Utilization of dimeric lignin model compounds by mixed bacterial cultures

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Summary. The degradation of dimeric phenylpropanoid lignin model compounds using mixed bacterial cultures was studied. The six model compounds contained the most common linkages of lignin: β-O-4, β-β, β-5, and β-1. The results indicate that it is possible to enrich bacteria which are able to degrade all these compounds. Bacteria were also able to use these dimers as the sole source of carbon for growth. In view of these results it seems probable that bacterial inability to degrade polymeric lignin is due to the physical properties such as the molecular size of lignin.

Introduction

Fungi are believed to be mainly responsible for returning of lignin into the carbon cycle while bacteria may be important for the degradation of small fragments arising from fungal degradation of lignin (Ander and Eriksson 1978; Crawford 1981). Several authors have reported partial degradation by bacteria of synthetic lignin models (DHP) (Trojanowski et al. 1977; Haider et al. 1978; Kaplan et al. 1980), in situ 14C-phenylalanine labeled lignin of poplar (Odier et al. 1981), douglas fir (Crawford 1978; Crawford and Sutherland 1979, 1980; Phelan et al. 1979) or spruce (Robinson and Crawford 1978) or 14C-labeled lignosulfonic acids (Haider and Trojanowski 1981).

At least two reasons could explain why bacteria degrade lignin only partially. Firstly, the permeability barrier of the bacterial cell membrane may exclude molecules of the size of lignin. Metabolism could hence occur only if effectuated by extracellular catalysts. Secondly, lignin may contain chemical bonds which cannot be cleaved by bacteria. Before taking on a study on the degradation of oligomeric lignin-born materials (industrial lignins) we studied the abilities of 11 mixed enrichment cultures of bacteria to utilize different types of dimeric phenylpropanoid model compounds. Degradation of the model compounds was determined by high performance liquid chromatography. The results are reported in this paper.

Materials and methods

Model compounds. The structures of compounds are presented in Fig. 1. 1-(3-methoxy-4-hydroxyphenyl)-2-(2-methoxyphenoxymethylene)-propane-1,3-diol (guaiacylglycerol-β-guaiacyl ether, compound I), was synthesized using the procedure described by Miksche et al. (1966). 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxymethylene)-propane-1,3-diol (veratrylglycerol-β-guaiacyl ether, compound II) was a gift from K. Lundquist, Chalmers University of Technology, Gothenburg. 1,2-bis(3-methoxy-4-hydroxyphenyl)-1,3-propanediol (compound III) was synthesized using a procedure described elsewhere (Alhonen et al. 1983; Brunow et al. 1981). d-pinoresinol (compound IV) was from the same source as compound II and

Fig. 1. The dimeric phenylpropanoid compounds used in the present study
d-1-syringaresinol (compound V) from T. Higuchi, synthesized by A. Kamaya, Institute of Wood Research, University of Kyoto. Dehydrodiosgenoicogenol (compound VI) was prepared from trans-isoeugenol according to Leopold (1950). The chemical identity and purity of each compound was confirmed with nuclear magnetic spectrometry and high-performance liquid chromatography.

Cultures. The 11 mixed cultures used in this study originated from inoculates collected in an area which was polluted by pulp and paper industry waste waters. The inoculates were enriched for 5 months at room temperature on ten different media composed of liquors from pulping industry: thermomechanical pulping waste water (1); E-stage spent liquor of oxygen bleaching (2); black liquor at pH 9 (3) and 7.5 (4); O-stage effluent of oxygen bleaching with bark chips at pH 9.5 (5), at pH 9.5 with NH4Cl (0.8 g/l), K2HPO4 (1.2 g/l), KH2PO4 (0.8 g/l), NaCl (0.04 g/l), and MgSO4·7H2O (0.1 g/l) (6), and at pH 9.5 without addition of nutrients (7). The oxygen bleaching liquor was ultrafiltered with a filter with a cut-off of 0.5,000 D and the permeate and retentate were used in enrichments 8 and 9, respectively, at pH 8. Culture 10 was obtained from a bioreactor described elsewhere (Salkinioja-Salonen et al. 1983).

Culture number 11 was forest soil without further enrichment. Waste waters from thermomechanical pulping contained 3.5–10 mg N/l and 0.16–23 mg P/l, bleaching liquors 6–8 mg N/l and 1.6–2.6 mg P/l, and black liquor ca. 14 mg N/l and ca. 2 mg P/l.

Dimer degradation experiments. The cultures were tested in the following way for their ability to utilize dimeric lignin model compounds as the sole source of carbon. A 50 μl inoculum of a mixed culture was diluted in 1 ml of a mineral salts medium containing (NH4)2SO4 (1.0 mg), KH2PO4 (5.0 mg), MgSO4·7H2O (0.1 mg), FeSO4·7H2O (0.01 mg) per milliliter, supplemented with 5 μl of a vitamin mixture (Sandman 1964) 1.0 μl of a trace element mixture (Bauchope and Eldred 1960) and 0.3 mg of compound I per milliliter of medium. The pH of the medium was adjusted to 7.0. The cultures were incubated aerobically without agitation in 5-ml tubes at 28°C and diluted after 7 days into a fresh medium (50 μl of culture into 1 ml of the same medium). These cultures were used as inocula for experiments with the dimeric phenylpropanoid compounds I–VI (Fig. 1).

The effects of the antibiotics chloramphenicol and cycloheximide (Sigma, St. Louis, USA) (0.10 mg/ml) were examined using 1 ml of the liquid medium as above with 0.15 mg/ml of compound I.

An uninoculated control sample was prepared for each test incubation.

Measurement of bacterial growth. Bacterial growth was estimated by the increase in turbidity at 420 nm after settlement of insoluble substrate and possible precipitates. Biomass was calculated from the turbidity as described in the literature (Koch 1981). The content of protein of the cultures grown on dimers could not be determined by the Lowry procedure because of interference by phenolic substrates and the quantity of biomass protein was too small for other methods of protein assay.

Chromatography. At the end of the incubation (7–12 days) 1 ml of acetonitrile was added per milliliter of culture to dissolve unused substrate and possible reaction products. Insoluble solids (bacterial debris) were then removed by centrifugation. The acetonitrile extract was analyzed with a high-performance liquid chromatograph (Micromeritics Instrument Corp., Norcross, GA, USA) connected to a variable wavelength UV/vis-detector. Rad-Pak C18 (5 or 10 μm) columns were used in an RCM-100 compression module (Waters Associates Inc., Milford, MA, USA). Acetonitrile and water mixtures containing 1% acetic acid were used as mobile phases.

Results

Tables 1 and 2 show how each of the cultures utilized the different model compounds. The percentage of degradation was calculated from the difference in the

Table 1. Degradation of model compounds I–VI

<table>
<thead>
<tr>
<th>Culture</th>
<th>Compound</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent degraded of input</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>17</td>
<td>81</td>
<td>100</td>
<td>42</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>100</td>
<td>49</td>
<td>24</td>
<td>17</td>
<td>4</td>
<td></td>
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<tr>
<td>3</td>
<td>100</td>
<td>100</td>
<td>94</td>
<td>80</td>
<td>62</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>100</td>
<td>91</td>
<td>100</td>
<td>30</td>
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<td>100</td>
<td>100</td>
<td>96</td>
<td>0</td>
<td>47</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>100</td>
<td>86</td>
<td>100</td>
<td>66</td>
<td>78</td>
<td></td>
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<tr>
<td>7</td>
<td>100</td>
<td>100</td>
<td>79</td>
<td>100</td>
<td>44</td>
<td>0</td>
<td></td>
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<td>8</td>
<td>100</td>
<td>24</td>
<td>22</td>
<td>95</td>
<td>39</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>53</td>
<td>20</td>
<td>0</td>
<td>38</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>100</td>
<td>95</td>
<td>100</td>
<td>37</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>62</td>
<td>6</td>
<td>0</td>
<td>8</td>
<td></td>
<td></td>
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<tr>
<td>Average</td>
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<td>97</td>
<td>73</td>
<td>65</td>
<td>64</td>
<td>42</td>
<td>18</td>
</tr>
</tbody>
</table>

Input of dimer (mg/ml) | 0.3 | 0.3 | 0.5 | 0.3 | 0.5 | 0.5

Incubation time (days) | 12  | 12  | 7   | 7   | 7   |

* Degradation is expressed as [(m0−mα)/m0] × 100%, where m0 = amount of dimer recovered from the uninoculated control sample, mα = remaining amount of dimer at the end of the incubation, = not determined

Table 2. Biomass yields for the cultures grown on lignin model dimers

<table>
<thead>
<tr>
<th>Culture</th>
<th>Compound</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biomass (μg/ml dry weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>52.6</td>
<td>2.5</td>
<td>77.2</td>
<td>38.1</td>
<td>67.2</td>
<td></td>
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<tr>
<td>2</td>
<td>87.7</td>
<td>35.5</td>
<td>12.5</td>
<td>0.0</td>
<td>51.0</td>
<td></td>
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<tr>
<td>3</td>
<td>126.4</td>
<td>73.8</td>
<td>118.3</td>
<td>7.7</td>
<td>70.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>96.0</td>
<td>50.4</td>
<td>39.2</td>
<td>46.8</td>
<td>47.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>35.2</td>
<td>52.8</td>
<td>4.5</td>
<td>80.2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>76.7</td>
<td>32.9</td>
<td>49.1</td>
<td>133.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>79.0</td>
<td>46.5</td>
<td>35.1</td>
<td>41.5</td>
<td>81.7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>52.0</td>
<td>10.1</td>
<td>22.6</td>
<td>30.8</td>
<td>47.7</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>17.8</td>
<td>7.9</td>
<td>2.1</td>
<td>38.3</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>86.0</td>
<td>79.0</td>
<td>121.2</td>
<td>60.5</td>
<td>61.6</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>14.0</td>
<td>-</td>
<td>27.5</td>
<td>0.0</td>
<td></td>
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<tr>
<td>Average</td>
<td></td>
<td>82.8</td>
<td>40.1</td>
<td>52.0</td>
<td>28.1</td>
<td>61.7</td>
</tr>
</tbody>
</table>

Biomass yields were calculated from culture turbidities (A600) (measured against uninoculated blank) according to Koch (1981)

= not determined
amount of dimer in incubated samples with and without inoculum. Compounds with a β-O-4 ether bond (I and II) were well degraded by the cultures. This was expected because cultures pregrown on compound I were used as inoculum and compounds I and II were closely related. However, the biomass yield was higher for compound I (average 82.8 µg/ml) than for compound II (average 40.1 µg/ml).

Figures 2 and 3 show that the maximum turbidity of the cultures was reached shortly after compound I had disappeared from the medium. The cultures grew to an optical density up to $A_{420} = 1.0$ (shown for compound I in Fig. 2). This is high considering the low substrate input (0.3 mg/ml). Many cultures grown on the other compounds (II—VI) were similarly dense.

The cultures grew turbid in a few days and in many cases no uv-absorbing (254 nm) compounds were left in the fully grown cultures, indicating disruption of aromatic rings. Examples of liquid chromatograms showing disappearance of the substrates are presented in Fig. 4.

Light microscopic and electron microscopic examination of the cultures showed that they were composed of bacteria exclusively. Table 3 shows how two antibiotics affected degradation of compound I. Chloramphenicol inhibited the degradation whereas cycloheximide had no effect. This confirmed the involvement of bacteria in the degradation; cycloheximide is known to affect eukaryotic cells and chloramphenicol prokaryotic cells.

The chromatograms showed that intermediate products were formed from compound III in several cultures. Two examples are presented in Fig. 5. Compound III used was pure erythro isomer. During incubation isomerization to threo isomer may have occurred. The peak area of the unincubated sample

<table>
<thead>
<tr>
<th>Culture</th>
<th>Percentage of degradation in the presence of</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No antibiotic</td>
<td>Chloramphenicol</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>57</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>41</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>26</td>
<td>100</td>
</tr>
<tr>
<td>Uninoculated control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Calculated as in the Table 1. Initial concentration of compound I was 0.15 mg/ml and incubation time 4 days

b Concentration 0.10 mg/ml
the combined area of the incubated control sample
with the shoulder. The ratio of the peak areas (main
peak : shoulder) in incubated control sample was
2 : 1.

Table 2 shows that biomass yield for compound
III averaged 52.0 μg/ml. The average biomass yields
for compounds IV and V were, 28.1 and 61.7 μg/ml,
respectively, despite their similar structures.

Compound V was metabolized somewhat less
efficiently (average 42%) than compound IV (aver-
age 64%). A pink colour emerged from compound V
after a few days' incubation in some of the cultures.
This may have been caused by accumulation of an
intermediate product with quinonic structure (ab-
sorption maximum at 522 nm). Degradation of
the compound V may have halted because of toxicity
of such a quinone to the bacteria.

Only three of the cultures degraded compound VI
to a significant degree. The biomass values were not
included in Table 2 because biomass assay was
disturbed by the insoluble substrate.

None of the compounds was completely resistant
against bacterial attack. Culture 6 metabolized each
of the six model compounds and produced biomass
using these compounds as the sole source of carbon
and energy. This culture was enriched by using
oxygen bleaching waste water at pH 9.5 with addition
of mineral salts.

Discussion

The most common linkages between the phenylpro-
panoid units of lignin are β-O-4-aryl ether, β-1, β-β,
β-5, and 5-5 bonds (Lai and Sarkinen 1971). One way
to study the biodegradability of lignin, is to use
phenylpropanoid dimers linked to each other with
one of these bonds. Bacterial degradation of such
lignin models has been mostly studied with dimers
containing the β-O-4 ether linkage (Trojanowski et
al. 1970; Crawford et al. 1973; Fukuzumi and
Katayama 1977; Katayama and Fukuzumi 1979b;
Rast et al. 1980; Odier et al. 1982). Compounds of
α,β-diaryl, pinoresinol and phenylecoumaran types
have also been studied (Katayama and Fukuzumi

The results presented here show that the inde-
dependently prepared cultures selected for growth on
one type of dimer [guaiacylglycerol-β-guaiacyl ether
(1)] also utilized other types of dimers. Dehydrodiso-
eugenol (VI) differed from the other phenylpropa-
noid dimers in that it was much less degraded.

Inspection of the cultures by light microscopy and
electron microscopy showed that they were com-
posed of bacteria. Some occasional fungal hyphae in
the primary cultures disappeared during continued
culture with guaiacylglycerol-β-guaiacyl ether (com-
pound I) as the carbon source. Absence of fungal
growth probably reflects the inability of fungi to use
ligninous materials as the sole source of carbon

Our results resemble those described by Fuku-
zumi and Katayama (1977) and Katayama and
Fukuzumi (1978, 1979a, b) who had isolated Pseu-
domonas putida (strain FK2) on 5,5'-dehydrovan-
illic acid and found that it was also capable of utilising
several different structurally unrelated dimers. This
coincidence may point at some type of genetic or
metabolic linkage in the biodegradation of these
dimeric lignin models.

The results (Table 2) show further that some
cultures not only degraded the different phenylpro-
panoid dimers so that the original compound com-
pletely disappeared, but biomass was also formed.
Although Katayama and Fukuzumi (1978, 1979a, b)
did not specify growth yields, it is obvious from their
data that their Pseudomonas strain could also use
several dimers as the source of carbon and energy.
According to Crawford et al. (1973) a Pseudomonas
strain E-3 was able to use compound 11 as its only
source of carbon and energy. A low biomass density
(10^7 cells/ml) was reported by Odier et al. (1981) for
bacteria grown on poplar lignin. In the other reports
where the degradation of dimeric, oligomeric or
polymeric lignin models was studied with bacteria, a
supplementing carbon source was always used (Tro-
The inability of bacteria to propagate efficiently on polymeric lignin might therefore be caused by the molecular weight of lignin or inaccessibility of the substrate to extra cellular enzymes. If bacterial metabolism of benzenoid compounds is confined to intracellular mechanisms, large molecules which are not transported into the cell cannot be metabolized. The permeability barrier of gram negative bacteria has been shown to be around 600–1,300 daltons for carbohydrates, polyethylene glycols, and peptides depending on the species (Payne and Gilvarg 1968; Nikiido and Nakae 1980). The permeability for lignin has not been determined. However, treatments of bacterial cells with organic solvents which are known to increase the permeability of cell membrane do not necessarily lead to decreased enzyme activity (Fukui and Tanaka 1982). This opens perspectives towards a lignin-based biotechnical process, since polymeric lignin can be converted into soluble oligomeric material by several known procedures, such as auto-oxidation under pressure (Sarkansen 1975), catalytic hydrolysis (Harris et al. 1938), acidolysis (Lundquist and Lundgren 1972; Lundquist 1976), hydrolysis (Lora and Wayman 1980), electrochemical oxidation or reduction (Brewer et al. 1948; Chun and Osteryoung 1981) or steam hydrolysis (Nimz et al. 1983).

References


Sandman V (1964) A description of some lignolytic soil bacteria and their ability to oxidize simple phenolic compounds. J Gen Microbiol 36: 171–188


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Note added in proof: This report is an extension and confirmation of the results reported earlier by Pellinen et al. (1983)