Development of High-Throughput Assays for the Detection of Rieske Dioxygenase Activity

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Background on Rieske Dioxygenases

- Enzymes are proteins that act as biological catalysts, speeding up the rate of biological reactions.
- Enzymes provide an environmentally benign alternative to traditional catalytic methods
- Rieske dioxygenases (RDO) are a class of enzyme that can perform the *cis*-dihydroxylation of aromatic compounds to form chiral *cis*-diols
- RDOs were discovered by Prof. David Gibson in soil bacteria, degrading aromatic pollutants from the environment[1]

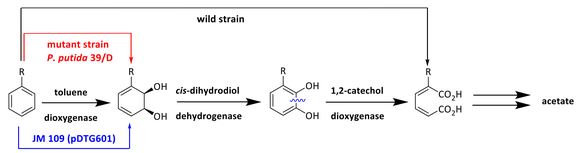

Figure 1: Degradation pathway of aromatic substrate with toluene dioxygenase with mutant/transgenic strains compared to wild strain.

- RDOs are multicomponent enzyme systems, transporting electrons from a reductase to a ferredoxin and finally to a catalytic oxidase
- RDOs perform *cis*-dihydroxylation with O_2 through a unique side-on binding modality, as both oxygen atoms are regio- and stereoseletively incorporated into the aromatic substrate[2]

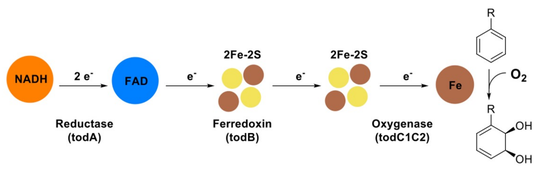

Figure 2: Metabolic pathway for the oxidation of toluene to its corresponding cis-dihydrodiol metabolite by the RDO toluene dioxygenase.[3]

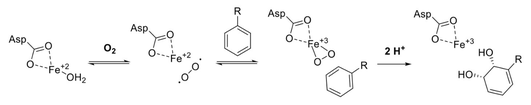

Figure 3: Mechanism of TDO-O dihydroxylation of aromatic compounds

Utility of Metabolites

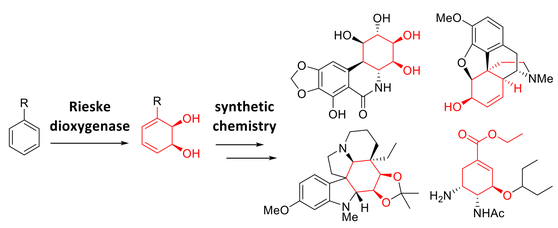

Figure 4: High-value compounds manufactured from RDO metabolites[4]

- RDO have been used to create valuable substances, but their utility is restricted by their substrate scope and strict selectivity[4]

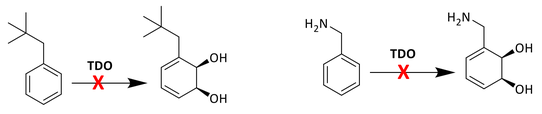

Figure 5: Examples of steric hindrance and electronic constraints of TDO substrate scope

- For RDO catalysts with expanded substrate scopes to be developed, a novel assay system must be developed.
- While assays to detect RDO metabolites exist, they are difficult and expensive to use or provide little information on the production of various metabolites[5]

Assay for Aromatic Dihydroxylation

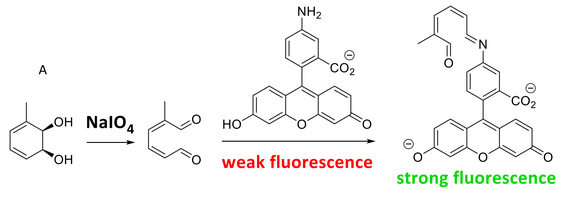

Figure 6: (A) Coupled reactions employed by the fluorescence-based assay system for the detection of cis-diol metabolites; (B) Concentration-dependent fluorescence response of the assay to the presence of cis-diol metabolites [6]

- Our initial assay design employed the oxidative cleavage of the *cis*-diol metabolites produced by active RDOs with sodium (meta)periodate to produce a dialdehyde analyte that can be detected through conjugation to the fluorescent amine-functionalized probe fluoresceinamine
- The conjugation of this probe to the dialdehyde analytes was shown to produce a strong, concentration-dependent fluorescence response[6]
- While this assay system was shown to be effective in detecting the metabolites produced from the dihydroxylation of many aromatic substrates, it demonstrated significantly reduced activity in the detection of the *cis*-diol metabolites produced from the dihydroxylation of aliphatic substrates.

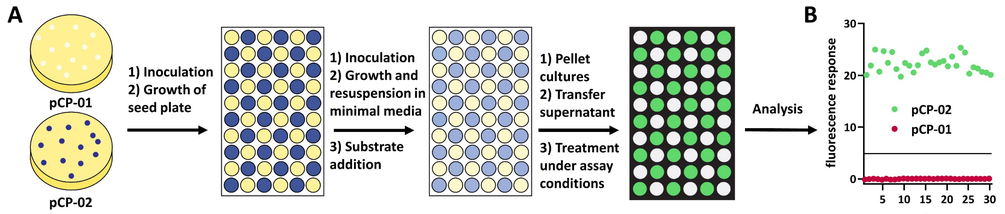

Figure 7: (A) Scheme demonstrating the procedure for the whole-cell checkerboard assay. (B) Fluorescence response of checkerboard assay with and without cultures expressing toluene dioxygenase

Assay for Aliphatic Dihydroxylation

Figure 8: Reaction schemes for synthesis of (A) ABAO probe and (B) MeO-ABAO probe[7]

- An alternate assay system was developed that utilizes the reactive 2-amino-benzamidoxime (ABAO) or 5-methoxy 2-amino-benzamidoxime (MeO-ABAO) probes to quantify the metabolites produced from the dihydroxylation of aliphatic olefins.[7]
- These probes react with the aliphatic aldehydes derived from the periodate oxidation of aliphatic *cis*-diol metabolites to produce a bicyclic analyte which has a significantly increased absorbance at 405 nm.

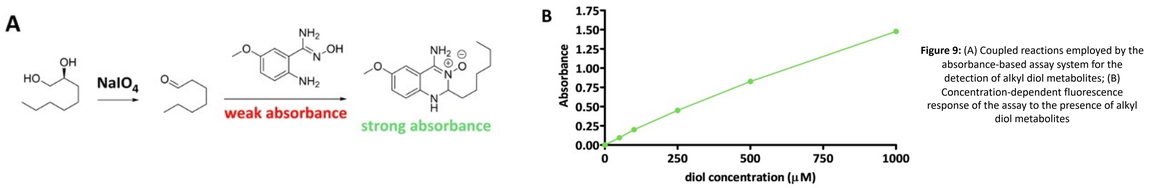

Figure 9: (A) Coupled reactions employed by the absorbance-based assay system for the detection of alkyl diol metabolites; (B) Concentration-dependent fluorescence response of the assay to the presence of alkyl diol metabolites

- The MeO-ABAO probe was shown to produce a stronger response than the ABAO probe, but is more expensive and time consuming to produce

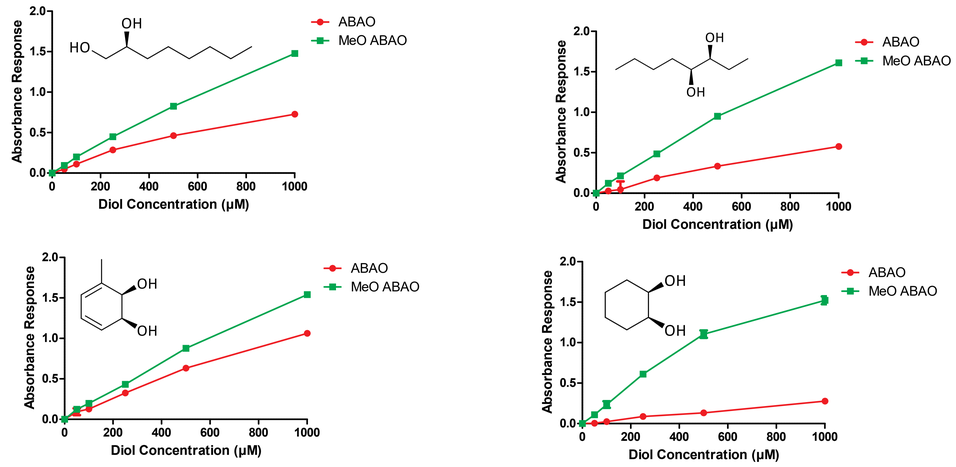

Figure 10: Concentration-dependent absorbance response of probes to various aromatic and aliphatic diols

Conclusions

- Two sensitive assay systems have been developed for detection of RDO activity.
- These assays use relatively low-cost and readily available reagents, and do not require specialized equipment.
- These assay systems are concentration-dependent
- Both assays work through the formation of imines from aldehydes created through the reaction of RDO metabolites with sodium (meta)periodate, creating a strong fluorescent or absorbent response
- The use of these assays will allow the engineering of RDO variants to better create many high-value compounds in a more sustainable way than through petroleum

Future Work

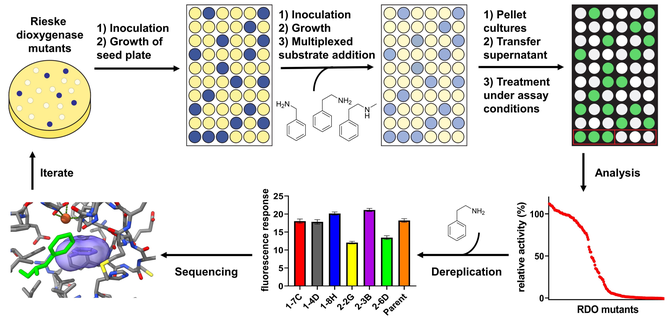

Figure 11: Schematic representation of the directed evolution process employed to engineer Rieske dioxygenase (RDO) variants with expanded substrate scopes.

- Application of these assays will enable the rapid and facile screening of engineered RDO variants
- The substrate scope of RDOs may be expanded by engineering enzyme variants able to catalyze the dihydroxylation of substrates that are be more sterically hindered or electronically constrained
- The substrate scope of RDOs may be expanded by engineering enzyme variants with increased reactivity towards aliphatic substrates

Acknowledgements

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