1. Introduction

- Lignocellulosic biomass, consisting of cellulose, hemicellulose and lignin, is among the best sources for renewable energy and biomaterials.
- Soil microorganisms – nature’s biofuelist – transform cellulose, hemicellulose and lignin to derive energy and nutrients by using specific enzymes (Fig. 1).
- Microbial enzymes are frequently used as biocatalysts to transform cellulose and hemicellulose into fuels and value-added products. However, a very few enzymes can catalyze lignin transformation.
- Thus, lignin, the most abundant aromatic biopolymer, is currently of little commercial value. Moreover, its recalcitrance contributes to the higher cost of biofuels.

2. Enzyme discovery

**Database search:**
- Sequenced bacterial genomes – For example, putative lignin modifying enzymes, such as, dye decolorizing peroxidases (DyPs), multi-copper oxidases (MCOs), aryl-alcohol oxidases (AAOs) were identified and are being characterized from *R. jostii* RH1.

**High-throughput screening methods:**
- Libraries of clones containing large insert DNA, representing bacterial genome or metagenome, are constructed using an appropriate host (for e.g., E. coli).
- Functional screening of libraries to identify clones actively transforming lignin.
- Characterization of clones to identify genes conferring lignin transformation.

3. Characterization and engineering of DypB from RH1A

**Belongs to the CDE superfamily of heme proteins (Fig. 3, left).**
- The first bacterial DyP characterized as ligninase.
- Catalytic efficiency ~10-fold lower than plant-type peroxidases such as HRP.  
- Oxidizes Mn(II); catalytic efficiency ~5,000-fold lower than fungal MnP.  

**Engineered DypB:**
- Substitution of Asn246 with alanine improved the Mn(II)-oxidation rates of DypB  80-fold (Fig. 3, right).
- The engineered variant transformed Kraft lignin and its fractions into mono-aromatics such as: 2,6-dimethoxybenzene and 4-hydroxy-3,5-dimethoxybenzaldehyde (Figs. 4-6).

4. Identification of a ligninolytic multi-copper oxidase (MCO) from a coal bed matagomone

- One of the clones (182-02-C21) that activated the biosensor was subjected to random transposon mutagenesis (Tn5).
- Disruption of one of the genes, encoding for a MCO, reduced the biosensor activation and lignin transformation (Fig. 7).
- The encoded MCO had 80% and 100% amino acid sequence identity with CopA of *Pseudomonas putida* and *P. stutzeri* ATCC 14405, bacteria that degrade a range of aromatic compounds. 

**CopA, a TAT-secreted MCO provides resistance to copper in *P. syringae*, a plant pathogen, and has been described as a pseudoc-lacue due to the requirement of exogenous Cu^{2+} for oxidase activity.**
- Interestingly, copA, located at one end of the fosmid clone, encoded protein had a 39-residue C-terminal truncation with respect to the pseudomonal MCO. This included the conserved motif (HCHXXHXXXXXLM/LF) required for the binding of type 1 (T1) and type 3 (T3) copper centers (Fig. 8).
- A full-length gene was amplified from the metagenomic DNA using to construct the library and was cloned to produce a poly-histidine tagged protein in E. coli.

5. Purification and preliminary characterization of CopA

- CopA was purified using affinity chromatography (Fig. 9, lane 4).
- Purified enzyme catalyzed the oxidation of ABTS and 2,6-DMP in the presence of added Cu(I) (Table 2).
- Interestingly, CopA also catalyzed the oxidation of 2,6-DMP in the absence of Cu(II) after a lag phase of ~10 min (Fig. 10).

6. CopA catalyzed lignin transformation

- Samples of HP-L™ were incubated for 3 hours with CopA in the absence and presence of exogenous Cu(II).
- GC-MS analysis revealed the appearance of new peaks, both in the presence and absence of Cu(II), as compared to the controls containing no enzyme (Fig. 11).
- Products identified using a catalogue search included 6-acetyl-2,5-dihydroxy-1,4-naphthoquinone (t, 10.8 min), 2,6-dimethoxybenzene-1,4-diol (t, 12.8 min), 4-hydroxy-3,5-dimethoxybenzoic acid (t, 13.4 min) and 4-hydroxy-3,5-dimethoxybenzoic acid (t, 17.2 min).

7. Summary

- We identified and characterized two bacterial enzyme, DypB and CopA, that catalyze lignin transformation.
- DypB was the first DyP-type peroxidase implicated in lignin transformation.
- Engineering of DyPb improved its efficiency to transform lignin.
- CopA was identified using a biosensor based HTS method.
- The transformation of lignin and the oxidation of 2,6-DMP by CopA are the first reported oxido-activities for this class of MCOs in the absence of exogenous Cu(II).
- On-going studies are aimed at elucidating the molecular basis for the activity of CopA as well as that of the truncated enzyme in whole cells.

8. References


9. Acknowledgements

Dr. William Mohr provided access to the GC-MS. Jo Ho cloned CopA.