Discovery and engineering of lignin modifying enzymes

Rahul Singh[1], Cameron R. Strachan[1,3], Karen Budwill[4], Steven J. Hallam[1,3] and Lindsay D. Eltis[1,2]

Departments of [1]Microbiology and Immunology, [2]Biochemistry and Molecular biology, The University of British Columbia, Vancouver, BC, V6T 1Z3 Canada,
[3]MetaMixis, Vancouver, BC, Canada, [4]Environment and Carbon Management Division, Alberta Innovates-Technology Futures, Edmonton, AB, Canada

1. Introduction

- Lignocellulosic biomass, consisting of **cellulose**, **hemicellulose** and **lignin**, is among the best sources for renewable energy and biomaterials.[1]
- Soil microorganisms – nature's biocatalyst – 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.

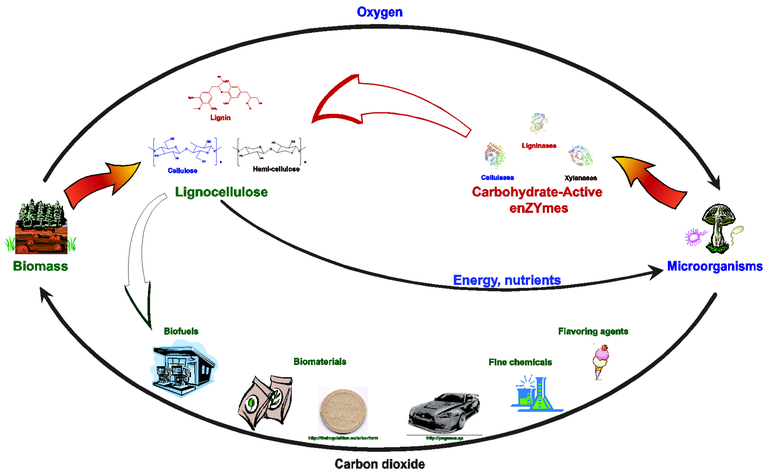


Figure 1. Components of the biomass and the enzymes involved in its deconstruction.

Lignin bio-degradation:

- The best characterized lignin-modifying organisms are fungal.[2] These produce lignin and manganese peroxidases that depolymerize lignin (*i.e.*, ligninases). However, the challenges associated with fungal genetics and protein production have limited the commercial applications.

Lignin degradation by bacteria

- Several classes of "lignolytic or lignin modifying" bacteria have been identified.[2] However, bacterial ligninases and lignin catabolic pathways are poorly characterized.
- The best characterized bacterial ligninases are dye-decolorizing peroxidases (DyP): DypB of *Rhodococcus jostii* RHA1[3] and DyP2 of *Amycolatopsis* sp. 75iv2.[4]
- Both catalyze the H_2O_2-dependent oxidation of Mn(II), albeit less efficiently than fungal MnPs.[4]
- Moreover, the next generation DNA-sequencing and high-throughput screening methods provides a great opportunity to discover lignin transforming enzymes from characterized and uncharacterized bacteria.

Overall aims: to characterize and engineer bacterial lignin-modifying systems.

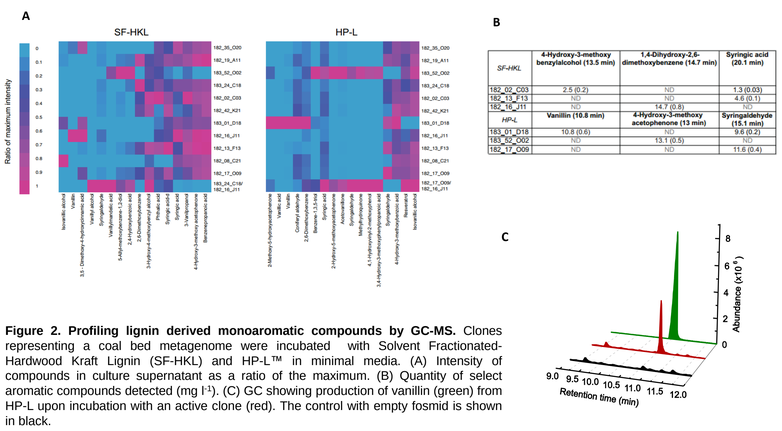
2. Enzyme discovery

Database search:

- Sequenced bacterial genomes – For example, putative lignin modifying enzymes, such as, dye decolorizing peroxidase (DyPs), multi-copper oxidases (MCOs), aryl-alcohol oxidases (AAOs) were identified and are being characterized from *R. jostii* RHA1.

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.



SF-HKL	4-Hydroxy-3-methoxy benzylalcohol (13.5 min)	1,4-Dihydroxy-2,6-dimethoxybenzene (14.7 min)	Syringic acid (20.1 min)
182_02_C03	2.5 (0.2)	ND	1.3 (0.03)
182_13_F13	ND	ND	4.6 (0.1)
182_16_J11	ND	14.7 (0.8)	ND
HP-L	**Vanillin (10.8 min)**	**4-Hydroxy-3-methoxy acetophenone (13 min)**	**Syringaldehyde (15.1 min)**
183_01_D18	10.8 (0.6)	ND	9.6 (0.2)
183_02_C002	ND	13.1 (0.5)	ND
182_17_C09	ND	ND	11.6 (0.4)

Figure 2. Profiling lignin derived monoaromatic compounds by GC-MS. Clones representing a coal bed metagenome were incubated with Solvent Fractionated-Hardwood Kraft Lignin (SF-HKL) and HP-L™ in minimal media. (A) Intensity of compounds in culture supernatant as a ratio of the maximum. (B) Quantity of select aromatic compounds detected (mg l^{-1}). (C) GC showing production of vanillin (green) from HP-L upon incubation with an active clone (red). The control with empty fosmid is shown in black.

- For example, using a biosensor based HTS method, we identified 24 clones representing a coal bed metagenome showing lignin transformation phenotype (Fig. 2).
- Transposon mutagenesis and bioinformatics analyses of the active clones highlighted the genes, encoding for enzymes such as multi-copper oxidase, aryl alcohol oxidase, conferring lignin transformation phenotype.

3. Characterization and engineering of DypB from RHA1

- 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.[3]
- Oxidizes Mn(II); catalytic efficiency ~5,000-fold lower than fungal MnP.[3]

Engineered DypB

- Substitution of Asn246 with alanine improved the Mn(II)-oxidation rates of DypB ~80-fold[5] (Fig. 3, right; Table 1).
- The engineered variant transformed Kraft lignin and its fractions into mono-aryls such as: 2,6-dimethoxybenzene and 4-hydroxy-3,5-dimethoxybenzaldehyde (Figs. 4-6).[5]

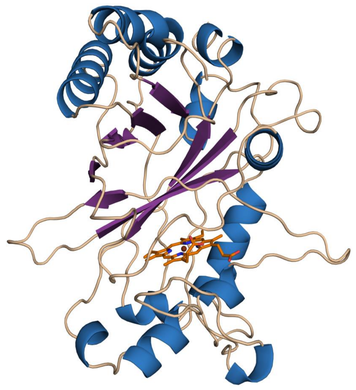


Table 1. Steady state kinetic parameters for Mn(II) oxidation by DypB, N246A and DyP2.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (x10^3 $M^{-1}s^{-1}$)
DypB	3.0 ± 0.3	0.49 ± 0.02	0.16 ± 0.01
N246A	17 ± 2	39 ± 3	2.3 ± 0.1
Dyp2	0.21 ± 0.03	24 ± 1	120 ± 20

Figure 3. Crystal structure of DypB (left) and the Mn(II)-binding site of the N246A variant (right) (X-ray crystallography in collaboration with the Murphy lab).

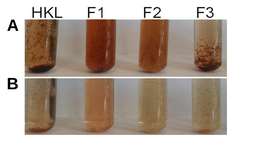


Figure 4. Incubation of HKL and its fractions with N246A. Reaction mixtures contained 0.25 mg/ml of a preparation of HKL , 100 nM N246A 20 mM $MnSO_4$, and 0.5 mM H_2O_2 (20 mM sodium malonate, pH 5.5, 15% DMSO) and were incubated at 30 °C for 60 min with (A) or without (B) enzyme.

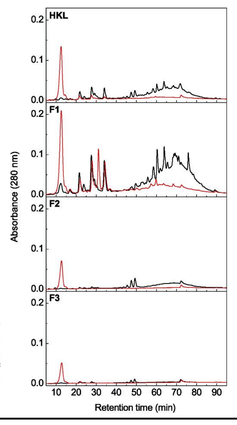


Figure 5. Chromatographic analyses of the reactions described for Figure 5. The soluble products were extracted and analyzed using reverse phase HPLC. The elution profiles of reactions incubated with (red traces) and without (black traces) N246A are shown.

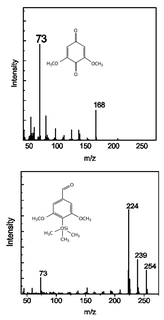


Figure 6. Structural characterization of transformation products obtained from HKL-F1. The mass spectra of the purified products eluting at t_r = 12 min (left) and t_r = 31 min (right) are shown. Structures corresponding to the fragmentation patterns are shown in inset.

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

- 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.[3,7]
- CopA, a TAT-secreted MCO provides resistance to copper in *P. syringae*, a plant pathogen, and has been described as a pseudo-laccase due to the requirement of exogenous Cu^{2+} for oxidase activity.[7]
- Interestingly, *copA,* located at one end of the fosmid clone, encoded protein had an 39-residue C-terminal truncation with respect to the pseudomonad MCO. This included the conserved motif (HCHXXXHXXXXM/L/F) 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 used to construct the library and was cloned to produce a poly-histidine tagged protein in *E. coli*.

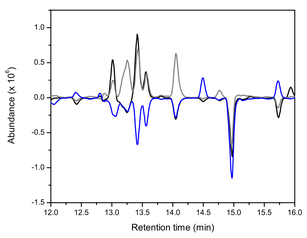


Figure 7. GC-MS of HP-L™ incubated with the clone 182-08-C21 (blue) and its Tn5 mutants at the position 4949 (black) and 55060 (black).

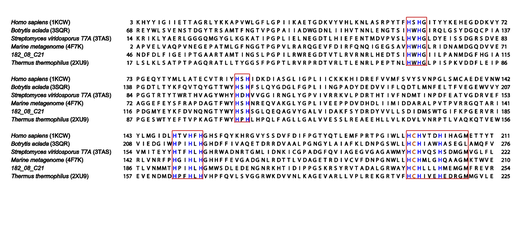


Figure 8. Structure based sequence alignment of CopA. The structure based multiple-sequence alignment was generated using 3D-Coffee. The structures of MCOs from six different organisms: *Homo sapiens* (PDB ID: 1KCW), *Streptomyces viridosporus* T7A (PDB ID: 3TAS), a marine metagenome (PDB ID: 4F7K), and *Thermus thermophilus* (PDB ID: 2XU9) were selected from the protein data bank. The resulting alignment was edited manually to show the structurally conserved regions

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(II) (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).

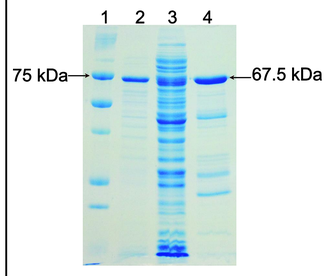


Figure 9. SDS-PAGE of CopA. Lane 1, protein molecular weight marker; lane 2, total protein; lane 3, soluble portion; lane 4, eluate from Ni-NTA column.

Table 2. Specific activity of CopA. The specific activity was determined by following the enzyme catalyzed oxidation of ABTS and 2,6-DMP. ABTS oxidation was monitored at 414 nm in 50 mM sodium acetate (pH 4.5), whereas oxidation of 2,6-DMP was monitored at 470 nm in 20 mM potassium phosphate (pH 8.0). The reactions were catalyzed by adding 30 nM purified CopA in the presence of 5 mM $CuSO_4$. All the measurements were performed in triplicates.

Substrate	Specific activity (nmole min^{-1} mg^{-1})
ABTS (pH 4.5)	1000 ± 25
2,6-DMP (pH 4.5)	700 ± 40
2,6-DMP (pH 8.0)	125 ± 10

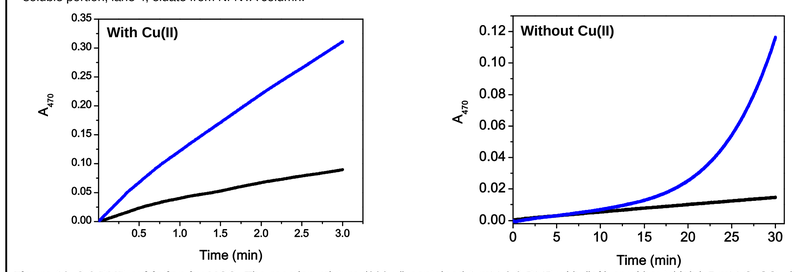


Figure 10. 2-6,DMP oxidation by MCO. The reaction mixture (200 µl) contained 1 mM 2,6-DMP with (left) or without (right) 5 mM $CuSO_4$, buffered at pH 8 in 20 mM sodium phosphate. The reaction was catalyzed by adding 30 or 200 nM CopA (without $CuSO_4$). The no enzyme control is shown in black.

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_r 10.8 min), 2,6-dimethoxybenzene-1,4-diol (t_r 12.8 min), 4-hydroxy-3-methoxybenzoic acid (t_r 13.4 min) and 4-hydroxy-3,5-dimethoxybenzoic acid (t_r 17.2 min).

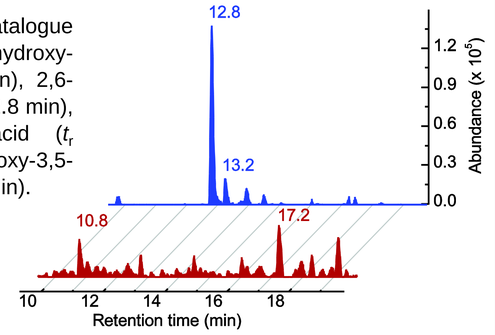


Figure 11. Gas chromatography of HP-L™ incubated with CopA. Difference chromatogram showing CopA catalyzed transformation of HP-L™ in the presence (blue) or absence (red) of $CuSO_4$.

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 oxidase 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

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9. Acknowledgements

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