Oxidation of Lignin-Related Aromatic Alcohols by Cell Suspensions of *Methylosinus trichosporum*

DOUGLAS O. MOUNTFORT,* DAVID WHITE, AND RODNEY A. ASHER

Cawthron Institute, Private Bag, Nelson, New Zealand

Received 30 June 1989/Accepted 22 October 1989

Cell suspensions of *Methylosinus trichosporum* oxidized the aromatic alcohols benzyl alcohol, vanillyl alcohol, and veratryl alcohol to the corresponding aldehydes, and with the exception of vanillyl alcohol, the aldehydes were further oxidized to the corresponding aromatic acids. No other transformation was observed, and the methoxyl moieties attached to the aromatic nucleus remained intact. More than 70% of the alcohol oxidized could be accounted for by aldehyde and/or acid. Investigation of the inhibitor kinetics of EDTA or p-nitrophenylhydrazine (specific for NAD⁺-independent methanol dehydrogenase in methylotrophs) on aromatic alcohol oxidation revealed noncompetitive inhibition in which the *V*₂₅₆ was decreased but the *K*ᵢ remained unchanged. The pattern of inhibition of aromatic alcohol oxidation matched that of methanol oxidation, and the *K*ᵢ values for all of the substrates were similar (12 to 16 mM). The results indicate that the initial step in the oxidation of aromatic alcohols was similar to that for methanol, and because oxidation was incomplete (i.e., only the corresponding aldehyde or acid was produced), there may be some biotechnological advantages in using whole cells of methylotrophs to facilitate aromatic biotransformations.

Obligatory methylotrophic bacteria are dependent on oxidation of methane, methanol, and in some cases, methylated amines or methylated sulfur compounds to provide energy for growth (1, 13), and there is now considerable documentation of the reactions involving successive two-electron steps for oxidation of methane and methanol.

The enzymes that catalyze the initial step in methane or methanol oxidation are nonspecific, and a considerable range of substrates can be oxidized by either enzyme system (1). For methane monoxygenase, these include N-alkanes, alkenes, and aromatic hydrocarbons, and methanol dehydrogenase can also oxidize a range of unsubstituted and unsaturated alcohols. This ability is not shared by enzymes that catalyze the further oxidation of 1-carbon compounds, and consequently, the more complex substrates are often only partially oxidized in one or several steps (13).

Although previous studies have shown that methanol dehydrogenase can catalyze the oxidation of primary alcohols (1), evidence is lacking on the ability of this enzyme to oxidize lignin-related aromatic alcohols. Furthermore, with the exception of cinnamyl alcohol oxidation by the facultative methylotroph *Pseudomonas* sp. strain M27 (4), aromatic alcohols have not previously been shown to be oxidized by whole-cell systems of methylotrophs.

In this report, we examine the oxidation of lignin-related aromatic alcohols based on the structure of benzyl alcohol (Fig. 1 shows the structural formulae) by whole-cell suspensions of *Methylosinus trichosporum*. We also describe novel bioconversions of these compounds which could be of biotechnological significance. Furthermore, we examine the effects of established inhibitors of methanol dehydrogenase on their oxidation.

**MATERIALS AND METHODS**

**Source of organism.** *M. trichosporum* OB3b was kindly provided by E. Taylor, Biotechnology Center, Cranfield Institute of Technology, Bedford, England.

**Growth of organism.** *M. trichosporum* was grown on nitrate salts medium with methanol or methanol as the carbon source (7, 10). The organism was adapted to grow on methanol by sequential transfer into media with progressively higher concentrations of methanol (0.1, 0.2, 0.5, and 1.2% [vol/vol]). Cultures for experiments were grown at 30°C in 50- to 500-ml butyl rubber-stoppered shake flasks containing 10 or 100 ml of medium, respectively, with methanol at 1.2% (vol/vol). Maintenance of cultures was achieved by weekly transfer of 20% of the volume of a 10-ml culture at full growth.

**Culture purity.** Cultures were routinely examined for purity by phase-contrast microscopy and by being streaked onto agar plates containing 15 mM glucose–3% (wt/vol) Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar medium.

**Cell suspension experiments.** Cells from 300 ml of culture were harvested at the end of the exponential phase of growth by centrifugation at 6,000 × g for 15 min at 2°C, washed twice with phosphate buffer (0.01 M; pH 7.0), and resuspended in the same buffer to a cell concentration of 2.5 mg (dry weight) per ml. The concentration of aromatic alcohol or methanol in cell suspension buffer was in the range of 0 to 13.3 mM. When the effects of nongaseous inhibitors were tested, these were added to medium immediately before resuspension.

Cell suspensions were added in 1-ml portions to 10-ml shake vials which were sealed with septum stoppers secured by aluminum serum cap closures (5). Gaseous inhibitors were added by gas-tight syringe immediately after the vials were sealed. The vials were incubated with shaking at 30°C for up to 20 h.

**Determination of methanol utilization.** After termination of the reaction by injection of 0.2 ml of 5 N H₂SO₄, methanol in the headspace of each reaction vial was determined by gas chromatography with a Porapak R column (1.2 m by 2 mm) connected to a flame ionization detector. The amount of methanol was determined by comparison of peak areas with those of standards.

**Determination of aromatic alcohols and their products.** Vials taken at various stages of the time course incubations...
were cooled, and the contents were centrifuged at 6,000 × g at 2°C for 15 min. Aromatic alcohols, acids, and aldehydes present in the supernatant were determined by high-performance liquid chromatography on an Alltech C18 column (15 cm by 4.6 mm). Separation was achieved with an eluant consisting of methanol–0.05 M sodium acetate, pH 4.0 (20:80 [vol/vol]) at a flow rate of 1.0 ml/min, and eluted aromatic compounds were detected at 275 nm with a Pye Unicam PU 4020 UV detector system. Compounds were identified and quantitated by comparison of retention times and peak areas with those of authentic samples.

Growth and dry-weight measurements. Growth was determined by optical density measurement at 600 nm, and to estimate dry weight, suspensions of M. trichosporium were washed once with distilled water and dried at 60°C to a constant weight.

RESULTS

Development of optimal conditions for oxidation of aromatic alcohols. The effects of cell concentration, pH, and shaking were examined to determine the optimal conditions necessary for oxidation of the alcohols. Figure 2 shows the effect of cell concentration on the oxidation of veratryl and vanillyl alcohols. The results showed that linearity existed between alcohol oxidation rate and cell dry weight up to a value of 2.5 mg (dry weight) per ml. Above this concentration, linearity was lost. Therefore, further experiments were conducted at this dry-weight concentration. When oxidation of the alcohols was determined over the pH range of 5.0 to 8.0, the optimum pH was 7.0. We also examined the effect of shaking and found that the most suitable shaking speed was 60 rpm. The optimal volume of a cell suspension in a shake vial was 1 ml.

Products of oxidation of aromatic alcohols. When vanillyl, benzylic, and veratryl alcohols were oxidized by cell suspensions of M. trichosporium, the corresponding aldehydes were formed, and with the latter two substrates, the aldehydes were further converted to the corresponding aromatic acids. Figure 3 shows the time course of the oxidation of veratryl alcohol. Maximal levels of veratraldehyde occurred after 1 h, and by 4 h, virtually all of the veratraldehyde was converted to veratic acid. In contrast, oxidation of benzyl alcohol resulted in formation of low levels of aldehyde, and the major product at all stages of incubation was benzoic acid.
TABLE 1. Yield of aromatic aldehyde or acid from oxidation of aromatic alcohols by whole-cell suspensions of \( M. \) trichosporium

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( \mu \text{mol of substrate utilized at}^a )</th>
<th>( \mu \text{mol of product at}^b )</th>
<th>Yield (( \mu \text{mol of product/} \mu \text{mol of substrate,} \times 10^2 ) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzy alcohol</td>
<td>2.6 4.7</td>
<td>0.7 ((-\text{CHO})) 0.4 ((-\text{CHO}))</td>
<td>26.9 8.5</td>
</tr>
<tr>
<td>Vanillyl alcohol</td>
<td>2.6 4.4</td>
<td>1.9 ((-\text{COOH})) 3.6 ((-\text{COOH}))</td>
<td>73.1 76.6</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>4.6 6.6</td>
<td>2.0 ((-\text{CHO})) 4.0 ((-\text{CHO}))</td>
<td>76.9 90.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( \mu \text{mol of substrate utilized at}^a )</th>
<th>( \mu \text{mol of product at}^b )</th>
<th>Yield (( \mu \text{mol of product/} \mu \text{mol of substrate,} \times 10^2 ) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzy alcohol</td>
<td>2.6 4.7</td>
<td>0.7 ((-\text{CHO})) 0.4 ((-\text{CHO}))</td>
<td>26.9 8.5</td>
</tr>
<tr>
<td>Vanillyl alcohol</td>
<td>2.6 4.4</td>
<td>1.9 ((-\text{COOH})) 3.6 ((-\text{COOH}))</td>
<td>73.1 76.6</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>4.6 6.6</td>
<td>2.0 ((-\text{CHO})) 4.0 ((-\text{CHO}))</td>
<td>76.9 90.9</td>
</tr>
</tbody>
</table>

\( ^a \) The initial substrate concentration in suspension media was 6.6 mM.

\( ^b \) The values shown are means of duplicate determinations for 1-ml cell suspension volumes.

\( ^c \) Compounds not produced from aromatic alcohol oxidation included protocatechuic acid and 3,4-hydroxybenzaldehyde (yield, <0.1%).

Yields of aromatic aldehyde and acid per mole of aromatic alcohol degraded after 1 and 4 h, respectively, are shown in Table 1. The data show that in most cases, >70% of the alcohol oxidized could be accounted for by the corresponding aldehyde and/or acid. Aldehyde was the major product in the early phase of the time course for veratryl and vanillyl alcohols and acid was the major product in the late phase for benzyl and veratryl alcohols. There was no evidence for the further degradation of aromatic acid, because prolonged incubation (10 h) showed that levels had not declined over those obtained after 4 h.

Comparison of the effects EDTA and \( p \)-nitrophenylhydrazine on the oxidation of methanol and aromatic alcohols. When EDTA or \( p \)-nitrophenylhydrazine was added to cell suspensions of \( M. \) trichosporium utilizing methanol or aromatic alcohols, inhibition of substrate utilization occurred. Double-reciprocal plots of oxidation rate versus [substrate] at different concentrations of either inhibitor revealed that inhibition was noncompetitive, as shown in Fig. 4 for veratryl alcohol oxidation. The apparent \( K_m \) for each substrate (see Table 2 for values) was unaffected by the inhibitor, but \( V_{max} \) was substantially decreased. When the rate of substrate oxidation was plotted against the inhibitor concentration (EDTA or \( p \)-nitrophenylhydrazine), inhibition of aromatic alcohol oxidation was found to be similar to that for methanol oxidation over the same range of inhibitor concentrations (Fig. 5). That the extents of methanol oxidation and aromatic alcohol oxidation were similarly affected by the inhibitor is also shown by the data in Table 2, which are the inhibitor concentrations that gave 50% inhibition with the various substrates.

Effect of EDTA and \( p \)-nitrophenylhydrazine on multiple product formation during aromatic alcohol oxidation by \( M. \) trichosporium. Benzyl and veratryl alcohols gave both the corresponding aldehydes and acids during the time courses of their degradations (Table 1). When an inhibitor (EDTA or \( p \)-nitrophenylhydrazine) was added to incubations, the ratio of acid to aldehyde increased as shown in Fig. 6 for benzyl alcohol oxidation. This increase was most apparent in the early stages of the time course and was found to occur with different substrate and inhibitor concentrations.

Effect of acetylene on oxidation of methanol and aromatic alcohols. No inhibition of methanol or aromatic alcohol oxidation was observed in the presence of acetylene, which was added to incubation vials in amounts ranging from 8 to 40 \( \mu \)mol. Likewise, treatment of cells with acetylene for 10 min, followed by sparging with air, resulted in no loss of activity.

**DISCUSSION**

This communication shows that aromatic alcohols may be converted to their corresponding aldehydes and acids by
whole-cell suspensions of *M. trichosporium*. No other transformation was observed with these aromatic substrates, and methoxyl groups associated with the aromatic nucleus remained intact.

With the exception of cinnamyl alcohol oxidation by the facultative methylotroph *Pseudomonas* sp. strain M27 (4), oxidation of aromatic alcohols based on the structure of benzyl alcohol has not been demonstrated before in methylotrophs, despite studies by previous workers showing the extensive range of other substrates utilized by these organisms (14). Our studies provide the first example of such transformations in obligate methylotrophs.

It is of interest that conversion of veratryl alcohol to veratraldehyde has previously been demonstrated as a measurement for ligninase activity in cell extracts of lignin-degrading organisms in which there is a requirement for H$_2$O$_2$ (11, 16, 17). Addition of H$_2$O$_2$ at levels similar to those used in the ligninase assay to whole-cell suspensions of *M. trichosporium* actually inhibited oxidation of veratryl alcohol (unpublished data). However, this does not rule out the possibility that there is a role for H$_2$O$_2$ in methylotrophs to facilitate transformations similar to those of ligninase along the lines first suggested by Harrington and Kallio (12), in which methanol is oxidized by the peroxidative action of catalase, although subsequent studies have suggested that such a reaction is unlikely (1).

It appears more likely that aromatic alcohols are oxidized by *M. trichosporium* via enzyme systems similar to those used for methanol oxidation. Similar sensitivities of aromatic alcohol oxidation and methanol oxidation to inhibition by EDTA and *p*-nitrophenylhydrazine, known inhibitors of NAD$^+$-independent methanol dehydrogenase (1, 2, 6, 8), is consistent with this view. EDTA and *p*-nitrophenylhydrazine probably act by blocking the transfer of electrons from methanol dehydrogenase to cytochrome *c* either by interaction with metal ions (8, 9) or by affecting binding of the enzyme to cytochrome *c* (6). NAD$^+$-linked dehydrogenases similar to those described for oxidation of coniferyl alcohol (15) were unlikely to have a role in the oxidations described here, since these show little or no sensitivity to EDTA and *p*-nitrophenylhydrazine. Methane monoxygenase, which has been suggested to have a role in methanol oxidation (10), was also unlikely to be involved, since rates of methanol and aromatic alcohol oxidation were unaffected by the presence of acetylene, an inhibitor specific for this enzyme.

The enzyme system for alcohol oxidation in *M. trichosporium* possesses several features which favor the use of whole cells in studies of inhibition kinetics. (i) Methanol dehydrogenase is located on the outside of the cytoplasmic membrane (1), thus allowing it to be accessible to inhibitors. (ii) The enzyme is linked to natural electron acceptors, whereas artificial electron acceptors would be necessary if the purified enzyme were used. (iii) The normal conformation of the enzyme is maintained through its association with the cytoplasmic membrane. In the case of features ii and iii, inhibitor sensitivity of methanol dehydrogenase is significantly decreased with the purified enzyme because of changes in conformation and loss of association with the natural electron acceptors (3). We are currently attempting to define the enzymes involved in aromatic alcohol oxidation with purified enzyme preparations from *M. trichosporium*.

There may be biotechnological advantages in using methylotrophs to facilitate bioconversion of aromatic alcohols to high-value products, such as vanillin. Not only is such a transformation carried out at an effective rate, but the product yields are satisfactory (>70%). Also, in the transformation producing vanillin, there is little or no oxidation of the aldehyde to an unwanted product. The fact that the conversions can be facilitated by whole cells obviates the need for enzymatic conversions whereby cell fractionation procedures would be required together with the need for expensive cofactors. In two-step oxidation of aromatic alcohols, such as benzyl alcohol, it is possible to improve production of the acid over aldehyde by addition of an inhibitor specific for alcohol dehydrogenase (i.e., EDTA), and this may be useful in bioconversion systems in which only the acid is required.

ACKNOWLEDGMENTS

We thank Don Grant for helpful criticisms during the preparation of the manuscript.

LITERATURE CITED


---

TABLE 2. $K_m$ values for oxidation of different alcohol substrates by whole-cell suspensions of *M. trichosporium* and inhibitor concentrations that gave 50% inhibition of activity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>EDTA concn (mM)</th>
<th>$p$-Nitrophenylhydrazine concn (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>15.6</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>11.5</td>
<td>1.7</td>
<td>2.8</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>16.6</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Vanillyl alcohol</td>
<td>12.2</td>
<td>1.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

FIG. 6. Time course of oxidation of benzyl alcohol with no added inhibitor (A) and with 1.6 µM $p$-nitrophenylhydrazine (B) by whole-cell suspensions of *M. trichosporium*. The conditions for incubation were as described in the text, and the initial substrate concentration was 6.6 mM. Symbols: ■, benzyl alcohol; ▲, benzaldehyde; ○, benzoic acid.