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Complete List of Authors:	Sobottka, Devin; University of Wisconsin-Eau Claire, Chemistry and Biochemistry Rooney, Grace; University of Wisconsin-Eau Claire, Chemistry and Biochemistry Thompson, Samantha; University of Wisconsin-Eau Claire, Chemistry and Biochemistry Ubbelohde, Jessica; University of Wisconsin-Eau Claire, Chemistry and Biochemistry Aldag, Ava; University of Wisconsin-Eau Claire, Chemistry and Biochemistry Lerch, Austin; University of Wisconsin-Eau Claire, Chemistry and Biochemistry Doyon, Tyler; University of Wisconsin-Eau Claire, Chemistry and Biochemistry

ARTICLE

Sustainable biocatalytic synthesis of substituted muconic acids

Devin J. Sobottka[a‡], Grace C. Rooney[a‡], Samantha P. Thompson[a], Jessica M. Ubbelohde[a], Ava M. Aldag[a], Austin R. Lerch[a] and Tyler J. Doyon[a*]

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The development of sustainable routes to organic building blocks is a critical endeavor for reducing the environmental impact of chemical synthesis. Biocatalysts are poised to play an important role in sustainable synthesis, as they perform highly selective reactions under mild conditions. The application of enzymes to organic synthesis requires an approach which is operationally simple, inexpensive to prepare, and reasonably scalable. In this work, we demonstrated the utility of a Type I ring-cleaving dioxygenase CatA (*P. putida* KT2440) for preparative-scale synthesis of muconic acid derivatives. Muconic acids are important precursors in the synthesis of polymers and commodity chemicals. In this work, we optimized the performance of CatA under millimolar substrate concentrations and characterized the activity of the enzyme with an array of catechol substrates. Furthermore, we developed a scalable platform using cellular lysates to produce diverse muconic acids, generating up to a gram of the desired product. A simple trituration procedure was utilized for the purification of these muconic acids that obviated the need for chromatographic purification and reduced overall solvent waste.

Introduction

Muconic acid (2,4-hexadiendioic acid, **2**) and its derivatives are important precursors for the synthesis of commodity chemicals. For example, the nylon feedstock compound adipic acid (**3**) has been generated from catalytic hydrogenation of muconic acid (Figure 1A, **2**-**3**).[1–5] These conjugated dienes have also been used as substrates in the synthesis of diethyl terephthalate (Figure 1B, **4**-**6**), showcasing their potential for downstream synthesis of complex molecules.[6] As a result of their potential as valuable organic building blocks, the search for sustainable and scalable routes to substituted muconic acids has been extensive. These efforts have primarily focused on the bioengineering of microorganisms that natively metabolize aromatic compounds.[7-11] This approach relies on enzymes to transform feedstock carbon sources (such as benzoic acid, **7**) into muconic acids via biotransformation (Figure 1C).[7–11] The scope of muconic acids that can be produced by metabolic engineering is generally quite limited, as all of the enzymes in the metabolic pathway must be able to process the substrate. In contrast, direct oxidation of catechols by non-heme iron ring-cleaving dioxygenases (RCDs) offers a streamlined route to structurally diverse muconic acids. Type I RCDs selectively cleave catechols in an "intradiol" fashion, generating the desired diacid product (Figure 1D, **11**-**13**). Furthermore, catechol substrates can be synthesized efficiently from commercially available salicylaldehydes using a Dakin oxidation procedure. This approach enables access to a wide variety of potential substrates for ring cleavage.[12]

Numerous type I RCDs have been identified and shown to catalyze oxidative ring-cleavage of catechols.[13,14] In general, characterization of enzyme performance in this reaction is achieved by measurement and comparison of substrate specificity values (k_{cat}/K_M) with a small panel of catechols under micromolar substrate concentrations.[13] While this data provides critical information for comparison of the biocatalytic properties of RCDs, it does not describe the catalyst behavior at the concentrations necessary for performing reasonable preparative scale reactions to isolate muconic acids (i.e. millimolar concentrations). Furthermore, most RCD characterization studies focus on reactions with only a few commercially available substrates, which falls short in evaluating the synthetic limitations of the enzyme. To address this gap in knowledge, we sought to characterize RCD activity at increased substrate concentrations with a more structurally diverse panel of catechols. We envisioned that this analysis would provide key insights into the capabilities and limitations of the biocatalyst. We also anticipated that this information would enable preparative scale biocatalytic synthesis of muconic acid derivatives, facilitating access to these valuable organic building blocks.

Biocatalysts play an increasingly important role in the selective and sustainable synthesis of organic compounds.[15,16] However, the practical application of enzymes to organic synthesis faces some barriers. For example, the enzyme should be expressed in high titers and active without extensive purification steps. The catalyst should also be able to process substrates at high concentrations so that overall reaction volumes are of a reasonable size. Furthermore, the ability to use alternative catalyst preparations, such as whole cells or clarified lysates, eases the burdens and associated cost of performing biocatalytic reactions.[17,18] Based on these criteria, we anticipated that catechol dioxygenase A (CatA) from *Pseudomonas putida* KT2440 would be a viable candidate for use in biocatalytic muconic acid synthesis.[19] CatA has been previously heterologously expressed in *E. coli* and shown to catalyze efficient oxidative cleavage of a small number of catechols at sub-millimolar concentrations using purified enzyme.[19] We sought to evaluate if this enzyme could catalyze efficient ring cleavage at synthetically relevant concentrations as

[a] *Department of Chemistry and Biochemistry, University of Wisconsin-Eau Claire, 101 Roosevelt Ave, Eau Claire, WI 54701, USA. Email: doyontj@uwec.edu*
**Corresponding Author*
‡These authors contributed equally to this work.
Electronic Supplementary Information (ESI) available: NMR spectra of synthesized compounds, synthetic procedures, biocatalyst preparation procedures, and UV-visible spectra. See DOI: 10.1039/x0xx00000x

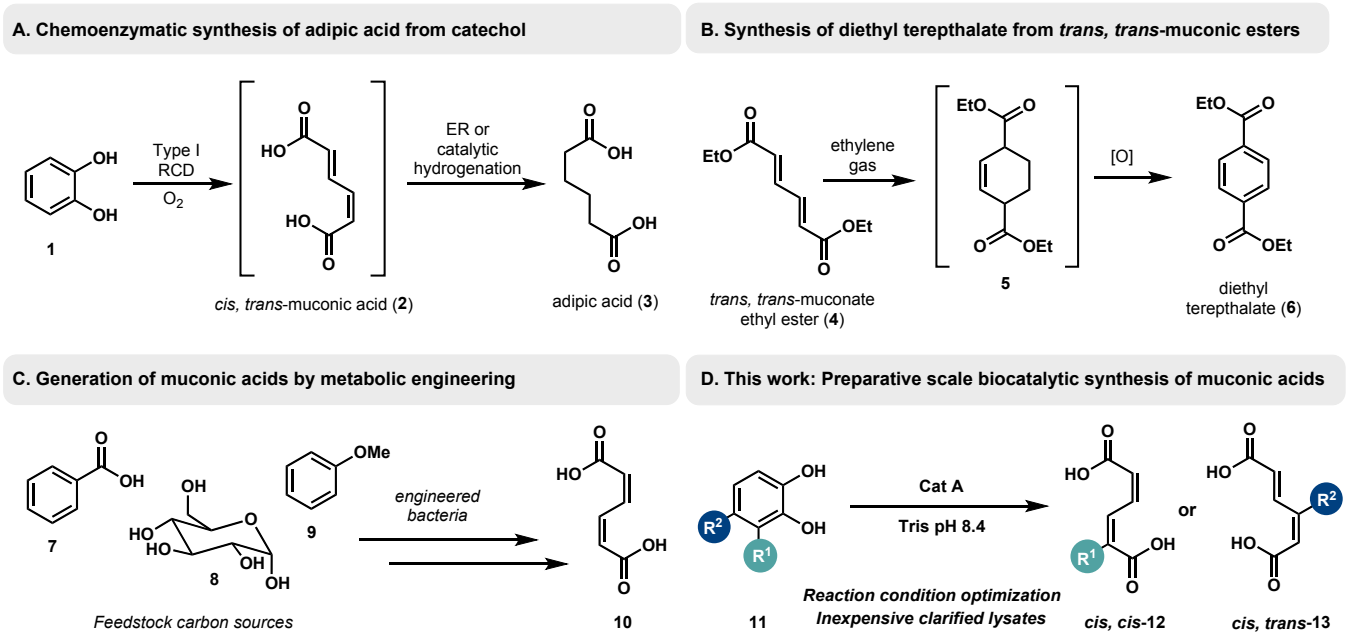


Figure 1. A. Chemoenzymatic synthesis of commodity chemical adipic acid (**3**) from catechol (**1**). B. Synthesis of commodity precursor diethyl terephthalate (**6**) from muconic ester (**4**). C. Engineering of microbes to produce muconic acid (**10**). D. This work: demonstrated preparative scale biocatalytic synthesis of substituted muconic acids. RCD = Ring-cleaving Dioxygenase. ER = "ene"-reductase.

well as to evaluate the scope and scalability of this biocatalytic method for muconic acid synthesis.

Results and Discussion

To access this biocatalyst, the gene for *catA* was codon optimized for *E. coli*, synthesized, and cloned into a pET-28a expression vector with an *N*-terminal 6 x His tag (Twist Biotechnology). The enzyme was heterologously expressed in BL21 (DE3) *E. coli* cells and the growth media was supplemented with ferric ammonium citrate (0.2 mg/mL) to ensure expression of *holo* CatA. The enzyme was purified to homogeneity by immobilized metal affinity chromatography, yielding 50-60 mg of protein per liter of cell growth (see Supplementary Information for details). The biocatalyst was used without further manipulation. Some wet whole cells harboring CatA were flash frozen and set aside for use in whole cell reactions.

To achieve preparative scale synthesis of muconic acids, we first sought to optimize the reaction of CatA with 4-methylcatechol (**14**) at 5 mM substrate concentration (Figure 2A). Previous work with CatA demonstrated that productive catalysis could be achieved with a Tris buffer at pH 8.0, providing a starting point for our analysis.[19] We envisioned that a screen of reaction pH conditions could provide improved yields of 3-methylmuconic acid (**15**). To thoroughly analyze the effect of pH on CatA activity, we generated buffers with pH values ranging from 6.8 to 10. Tris base was used to achieve pH values ranging from 6.8-8.8 and sodium carbonate was used to achieve pH values from 9-10. Product yields (Figure 2C) generally increased as pH was increased from 6.8 to 8.4, then began to decrease at pH values from 8.6 to 9.0, suggesting that the optimal pH for CatA is 8.4. Interestingly, a slight uptick in yield was observed with carbonate buffer at pH 9.4. We hypothesize that the observed increase in yield at high pH likely results from deprotonation of the catechol, which is a proposed step in the mechanism of Type I RCD ring cleavage.[20] While this trend in enzyme activity was noteworthy, Tris pH 8.4 was observed to provide the highest yield of 3-methylmuconic acid (**15**). We therefore chose to use Tris pH 8.4 for further reactions with CatA.

Following optimization of reaction conditions with model substrate **14**, we sought to characterize the ability of CatA to undergo productive catalysis with an array of catechols (Figure 2D, see **1**, **14**, **17**-**27**). We anticipated that this analysis would provide key information about the synthetic capabilities of CatA and enable the development of a preparative scale reaction platform. Analytical scale reactions with purified CatA were performed at a synthetically relevant substrate concentration (5 mM) for 4 h under pH optimized conditions. Reactions with catechol (**1**) proceeded efficiently under these conditions, resulting in product yields of greater than 99%. Catechols with substitution at the 4-position (Figure 2D, see **14**, **17**-**21**) readily underwent oxidative cleavage to produce 3-substituted muconic acids in moderate to high yields. Larger, more sterically demanding substitutions (see **17** and **18**) had the most notable impact on catalysis, reducing the number of turnovers observed. Catechols with halogens at *C*4 (**19**-**21**) were oxidized with moderate yields. Catechols with strongly electron-withdrawing *C*4-substituents did not undergo productive catalysis with CatA (Supplemental Information Figure S4). For example, 4-nitrocatechol (**S1**), 4-cyanocatechol (**S2**), and 3,4-dihydroxybenzoic acid (**S3**) were not processed by CatA, demonstrating some limitations of the biocatalyst in reacting with deactivated aromatic systems.

Catechols with substitution at *C*3 were also processed by CatA, albeit in a more limited fashion. 3-methylcatechol (**22**) was oxidized efficiently by CatA, resulting in a high yield of 2-methylmuconic acid (90%). However, 3-ethylcatechol (**23**) was a poor substrate for CatA, demonstrating the effects of steric bulk on CatA activity. Increasing

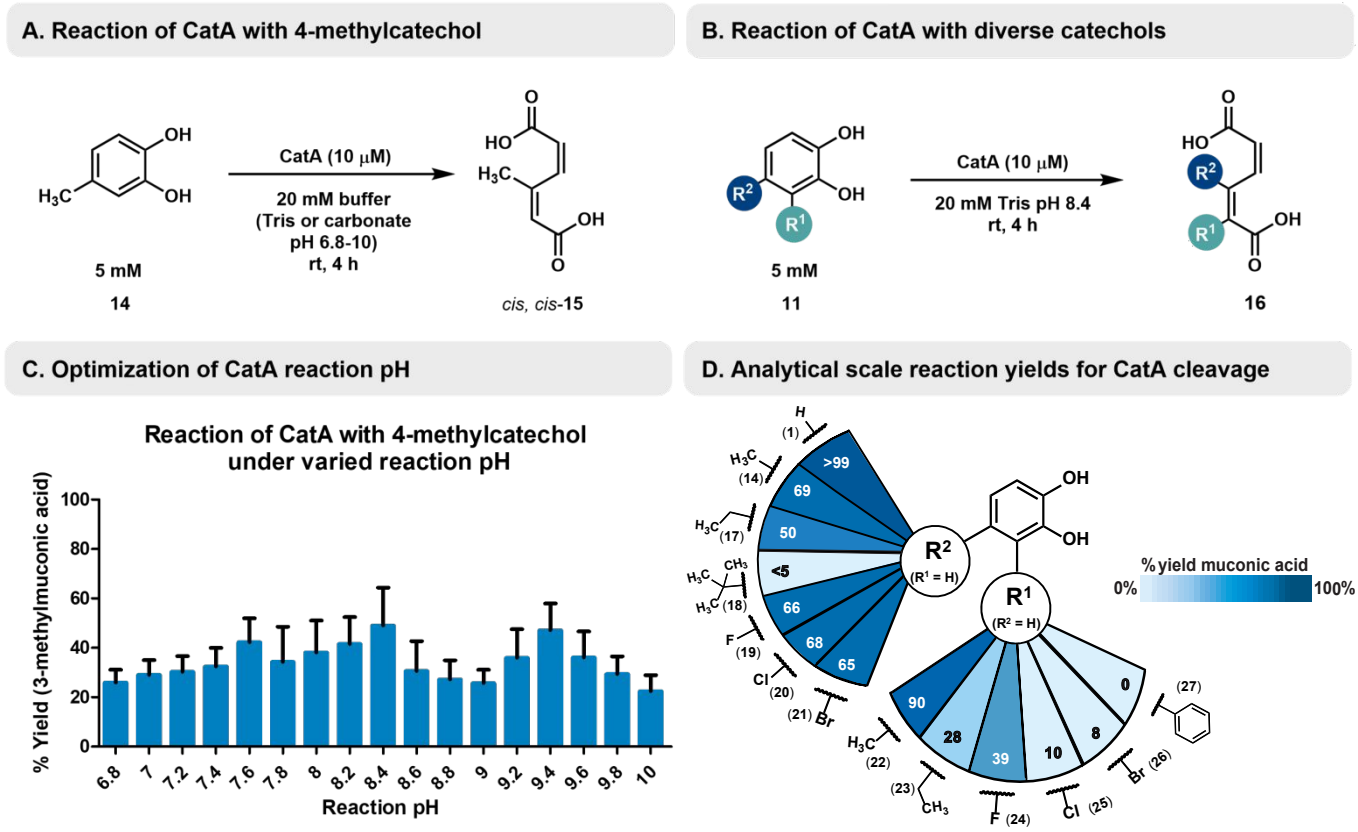


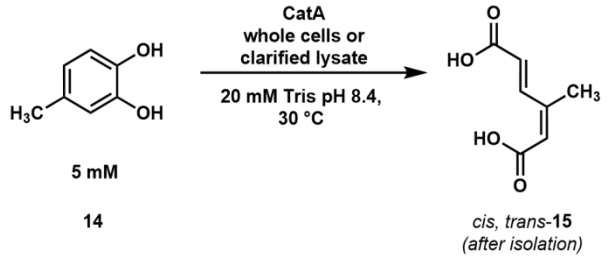
Figure 2. Optimization of CatA-catalyzed oxidative cleavage and evaluation of the substrate scope. A. Reaction scheme for optimization of CatA-catalyzed oxidative cleavage of 4-methylcatechol (**14**). B. Reaction scheme for analytical scale reactions with substituted catechols. C. Optimization of buffer pH conditions for reactions of CatA with 4-methylcatechol (**14**). D. Evaluation of the substrate scope of CatA-catalyzed ring cleavage using purified enzyme. Reaction conditions: 5 mM substrate, 20 mM Tris (pH 6.8-8.8) or sodium carbonate buffer (pH 9-10), 10 μM purified CatA, room temperature (rt), 4 h reaction time. Reactions were performed in triplicate and recorded values represent the mean of those trials. Error bars represent the standard deviation of the data. Reactions were quenched by the addition of 2 volumes of methanol and centrifuged at 17,000 x *g* for 10 min. Reaction yields were determined by measuring the absorbance of a dilute reaction mixture (50 x dilution) at 260 nm and calculation of the product concentration was achieved using the Beer-Lambert equation. Product molar absorptivity values were obtained from literature sources[19] or calculated using the method outlined by Dorn and Knackmuss.[23]

substituent bulk at *C3* to a phenyl group (**27**) was further deleterious as no turnover was observed with 3-phenylcatechol (**27**). Low yields were also observed for catechols with halogens at the *C3*-position. For example, 3-fluorocatechol (**24**), 3-chlorocatechol (**25**), and 3-bromocatechol (**26**) were poor substrates for oxidation by CatA. This trend demonstrated that small modifications to the substrate at *C*3 had a significant impact on observed CatA activity. Catechols with other polar substituents at *C*3 were not substrates for CatA, including 2,3-dihydroxybenzoic acid (**S4**), 2,3-dihydroxybenzaldehyde (**S5**), 3-methoxycatechol (**S6**), and 3-(aminomethyl)benzene-1,2-diol hydrochloride (**S7**), demonstrating that CatA activity is impacted by structural perturbations at this position.

Following substrate screening with purified CatA, we sought to develop a biocatalytic platform for preparative scale muconic acid synthesis. We envisioned that a sustainable and scalable route to muconic acids could be achieved through the use of wet whole cells or clarified lysates harboring CatA. These enzyme preparations are inexpensive, simple to prepare, and obviate the need for protein purification. Toward this end, we initially performed analytical scale test reactions using wet whole cells harboring overexpressed CatA (Figure 3A and 3C). Reactions with 4-methylcatechol (**14**) were carried out at varying concentrations of wet whole cells (0.5-4% w/v). Gratifyingly, we observed an increase in product concentration over time, demonstrating that CatA was active in a whole cell format. Increasing wet whole cell loading from 0.5% to 2% w/v resulted in a commensurate increase in analytical yield to >99%. These experiments defined the cell loading and incubation time required to achieve high yields, clearing the way for attempting preparative scale reactions and isolation of the desired muconic acid product.

We then performed a milligram-scale reaction with 4-methylcatechol (**14**) using 2% w/v wet whole cells. After 16 h, UV-vis analysis of the crude reaction mixture showed high conversion to product (72%). The reaction was quenched with 3 volumes of acetone and the mixture was centrifuged for 20 min at 4,000 *x g* to remove cellular debris. The resulting supernatant was acidified with 2N sulfuric acid to pH 2.0 and extracted 3 times with ethyl acetate. The organic fractions were combined, and the solvent was removed under vacuum to yield a crude solid. Initially, we aimed to purify the desired product by chromatographic separation, but we were stymied by the inherent challenges in purifying amphipathic diacids. To overcome this challenge and improve the sustainability of the

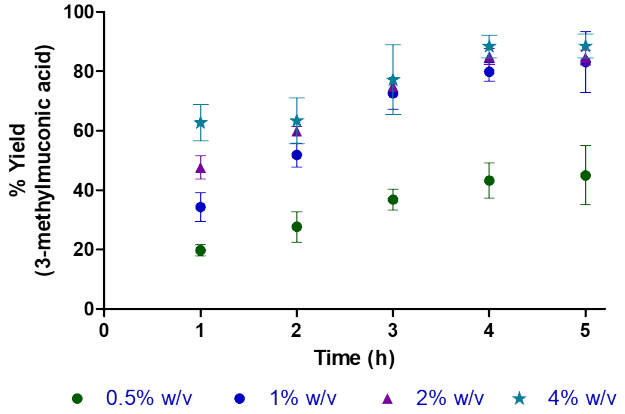
A. General reaction of CatA with 4-methylcatechol



B. Effects of catalyst preparation on isolated yields

Entry	Catalyst loading procedure	isolated yield of 15
1	whole cells, no pellet extraction	7%
2	whole cells with pellet extraction	26%
3	clarified cell lysate	90%

C. Reactions of 14 with whole *E. coli* cells harboring CatA



D. Reactions of 14 with clarified lysates harboring CatA

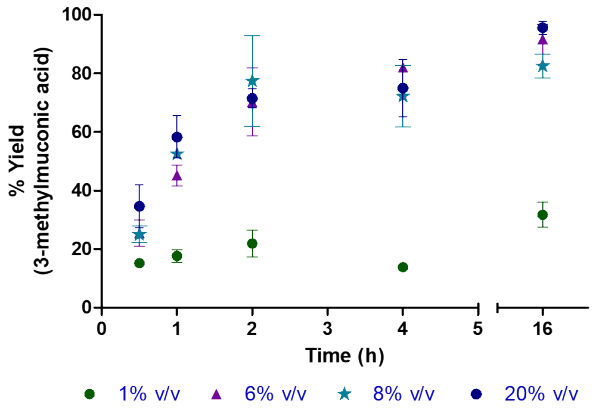


Figure 3. Evaluation and optimization of catalyst-delivery methods for preparative-scale synthesis of muconic acids. A. Reaction scheme for optimization of catalyst delivery in CatA-catalyzed oxidative cleavage of 4-methylcatechol (**14**). B. Comparison of isolated yields of 3-methylmuconic acid (**15**) using different catalyst delivery and recovery methods. C. Reaction progress curves for reactions of whole, wet *E. coli* cells harboring CatA with 5 mM compound **14**. Cell concentration is given as a weight/volume (w/v) percentage based on the total reaction volume. D. Reaction progress curves for reactions of clarified cell lysates harboring CatA with 5 mM compound **14**. Lysates were prepared from a suspension of 250 mg/mL whole, wet *E. coli* cells in Tris pH 8.4 (50 mM) with 50 mM NaCl. Final lysate concentration is given as a volume/volume (v/v) percentage based on the total reaction volume. Reaction conditions: 5 mM substrate, 20 mM Tris (pH 8.4), room temperature (rt), 4 h reaction time. Reactions were performed in triplicate and recorded values represent the mean of those trials. Error bars represent the standard deviation of the data. Reactions were quenched by the addition of 2 volumes of methanol and centrifuged at 17,000 x *g* for 10 min. Reaction yields were determined by measuring the absorbance of a dilute reaction mixture (50 x dilution) at 260 nm and calculation of the product concentration was achieved using the Beer-Lambert equation. Product molar absorptivity values were obtained from literature sources[19] or calculated using the method outlined by Dorn and Knackmuss. [23]

reaction sequence, we developed a purification method that avoided the solvent waste associated with liquid chromatography. After screening product solubility in several organic solvents, we discovered that muconic acids possessed low solubility in small amounts of cold acetonitrile (MeCN), while the remaining contaminants were quite soluble. To purify the muconic acid, the crude product mixture was triturated with cold MeCN, the supernatant was then removed by suction, and the resulting solid was dried under reduced pressure, producing clean (2*Z*, 4*E*)-3-methylmuconic acid (**15**).

Despite our success in isolating and purifying compound **15**, the yield for the reaction was quite low (7%, see Figure 3B, entry 1). We hypothesized that the low yield could result from *E. coli* cells sequestering the amphipathic 3-methylmuconic acid (**15**). We envisioned that extraction of the insoluble cellular debris could improve overall isolated yields by solubilizing any trapped product. Extraction of the pelleted cell debris with ethyl acetate at pH 2.0 improved the isolated yield to 26% (Figure 3B, entry 2), but did not achieve a value commensurate with the observed conversion of the crude reaction mixture (>70%). We hypothesized that the removal of insoluble cellular components from the reaction mixture could reduce product sequestration and improve isolated yields. We envisioned that clarified cell lysates harboring CatA could achieve this goal, while also avoiding arduous and expensive catalyst purification steps.

Clarified lysates were produced by resuspending whole *E. coli* cells in a buffer containing 50 mM Tris pH 8.4 and 50 mM NaCl at a concentration of 250 mg wet cells/mL. The cells were lysed by sonication and the lysate was then clarified by centrifugation (see Supplemental Information). The resulting lysate was flash frozen and used without further modification in reactions. To test the viability of this catalyst preparation for oxidative cleavage of catechols, analytical scale reactions were performed using various concentrations of clarified CatA lysate (Fig 3D). Time trial

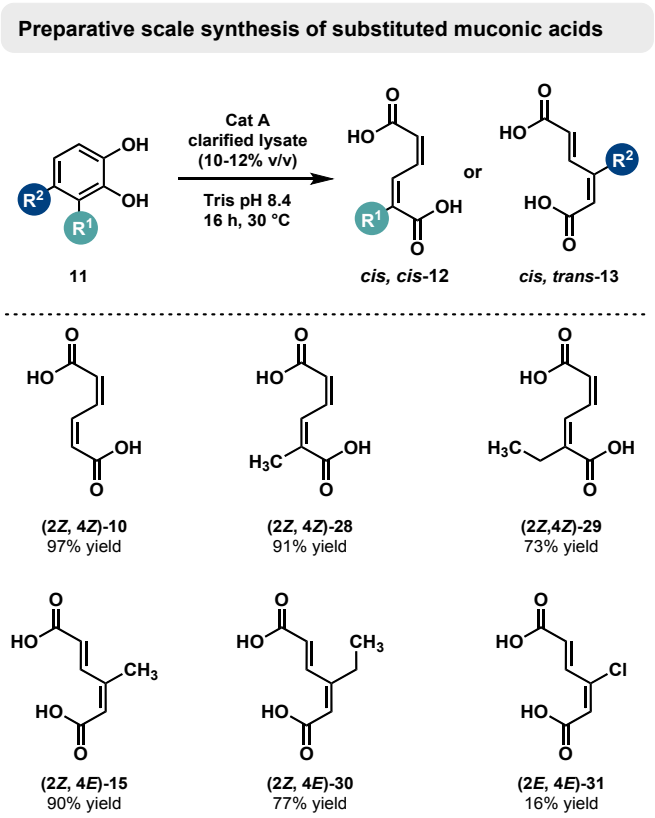


Figure 4. Preparative scale biocatalytic synthesis of substituted muconic acids. Conditions: 5 mM catechol substrate, 10-12% v/v clarified cell lysate harboring CatA, 50 mM Tris pH 8.4, 30 °C, 16 h, 60 rpm shaking.

experiments showed that high yields could be achieved by incubation with 6% v/v lysate, demonstrating that the catalyst was active within this preparation. We then sought to determine if the use of clarified lysates would improve isolated yields of **15**. To test this, we performed a preparative scale reaction using 10% v/v clarified lysate (Figure 3B, entry 3). Analysis of the crude reaction mixture by UV-vis spectroscopy revealed high turnover (96% analytical yield). After workup and trituration of the crude solid with MeCN, we observed a significant increase in the isolated yield of compound **15** to 90%. This represented a 64% increase when compared to the whole cell method (Figure 3B, entry 3). This improvement highlights the importance of screening catalyst preparations when designing a preparative scale biocatalytic platform.

With a reliable method for catalyst delivery and product isolation in hand, we sought to demonstrate milligram scale biocatalytic synthesis of a variety of substituted muconic acids. A reaction with catechol (**1**) at 10% v/v lysate generated the (2*Z*, 4*Z*)-isomer of muconic acid (**10**) in high yield (97%). Under the same conditions, the reaction with 3-methylcatechol (**22**) was high yielding (91%) and exclusively produced the *cis, cis*-isomer of compound **28**. In comparison, initial reactions with 3-ethylcatechol (**23**) proved to be more challenging, resulting in low isolated yields when using 10% v/v lysate. We envisioned that increasing catalyst loading slightly could improve the isolated yield of (2*Z*, 4*Z*)-2-ethylmuconic acid (**29**). To test this hypothesis, oxidative cleavage of **23** was performed at 12% v/v lysate. After isolation and purification, we obtained compound **29** in good yield (73%). To the best of our knowledge, this is the first reported isolation and characterization of the free acid of **29**. However, it should be noted that the methyl ester of cis, cis-**29** has been previously reported.[21]

Preparative-scale reactions with catechols possessing substitution at *C*4 yielded the *cis, trans*-isomer of the resulting muconic acids (see **15**, **30** and **31**). Acid-catalyzed isomerization of *C*3-substituted muconic acids is a known phenomenon, producing the *cis*, *trans*-isomer after acidic workup and extraction.[6] 3-methylmuconic acid (**15**) and 3-ethylmuconic acid (**30**) were both isolated in good yield using 10% v/v CatA lysate. In comparison, the synthesis of 3-chloromuconic acid (**31**) was low yielding, despite observing high substrate conversion by UV-vis analysis. This was primarily due to competing intramolecular lactonization that has been previously observed in 3-halomuconic acids.[22] Due to their inherent reactivity, these molecules are challenging to isolate in the diacid form.[22] Nevertheless, we were able to obtain compound **31** in 16% yield using 10% v/v lysate.

Finally, we sought to demonstrate the feasibility of larger scale reactions using CatA lysates. A gram-scale reaction was performed with catechol (**1**) at 8% v/v lysate (see Figure S2). To reduce the overall volume of the reaction and catalyst use, substrate concentration was increased to 10 mM. This led to an expected reduction in turnover when compared to reactions with 5 mM substrate loading. This reaction yielded 1.002 grams (52%) of the desired cis, cis-muconic acid (**10**). A second larger scale reaction (Figure S3) was performed with 4-methylcatechol (**14**) at 10% v/v lysate loading. This reaction provided 880 mg of (2*Z*, 4*E*)-3-methylcatechol (**15**, 47% yield), further demonstrating that large quantities of muconic acid products can be accessed via biocatalytic oxidation. As with the previous reactions, purification was achieved by trituration with cold MeCN, which resulted in clean material for spectral characterization.

Experimental

Materials and methods

Chemicals and reagents were purchased from commercial suppliers (Sigma-Aldrich, Fisher Scientific, VWR, Twist Biosciences) and used without further purification unless otherwise noted. Some substrates were commercially available, with the exception of **19**-**21** and **25**-**27**, which were synthesized using procedures outlined in the Electronic Supporting Information document. BL21 (DE3) *E. coli* cells were made chemically competent by $CaCl_2$ protocol and transformed with the relevant construct by heat-shock technique. A New Brunswick I26R, 120 V/60 Hz shaker incubator (Eppendorf) was used for cell growth. Optical density and UV-vis measurements were collected on a Cary 50 or Cary 60 UV-vis spectrophotometer using a 1.0 mL quartz cuvette. Preparative flash chromatographic separations were performed on an Isolera One Flash Purification system (Biotage). High resolution mass spectrometry (HRMS) data were collected on an Agilent 6230 TOF LC/MS accurate-mass time-of-flight instrument (supported by NSF CHE-1429616 from Ripon College in Ripon, WI) with samples ionized by electrospray ionization. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE II-400 MHz spectrometer equipped with a 5 mm BBO/Z gradient broadband probe. ^{1}H chemical shifts are reported in ppm (δ) relative to the solvent resonance (i.e., δ $CDCl_3$ 7.26 ppm, δ acetone-d6 2.05 ppm, or δ MeOD 3.31 ppm). ^{13}C NMR data were acquired with ^{1}H decoupling

and chemical shifts are reported in ppm (δ) relative to the solvent resonance (δ $CDCl_3$ 77.16 ppm, δ acetone-d6 29.84 ppm, δ MeOD 49.00 ppm). Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), doublet of doublets (dd), triplet (t), quartet (q), multiplet (m)], coupling constants [Hz], integration). All NMR spectra were recorded at ambient temperature (20–25 °C).

***In vitro* analytical-scale reactions**: For reaction condition optimization, each reaction (100 µL total volume) contained 20 mM buffer (Tris or carbonate) pH 6.8-10.0 (2.0 µL of a 1 M solution), 5 mM substrate (5.0 µL, 100 mM stock), 10 µM CatA (5.88 µL, 170 µM stock), and deionized water to a final volume of 100 µL. After optimization of reaction pH conditions, Tris pH 8.4 was used in all further reactions. Reactions were carried out at 30 °C for 4 h and quenched by the addition of 2 volumes of methanol. Precipitated biomolecules were pelleted by centrifugation (17,000 x g, 10 min). The supernatant was analyzed by UV-vis spectrophotometry and the reaction yield was obtained by the method described below in this section.

Spectrophotometric quantification of percent yield: To quantify the muconic acid product yield in CatA catalyzed reactions, a 100 µL reaction aliquot was quenched by the addition of 2 volumes of methanol and centrifuged at 17,000 x g for 10 min. The resulting supernatant was diluted so that the absorbance at 260 nm would not exceed the linear range for the spectrophotometer (typically a 50 x to 100 x dilution factor). The dilution mixture for this process was 20 mM Tris pH 8.4 to maintain the protonation state (and prevent isomerization) of the muconate product. Using a quartz cuvette, the spectrophotometer was baselined against the dilution mixture before the measurement of A260 for the reaction mixture. Product concentration was calculated using the Beer-Lambert equation and multiplied by the dilution factor to determine the product yield. Product molar absorptivity values were obtained from literature sources[19] or calculated using the method outlined by Dorn and Knackmuss.[23]

General procedure for whole cell preparative scale biocatalytic reactions: A 1 or 2 L (scale-dependent) Fernbach flask was charged with deionized water and Tris buffer at a final concentration of 50 mM (pH 8.4). The corresponding catechol, dissolved in deionized water (200 mM stock, 5 mM final concentration) was added to the solution. The reaction was initiated upon the addition of wet *E. coli* cells harboring overexpressed CatA (20 mg/mL final concentration, 2% w/v). The reaction vessel was placed in the shaking incubator (gentle shaking) at 30 °C for 16 h. Product formation was monitored by dilution and evaluation of absorbance at 260 nm, with quantification as described for analytical scale reactions. After reaction completion, the mixture was quenched with an equivalent volume of acetone and centrifuged (4,000 rpm, 15 min) to remove cellular debris. Supernatant was transferred to a clean beaker, acidified with 2N H_2SO_4 and extracted with ethyl acetate (3 x 30 mL). The remaining cell debris was resuspended in acidic water (pH < 2.0, H_2SO_4) and extracted with a 1:1 mixture of hexanes and ethyl acetate. The organic fraction was filtered to remove particulate matter and combined with organic layers from the supernatant extraction. The organic layer was dried over anhydrous Na_2SO_4 and the volume was reduced by rotary evaporation. Products were purified by repeat trituration with cold MeCN. The remaining solid was dried under vacuum and analyzed directly without further purification.

General procedure for preparative scale biocatalytic reactions with clarified cell lysates: A 1 or2 L (scale-dependent) Fernbach flask was charged with deionized water and Tris buffer at a final concentration of 50 mM (pH 8.4). The corresponding catechol, dissolved in deionized water (200 mM stock, 5 mM final concentration) was added to the solution. The reaction was initiated upon the addition of clarified cell lysates harboring expressed CatA (8-10% v/v). The addition of substrate and catalyst was performed nearly simultaneously so as to prevent non-productive oxidation of the catechol. The reaction vessel was placed in the shaking incubator (gentle shaking) at 30 °C for 16 h. Product formation was monitored by dilution and evaluation of absorbance at 260 nm, with quantification as described for analytical scale reactions. After reaction completion, the mixture was quenched with an equivalent volume of acetone and centrifuged (4,000 rpm, 10 min) to remove denatured protein materials. The supernatant was transferred to a clean beaker, acidified with 2N H_2SO_4 and extracted with ethyl acetate (3 x 30 mL). The organic layer was dried over anhydrous Na_2SO_4 and the volume was reduced by rotary evaporation. The isolated solids were purified by repeat trituration with cold MeCN. The remaining solid was dried under vacuum and analyzed directly without the need for further purification.

Further details regarding biocatalyst preparation, compound synthesis procedures, and full compound analysis, including NMR and UV-visible spectra, can be found in the Electronic Supplemental Information (ESI) document.

Conclusions

We have established conditions for productive catalysis with biocatalyst CatA at synthetically relevant concentrations and have characterized the scope of its reactivity with diverse catechols. Furthermore, we demonstrated the feasibility of preparative-scale synthesis and isolation of substituted muconic acids, up to gram scale, with clarified lysates of CatA. We also developed a simple procedure for purification of the muconic acids by trituration of the product with cold acetonitrile. This method obviates the need for chromatographic purification, which minimizes solvent waste and reduces the environmental impact of the desired transformation. We envision that this sustainable biocatalytic approach to muconic acid synthesis will contribute to ongoing efforts to efficiently access these important organic building blocks.

Author Contributions

DJS and GCR developed the whole cell and lysate biocatalytic methods and performed preparative scale biocatalytic reactions and data analysis. SPT and AMA performed *in vitro* reactions and data analysis. JMU and ARL performed preparative scale reactions using lysates. TJD supervised the project, synthesized substrates and muconic acids, performed data analysis, and wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

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Data Availability Statement for “Sustainable biocatalytic synthesis of substituted muconic acids**”:**

Further details regarding biocatalyst preparation, compound synthesis procedures, and full compound analysis, including NMR and UV-visible spectra, can be found in the Electronic Supplemental Information (ESI) document.