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Degradation and dehalogenation of monochlorophenols by the phenol-assimilating yeast *Candida maltosa**

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Abstract

The phenol-assimilating yeast *Candida maltosa* is able to degrade monochlorophenols but cannot grow on these substrates. 3- and 4-chlorophenol were broken down very rapidly by phenol-grown cells under the formation of 4-chlorocatechol, 5-chloropyrogallol and 4-carboxymethylenebut-2-en-4-olide with concomitant release of chloride.

2-Chlorophenol was partially converted into cis,cis-2-chloromuconic acid via 3-chlorocatechol which was also obtained from 3-chlorophenol in low amounts. No further metabolites containing chloride were found.

The dehalogenation step in the chlorophenol degradation is the cycloisomerization of the cis,cis-chloromuconic acid to 4-carboxymethylenebut-2-en-4-olide in the ortho fission pathway.

Introduction

Chlorophenols have been classified as priority pollutants (Keither & Telliard 1979). Terrestrial and aquatic ecosystems are contaminated all over the world by these toxic compounds. The degradation of mono-, di- and polychlorophenols by bacteria has been frequently reported (for recent reviews see: Chaudhry & Chapalamadugu 1991; Häggblom 1990; Müller & Lingens 1986; Reineke & Knackmuss 1988).

The white rot fungus *Phanerochaete chrysosporium* has been shown to be capable of oxidative dechlorination and mineralization of chlorophenols (Hammel & Tardone 1988; Mileski et al. 1988; Valli & Gold 1991). Highly toxic chlorinated dibenzo-p-dioxins and dibenzofurans are formed in a peroxidase-catalyzed oxidation of trichlorophenols by this and other fungi (Dec & Bollag 1990; Öberg et al. 1990; Svenson et al. 1989; Wagner et al. 1990).

Many yeasts utilize phenol as a sole carbon and energy source for growth (Hofmann & Schauer 1988). However, it has been shown that *Candida tropicalis* metabolizes 3- and 4-chlorophenol only in the presence of glucose (Krug et al. 1985). 2-Chlorophenol and more highly chlorinated phenols are not metabolized by this yeast. Phenol-grown cells of *Rhodotorula glutinis* oxidize 2-, 3- or 4-chlorophenols, 4-bromophenol, 3,4-dichlorophenol and 2,4-dibromophenol (Walker 1973), but these two yeasts and also *Trichosporon cutaneum*

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(Hasegawa et al. 1990) cannot grow on any of the chlorophenols or bromophenols.

Despite considerable progress in the understanding of the chlorophenol degradation mechanisms in bacteria and white rot fungi, no information is available about dehalogenation steps and the reasons for the differences in the degradation of various chlorophenols by yeasts.

In this study we investigated the kinetics of monochlorophenol degradation and the formation of chlorinated and dechlorinated metabolites by the yeast *Candida maltosa* SBUG 700 known as a phenol- and hydrocarbon-utilizing organism (Hofmann & Schauer 1988).

Furthermore, we tested the conversion of monochlorophenols and 4-chlorocatechol by the phenol hydroxylase in crude extracts from phenol-grown cells of *Candida maltosa*. This enzyme catalyzes the primary attack during phenol degradation by yeasts (Gaal & Neujahr 1979; Neujahr & Gaal 1973). We assumed that the phenol hydroxylase determines the possibility and rate of chlorophenol degradation.

Materials and methods

Organisms and culture conditions

The yeast *Candida maltosa* SBUG 700 was obtained from the culture collection of the Institut für Angewandte und Technische Mikrobiologie, Universität Greifswald.

The yeast was cultivated in shake flasks with a mineral salt medium (Hofmann & Vogt 1987) containing yeast extract (0.2%), glucose (1%) and phenol (0,025%) at 30° C for 24 hours. The initial pH value of the medium was 4.5. Afterwards phenol was added (0.05%) and the yeast was cultivated for another 16 hours. The phenol-induced cells were used for the degradation experiments.

Investigation of chlorophenol degradation

To investigate chlorophenol degradation, phenol-induced cells were harvested by centrifugation (6.000 * g, 10 min), washed twice with 67 mM potassium dihydrogen phosphate and resuspended in the same solution (20 g wet weight cells per l). After incubating the cells at 30° C with aeration rate of 100 l * h^{-1} * l^{-1} for 10 min, one of the monochlorophenols (100 mg per l) was added. Samples were taken before and immediately after the addition of the chlorophenols and at defined times afterwards. Cells in the samples were separated by centrifugation (6.000 * g, 10 min).

Chemical analysis of substrates and metabolites

In the cell-free supernatants the phenol content was measured by a colorimetric method (Der Yang & Humphrey 1975) or together with degradation products by reversed phase HPLC using the following equipment: two pumps M 6000 A connected to a gradient programmer M 680 and a WISP 710B injector (Waters), a photo diode array detector 1040 A (Hewlett Packard) and an ODS-1 column, 125 * 4 mm, 5 μm particles (Chromatography Service, Langerwehe, Germany). The metabolites were separated by gradient elution. The initial solvent was 0,1% phosphoric acid, the final one was acetonitril/0.1% phosphoric acid (6 : 4, v/v). A linear gradient over 20 min was used at a flow rate of 1.0 ml per min.

For gas chromatography/mass spectrometry (GC/MS), the metabolites were extracted from the culture liquid with ethyl acetate. To enhance the recovery of acidic metabolites, the culture medium was acidified to pH 2.0 with 1 N HCl before extraction. The extracts were dried with anhydrous Na_2SO_4. The solvent was removed in a rotary evaporator under reduced pressure. Afterwards metabolites were derivatized by silylation using N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) at 100° C for 10 min in a screw-cap vial.

GC/MS was performed using a Kratos mass spectrometer MS 25 (Kratos, Manchester) combined with a Carlo Erba 2100 gas chromatograph. Samples of the reaction mixture were injected onto a Chrompak CP Sil 5 quartz capillary column (25 m * 0.32 mm) with He as carrier gas using a split ratio of 30 : 1 (Gupta et al. 1986). Spectra were obtained at

70 eV over a scan range of 35 to 600 daltons. Intermediates were identified after interpretation of mass spectra and UV spectra by comparison with authentic compounds.

Chloride ion concentrations in the culture supernatants were measured by the Spectroquant reagent kit (Merck, Darmstadt).

Growth test for chlorophenols

Phenol induced cells of *Candida maltosa* SBUG 700 were cultivated in mineral salt medium (see above) containing yeast extract (0.2%) and 100 mg per l of one of the chlorophenols. Growth was followed by measuring the optical density at 700 nm ($O.D._{700}$).

Assay of phenol hydroxylase

To prepare cell-free extracts, phenol-induced cells were harvested by centrifugation (6.000 * g, 10 min) and washed twice in 50 mM potassium phosphate buffer pH 7.6 containing 1 mM 2-mercaptoethanol, 0.1 mM EDTA and 2 μM FAD. The washed cell pellet (about 500 mg wet weight) was suspended in 5 ml of the same buffer and frozen in a cell of an X-press at $-20°$ C. Cell-free extracts were obtained by two passages of the frozen cells through the X-press and centrifugation (20.000 * g, 4° C, 20 min). The supernatants were used as crude extracts.

The concentration of protein in the crude extracts was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

The activity of phenol hydroxylase was assayed at 340 nm and at 30° C in a spectrophotometer Uvicon 930 (Kontron Instruments, Germany) by modifying the method of Neujahr & Gaal (1973). The standard assay (volume = 2.0 ml) contained 100 μmoles potassium phosphate buffer pH 7.6, 0.4 μmoles NADPH, crude extract (about 1 mg protein). The reaction was started by addition of phenol, one of the chlorophenols, catechol or 4-chlorocatechol (1.0 μmol each).

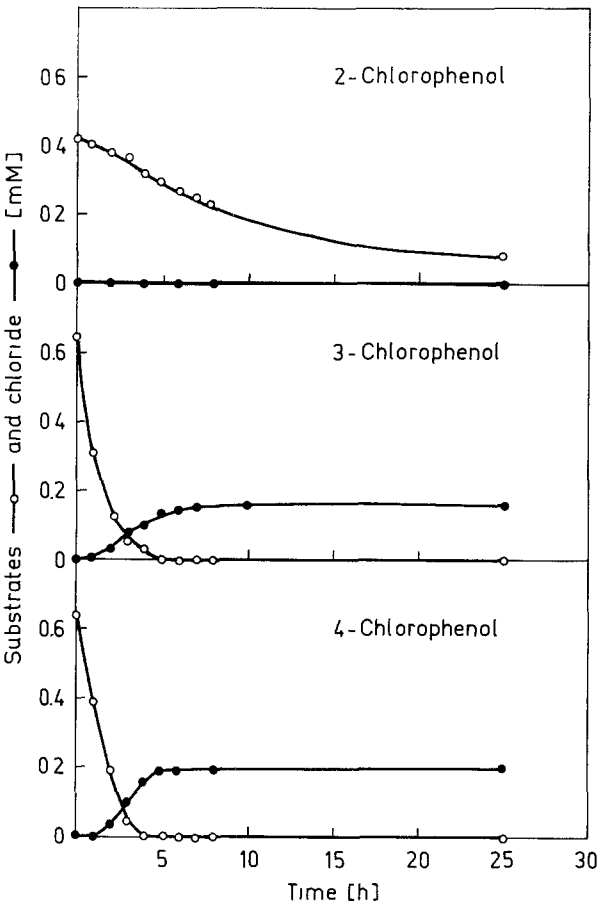

Fig. 1. Kinetics of monochlorophenol degradation and chloride release by phenol- grown cells of *Candida maltosa* SBUG 700

Results presented in this paper are based on the means of at least three independent assays.

Chemicals

2-Chlorophenol, 3-chlorophenol, 4-chlorophenol, and catechol were obtained from Merck, Darmstadt. 4-Chlorocatechol was synthesized according to Dakin (1909) and 5-chloropyrogallol by a method described by Horner & Goewecke (1961). 3-Chlorocatechol and cis, cis-2-chloromuconic acids were gifts from Dr. E. Schmidt (Wuppertal). Other chemicals were of the highest purity available.

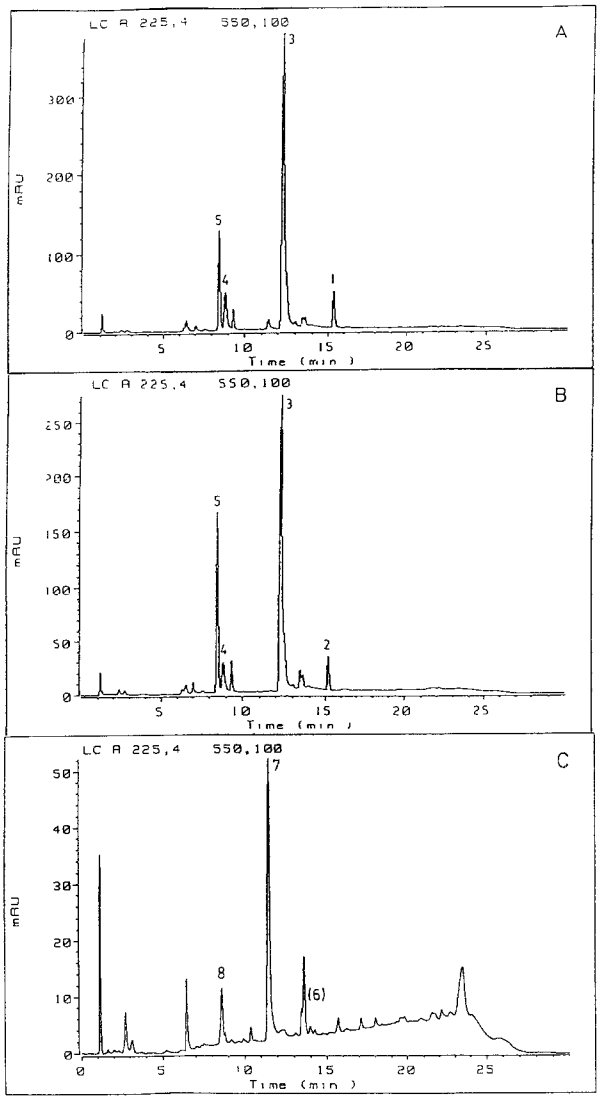

Fig. 2. HPLC elution profiles of degradation products from (A) 3-chlorophenol, (B) 4-chlorophenol, and (C) 2-chlorophenol formed and of metabolites excreted by *Candida maltosa*. The culture liquids for analyses were harvested 2 hours (A + B) and 24 hours (C) after the start of the experiments. 1: 3-Chlorophenol, 2: 4-chlorophenol, 3: 4-chlorocatechol, 4: 5-chloropyrogallol, 5: 4-carboxymethylenebut-2-en-4-olide, 6: 2-chlorophenol, 7: 3-chlorocatechol, 8: cis,cis-2-chloromuconic acid.

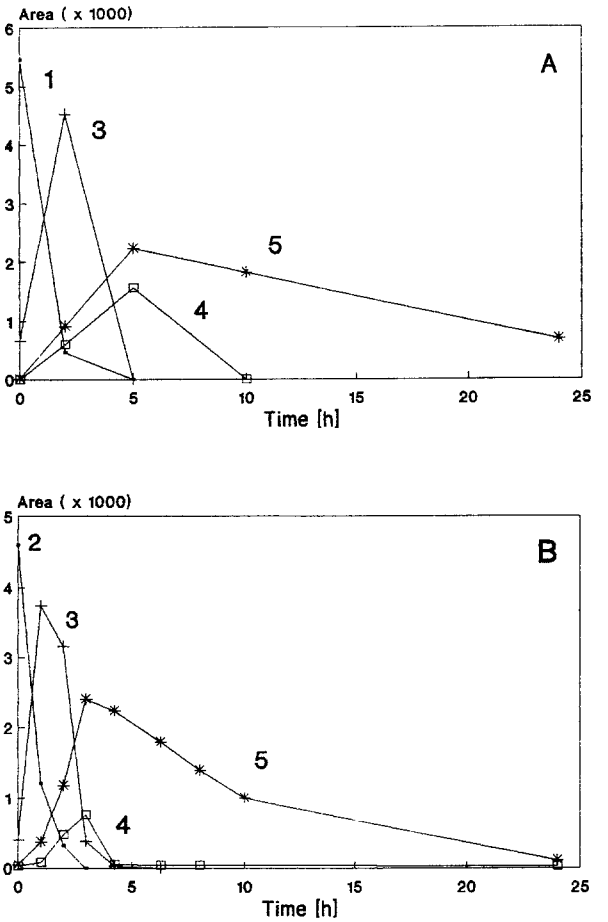

Fig. 3. Kinetics of product accumulation and decomposition in the culture medium of *Candida maltosa* in the presence of (A) 3-chlorophenol and (B) 4-chlorophenol. 1: 3-Chlorophenol, 2: 4-chlorophenol, 3: 4-chlorocatechol, 4: 5-chloropyrogallol, 5: 4-carboxymethylenebut-2-en-4-olide.

Results

Candida maltosa could not grow on chlorophenols as a sole carbon and energy source. The increase of the $O.D._{700}$ was even lower in the Erlenmeyer flasks in which the cells were incubated in the presence of chlorophenols than in assays without chlorophenols and only with yeast extract.

In all cases, cells of *Candida maltosa* pregrown in the absence of phenol could not degrade any chlorophenol.

As shown in Fig. 1, about 20% of 3- and 4-chlorophenol and more than 40% of 2-chlorophenol disappeared immediately and were probably absorbed by or transported into the yeast cells after addition of the compounds to the cell suspension. Afterwards, 3- and 4-chlorophenol were broken down very rapidly and were below the detection limit after 5 hours (Figs. 1 and 3). The concentra-

Table 1. Characterization of substrates and products of monochlorophenol degradation by *Candida maltosa*.

Compound	HPLC retention time (min)	UV-absorption maxima (nm) from HPLC runs	Characteristic mass ions m/e (%) (TMS-Derivative)
2-Chlorophenol	14.5	215, 275	n.d.
3-Chlorophenol	15.8	219, 275	n.d.
4-Chlorophenol	15.6	227, 280	200/202 (42/15) M^+; 185/187 (100/39) M-CH_3; 73(45) TMS.
3-Chlorocatechol	11.4	230, 277	288/290 (53/22) M^+; 273/275 (13/4) M-CH_3; 200/202 (5/2); 185/187 (8/5; 73(> 100)
4-Chlorocatechol	12.3	225, 285	288/290 (15/5) M^+; 273/275 (3/1) M-CH_3; 200/202 (2/0.6); 185/187 (4/1.5); 73(100).
4-Carboxymethylenebut-2-en-4-olide	8.4	277	212(1) M^+; 197(12) M-CH_3; 153 (35); 123(5); 109(10), 77(38); 75(58); 44(100).

n.d.: not determined.

tion of 2-chlorophenol decreased only slowly and its amount disappearing from the medium over 25 hours could be detected mostly in the exit gas. However, minor amounts were converted into cis,cis-2-chloromuconic acid via 3-chlorocatechol (see Fig. 2C).

An increase of Cl^--ions was measured during the incubation of *Candida maltosa* in the presence of 3- or 4-chlorophenol as long as the chlorophenols were degraded (Fig. 1). The final concentration of 0.15 mM in the case of 3-chlorophenol and 0.19 mM in the case of 4-chlorophenol in the assays corresponded to 19.8% and 24,7% of the chloride bound to the degraded chlorophenols.

No formation of chloride ions was found in the assays of *Candida maltosa* with 2-chlorophenol.

During incubation of *Candida maltosa* in the presence of 3-chlorophenol or 4-chlorophenol, beside the substrates a number of compounds was detected in the culture fluids using reversed phase HPLC (Fig. 2A and B). Among them, 3- and 4-chlorocatechol, and 4-carboxymethylenebut-2-en-4-olide were identified by GC/MS. Surprisingly, we detected also 5-chloropyrogallol but no chloromuconic acid in the extracts. These results were confirmed by HPLC and comparison of the UV absorption spectra with those of authentic chemicals (Table 1).

The remaining metabolites shown in Fig. 2 were not yet identified. However, mass spectrometry revealed that they did not contain chlorine.

The decrease of 3- and 4-chlorophenol in the culture medium was accompanied by a successive accumulation of 4-chlorocatechol and 5-chloropyrogallol. Already 10 hours after the start of the degradation experiments no chlorinated metabo-

Table 2. Turnover of NADPH + H^+ by the enzyme phenol hydroxylase in crude extracts from phenol-grown yeasts.

Substrate	*Candida maltosa* SBUG 700	*Candida tropicalis* (Krug et al. 1985)	*Trichosporon cutaneum* (Neujahr & Gaal 1973)
Phenol	100[1]	100[2]	100[3]
2-Chlorophenol	9	13	10
3-Chlorophenol	67	54	26
4-Chlorophenol	47	65	29
Catechol	78	92	58
4-Chlorocatechol	83	25	–

1: 0.285 μmoles * min^{-1} * mg^{-1} protein, 2: 0.69 μmoles * min^{-1} * mg^{-1}, 3: highly purified enzyme.

lites could be detected. The disappearance of 4-carboxymethylenebut-2-en-4-olide from the medium, however, required a relatively long period of time (Fig. 3A and B).

It was assumed that the phenol hydroxylase determines whether a monochlorophenol is metabolized by the yeast or not, and this enzyme is also responsible for the formation of chloropyrogallol. In order to examine this assumption, we investigated the activity of this enzyme in crude extracts from phenol-grown *Candida maltosa* cells in the presence of monochlorophenols and 4-chlorocatechol. The results are presented in Table 2. A high activity of this enzyme was measured in the presence of 3- and 4-chlorophenol. In comparison, 2-chlorophenol was transformed by the phenol hydroxylase in the crude extracts only very slowly. A high activity was also found with catechol and even with 4-chlorocatechol as substrates for the hydroxylation reaction (Table 2).

Discussion

Although *Candida maltosa* like many other yeasts utilizes phenol as a sole carbon and energy source (Hofmann & Schauer 1988), growth of this yeast on monochlorophenols was not expected. From previous publications it was known that other yeasts are unable to grow on chlorophenols (Krug et al. 1985; Walker 1973).

The increase of the chloride concentration in the incubation assays of *Candida maltosa* with 3- and 4-chlorophenol (Fig. 1) provided the first evidence of a dehalogenation activity in yeasts. Accumulation of Cl^--ions in the yeast cells could be the reason for the only partial release of chloride from the chlorophenols into the medium. A chloride transport system had been found in the vacuolar membrane of *Saccharomyces cerevisiae* (cf. Klionsky et al. 1980). The formation of chlorinated dead-end metabolites during the degradation of monochlorophenols must be ruled out as an explanation for the insufficient recovery of chloride from the substrates. Chlorocatechol and chloropyrogallol were the only chlorinated metabolites identified. They disappeared from the culture medium during the degradation experiments. All other so far not identified compounds shown in Fig. 2A and B were free of chlorine.

The occurrence of 4-chlorocatechol, minor amounts of 3-chlorocatechol and of 4-carboxymethylenebut-2-en-4-olide in the culture fluids and relatively high activities of phenol hydroxylase in crude extracts from phenol-grown cells of *Candida maltosa* in the presence of 3- and 4-chlorophenol are evidence for the degradation of monochlorophenols via ortho fission of the phenol assimilation pathway (cf. Gaal & Neujahr 1979).

No cis,cis-3-chloromuconic acid could be detected, obviously due to rapid cycloisomerization into 4-carboxymethylenebut-2-en-4-olide under release of chloride. This dehalogenation mechanism was already described for *Pseudomonas* sp. B13 by Knackmuss & Hellwig (1978).

5-Chloropyrogallol is not a constituent of the ortho fission pathway. Its occurrence, however, resulted from the hydroxylation of the metabolite 4-chlorocatechol accumulated during degradation of 3- and 4-chlorophenol.

The results presented in Table 2 indicate that the phenol hydroxylase determines the degradation rate of the various monochlorophenols. A chlorine atom in ortho position to the phenolic OH-group is obviously a strong obstacle for this hydroxylase reaction. Although small amounts of 3-chlorocatechol and cis,cis-2-chloromuconic acid were found as metabolites of 2-chlorophenol, no chloride release and other indications of further degradation were observed.

Acknowledgements

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Note added in proof

An additional chlorine-containing metabolite was

tentatively identified as the monoethylether of 5-chloropyrogallol.

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