11, 16]. *Pleurotus ostreatus*, *Coriopsis polyzona*, *Phanerochaete chrysosporium* and *Trametes versicolor* were all able of significant PCB degradation with low congener specificity, and removal rates did not correlate with the degree of chlorine substitution [10, 17]. Their removal capacity seems to require different adaptation systems according to the fungal species [11, 18, 19]. In order to develop an effective mycoremediation process it is essential to look for low-cost culture conditions that improve fungal biodegradation [20, 21].

*Pleurotus sajor-caju* is an edible white-rot fungus [22]. This basidiomycete and its enzymes, mainly Lac, were involved in the removal of phenols and the decolorization of dyes and paper mill effluents [23, 24, 25]. *P. sajor-caju* LBM 105 has demonstrated potential to be applied in PCBs bioremediation

[26]. The aim of this work was to evaluate the feasibility of applying *P. sajor-caju* LBM 105 in mycoremediation of high PCBs concentrations. In order to approach different aspects of the process, we focused on the study of PCBs fungal removal capacity and the associated toxicity reduction, the changes in morphology and laccase expression caused by the addition of this pollutant and the cost of the treatment under two different nutritional conditions.

## 2. Materials and Methods

### 2.1 Materials

The investigation was performed using transformer oil containing a mixture of Aroclor 1242, 1254 and 1260, obtained from Kioshi S.A. (Buenos Aires, Argentina). All the solvents were purchased from Merck or Cicarelli (Santa Fe, Argentina) and were of p.a. quality, trace analysis quality or gradient grade. All the chemicals used for the biochemical studies were obtained from Merck or Britania (CABA, Argentina).

### 2.2 Microorganism, media and inoculum preparation

*P. sajor-caju* LBM 105 was isolated from Eldorado, Misiones, Argentina and provided by the Faculty of Forestry Sciences, National University of Misiones, Argentina. Two types of liquid media were used in this study: a natural medium (MEM) consisting of malt extract, 12.7 g; corn steep liquor, 5 g; distilled water up to 1 L [27] and a nitrogen-limited mineral medium (GA) containing glucose, 10 g; asparagine monohydrate, 0.5 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g;  $\text{H}_2\text{KPO}_4$ , 0.5 g;  $\text{HK}_2\text{PO}_4$ , 0.6 g;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.4 mg;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.1 mg;  $\text{H}_3\text{BO}_3$ , 0.1 mg;  $\text{CaCl}_2$ , 0.1 g;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.02 mg;  $\text{FeCl}_3$ , 1 mg;  $\text{ZnCl}_2$ , 3.5 mg; thiamine hydrochloride, 0.1 mg; Tween 80, 1.7 mmol; distilled water up to 1 L, final pH: 6.5 [28]. Erlenmeyer flasks (250 mL) with 20 mL of medium were autoclaved at 105°C for 20 min to prevent microbial contamination. Each Erlenmeyer flask was inoculated with 1 agar plug (5 mm diameter) of the fungus grown for 7 d on MEA plates (malt extract, 12.7 g L<sup>-1</sup>; agar, 20 g L<sup>-1</sup>). At the beginning of the cultivation, each flask was spiked with 4000 µg of PCBs in acetone. Unspiked and abiotic cultures were used as controls. Cultures spiked with acetone without PCBs were used as controls of this solvent. Heat-killed controls were obtained by the addition of PCBs to autoclaved 7-d

old fungal cultures. All cultivations were carried out in duplicate at  $29 \pm 1^\circ\text{C}$  under static conditions for 35 d. The samples were harvested weekly and the mycelium was separated from the supernatant by centrifugation at 8000 rpm for 10 min.

### 2.3 PCBs determination

The PCBs were extracted from cultures supernatants by washing three times with 20 mL of hexane under agitation (250 rpm,  $25 \pm 1^\circ\text{C}$ ) for 30 min [29]. The samples were then cleaned up with sulfuric acid and centrifugation (8000 rpm). Quantitative analyses of PCBs were performed using a gas chromatograph (Agilent 6890) equipped with a HP1 capillary column (Length: 30 m, ID: 0.23 mm, Film: 0.25  $\mu\text{m}$ ) and an electron capture detector ( $\mu\text{ECD}$ ). The abiotic losses were determined from cell-free controls incubated in parallel with fungal cultures. The reference material was 50  $\mu\text{g g}^{-1}$  of AccuStandad Aroclor 1242, 1254 and 1260. The removal percentages were obtained by comparing the treated samples with the heat-killed controls, and were adjusted in function of time to:

$$y^2 = at$$

Where  $y$ : is the removal percentage,  $a$ : the regression coefficient and  $t$ : the incubation period (in d).

### 2.4 Toxicity assay

To assess the bioremediation efficiency, *Ganoderma* sp. LBM 001 was used as toxicity indicator. This strain was severely affected by the presence of PCBs in previous studies [26]. The fungus was cultivated in 90 mm diameter agar plates containing sugarcane bagasse-based medium [26] supplemented with 100  $\mu\text{L}$  crude extracts from 35 d cultures of *P. sajor-caju* LBM 105 grown in GA or MEM media with PCBs addition. Crude extracts were spread on the surface of the plates using a Drigalski spatula. The radial growth was measured every 48 h for 16 d. Fungal growth was modeled by using a logistic equation [26]. Fungal growth  $\tau$  was standardized as  $\Delta\tau_1 = \tau\text{T} - \tau\text{C}$  and  $\Delta\tau_2 = \tau\text{PCB} - \tau\text{C}$ , where  $\tau\text{T}$  were the values from medium with PCBs treated with the fungus,  $\tau\text{PCB}$  were the values from medium with PCBs without fungal treatment and  $\tau\text{C}$  were the values from control culture without PCBs to ignore the effect of nutrient components. A positive value of  $\Delta\tau$  proved fungal growth inhibition in response to PCBs and/or toxic metabolites.

### 2.5 Effect of PCBs on growth and morphological appearance of the mycelium

To remove the attached PCBs, the mycelium was washed three times with a solution of hexane-acetone (10:6) during 15 min at 250 rpm, and then dried at  $105 \pm 1^\circ\text{C}$  up to constant weight.

Morphological changes due to PCBs addition were evaluated by direct observation and scanning electronic microscopy (SEM). For the microscopic analysis, mycelium grown for 14 d was separated from the supernatant. The hyphae were then fixed with a solution containing formaldehyde, alcohol and acetic acid (10:50:5). After drying at critical point with  $\text{CO}_2$  and gold-metalized, the surface was observed with a scanning electron microscope JEOL 5800LV. To evaluate the effect of PCBs on mycelia, hyphal diameter was measured in fifty hyphae using ImageJ software.

### 2.6 Analytical determinations in culture supernatants

The extracellular proteins from the supernatant were measured by the Lowry procedure [30], with bovine serum albumin as standard. Remaining glucose in the supernatant was determined by the glucose-oxidase/peroxidase method [31]. Enzymatic determinations: Lac activity was measured at  $30 \pm 1^\circ\text{C}$  using 5 mM of 2,6-dimethoxyphenol (DMP) as substrate in sodium acetate buffer 0.1M (pH 3.6). The changes in absorbance were evaluated at 469 nm ( $\epsilon_{469} = 27.5 \text{ mM}^{-1} \text{ cm}^{-1}$ ) [32]. Peroxidase activity was determined at  $30 \pm 1^\circ\text{C}$  by monitoring the oxidation of 5 mM of DMP in sodium acetate buffer 0.1 M (pH 3.6) and  $\text{H}_2\text{O}_2$  0.1 mM at 469 nm. The enzyme activities were expressed in international units (U), defined as the amount of enzyme required to produce  $1 \mu\text{mol}$  of product  $\text{min}^{-1}$  at  $30 \pm 1^\circ\text{C}$ . The activity was reported as  $\text{U L}^{-1}$ . The peroxidase activity was always corrected for Lac activity.

### 2.7 Polyacrylamide gel electrophoresis (PAGE) and Lac activity staining of gels

The secreted protein profile was observed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% w  $\text{v}^{-1}$ ) [33]. Proteins were stained with  $2 \text{ g L}^{-1} \text{ AgNO}_3$  [34]. To determine the presence of Lac isoenzymes, the culture supernatant was subjected to native polyacrylamide gel electrophoresis (ND-PAGE, 12% w  $\text{v}^{-1}$ ). After proteins separation, the gel was

incubated in 0.1 M sodium acetate buffer (pH 3.6) containing 5 mM DMP for Lac activity detection [27, 35]. After 10 min incubation, the solution was discarded, and the gel was immediately scanned with a Scanner HP Deskjet F300. To assess Lac isoenzymes molecular weight, an electrophoretic separation by SDS-PAGE (12% w v<sup>-1</sup>), followed by a subsequent re-naturalization with Triton X-100 0.2% v/v and detection technique was performed as previously described [27, 35, 36] and compared with a molecular weight marker (Prestained Kaleidoscope SDS-PAGE standard broad range, BioRad). The electrophoretic run was performed at 120 V for 120 min in 1.5 M Tris–Glycine buffer (pH 8.3).

### *2.8 Isolation of total RNA and Lac fragment amplification*

RNA was extracted from cultures harvested at 14, 21 and 28 d in GA and control media by centrifugation. The RNA was isolated with Tri-Reagent (Sigma-Aldrich) following manufacturer's instructions and re-suspended in 20 µL of RNases-free sterile water. To confirm the quality of the isolated RNA, a 0.8% (w/v) agarose gel electrophoresis was performed. Total RNA was spectrophotometrically quantified at 260 nm. Samples were treated with RNase-Free DNase (Promega) at 37°C for 30 min, halting the reaction with Stop Solution incubated at 65°C for 10 min, following the manufacturer's protocol. In order to obtain the double-stranded cDNA, 100 pmol of oligo (dT) primer was added to the samples treated with DNases. The reverse transcription was carried out at 42°C for 60 min in a 20 µL reaction containing: 5X Reaction Buffer (Tris-HCl pH 8.3 250 mM, KCl 250 mM, MgCl<sub>2</sub> 20 mM, DTT 50 mM), dNTPs 10 mM of each one, RNase inhibitor (ARNsin) 20 U, RevertAid-Reverse Transcriptase (Thermo Scientific) 200 U.

The PCR technique was performed at a final reaction volume of 20 µL containing 2 µL of cDNA from the previous step, 1X Taq polymerase Buffer (10X Tris-HCl pH 8.8 750 mM, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 200mM, Tween 20 0.1% (v/v)), MgCl<sub>2</sub> 2.5 mM, dNTP mix 200 µM, 10 pmol of each primer and 0.5 U Taq polymerase (Inbio Highway). The primers used for Lac fragment amplification were previously described by D'Souza et al. [37] and for internal control, an actin fragment was amplified using the primers described by Luis et al. [38]. The cycling conditions were 5 min 95°C, 40 cycles of 20, 40 and 40 s at 95°C, 50°C and 72°C respectively and final extension of 15 min 72°C. Actin and Lac fragments from genomic DNA were also amplified as positive reaction controls. The results of the



amplification were verified by agarose gel electrophoresis (2% w/v) stained with ethidium bromide and photographed with a Canon Power Shot G10 digital camera. The images were analyzed by densitometry. To confirm PCR specificity, the PCR products were sequenced.

### 2.9 Cost-effectiveness study

In order to evaluate both culture media, a cost-effectiveness study was carried out. Culture media costs were assessed based on their composition. The relationship between PCBs removal and incubation period was calculated by simple regression. Therefore, the treatment cost was estimated considering removal period and PCBs concentration in the culture medium.

### 2.10 Statistical analysis

Data was processed using Microsoft Excel and the statistical analysis was performed using Graphpad Prism (5.01) for Windows (GraphPad Software, San Diego California USA). The results were presented as the mean  $\pm$  standard deviation. Statistical significance values were evaluated using one-way analysis of variance. A  $p < 0.05$  value was considered statistically significant.

## 3. Results

### 3.1 *P. sajor-caju* LBM 105 capacity of removing and detoxifying PCBs

The time courses of PCBs removal in both MEM and GA media are shown in Fig. 1. The results are displayed as the sums of Aroclors compared with heat-killed controls. Culture media spiked with acetone revealed that this solvent did not show signs of toxicity towards the strain. *P. sajor-caju* LBM 105 removed 34.8% of the PCBs in MEM medium after 7 d of treatment. Conversely, no significant decrease in PCBs concentration was observed within the first 21 d of incubation in GA medium. This strain was capable of removing 97.7% and 91.7% of PCBs (217 mg L<sup>-1</sup> initial concentration) in MEM and GA media respectively after 35 d. The ability of *P. sajor-caju* LBM 105 to remove Aroclor 1242, 1254 y 1260 is depicted in Figs. 1B and C. There were no significant differences between the removal percentages of diverse congeners in MEM medium ( $p > 0.05$ ). However, these percentages diminished with the increased degree of chlorination of the congeners in GA medium at 21 d ( $p < 0.05$ ). To

evaluate the production of harmful metabolites, a microbial toxicity test was performed using *Ganoderma* sp. LBM 001. This strain proved to be sensitive to PCBs in previous studies (Sadañoski et al. 2018). The results indicated that the treatment with *P. sajor-caju* LBM 105 increased the radial growth of the biomarker demonstrating a reduction of toxicity as depicted in Table 1.

### 3.2 *P. sajor-caju* LBM 105 responses towards PCBs addition

Mycelium morphology did not present differences at macroscopic level (data not shown). Fig. 2 shows the effect of PCBs on the mycelium of *P. sajor-caju* LBM 105 in MEM and GA media. SEM observation revealed that *P. sajor-caju* LBM 105 hyphae showed the presence of ripples and protrusions at the surface in both media with and without PCBs. Mycelia in GA appeared wrapped by extra layers. However, these additional layers were not observed in the same medium supplemented with PCBs, the micrographs showed a spaced and linear filamentous mycelium. Nevertheless, hyphal diameter increased with PCBs addition ( $p < 0.05$ ). Fig. 3 depicts growth, glucose consumption, extracellular protein, and Lac secretion of *P. sajor-caju* LBM 105 in both media. The tolerance of this fungus to PCBs was assessed by comparing the mycelial dry weight attained both in the presence or absence of PCBs. Although the growth kinetics was slower in both media in the presence of the pollutant, the values of biomass detected at 21 d in MEM and 28 and 35 d in GA were similar with or without PCBs ( $p < 0.05$ ). Secreted extracellular proteins increased with PCBs in MEM medium. Under the conditions assayed in the present work, peroxidase activity was not detected. Highest Lac titers were detected after 14 d of incubation in MEM medium and were similar in the presence or absence of PCBs ( $p < 0.05$ ). In contrast, the peak of Lac activity was detected at 14 d in GA medium in the absence of PCBs and after 21 d of incubation in the medium amended with the pollutant. To verify the possible presence of Lac isoforms and their differential response to PCBs, SDS-PAGE analyses with DMP were carried out. Lac isoenzymatic pattern varied with culture media, incubation period and the presence or absence of PCBs. A Lac of high electrophoretic mobility was distinguished after 7 d of incubation both in absence and presence of PCBs in MEM and at 21 d in GA amended with PCBs (data not shown). In most of the other conditions assayed a diffuse Lac band with lower electrophoretic mobility was detectable in most of the other conditions assayed. This band showed a

clear increment at 14, 21 and 28 d in MEM medium with the pollutant, but also in GA medium at 14 and 21 d in cultures without PCBs and at 28 d in PCBs supplemented GA medium. Based on SDS-PAGE, the molecular weights of Lac isoenzymes were estimated to be between 60 and 80 kDa (Fig. 4 A, C). Peroxidase activity could not be detected. Fig. 4 B, D depicts the protein profile obtained from culture supernatants of *P. sajor-caju* LBM 105 without PCBs (-) and with PCBs (+) using SDS-PAGE 12% w/v in MEM (A) and GA (B) media after different incubation periods. SDS-PAGE showed an increase of all protein fractions especially between 45 kDa and 78 kDa. Biochemical studies showed that the kinetics of Lac secretion varied in GA medium with the addition of PCBs (Fig. 3). Accordingly, as shown in Fig. 5, Lac mRNA increased 16% and 91% at 21 and 28 d respectively when PCBs were added to the culture medium, due to increased levels of Lac gene transcription.

### 3.3 Bioremediation cost-effectiveness

Synthetic culture medium (GA) resulted more economically convenient than the natural MEM medium in order to grow *P. sajor-caju* LBM 105 (Table 2). PCBs removal was modelled as function of time through a simple regression. The general regression model was  $y^2 = k*t$ , where  $y$  depicts PCBs degradation,  $t$  is the time (d) and  $k$  represents an apparent removal rate ( $d^{-1}$ ).

Model equation 1 for MEM was:  $y_{MEM} = (0,025234*t)^{1/2}$  with a correlation coefficient of 0.96.

Model equation 2 for GA was:  $y_{GA} = (0,0220119*t)^{1/2}$  with a correlation coefficient of 0.89.

The value of  $y$ , the removal period and the mass of PCBs were used to evaluate the cost-effectiveness of the process (Table 3).

## 4. Discussion

Considering that PCBs pollution is typically caused by mixtures of congeners [39], we evaluated the removal capacity of *P. sajor-caju* LBM 105 using transformer oil containing a mixture of Aroclor 1242, 1254 and 1260. *P. sajor-caju* LBM 105 was able to tolerate high concentrations of PCBs (approx. 200 ppm) and proved high efficiency in the removal of the pollutant in liquid culture (more than 90% after 35 d of incubation). This percentage of PCBs removal is comparable to those previously obtained with other ligninolytic fungi [12, 16, 18, 40, 41] or strains isolated from PCBs-

polluted soils [29, 42]. Nevertheless, fungal PCBs removal is generally affected by initial PCBs concentration [42] and to our best knowledge only Ruiz Aguilar et al. [41] assayed PCBs removal by white-rot fungi in liquid culture at higher concentrations.

Culture conditions demonstrated to influence fungal growth and enzymatic production [15]. As shown in Fig. 1, nutrient availability affected *P. sajor-caju* LBM 105 capability to remove PCBs, being faster and more efficient in the complex medium than in the N-limited one. Similar results were reported by Plačková et al. [16] with *T. versicolor*. This fungus removed higher concentrations of PCBs in malt extract glucose medium than in a mineral medium. Nevertheless, other strains of white-rot fungi were capable of removing PCBs in mineral media more efficiently than in rich media [12].

Although bioremediation is pointed out as a good solution for the removal of PCBs from contaminated environmental matrixes [43], there is scarce knowledge on its ecotoxicological impact.

Mycoremediation of PCBs could potentially lead to the formation of toxic metabolites, such as chlorobenzoic acids, whose accumulation might affect PCBs biotransformation [44]. However, a toxicity test employing *Ganoderma* sp. LBM 001 demonstrated that *P. sajor-caju* was able to decrease PCBs toxicity in both liquid media. In accordance with our findings, Čvančarová et al. [12] also reported a reduction in PCBs toxicity in liquid media with *P. ostreatus*.

The ability of fungal strains to grow in the presence of aromatic compounds and other pollutants is associated with the development of mechanisms that favor their adaptation to diverse substrates, being crucial for their application in bioremediation [45, 46]. It is important to evaluate mycelial changes caused by PCBs presence because it is known that fungal mycelia control the flow of nutrients maintaining ecological equilibrium [47]. *P. sajor-caju* macroscopic morphology was not affected in PCBs media. However, electron microscopy images showed an increase in hyphal diameter in response to PCBs addition. The fungal cell wall is a highly dynamic structure and is regulated depending on internal and external stimuli [48]. Cell wall strengthening has been linked to the successful growth of the halophilic yeast *Wallemia ichthyophaga* at high salinities [49]. Cell wall stress response in *Aspergillus niger* involved increased deposition of chitin to ensure cell wall integrity [50]. The mycelia in GA (nitrogen limited medium) appeared enclosed by extra layers; likewise, under nitrogen limitation, SEM images showed that the mycelium of *P. chrysosporium* appeared covered by

abundant quantities of polysaccharides [51]. However, these additional layers were not detected in the same medium supplemented with PCBs. In contrast, Hong et al. [18] observed that PCBs led to the formation of extra layers attached to the fungal surface of *Ceriporia* sp. Exopolysaccharides (EPS) provide protection against environmental stresses [52]. As the PCBs mixture was added to the cultures at the start of the incubation, the fungus was exposed to the toxic influence of both PCBs and their metabolites during the whole incubation period. The toxic stress of the long exposition to PCBs could be responsible for the slow growth in the media with the pollutant within the first days of incubation [53]. Extracellular protein secreted increased in the presence of PCBs in MEM media. Hong et al. [18] also detected a rise in extracellular protein secretion after the addition of Aroclors in *Ceriporia* cultures, suggesting that various extracellular enzymes might have an effect on biodegradation of Aroclors, principally ligninolytic enzymes. *P. sajor-caju* produced the highest amounts of Lac in the simultaneous presence of high nitrogen and carbon concentrations in the medium, concordant with the fact that its ligninolytic system is expressed constitutively. The intensity of the bands displayed in the zymograms was related to the enzymatic activity detected. ND-PAGE revealed Lac enzymes with high electrophoretic mobility on 7 and 21 d in MEM and GA media respectively. It was not possible to determine the molecular weight of these fractions on SDS-PAGE, perhaps because the enzymes could not renature their catalytic domains after treatment with Triton X-100. Mostly, Lac enzymes have molecular weights between 60 and 80 kDa. Nakamura and Go [54] described Lacs of low molecular weight in different fungi, including a Lac of 34 kDa in *Pleurotus eryngii*. PCBs were reported as Lac inducers in *P. ostreatus* [55, 56], *T. versicolor* [16] and *Ceriporia* sp. ZLY-2010 [18]. In the present work, Lac transcript levels were higher in presence of PCBs than in their absence. This increase was mainly observed at 21 and 28 d of culture, in correlation with Lac titers detected. Xenobiotic response elements (XRE) appeared in promoter regions of different white-rot fungal Lac suggesting that transcription of these Lac genes might be activated by aromatic compounds [57]. However, although Lac mRNA was detected at 14 d of incubation, Lac activity could not be measured in PCBs media. On the contrary, previous reports showed high Lac titers but low transcript levels in PCBs presence, indicating either an increased rate of Lac synthesis or an inespecificity of the biochemical reaction [56]. These results indicate that the presence of PCBs modifies Lac expression at the transcriptional level,

perhaps in response to specific transcription factors. Likewise, other factors seemed to be involved, such as the stability of the transcript and translational and post-translational processes, or trafficking and protein folding [58, 59]. Lac secretion in a shorter period of time might be an important factor increasing not only PCBs degradation efficiency but also PCBs tolerance as demonstrated in a previous study [26]. Many authors investigated the possible association between Lac production and PCBs degradation capability [12, 16, 56, 60, 61]. Čvančarová et al. [12] found no correlation between ligninolytic production and PCBs degradation efficiency. In contrast, PCBs stimulated Lac production by *P. ostreatus* promoting the degradation of PCBs with a high degree of chlorination and it was not detected peroxidase activity in culture supernatants [56]. Nevertheless, peroxidases [62] and cytochrome P450 monooxygenase [63] might be involved in the oxidation of PCBs prior to their degradation, as demonstrated in the case of polycyclic aromatic hydrocarbons.

Table 3 shows PCBs removal as a function of time fitted to the obtained model (Equations 1 and 2). The same table displays bioremediation costs considering the price of culture media, PCBs mass and incubation period. Thus, it demonstrates that the cost to achieve almost complete removal diminishes by increasing the incubation period. Mycoremediation of PCBs with *P. sajor-caju* LBM 105 cultivated in GA medium exhibited a lower cost for PCBs removal per gram and day ( $0.69 \text{ \$ g}^{-1}\text{d}^{-1}$ ) than when using MEM medium (mainly due to the price of medium components).

In conclusion, *P. sajor-caju* (LBM 105) was capable of removing high concentrations of PCBs in liquid conditions and diminishing their toxicity. Changes in its morphology were observed, as well as an increase in hyphal diameter. PCBs addition also augmented Lac activity and Lac expression at the transcriptional level. The mycoremediation of PCBs with *P. sajor-caju* LBM 105 cultivated in N-limited liquid GA medium demonstrated to be an effective low-cost alternative technique for PCBs treatment. Based on an exhaustive review of research literature, this work can be considered as the first study about *P. sajor-caju* potential for low-cost bioremediation of high PCBs concentrations.

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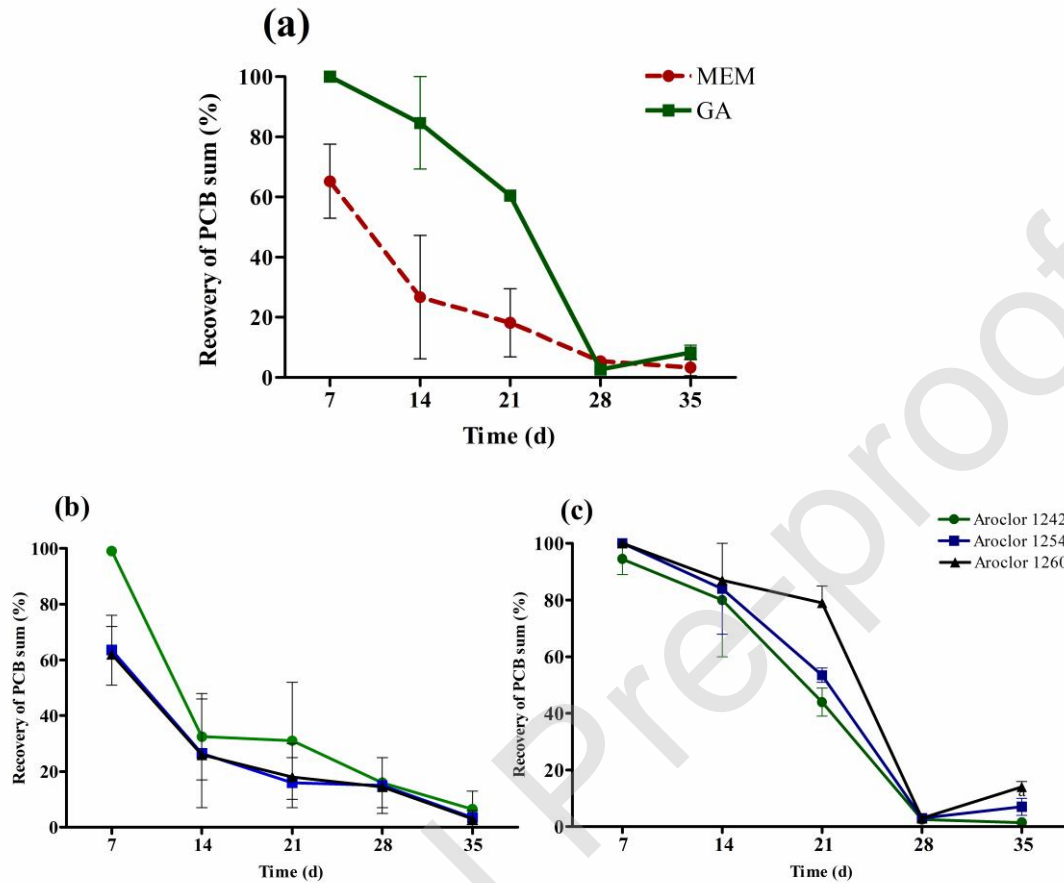
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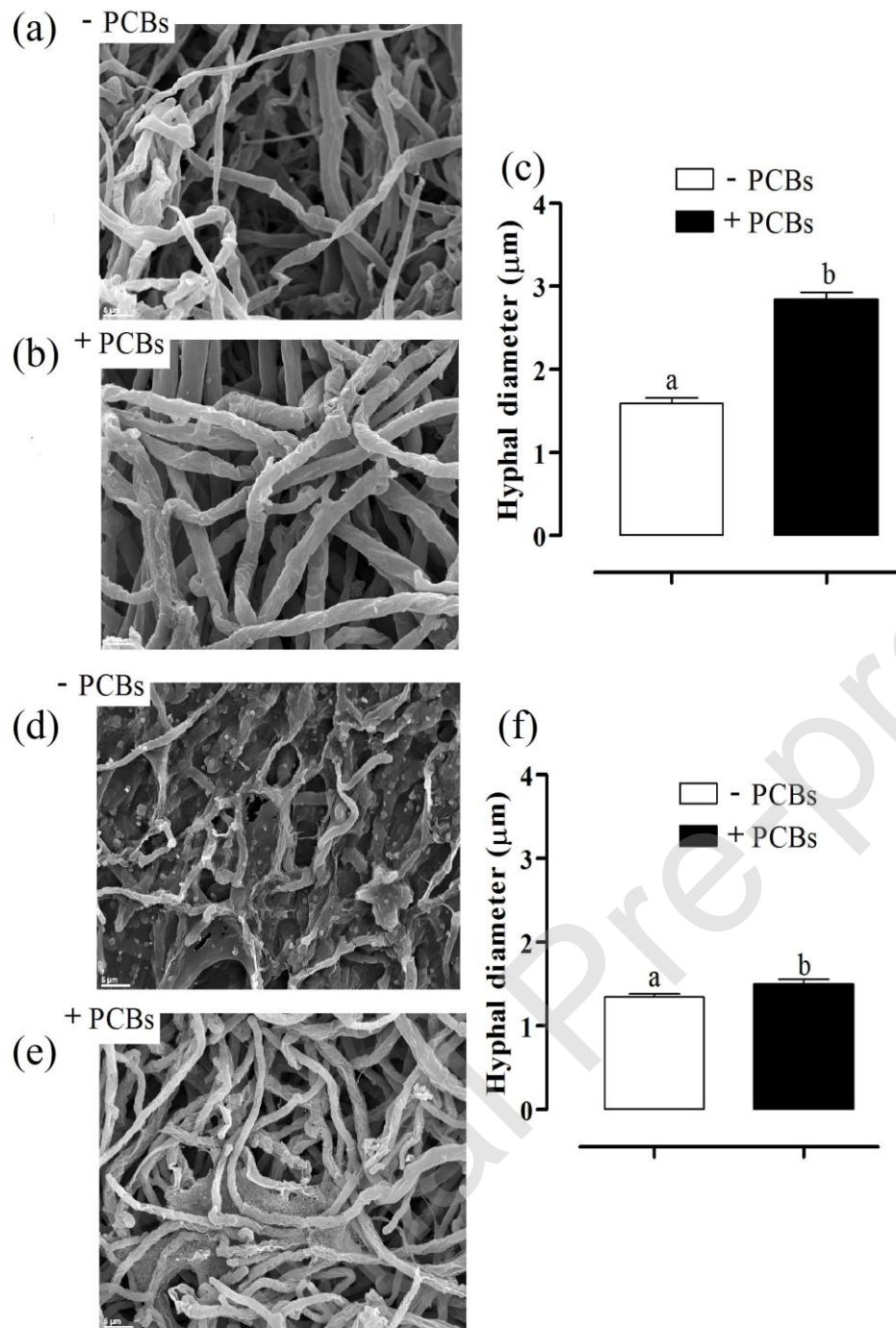
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**Fig. 1** Removal of PCBs after treatment with *P. sajor-caju* LBM 105. A. Recovery of the sum of PCBs. B-C. Recovery of the sum of Aroclors 1242, 1254 and 1260.



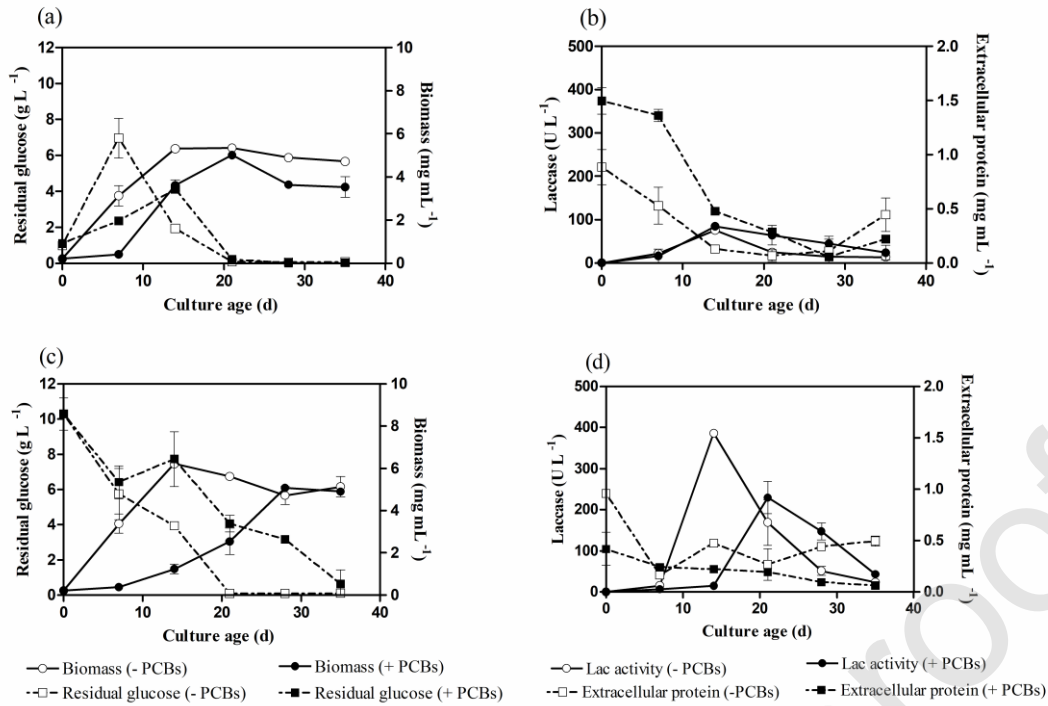
**Fig. 2** *P. sajor-caju* LBM 105 micromorphology after 14 d of incubation in absence (-) or presence (+) of PCBs ( $217 \text{ mg L}^{-1}$ ). SEM micrographs of mycelia in A-C. MEM culture media and D-F. GA culture media. Bar =  $5 \mu\text{m}$ . C, F. Hyphal diameter. Means with different letters are significantly different ( $p < 0.05$ ).



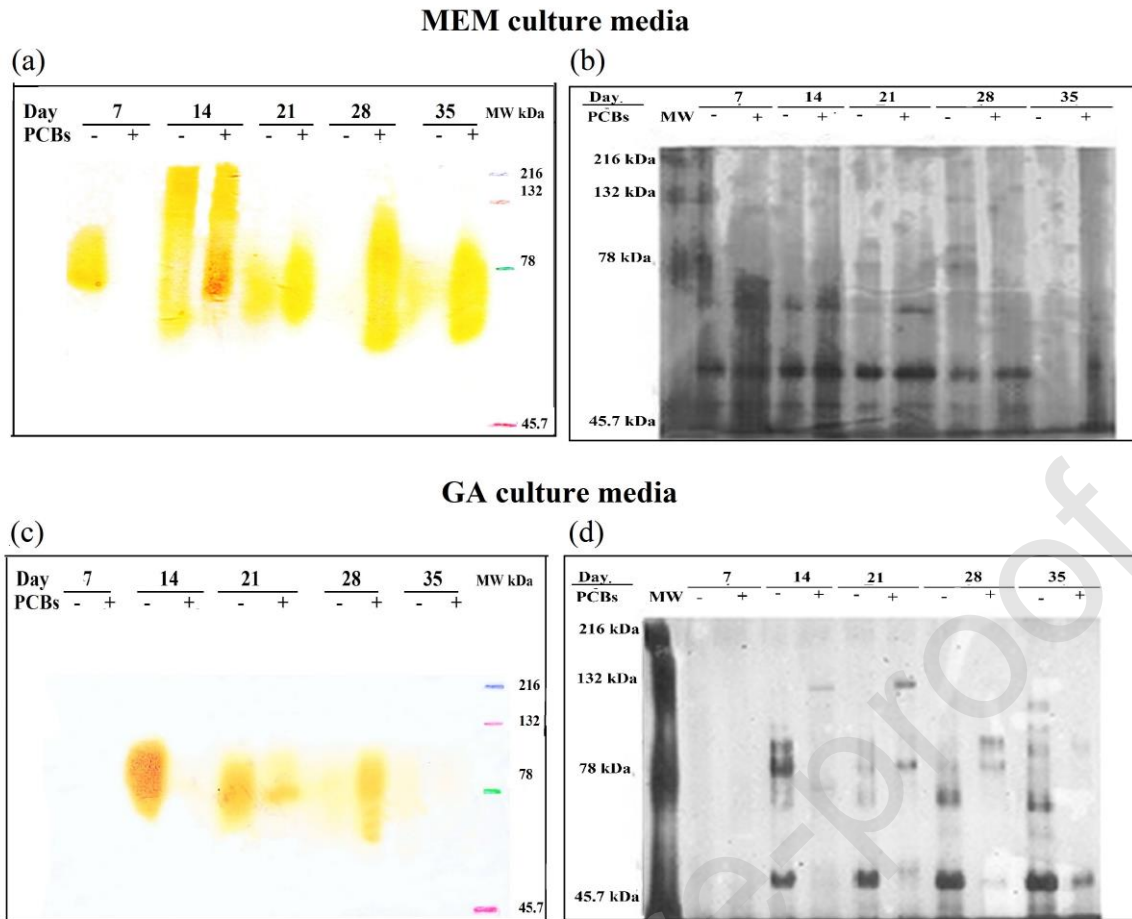


**Fig. 3** Effect of PCBs on growth, glucose consumption, protein and Lac secretion in A-B. MEM and C-D. GA culture media.

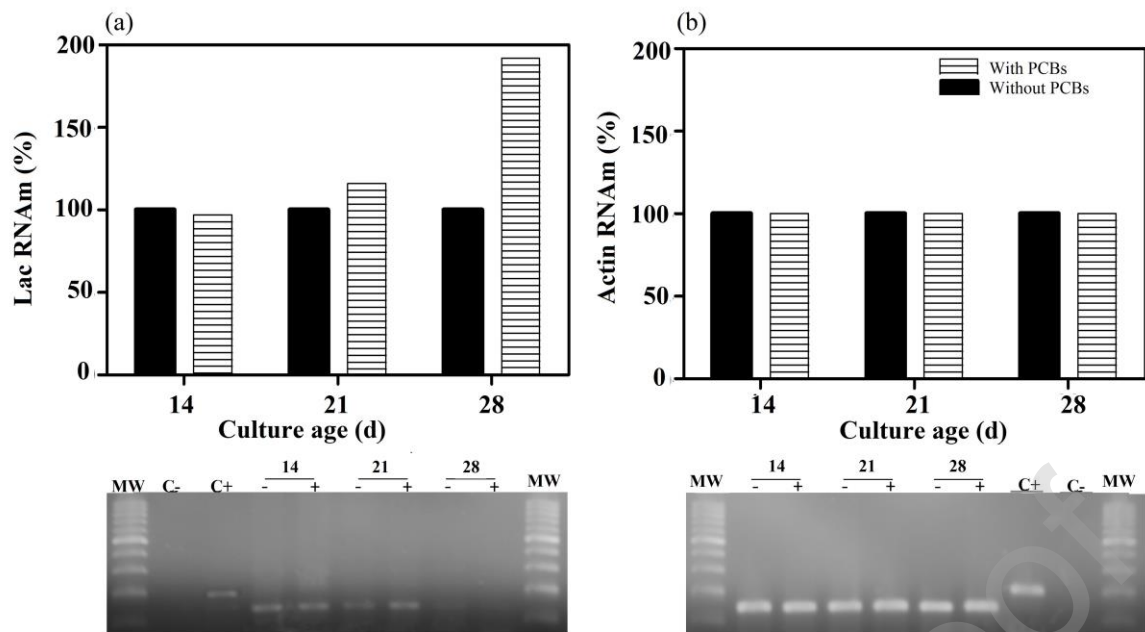




**Fig. 4** Effect of PCBs on Lac and protein profile of *P. sajor-caju* LBM 105 without (-) and with (+) PCBs at 7, 14, 21, 28 and 35 d. A, C. SDS-PAGE (12% wt v<sup>-1</sup>) revealed with DMP substrate. B, D. SDS-PAGE (12% wt v<sup>-1</sup>) after silver nitrate staining. MW: molecular weight marker.



**Fig. 5** Semi quantitative RT-PCR for mRNA of Lac (left) and actin mRNA (right). Three independent experiments were performed with RNA isolated from mycelium grown in absence (-) or presence of 217 mg L<sup>-1</sup> PCBs (+) during 14, 21 and 28 d in GA medium. The densitometry graph is shown above, and the matched products of RT-PCR separated using electrophoresis in agarose 2% (w v<sup>-1</sup>) are shown below. The positive control (C+) corresponds to the 200 bp fragment obtained from genomic DNA (amplification control) and negative control (C-) corresponds to the amplification without the presence of cDNA or genomic DNA respectively. MW: molecular weight marker.



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**Table 1.** Reduction of toxicity during PCBs treatment with MEM and GA using *Ganoderma* sp. LBM 001 as toxicity indicator.

Treatment	$\Delta\tau_1$	$\Delta\tau_2$	Toxicity reduction (%)
MEM	2.59	2.85	9.12
GA	1.31	1.46	10.27

**Table 2.** Cost of MEM and GA culture media.

Medium component	Unit cost \$ kg <sup>-1</sup>	GA		MEM	
		g L <sup>-1</sup>	Total cost \$ kg <sup>-1</sup>	g L <sup>-1</sup>	Total cost \$ kg <sup>-1</sup>
Glucose	239.82	10.000	2.433	-	-
Asparagine	1062.38	0.5000	0.539	-	-
MgSO <sub>4</sub> .7H <sub>2</sub> O	347.27	0.5000	0.176	-	-
H <sub>2</sub> KPO <sub>4</sub>	362.66	0.5000	0.184	-	-
HK <sub>2</sub> PO <sub>4</sub> .3H <sub>2</sub> O	499.73	0.6000	0.304	-	-
H <sub>3</sub> BO <sub>3</sub>	251.68	0.0001	0.000	-	-
CaCl <sub>2</sub>	666.71	0.1000	0.068	-	-
CuSO <sub>4</sub> .5H <sub>2</sub> O	597.74	0.0004	0.000	-	-
MnCl <sub>2</sub> .4H <sub>2</sub> O	716.32	0.0001	0.000	-	-
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	2758.80	0.00002	0.000	-	-
FeCl <sub>3</sub>	166.98	0.001	0.000	-	-
ZnCl <sub>2</sub>	13128.50	0.0035	0.047	-	-
Tiamine HCl	1183.38	0.0001	0.000	-	-
Tween 80	210.62	2.227	0.476	-	-
Malt extract	1495.56	-	-	12.70	19.336
Corn steep liquor	228.70	-	-	5.00	1.164
Water	0.07	985.57	0.067	982.30	0.067
<b>Total</b>		1000.00	<b>\$ 4.295</b>	1000.00	<b>\$ 20.567</b>

**Table 3.** Cost-effectiveness study of PCB bioremediation in MEM and GA culture media.

<b>Culture Time (d)</b>	<b>PCBs Removal Fit</b>		<b>Bioremediation Cost</b>	
	$y_{MEM}$	$y_{GA}$	<b>MEM</b> (\$ g <sup>-1</sup> d <sup>-1</sup> )	<b>GA</b> (\$ g <sup>-1</sup> d <sup>-1</sup> )
7	0.420	0.392	34.95	7.81
14	0.594	0.555	12.35	2.76
21	0.728	0.679	6.72	1.50
28	0.840	0.785	4.36	0.97
35	0.939	0.877	3.12	0.69

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