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Bacterial monooxygenase enzymes catalyze a regiospecific single-step hydroxylation of diphenylacetylene to yield meta- and para-hydroxydiphenylacetylene.

Synthesis of high performance polymers and composites often requires feedstocks that are difficult to synthesize with traditional chemistry. Regio- and stereospecific functionalization of aromatic compounds can be particularly challenging. Here we describe a biocatalytic approach to the production of two isomers of the endcapping agent, hydroxydiphenylacetylene (HDPA) via single-step regioselective monooxygenation (Fig. 1). This type of endcap can be used in the formation of phenylethyl terminated reactive oligomers, which have received much attention over the past decade due to their high thermal stability and excellent mechanical properties. The biocatalytic route provides an alternative single-step method for synthesis and has the added advantage of being environmentally benign, in contrast to the harsh conditions and reagents used in conventional chemical synthesis.

Multicomponent dioxygenases can catalyze the conversion of aromatic substrates to cis-dihydrodiol intermediates which undergo dehydrogenation to phenolic products. For example, a mutant derived from a toluene-degrading organism accumulated a cis-dihydrodiol derivative of diphenylacetylene (DPA) which could be converted to meta-HDPA or ortho-HDPA by treatment with acid or base. The para-HDPA isomer cannot be formed by the above method because of the 2,3-position of the initial hydroxylation.

Bacterial multicomponent monooxygenases are a family of nonheme, di-iron enzymes capable of using molecular oxygen to hydroxylate a variety of organic compounds that allow bacteria to use the compounds as the sole source of carbon and energy. Monooxygenase enzymes catalyze a single-step direct hydroxylation of aromatic rings, often in a highly regiospecific manner. The substrate range of many of the monooxygenases is broad and the enzymes will oxidize a wide range of aromatic and aliphatic substrates to form hydroxylate a variety of organic compounds that allow bacteria to use the compounds as the sole source of carbon and energy.

We tested a variety of bacterial monooxygenase enzymes for the ability to catalyze the direct monooxygenation of DPA. The biotransformation of DPA was investigated with recombinant strains expressing various toluene monooxygenase, all are capable of transforming toluene to cresol, and the isomer obtained is enzyme-specific (Table 1). All of the recombinant strains tested catalyzed the transformation of DPA to HDPA with varying degrees of efficiency and regioselectivity. The isomer of HDPA could be correlated to the type of monooxygenase (Table 1).

Toluene-4-monooxygenases catalyzed the formation of predominantly meta-HDPA. In contrast, toluene-2-monooxygenases formed predominantly para-HDPA. Initial experiments with recombinant organisms indicated that toluene monooxygenase enzymes can catalyze the hydroxylation of DPA, but the overall yields were low. A second strategy was pursued to obtain bacterial strains that catalyze higher DPA conversion rates and product yields. A collection of 226 toluene-degrading isolates was screened for the ability to transform DPA; 39 produced HDPA. Ralstonia picketti JS757 produced the highest yield. When grown with toluene as the sole carbon source, it converted 1 mM DPA to 0.41 (± 0.022) mM meta-HDPA and 0.29 (± 0.021) mM para-HDPA (Fig. 2A). The initial product formation rates (first 2.5 h) were 1.25 and 0.86 nmol min⁻¹ g⁻¹ dry weight for the meta- and para-isomers, respectively. The turnover of substrate was not complete and some residual DPA was detected at the end of the reaction, traces of uncharacterized polar products also accumulated during the transformations.

We speculated that a monooxygenase enzyme catalyzed the monohydroxylation of DPA in strain JS757 and tested toluene-grown cells for the ability to oxidize intermediates of various toluene assimilation pathways. Oxygen uptake studies showed that strain JS757 rapidly oxidised intermediates of a toluene monooxygenase pathway (all cresol isomers) but did not oxidise intermediates of a dioxygenase pathway (toluene or benzene cis-dihydrodiol). The alkene monooxygenase from Xanthobacter strain PY2 is a soluble di-iron monooxygenase known to catalyze the oxidation of HDPA (Fig. 2).

Table 1 Screening of recombinant monooxygenase strains for conversion of diphenylacetylene

<table>
<thead>
<tr>
<th>Enzyme function</th>
<th>Recombinant strain</th>
<th>Product of toluene transformation</th>
<th>Product of DPA transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene-2-</td>
<td>E. coli T1</td>
<td>o-cresol</td>
<td>para-HDPA (77%)</td>
</tr>
<tr>
<td>monooxygenase</td>
<td>(pTKW[ROM])</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. coli Top106</td>
<td>o-cresol</td>
<td>para-HDPA (52%)</td>
</tr>
<tr>
<td></td>
<td>(pJS407) Tol-2mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene-4-</td>
<td>E. coli T1</td>
<td>p-cresol</td>
<td>meta-HDPA (64%)</td>
</tr>
<tr>
<td>monooxygenase</td>
<td>(pTKW[ROM])</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. coli Top106</td>
<td>p-cresol</td>
<td>meta-HDPA (69%)</td>
</tr>
<tr>
<td></td>
<td>(pJS409) Tol-4mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tol/xylene</td>
<td>E. coli T1</td>
<td>o-, m- and p-cresol</td>
<td>para-HDPA (69%)</td>
</tr>
<tr>
<td>monooxygenase</td>
<td>(pTKW[ROM])</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This study. See ESI HDPA: hydroxidiphenylacetylene, DPA: diphenylacetylene. Major product isomer shown, residual percentage is alternate isomer, either meta- or para- in all cases; ortho-HDPA was not detected.
Recombinant strains were cultured at 37 °C in Luria-Bertani medium (Difco) containing glycerol (1%) and ampicillin (100 mg mL⁻¹) or kanamycin (50 mg mL⁻¹) to maintain plasmid selection. All of the strains were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) during exponential growth. R. pickettii strain JS757 was grown in minimal salts medium at 30 °C, pH 7.0 with toluene in the headspace. Xanthobacter strain Py2 was cultured as described previously.¹⁰ In a typical biotransformation reaction, cells were harvested in late exponential phase by centrifugation and then washed and suspended in fresh medium to give a 10-fold concentration. DPA was added to a final concentration of 1 mM and pyruvate (2 mM) was added as a carbon and energy source. The concentration of products and reactants were monitored by HPLC (Hewlett Packard 1100). Compounds were separated on a Chromolith RP-18 column (100-4.6 mm, Merck) or a Supelcosil LC-ABZ + PLUS column with an acetonitrile-water gradient. For product purification, the reaction mixture was filtered through glass wool to remove any residual DPA crystals and then passed through a C18 solid phase extraction column (Waters, 12 g). Products were eluted in acetonitrile and the excess solvent removed in a rotary evaporator under reduced pressure. The two HDA isomers were separated by preparative scale HPLC with a Supelcosil LC-ABZ column (25 mm × 10 mm, 5 μm, Supelco) with a mobile phase as above. DPA is insoluble in water and was therefore difficult to quantify during the reaction. Conversion calculations were based on product concentration and initial DPA concentration. NMR analysis was performed in acetone- and NMR spectra were recorded on a Varian Inova spectrometer equipped with a 5 mm indirect detection probe, operating at 500 MHz for 1H and at 125 MHz for 13C. Chemical shifts were consistent with proposed structures and previous literature reports.²⁴⁻³²

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Notes and references

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6 Isolated from contaminated soil samples by enrichment with tolune.

7 The identification of isolate JS757 was based on morphological studies and partial 16S RNA sequencing (Midi Labs), which showed a 99.5% sequence identity with Ralstonia picketti.


