Expression, purification, and expression of ring-cleaving dioxygenases for synthesis and bioremediation

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Introduction

The development of sustainable routes to organic building blocks is a critical endeavor for reducing the environmental impact of traditional organic chemical synthesis. Biocatalysts offer an alternative method to facilitate sustainable synthesis, as they perform highly selective reactions at an increased rate. **Ring-cleaving dioxygenases (RCDs)** are a class of enzymes responsible for selectively breaking open the ring of benzene derivatives to provide a carbon source for microorganisms in bioremediation.

To access the biocatalysts, many microbiology methods were utilized. The *E. coli* cells were transformed to contain the desired gene; the cells were then grown until there optical density was at the ideal value for cell viability (0.6-0.8) and induced with IPTG to facilitate protein expression. After heterologous expression, the enzyme was purified to homogeneity by immobilized metal affinity chromatography.

We continue to analyze RCD types (type I, II, and III)[1] through endpoint screening and product isolation using various substituted catechols. We envision that this approach to muconic acid synthesis will contribute to ongoing efforts to streamline synthesis of these important organic building blocks and reduce the usage of fossil fuels for organic synthesis.

Types of RCDs

Intradiol RCD (type I)

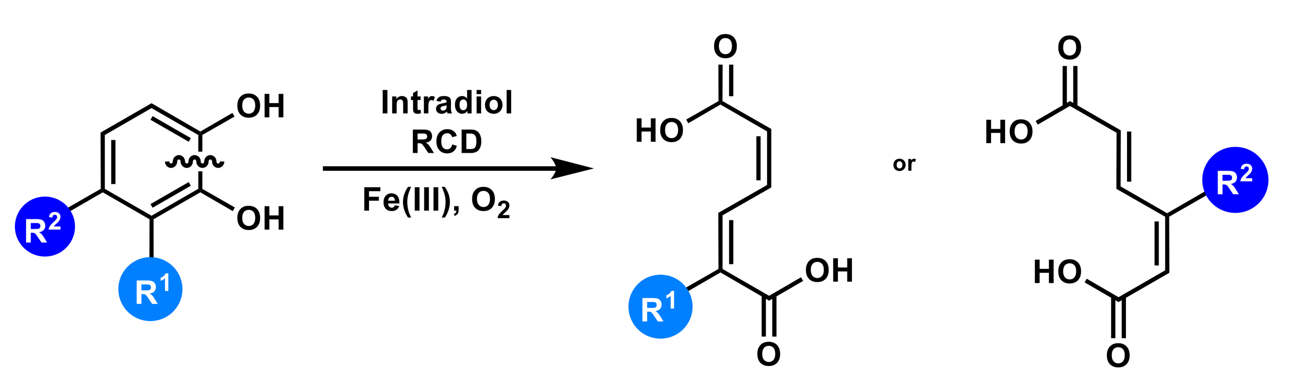


Figure 1. Intradiol oxidative cleavage with Fe^{+3} as the cofactor.

Extradiol RCD (type II)

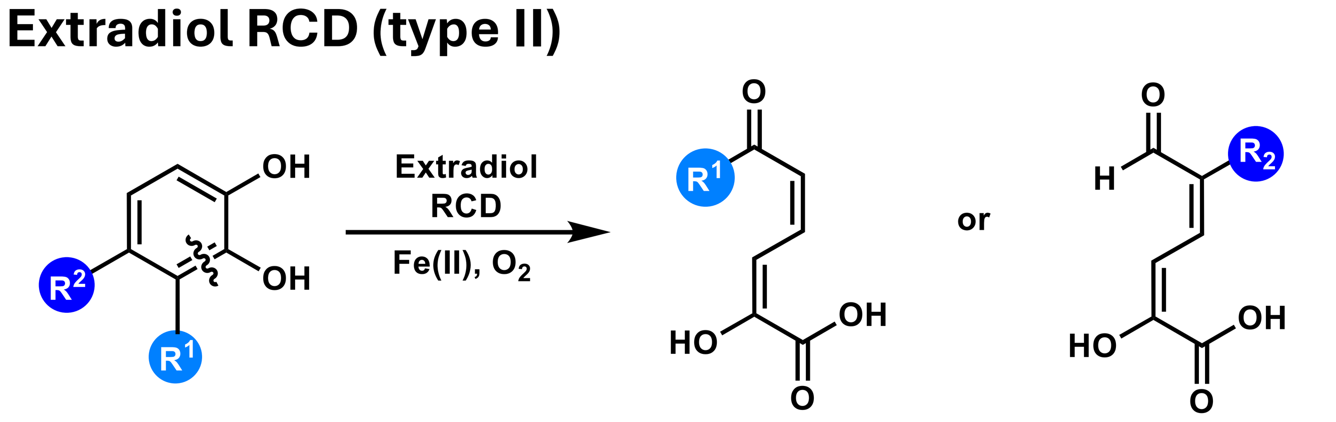


Figure 2. Extradiol oxidative cleavage with Fe^{+2} or other divalent metals (Mn^{+2}, Co^{+2}).[1]

GDO/HGDO/PDO (type III)

Gentisate 1,2-dioxygenase (GDO) produce C7-diacids, Homogentisate 1,2-dioxygenase (HGDO) produce C8-diacids, and PHQ 1,2-dioxygenase (PDO) produce C6-aldehydes.[2]

E. coli Outgrowth and Purification

Transformation

1. Incorporate the purified DNA of the desired dioxygenase
   a. BL21 DE3 competent E. coli cells + dioxygenase DNA
   b. Heat shock
2. Grow on antibiotic containing media overnight
3. Utilize individual colonies for outgrowth

Outgrowth

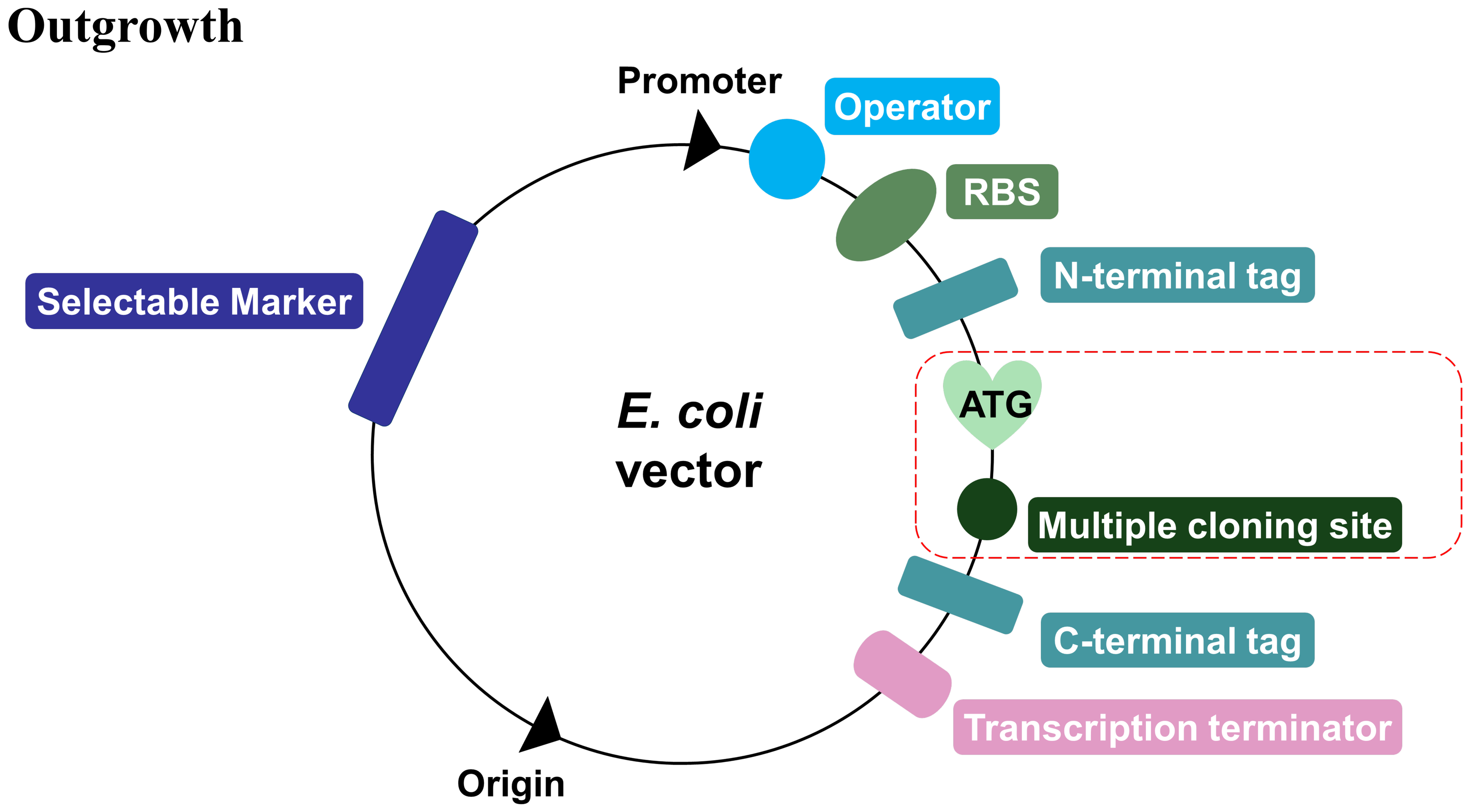
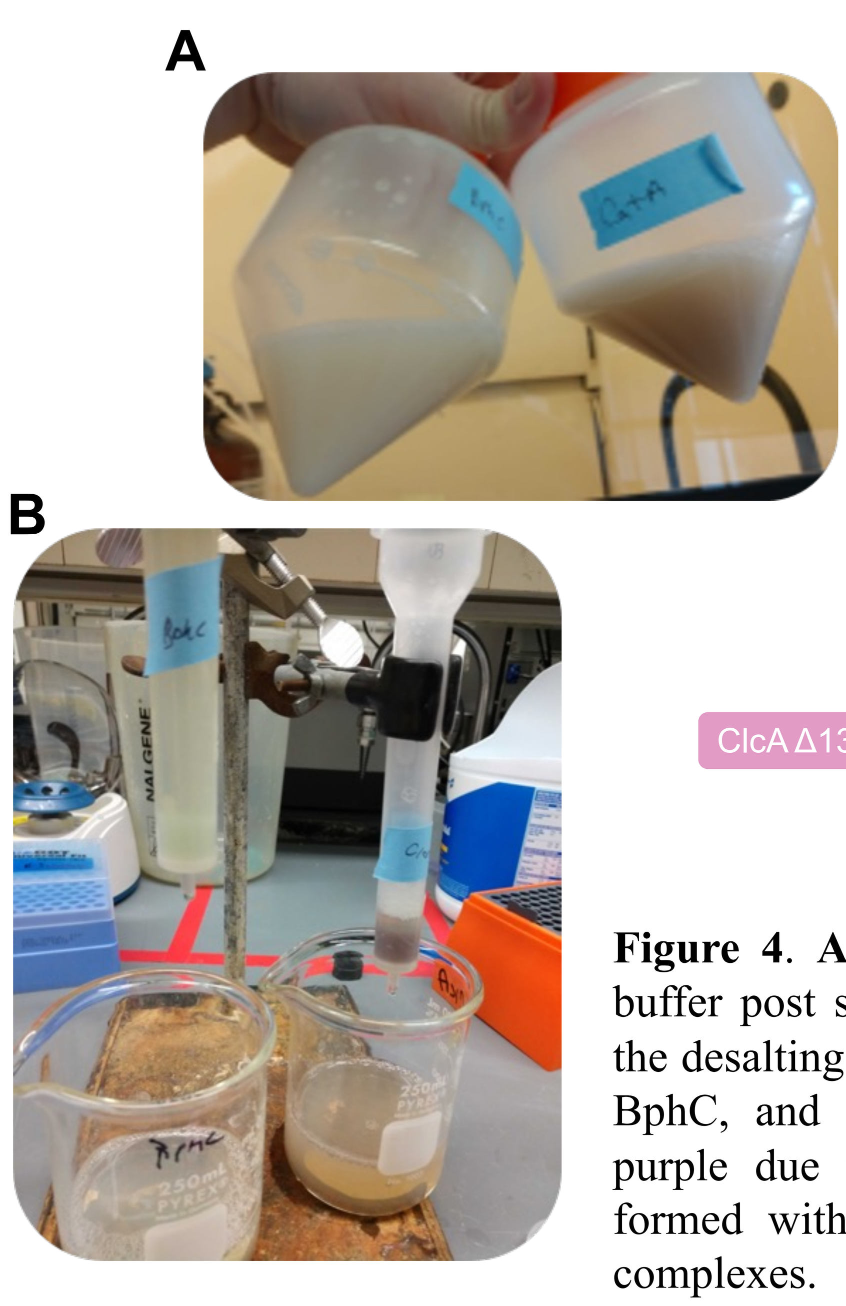


Figure 3. depicts an E. coli vector with the incorporation of the selectable marker from the transformation process. The selectable marker results in antibiotic resistance in the cells that successfully incorporated the enzyme. The ribosome binding site sequence (RBS) is depicted in dark green. ATG refers to the start codon. The red dashed box is where the DNA sequence is cloned into the plasmid DNA.

Purification



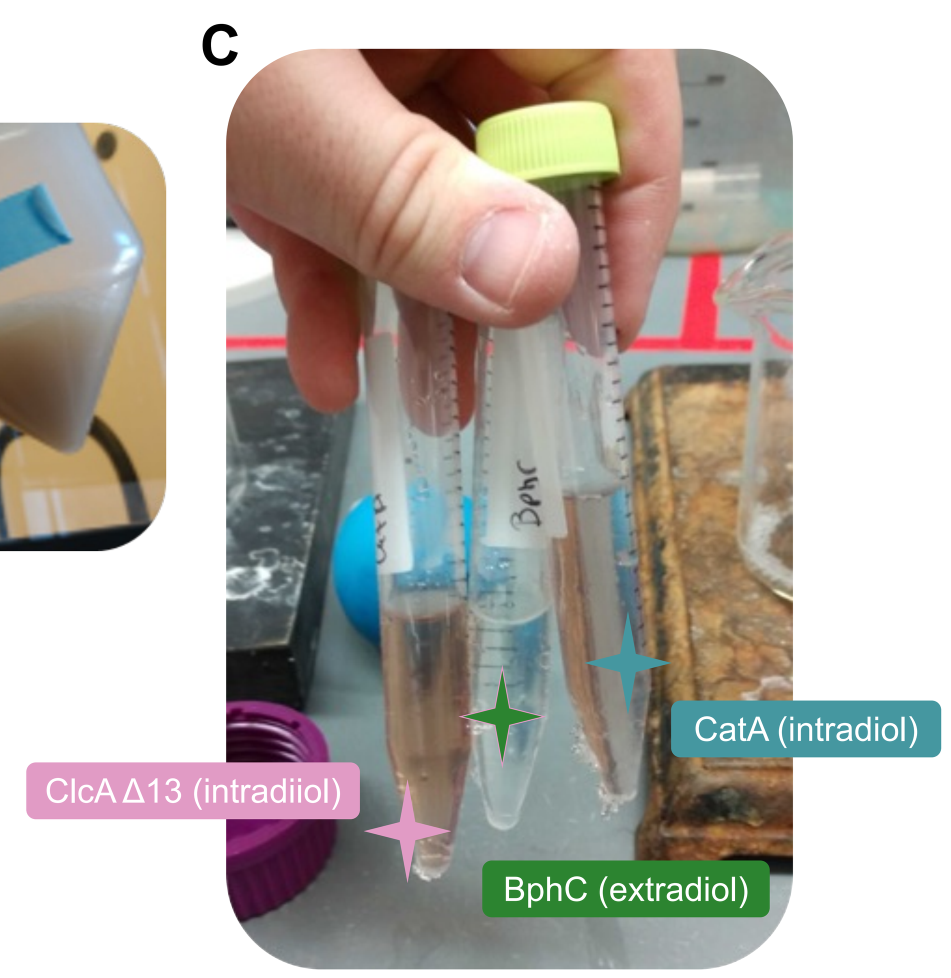


Figure 4. **A** shows the whole cells suspended in lysis buffer post sonication, prior to centrifugation. **B** depicts the desalting columns. **C** from left to right has ClcA Δ13, BphC, and CatA. ClcA Δ13 and CatA being slightly purple due to metal-ligand charge transfer complexes formed with Fe^{+3}. BphC is Fe^{+2} and does form these complexes.

Protein Concentrations

Enzyme[1]	Type	Trial[2]	Yield
BphC	II	1*	38 mg/L
		2	13.14 mg/L
		3	9.89 mg/L
ClcA	I	1*	73 mg/L
CatA	I	1*	75 mg/L
		2	25 mg/L
DfdB	II	1	2.47 mg/L
		2	4.389 mg/L

[1]all proteins were purified under the same conditions. The only variance is when the iron is added and the type of iron (Fe^{+2} or Fe^{+3}). [2]Trials with an asterisk indicate purification preformed at a different institution by the principal investigator Dr. Doyon.

Future Directions

1. Continue to explore the enzymatic library. Including deeper exploration into type III dioxygenases.
2. Utilize isolated muconic acids
3. Optimize a reduction pathway to create adipic acid from muconic acid using alkene reductases.

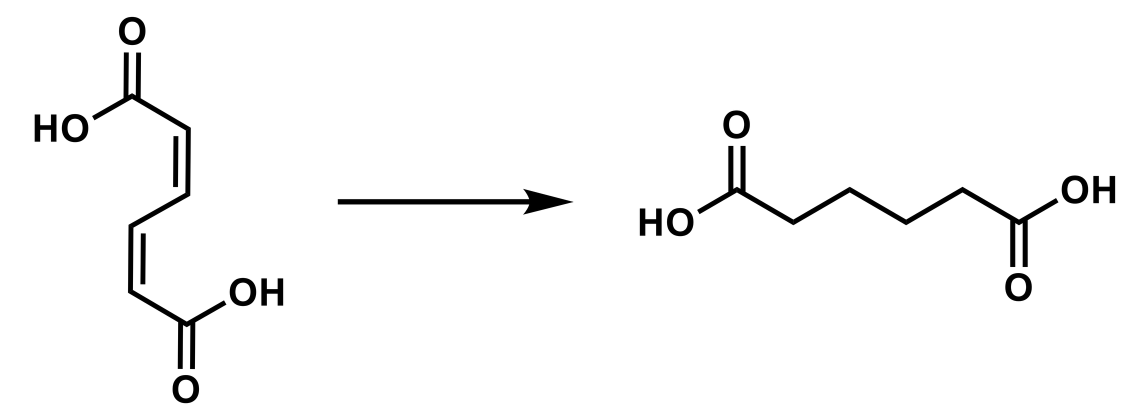


Figure 5. muconic acid (L) adipic acid (R)

Conclusions

The world of enzymology is constantly changing and evolving. The utilization of ring cleaving dioxygenases for industrial production and waste bioremediation is in its infancy. Thus, there is much more to learn about these transformative enzymes.

Acknowledgements/References

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(1) A new thermophilic extradiol dioxygenase promises biodegradation of catecholic pollutants. *Journal of Hazardous Materials* **2021**, *422*, 126860. https://doi.org/10.1016/j.jhazmat.2021.126860.

(2) *ACS Catal.* **2022**, 12, 6191−6208.