

Degradation of Pentachlorophenol by *Mycena avenacea* TA 8480 – Identification of Initial Dechlorinated Metabolites

Stefan Kremer, Olov Sterner*, and Heidrun Anke

Universität Kaiserslautern, LB Biotechnologie, Paul-Ehrlich-Straße 23,
D-W-6750 Kaiserslautern, Bundesrepublik Deutschland

* University of Lund, Department of Organic Chemistry 2, S-221 00 Lund, Sweden

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Cultures of the basidiomycete *Mycena avenacea* TA 8480 were shown to metabolize pentachlorophenol (PCP), 2,3,5,6-tetrachloro-*p*-hydroquinone (TeCHQ), and 2,3,5,6-tetrachloro-*p*-benzoquinone (TeCBQ). The first metabolite of the PCP degradation pathway was identified as TeCBQ which in a second reaction is reduced to the hydroquinone TeCHQ. Subsequently dechlorination of TeCHQ yielded 3,5,6-trichloro-2-hydroxy-*p*-benzoquinone (TCOHBQ). The specific degradation rate for PCP was $1.4 \text{ mg} \times \text{g dry mycelia}^{-1} \times \text{day}^{-1}$. The initial dechlorination rate of TeCHQ was $5.9 \text{ mg} \times \text{g dry mycelia}^{-1} \times \text{hour}^{-1}$. None of the compounds supported growth of the fungus.

Introduction

In recent years bacterial metabolism and degradation of pentachlorophenol (PCP) have been described under laboratory conditions [1–10] and in the environment [11–13]. Pathways for aerobic and anaerobic degradation have been proposed [11, 14–17] and enzymes characterized [18].

The metabolism of PCP by deuteromycetes has been studied to some degree. Especially fungi of the genus *Trichoderma* showed significant degradation of sodium pentachlorophenate [19] or metabolization to pentachloroanisole [20]. Although basidiomycetes are known to produce chlorinated aromatic secondary metabolites [21–25] no information is available regarding degradation of these natural compounds by the producing organism and only few studies were carried out on the degradation of PCP. Most of these dealt with the degradation of PCP by the white rot fungi *Phanerochaete chrysosporium* Burds. in nitrogen-limited cultures and in soil [26–28]. Lignin peroxidases and manganese peroxidases were found to catalyze the initial *para*-dechlorination of PCP to TeCBQ [27, 29]. The release of $^{14}\text{CO}_2$ from labelled PCP indicated a complete degradation [27, 30].

In the present paper the degradation of PCP by a non white rot basidiomycete, *Mycena avenacea*,

will be described together with the characterization of the initial metabolites in the degradation pathway.

Experimental

Chemicals

Pentachlorophenol (PCP) and 2,3,5,6-tetrachloro-*p*-hydroquinone (TeCHQ) were obtained from Riedel-de Haen AG, Seelze, Germany. 2,3,5,6-Tetrachlorobenzoquinone (TeCBQ) was purchased from Sigma Chemicals. Acetonitrile for HPLC analysis was obtained from Zinsser Analytik, Frankfurt, Germany.

Organism

Mycelial cultures of *Mycena avenacea* (Fries), sensu Schroeter TA 8480 were obtained from spore prints from specimens collected in Wallhalben, Germany. *M. avenacea* is a small agaric with a yellow to ochre hat up to 20 mm in diameter. The lamellae are greyish and their edges slightly brown. The white spores measure $10\text{--}11 \times 5.5 \text{ }\mu\text{m}$.

Cultivation of *M. avenacea*

The strain was maintained on YMG agar composed of (g/l): yeast extract (4), malt extract (10), glucose (4), and stored at 4°C . Subcultures were made every 12 month.

Cultures used for inoculation were grown on YMG agar at 21°C for three weeks. Ten agar plugs (4 mm in diameter) were used to inoculate

Reprint requests to H. Anke.

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250 ml medium in 1000 ml Erlenmeyer flasks. Incubations were carried out on a rotary shaker at 21 °C in the dark. Each culture was incubated for two weeks before PCP, TCHQ or TCBQ were added aseptically.

Determination of metabolites

For the extraction, isolation and identification of PCP and its metabolites 10 ml samples were withdrawn. The mycelia were extracted twice with methanol in a ultrasonic bath. The culture filtrate was extracted with three equal volumes of ethyl acetate. In order to obtain more hydrophilic metabolites the culture broth was extracted with three equal volumes of butanol. The solvent was evaporated under reduced pressure at 40 °C. The residue was dissolved in 1 ml methanol and analyzed by HPLC.

High performance liquid chromatography (HPLC) analysis was carried out on a Merck-Hitachi liquid chromatograph L-6200 using a RP 18 Lichrosphere column (inner dimensions 125 × 4.6 mm, particle size 5 µm). The metabolites were separated by using a 20 min linear gradient of 0.1% phosphoric acid–acetonitrile (95:5 to 0:100) at a solvent flow rate of 1 ml/min. UV detection operated with dual channel analysis at 210 and 280 nm with a Merck-Hitachi photodiode array detector.

Isolation of 3,5,6-trichloro-2-hydroxybenzoquinone

After 2 h of incubation the mycelium and culture broth were separated. The culture filtrate was extracted with butanol and the extract was concentrated to dryness. The residue was washed four times with toluene to remove lipophilic compounds. The toluene was discarded. The crude extract was dissolved in MeOH and applied onto Sephadex LH 20 column (400 mm × 24 mm). Final separation was achieved by HPLC on a RP 18 semipreparative column (inner dimensions 250 mm × 10 mm, particle size 10 µm) using a 30 min linear gradient of H₂O–MeOH (90:10 to 0:100).

Mass spectrometry

The high resolution mass spectrum was recorded with a Jeol SX 102 mass spectrometer. Operating conditions were as follow: emission current,

70 eV; electron multiplier voltage, 1000 V; ionization chamber temperature was 250 °C.

Results

During the screening of basidiomycetes for degradation of the fungicide PCP, *Mycena avenacea* TA 8480 was found to tolerate 20 µg/ml of PCP. In following experiments the minimal inhibitory concentration (MIC) of PCP towards strain TA 8480 was determined to 150 µg/ml in YMG medium. PCP was degraded but could not support growth of the fungus as a sole carbon source.

The disappearance of pentachlorophenol in cultures of *M. avenacea* is shown in Fig. 1. The strain was preincubated 14 days in YMG medium at 21 °C before 100 mg PCP/l were added. At this stage of the fermentation glucose had been completely consumed. After 9 days the PCP concentration had decreased from 100 mg/l to 11.5 mg/l. Even at prolonged incubation no further decrease in the PCP concentration could be observed. Upon incubation of dead mycelia (boiled for 10 min) with PCP only a slight reduction (<10%) of the PCP concentration was observed. The same results were obtained when uninoculated medium was incubated with PCP. This decrease of PCP may be due to absorption to glass ware or to stripping. No metabolites could be found in these control experiments.

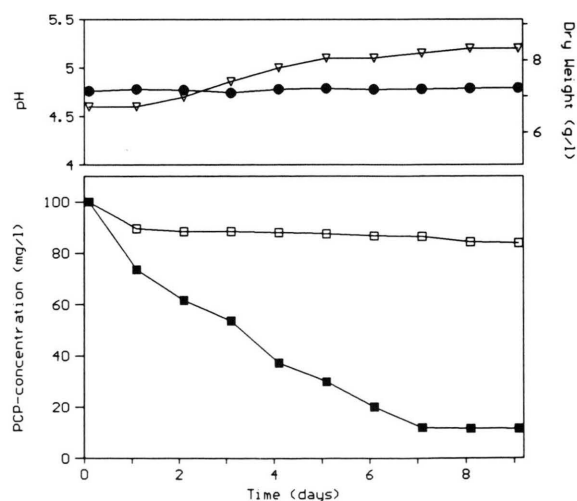


Fig. 1. Degradation of pentachlorophenol by *Mycena avenacea* TA 8480 in YMG medium. ■—■, PCP concentration; □—□, PCP concentration in control culture; ▽—▽, pH; ●—● mycelial dry weight.

During incubation with PCP no increase of mycelial weight could be observed. This is in accordance with the fact, that PCP did not support growth.

In order to identify degradation metabolites from culture broth and mycelia, extracts were separated by HPLC and newly formed compounds were monitored by UV absorption at 210 and 280 nm. Figs. 2a and 2b show HPLC elution profiles of PCP and its metabolites obtained from the culture filtrate and mycelium after two days of incubation. Among the six major peaks shown in Fig. 2a, three have been identified as dechlorination products of PCP, namely 2,3,5,6-tetrachloro-hydroquinone (TeCHQ), 2,3,5,6-tetrachlorobenzoquinone (TeCBQ) and 3,5,6-trichloro-2-hydroxybenzoquinone (TCOHBQ). The remaining compounds (FM1, 3–6), with the exception of FM2 (retention time 13.9 min) are fungal metabolites which were also found in cultures of *M. avenacea* without PCP added. The metabolites were identified by their retention times and UV spectra as well as with the help of authentic standards (TeCHQ and TeCBQ). During the whole incubation period two thirds of the remaining PCP were found in the filtrate and one third was extracted from the mycelium. The degradation rate

of PCP was $1.4 \text{ mg} \cdot \text{g}^{-1} \cdot \text{day}^{-1}$. TeCHQ and TeCBQ were both located in the culture filtrate.

When TeCHQ or TeCBQ were added to pre-grown cultures of *M. avenacea*, the formation of TCOHBQ was observed. In Fig. 3a, a rapid disappearance of TeCHQ and the formation of TeCBQ and TCOHBQ can be seen. The degradation of TeCBQ is much slower as shown in Fig. 3b. Within the first two hours, TeCHQ is almost the sole reaction product. Then, concurrently with its disappearance TCOHBQ is formed. This strongly supports the reaction sequence given in Fig. 5. The specific initial dechlorination rate of TeCHQ to TCOHBQ was $5.9 \text{ mg} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$. Compared to PCP, the dechlorination of TeCHQ is 150-fold faster. The experiments were carried out with 7 g mycelial dry weight/l. 93.8% ($\pm 2.0\%$) of the TeCHQ was found in the culture filtrate, the remainder was extracted from the biomass. TeCBQ and TCOHBQ were exclusively found in culture fluid. When boiled mycelia (10 min, 100°C) were incubated with TeCHQ or TeCBQ no dechlorination occurred.

TCOHBQ was isolated and identified from cultures with TeCHQ added. The mass spectrum of the dark-purple compound is given in Fig. 4. The mass of the high resolution mass spectrum

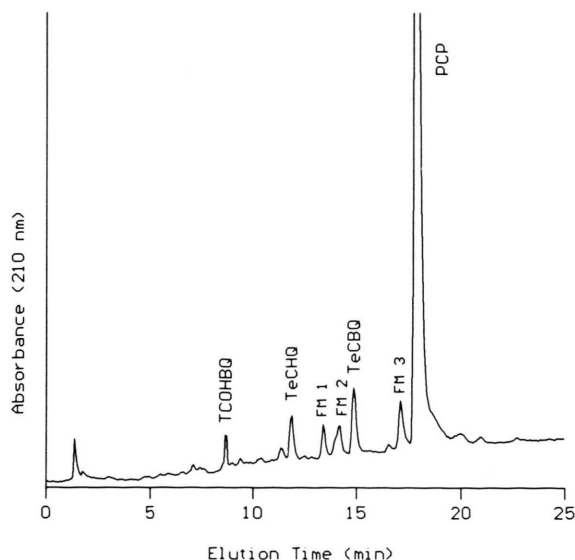


Fig. 2a. HPLC elution profile from culture filtrate extracts after two days of incubation with 100 mg PCP/l . FM: unknown fungal metabolites.

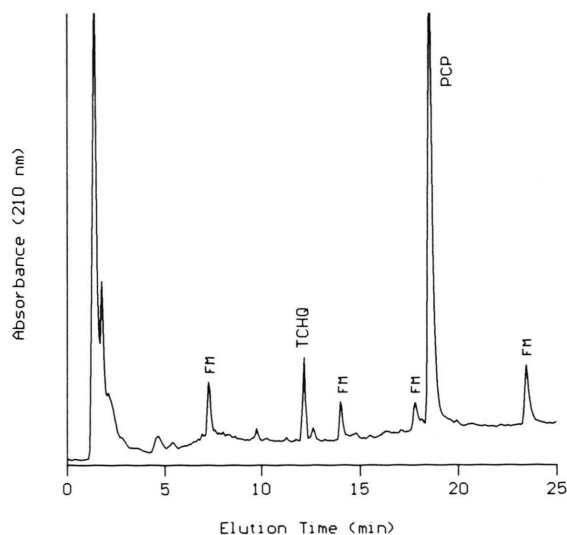


Fig. 2b. HPLC elution profile from mycelial extracts after two days of incubation with 100 mg PCP/l . FM: unknown fungal metabolites.

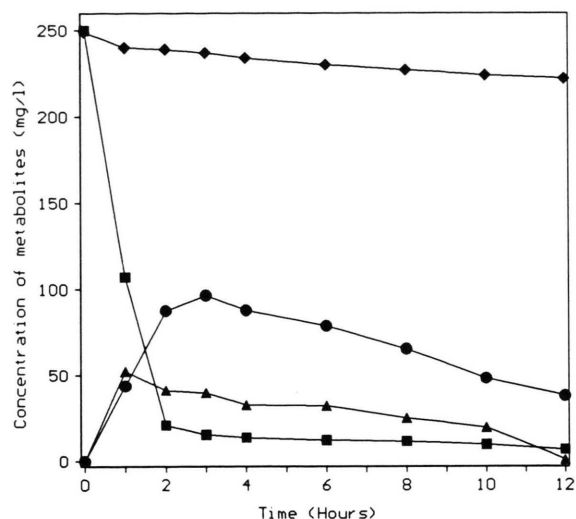


Fig. 3a. Metabolism of 2,3,5,6-tetrachlorohydroquinone by *Mycena avenacea*. The culture was grown in YMG medium for two weeks before the xenobiotic was added. ■—■, 2,3,5,6-tetrachlorohydroquinone; ▲—▲, 2,3,5,6-tetrachlorobenzoquinone; ●—●, 3,5,6-trichloro-2-hydroxy-benzoquinone; ◆—◆, control culture.

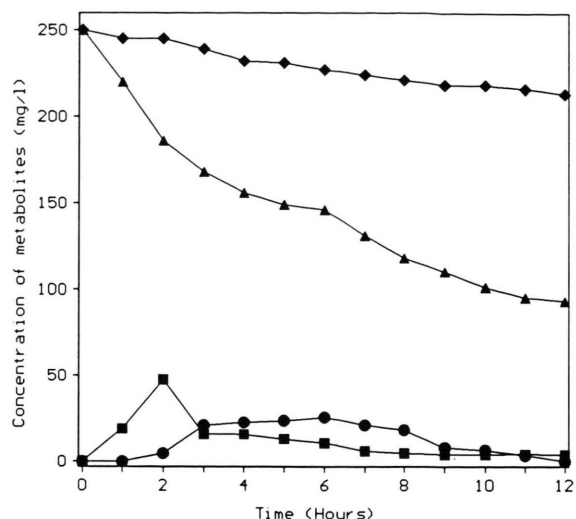


Fig. 3b. Metabolism of 2,3,5,6-tetrachlorobenzoquinone by *Mycena avenacea*. The culture was grown in YMG medium for two weeks before the xenobiotic was added. ■—■, 2,3,5,6-tetrachlorohydroquinone; ▲—▲, 2,3,5,6-tetrachlorobenzoquinone; ●—●, 3,5,6-trichloro-2-hydroxy-benzoquinone; ◆—◆, control culture.

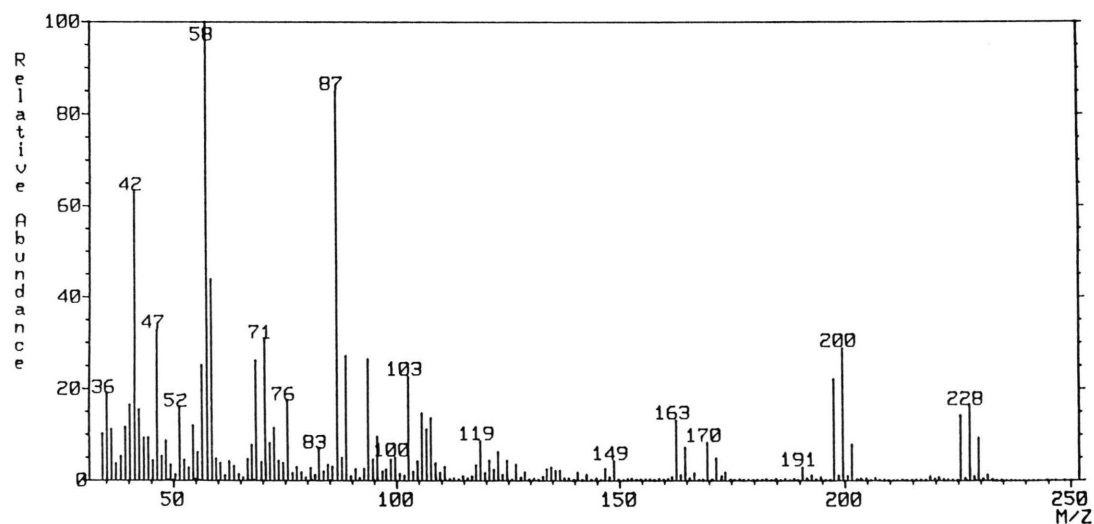


Fig. 4. Mass spectrum of 3,5,6-trichloro-2-hydroxybenzoquinone.

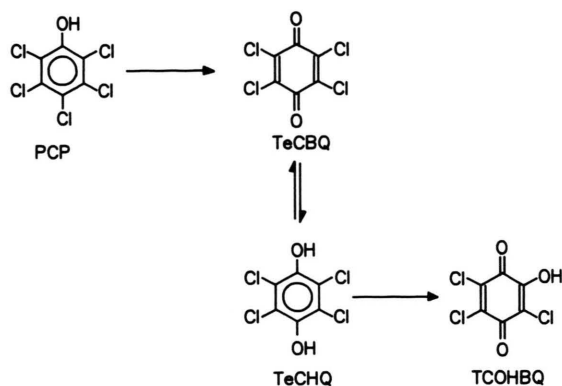


Fig. 5. Proposed initial pathway of PCP degradation by *Mycena avenacea* TA 8480.

(225.8994) is consistent with the formula $C_6HO_3Cl_3$. TCOHBQ is easily soluble in methanol, acetone and butanol, poorly soluble in ethyl acetate and insoluble in toluene and cyclohexane. The substance is stable in methanol at 4 °C for more than 8 weeks.

Incubation of TeCHQ with mycelia-free culture broth from two week old cultures of *M. avenacea* resulted also in the formation of TCOHBQ. The conversion of TeCBQ was neglectable (<2%). Heating to 100 °C for 10 min destroyed this dechlorination activity. Dechlorination of PCP could not be found under these conditions.

Discussion

This is the first report on the dechlorination of PCP by a non white rot basidiomycete. *Mycena avenacea* is a saprophytic fungus which grows on decaying wood and plant materials. *M. avenacea* tolerates PCP concentrations up to 150 mg/l, without being able to use it as a sole carbon source. *P. chrysosporium* and *P. sordida* have been reported to grow in soil containing 400 mg PCP/kg [30]. *Cephaloscyus fragrans* strains could be adapted to concentrations of 280 mg/l [19]. Tolerance towards PCP however is not too common among basidiomycetes since during the screening of 200 strains representing 45 genera less than 10% of the strains were found to grow in the presence of 20 mg PCP per liter.

So far, we have identified three dechlorination products of PCP in cultures of *M. avenacea*. Bio-

transformation of PCP to pentachloroanisole and TeCHQ to tetrachloro-4-methoxyphenol and tetrachloro-1,4-dimethoxybenzene was not observed. O-Methylation of PCP and TeCHQ has been found in PCP-degrading cultures of *Rhodococcus* sp. and *Mycobacterium* sp. [31]. Mono-, di-, tri- and tetrachlorophenols, generated by reductive dechlorination in bacteria [32, 33], were not found either.

The first step in all bacterial degradation processes of PCP is the hydrolytic *para*-hydroxylation yielding TeCHQ [14, 16–18, 33]. Dechlorination of PCP by extracellular lignin peroxidase from *P. chrysosporium* however, yielded TeCBQ [27, 29]. In a similar reaction 2,4-dichlorophenol was dechlorinated to 2-chlorobenzoquinone, which after reduction to the hydroquinone was completely dechlorinated by manganese and lignin peroxidases from *P. chrysosporium* [34]. Likewise, in cultures of *M. avenacea* the benzoquinones and not the hydroquinones are the reaction products of the oxidative dechlorination steps. Two days after the addition of PCP, TeCBQ and TeCHQ were found in the culture filtrate of *M. avenacea*. In addition TeCHQ was found in the mycelium. In the next dechlorination step TeCHQ is converted to TCOHBQ as can be deduced from Fig. 3a and 3b. The conversion of TeCHQ to TCOHBQ is ten times faster than the conversion of TeCBQ. In addition, formation of TCOHBQ from TeCBQ is only observed after the reduction to TeCHQ (Fig. 3b).

Among the not yet identified fungal metabolites (FM 1–6) present in the HPLC diagrams (Fig. 2a and 2b) FM2 seems to be a degradation product. It is currently being isolated.

TCOHBQ has not yet been identified as a dechlorination product of PCP. The corresponding hydroquinone has been postulated in a degradation scheme of TeCHQ by *Rhodococcus* sp. CP-2 [32].

Some of the dechlorination activity could be detected in the culture broth. Incubation of mycelia-free culture filtrate from a two week old culture with TeCHQ gave rise to TCOHBQ, whereas the transformation of TeCBQ was very low *e.g.* less than 2% compared to 22% in the case of TeCHQ. PCP was not dechlorinated under these conditions. Whether this enzymatic conversion in the culture filtrate is due to an exoenzyme or due to

lysis of mycelia in the idiophase has not yet been investigated as well as the nature of the enzymes involved.

In summary the initial steps of the degradation scheme of PCP by *M. avenacea* resulting from our results are shown in Fig. 5. PCP by oxidative dechlorination is slowly converted to TeCBQ, which after reduction to the hydroquinone, is rapidly

converted in a second oxidative dechlorination step to TCOHBQ. The elucidation of the further degradation sequence is in progress.

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