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Metabolism of phenol, chloro- and nitrophenols by the *Penicillium* strain *Bi 7/2* isolated from a contaminated soil

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Abstract

The *Penicillium* strain *Bi 7/2* able to grow on phenol as sole source of carbon and energy was isolated from a contaminated soil in Bitterfeld (East Germany). The strain is adapted to high phenol concentrations. Spores germinated still at a phenol concentration of 1.5 g/l. Phenol is degraded by the ortho-pathway with catechol as first intermediary product. The *Penicillium* strain metabolizes 4-, 3- and 2-chlorophenol with decreasing rates with phenol or glucose as cosubstrate. In the case of 4-chlorophenol 4-chlorocatechol was detected as intermediary product, further degraded as indicated by release of about 35% of the bound chlorine of the aromatic molecule. The strain also cometabolically metabolizes 4-, 3- and 2-nitrophenol. The final product of 3- and 4-nitrophenol is 4-nitrocatechol.

Introduction

Several fungi possess the capacity to metabolize phenol and other aromatic compounds. The understanding of the biodegradation potential requires comprehensive knowledge about activities of single members of natural communities and used metabolic pathways. Whereas we are well-informed about activities of aerobic bacteria, the respective knowledge about fungi is limited. In soils the standing stock of filamentous fungi is about tenfold higher than of bacteria (Yanagita 1990). Therefore we focused our studies on autochthonous fungi of contaminated soils. Most of these fungi degrade the xenobiotics by cometabolic reactions (Bollag 1972). Only a few fungi are able to utilize phenol as sole source of carbon and energy. In previous studies were reported that yeasts of the genera *Trichosporon, Candida, Torulopsis* and *Rhodotorula* are able to grow with phenol (Harris et al. 1959; Neujahr & Varga 1970). Mycelial fungi, however, have been largely neglected in studies of phenol catabolism. Anselmo et al. (1984, 1985, 1989) described the growth of *Fusarium flocciferum* with phenol as sole carbon source. *F. flocciferum* showed significant growth at concentrations up to 1 g/l after stepwise adaptation to higher concentrations. Scow et al. (1990) investigated the mineralization of phenol at low concentrations by a *Penicillium* strain isolated from silt loam that had been amended with 50 µg phenol per g. Reports on the metabolism of chloro- and nitrophenols by fungi are scanty. Walker (1973) described the formation of 4-chlorocatechol by *Rhodotorula glutinis* within the metabolism of 3- and 4-chlorophenol, however the ring was not dechlorinated. Laccase from various white-rot fungi oxidized chlorophenols in in vitro experiments, but did not attack nitrophenols (Claus & Filip 1990).

Fusarium oxysporum reduced 2,4-dinitrophenol to 2-amino-4-nitrophenol or 4-amino-2-nitrophenol according to Madhosnigh (1958).

The main aim of this work was to isolate autochthonous mycelial fungi from a contaminated soil and to analyze the capacity to metabolize phenol and chloro- and nitro-substituted derivatives.

Materials and methods

Isolation of soil fungi

Samples from a soil contaminated with aromatic hydrocarbons (phenols and benzenes up to 500 mg/kg) near an industrial effluent were collected in Bitterfeld (East Germany). The soil fungi present in these samples were isolated on a selective soil extract agar. It contained the extract of 1 kg garden soil in 1 l distilled water with 30 mg penicillin chloride, 30 mg streptomycine sulphate, 10 mg chloramphenicol and 100 mg phenol (Kreisel & Schauer 1987). Stock cultures of the isolated soil fungi were maintained on malt agar.

Selection of phenol-utilizing species

All isolated fungi were tested in pure culture for their ability to grow on phenol. 250 mg/l phenol were added as sole carbon source to a Czapek Dox basal medium (Kreisel & Schauer 1987). 100 ml of the medium were inoculated with 1 ml of a spore suspension in 500 ml-flasks. The cultures were incubated at 24°C under submerged conditions (160 rpm). Mycelial growth was estimated by dry weight measurement as described by Calam (1968).

Culture conditions for cometabolism of phenol, chloro- and nitrophenols

These studies were carried out in Czapek Dox medium with 10 g/l glucose. The cometabolism of chloro- and nitrophenols were also investigated with phenol as cosubstrate (500 mg/l). Growth conditions were those described above. To detect dechlorination of 4-chlorophenol a medium without chloride was used. The medium was composed of: 3 g/l sodium nitrate, 1.2 g/l dipotassium hydrogenphosphate, 0.5 g/l potassium nitrate, 0.5 g/l magnesium sulphate, 0.1 g/l yeast extract and 0.01 g/l iron (II) sulphate.

Chemical and physical analyses

A Merck-Hitachi HPLC (L-6200/D-2500) with a variable wavelength absorbance detector (model UV-4200) operating at 280 nm and fitted with a Merck LiChrospher 5-µm RP-18 column (125× 4.6 mm ID) was used to detect the various phenols and catechols by using isocratic conditions (solvent: ammonium acetate 30 mM/acetonitrile/methanol, 56:34:10, vol/vol/vol) at a solvent flow rate of 2 ml/min (Alacron 1987).

Metabolites were extracted with diethylether, evaporated to dryness and dissolved in methanol. They were collected by HPLC from repeated injections of the methanolic extracts or separated by TLC on RP-18 plates (Merck RP-18W UV-254, 5× 20 cm) with the same solvent as described for HPLC. Developed chromatograms were examined under UV-light. The several compounds were scraped off and extracted with methanol/water (1:1, vol/vol).

The UV-visible absorption spectra of the metabolites were determined by using a Hewlett Packard series 1050 HPLC with a 1040M series II DAD-detector.

Mass spectral analyses were performed on a high-resolution mass spectrometer model AMD-402/AMD-Intectra (Harbstedt).

Chloride concentrations in the culture fluid were determined photometrically with Merck Microquant chloride test.

The ring cleavage test was performed according to Ottow & Zilg (1969).

Chemicals

All phenols, catechol and 4-nitrocatechol were purchased from Fluka Chemie AG, Neu Ulm with p.A.

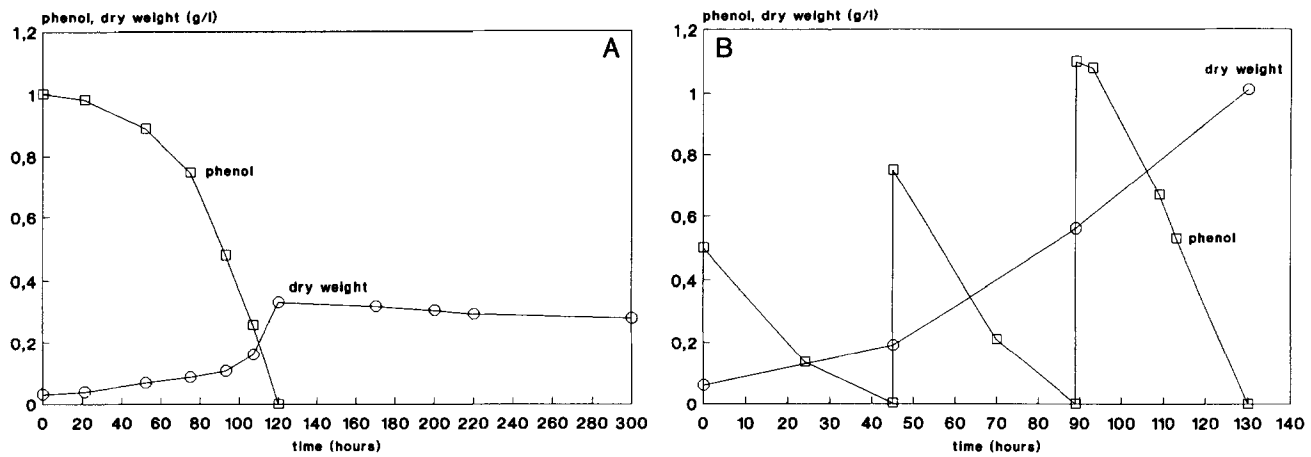

Fig. 1. (A): Growth and utilization of phenol by *Penicillium* sp. *Bi 7/2* in submerged culture. (B): Growth and utilization of phenol as sole carbon source under fed-batch conditions.

or puriss. purity grade. 4-Chlorocatechol was prepared by chlorination of catechol by sulfuryl chloride.

Results

Isolation of soil fungi

Six mycelial soil fungi were isolated from contaminated soil samples of Bitterfeld by using soil extract agar. Three of them were identified as *Penicillium* sp.. Besides the genera *Mucor, Fusarium* and *Alternaria* a sterile mycelium could be determined as well (Table 1).

Table 1. Fungi isolated from contaminated soil samples (phenols and benzenes up to 500 to mg/kg) in Bitterfeld (Germany) and their capability to utilize phenol as sole carbon source (phenol concentration 250 mg/l) or to cometabolize phenol with glucose as cosubstrate (initial concentration of phenol 250 mg/l, glucose 10 g/l, cultivation period 20 days).

Genus	Strain signation	Utilization of phenol as sole carbon source	% Phenol cometabolized during 20 days	Final dry weights [mg/ml]
Penicillium	Bi 4/1	–	100	2.5
Penicillium	Bi 7/1	–	54	2.7
Penicillium	Bi 7/2	+	100	3.1
Mucor	Bi 7/3	–	6	2.2
Alternaria	Bi 7/4	–	100	2.2
Mycelium sterile	Bi 6	–	44	2.0
Controls	–	–	3	–

Utilization of phenol

Among the isolated fungi one strain (*Penicillium* sp. *Bi 7/2*) was highly active and used phenol as sole source of carbon and energy (Fig. 1A, 1B). The strain reached a yield coefficient of about 0.4 under fed-batch conditions. The isolated strain *Penicillium* sp. *Bi 7/2* was adapted to relatively high phenol concentrations, because of its heritage from a phenol contaminated soil. A gradual subcultivation at higher phenol concentrations in the lab was not necessary. Spores of the strain formed on malt agar plates germinated in submerged cultures still at phenol concentrations up to 1.5 g/l.

The intermediate formation of catechol in the culture fluid was demonstrated by a characteristic colour reaction according to Arnov (1937) and by HPLC-analysis. The temporarily formed catechol was only detectable for some hours. By the ring cleavage test according to Ottow & Zolg (1969) it was demonstrated that the ortho-pathway was used which is typical for fungal degradation of aromatic acids and phenols (Rochkind-Dubinsky et al. 1987).

Metabolism of chlorophenols

The strain *Penicillium* sp. *Bi 7/2* metabolized 2-, 3- and 4-chlorophenol with glucose or phenol as cosubstrate. 4-Chlorophenol was degraded with the highest rate (Figs 2, 3). If 4-chlorophenol was added

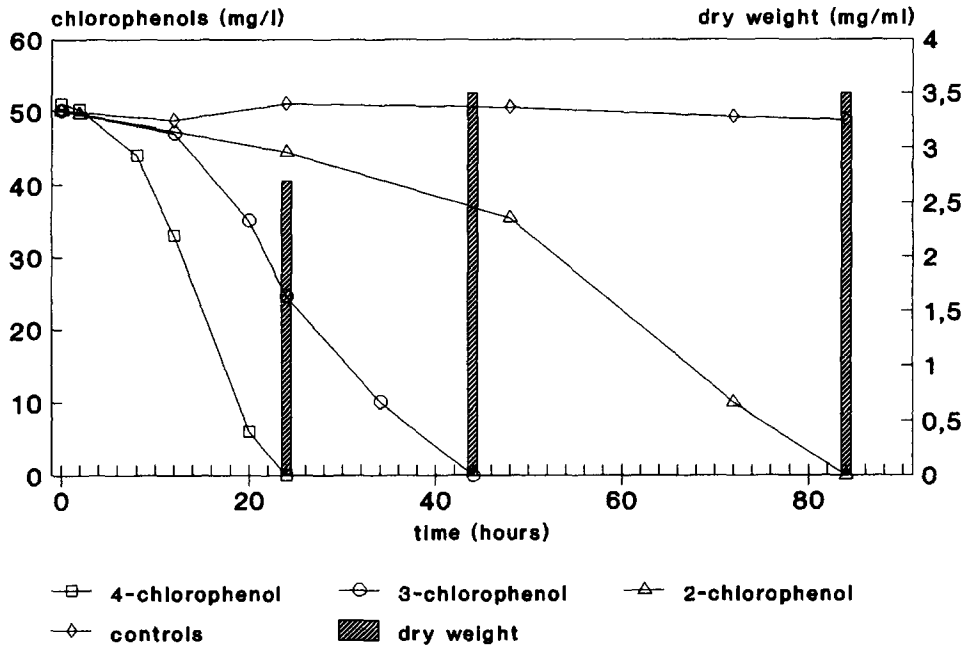

Fig. 2. Cometabolic degradation of various monochlorophenols with glucose as cosubstrate (10g/l). The columns show the mycelial dry weight after complete turnover of the respective phenols. The flasks were inoculated with spores.

as sole carbon source (after growth on phenol) a metabolite was temporarily detected. After extraction of the culture fluid with diethylether and isolation of the metabolite by TLC and HPLC, the UV-visible spectrum and the mass spectrum were determined. The UV-visible spectra of the isolated compound and 4-chlorocatechol were nearly identical. The compound was also identified as 4-chlorocatechol by analysis of the mass spectrum (M = 144). 4-Chlorocatechol was transformed to dark brown products in subsequent oxidative polymerisation reactions. Simultaneously with the oxidative poly-

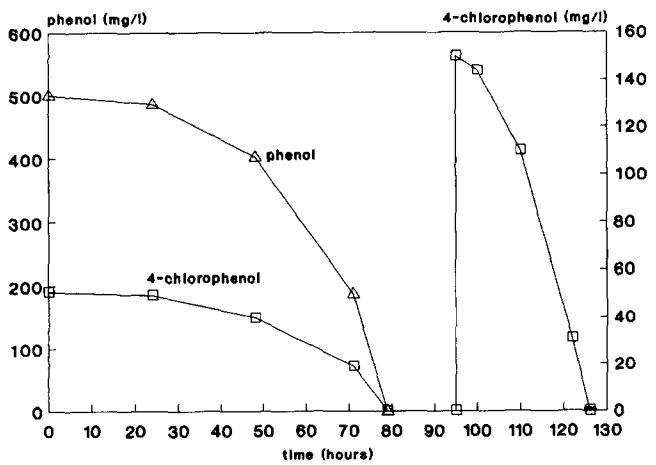

Fig. 3. Degradation of 4-chlorophenol with phenol as cosubstrate. In the second phase of the experiment only 4-chlorophenol was added. Final dry weight: 0.23 mg/ml.

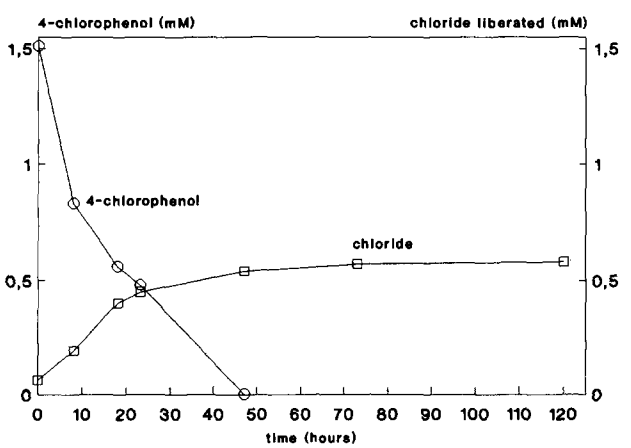

Fig. 4. Degradation and partial dechlorination of 4-chlorophenol (200 mg/ml). The mycelium grew on phenol as sole carbon source. The basal medium did not contain chloride.

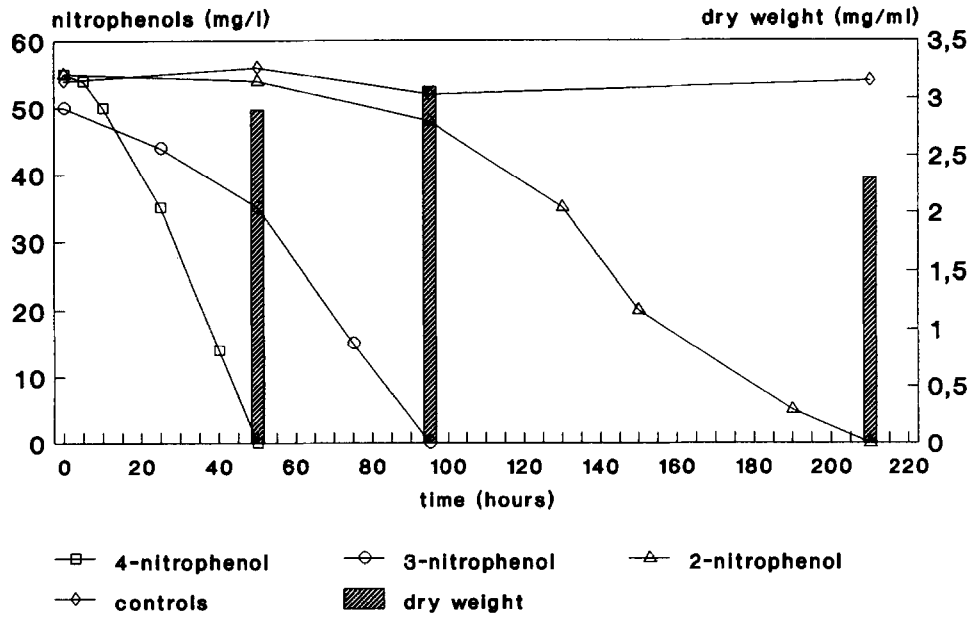

Fig. 5. Cometabolism of various mononitrophenols with glucose as cosubstrate (10g/l).

merisation the ring was partly dechlorinated (Fig. 4). About 35% of the covalently bound chlorine was released as chloride.

Metabolism of nitrophenols

2-, 3- and 4-nitrophenol were also cometabolized by *Penicillium* sp. *Bi 7/2,* but with a lower rate than chlorophenols (Fig. 5). During the linear degradation-period on an average 9mg 4-nitrophenol but 19mg 4-chlorophenol per g dry weight and day were metabolized with glucose as cosubstrate. If phenol was used as cosubstrate it had been degraded before 4-nitrophenol was attacked (Fig. 6). A final metabolite was accumulated in the medium with a characteristic red colour. The UV-visible spectrum was identical to that of 4-nitrocatechol. The metabolite was identified as 4-nitrocatechol by mass spectrum analysis, too (M = 155). 4-Nitrocatechol was also detected in experiments with 3-nitrophenol as substrate.

Discussion

With phenols and other aromatic hydrocarbons contaminated soils of industrial areas contain filamentous fungi able to metabolize phenol. A highly active strain belongs to the genus *Penicillium.* Some metabolic capabilities of the *Penicillium* strain *Bi 7/2* growing on phenol as sole source of carbon and energy were analyzed. The strain is relatively resistent to phenol, spores germinated without adaptation at concentrations up to 1500mg/l. The comparison of the specific degradation rate of this *Penicillium* strain with *Pseudomonas putida* (Janke & Fritsche 1981) indicated that the rates are in the same magnitude of 500–1000mg phenol per g dry weight and day. The strain degrades phenol by the ortho-pathway as investigated with the ring cleavage test according to Ottow & Zolg (1969).

In the literature there are only few reports on phenol degradation by mycelial fungi. Cain (1958) and Anselmo & Novais (1984) described *Fusarium* strains able to degrade phenol at a lower rate than *Penicillium* sp. *Bi 7/2.*

The investigated *Penicillium* strain degraded and partly dechlorinated monochlorophenols under cometabolic conditions. 4-Chlorophenol was de-

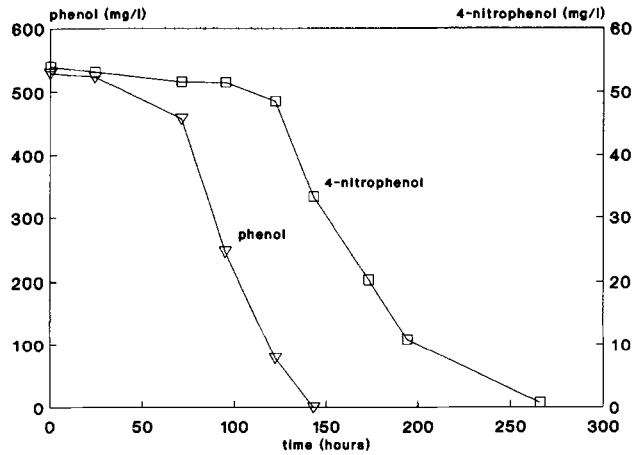

Fig. 6. Cometabolism of nitrophenol with phenol as cosubstrate. Final mycelial dry weight 0.14 mg/ml.

graded at a higher specific rate than 3- and 2-chlorophenol. Phenol and 4-chlorophenol were simultaneously metabolized at the same rate. The higher toxicity of chlorinated phenols caused us to apply these compounds with a tenfold lower concentration than phenol. An intermediate accumulation of 4-chlorocatechol was detected by UV- and mass-spectroscopy. In the course of further degradation, a partial dechlorination up to 35% of the covalently bound chlorine took place. The formation of 4-chlorocatechol was connected with the production of brown substances precipitating in the culture medium. An accumulation of 4-chlorocatechol as dead-end product like during the 4-chlorophenol degradation by *Rhodoturula glutinis* did not occur (Walker 1973). The dechlorination of chlorobenzoic and chlorophenoxyacetic acids by *Aspergillus niger* and *Aspergillus japonicus* was described by Shailubhai et al. and Sahasrabudhe et al. (1983, 1984, 1985). The same authors reported that cell-free extracts of *Aspergillus niger* dechlorinated 4-chloro- and 2,4-dichlorophenol. The liberation of chloride occurred during the first hydroxylation step prior to ring cleavage. A formation of 4-chlorocatechol was not observed (Sahasrabudhe et al. 1987).

Monosubstituted nitrophenols were degraded with about half of the specific activities in comparison to chlorophenols. In an experiment with simultaneous application of phenol and 4-nitrophenol, the degradation occurred sequentially, with that of 4-nitrophenol starting after the exhaustion of phenol. As degradation product 4-nitrocatechol was detected. The formation of nitrocatechols has not been demonstrated for fungi, but it is known for various bacteria (Cain 1958; Tewfik & Evans 1966; Raymond & Alexander 1971).

The further degradation process of halo- and nitrophenols is under investigation. First studies with 4-fluorophenol indicate that 4-carboxymethylene-but-2-en-4-olid is formed.

The results of this study indicate that soils contaminated by waste products of chemical industry contain fungi which are active in the metabolism of phenols. Additional work is needed to determine the significance of these fungi for self-purification of soil or remediation technologies.

Acknowledgements

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