Novel aerobic perchloroethylene degradation by the white-rot fungus *Trametes versicolor*

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Abstract

Perchloroethylene (PCE) is one of the most important groundwater pollutants around the world. It is a suspected carcinogen and is believed to be rather recalcitrant to microbial degradation. We report here, for the first time, aerobic degradation of PCE by the white rot fungus, *Trametes versicolor*, to less hazardous products. Aerobic degradation rate of PCE was 2.04 to 2.75 × 10-4 μmol h-1 mg dry weight of fungal biomass. Trichloroacetyl chloride (TCAC) was identified as the main intermediate using [2-^{13}C]PCE as the substrate. Chloride release was stoichiometric with PCE degradation. Re-oxygenation of the cultures resulted in increased PCE degradation as well as a corresponding increase in chloride release. These results suggest that better degradation rates can be achieved by appropriate optimization of culture conditions. Additionally, our studies using 1-aminobenzotriazole (ABT), an inhibitor of cytochrome P-450, suggested that cytochrome P-450 system is involved in PCE degradation by *T. versicolor*. These results are of particular interest because both the involvement of cytochrome P-450 system in PCE degradation as well as TCAC production from PCE has been reported to date only in mammalian systems, but not in bacteria or fungi.

1. Introduction

Perchloroethylene (PCE), an effective degreasing solvent and fumigant, is among the most frequently detected recalcitrant xenobiotic pollutant in soil and groundwater around the world. In fact, PCE has been shown to be present most of the hazardous waste sites on the U.S. Environmental Protection Agency National Priority List (*1*).

Although the reductive dechlorination of PCE has been achieved by bacterial consortiums under anaerobic conditions some two decades ago (*2- 5*), description of axenic cultures capable of degrading PCE have been more recent. These axenic cultures belong to four different metabolic groups: halorespirers, acetogens, methanogens and facultative anaerobes (*6*). Detailed studies on the mechanisms of reductive dehalogenation of PCE have been initiated and several reductive dehalogenases involved in PCE degradation have been purified (*7-10*). Co-metabolic dehalogenation, expected for acetogens and methanogens, results in one step conversion of PCE to TCE and the release one chlorine moiety. Most of the halorespirers, which are able to gain energy from the dehalogenation reaction, and facultative anaerobes continues further conversion of PCE to less chlorinated compounds. To date, only *Dehalococcoides ethenogenes* strain 195 has been known to degrade PCE to the non-toxic compound ethene (*11*), while most other natural biotic and abiotic processes degrade PCE to result in toxic products (such as cis-dichloroethylene) and carcinogenic intermediates (such as vinyl chloride) (*12-16*).

For many years, PCE was thought to be non-biodegradable in the presence of oxygen, but PCE degradation by *Pseudomonas stutzeri* OX1, involving a toluene-o-xylene

67 monooxygenase was reported recently (*17, 18*). The evidence for degradation was primarily
68 based on the quantification of chloride ions released into the medium, but the reaction
69 products were not identified. Also, Enzien et al (*19*) observed dehalogenation of PCE under
70 bulk aerobic conditions but they suggested that anaerobic microsites were the sites for
71 dehalogenation.

72 White rot fungi are able to degrade lignin present in woody plants using nonspecific
73 enzymes systems as exemplified by lignin peroxidases (LiP), manganese peroxidases
74 (MnP) and laccases (Lac). These enzymes use free radicals mechanisms to catalyze the
75 degradation of a wide variety of chloroaromatic pollutants (*20-22*). However, subsequent
76 studies have demonstrated that the mechanism of degradation of some pollutants are not
77 linked to the production of the peroxidase system and reported the involvement of alternate
78 oxygenases, particularly P-450 monooxygenases (*23-25*).

79 In this report, we demonstrate for the first time the ability of fungus to degrade PCE
80 under aerobic conditions using a degradation mechanism that has not been demonstrated in
81 microbes to date. We present here our results on PCE degradation rate, the reaction
82 products obtained, and a suggested mechanism for PCE degradation used by *T. versicolor*.
83 The feasibility for improvement in the degradation rate by reoxygenation of cultures of this
84 fungus has also been studied.

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2. Materials and methods

2.1. Fungal strains and chemicals. *T. versicolor* (ATCC#42530) was maintained by subculturing on 2% malt extract agar slants (pH 4.5) at room temperature. Subcultures were routinely made every 30 days. PCE was obtained from Sigma-Aldrich Co. (St. Louis, MO) and [2-^{13}C]PCE (99%) was from Isotec (Miamisburg, OH).

2.2. Media and cultures. Defined medium contained 8 g/L glucose, 498 mg/L N as ammonium tartrate, 10 and 100 mL/L of a micro and macronutrient solution, respectively (*26*), and 1.168 g/L of 2,2-dimethylsuccinate buffer, unless otherwise indicated. pH was adjusted to 4.5.

A mycelial suspension of *T. versicolor* was obtained by inoculation of four 1 cm^2 area agar plugs from the growing zone of fungus on malt agar (2%) to a 500 ml Erlenmeyer flask containing 150 ml of malt extract medium (2%) at pH 4.5. Flasks were incubated at 25ºC on an orbital shaker (135 rpm, r=25 mm). After 4-5 days, the dense mycelial mass was ground with a X10/20 homogenizer (Ystral GmbH, Dottingen, Germany). This blended mycelial suspension was used as the inoculum. Pellets of *T. versicolor* were produced by using 1 ml of the mycelial suspension to inoculate 250 ml of malt extract medium (2% malt extract, pH 4.5) in a 1 litre Erlenmeyer flask. This was shaken (135 rpm, r=25 mm) at 25ºC for 5-6 days. Subsequently pellets formed by this process were washed with sterile deionized water.

2.3. PCE degradation experiments. All the experiments were performed using 125-ml serum bottles sealed with Teflon-coated grey butyl rubber stoppers (Wheaton, Millville, N.J.) and aluminium crimps (Baxter Scientific Products, McGaw Park, Ill). Each bottle was inoculated with 2 g of wet pellet of *T. versicolor* (equivalent to 5.0 g/l dry weight). 10-ml of liquid medium was added to each inoculated bottle and subsequently was oxygenated for 1 min (30 KPa) and sealed immediately. Then, 20 µL of a solution of PCE in ethanol was added by means of a pressure-lok gas-tight syringe (VICI Precision Sampling, Baton Rouge, LA) through the stoppers to give 5 mg/L PCE in the liquid media. The bottles were shaken vigorously for 30 min in an inverted position (to minimize gas leakage) and subsequently were incubated at 25ºC on an orbital shaker (135 rpm, r=25 mm), also in an inverted position. In those cases where reoxygenation took place, 5 ml of pure oxygen was added by means of a pressure-lok gas-tight syringe through the stoppers.

Each experiment included uninoculated and heat-killed controls. Heat-killed controls consisted of autoclaved cultures that had been pre-grown for 7 days under conditions identical to those of the experimental cultures. Percent degradation at a specified interval was calculated by comparing concentration in the uninoculated blanks with those in the experimental bottles. All degradation values were corrected for the sorption values determined using the heat-killed controls. PCE concentration values were also corrected considering the water volume added with pellets. Each bottle was sacrificed at each time point for analysis.

2.4. Experiments with cytochrome P-450 inhibitor. For those microcosms that were tested with the cytochrome P-450 inhibitor 1-aminobenzotriazole (ABT), a final

concentration of 1 mM ABT was present in 10 ml of defined medium with 2 g of *T. versicolor* pellets, as described above. The bottles were incubated at 25ºC on an orbital shaker (135 rpm) for 7 days. Heat-killed and inhibitor-free controls were included in triplicate in this experiment.

2.5. PCE analysis. The concentration of PCE was determinated by static headspace gas chromatography. All samples were equilibrated at 25ºC before analysis. A 1 ml liquid sample from each experimental bottle was transferred to 4 ml sodium azide solution (1%) in a 10 ml vial and sealed immediately with a teflon coated stopper. The vial was placed in a headspace sampler Agilent 7964 (Agilent Technologies, Palo Alto, CA) and was heated to 85ºC for 50 min. Subsequently, a 1-mL headspace sample was injected automatically into a gas chromatograph (Agilent 6890N) equipped with a column Agilent HP-5 (30 × 0.32× 0.25) and a flame ionization detector.

The GC operating conditions were as follows: column temperature, 40ºC (2 min), slope 4ºC/min, 50ºC (1 min), slope 10ºC/min, final temperature: 160ºC; injector temperature, 125ºC; flame ionization detector temperature, 260ºC; and carrier gas He at 7 psi pressure. Data was acquired and quantified by Millennium 32 software (Waters, Milford, MA).

Total amount of PCE in the experimental bottles and its concentration in liquid media were determined by comparing peak areas with those of external standards and by using Henry´s law constant reported previously (*27*) and verified in our laboratory.

2.6. GC-MS analysis. GC-MS measurements were performed injecting a 1-mL headspace sample automatically from a headspace sampler Agilent 7964 (Agilent Technologies, Palo Alto, CA) using same conditions as those described above, to an Agilent 6890 (Agilent Technologies, Palo Alto, CA) gas chromatograph coupled with an Agilent 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA). The samples were injected on capillary column Agilent HP-5MS (30 × 0.25× 0.25), and helium was used as the carrier gas. The temperature program was 40ºC (2 min), slope 4ºC/min, 50ºC (1 min), slope 10ºC/min, final temperature: 200ºC and injector temperature, 125ºC. The following MS conditions were used: ionization mode, EI^+; ionization energy, 70 eV; mass range, m/z 35 to 220.

Intermediates were identified using the Wiley 275 Mass Spectral Library (John Wiley & Sons, New York, NY; purchased from Hewlett Packard, Palo Alto, CA) and by comparison of the mass spectra with those of a commercially available pure compound.

2.7. NMR analysis. The identification of PCE degradation intermediates was done using [2-^{13}C]PCE in experiments similar to those described above followed by nuclear magnetic resonance (NMR) analysis. NMR spectra were recorded in a BRUKER AV500 spectrometer equipped with a high-sensitivity cryogenically cooled TCI probe and operating at 100.62 MHz for ^{13}C.

The sample used was prepared by adding 50µl of D_2O (as a source of deuterium to lock the sample into the magnet) in a 500µl of a 31-day old liquid sample. ^{1}H-decoupled ^{13}C spectra were recorded using the inverse-gated method with 5 seconds of pre-scan delay. Data were processed using an exponential window function (line broadening of 2 Hz) prior

177 to Fourier Transformation. The resulting NMR spectra were compared with those of non-
178 labelled standards to confirm the presence or absence of possible target compounds.
179
180 **2.8. Other analyses.** Mycelial dry weights were determined by vacuum filtering the
181 cultures with preweighed glass filters (47-mm-diameter). The filters containing the
182 mycelial mass were placed in glass dishes and dried at 100ºC to constant weight.
183 The concentration of chloride ions released during PCE degradation was measured
184 by an ionic chromatograph Dionex ICS-1000 equipped with a conductivity detector
185 (Dionex, Wommelgem, Belgium), using a 4-mm anionic exchanger column, IonPack AS9-
186 HC (also from Dionex). The volume of injection was 25 µL and the mobile phase was
187 9 mmol/L sodium carbonate solution with a flow rate of 1 ml/min.
188 Laccase activity was measured using a modified version (*28*) of the method for the
189 determination of manganese peroxidase (*29*), where 2,6-dimethoxyphenol (DMP) was
190 oxidized by laccase in the absence of a cofactor. Conversely, oxidation by manganese
191 peroxidase (MnP) requires the presence of H_2O_2 and catalytically active Mn^{2+}. One activity
192 unit (AU) was defined as the number of micromoles of DMP oxidized per minute. The
193 DMP extinction coefficient was 10000 M-1 cm-1.
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195

196 ## 3. Results

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198 ### 3.1. Degradation of PCE by *T. versicolor* and identification of the primary
199 intermediate.

200 Results presented in Figure 1 and 2 show that substantial PCE degradation observed in
201 experimental flasks was accompanied by the production of a product which was
202 subsequently identified as chloroform (see below). The measured rate of PCE degradation
203 was between 2.04 and 2.75 × 10^{-4} μmol $h^{-1}mg^{-1}$ dry weight of biomass during the first three
204 days of incubation. Neither the disappearance of PCE nor the production of any
205 intermediate was observed in heat-killed controls and uninoculated bottles.

206 Laccase activity is strongly inhibited by the addition of PCE, obtaining a maximum
207 enzyme activity of 45.5 ± 4.3 AU/l (3rd day of incubation) and 16.2 ± 7.1 the remaining
208 period. In control cultures under identical growth condition but without PCE the maximum
209 enzyme activity was 226.2 ± 13.5 AU/l.

210 The intermediate detected during PCE degradation was identified as chloroform
211 based on GC-MS analysis and using the Wiley 275 Mass Spectral Library. The mass
212 spectrum of chloroform is characterized by the peaks at *m/z* 47, 83 and 118 (Figure 3A).
213 This mass spectrum coincided fully with that obtained during PCE degradation experiment
214 (Figure 3B). In stable isotopic-labelling degradation experiments with [2-^{13}C]PCE,
215 chloroform carried the ^{13}C label. Their mass spectra showed a shift of 1 atomic mass unit
216 compared to the chloroform obtained from non labeled PCE (47→48, 83→84, 118→119)
217 as shown in Figure 3C.

3.2. Identification of intermediate by nuclear magnetic resonance (NMR). Appearance of chloroform was not reported in previous PCE biodegradation reports. Our working hypothesis was that chloroform was formed by an abiotic process possibly from another primary intermediate during static headspace gas chromatography analysis. Therefore, cultures spiked with 5 mg/L of [2-^{13}C]PCE were analyzed by $^{13}C\{^{1}H\}$ NMR. The presence of [^{13}C]trichloroacetic acid ([^{13}C]TCA) was confirmed by the presence of an AX spin system consisting of two doublet resonances at chemical shifts of 167.1 and 95.4 ppm with a J(CC) coupling value of 61Hz (Figure 4B). These resonances agreed with the NMR spectrum obtained from a sample of commercially available, non-labeled TCA. In addition, the doublet splitting of these resonances confirms that TCA arises from the initial [2-^{13}C]PCE. On the other hand, while NMR signals from [2-^{13}C]PCE, and non-labeled α- and β-glucose and ethanol were clearly visible in heat-killed controls, TCA resonances were not observed in these cultures (Figure 4A).

3.3. Inhibition study with ABT. The addition of 1-aminobenzotriazole (ABT), a known inhibitor of cytochrome P-450 system (*30*), to cultures containing 5 mg/L of PCE resulted in total inhibition of PCE degradation whereas in inhibitor-free controls, PCE degradation and chloride release was seen (Table 1). No PCE degradation was observed in heat-killed controls. In parallel experiments, ABT did not affect cell yields of *T. versicolor* (data not shown)

240 **3.4. Effect of oxygenation on PCE degradation and chloride release.** Since *T. versicolor*
241 is an aerobic organism, we hypothesized that oxygen depletion may be a significant
242 limitation on PCE degradation in the closed culture vessels employed in this study,
243 necessitated by the fact that PCE is highly volatile. The observed plateau in PCE
244 degradation observed after 6 days of incubation is consistent with this idea. Therefore, one
245 set of parallel cultures were re-oxygenated after 4 days of incubation. These re-oxygenated
246 cultures showed a slight increase in PCE degradation as well as in the amount of chloride
247 ions released. (Table 2).
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4. Discussion

Involvement of cytrochrome P-450 in PCE degradation has so far been reported in mammalian systems only. No anaerobic or aerobic bacteria were shown to catalyze P-450 mediated degradation of PCE. The results presented here constitute the first demonstration of PCE degradation under aerobic conditions by fungi in general, and more specifically the white-rot fungus *T. versicolor*. That PCE degradation observed is biological is supported by the following observations: (1) increase in PCE degradation occurs during the first three days, which coincides with the product formation (see Figure 1 and 2); (2) the stoichiometry between μmol of chloride released and μmol of PCE degraded was comparable to the theoretical ratio of 1:1 (see Table 2); (3) dechlorination of PCE does not occur in heat-killed controls and in uninoculated bottles; and (4) Oxidative degradation of PCE and concomitant chloride release and TCAC production was inhibited in the presence of 1-aminobenzotriazole (ABT), a known cytochrome P-450 inhibitor.

The results on PCE disappearance, concomitant chloride release, and [2-^{13}C]PCE experiments show that PCE is degraded by *T.versicolor* to trichloroacetyl chloride (TCAC), which is rapidly hydrolyzed in water (abiotically) to TCA. The later compound undergoes spontaneous intramolecular rearrangement at elevated temperature (85°C), which occurs when the culture sample is heated during gas chromatography, and forms chloroform. This abiotic formation of chloroform is some what analogous to the formation of trichloroacetaldehyde (chloralhydrate) from trichloroethylene (*31*). Formation of

chloroform from TCAC in this study (Figure 2) was independently corroborated by injecting pure TCAC with deionized water using static head space gas chromatography.

It is note worthy that vinyl chloride and dichloroethene, frequently seen products of reductive dehalogenation by bacteria under anaerobic conditions, were not observed as products of PCE degradation by *T. versicolor*. In mammalian systems, cytochrome P-450 mediated oxidation of PCE results in the formation of TCAC via epoxy-PCE, which reacts subsequently with amino groups in macromolecules or with water to give trichloroacetic acid (TCA) (*32, 33*). Thus, PCE degradation by *T. versicolor* appears to be analogous to the mammalian systems and is quite different from PCE degradation aerobic and anaerobic bacteria described to date. Furthermore, unlike PCE and its products of vinyl chloride and dichloroethene seen in bacterial systems, TCA produced from PCE by cultures of *T. versicolor* is not considered to be a suspected carcinogen, is far less toxic, and is readily degraded by other organisms in the environment. This is a distinct advantage with the white-rot fungus *T. versicolor* in comparison to many of the bacterial systems for degradation of PCE.

Enzymes of the P-450 super family are found in a wide range of prokaryotic and eukaryotic organisms and have been well characterized, regarding their function, regulation, and expression (*34, 35*). In mammalian systems, PCE is known to be metabolized by both cytochrome P-450- and glutathione-dependent biotransformation pathways, leading to the generation of reactive metabolites which may covalently bind to cellular macromolecules (*32, 33, 36, 37*). In contrast to this, relatively little is known about the basic biochemistry of fungal P-450 systems, in spite of the fact that P-450 has been known for several years to play a key role in the biotransformation of various

294 environmental pollutants (*24, 25, 38, 39*) by white-rot fungi. Our experiments with
295 cytochrome P-450 inhibitor, ABT suggest that *T. versicolor* degrades PCE by the pathway
296 presented in Figure 5. Our data supporting the formation of TCAC and TCA as successive
297 degradation products of PCE is supported by the earlier precedent of PCE transformation to
298 TCAC in mammalian systems. Recent reports on the identification and characterization for
299 the first time of P-450 encoding genes in *T. versicolor* (*40*), and the reported involvement
300 of P-450 in this organism in metabolizing recalcitrant dibenzothiophene derivatives (*41*), is
301 also consistent with the results of this study suggesting the involvement of P-450 in the
302 PCE degradation pathway shown in Figure 5.

303 Three major families of lignin-modifying enzymes, lignin peroxidases (LiP),
304 manganese peroxidases (MnP), and laccases have been recognized (*42*). These enzymes are
305 relatively non-specific with respect to substrate and one or more of these enzymes are
306 known to catalyze the degradation of a wide variety of chroaromatic pollutants (*42*). No
307 LiP or MnP activity was detected in the extracellular culture fluid in any of the *T. versicor*
308 cultures in this study. Laccase activity was found but the level of its activity in PCE
309 cultures was much less than that observed in uninoculated control cultures without PCE.
310 Addition of laccase in vitro to TCAC containing reaction mixtures, in the presence or
311 absence of mediators such as 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
312 diammonium salt (ABTS), hydrate 1-hydroxy-benzotriazol (HOBT), voluric acid (VA) and
313 3,5-dimethoxy-4-hydroxyacetophenol (DMHAP) [data not shown], did not result in TCAC
314 degradation. Furthermore, adding TCAC (5 mg/L) to growing cultures of *T. versicolor* did
315 not result in its degradation. These results indicate that TCAC (or TCA) are not degraded

316 further by *T. versicolor* cultures; however, both TCA and TCAC are known to be
317 efficiently degradable by pure and mixed cultures of other microbes (*43-47*).

318 Compared with dehalorespirers, co-metabolic PCE dechlorination processes
319 proceed at a much lower rate. The rates of PCE dechlorination (to TCE) by
320 *Methanosarcina sp.* and *Acetobacterium woodii* were 3.5×10^{-5} and 3.6×10^{-3} μmol h^{-1} mg
321 $protein^{-1}$, respectively (*48*). In comparison to this, PCE dechlorination rates coupled to
322 dehalorespiration in *Dehalospirillum multivorans* and *Dehalococcoides ethenogenes* strain
323 195 were 3 (*49*) and 4.14 μmol h^{-1} mg $protein^{-1}$ (*11*), respectively. Considering that 10% of
324 the mycelial dry weight of filamentous fungi is protein (*50*), the PCE dechlorination rate (to
325 TCAC) by *T. versicolor* was calculated to be 2.04 to 2.75×10^{-3} μmol h^{-1} mg dry weight of
326 $biomass^{-1}$, which is closer to that of bacteria which degrade PCE co-metabolically but lower
327 than that of dehalorespirers.

328 In this study, we observed that much of the growth in *T. versicolor* cultures occurs
329 in the first three days and much of the oxygen gets depleted in these cultures around this
330 time (data not shown) resulting in lower PCE degradation during the rest of the incubation
331 period. In an earlier study, similar cultures grown in serum bottles with the white-rot
332 fungus *P. chrysosporium* showed 95.4% consumption of available oxygen in 5 day-old
333 cultures (*51*). This indicated the importance of re-oxygenation for potential improvement of
334 the PCE degradation by *T. versicolor*. Consistent with this, cultures that were re-
335 oxygenated after four days of cultivation showed higher level of degradation and chloride
336 release than control cultures (Table 2) but not as much as one would have expected
337 suggesting that there may also be other factor(s) that might be contributing to the limitation
338 of PCE degradation in older cultures.

339 The results of this study open-up an interesting new area for detailed studies on the
340 physiology, biochemistry, and molecular biology of aerobic degradation of PCE by white-
341 rot fungi, a group that has been and is being studied intensively for their versatility in
342 degrading a variety of chlorinated environmental pollutants (*42*). Such studies are
343 particularly important since most of the work to date primarily focused on bacterial PCE
344 degradation by reductive dehalogenation. Given the fact that *T. versicolor* is an ubiquitous
345 fungus worldwide, it would be an interesting model for further studies and it could
346 potentially be an important organism in the future for bioremediation of PCE-contaminated
347 environments.

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TABLE 1. Effect of the cytochrome P-450 inhibitor ABT on PCE degradation by *T. versicolor*[a]

Culture Treatment	PCE in the serum bottle (µmol)	Cl^- generated (µmol)
ABT-free cultures	1.95 ± 0.05	0.8 ± 0.3
Cultures containing 1mM ABT	3.01 ± 0.21	-
Heat-killed controls	2.62 ± 0,23	-

[a]Details regarding conditions of the experiments are found in Materials and Methods. Values represent means ± standard deviations for triplicates.

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TABLE 2. Effect of re-oxygenation on PCE degradation and the release of chloride ions in cultures of *T. versicolor* [a].

Treatment	PCE degraded (µmol)	Cl^- generated (µmol)
Re-oxygenated cultures	1.4 ± 0.08	1.4 ± 0.6
Non-re-oxygenated cultures	1.2 ± 0.03	1.1 ± 0.2

[a]Culture conditions were as described in the legend to Figure 1. Degradation was measured at 2-day intervals and one serum bottle was sacrificed at each time point for analysis. On the 4th day of cultivation, 5 ml of pure oxygen was added in the re-oxygenated bottles. Values presented are means ± standard deviations for three samples analyzed after 8-days of incubation when %PCE degradation for re-oxygenated bottles has reached a plateau. All values are corrected for sorption values obtained with parallel heat-killed controls.

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FIGURE LEGENDS

FIGURE 1. Degradation of PCE by *T. versicolor* [a].

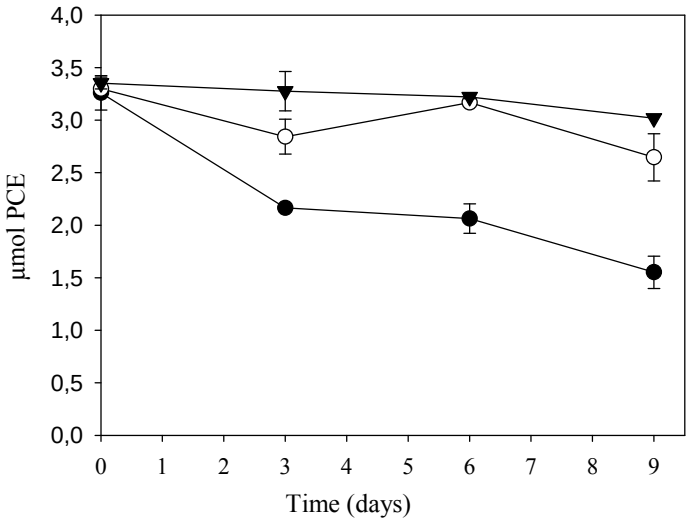
FIGURE 2. Appearance of an intermediate identified primary as chloroform during PCE degradation by *T. versicolor* [b].

FIGURE 3. Mass spectra of chloroform. (A) Mass spectrum of commercially available chloroform. (B-C) Mass spectra of chloroform produced in degradation experiments with non-labelled PCE (B) and [2-^{13}C]PCE (C).

FIGURE 4. NMR spectra for identification of PCE-degradation reaction products. Spectra for heat-killed controls (A) and cultures of *T. versicolor* (B) spiked with 5 mg/L of [2-^{13}C]PCE.

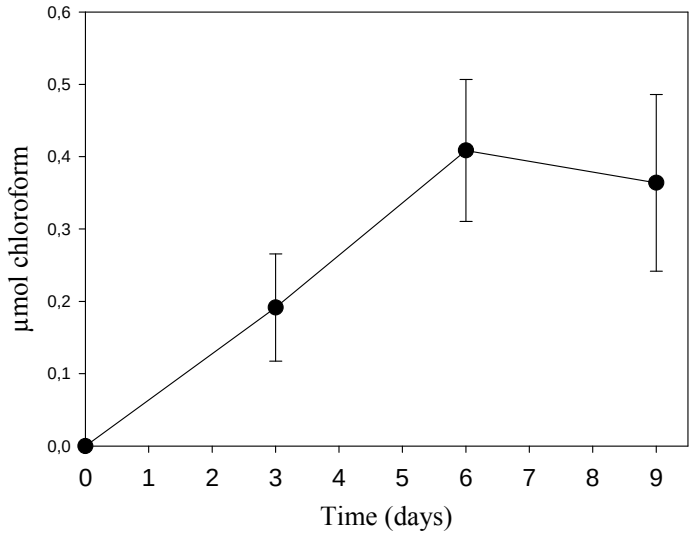
FIGURE 5. Suggested pathway for PCE degradation to Trichloroacetic acid (TCA) by the white rot fungus *T. versicolor*.

FIGURE 1



[a] 10-ml of liquid media and 2 g of wet *T. versicolor* pellet were added in each serum bottle. PCE concentration was 5 mg/L in the liquid media. Cultures were incubated in shaken conditions (135 rpm) at 25ºC in serum bottles sealed with Teflon-coated stoppers. Values plotted are means ± standard deviations for triplicate cultures. Individual bottles were sacrificed at each sampling time. Symbols mean µmol of chloroform (□), and µmols of PCE in uninoculated bottles (▼), in heat-killed controls (○), and experimental bottles (●).

402 **FIGURE 2**



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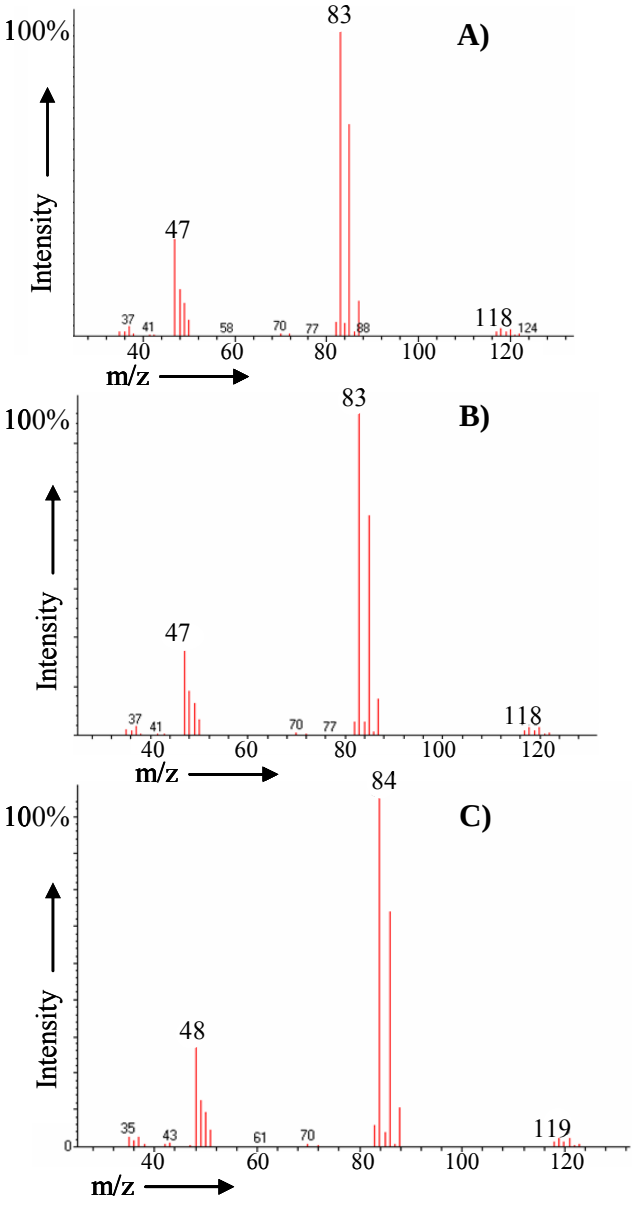
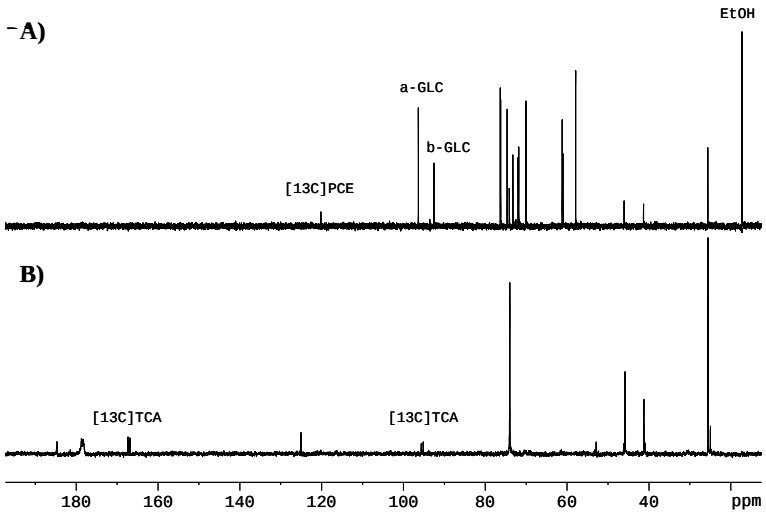
405 [a] Culture conditions are the same than those described in Figure 1.

FIGURE 3



[a] Culture conditions were as described in the legend for Figure 1. Samples were analyzed after 7 days of incubation.

FIGURE 4



[a] Culture conditions were as described in the legend for Figure 1, but [2-^{13}C]PCE was used in this case. Samples were analyzed after 7 days of cultivation.

445 **FIGURE 5**

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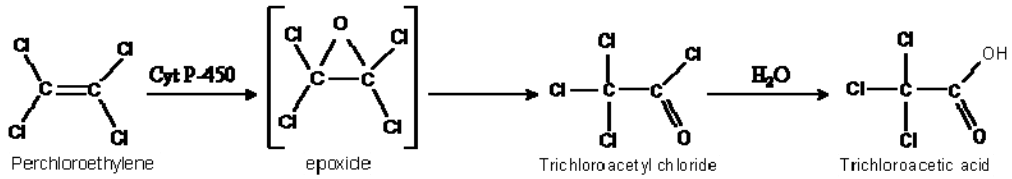

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