Degradation of Organochlorine Compounds in Spent Sulfite Bleach Plant Effluents by Actinomycetes

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Actinomycetes isolated from different soil samples were tested for their abilities to utilize spent sulfite bleach effluents from a paper mill. Degradation and dechlorination of the chlorinated compounds in the effluents of the first two bleaching stages, i.e., chlorination stage [(C + D)_{tot}] and alkaline extraction stage (E_{10}), were monitored by determining total organic carbon (TOC) and activated-carbon-adsorbable organic-bound halogen (AOX). The isolates showed increased degradation rates after repeated incubations in the effluent-containing medium. Separation of the culture supernatants by ultrafiltration into three fractions of different molecular weights revealed substantial AOX and TOC reductions in the low-molecular-weight fraction. The AOX values of the higher-molecular-weight fractions were also reduced. Extracellular peroxidase and cell wall-bound catalase activities were produced during growth of the microorganisms on effluent.

MATERIALS AND METHODS

Microorganisms and culture maintenance. Actinomycetes were isolated by selective isolation procedures (16, 17) from 16 soil samples obtained from various places in Southeast Asia and southern Europe. Stock cultures of the isolates were maintained at 4°C after growth and sporulation at 30 or 37°C on MS agar (20 g of mannitol, 20 g of soya, and 20 g of agar per liter of deionized water), DSM (German Collection of Microorganisms, Braunschweig) medium 53 corynebacterium agar (10 g of casein peptone [tryptic digest], 5 g of yeast extract, 5 g of glucose, 5 g of NaCl, and 15 g of agar [adjusted to pH 7.2 to 7.4] per liter of deionized water), no. 65 streptomyces medium (4 g of glucose, 4 g of yeast extract, 10 g of malt extract, and 12 g of agar [adjusted to pH 7.2] per liter of deionized water), or no. 83 Czapek peptone agar (30 g of saccharose, 2 g of KNO_{3}, 1 g of K_{2}HPO_{4}, 0.5 g of MgSO_{4} · 7H_{2}O, 0.5 g of KCl, 0.01 g of FeSO_{4} · 7H_{2}O, 2 g of yeast extract, 5 g of peptone, and 15 g of agar [adjusted to pH 7.3] per liter of deionized water). Liquid cultures consisted of 0.3 g of NaNO_{3}, 0.66 g of K_{2}HPO_{4} · 3H_{2}O, 0.01 g of CaCl_{2}, 5 g of glucose, 5 g of D-mannitol, 2 g of yeast extract, 1 ml of trace element solution (11), and 8.75 ml of 0.5 M phosphate buffer (pH 6.0) per liter of deionized water. Arthrobacter picolinophilus (DSM 20665) was obtained from the German Collection of Microorganisms and maintained at 4°C after growth at 30°C on DSM medium 53.

Screening procedure. Experiments were carried out with pure cultures. Sampling and substrate additions were made under aseptic conditions, and appropriate noninoculated controls were included. Screening for growth on sulfite spent bleach plant effluents from the first two stages of a conventional bleaching sequence was performed on agar plates containing a mixture of 166 ml of E_{10}-O-stage effluent, 333 ml

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of (C+D)red-stage bleach effluent. 500 ml of double-distilled water, and 20 g of agar per liter of medium. Isolates growing on this medium were subcultured on the same medium to obtain spores to be used as inocula for liquid cultures. The liquid medium for degradation experiments contained 14.8 g of Na₂HPO₄·2H₂O, 2.3 g of KH₂PO₄, 5 g of yeast extract, 2 g of (NH₄)₂SO₄, 0.05 g of Ca(NO₃)₂, 1 ml of trace element solution (11), 333 ml of (C+D)red-stage bleach effluent, 166 ml of E₁₀-stage bleach effluent, and 500 ml of double-distilled water per liter. The E₁₀/C+D ratio of the medium reflects the conditions found in the effluents of the paper mill. Liquid cultures for degradation experiments and production of extracellular enzymes were performed in 500-ml shaking flasks with 180 ml of medium at 30 or 37°C, respectively, and 150 rpm. The inoculum size for isolates growing as single cells gave a final cell density of about 10⁸/ml of medium. The inoculum sizes of isolates forming mycelial pellets could not be determined. Autoclaved media were used for adaptation and maintenance of the isolates. Cultures used for measuring AOX, total organic carbon (TOC), and DOC reductions and extracellular peroxidase activities contained nonautoclaved bleach effluents. The media had a pH of 7.2, which was readjusted with 3 N HNO₃ every 24 to 36 h.

**Substrates and chemicals.** Bleach effluents were from a paper mill (Cellulose Attitzhal AG, Luterbach, Switzerland). (C+D)red-stage bleach effluent contained spruce and beech chlorolignins, and E₁₀-stage bleach effluent contained beech chlorolignin only. The distribution of AOX, TOC, and color unit (CU) values in the molecular weight fractions of the two bleach effluents is given in Table 1. The pulp mill used a bleaching sequence of (C+D)red, E₁₀, (H+D), E, and then D. All other chemicals used were from Aldrich, Merck, or Sigma and were analytical grade.

**Enzyme assays. (i) Peroxidase and phenol oxidase.** After being separated from the cells by centrifugation and pH adjustment to pH 7.0, culture supernatants were routinely assayed for peroxidase and phenol oxidase activities. All assays were performed at 40°C. Final volumes of 1.0 ml of the reaction mixtures contained (i) 100 mM phosphate buffer (pH 7.0), 1 mM L-3,4-dihydroxyphenylalanine (l-DOPA), and 4 mM H₂O₂ (23); (ii) 100 mM acetic acid-acetate buffer (pH 5.5), 48 μM o-dianisidine, and 4 mM H₂O₂ (3); (iii) 50 mM morpholinencethanesulfonic acid (MES)-NaOH buffer (pH 5.5), 320 μM syringaldazine (dissolved in ethanol; the final ethanol concentration in the assay was 10%), and 4 mM H₂O₂ (4); (iv) 100 mM sodium succinate buffer (pH 5.5), 82 mM 4-aminoantipyrine, 1 mM 2,4-dichlorophenol, and 4 mM H₂O₂ (23) and (as part of all assays) 750 μM of enzyme solution. The reaction was initiated by the addition of hydrogen peroxide solution for peroxidase assays, and the increases in A₄₉₂, A₄₂₅, A₅₂₅, and A₅₄₀, respectively, were monitored for 5 min, starting 30 s after the addition of hydrogen peroxide. Phenol oxidase assays were started by the addition of enzyme solution. One unit of enzyme activity was expressed as the conversion of 1 μmol of substrate per min. The following extinction coefficients were used: l-DOPA ε₄₉₂, 3,088 M⁻¹ cm⁻¹; o-dianisidine ε₄₆₀, 24,039 M⁻¹ cm⁻¹; syringaldazine ε₅₂₅, 31,659 M⁻¹ cm⁻¹; and 2,4-dichlorophenol ε₅₄₀, 21,647 M⁻¹ cm⁻¹.

(ii) Catalase. Catalase activity in culture supernatants and cell suspensions was measured with a Rank Brothers (Bottisham, England) Clark-type oxygen electrode. Appropriate volumes of enzyme preparation or cell suspension were brought to 50°C and degassed with nitrogen. After dilution with 50 mM phosphate buffer (pH 7.0) to a final volume of 2 ml in a temperature-controlled measuring cell, the reaction was initiated by the addition of 20 μl of 0.1 M sodium perborate solution. Reading of the electrode current started 15 s after the addition of sodium perborate. Calibration of the oxygen electrode was done with catalase (from beef liver; catalog no. 106810; Boehringer Mannheim GmbH) and various amounts of sodium perborate (8, 24). Cells were treated for 30 s by ultrasonication at 50 W (Branson Sonifier B-12; Branson, Danbury, Conn.) in a 50 mM phosphate buffer (pH 7.0) containing 0.3% CHAPS (3-[3-cholamidopropyl]dimethylammonio-1-propane sulfonate; Serva, Heidelberg, Germany) to release cell wall-bound catalase.

**Chemical analysis.** Determination of AOX in the culture supernatant was done after removal of the cells by centrifugation. Organochlorine compounds associated with cells or mycelium during the growth in bleach plant effluent-containing medium were determined by direct application of washed cells to the AOX analyzer. Analysis was performed (according to DIN 38409, part 14 [1a]) with an AOX analyzer (Euroglas, Delft, The Netherlands).

Determinations of TOC and DOC were performed simultaneously with a Hydromat (Westhoff GmbH Meßtechnik, Bochum, Germany). In contrast to the standard TOC and DOC analysis methods, filtration of the sample through 0.45-μm-pore-size membranes was omitted because of adsorption of AOX material on the membrane.

Culture supernatants were separated by molecular weight into three fractions by using ultrafiltration in a 400-ml Amicon cell (W. R. Grace & Co., Amicon Div., Danvers, Mass.) with Amicon membranes PM 10 (10,000 cutoff) and YM 2 (1,000 cutoff). Samples were adjusted to pH 7.0 and filtered through 8-μm-pore-size cellulose nitrate membranes (Sartorius, Göttingen, Germany) prior to ultrafiltration. The medium was concentrated 10-fold and washed four times by refilling the cell with water purified with a NANOpure system (Barnstead Thermoline Corp., Dubuque, Iowa) to its original volume and then concentrated. The efficiency of the washing steps was controlled by measuring the conductivity of the filtrate.

### Table 1. AOX, TOC, and CU contents of fractions of E₁₀- and (C+D)red-stage bleach effluents

<table>
<thead>
<tr>
<th>Mol wt</th>
<th>E₁₀</th>
<th>(C+D)red</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOXb</td>
<td>120.9</td>
<td>98.6</td>
</tr>
<tr>
<td>L</td>
<td>15.2</td>
<td>21.2</td>
</tr>
<tr>
<td>M</td>
<td>49.9</td>
<td>40.2</td>
</tr>
<tr>
<td>H</td>
<td>45.2</td>
<td>31.1</td>
</tr>
</tbody>
</table>

| TOC   | 1,295 | 653    |
| L      | 470   | 438    |
| M      | 405   | 125    |
| H      | 420   | 90     |

| CU    | 6,194 | 3,152  |
| L      | 470   | 432    |
| M      | 1,163 | 784    |
| H      | 4,561 | 1,936  |

a After adjustment to pH 7.0 and filtration through 8-μm-pore-size cellulose nitrate filters, the filtrate was separated into three molecular weight fractions, i.e., L (<1,000), M (1,000 to 10,000), and H (>10,000), by ultrafiltration through Amicon membranes PM 10 and YM 2.

b In mg of Cl liter⁻¹.

c In mg of C liter⁻¹.
CU were measured according to the Canadian Pulp & Paper Association standard method (1b).

RESULTS

Isolation of actinomycetes. Isolation of actinomycetes from 16 different soil samples resulted in 217 cultures, mainly streptomycetes, growing at 30 and 37°C.

Screening and adaptation on bleach plant effluents. A total of 208 actinomycete isolates and *A. picolinophilus* were tested for growth on agar plates containing bleach plant effluents. Of these isolates, 66 did not grow on the agar plates. *A. picolinophilus* and 105 of the isolates which grew well on the agar plates were selected for further tests in liquid medium. Measurement of the remaining AOX contents at the end of the first and third incubation periods of 21 days each revealed for isolates BW 42, BW 108, BW 154, BW 171, BW 184, and BW 212 and for *A. picolinophilus* an increase in degradation rates (Fig. 1). Release of organically bound chlorine increased during the adaptation period from 12 to 45%, corresponding to reductions of 7.4 to 25 mg of Cl liter⁻¹, respectively. Isolate BW 184 showed the highest reduction (27.2%, or 14.4 mg of Cl liter⁻¹) in AOX content during the first incubation period. Uninoculated controls showed a 10% decrease in AOX content due to autohydrolysis within 21 days.

Time course of AOX, DOC, and DOC reductions. Figure 2 shows the decrease of AOX in the culture supernatants of *A. picolinophilus* and isolates BW 22, BW 93, BW 184, and BW 214 and the corresponding TOC and DOC values of isolate BW 214. Most of the microbial AOX degradation, about 18 to 28%, corresponding to 9 to 13.8 mg of Cl liter⁻¹, occurred during the first 24 h. Further reductions of 12% (9 mg of Cl liter⁻¹) could be measured during the following 48 to 72 h. A slight increase of 2 to 3 mg of Cl liter⁻¹ in the culture supernatants was observed at the end of the incubation period. Figure 2 includes TOC and DOC values for isolate BW 214. Major reductions took place during the first 24 h, when TOC values decreased to 35.5% of the initial value (from 2,250 to 800 mg of Cl liter⁻¹) and DOC decreased to 36.8% of the initial value (from 5,950 to 2,190 mg of O₂ liter⁻¹). AOX, TOC, and DOC values behaved similarly over the whole incubation period. The same pattern was observed for the other isolates (data not shown).

Some cultures were inoculated with a spore suspension (AOX-free inoculum) to estimate the amount of loss of AOX in the supernatant due to association with the cells by adsorption, undegraded intracellular metabolites, or entrapment in the microbial mycelium. The results indicated that only 3.5 to 4.9% of the initial AOX content becomes associated with the cells.

Changes in AOX, TOC, and CU contents of three bleach effluent molecular weight fractions. Ultrafiltration of the two bleach effluent fractions and the medium resulted in three molecular weight fractions: L (<1,000), M (1,000 to 10,000), and H (>10,000). Table 2 lists the AOX, TOC, and CU contents of the culture supernatant molecular weight fractions at the end of a 21-day incubation. AOX values decreased in all three molecular weight fractions. AOX values in the L fractions decreased as much as 35 to 59% (4.7 to 7.9 mg of Cl liter⁻¹), and the AOX for *A. picolinophilus* was below the detection limit. The corresponding TOC values decreased by 68.8 to 94.4% (1,035 to 1,421 mg of C liter⁻¹). Expressed as a percentage, this decrease is much higher than that for the corresponding AOX values. In the M fractions, AOX values decreased by 28 to 70% (4.6 to 9.2 mg of Cl liter⁻¹). The corresponding TOC values decreased similarly, by 61.3 to 82.7% (230 to 310 mg of C liter⁻¹). AOX values in the H fractions decreased by 10 to 30% (1.7 to 6.6 mg of Cl liter⁻¹), whereas the TOC values increased up to 228.2% (from 195 to 445 mg of C liter⁻¹) of the initial value. All TOC values for H fractions at the end of the 21-day incubation were above the initial value.

The total CU in the culture supernatants of all isolates increased during the incubation period. The increases in total CU varied between 790 and 2,260 CU. L- and M-frac-
TABLE 2. AOX, TOC, and CU contents of molecular weight fractions of bleach effluent medium at the end of 21-day incubation

<table>
<thead>
<tr>
<th>Mol wt fraction</th>
<th>Incubation witha:</th>
<th>A. picolinophilus</th>
<th>BW 42</th>
<th>BW 93</th>
<th>BW 154</th>
<th>BW 171</th>
<th>BW 184</th>
<th>BW 212</th>
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<tbody>
<tr>
<td></td>
<td>Controlb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOXc</td>
<td>L</td>
<td>13.5</td>
<td>ND</td>
<td>6.8</td>
<td>6.1</td>
<td>6.1</td>
<td>5.6</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>16.0</td>
<td>4.8</td>
<td>11.2</td>
<td>10.3</td>
<td>10.9</td>
<td>11.2</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>19.3</td>
<td>15.3</td>
<td>16.4</td>
<td>12.7</td>
<td>12.9</td>
<td>13.6</td>
<td>12.6</td>
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<tr>
<td>TOCd</td>
<td>L</td>
<td>1,505</td>
<td>113</td>
<td>140</td>
<td>98</td>
<td>104</td>
<td>95</td>
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<td></td>
<td>M</td>
<td>375</td>
<td>75</td>
<td>100</td>
<td>85</td>
<td>90</td>
<td>90</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>195</td>
<td>445</td>
<td>215</td>
<td>285</td>
<td>315</td>
<td>335</td>
<td>245</td>
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<tr>
<td>CU</td>
<td>Total</td>
<td>4,179</td>
<td>4,967</td>
<td>5,063</td>
<td>5,711</td>
<td>6,281</td>
<td>5,147</td>
<td>6,436</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>1,134</td>
<td>418</td>
<td>431</td>
<td>446</td>
<td>362</td>
<td>361</td>
<td>704</td>
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<tr>
<td></td>
<td>M</td>
<td>1,731</td>
<td>849</td>
<td>1,182</td>
<td>996</td>
<td>1,030</td>
<td>1,011</td>
<td>916</td>
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<tr>
<td></td>
<td>H</td>
<td>1,314</td>
<td>4,746</td>
<td>3,356</td>
<td>3,620</td>
<td>4,318</td>
<td>4,909</td>
<td>3,526</td>
</tr>
</tbody>
</table>

a All strains and the control were incubated at 30°C. ND, not detected.
b Uninoculated medium.
c In mg of Cl liter−1.
d In mg of C liter−1.

tion CU decreased by 430 to 1,000 CU, but H-fraction CU increased by 2,042 to 3,595 CU.

Extracellular enzyme activities. Decreases in AOX and TOC values during growth on bleach effluents for compounds with molecular weights above 1,000 were expected to involve extracellular or cell wall-bound enzymes. Isolates growing on bleach plant effluents were analyzed for extracellular peroxidase and phenol oxidase activities, with L-DOPA, syringaldazine, o-dianisidine, and 2,4-dichlorophenol used as substrates. Of 106 organisms, 47 showed peroxidase activities in the culture supernatants with L-DOPA, reaching activity levels of up to 24 mU ml−1. Only a few also exhibited low activities with 2,4-dichlorophenol, o-dianisidine, or syringaldazine as substrate, with activity levels of 0.5 to 5 mU ml−1. Figure 3 shows the time courses of peroxidase activities with L-DOPA as substrate in culture supernatants of isolates BW 1, BW 9, BW 42, and BW 206 grown on the bleach effluents. Maximum L-DOPA-peroxidase activities were reached during the exponential growth phase after 24 to 48 h of incubation. The appearance of this peroxidase activity and the reductions in AOX and TOC values coincided. The peroxidase activity measured with 2,4-dichlorophenol as substrate reached its maximum during the late exponential growth phase about 96 h after inoculation. Most of the isolates showed extracellular phenol oxidase activity with 2,4-dichlorophenol as substrate, reaching activity levels of up to 3 mU ml−1. Only a few also exhibited phenol oxidase activities when L-DOPA or syringaldazine was used as substrate. Phenol oxidase activity reached its peak levels at 3 to 7 days after inoculation during the late exponential and stationary growth phases.

Catalase activities could also be observed in culture supernatants. Maximum activity of 1.5 U ml−1 was measured in the culture supernatant of BW 42 grown on bleach effluent-containing medium. Assays with cell suspensions showed that catalase activities were also associated with the cells. Only a small proportion of this catalase activity could be released from the cells by ultrasonic treatment in phosphate buffer containing 0.3% CHAPS.

DISCUSSION

The data presented in this paper demonstrate the ability of actinomycetes to degrade and partly dehalogenate chlorinated compounds in bleach effluents. The strains were isolated under nonspecific conditions and had not previously been in contact with bleach effluents. After repeated incubations of several of the strains in bleach effluent-containing medium, increased AOX reduction rates were found, indicating the ability of the organisms to adapt to the conditions prevailing in the medium. This AOX reduction in the supernatants is not due to adsorption to the cells or autohydrolysis of unstable organochlorine bonds. The characterization of the degraded bleach effluents which were separated into three fractions by molecular weight revealed different patterns of AOX and TOC reductions. Major changes occurred in the L and M fractions, in which the AOX, TOC, and CU values were reduced during the incubation period. The bleach effluents and the yeast extract,
which serves as an additional C source, contribute to the TOC value measured in the medium. Therefore, the initial molar TOC/AOX ratio, an indicator of the average degree of halogenation, was calculated from the TOC values of the corresponding bleach effluent molecular weight fractions. Assuming a total consumption of yeast extract in the L and M fractions during the incubation period, the TOC/AOX ratios increased in the L fractions in cultures of *A. pichinophilus* and isolate BW 42 and in all of the M fractions. This indicates a lower average degree of halogenation of the remaining compounds in the medium after treatment with the organisms. The ratio stayed constant in the L fractions for all other isolates. With the exception of that of *A. pichinophilus*, no L fractions showed total AOX reductions.

O-Methylation of phenolic compounds, which can be constitutively performed by actinomycetes, leads to the formation of chloroveratroles and chloroanisoles (1, 20, 21). These conversions could have occurred in the medium and contributed to the remaining AOX in the L and M fractions. In the H fractions, AOX reductions could be observed, but TOC values were increased. This indicates that the TOC reductions detected in the other fractions could be partly due to condensation reactions. Polymerization leading to compounds of higher molecular weights has not yet been reported to occur by treatment of bleach effluents with actinomycetes. Other groups (1, 7, 20) have reported formation of chloroguaniaocls and its O-methylation products by treatment of bleach effluent fractions above 1,000 molecular weight (H+M) with *Arthrobacter* spp. and other actinomycete species. Eriksson et al. (7) reported only minor TOC reductions in H+M chloroguaniacls during a 40-day incubation. Another indication for modifications in the H fractions and/or condensation of low-molecular-weight compounds is the observed increase in TOC. Replacement of chloride substituents by hydroxy groups and formation of quinones increases the TOC of the chloroguaniaocls. Chromophore contents in the M and L fractions were substantially reduced despite a strong increase in overall TOC. Compounds in the H fractions are most likely attacked by extracellular or cell wall-bound enzymes. These modifications also alter the solubility and hydrophobicity of the compounds. The different degradation patterns found in the three molecular weight fractions could indicate the involvement of enzymes in the degradation and dehalogenation of bleach effluents.

Four phenol oxidase-peroxidase substrates were used for the determination of extracellular enzyme activities in the culture supernatants of organisms grown on bleach effluents. Mainly peroxidases were found when L-DOPA was used as substrate, and low levels of peroxidases and phenol oxidases could be detected when 2,4-dichlorophenol was used as substrate. The isolates grown on chloroguaniacls showed peaks of peroxidase activity during their growth phase after 24 to 48 h of incubation when L-DOPA was used as substrate. Major AOX and TOC reductions occurred during the same period. The 2,4-dichlorophenol-peroxidase and 2,4-dichlorophenol–phenol oxidase activities appeared during the late exponential and stationary growth phases, during which only minor AOX reductions occurred, indicating no major role for these phenol oxidases and peroxidases in the degradation of the chlorinated compounds in bleach effluents under cometabolic conditions. Horseradish peroxidase and a laccase from streptomycetes are known to polymerize chlorophenols with the release of chloride (5).

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**REFERENCES**

18. Leuenberger, C., R. Coney, J. W. Graydon, E. Molnár-Kubica,
25a.van Loon, W. M. L. M. Personal communication.