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Biotransformation of halophenols by a thermophilic *Bacillus* sp.

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Abstract

The thermophilic *Bacillus* sp. A2 transformed various halophenols. 2-Chlorophenol, 2-bromophenol, 3-bromophenol and 2-fluorophenol were transformed under resting cell conditions at 60 °C to 3-chlorocatechol, 3-bromocatechol, 4-bromocatechol and 3-fluorocatechol, respectively. The hydroxylation of 3-bromophenol occurred at the proximal and distal position relative to the halogen substituent. In complex medium this strain completely transformed 2-chlorophenol and 2-bromophenol at concentrations up to 1 mM. Concomitantly, an accumulation of oxygen- and temperature sensitive halocatechols was observed. 3-Chlorocatechol possesses a half-life of 11.5 h at 60 °C and is therefore readily decomposed during incubation. The hydroxylating system was present in phenol-grown cells but not in glucose-grown cells. The hydroxylase activity could also be induced by 2-chlorophenol. The product, 3-chlorocatechol, is not a substrate for the catechol 2,3-dioxygenase.

Abbreviations: 2-CP – 2-chlorophenol, DCP – dichlorophenol, TCP – trichlorophenol, tetraCP – tetrachlorophenol, MIC – minimal inhibitory concentration, CF – chloride-free, CFG – chloride-free plus glucose, CFGY – chloride-free plus glycerol, CFP – chloride-free plus phenol, CAM – chloramphenicol

Introduction

Phenol belongs to the EPA list of high priority pollutants and is produced in large quantities. Halogenated analogues are used for the production of microbiocides in general. The microbiocidal effects of phenol can be increased by halogenation. To date, the combined alkylated and halogenated derivatives have attained the greatest practical importance as active ingredients in disinfectants (e.g. 4-chloro-3-methylphenol, MIC *B. subtilis*: 150 mg/l).

However, this engineered biocidal activity leads to environmental problems if high concentrations of highly toxic compounds are released and the combined action of photochemical, chemical and biological degradation mechanisms are too slow in comparison with the rate of toxification (Hwang et al. 1986). Therefore, there is considerable interest concerning the biodegradability of these compounds.

During recent years several bacteria have been discovered, that grow at high temperatures up to above 100 °C. These organisms have gained great biotechnological interest (Van der Oost et al. 1996). If such bacteria had the ability to degrade environmental pollutants, they would form ideal tools for the biological treatment of hot waste water streams or exhaust gases, which cannot be treated at lower temperatures. To date information on the ability of thermophilic microorganisms to degrade environmental pollutants, especially chlorinated compounds, is very limited.

Under mesophilic conditions several pathways have been elucidated for the aerobic biodegradation of chlorophenols. Mono- and dichlorophenols are oxygenated to chlorocatechols, whereas the higher chlorinated phenols are hydroxylated to form chlorinated hydroquinones (Commandeur & Parsons 1990). In contrast, there are no detailed data about the aerobic degradation pathways of halophenols under thermophilic conditions.

Several phenol-degrading thermophiles have been reported, all of them belonging to the genus *Bacillus* (Buswell 1975; Gurujeyalakshmi & Oriel 1989; Yanase et al. 1992). Although there is an unclear taxonomic situation concerning the thermophilic members of this genus, most of the isolates seem to be *Bacillus stearothermophilus* species. The majority of these strains needs complex supplements for growth on phenol and the best documented strain has an optimal growth temperature of 55 °C.

The recently isolated thermophilic *Bacillus* sp. A2 has an optimal growth temperature of 60–65 °C and is able to use phenol as sole carbon and energy source (Mutzel et al. 1996). Here we describe that this strain is able to transform various halophenols, a feature not described for other thermophilic bacilli. The conditions for these transformations as well as the isolation and identification of several transformation products are presented. Finally, the fate of the halocatechols produced under thermophilic conditions was investigated.

Table 1. Cometabolic transformation with resting or growing cells of *Bacillus* sp. A2. The chlorophenols were added to separate vials at 1 mM final concentration

Substrates	Phenol-grown resting cells transformation after 8 h (μM)	Growing-cells in CFG medium
Phenol	194	+
2-Chlorophenol	443	+
3-Chlorophenol	-	-
4-Chlorophenol	-	-
2-Bromophenol	365	+
3-Bromophenol	21	+
4-Bromophenol	-	-
2-Fluorophenol	102	-
3-Fluorophenol	-	-
4-Fluorophenol	-	-
4-Iodophenol	-	-

- no transformation observed, values corrected for abiotic loss.
+ transformation observed.

Materials and methods

Bacteria, media and culture conditions

The phenol-degrading *Bacillus* sp. A2, recently isolated by Mutzel et al. (1996), was maintained at 60 °C on solidified CF (chloride free) medium containing 1 mM phenol as sole carbon and energy source. Growth was measured photometrically at 540 nm and correlated with direct microscopic cell count. The chloride-free mineral salts medium (CF) contained (per litre of deionized water) 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$ and 0.2 g $MgSO_4 \times 7H_2O$. After adjusting the pH to 6.5 with 6 M H_2SO_4, the solution was autoclaved and the following sterile solutions were added (per litre of medium): 5 ml of trace element solution [consisting of (per litre): 24.0 g EDTA, 4.0 g NaOH, 2.0 g $MgSO_4 \times 7H_2O$, 0.8 g $ZnSO_4 \times 7H_2O$, 0.8 g $MnSO_4 \times 4H_2O$, 0.2 g $CuSO_4 \times 5H_2O$, 4.0 g $Fe_2(SO_4)_3$, 0.2 g $Na_2MoO_4 \times 2H_2O$, 20.0 g Na_2SO_4, 1.0 ml H_2SO_4 conc.] and 10 ml of vitamin solution (Wolin et al. 1964). In all experiments the halophenols were added at a final concentration of 1 mM. In addition glucose (0.5 mM), glycerol (1 mM) or phenol (1 mM) were added to CF medium yielding CFG, CFGY and CFP medium, respectively. For growing cells experiments a Luria-Bertani (LB) medium (Sambrook et al. 1989) was used (10.0 g yeast extract (Merck), 5.0 g tryptone (Oxoid) and 5.0 g NaCl per litre). All incubations were carried out at 60 °C. Screw-capped vials equipped with a teflon-lined rubber septum were used to prevent evaporation during incubation.

Test for growth on different substrates

The substrates listed in Table 1 were tested as sole carbon and energy source by incubating strain A2 in CF medium. Sterile controls permitted the determination of abiotic substrate loss especially due to elevated temperatures. Every 24 hours of incubation, the optical densities were determined, microscopic examinations and viable cell counts on LB agar were conducted. Substrate concentrations were measured using HPLC.

Induction of phenol hydroxylase and catechol 2,3-dioxygenase

For the induction experiments cells grown in LB, CFP and CFG were centrifuged and resuspended in phosphate buffer (0.05 M, pH 7.0) to a final cell concentration of 1.5×10^8/ml. After addition of phenol, 2-chlorophenol, catechol or 3-chlorocatechol to the resuspended cells the phenols were monitored using HPLC. The products of the catechol 2,3-dioxygenase were measured spectrophotometrically at 375 nm. In additional experiments protein synthesis was inhibited by the addition of chloramphenicol at a concentra-

tion of 50 μg/ml. After 10 min of incubation with the inhibitor, the same measurements of phenol/catechol consumption were conducted as described above.

Cometabolic transformation with growing or resting cells

For the cometabolic transformation experiments with growing cells 3 ml of LB, CFG, CFP or CFGY medium containing the halophenols or phenol at 1 mM concentration were inoculated with strain A2. The vials were incubated on a rotary shaker and samples of 200 μl were withdrawn for HPLC analysis and for the identification of metabolites using GC-MS.

For the resting cells experiments phenol-grown cells from a continuous fermentation were centrifuged, washed with phosphate buffer (50 mM, pH 7.0) and resuspended to a final cell concentration of 1×10^9/ml. Halophenols or phenol were added to separate vials at 1 mM final concentration. The vials were incubated and samples were withdrawn after 6, 8, 10 h for analysis.

Analytical measurements

Substrates and metabolites were analysed by HPLC using a diode-array-detector (Waters/Millipore, model 994) in culture supernatant clarified by centrifugation. Identification was done by comparing UV spectra and retention times with those of reference substances. Reversed-phase HPLC was carried out on a LiChrospher RP18 column (4.6 × 150 mm) using methanol/water/acetic acid (500/500/10, v/v/v) as eluent with a flow rate of 1 ml/min. Substances were detected at a wavelength of 270 nm. For analysis of the higher chlorinated phenols the mobile phase was changed to methanol/water/acetic acid (700/300/10, v/v/v).

For the identification with the GC-MS system the metabolites were extracted from the culture medium. 0.5 ml of culture liquid was centrifuged and the supernatant, acidified with 6 M H_2SO_4, was extracted three times with equal volumes of ethyl acetate. The combined extracts were dried over Na_2SO_4 and the solvent was removed by evaporation. The residues were redissolved in small amounts of ethyl acetate for GC-MS analysis, which was performed on a HP G1800A GCD System equipped with a FS-capillary column (HP5) of 30 m × 0.25 mm dimensions. Injector and detector temperatures were 250 °C and 280 °C, respectively, and the column temperature was raised from 70 °C to 150 °C at a rate of 10 °C/min with an initial holding time of three minutes. The mass spectra were obtained at 70 eV.

Decomposition of 3-chlorocatechol at higher temperatures

Solutions of 3-chlorocatechol (0.1 mM) in CF mediums or phosphate buffer (0.05 M, pH 7.0) were incubated at the following temperatures: 40 °C, 50 °C, 60 °C, 70 °C. The decrease in concentration was measured by HPLC. The solutions were incubated on a rotary shaker.

Chemicals

Phenol and all halophenols except 2,3,4,5 tetrachlorophenol were purchased from Fluka (Buchs, CH). Tetrachlorophenol was obtained from Lancaster (Mühlheim, Germany). 3-Chlorocatechol was generously provided by Dr. S. Fetzner (Institute for Microbiology, University Hohenheim).

Results

Utilisation of halophenols as sole growth substrate

Strain A2 was incubated with the halophenols listed in Table 1 and nine higher chlorinated phenols (2,3-DCP/ 2,4-DCP/ 2,5-DCP/ 3,4-DCP/ 2,3,4-TCP/ 2,4,5-TCP/ 2,4,6-TCP/ 3,4,5-TCP/ 2,3,4,5-TetraCP). None of the tested halophenols supported growth even after 4 days incubation at 60 °C. Under identical conditions with phenol as substrate, growth and phenol degradation occurred within 12 h. The halophenol concentrations showed no significant decrease. Abiotic losses accounted for less than 1% of the initial concentration. Free spores were observed microscopically after 12 h.

Induction of phenol hydroxylase and catechol 2,3-dioxygenase in whole cells

The transformation of phenol, 2-chlorophenol, catechol and 3-chlorocatechol with phenol-grown, LB-grown and glucose-grown cells was tested (Table 2). Phenol-grown and LB-grown cells were able to transform phenol, 2-chlorophenol and catechol even in the presence of chloramphenicol. 3-Chlorocatechol was not transformed. Glucose-grown cells were able to transform phenol and 2-chlorophenol but only in

Table 2. Inducation of phenol hydroxylase and catechol 2,3-dioxygenase activities in whole-cells of *Bacillus* sp. A2

Substrate	Growth substrate		
	Phenol	LB	Glucose
Phenol	+	+	+
Phenol + CAM	+	+	-
2-CP	+	+	+
2-CP + CAM	+	+	-
Catechol	+	+	-
Catechol + CAM	+	+	-
3-Chlorocatechol	-	-	-
3-Chlorocatechol + CAM	-	-	-

+: transformation observed, -: no transformation observed.

the absence of chloramphenicol. Catechol and 3-chlorocatechol were not transformed in any case by these cells. These results suggest, that the enzymes for the degradation of phenol were induced in phenol and LB-grown cells. Only in glucose-grown cells the enzymes were repressed. However, they could be readily induced in the presence of glucose by the addition of phenol or 2-chlorophenol. The catechols did not induce any of the enzymes.

Transformation under resting cell conditions and with growing cells

Cells from a continuous culture with phenol as sole source of carbon and energy were used for the resting cell experiments. These cells contain an induced phenol degradation pathway and therefore phenol-hydroxylase activity. In another series of experiments each of the halophenols was added separately to growing cells in CFG medium. In this case the phenol-hydroxylase was not induced. With resting cells 2-chlorophenol, 2-bromophenol, 3-bromophenol and 2-fluorophenol were transformed (Table 1). The highest transformation was detected for 2-chlorophenol (44%) and the lowest for 3-bromophenol (2.1%) after 8 h. Of the monohalophenols tested with the exception of 3-bromophenol, only the *ortho* halogenated phenols were transformed. None of nine higher chlorinated phenols (2,3-DCP/ 2,4-DCP/ 2,5-DCP/ 3,4-DCP/ 2,3,4-TCP/ 2,4,5-TCP/ 2,4,6-TCP/ 3,4,5-TCP/ 2,3,4,5-TetraCP) served as transformation substrates.

The non-induced growing cells in CFG medium transformed phenol, 2-chlorophenol, 2-bromophenol and 3-bromophenol after 6 h. However, even after 12 of incubation no transformation of 2-fluorophenol was detected. No decrease in viable cell numbers was observed in these experiments with the halophenols present at concentrations of 1 mM. These results suggest, that in addition to phenol, 2-chloro-, 2-bromo, and 3-bromophenol can induce the phenol hydroxylase but not 2-fluorophenol.

Table 3. GC and MS data of the isolated halocatechols formed cometabolically by *Bacillus* sp. A2

Compound	GC retention times [min]	m/z	Assignment
3-Chlorocatechol	9.13	146/144	M^+
		126	M^+-H_2O
		108	M^+-HCl
3-Bromocatechol	10.56	190/188	M^+
		172/170	M+-H_2O
		108	M^+-HBr
4-Bromocatechol	12.96	190/188	M^+
		172/170	M^+-H_2O
		108	M^+-HBr
3-Fluorocatechol	7.76	128	M^+
		108	M^+-HF
		80	M^+-HF-CO

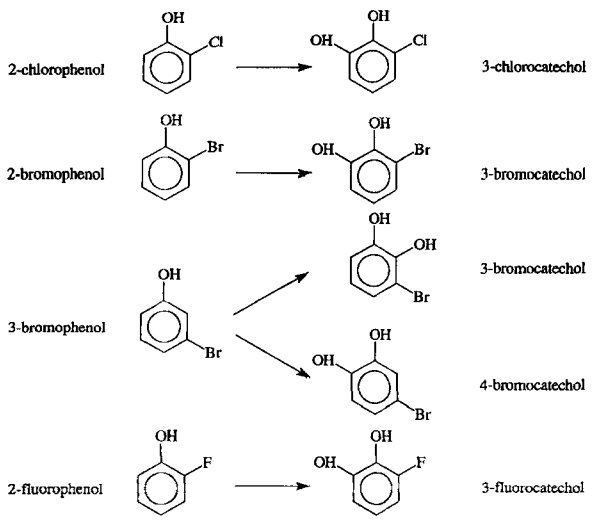

Figure 1. Structures of the halogenated phenols transformed by *Bacillus* sp. A2 and the products hydroxylated by phenol hydroxylase.

Isolation and identification of metabolites

The halophenols transformed to the corresponding halogenated catechols are shown in Figure 1. 2-

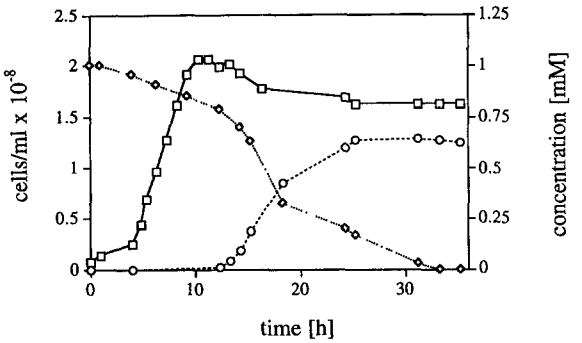

Figure 2. Transformation of 1 mM 2-chlorophenol at 60 °C by growing-cells of *Bacillus* sp. A2. - - - □ - - - cells, - - - ◊ - - - 2-chlorophenol, - - - ○ - - - 3-chlorocatechol.

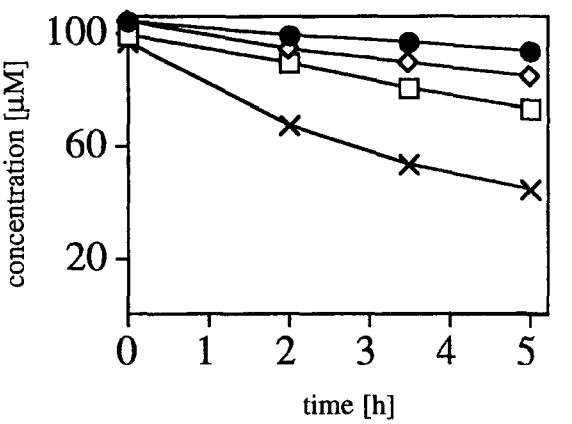

Figure 3. Decomposition of 3-chlorocatechol at different temperatures in chloride-free medium. - - - ● - - - 40 °C, - - - ◊ - - - 50 °C, - - - □ - - - 60 °C, - - - × - - - 70 °C.

Chlorophenol was transformed into 3-chlorocatechol, 2-bromophenol into 3-bromocatechol, 3-bromophenol into 3-bromocatechol and 4-bromocatechol, 2-fluorophenol into 3-fluorocatechol. The molecular ions show the typical isotopic ratios for the chloro- and bromocatechols. 3-Bromophenol is hydroxylated at the 2-position as well as at the 6-position, leading to a mixture of 3-bromocatechol and 4-bromocatechol. The former is the predominant metabolite.

The identification of 4-bromocatechol was possible due to the enhanced occurrence of M^+-H_2O and the reduced formation of M^+-HBr ions compared to 3-bromocatechol (Knuutinen & Korhonen 1983). For the 3-fluorocatechol we propose a fragmentation pattern with a loss of HF and following release of CO resulting in the observed ion of 80 m/z.

The GC retention times increased with increasing molecular mass of the halogen substituent. But also the bromocatechol isomers, which have an identical molecular mass, were well separated (Table 3).

Complete transformation of 2-chlorophenol to 3-chlorocatechol

To elucidate whether it is possible to obtain a complete biotransformation of initially 1 mM 2-chlorophenol three media compositions (LB, CFG, CFGY) were compared. After 12 h of incubation the 2-chlorophenol concentrations for the three media were the following: 0.75 mM, 0.89 mM and 0.95 mM, respectively. LB medium was chosen for further batch cultivation.

As shown in Figure 2 the stationary growth phase was reached after 15 h and between 15 h and 20 h the maximal 2-chlorophenol transformation rate was observed. After 33 h no more 2-chlorophenol was detected. Under identical conditions complete transformation was observed when 2-chlorophenol was substituted with 2-bromophenol.

The transformation of 2-chlorophenol results in an accumulation of 3-chlorocatechol in the medium, but no equimolar quantities of the chlorinated catechol were present at any time. For example after 15 h the 3-chlorocatechol concentration was 0.2 mM whereas 0.4 mM 2-chlorophenol had already been transformed. This apparent contradiction can be explained by the autoxidation of halocatechols. 3-Chlorocatechol is not stable at higher temperatures (Figure 3). For example at 70 °C 30% are decomposed within 2 h while at 40 °C only 5% loss was detected when incubated in CF medium.

The decomposition was determined to be a first order reaction as seen by the linear relationship between the logarithm of concentration versus time. There was no significant difference when using phosphate buffer. We assume that there is no contribution of the additional salts to the decomposition reaction.

The half-life times obtained for 40 °C, 50 °C, 60 °C, and 70 °C were 30.9 h, 17.2 h, 11.5 h and 4.5 h, respectively. The rate of decomposition of 3-chlorocatechol was not influenced by the addition of 2-chlorophenol or phenol. The phenols were stable at all incubation temperatures.

Discussion

Only a few reports have been published on the degradation of phenolic substances in the thermophilic temperature range and they are restricted to aerobic processes (Buswell 1975; Gurujeyalakshmi & Oriel 1989; Adams & Ribbons 1988). In the majority of cases phenol could only be degraded with the supplementation of culture medium with yeast extract and peptones. The effects of these complex components on the induction of the phenol hydroxylase and catechol 2,3-dioxygenase remained unclear.

Yanase et al. (1992) described two isolates, a *Bacillus* sp. and strain 501 assigned to the family of *Rhizobiaceae*, capable of phenol degradation with optimal growth temperatures of 50 °C. The latter was able to transform various monohalophenols under resting cell conditions at 50 °C, but no metabolites were analysed. Halogenated phenols could not serve as growth substrates for these isolates, suggesting a higher toxicity, failure of induction of the catabolic enzymes or simply the absence of suitable enzyme machinery for the halogenated phenols. In *Bacillus* sp. A2 we detected a sufficient cell viability and the presence of a hydroxylase with activity towards some halophenols.

A number of aerobic mesophiles have been shown to degrade halogenated phenols especially chlorophenols, and the corresponding pathways have been elucidated (Li et al. 1991; Schmidt & Knackmuss 1980; Knackmuss & Hellwig 1978). Generally speaking, mono- and dichlorophenols are channelled via their chlorocatechols into a productive ortho-cleavage, with dechlorination only after ring cleavage, whereas higher chlorinated phenols are dechlorinated prior to the ring fission through hydroxylating and/or reductive reactions (Häggblom 1990). Janke et al. (1989) studied a *Rhodococcus* strain that hydroxylated chlorophenols to chlorocatechols. Only 4-chlorocatechol was produced from 3-chlorophenol. On the other hand, *Pseudomonas* sp. B13 converted 3-chlorophenol to a mixture of both 3- and 4-chlorocatechol and resembles in this respect the *Bacillus* sp. described here.

Non-induced cells of several strains of the genus *Rhodococcus* were found to hydroxylate mono-, di- and trichlorophenols at the ortho position to chlorocatechols, which accumulated in the culture broth (Häggblom et al. 1989). Non-induced cells of the thermophilic *Bacillus* strain A2 hydroxylate different substrates without metabolising the products further indicating a comparable broad substrate specificity of the first enzyme. The operating hydroxylase is induced by several halophenols. This gratuitous and incomplete metabolism of chloroaromatics is in good correlation with the fact that there is no known history of previous exposure to chlorinated aromatics for this isolate. The recombinant *E. coli* (containing a plasmid with the phenol hydroxylase gene from a thermophilic *Bacillus*) produced by Kim and Oriel (1995) also showed inducibility by substituted phenols, especially 2-chlorophenol, which did not serve as growth substrate for the wild type.

Many examples exist for pure cultures which cannot use halophenols as growth substrate but are able to transform them into dead end products, especially halocatechols (Bartels et al. 1984). The transformation of a non growth substrate by growing cells in the presence of a growth substrate or by resting cells in the absence of a growth substrate with or without an electron donor present is defined as cometabolism (Horvath 1972). The corresponding principles depending on the presence or absence of growth substrate and electron donor have been modelled in the case of phenol cometabolism (Criddle 1993).

The most important features for resting cells are: loss of activity due to endogenous decay, transformation of the non growth substrate and therefore depletion of reducing power in the case of NAD(P)H dependent hydroxylations and product inhibition caused by accumulated metabolites exerting inactivation. Janke et al. (1989) have shown that the primary function of the cosubstrates in the cometabolic turnover of aniline, phenol and some chlorinated derivatives was to provide reducing power for the first step of hydroxylation.

These findings prompted us to further stimulate the cometabolic transformation using growing cells providing reducing power for the hydroxylase. Indeed, in contrast to resting cells we achieved a complete transformation of 2-chlorophenol and 2-bromophenol into the corresponding halogenated catechols. The results of these experiments, however, pointed to an interesting question about the fate of the catechols. Chlorocatechols normally inhibit *meta*-cleaving dioxygenases. Bartels et al. (1984) detected the formation of 2-hydroxypenta-2,4-dienoic acid using 3-chlorocatechol as substrate. They suggested 5-chloroformyl-2-hydroxypenta-2,4-dienoic acid to be the actual suicide product of *meta*-cleavage. In contrast, with *Pseudomonas putida* 3-chlorocatechol itself was a potent inhibitor of the catechol 2,3-dioxygenase (Klecka & Gibson 1981). Further investigations with the purified dioxygenase could clarify the inactivation

of the catechol 2,3-dioxygenase of the *Bacillus* sp. A2 by 3-chlorocatechol.

At higher temperatures the labile 3-chlorocatechol is oxidised and polymerised much faster to a brownish undefined polymer than under mesophilic conditions. These reactions can lead to a complete removal of halogenated phenols under cometabolic conditions often encountered in remediation processes and waste water treatment. Upon the addition of extra growth substrates the toxic 2-chlorophenol was transformed into 3-chlorocatechol and finally removed by the polymerisation reaction. During the transformation of 1 mM 2-chlorophenol to 3-chlorocatechol concentration never exceeded 0.6 mM. Since growth was not inhibited during these experiments, the polymer formed seemed not to have an inhibitory effect on the growth of *Bacillus* sp. A2. Therefore, under thermophilic conditions in contrast to mesophilic conditions the cometabolic conversion of chlorophenols to chlorocatechols causes no problems.

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