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Transformation and mineralization of halophenols by *Penicillium simplicissimum* SK9117*

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Abstract

The metabolism of monohalophenols by *Penicillium simplicissimum* SK9117, isolated from a sewage plant was investigated. In submerged cultures, 3-, 4-chlorophenol, and 4-bromophenol were metabolized in the presence of phenol. 3-Chlorophenol was transformed to chlorohydroquinone, 4-chlorocatechol, 4-chloro-1,2,3-trihydroxybenzene, and 5-chloro-1,2,3-trihydroxybenzene. With 4-chlorophenol only 4-chlorocatechol was observed as transient product. A release of chloride ions was not observed. Whereas monobromo-, and monochlorophenols could not support growth as sole carbon and energy source, growth and release of fluoride ions were observed with monofluorophenols as substrates. In presence of phenol, the degradation of all monofluorophenols was enhanced. Substrate and cosubstrate disappeared simultaneously. 3-Fluorophenol and 4-fluorophenol were completely mineralized as shown by the equimolar release of fluoride ions.

Introduction

Phenol and halogenated phenols are a group of widespread pollutants in water and soil. They arise as wastes from the chemical, pharmaceutical, or petrochemical industries, and chlorophenols are used as disinfectans and fungicides.

Contrary to aerobic degradation of phenol and halophenols by bacteria, informations about the degradation of these compounds by fungi are scarce. Nevertheless the few investigations show, that yeasts (Hofmann & Schauer 1988) and also mycelial fungi (Anselmo & Novais 1984; Anselmo et al. 1985; Hofrichter & Scheibner 1993; Scow et al. 1990) are able to use phenol as sole carbon and energy source.

Phenol grown cells of the yeast *Rhodotorula glutinis* were able to oxidize 3-, and 4-chlorophenol and 4-bromophenol to 4-chlorocatechol and 4-bromocatechol, respectively (Walker 1973). *Candida maltosa* converted 2-chlorophenol to 3-chlorocatechol and *cis,cis*-2-chloromuconic acid. With 3-, and 4-chlorophenol, 3-chlorocatechol, 4-chlorocatechol, 5-chloro-1,2,3-trihydroxybenzene, and 4-carboxymethylenebut-2-en-4-olide were detected (Polnisch et al. 1992). Phenol induced cells of the yeast *Candida tropicalis* metabolized 3-, and 4-chlorophenol (Krug et al. 1985). In the presence of glucose or phenol, *Penicillium frequentans* metabolized monochloro-, and monofluorophenols. During the metabolism of *meta-* or *para-* substituted chloro-, and fluorophenols the corresponding catechols and 4-carboxymethylenebut-2-en-4-olide appeared as intermediary metabolites, and chloride and fluoride ions were released into the medium (Hofrichter et al. 1992, 1993, 1994). Neujahr and Varga (1970) observed an oxidation of all monofluorophenols by phenol or resorcinol adapted cells of *Trichosporon cutaneum.*

These investigations show that yeasts and fungi have the capabilities to transform, or mineralize cometabolically some monohalogenated phenols but none of the organisms could utilize these compounds as growth substrates.

In this study we investigated the mineralization of monofluorophenols and the transformation

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of monobromo-, and monochlorophenols by the deuteromycete *Penicillium simplicissimum* SK9117 which was selected because of its growth on phenol (8.5 mM) as sole carbon and energy source. The pathway by which phenol is mineralized was elucidated and metabolites formed from monohalophenols were isolated and characterized, among them chlorohydroquinone and 4-chloro-1,2,3-trihydroxybenzene which never before have been isolated from fungal cultures.

Material and methods

Organism and culture conditions

The fungus used in this study was isolated from a sample of the sewage plant of the city of Kaiserslautern (Germany) and was determined as *Penicillium simplicissimum* SK9117. On malt extract agar plates at 25°C, the strain formed rapidly growing green and powdery colonies due to the production of conidia. At 37°C, growth was reduced. The rough walled conidiophores (400–450 μm x 2,5–3 μm) bearing regular verticils of 3 metulae (12–15 μm x 2,5–3 μm) are characteristic for the species (Pitt 1979). Each metula carried at least four flask-shaped phialides (8 μm x 2 μm) which produced long chains of elliptical–subglobose conidia (3 μm x 2–2,5 μm).

Stock cultures of the strain were maintained on YMG agar containing (in grams per liter): glucose, 4; malt extract, 10; yeast extract, 4 and agar 20. The pH was adjusted to 5.6 before sterilization.

Culture conditions for cometabolism and mineralization of monohalophenols

The mineralization of monohalophenols was investigated in submerged cultures in 500 ml flasks with 200 ml of mineral salt medium MM1 containing (in grams per liter): $(NH_4)_2SO_4$, 1; $CaCl_2$ x 2 H_2O, 0.1; NaCl, 0.1; $MgSO_4$ x 7 H_2O, 0.5; K_2HPO_4, 0.125; KH_2PO_4, 0.875 and 10 ml/l trace element solution (Wedding & Kentrick 1959). In the case of monofluorophenols 200 ml of modified mineral salt medium MM2 (Kaufman et al. 1973) was used. The media contained 0.5 mM of the appropriate halogenated compound as sole carbon and energy source. Under cometabolic conditions 1 mM of phenol was added. In all media the pH was adjusted to 5.6. Monohalophenols and phenol were added aseptically after sterilization (121°C, 20 min). Five inoculation plugs (4 mm diameter) of one week old cultures on mineral salt agar (2% agar), containing the same phenolic compounds as the submerged cultures, were used. The cultures were incubated at 25°C and 120 rpm.

Chemical and physical analyses

A Merck-Hitachi high pressure liquid chromatograph (HPLC) equipped with a Merck-Hitachi photodiode array detector operated at 210/280 nm and fitted with a Merck-LiChrospher 100RP-8 end-capped column (125 mm by 4 mm [inside diameter], E. Merck, Darmstadt, Germany) was used to determine the concentration of monochloro-, monobromophenols, phenol, and metabolites in the culture filtrates. For the separation a gradient of phosphoric acid (0.1%) : acetonitrile [vol/vol] was used: 0–7,5min: 80:20 to 65:35; 7,5–8,5 min: 65:35 to 0:100. Phenol and monofluorophenols, respectively, were eluted with the following phosphoric acid (0.1%) : acetonitrile [vol/vol] gradient: 0–5 min: 98:2 to 95:5; 5–10 min: 95:5 to 0:100. The flow rates were 2 ml/min.

Chloride and fluoride concentration in the culture fluids were determined with the Spektroquant tests (E. Merck) in a UV-VIS spectrometer (model PU8800 Philips).

For the isolation of metabolites, mycelia and culture broth were separated. The culture filtrate was extracted with ethyl acetate and the extract was concentrated to dryness. For the separation of metabolites the Merck-Hitachi HPLC was equipped with a Merck-LiChrospher 100RP-18 column (250 mm by 10 mm [inside diameter]. Separation was performed using two different isocratic methods at a flow rate of 4 ml/min: water : methanol 77:23 [vol/vol] (method 1) and water : methanol 55:45 [vol/vol] (method 2), respectively.

Mass spectral analyses were performed with a JEOL SX-102 mass spectrometer. The spectra were recorded with electron impact ionization at 70 eV. The ionization chamber temperature was 250°C, and the electron multiplier voltage was 1.000 V. The ^1H nuclear magnetic resonance (NMR) measurements were carried out at 500 MHz in chloroform-d_1 with a Bruker ARX500 spectrometer, with the solvent signal (at 7.26 ppm) as reference.

Chemicals

Monohalophenols and 2-chloro-1,4-benzoquinone were purchased from Aldrich-Chemie, Steinheim,

Germany. Phenol was obtained from E. Merck. These and all other chemicals used were of highest purity available. Chlorohydroquinone was prepared by reduction of 2-chloro-1,4-benzoquinone with sodium dithionite.

High pressure liquid chromatography-grade solvent acetonitrile was obtained from Zinsser Analytik GmbH, Frankfurt, Germany and methanol from Riedel-de-Haën AG, Seelze, Germany.

Results

Mineralization of phenol

Penicillium simplicissimum SK9117 was able to mineralize phenol in submerged cultures up to 8.5 mM. During the degradation catechol, hydroquinone, and *cis,cis*-muconic acid were identified as intermediary metabolites. With decreasing pH citric acid, glutaric acid, and succinic acid were detected in the culture filtrates. The metabolites identified show that phenol is mineralized via the β-ketoadipate pathway as previously described by Gaal & Neujahr (1979).

Transformation and mineralization of monohalophenols

Penicillium simplicissimum SK9117 transformed 3-, and 4-chlorophenol in presence of phenol. As shown in Fig. 1A 83% of 3-chlorophenol were converted within 22 days. After phenol was used up, the 3-chlorophenol concentration decreased only slowly and stagnated after 22 days. With the disappearence of 3-chlorophenol a metabolite accumulated in the culture medium and later was identified as 4-chlorocatechol. After 26 days 0.19 mM of 4-chlorocatechol were detected in the culture broth. This corresponds to 38% metabolization. 4-Chlorophenol was completely cometabolized within 8 days (Fig. 1B). Substrate and cosubstrate were simultaneously metabolized. 4-Chlorocatechol accumulated only transiently in the culture medium on day 8 of incubation. During the cometabolism of 3-, and 4-chlorophenol no chloride ions were released into the culture medium. 2-Chlorophenol was not metabolized under these conditions, although phenol was mineralized within 5 days (data not shown).

P. simplicissimum SK9117 could not utilize monochlorophenols and monobromophenols as sole carbon and energy source. Neither fungal growth nor a change of the initial monohalophenol concentration in comparison with the controls were detectable. Under cometabolic conditions, 4-bromophenol was the only bromophenol metabolized. After 22 days of incubation, phenol was no longer detectable in the culture medium. 90% of 4-bromophenol were transformed within 28 days (data not shown). The release of bromide ions was not investigated. In the ethyl acetate extract of the culture filtrate a metabolite with a catecholic UV-spectrum (elution time 4.67 min) was detected. The isolation of this metabolite is still in progress. A cometabolic transformation of 2-, and 3-bromophenol was not observed. In the presence of 2-bromophenol the phenol was used up within 15 days whereas 3-bromophenol inhibited the utilization of phenol and thus the growth of the fungus.

In experiments with 2-, 3-, or 4-fluorophenol growth of *P. simplicissimum* SK9117 was observed even in the absence of phenol. After 22 days 2-

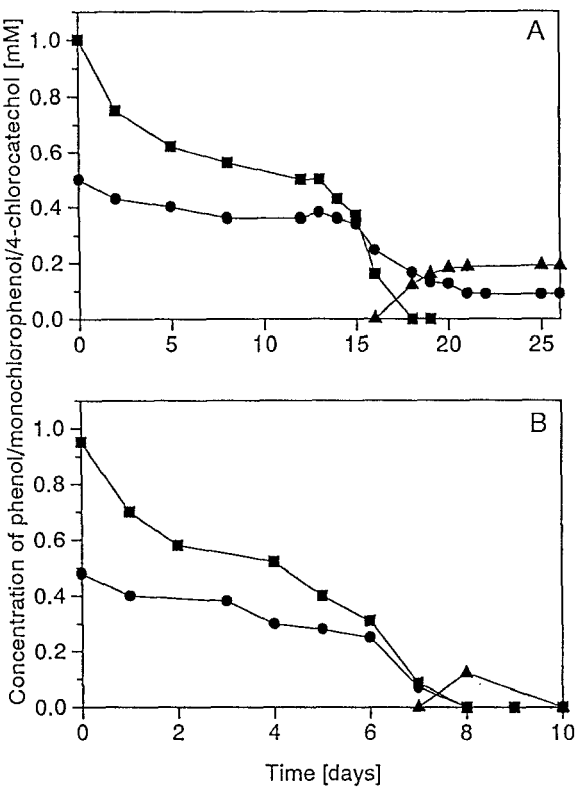

Fig. 1. Disappearance of 3-chlorophenol (A), 4-chlorophenol (B) and formation of 4-chlorocatechol in the presence of phenol as substrate in cultures of *Penicillium simplicissimum* SK9117, incubated in MM1 at 25°C and 120 rpm. Inoculum: 5 cylindrical plugs (4 mm diameter) of one week old precultures on MM1 (2% agar) containing 0.5 mM of 3- or 4-chlorophenol, respectively and 1 mM of phenol. Symbols: ■ phenol; ●, monochlorophenols; ▲, 4-chlorocatechol.

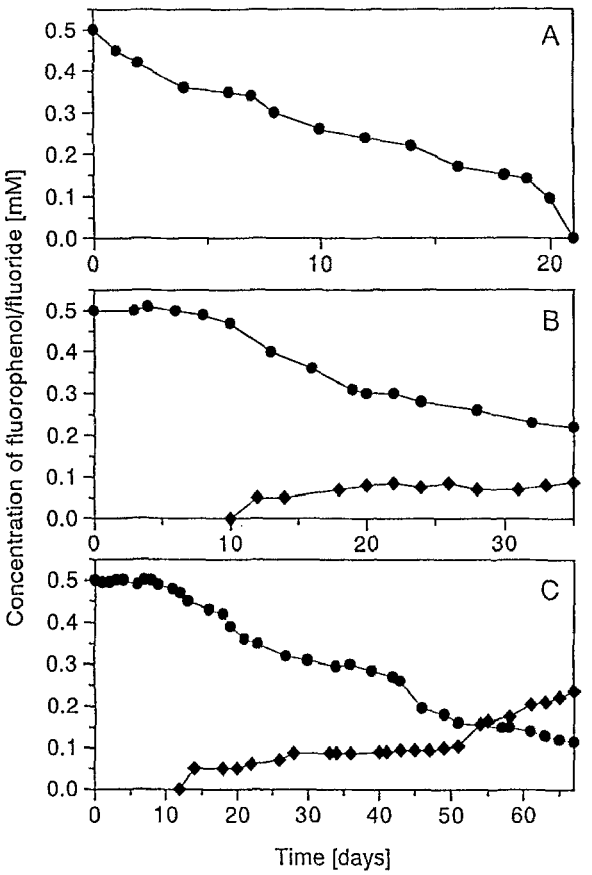

Fig. 2. Decrease of 2-fluorophenol (A), 3-fluorophenol (B), 4-fluorophenol (C) and release of fluoride ions in cultures of *Penicillium simplicissimum* SK9117, incubated in MM2 at 25°C and 120 rpm. Inoculum: 5 cylindrical plugs (4 mm diameter) of one week old precultures on MM2 (2% agar) containing 0.5 mM of the appropriate monofluorophenol. Symbols: ●, monofluorophenols; ♦, fluoride.

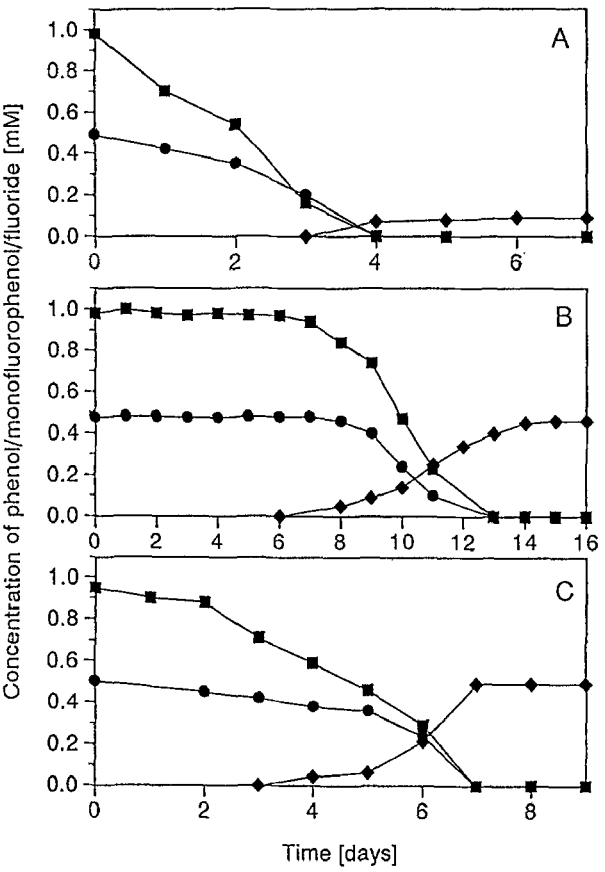

Fig. 3. Degradation of 2-fluorophenol (A), 3-fluorophenol (B) and 4-fluorophenol (C) in the presence of phenol as substrate and release of fluoride ions in cultures of *Penicillium simplicissimum* SK9117, incubated in MM2 at 25°C and 120 rpm. Inoculum: 5 cylindrical plugs (4 mm diameter) of one week old precultures on MM2 (2% agar) containing 0.5 mM of the appropriate monofluorophenol and 1 mM of phenol. Symbols: ■, phenol; ●, monofluorophenols; ♦, fluoride.

fluorophenol disappeared completely in the culture medium (Fig. 2A), but no release of fluoride ions was detected. A metabolite with a UV-spectrum similar to catechol accumulated in the culture medium and is currently being isolated. Upon incubation of the fungus with 0.5 mM 3-fluorophenol the concentration decreased slowly to 0.22 mM within 35 days and a release of fluoride ions into the culture medium to a final concentration of 0.09 mM was measured (Fig. 2B). As shown in Fig. 2C, the turnover of 4-fluorophenol by *P. simplicissimum* SK9117 was similar to that of 3-fluorophenol. Within 65 days the 4-fluorophenol concentration decreased to 0.12 mM and the fluoride concentration increased to 0.24 mM, i.e. 48% of the fluorine were released into the culture medium. It was possible to subcultivate the cultures with each monofluorophenol or glucose, respectively, on plate as well as in submerged cultures.

Under cometabolic conditions the turnover of all monofluorophenols was enhanced. Monofluorophenols and phenol were always simultaneously metabolized (Fig. 3A, B, C). After incubation periods of 4 days (2-fluorophenol), 13 days (3-fluorophenol) and 7 days (4-fluorophenol), respectively, the compounds were no longer detectable in the culture medium. In cultures with 2-fluorophenol the release of fluoride ions was incomplete, and reached a final concentration of 0.09 mM after 6 days (Fig. 3A). The same metabolite as in the mineralization experiments was detected. In the case of 3-, and 4-fluorophenol the disappearence of the monofluorophenols was accompanied with an equimolar release of fluoride ions (Fig. 3B, C).

Table 1. Electron impact mass spectral data for the metabolites obtained from the conversion of 3-chlorophenol by *Penicillium simplicissimum* SK9117.

Metabolites	MS (m/z) (% relative intensity)
4-Chlorocatechol	146 (32), 144 (100) [M^+], 126 (6), 115 (7), 98 (20), 81 (11), 79 (5), 63 (25), 51 (15)
4-Chloro-1,2,3-trihydroxy-benzene	162 (32), 160 (100) [M^+], 142 (10), 106 (20), 79 (18), 53 (12)
5-Chloro-1,2,3-trihydroxy-benzene	162 (32), 160 (100) [M^+], 131 (9), 114 (27), 86 (17), 79 (23), 51 (11)

Table 2. ^{1}H-Nuclear magnetic resonance spectral data (500 MHz) of metabolites formed from 3-chlorophenol by *Penicillium simplicissimum* SK9117. The spectra were recorded in chloroform-d_1 with the solvent signal (at 7.26 ppm) as reference. The coupling constants (J) are given in Hz.

Position	δ_H: multiplicity; J		
	4-Chlorocatechol	4-Chloro-1,2,3-trihydroxy-benzene	5-Chloro-1,2,3-trihydroxy-benzene
3-H	6.71; d; $J_{3,5}$=2.4	–	–
4-H	–	–	6.31; s
5-H	6.58; dd; $J_{3,5}$=2.4, $J_{5,6}$=8.5	6.58[a]; d; $J_{5,6}$=8.9	–
6-H	6.63; d; $J_{5,6}$=8.5	6.27[a]; d; $J_{5,6}$=8.9	6.31; s

Isolation and identification of metabolites

For the isolation of 4-chlorocatechol a 2 l culture of *P. simplicissimum* containing 0.5 mM 3-chlorophenol and 1 mM phenol, was incubated under the same conditions as described. The course of the phenol and 3-chlorophenol decrease and the accumulation of 4-chlorocatechol was nearly identical to that shown in Fig. 1A. A complete turnover of 3-chlorophenol was achieved by a second addition of 1 mM phenol and an increase of the 4-chlorocatechol concentration was observed. 4-Chlorocatechol was isolated from the ethyl acetate extract of the culture filtrate by preparative HPLC using isocratic method 2. The elution time for 4-chlorocatechol was 12.06 min. Three additional metabolites with elution times of 11.10, 12.58 and 16.08 min, respectively, were isolated using isocratic method 1. The metabolite at 11.10 min was identified as chlorohydroquinone by comparison of the UV-spectral characteristics and the HPLC retention time with an authentic standard.

The determination of the structures of the other three metabolites was carried out with mass spectroscopy and ^{1}H NMR spectrometry. The MS data are shown in Table 1, and the NMR data in Table 2.

The molecular ion of 4-chlorophenol in the EI mass spectrum shows the typical isotope pattern for a compound containing one chloro atom (M^+ +2 is approximately one third of M^+), and the exact mass of M^+ obtained by high resolution measurements (243.9963) is in agreement with the elemental composition $C_6H_5O_2{}^{35}Cl$ (requires 243.9978). The ^{1}H NMR data are typical for a 1,2,4-trisubstituted benzene, and as the product is obtained from both 3- and 4-chlorophenol it must be 4-chlorocatechol.

The mass spectra of the metabolites eluting at 12.58 and 16.08 min both contained a molecular ion at m/z 160, corresponding to the elemental composition $C_6H_5O_3{}^{35}Cl$ according to high resolution measurements, but show different fragmentation patterns (Table 1). The metabolite eluting at 12.58 min was determined as 4-chloro-1,2,3-trihydroxybenzene by NMR-, and UV-spectral data. Because the NMR data could not distinguish between 4-chloro-1,2,3-trihydroxybenzene and 3-chloro-1,2,4-trihydroxybenzene, determination of structure was achieved by comparison of UV-spectra. The metabolite, 1,2,3-trihydroxybenzene, and 5-chloro-1,2,3-trihydroxybenzene exhibit nearly identical UV-spectra, lacking a second maximum at 290 nm, which is char-

acteristic for 1,2,4-trihydroxy-substitution. The second metabolite eluting at 16.08 min was identified by NMR data as 5-chloro-1,2,3-trihydroxybenzene. Because of symmetry, the compound exhibited only a single proton resonance at 6.31 ppm (Table 2).

Discussion

The filamentous fungus *Penicillium simplicissimum* SK9117 is capable to utilize phenol and fluorinated phenols as sole carbon and energy source. The assimilation of various non halogenated phenolic and aromatic compounds including phenol has also been described for other strains of *Penicillium simplicissimum* (Jong et al. 1990; Patel et al. 1990). Monobromo-, and monochlorophenols could not support growth of strain SK9117. This is in accord with investigations obtained for the yeasts *Candida maltosa* (Polnisch et al. 1992), *Candida tropicalis* (Krug et al. 1985), *Rhodotorula glutinis* (Walker 1973), *Trichosporon cutaneum* (Hasegawa et al. 1990) and the deuteromycete *Penicillium frequentans* (Hofrichter et al. 1992; 1993; 1994) all of which could not utilize monohalophenols as growth substrates.

In presence of phenol, 2-chlorophenol was also not degraded by *P. simplicissimum* SK9117, or *C. tropicalis* (Krug et al. 1985). In contrast to these results, the compound was transformed to 3-chlorocatechol, and *cis,cis*-2-chloromuconic acid by phenol grown cells of *C. maltosa* (Polnisch et al. 1992) and to 3-chlorocatechol by *P. frequentans* (Hofrichter et al. 1992; 1994).

3-Chlorophenol was cometabolically converted by *P. simplicissimum* SK9117, without a release of chloride ions, to chlorohydroquinone, 4-chloro-1,2,3-trihydroxybenzene, 5-chloro-1,2,3-trihydroxybenzene, and 4-chlorocatechol as major metabolites which accumulated in the culture medium. Chlorohydroquinone and 4-chloro-1,2,3-trihydroxybenzene were identified for the first time as fungal transformation products within the metabolism of chlorophenols. 4-Chlorophenol was cometabolized faster than the 3-analogue. 4-Chlorocatechol could only be detected temporarily in the culture filtrate. Surprisingly no other of the known chlorinated metabolites were observed, although no chloride ions were released. Based on the identified metabolites a proposed pathway for the metabolism of monochlorophenols by *P. simplicissimum* is shown in Fig. 4. Contrary to the results obtained for strain SK9117, phenol grown

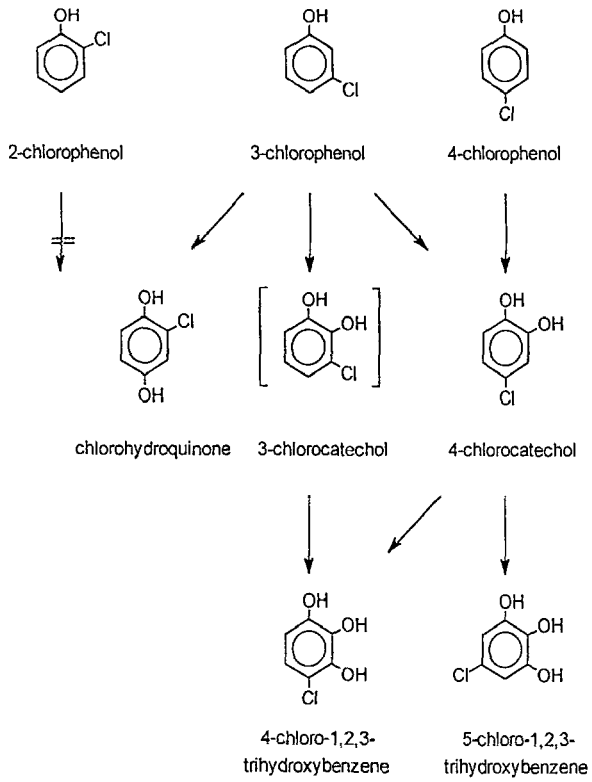

Fig. 4. Proposed pathway for the metabolism of monochlorophenols by *Penicillium simplicissimum* SK9117.

cells of *C. maltosa* were able to degrade 3-, and 4-chlorophenol with a release of chloride ions. 3-, 4-Chlorocatechol, 5-chloro-1,2,3-trihydroxybenzene, and 4-carboxymethylenebut-2-en-4-olide were identified as metabolites (Polnisch et al. 1992). Phenol grown mycelium of *P. frequentans* also degraded 3-, and 4-chlorophenol. Except 5-chloro-1,2,3-trihydroxybenzene, the same metabolites as for *C. maltosa* were detected. However, both chlorophenols were under these culture conditions or in presence of glucose or phenol as substrates only partially dechlorinated (Hofrichter et al. 1992; 1993; 1994).

Under cometabolic conditions only the *para*-substituted bromophenol was nearly completely transformed by *P. simplicissimum* SK9117. During the metabolization a not yet identified metabolite accumulated. Whether this metabolite is 4-bromocatechol as described by Walker (1973) and Hofrichter et al. (1994) for the metabolization of 4-bromophenol by *Rhodotorula glutinis* and *P. frequentans*, respectively, is at present not clear.

P. simplicissimum was able to grow with monofluorophenols as sole carbon and energy source. This

is the first report of a fungus growing on such compounds. However, the compounds were poor substrates as shown by a weak growth and only a partial defluorination of 3-, and 4-fluorophenol within long incubation periods. No liberation of fluoride ions could be observed during the growth with 2-fluorophenol. Only in the presence of phenol, a partial release of fluoride ions and the appearance of a "catechol-like" metabolite was observed. Whether the latter is 3-fluorocatechol which was identified with 2-fluoromuconic acid as metabolites in *P. frequentans* cultures (Hofrichter et al. 1992; 1994), remains to be elucidated. Under cometabolic conditions, 3-, and 4-fluorophenol were completely mineralized by *P. simplicissimum* as shown by the equimolar release of fluoride ions. Similar results were obtained during the cometabolism of these fluorophenols by *P. frequentans* (Hofrichter et al. 1992; 1994).

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