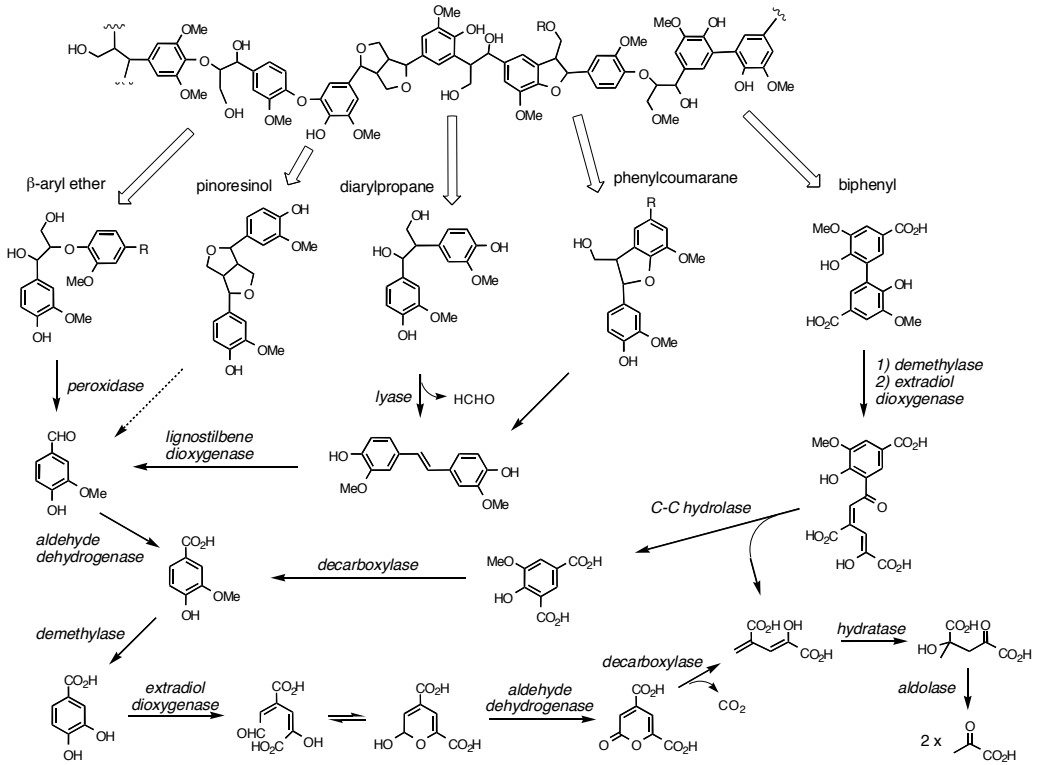
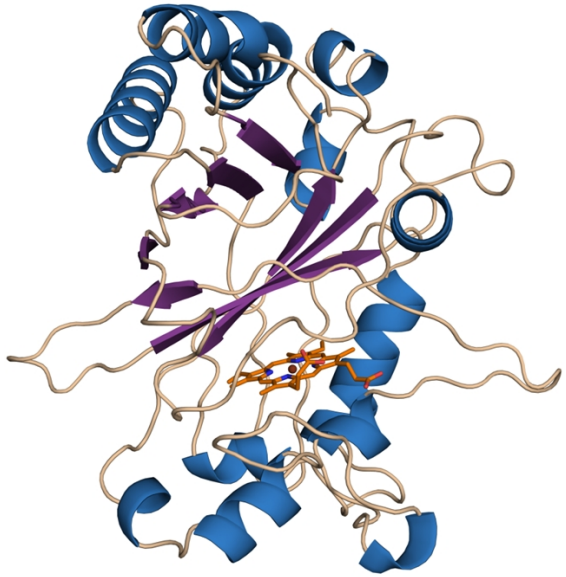
Bacterial Enzymes for Lignin Valorisation

Prof. Tim Bugg

Department of Chemistry, University of Warwick





THE UNIVERSITY OF WARWICK

Lignocellulose-Based Bio-refinery Concept

Biomass

2nd Generation Bioethanol Process

Thermochemical pretreatment

Lignin

Cellulose

Hemi-cellulose

cellulases

xylanases

Glucose

Xylose

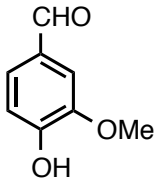
fermentation

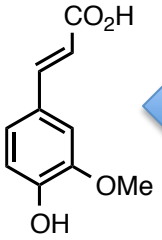
Ethanol

distillation

Biofuel

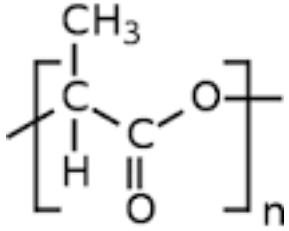
Organic Acids





Vanillin 14-15 $/kg

Ferulic acid 85-100 $/kg



Polylactic acid
Bio-based polymer $3-6/kg

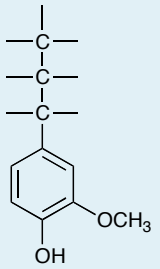
Lactic acid 1.3-1.6 $/kg

Succinic acid 6-9 $/kg

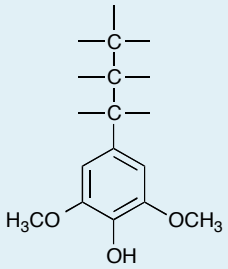
EtOH 0.5-1 $/litre

Molecular Structure of Lignin

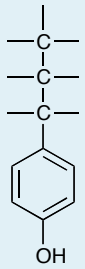
- High molecular weight aromatic polymer
- Heterogenous structure, highly cross-linked
- Contains aryl-C3 units:
- Different substitution patterns in different plant types:



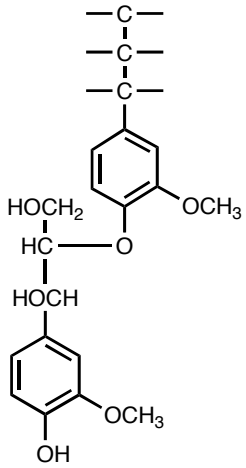
guaiacyl unit (G)



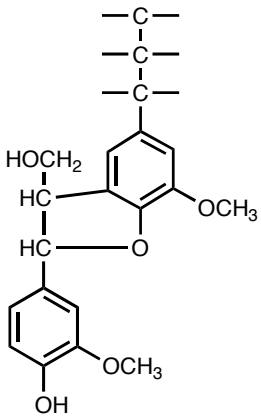
syringyl unit (S)



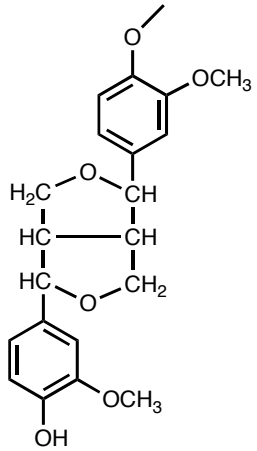
p-hydroxyphenyl unit (H)



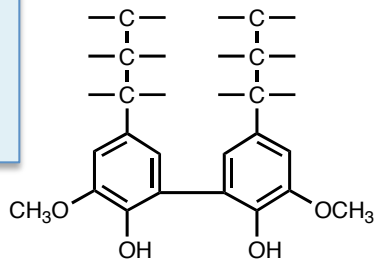
β-aryl ether



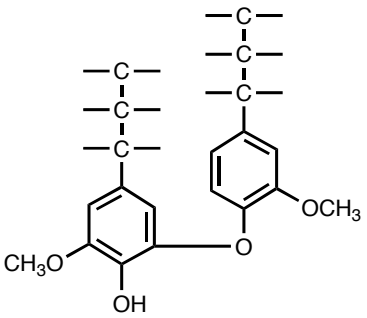
phenylcoumarane



pinoresinol



biphenyl



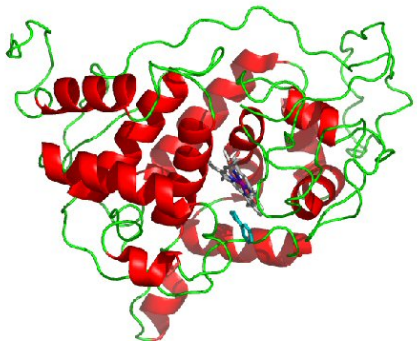
diaryl ether

Potential source of aromatic products

Fungal Enzymes for Lignin Oxidation

Lignin peroxidase

P. chrysosporium

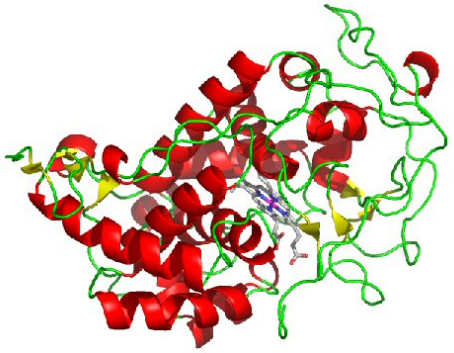


Heme Fe enzyme
Oxidant H_2O_2

Able to oxidise
Lignin model cpds
Cα-Cβ cleavage
Cα oxidation

Manganese peroxidase

P. chrysosporium

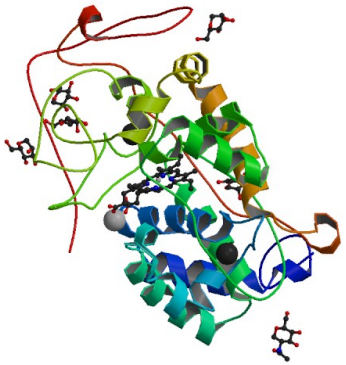


Heme Fe enzyme
Oxidant H_2O_2

Oxidises Mn(II) to Mn(III), oxidant for lignin

Versatile peroxidase

Pleurotus eryngii

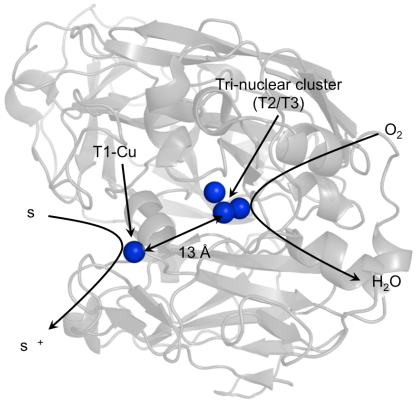


Heme Fe enzyme
Oxidant H_2O_2

Oxidises Mn(II) or lignin

Laccase

Trametes versicolor



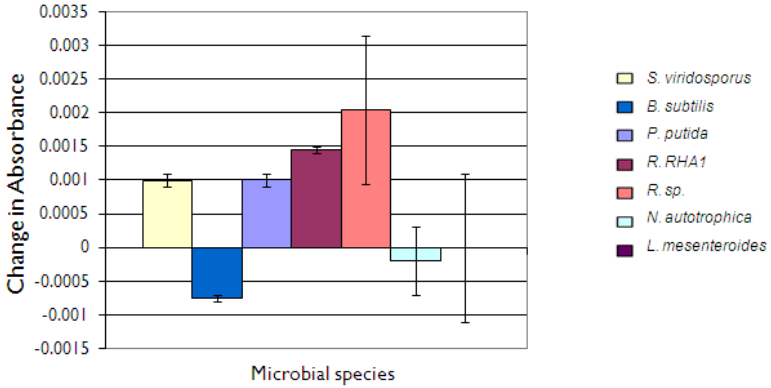
Multi-copper enzyme
Oxidant O_2

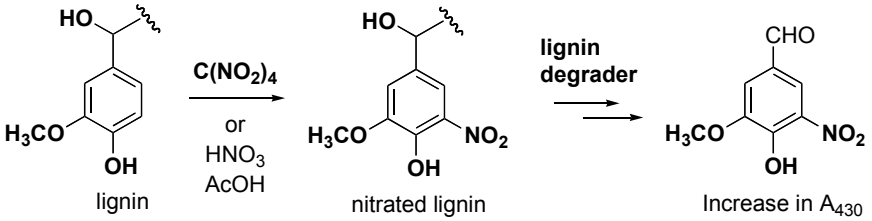
Able to oxidise wide range of phenols, using redox mediator

Bacteria – Ramachandra et al., *Appl. Env. Microbiol*. 1988, **54**, 3057 report that *Streptomyces viridosporus* contains extracellular lignin peroxidase activity, but no gene identified. Reports that strains of *Nocardia*, *Rhodococcus* have lignin oxidation ability.

Possible advantages: easier protein expression, genetic tools available, thermophilic enzymes?

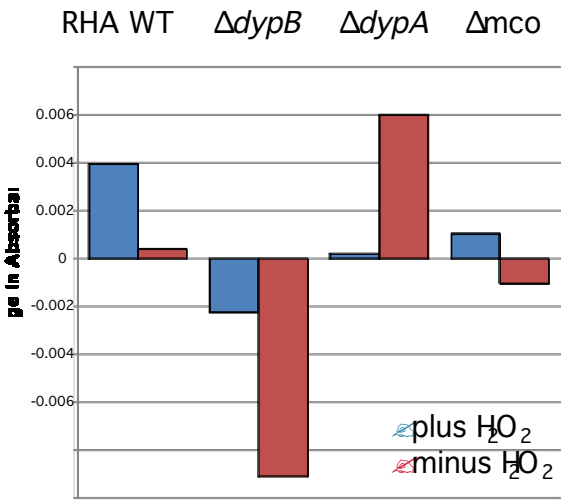
Identification of *Rhodococcus jostii* Peroxidase DypB



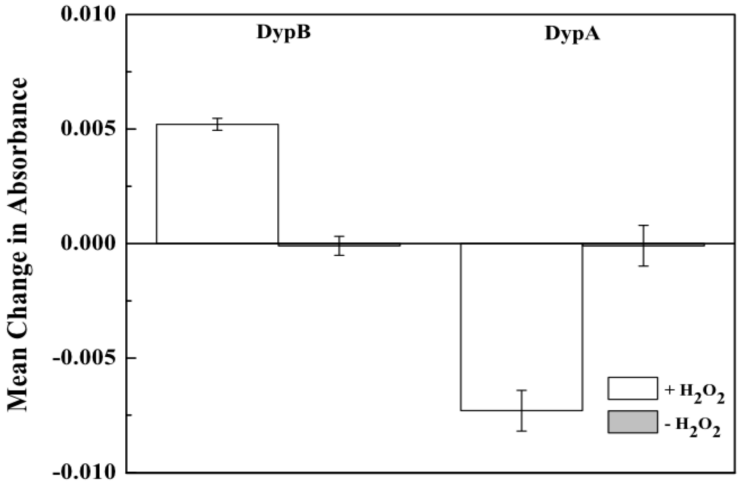


M. Ahmad et al, *Mol. Biosystems*, 2010, **6**, 815-821

2 unannotated peroxidase genes found in *R. jostii* genome, *ΔdypB R. jostii* mutant shows loss of activity:

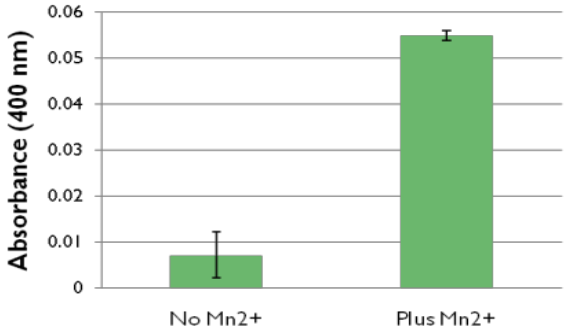


Recombinant DypB is active in nitrated MWL assay



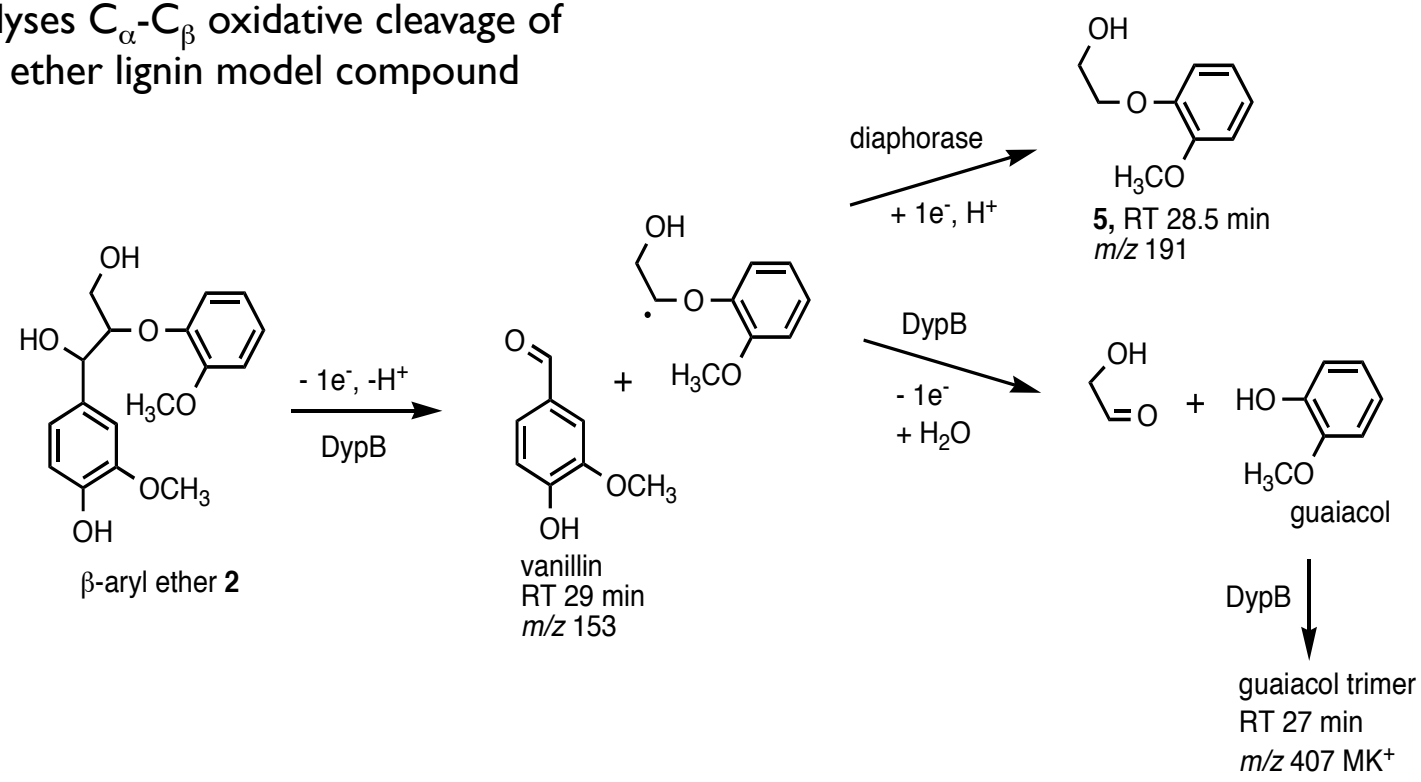
Biochemical Properties of DypB

- Activity with ABTS is enhanced by 1 mM $MnCl_2$



- Time-dependent activity observed with Kraft lignin (565 nm) and lignocellulose (in presence of Mn^{2+})

- catalyses C_α-C_β oxidative cleavage of a β-aryl ether lignin model compound

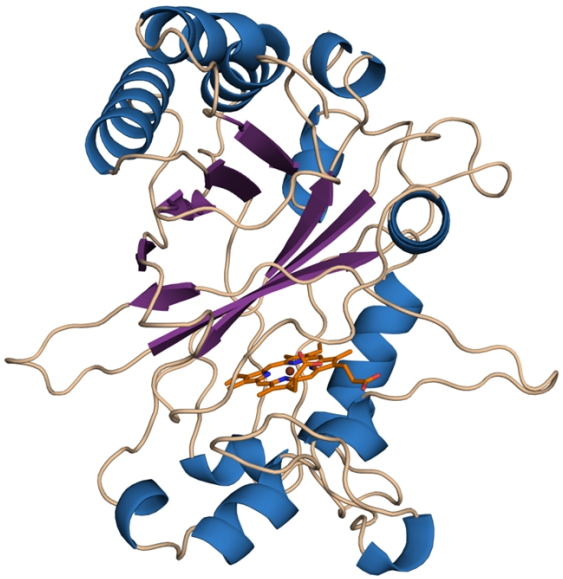


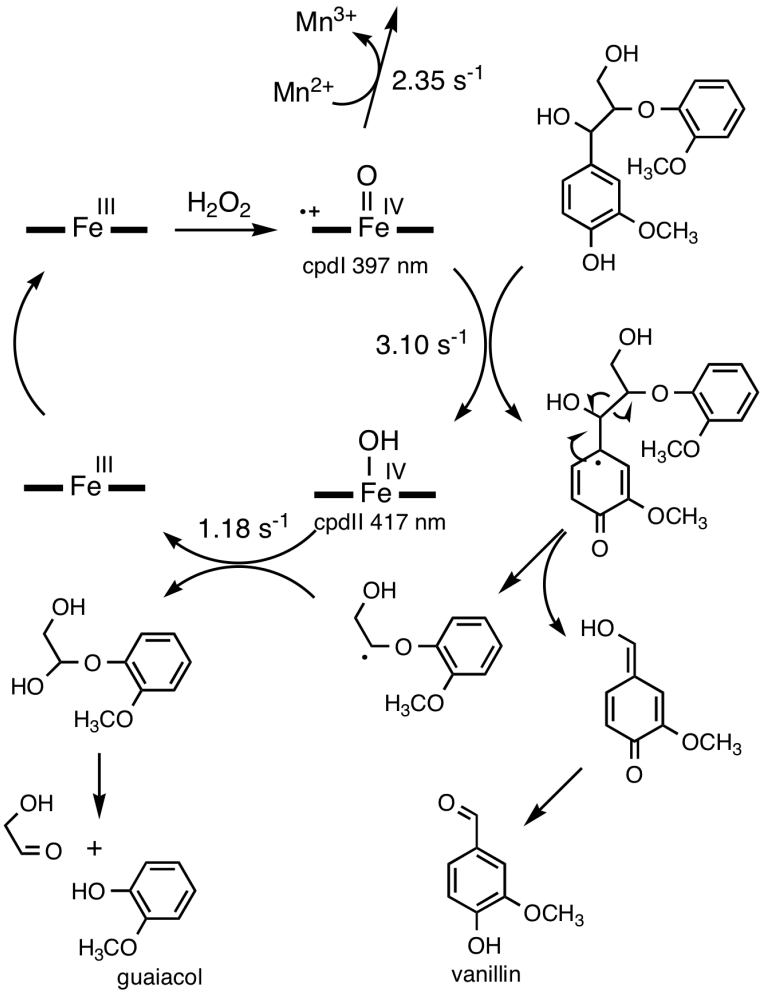
Catalytic cycle for DypB

- formation of compound I (397 nm) and compound II (417 nm) observed using stopped flow kinetics

- rate constants measured for oxidation of β-aryl ether and Mn(II)

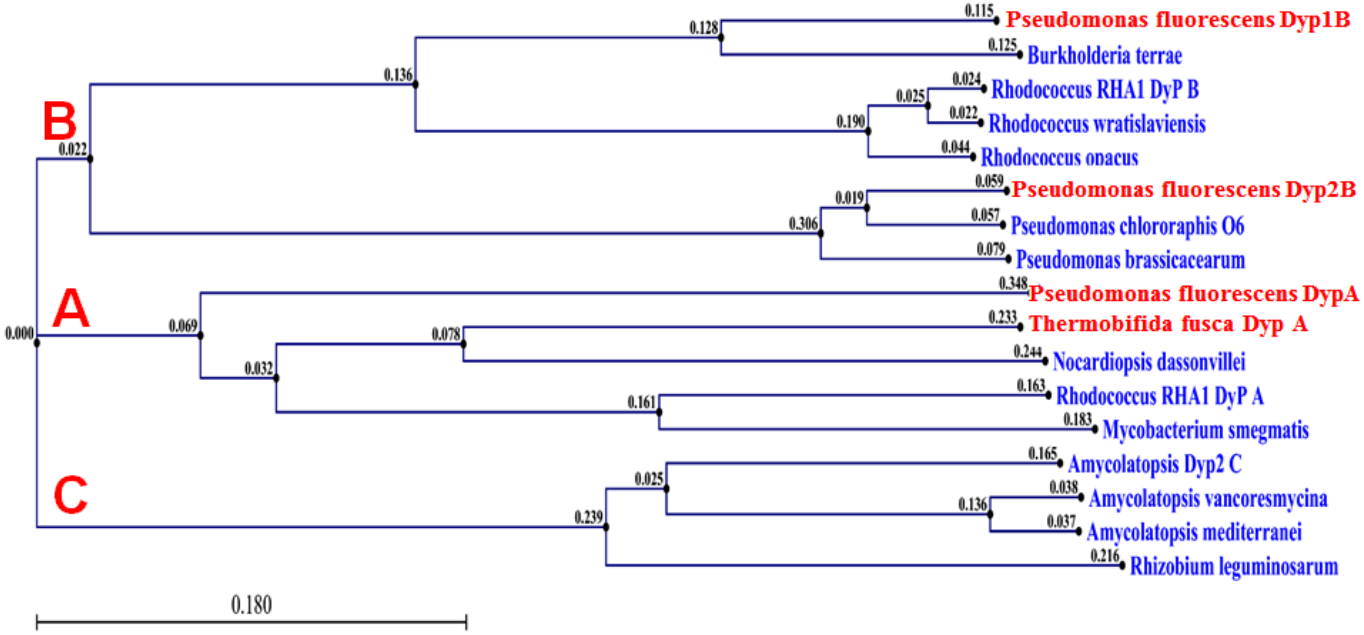
- Crystal structure of *R. jostii* DypB determined in collaboration with Prof. L. Eltis (UBC, Canada)



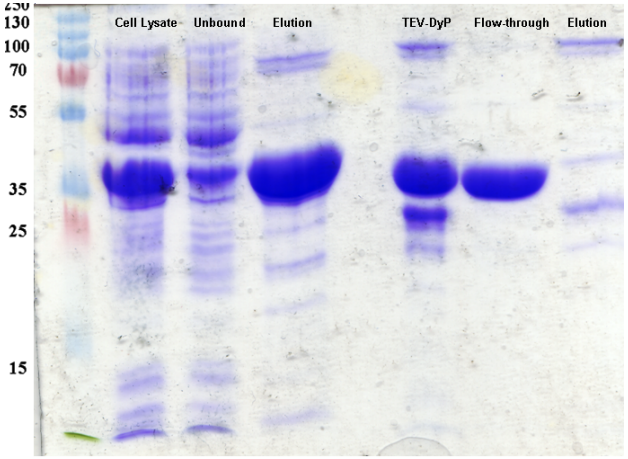


M. Ahmad, E.M. Hardiman et al., *Biochemistry*, 2011, **50**, 5096
J.N. Roberts et al., *Biochemistry*, 2011, **50**, 5108.

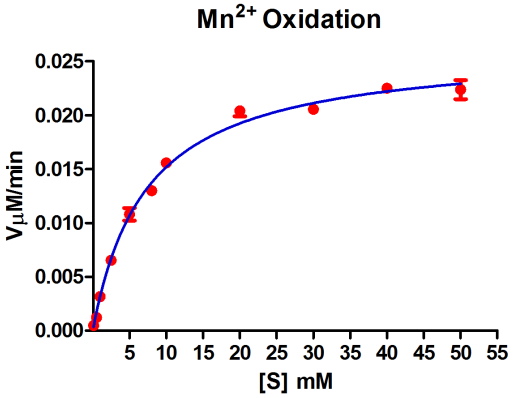
3 DyP Peroxidases present in *Pseudomonas fluorescens* Pf-5

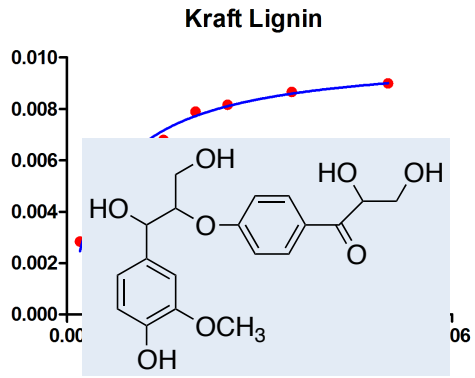


Expression & purification of
P. fluorescens DyP1B

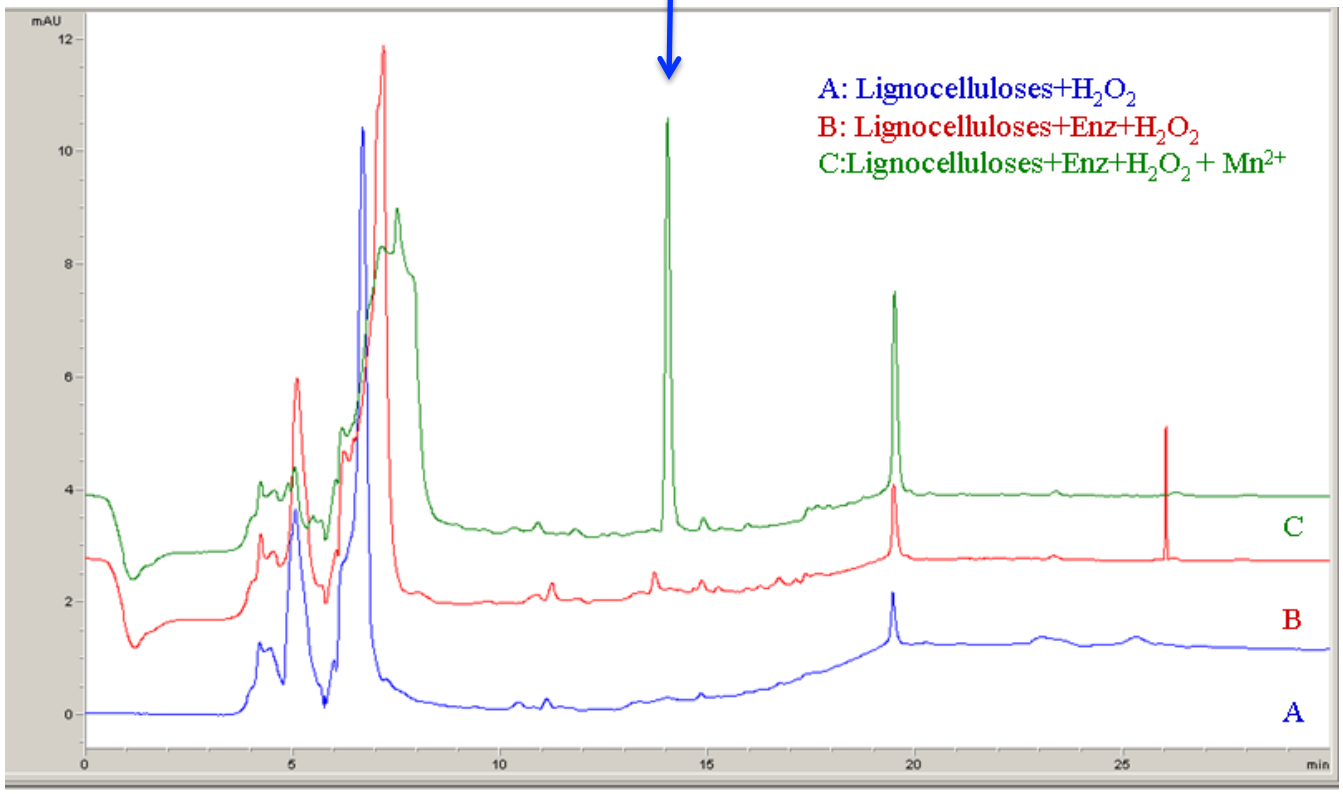


P. fluorescens DyP1B oxidises Mn^{2+}, Kraft lignin and lignocellulose



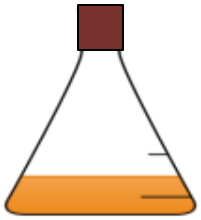


Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
ABTS	1.13	13.5	1.2×10^4
Mn(II)	7.3	2.4	3.3×10^2
Phenol	1.02	1.2	1.2×10^3
Kraft lignin	0.006	0.9	1.4×10^5

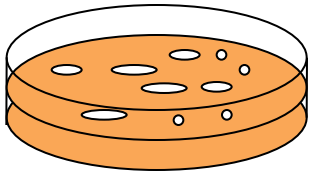


Screening for Novel Lignin Degrading Strains

Sampling

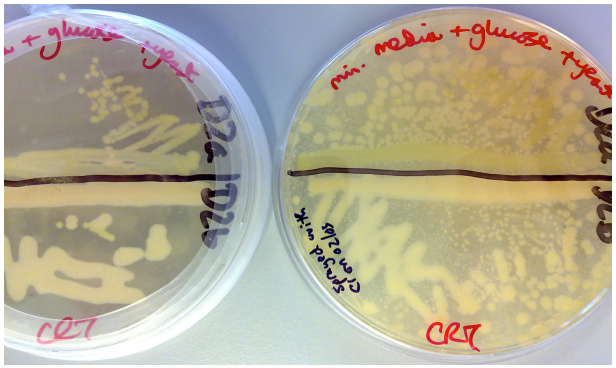


Culturing –
selective medium



Plating

Nitrated lignin
spray assay



Pure cultures

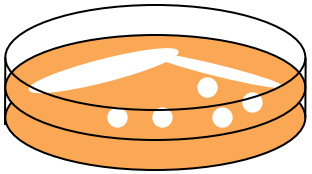


Plate assay

- Identification bacterial species (e.g.16S rRNA)
- Purify and identify extracellular enzymes
- Recombinant expression

Identification of Isolates Obtained from Screening

9 mesophilic isolates identified from soil enrichment
2 thermotolerant isolates from screening of composted wheat straw at 45 °C

Strain	Identification from 16S rRNA sequencing	Bacterial family	Activity in nitrated lignin assay (mAU)	
			+ H_2O_2	-H_2O_2
A1.1	*Microbacterium phyllosphaerae*	Actinobacteria	1.9	2.7
A1.2	*Microbacterium marinilacus*	Actinobacteria	1.7	0.6
A2.1	*Microbacterium marinilacus*	Actinobacteria	0.9	1.4
A4.3	*Ochrobactrum pseudogrignonense*	α-Proteobacteria	1.2	4.7
A5.1	*Rhodococcus erythropolis*	Nocardioform	1.6	1.9
A5.2	*Microbacterium oxydans*	Actinobacteria	1.1	5.4
B5.3	*Micrococcus luteus*	Actinobacteria	1.9	1.1
C4.1	*Ochrobactrum rhizosphaerae*	α-Proteobacteria	2.0	1.9
E1.1	*Micrococcus luteus*	Actinobacteria	0.9	1.1
T1	*Rhizobiales sp.*	α-Proteobacteria	0.2	2.0
T2	*Sphingobacterium sp.*	Bacteroides	9.0	30

C.R. Taylor, E.M. Hardiman et al., *J. Appl. Microbiol.*, 2012, **113**, 521.

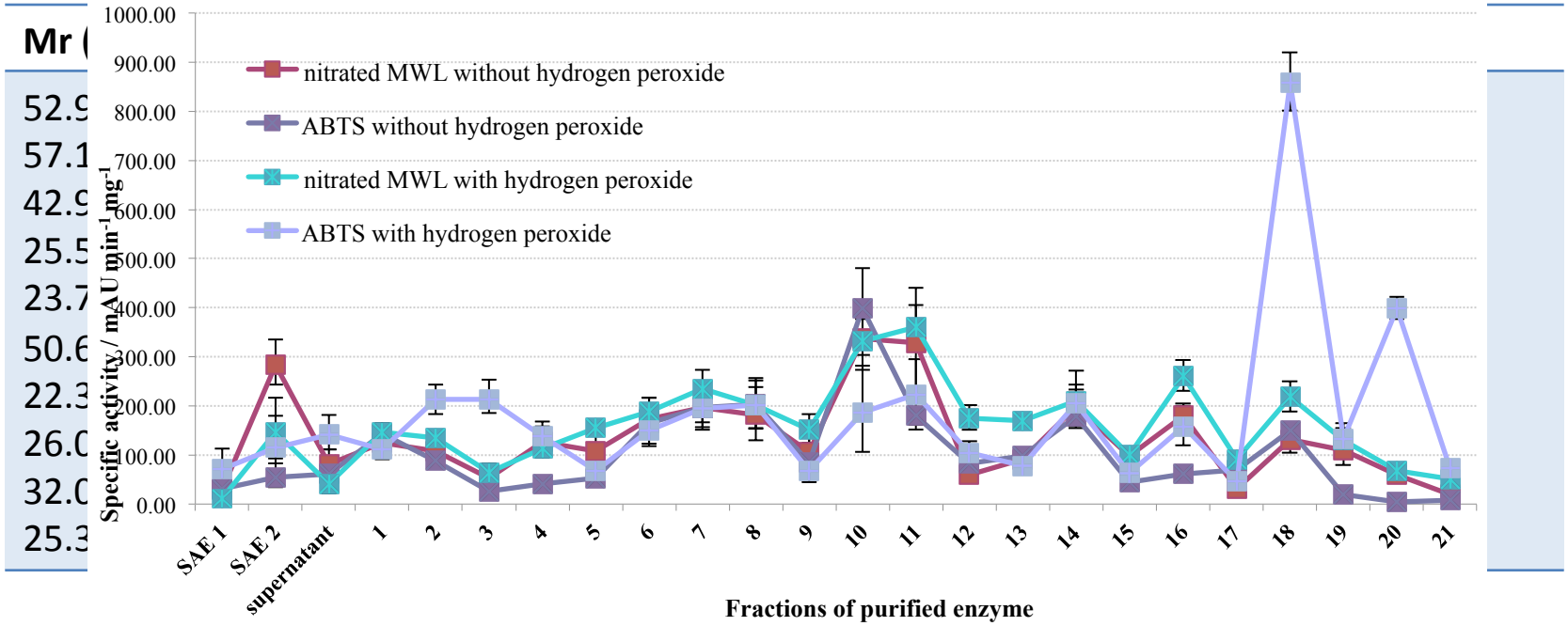
Possible Enzyme Activities from *Sphingobacterium* sp. T2 Genome Sequence

3.2 Mb genome **No dyp-type peroxidase!**

Predicted peroxidases:

- Cytochrome c peroxidase
- Glutathione peroxidase
- Thiol peroxidase
- Catalase/peroxidase
- Catalase

Proteomic data from extracellular fractions active in nitrated lignin assay:

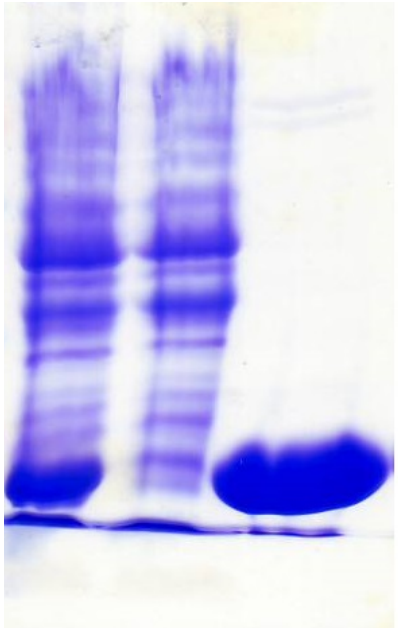


Reaction of *Sphingobacterium* MnSOD2 with Organosolv lignin

Mn SOD1 purified

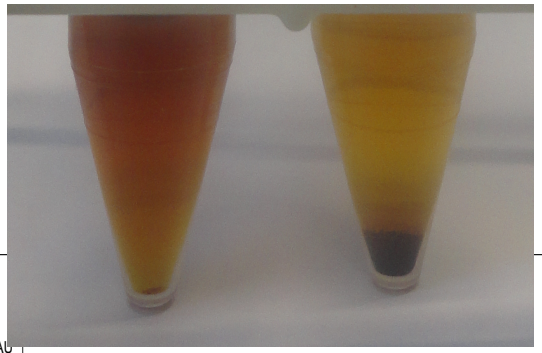
Mn SOD2 Extract

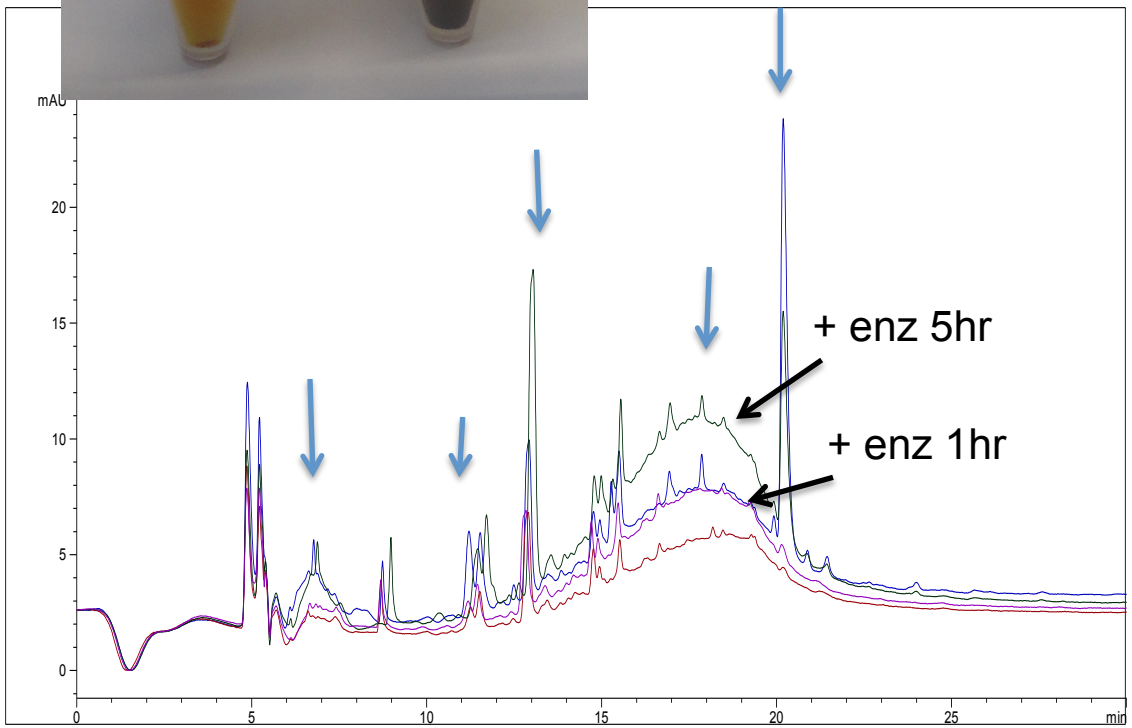
purified



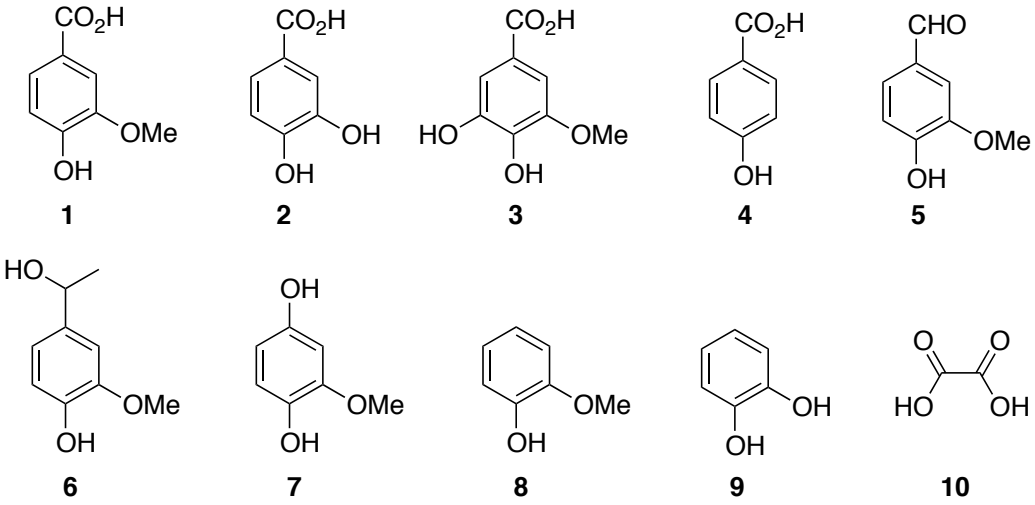
Reaction of MnSOD2 with Organosolv Lignin

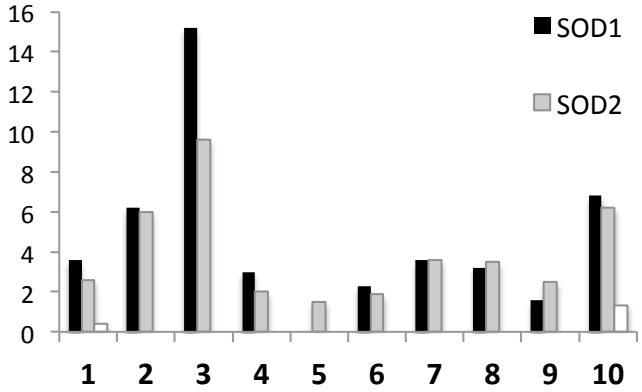
+ Enzyme -Enzyme

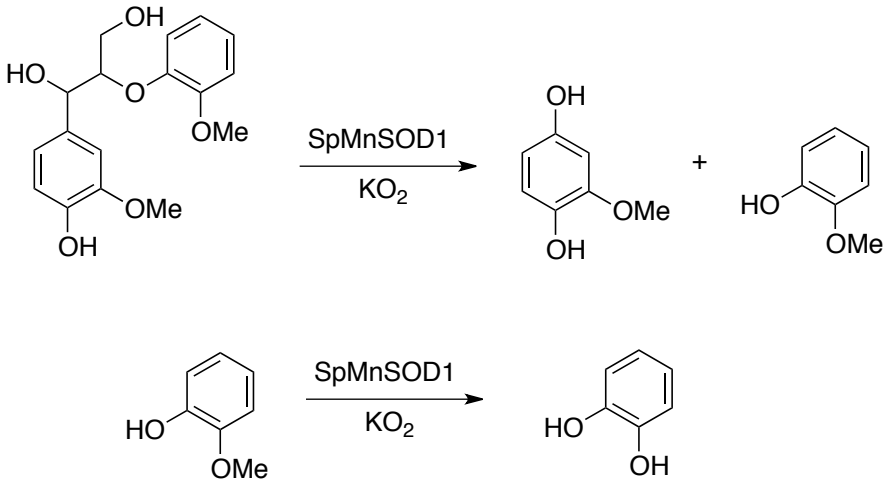




Reaction Products Identified from MnSOD Treatment of Organosolv Lignin

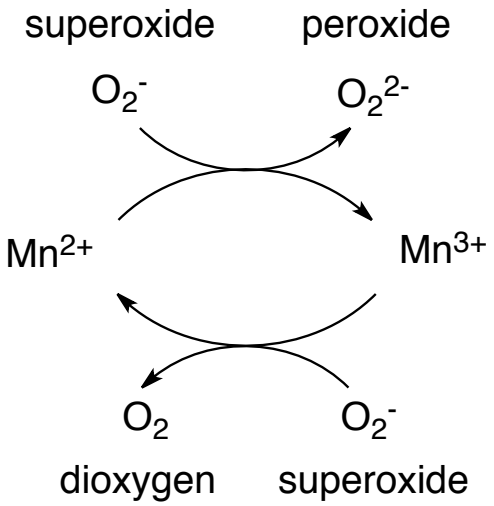






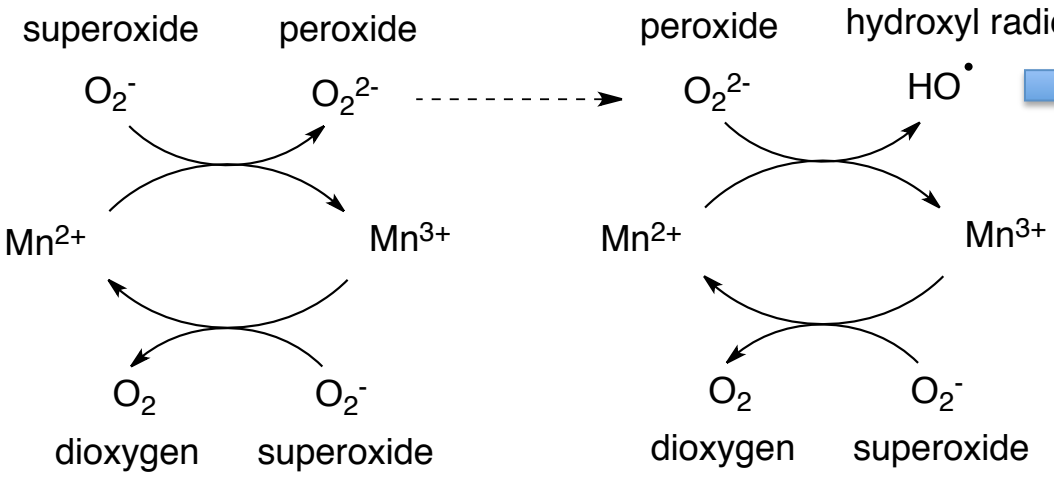
How could Superoxide Dismutase Attack Lignin?

Catalytic cycle of MnSOD



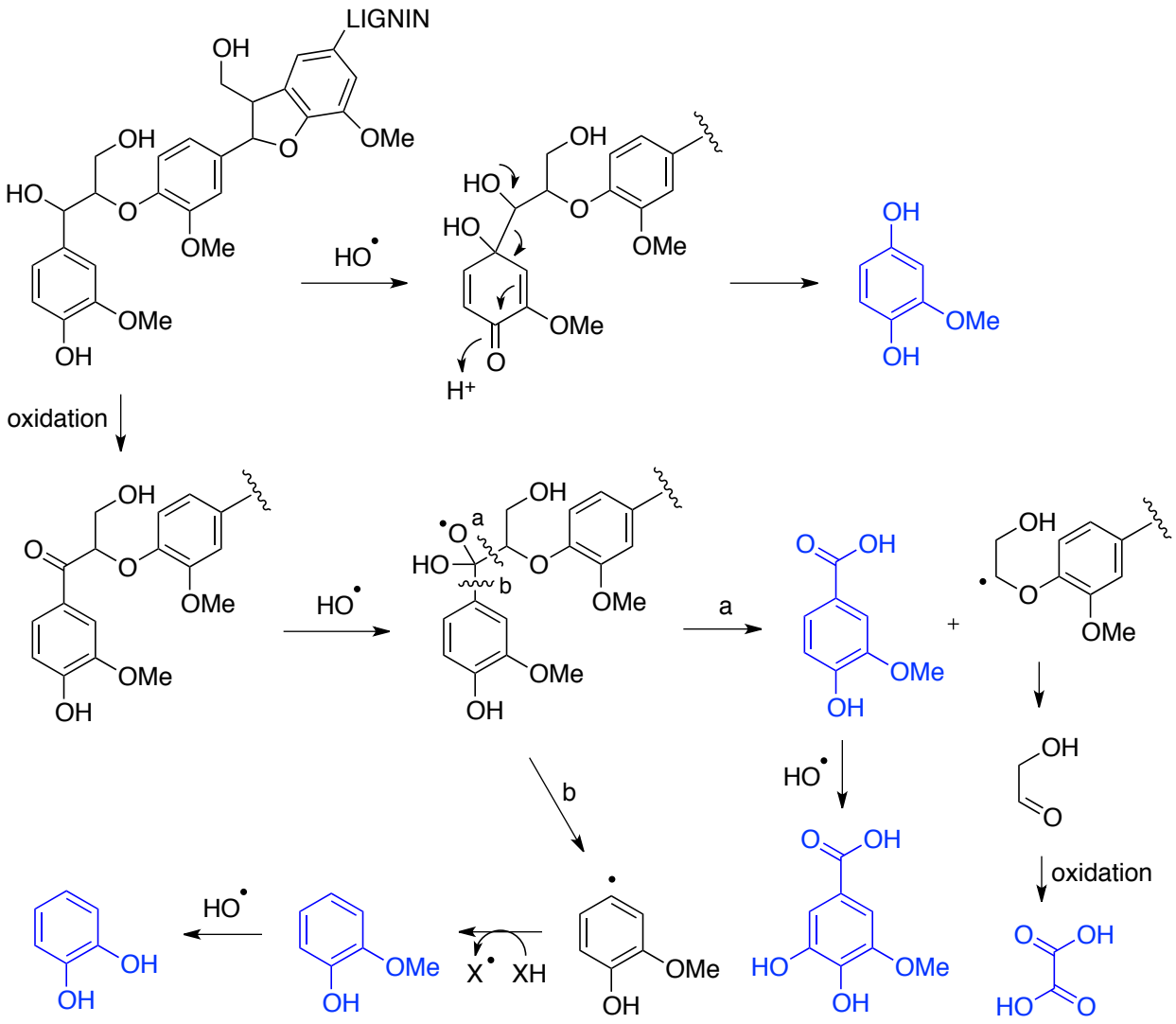
attacks lignin?

Possible modification:



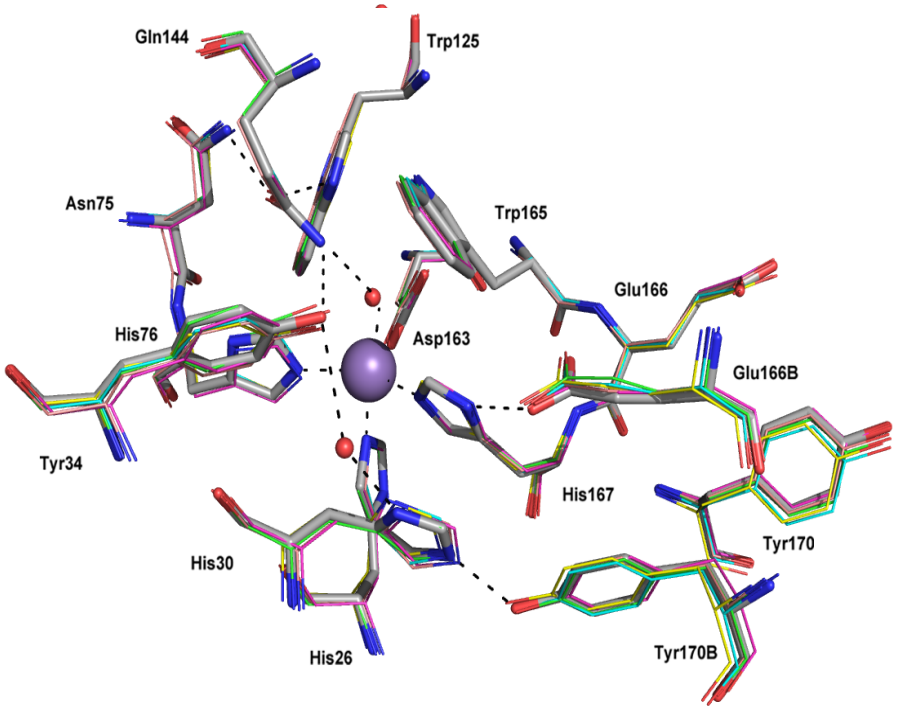
attacks lignin?

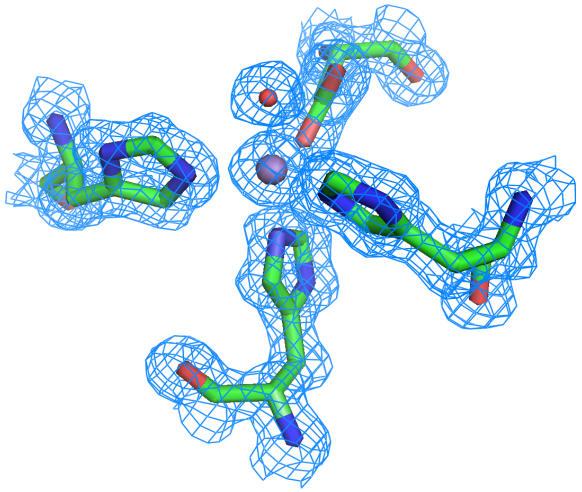
Hypothesis for MnSOD-catalysed Cleavage of Organosolv Lignin



1.35 Å Resolution Structure of *Sphingobacterium* sp. T2 MnSOD1

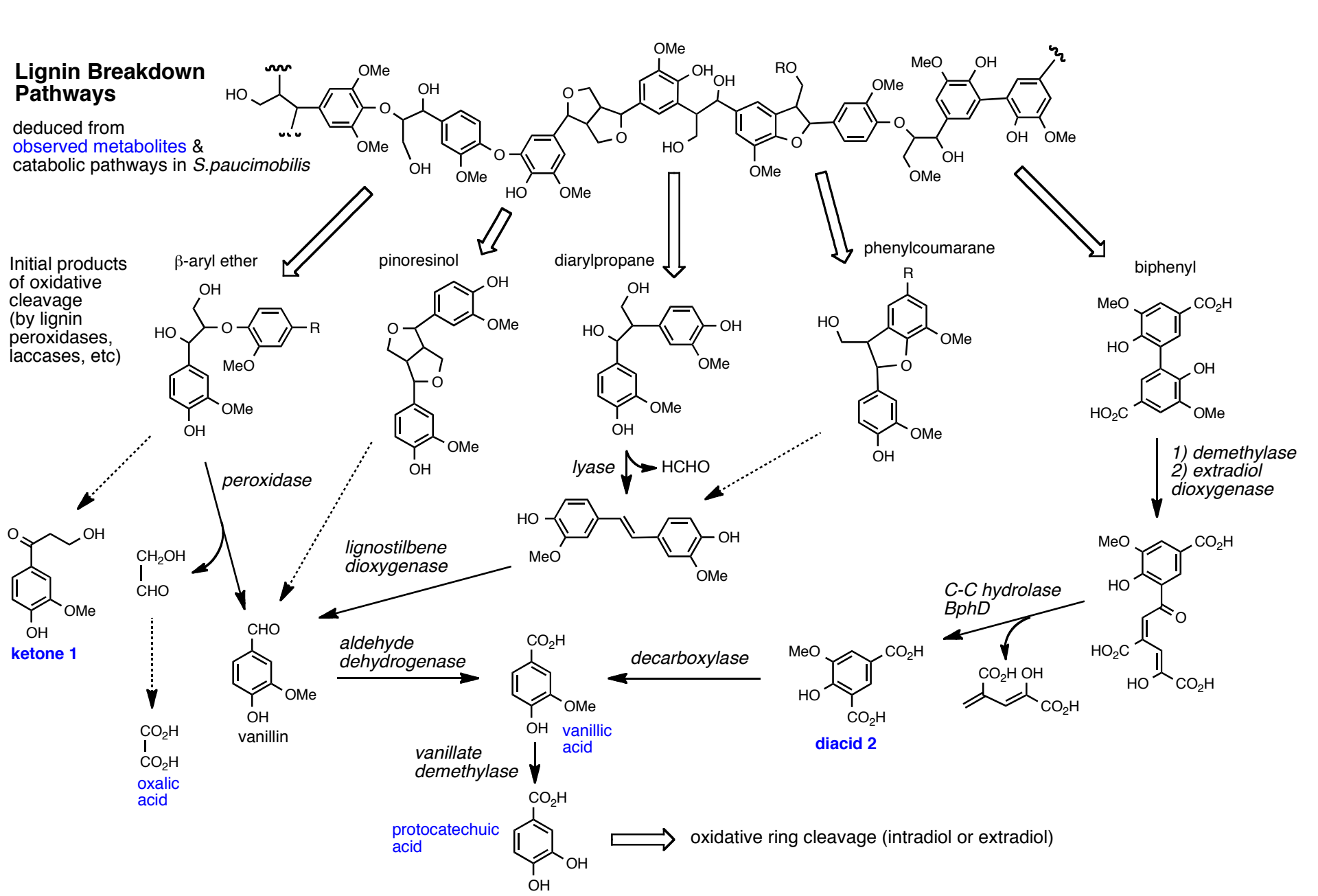
(with Prof. V. Fülöp, Dr. D. Rea, Univ. Warwick)





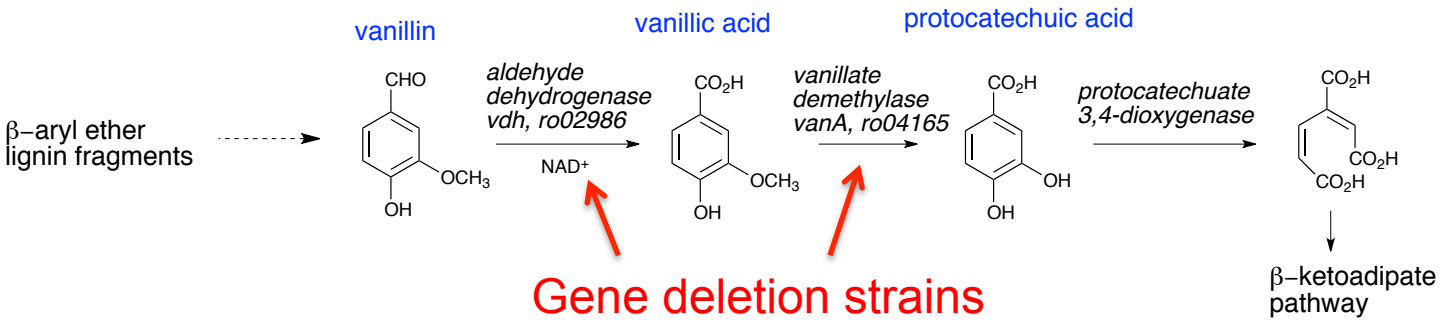
Only minor differences from structures of *E. coli*, *T. thermophilus* MnSOD

Reactivity with organosolv lignin: 5-6% product yield obtained for *Sphingobacterium* MnSOD

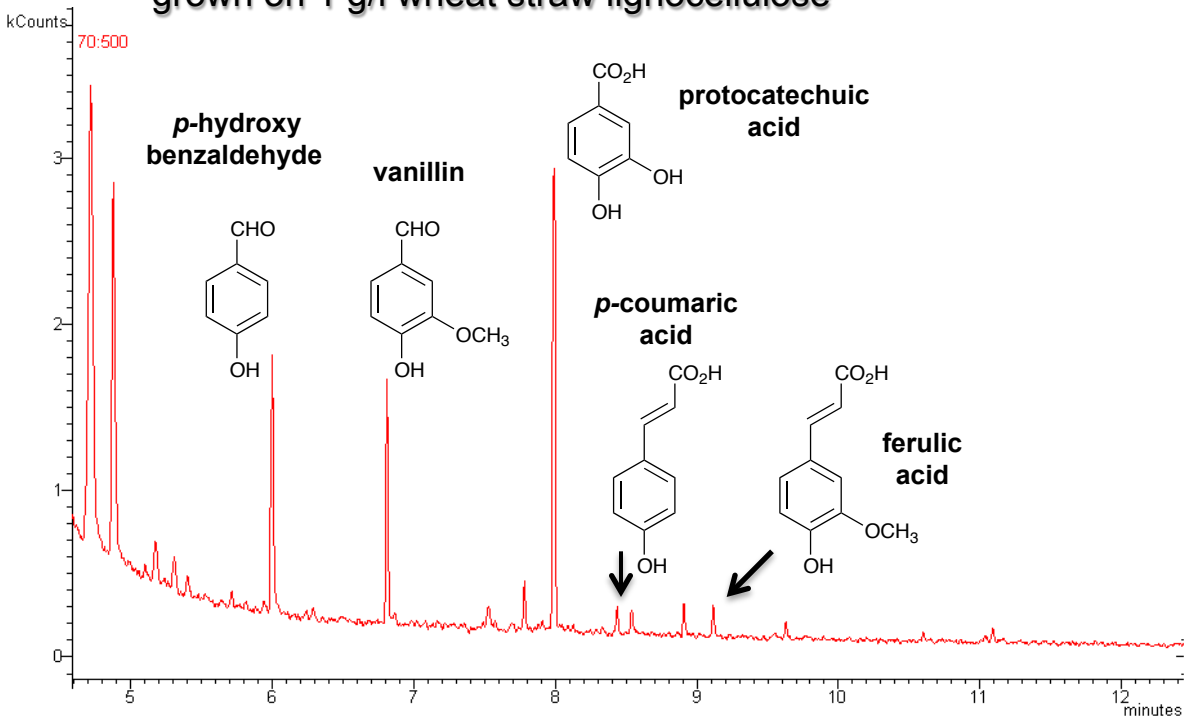


Can these pathways be manipulated via gene knockouts or enzyme inhibition?

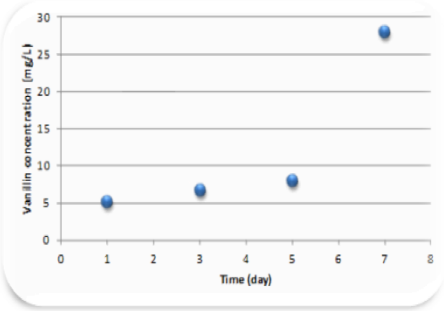
Gene Knockouts in Vanillin Catabolic Pathway in *Rhodococcus jostii* RHA1

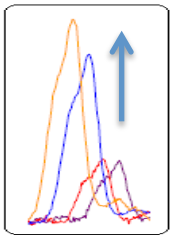


GC-MS Analysis of organic extract from *R. jostii Δvdh* mutant grown on 1 g/l wheat straw lignocellulose



Increase of vanillin peak vs time using LC-MS



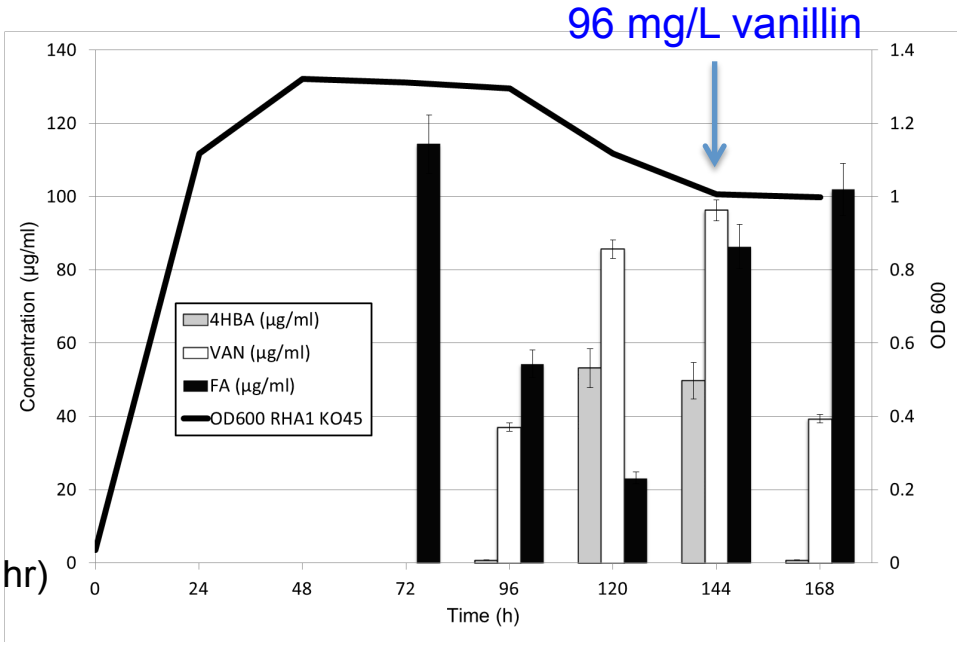


Yield 28 mg/litre

Yields of metabolites from RHA045 mutant:

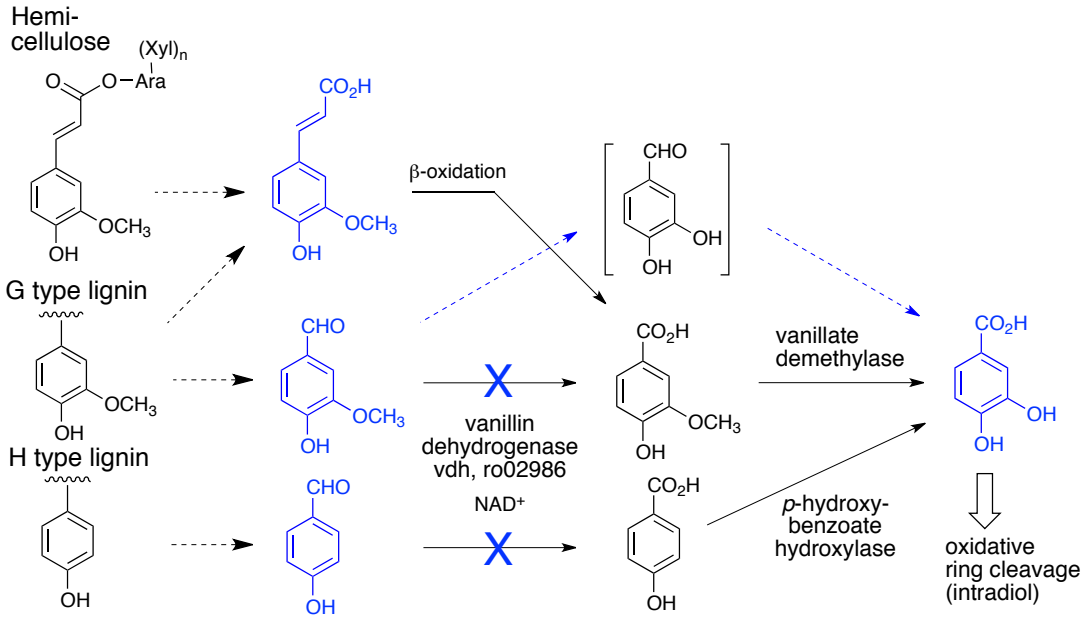
Minimal M9 medium supplemented with 2.5% wheat straw lignocellulose
Yields from LC-MS analysis.
VAN vanillin; 4HBA 4-hydroxybenzaldehyde
FA ferulic acid

Other carbon sources:
0.1% beech xylan: no vanillin observed
0.5% industrial Kraft lignin: 13 mg/L VAN (120 hr)



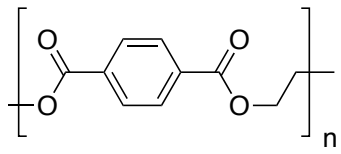
Rationalisation of catabolic pathways:

Growth on M9/0.1% ferulic acid produces >100 mg/L vanillic acid via β-oxidation, but not vanillin

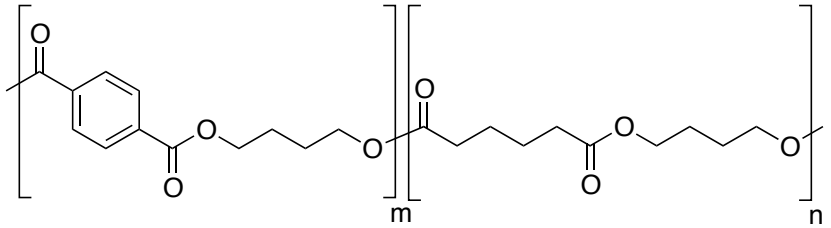


Generation of Aromatic Dicarboxylic Acids from Microbial Lignin Breakdown

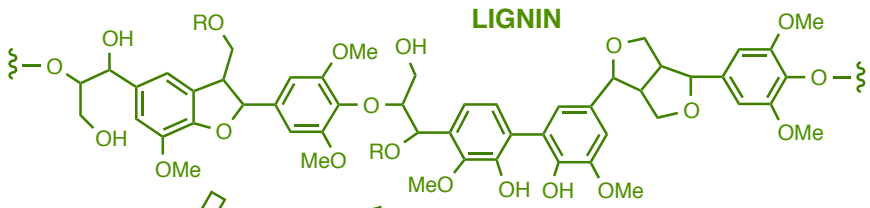
PET (polyethylene terephthalate)

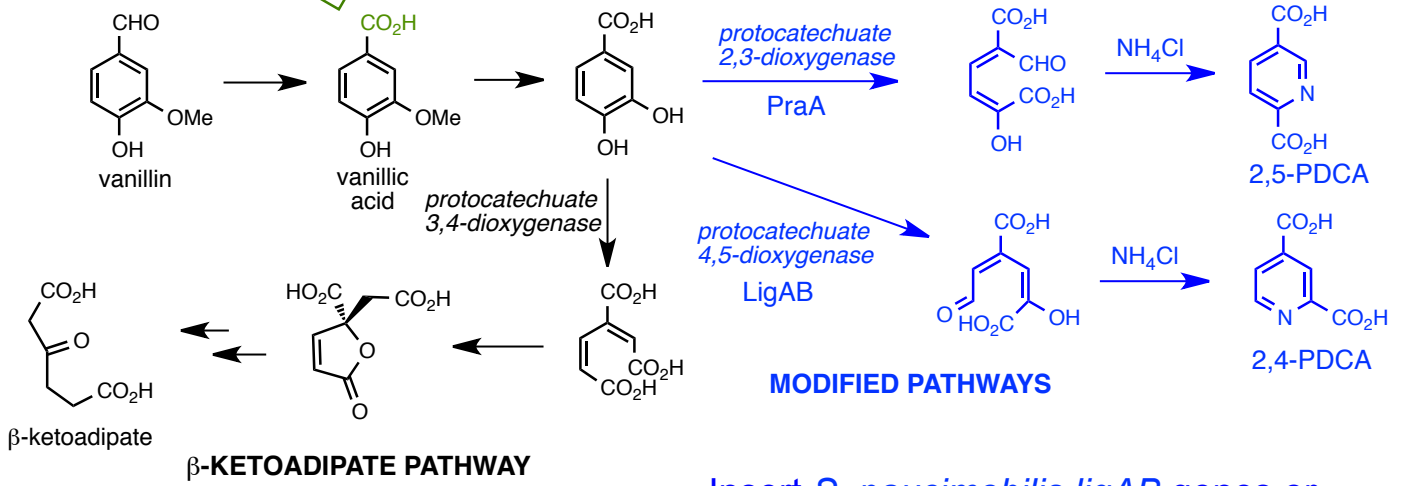


PBAT (polybutyrate adipate terephthalate)

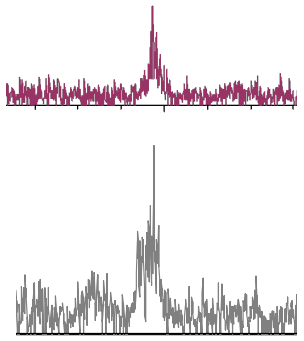


with Biome Bioplastics Ltd



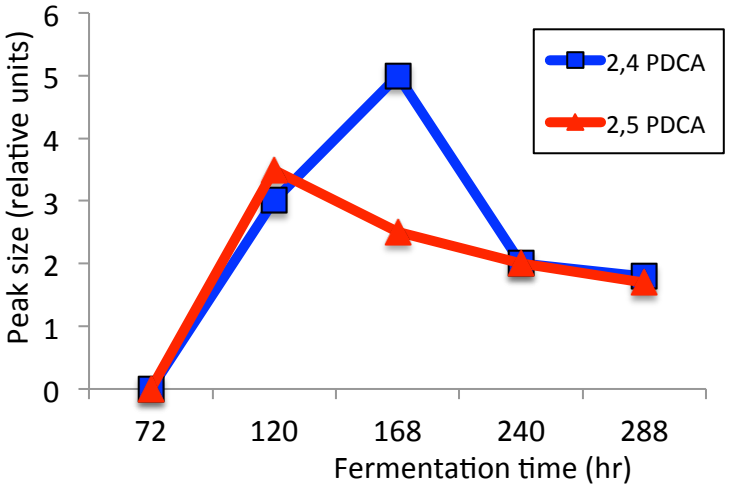


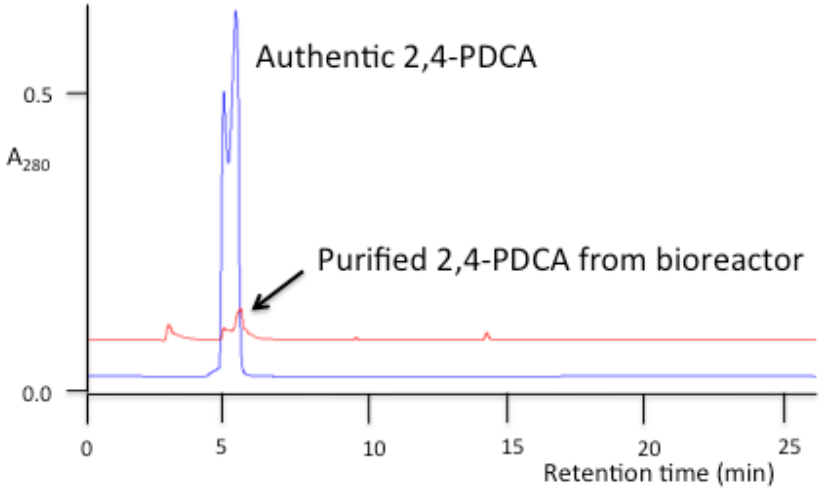
Identification by LC-MS from M9/1% wheat straw:



Insert *S. paucimobilis ligAB* genes or *Paenibacillus praA* gene into *Rhodococcus jostii* RHA1 using inducible pTipQ2 expression vector

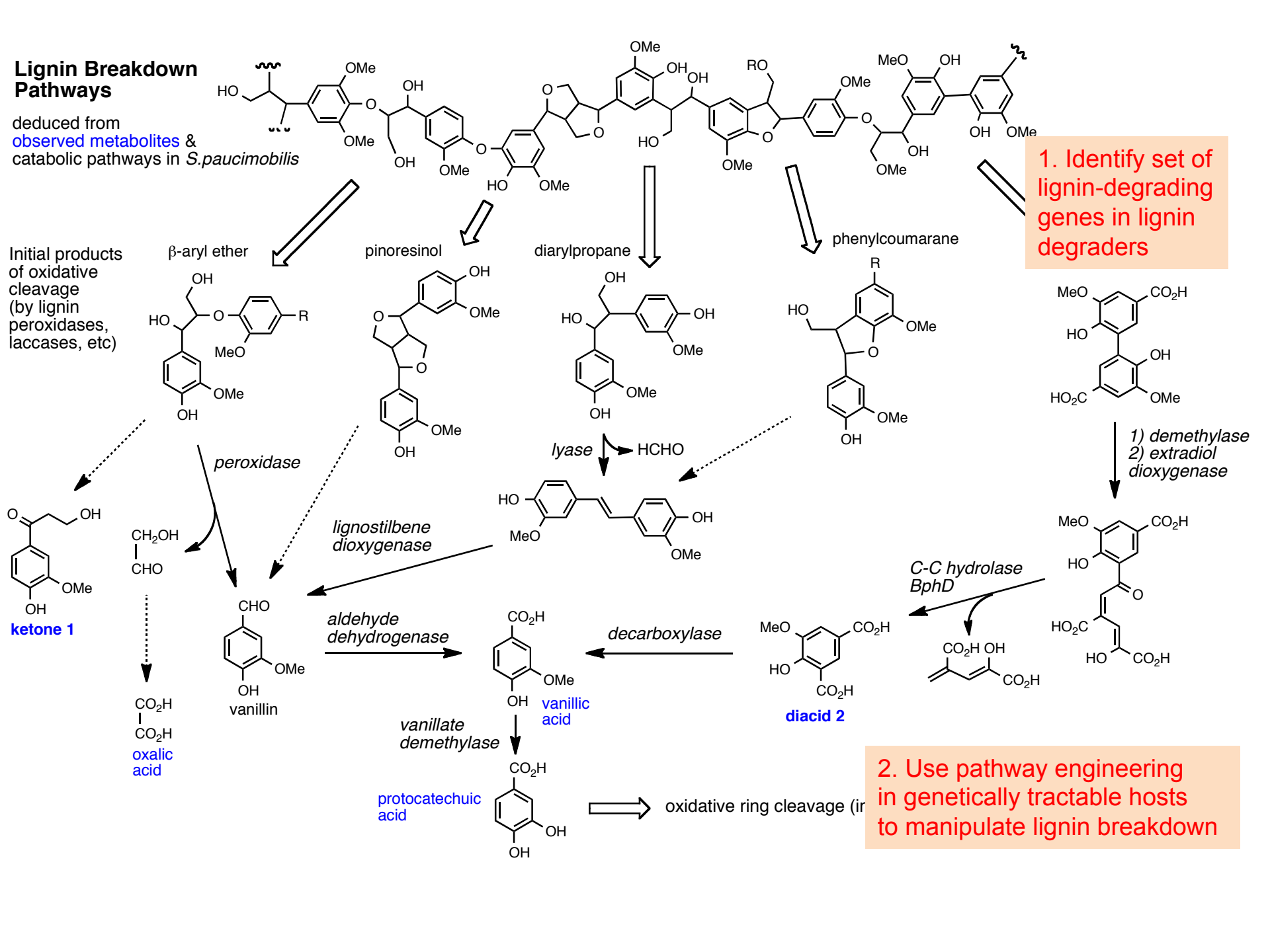
Production of Pyridine Dicarboxylic Acids by Fermentation





Construct	Product	Scale	Carbon source for M9 minimal media		
			0.1% vanillic acid	1% (w/v) chopped wheat straw	0.5% Kraft lignin
R. jostii pTipQC2-ligAB	2,4-PDCA	50 mL	112 mg/L (7 days)*	90 mg/L (7 days)*	NT
		2.5 L bioreactor	NT	125 mg/L (9d)* 102 mg/L (9d)#	53 mg/L (9 days)#
R. jostii pTipQC2-praA	2,5-PDCA	50 mL	80 mg/L (5 days)*	79 mg/L (5 days)*	NT
		2.5 L bioreactor	NT	106 mg/L (9d)* 65 mg/L (9d)#	NT

Yield estimated by *LC-MS analysis #product isolated by chromatography (UV-vis analysis)


Lignin Breakdown Pathways
deduced from observed metabolites & catabolic pathways in S.paucimobilis
1. Identify set of lignin-degrading genes in lignin degraders
Initial products of oxidative cleavage (by lignin peroxidases, laccases, etc)
β-aryl ether
pinoresinol
diarylpropane
phenylcoumarane
peroxidase
lyase
HCHO
1) demethylase
2) extradiol dioxygenase
lignostilbene dioxygenase
C-C hydrolase BphD
aldehyde dehydrogenase
decarboxylase
vanillate demethylase
ketone 1
oxalic acid
vanillin
vanillic acid
diacid 2
protocatechuic acid
oxidative ring cleavage (ir
2. Use pathway engineering in genetically tractable hosts to manipulate lignin breakdown

Acknowledgements

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Rahman Rahmanpour (3rd yr PhD)
Goran Mahmoud (3rd yr PhD)

Prof. Lindsay Eltis (UBC, Canada)
Paul Mines, Paul Law (Biome Bioplastics)

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Up to £50,000 in project initiation funding to help identify and scope commercial and scientific challenges

£10,000 Business Interaction Vouchers to encourage initial collaborations between industry and academia

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