Isolation of Phenol-Degrading *Bacillus stearothermophilus* and Partial Characterization of the Phenol Hydroxylase

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*Bacillus stearothermophilus* BR219, isolated from river sediment, degraded phenol at levels to 15 mM at a rate of 0.85 µmol/h (4 x 10⁶ cells). The solubilized phenol hydroxylase was NADH dependent, exhibited a 55°C temperature optimum for activity, and was not inhibited by 0.5 mM phenol.

Phenols present in the effluents of oil refineries, petrochemical plants, and other industrial processes are hazardous pollutants and a continuing waste treatment concern (16). A number of mesophilic microorganisms have been reported to degrade phenol at low concentrations, including *Pseudomonas* spp. (3), *Alcaligenes* spp. (9), *Streptomyces setonii* (1), and the yeasts *Trichosporon cutaneum* and *Candida tropicalis* (14, 15). Hydroxylation of phenol, the first step in phenol degradation, has been shown to be strongly substrate inhibited in the few cases studied. Janke et al. (10) showed inhibition of phenol hydroxylase activity in whole *Pseudomonas* cells at 0.25 mM phenol, and Neujahr and Gaal (14) demonstrated phenol hydroxylase inhibition above 0.25 mM phenol with highly purified enzyme from *T. cutaneum*.

It is recognized that the thermostable enzymes from thermophiles are also resistant to chemical denaturation (4). This suggests that if suitable pathways exist, thermophilic biotransformation of environmental pollutants might be possible at concentrations toxic to mesophiles. Although little is known about aromatic pathways in thermophiles, Buswell and Twomey (8) isolated a strain of *Bacillus stearothermophilus* capable of phenol degradation which utilized an NADH-dependent phenol hydroxylase. Phenol inhibition of the hydroxylase was not studied, but the strain was growth inhibited by phenol concentrations above 5 mM. We have initiated further studies of *B. stearothermophilus* in the hope of identifying strains with enhanced capability for phenol degradation.

An examination of phenol utilization was first carried out on *B. stearothermophilus* strains obtained from various culture collections. Growth was tested on phenol-bearing DP minimal plates (7) containing (per liter) K₂HPO₄, 0.5 g; NH₄Cl, 1.0 g; MgSO₄·7H₂O, 20 mg; yeast extract, 0.2 g; Casamino Acids, 0.1 g; trace elements (2), 1 ml; and agar, 20 g (pH 7.2). When DP plates containing 5 mM phenol were used, none of the nine strains tested demonstrated enhanced growth compared with the low level of growth observed on DP plates without phenol. This indicated that phenol utilization at this concentration was not a common characteristic. An isolation program at 55°C was then initiated for phenol-degrading strains, in which 1 g of soil or sediment was suspended in 1/100-strength L broth containing 5 mM phenol. By using this procedure, strain BR219 was isolated from Tittabawassee River sediment obtained near Midland, Mich. Identification of BR219 carried out by standard procedures (5) revealed typical characteristics for *B. stearothermophilus*, including rod morphology, oxidase activity, positive Gram stain, catalase activity, oxidase activity, gelatin hydrolysis, casein hydrolysis, and growth in 3% NaCl. Negative tests included starch hydrolysis, indole formation, citrate utilization, sodium azide utilization, anaerobic growth, and growth in 5% NaCl. A maximum growth temperature of 75°C was determined. Colonies sprayed with catechol following growth on DP plates containing 5 mM phenol developed a yellow color, indicating probable formation of 2-hydroxyxymuconic semialdehyde and the utilization of a *meta* phenol degradation pathway (6). Corresponding catechol 2,3-dioxygenase activity was determined in sonicated cell extracts by the method of Kojima et al. (11) but was not further characterized.

Growth and phenol utilization by *B. stearothermophilus* BR219 was measured at 55°C in 300-ml Erlenmeyer flasks containing 40 ml of DP medium and varied concentrations of phenol. The flasks were shaken in a New Brunswick G76 Gyrotory shaker. Residual phenol was measured by the colorimetric method of Martin (13) and organism culture density was measured with a Klett and Summerson colorimeter. BR219 could grow on phenol at levels up to 15 mM, with 10 mM giving optimal growth and complete phenol utilization (Fig. 1). BR219 thus degrades phenol optimally at higher concentrations than those possible for many mesophiles. For instance, Yang and Humphrey (17) found that both *Pseudomonas putida* and *T. cutaneum* were strongly growth inhibited above 0.5 mM phenol.

To investigate properties of the phenol hydroxylase, 2-liter exponential-phase cultures of BR219 in DP medium with 5 mM phenol were harvested, washed with 0.1 M phosphate buffer (pH 7.6), and resuspended in 20 ml of the same buffer prior to a 2-min sonication at 4°C with a Cole Parmer 4710 ultrasonic homogenizer. The homogenized suspension was centrifuged at 26,890 x g for 20 min, and protein concentration in the extract was measured by the method of Lowry et al. (12). Phenol hydroxylase activity was measured by phenol disappearance in a 1-ml reaction mixture containing 100 µM phenol, 1 mM NADH, and 100 mM Na₂HPO₄ buffer (pH 7.6). After the addition of 100 µl of enzyme extract to 1 ml of reaction mixture and incubation for 15 min at 55°C, the reaction was stopped by the addition of 12 µl of 2% 4-aminoantipyrine followed by 40 µl of 2 N ammonium hydroxide and 40 µl of 2% potassium ferricyanide, and the final volume was increased to 2 ml with water. The A₅₅₀ was read and compared with those of phenol standards after 20 min of incubation at room temperature. In
FIG. 1. Growth kinetics and phenol degradation by strain BR219. (A) Growth; (B) phenol degradation. Phenol concentrations were 0 (A), 5 (●), 10 (○), 15 (■), and 20 (□) mM.

this assay, maximum activity was found at 55°C (data not shown), indicating the expected thermostability of the enzyme. One unit of enzyme activity is therefore defined as 1 μmol of phenol conversion per min at 55°C. BR219 grown in DP medium containing succinate rather than phenol did not display phenol hydroxylase activity, suggesting induction of enzyme synthesis by phenol. The hydroxylase activity was independent of added NADPH and flavin adenine dinucleotide but was dependent on NADH addition, yielding a $K_m$ value of 263 μM (Fig. 2). Hydroxylase activity was dependent on phenol, with a measured $K_m$ of 66 μM and no discernible phenol inhibition up to a 0.5 mM concentration, the highest level for which the phenol disappearance assay could be reliably used (Fig. 2). Inhibitors for the enzyme are listed in Table 1. The hydroxylase resembled that reported by Buswell and Twomey (8) in utilization of NADH and inhibition by o-phenanthroline and differs from that of T. cutaneum (14) in sensitivity to EDTA and lack of stimulation by flavin adenine dinucleotide and NADPH. The lack of enzyme inhibition in 0.5 mM phenol differs from that reported for phenol hydroxylase from T. cutaneum (14) and P. putida (10) and provides encouragement for further purification and characterization of BR219 enzymes. It will also be of interest to examine thermophiles for other enhanced bioconversion capabilities.

LITERATURE CITED


TABLE 1. Effect of inhibitors on phenol hydroxylase activity

<table>
<thead>
<tr>
<th>Inhibitor (0.1 mM)</th>
<th>Enzyme activity (U/ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper sulfate</td>
<td>0.32</td>
<td>91</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>2.11</td>
<td>43</td>
</tr>
<tr>
<td>Mercuric chloride</td>
<td>0.48</td>
<td>87</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1.92</td>
<td>48</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>0.49</td>
<td>87</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.84</td>
<td>77</td>
</tr>
<tr>
<td>Diethylthiocarbamate</td>
<td>3.69</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
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<td>0</td>
</tr>
</tbody>
</table>

FIG. 2. Phenol and NADH substrate affinities of phenol hydroxylase. Phenol hydroxylase assays were performed as indicated in the text. (A) NADH varied, 100 μM phenol; (B) phenol varied, 1 mM NADH.